INSTITUTO POLITÉCNICO DE BRAGANÇA
Escola Superior Agrária

Evaluation of a Filter System to Harvest Plasma for Total Protein Measurement in Newborn Calves of the Holstein Breed

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Dissertação apresentada à Escola Superior Agrária de Bragança para obtenção do Grau de Mestre em Tecnologia da Ciência Animal

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Bragança
2018

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ACKNOWLEDGMENT

At the first moment, I would like to thank my family who, even for miles away, has always been with me, supporting me and encouraging me on this journey. Without your love I would not get here. I would also like to thank my friends, by the force transmitted through a hug or a message of affection.

I could not fail to thank the Polytechnic Institute of Bragança for its teaching structure and the opportunity to study again in another country. Special thanks to my counselor, Dr. Ramiro Valentim, especially for the opportunity, learning, patience and for believing in me. Besides being an excellent professional, it is admirable how he leads a team, surrounded by a great friendship. I also thank the team of the Animal Reproduction Laboratory, including academics, teachers and staff, in which we have created a bond of friendship.

It was a year of development of the thesis at Freie Universitat Berlin, where I met excellent professionals who also became friends, made me feel at home. I could not fail to mention my intern counselor, Dr. Heuwieser, and also my internship supervisor, Dr. Stefan. Thank you for your patience and learning. You have my admiration.

Finally, I also leave my thanks to my German family in which they made me feel part of the family and showed me the culture of the country. Thank you for your kindness and attention.

Thank you to all who took part in this walk, you were very important.

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LIST OF ABBREVIATIONS

AUC – Area Under the Curve

CC – Coliform Count

CFU/mL – Colony Forming Unit/Milliliter

CNS – Coagulase - Negative Staphylococci

ELISA – Enzyme-Linked Immune Sorbent Assay

ES – Environmental *Streptococci*

FPT – Failure of Passive Transfer

g – Gram

g/dL – Gram/deciliter

g/L – Grams/liter

Ig's – Immunoglobulins

IgA –Immunoglobulin A

IgD – Immunoglobulin D

IgE – Immunoglobulin E

IgG – Immunoglobulin G

IgM –Immunoglobulin M

Kg – Kilogram

Log_{10} – $Logarithm_{10}$

mg/mL – Milligram/milliliter

mL – Mililiter

NC – Noncoliform Count

RID – Tadial Immune Diffusion

ROC – Receiver Operating Characteristic

SA – *Staphylococcus aureus*

SAG – *Streptococcus agalactiae*

SD – Standard Deviation

Se – Sensitivity

Sp – Specificity

SPC – Standard Plate Count

STP – Serum Total Protein

TIA – Turbidometric Immuno Assay

TP – Total Protein

vs. – *Versus*

μg/dL – Micrograms/deciliter

°C – Degree Celsius

% – Percentage

ABSTRACT

The objective of this study was to evaluate a filter system to harvest plasma in order to assess failure of passive transfer (FPT) in newborn calves. Blood samples (n = 227) were collected from Holstein calves aged 1 to 6 days, from 4 commercial dairy herds in the northeast of Germany. Serum IgG concentration was determined in serum using an ELISA test. Failure of passive transfer was defined as IgG concentrations ≤ 10 mg/mL and used as a gold standard. An optical refractometer and 2 different digital BRIX refractometers were used to assess FPT in serum or plasma. Serum was obtained through centrifugation. Plasma was either obtained through a filter system or centrifugation. A receiver operating characteristic curve analysis was used to determine an optimum threshold for each of the 3 devices using different media. Sixty-seven (30%) calves had FPT. For device 1, the optimum threshold was 8.9% (Se 82.1%; Sp 63.8%; AUC 0.81), 9.4% (Se 76.1%; Sp 73.7%; AUC 0.80), using serum and centrifuged plasma, respectively. For device 2, the optimum threshold was 8.7% (Se 74.6%; Sp 76.2%; AUC 0.83), 9.5% (Se 80.6%; Sp70.6%; AUC 0.83), 9.2% (Se 58.2%; Sp 87.5%; AUC 0.80) using serum, centrifuged plasma, and filtered plasma, respectively. For device 3, the optimum threshold was 5.6 g/dL (Se 70.1%; Sp 80.0%; AUC 0.85), 6.3 g/dL (Se 82.1%; Sp 68.1%; AUC 0.84), 6.0 g/dL (Se 56.7%; Sp 90.0%; AUC 0.80) using serum, centrifuged plasma, and filtered plasma, respectively. Based on 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.

Keywords: Calves, colostrum, failure of passive transfer.

RESUMO

O objetivo deste estudo foi avaliar um sistema de filtragem para recolher plasma para avaliar a falha na transferência passiva (FTP) em vitelos recém-nascidos. As amostras de sangue (n = 227) foram recolhidas a partir de vitelos da raça Holstein (1 a 6 dias de idade), de 4 explorações leiteiras comerciais localizadas no nordeste da Alemanha. A concentração sérica de IgG foi determinada no soro utilizando o método ELISA. A falha da transferência passiva foi definida como concentrações de IgG ≤ 10 mg/mL e utilizada como padrão. Um refratómetro óptico e dois refratómetros digitais BRIX foram usados na avaliação do FTP no soro ou plasma. O soro foi obtido por centrifugação. O plasma foi obtido através de um sistema de filtração ou de centrifugação. Utilizou-se uma análise de curva característica de operação do receptor para determinar um limiar ótimo para cada um dos 3 dispositivos que utilizam meios diferentes. Sessenta e sete (30%) bezerros tinham FTP. Para o dispositivo 1, o limiar ótimo foi de 8,9% (Se 82,1%; Sp 63,8%, AUC 0,81), 9,4% (Se 76,1%; Sp 73,7%, AUC 0,80), utilizando soro e plasma centrifugado, respectivamente. Para o dispositivo 2, o limiar ótimo foi de 8,7% (Se 74,6%, Sp 76,2%, AUC 0,83), 9,5% (Se 80,6%, Sp70,6%, AUC 0,83), 9,2% (Se 58,2%; Sp 87,5%, AUC 0.80) utilizando soro, plasma centrifugado e plasma filtrado, respectivamente. Para o dispositivo 3, o limiar ótimo foi de 5,6 g/dL (Se 70,1%; Sp 80,0%, AUC 0,85), 6,3 g/dL (Se 82,1%; Sp 68,1%; AUC 0,84), 6,0 g/dL (Se 56,7%; Sp 90,0%; AUC 0,80) utilizando soro, plasma centrifugado e plasma filtrado, respectivamente. Com base na AUC, os 3 dispositivos produziram características de teste comparáveis para identificar vitelos com FTP. Em conclusão, um sistema de filtro pode ser usado para facilitar a avaliação de FTP como técnica de ponto de tratamento em vitelos sem a necessidade de centrifugação no soro.

Palavras-chave: colostro, falha na transferência passiva, vitelos.

INTRODUCTION

The dairy market is growing all over the world. In the year 2005, world milk production averaged 650 millions of tons, increasing by 26% until 2015 (IDFA, 2017). According to FAO (2016) the Europe Union is the second largest producer of milk, overdue only by Asia.

Feeding and management programs for calves are important in determining their health and survival as they are designed to help ensure that calves reach their biological growth potential (BAMN, 2017). To achieve the expected result of a dairy production, special care is required in the management of animals and, for this, the key factor is to achieve early and adequate intake of high quality colostrum to deliver maternal immunoglobulins (**Ig's**), nutrients, and non-nutrient factors that have been shown to impact the metabolism and the future performance of the neonatal calf (Van Amburgh, 2017). Continuous monitoring of successful passive transfer of IgG in newborn calves should be a crucial component of a sound colostrum management (DCHA, 2016).

For the assessment of a successful passive transfer a validated method is to use the concentration of serum total protein (**TP**) of calves aged at least 24 hours until 7 days using refractometry (Deelen et al., 2014). The failure of passive transfer (**FPT**) in calves is defined as serum IgG concentrations below 10 mg/mL (Godden, 2008). Prevalence of FPT ranged from 1.3 to 36% in the modern dairy industry (Lee at al., 2008; Morrill et al., 2013; Deelen et al., 2014; Elsohaby et al., 2015; Hernandez et al., 2016). Both optical and digital refractometry were highly correlated with the gold standard to assess FPT using the concentration of Ig in the serum of calves. Thresholds to define successful passive transfer for optical refractometer and digital BRIX refractometer were 5.5 g/dL and 8.4%, respectively (Weaver et al., 2000; Deelen et al., 2014). A calves' passive transfer status and the amount of liquid nutrition consumed during the preweaning period plays an important role in resistance to disease and growth (BAMN, 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 (Wallace et al., 2006). In human medicine a filter system was developed for point of care devices to separate plasma from blood cells (Wang et al., 2012). This system was shown to be efficient in separating plasma from cells and has several advantages, such as being portable, quick and requiring only small amounts of blood (Crowley et al., 2005; Chen et al., 2009). However, it has not been validated in veterinary medicine.

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

I – LITERATURE REVIEW

1. Colostrum

Colostrum is a fluid secreted by the female mammary gland in the first 24 hours after calving (Jaster, 2005), in which the main function is to meet the complete nutritional needs of the newborn (McGrath et al., 2016). The milk produced by the third day after the first milking is called transitional milk, after the first milking changes occur in the composition (BAMN, 2001). During the dry period, about two months before giving birth, the mammary gland begins a rebuilding phase (Hurley and Theil, 2011). Colostrum formation begins a few weeks before delivery, under the influence of lactogenic hormones such as prolactin, and abruptly terminates after calf birth (Godden, 2008).

The first food is very important for the development of the calf, both for immunity and body weight gain (Rebelatto and Weiblen, 1992). Faber et al. (2005) published a paper describing the importance of colostrum for the development of the newborn. They compared feeding 4 *vs*. 2 liters of maternal colostrum to 68 Brown Swiss heifer calves. The calves that received 4 liters of colostrum (classified as high quality) grew faster (1.03 *vs*. 0.80 kg/day), had fewer sick days (5 *vs*. 8 days/calf), and produced 550 kg of additional milk during two entire lactations. Feitosa et al. (2001) described the importance of transferring Ig's to newborn animals. In their study seven newborn (n = 40) calves died due to passive transfer failure. The mean total protein concentration of those animals was 4.7 g/dL, while the survivors presented an average of 7.0 g/dL.

1.1. Composition

Colostrum contains many important substances for calf health such as energy, cytokines, growth factors, and increased levels of vitamins and minerals (McGrath et al., 2016). The concentration of these compounds, especially of antibodies and proteins, decrease after the first day of milking and an increase in lactose concentration occurs (McGrath et al., 2016; TCRM, 2017) (Table 1).

The composition and physical characteristics of colostrum vary due to several factors, including individuality, race, parity, pre partum feeding, duration of dry cow period, postpartum milking time, among others (Weaver et al., 2000). In fact, as shown in the Table 1, the concentration of lactose is low in colostrum and changes inversely to other nutrients, such as fats and proteins, emphasizing the importance of providing colostrum on the first day of life. McGrath et al. (2016) refers that the lactose concentration reaches a normal

concentration within 7 days postpartum. Colostrum also has higher energy content than normal milk which is important since newborn calves lack large reserves of fat (Lorenz et al., 2011).

Table 1. Composition of colostrum, transition milk and milk (TCRM, 2017)

	Milking number			
	1	**2**	**3**	**11**
	Colostrum	**Transition milk**		**Milk**
Total solid (%)	23.9	17.9	14.1	12.5
Fat (%)	6.7	5.4	3.9	3.9
Protein (%)	14.0	8.4	5.1	3.1
Antibodies (%)	6.0	4.2	2.4	0.09
Lactose (%)	2.7	3.9	4.4	4.9
Minerals (%)	1.11	0.95	0.87	0.74
Vitamin A (µg/dL)	295	190	113	34

The growth and performance of the calf are related to a large number of factors, such as feeding (Miller-Cushon and DeVries, 2015). Growth is regulated by the daily intake of protein and energy found in milk (Faber et al., 2005). Therefore, after the supply of colostrum as the first feeding, it is very important to continue providing milk in the first days of life, with the introduction of a new diet (Miller-Cushon and DeVries, 2015), until the rumen starts supplying energy and microbial proteins in quantities sufficient to maintain growth (BAMN, 2017).

2. Immunoglobulins

Antibodies or Ig's are proteins normally found in the bloodstream and are vital components of the immune system, but they are not present in the calf circulation of newborns because they cannot cross the placenta during gestation (Wattiaux, 1996). The bovine placenta forms a syncytium between the maternal endometrium and the fetal trofoectoderm separating the blood circulation of the fetus and the mother and preventing the transmission of Ig's in uterus (Weaver et al., 2000).

Thus, the newborn acquires Ig's through colostrum, where they are transported through the mammary epithelial cells by receptor-mediated mechanisms and transferred out of the mammary gland by ejection of colostrum during suction (Hurley and Theil, 2011). In other

words, the antibodies will be absorbed after ingestion of colostrum (Wattiaux, 1996). The release of Ig's from the blood serum to the mammary glands begins two to three weeks before delivery (Heinrichs and Elizondo-Salazar, 2008). These Ig's are derived from various sources and represent the natural immunity acquired over the life of the cow due to exposure to antigens and therefore with a response of their immune system (Hurley and Theil, 2011). Several studies have shown that without an adequate amount of antibodies in the blood, the mortality of newborn calves increases dramatically in the first days and weeks of life (Lorenz et al., 2011; Meganck, 2014).

There are different classes, what differs from Ig's are the chemical structure, number of binding sites and antigen function (Jefferis et al., 2006). The main classes of Ig's found in blood serum are IgG, IgA and IgM (Cole and Bowen, 1976). Immunoglobulin M is the class that appears initially when an organism is exposed to an antigen for the first time, in which it has a lower potential in the defeat of an infection (Hurley and Theil, 2011). Immunoglobulin A is the major class of Ig found in mucous secretions and prevents mucosal infections by binding microbes whereas IgG is the primary Ig class found in colostrum and milk (Hurley and Theil, 2011) and constitutes approximately 85% of the Ig's in colostrum (Butler, 1983). IgG is produced by plasma cells in the spleen, lymph nodes and bone marrow. Because it is the smallest of Ig, IgG can migrate more easily from blood vessels than other isotypes (Tizard, 2013).

There are other classes such as IgE and IgD, which are found in lower concentration in the blood serum (Cole and Bowen, 1976). Immunoglobulin E is responsible for immunity against parasites and for allergy response and IgD is found on the surface of immature lymphocytes (Tizard, 2013). Godden (2008) reported that IgE is an important element in the defense against microorganisms in the first weeks of life and in particular against intestinal parasites.

3. Passive Transfer

Passive transference is defined by the transfer of Ig's from the cow to the calf through the colostrum, being important for the protection of the neonate against infectious diseases (McGuirk and Collins, 2004). The FPT makes the calf more susceptible to the development of diseases (Weaver et al., 2000), which may interfere with farm productivity, neonatal mortality increases due to diarrhea, respiratory diseases, increased veterinary costs, and use of antibiotics (Meganck et al., 2014). This may also reflect calf growth rate, daily weight gain and also milk production (USDA, 2008; DeLaval, 2014). Therefore, it is essential to provide

adequate amount of high quality colostrum in the first days of life, so that the calf develops his own immune system through passive immunity (Stelwagen et al., 2009). Almost 60% of maternal colostrum on farms is inadequate, increasing the risk of mortality of newborns (Morrill et al., 2012).

To minimize FPT in newborn calves, colostrum should be ingested within the first 24 hours of life so that it to absorbed Ig's, in which the equivalent of 10% of its body weight should be ingested within the first two hours of life (DCHA, 2016). It is very important that the newborn feed within this period, because that is when the digestive system can absorb large molecules, such as intact antibodies (Quigley and Mills, 2004) (Figure 1). This is because colostrum contains enzymes that inhibit digestive enzymes, which already have limited activity, present in the abomasum and small intestine of calves, allowing antibodies to reach the small intestine and be absorbed (BAMN, 2001). Factors such as the method and volume of administration of colostrum and the time of ingestion will also influence the success of passive transfer (Weaver et al., 2000).

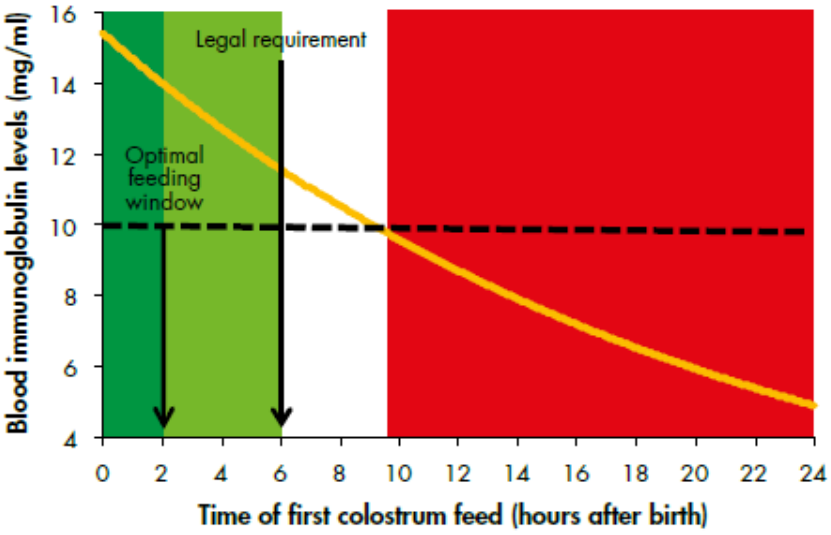

Figure 1. Calf survival by serum IgG concentration (AHDB, 2015).

Figure 1 shows that the ability of the calf to absorb Ig's is greater in the first two hours after birth. After six hours this skill decreases progressively. Therefore, the calf will have a higher concentration of circulating IgG in the blood if fed early. It happens because a calf's gastrointestinal tract has the ability to temporarily absorb large molecules, including Ig's, during the first 12 to 24 hours of life (Beam et al., 2009). These antibodies will remain in the bloodstream until around the 4th month when calves will be able to produce their own antibodies. In this phase, passive antibodies (absorbed via colostrum) are eliminated and exchanged for antibodies produced by the animal (Oliveira, 2012).

Colostrum should be of high quality, with an IgG concentration ≥ 50 g/L (McGuirk and Collins, 2004; Godden, 2008). The quality of the colostrum may vary according to the age of the cow, time of the first milking since calving, udder leakage before delivery, duration of the dry period, breed and feeding (DeLaval, 2014; TCRM, 2017).

The DCHA (2016) refers that the TP concentration in the blood serum of animals from 2 to 7 days of age should be ≥ 5.2 g/dL and the IgG concentration ≥ 10.0 mg/mL (Godden, 2008). Vogels et al. (2013) and Elsohaby et al. (2015) performed studies to determine the prevalence of failure to transfer passive immunity in calves. They found that 38% of the animals had a low TP concentration in serum (≤ 5.0 g/dL) and 27.5% of the animals had a low IgG in serum (≤ 10.0 mg/mL).

Weaver et al. (2000) pointed out the factors mentioned above, namely colostrum intake per calf, method and volume of administration of colostrum, ingested colostrum Ig's. They also cited that the metabolic status of the newborn calf and the stress experienced by the calf during calving are factors that affect the absorption of colostrum Ig's.

Continuous monitoring of successful passive transfer of IgG in newborn calves should be a crucial component of a sound colostrum management (DCHA, 2016). Only 6.2% of dairy farms, however, routinely monitories FPT on a regular basis (USDA, 2016).

3.1. Methods for Assessing Passive Immunity

There are tests available that evaluate serum IgG concentrations such as radial immune diffusion (RID) and enzyme-linked immune sorbent assay (ELISA), but these tests cannot be considered on-farm because they are technically demanding and costly (Davis and Giguère, 2005; Hogan et al., 2015). A practical alternative that can be performed at the farm for passive transfer monitoring is the refractometer, which is an indirect measure of the TP (Deelen et al., 2014).

Tests to evaluate the concentration of Ig's can be done on calves with a minimum age of 24 hours to 7 days (McGuirk and Collins, 2004). The refractometer is used on dairy farms to evaluate colostrum quality, estimation of serum IgG concentration in neonatal calves, and non-salable milk evaluation of total solids for calf nutrition (Floren et al., 2016). There are two types of refractometers, optical and digital. Both determine the density of a liquid through a beam of light that shines through the sample, that is, it measures the amount of light that is refracted by the TP of the sample (Quigley et al., 2013). Thus, the result estimates the passive transfer of Ig's (Wallace et al., 2006). Several studies have already proven that these methods are advantageous. One of such study was conducted by Quigley et al. (2013) who reported

that it is a cheap, fast method, requires minimal equipment and easy-to-learn technique. The optical and the digital refractometry were highly correlated with the gold standard to assess FPT using the concentration of Ig in the serum of calves (McCracken et al., 2017). Thresholds to define successful passive transfer for the optical refractometer and the digital BRIX refractometer were 5.5 g/dL and 8.4%, respectively (Weaver et al., 2000; Deelen et al., 2014).

Another method that measures the quality of colostrum is the colostrometer, which measures the specific gravity of colostrum and estimates the total gamma globulin based on statistical relationship and allows estimating the quality of colostrum based on the linear correlation between immunoglobulin concentration and its density (Godden, 2008; Weaver *et al*. 2000). Colostrum containing more than 49.8 mg/mL of globulins is considered to be of good quality (Fleenor and Stott, 1980).

In a recent publication Bartens et al. (2016) showed that the Brix refractometers provided the most accurate assessment of colostrum quality of the evaluated devices and demonstrated excellent repeatability accuracy. This is probably because the refractometers, as the optical as the digital, are precise instruments and the results are practically instantaneous, that can be used with the sample in temperature without there are distortions of results (Quigley et al., 2013). Mechor et al. (1991) reported that the temperature at which the measurement is carried out influences the readings carried out, when the test is carried out in low temperature conditions an overestimation of the Ig's concentration occurs and when it is carried out under high temperature conditions there is an underestimation of that concentration.

4. Management

A well-managed colostrum program is the most important step in reducing disease in neonatal calves on farms (Smith and Foster, 2007). For many years this subject has been studied by several researchers such as Wells et al. (1996). These researchers confirmed that the first feeding method is essential for calf survival, in which the colostrum supply can prevent up to 31% of the calves prevalence up to 21 days of age.

At the first step after birth, calf should be separated from the mother because allowing a calf to feed colostrum directly from its dam in the first hours of life can present many problems, such as increasing the risk that the calf does not get adequate amounts of colostrum (DeLaval, 2014). When a calf feeds on its dam, it is not possible to measure the amount of colostrum consumed or to estimate the quality of the ingested antibodies (USDA, 2008). A study by Franklin et al. (2003) showed that the newborn does not absorb sufficient amounts of

IgG when fed directly from the mother, that is, the minimum concentration of circulating Ig's in the blood is not enough to immunize the animal (threshold 5.0 g/dL). Separation may also prevent calf from ingesting feces, bedding or other environmental contaminated material from the cow (USDA, 2008).

Colostrum should be the first food consumed by the calf, should not ingest any food or even water before (Oliveira, 2012). As previously mentioned, colostrum should be given the equivalent of 10% of the live weight of the animal in the first 24 hours of life (DCHA, 2016), in which 4 liters of colostrum are normally offered (Godden, 2008). These processes are very important not to expose the animal to pathogenic agents, if bacteria reach the intestine before colostrum, they usually enter the blood and can prevent the antibodies from reaching the calf's blood or even bring the animal to death (DeLaval, 2014).

Colostrum of cows with mastitis should not be provided, since calves are extremely vulnerable to disease during the first days of life, therefor, the use of a commercial colostrum supplement is recommended if colostrum is of poor quality or insufficient for the calf (DeLaval, 2014).

In other words, there are 4 key factors that contribute to the success of passive immunity: colostrum feeding with high immunoglobulin concentration, colostrum volume, time to feed the calf after birth and minimizing colostrum bacterial contamination (Weaver et al., 2000; Johnson et al., 2007; Godden, 2008).

Incorrect management can lead to death of the calf, in which 39% of mortality is related to passive transfer failure (Tyler et al., 1999). Therefore, it should offer the right amount of colostrum and also a quality food, since neonates with low passive immunity had higher mortality rates, lower weight gain and lower performance (Rebelatto and Weiblen, 1992).

4.1. Methods Feeding Colostrum

As before, allowing the calf to feed directly from the mother is not the best method. There are some methods to feed the newborn with colostrum, such as: nipple bottle, bucket and tube feeding (Moran, 2002; Lorenz et al., 2011; TCRM, 2017).

The most practical form of feeding is the bucket, but, like bottle feeding, it can take time and trouble when calves are not motivated to drink, thus increasing the risk of FPT of immunity (Moran, 2002). However, when fed with bottle colostrum, calves rarely drink more than 2.5 liters voluntarily (Kaske et al., 2005). For this reason, some dairy farms have recently introduced esophageal tube feeding a way to ensure that calf ingests the right amount of

colostrum (Laestander, 2016). In the US feeding colostrum by esophageal feeder is used as a routine measure in about 14% of dairy heifer calves (USDA, 2007).

Feeding with an esophageal tube gives the opportunity to control the quantity fed, the quality of the milk fed and when the calf is feed, which is, according to some, the most important aspects in regard to failure of passive transfer (Weaver et al., 2000). But some of the reported disadvantages with using an esophageal tube is that there is a risk of feeding the calf too much and too fast, hurting the mouth, throat and esophagus and a risk of getting fluid in the calf's lungs (Quigley and Mills, 2004; Kaske et al., 2005).

If the only alternatives are the nipple bottle and the bucket, the best option is the nipple bottle because the use of a nipple bottle to feed the calves allows the staff to ensure that the calves be fed a specific amount of colostrum as well as allowing the calves to perform the natural behavior of suckling and thus benefit from the positive effects of suckling (Laestander, 2016) (Figure 2).

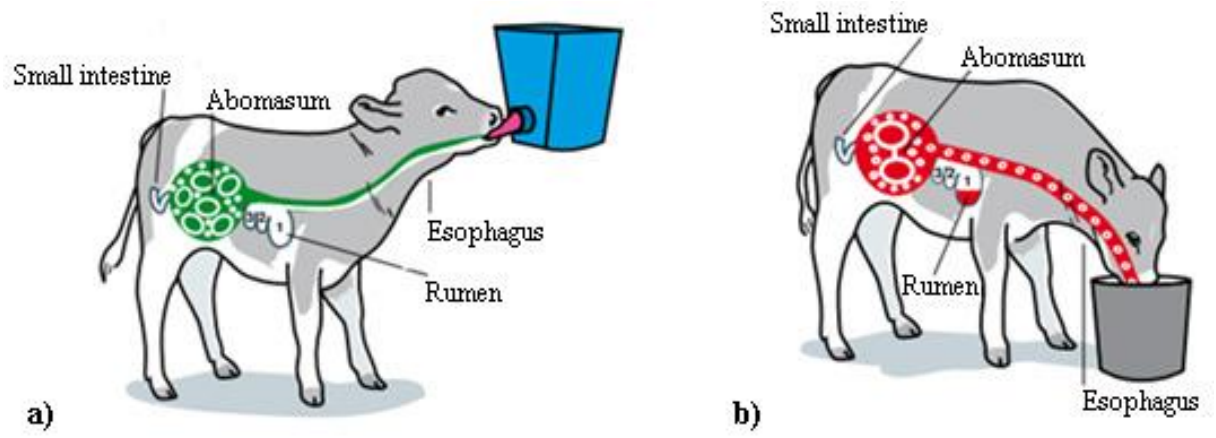

Figure 2. Illustration of two methods of supplying colostrum (DeLaval, 2014)**;** a) Colostrum feeding nipple bottle method and b) Colostrum feeding bucket method.

Figure 2 shows simple methods of supplying colostrum. When calves are drinking or sucking colostrum, it will bypass the rumen and flow directly into the abomasum (it is the only one of the calf's four stomachs that is functionally developed at birth) through the esophageal groove (the channel between esophagus and abomasum) (DeLaval, 2014). Studies describe that providing quality colostrum and the volume that meets the needs of the animal, the result with nipple bottle and tube feeding methods will be the same (Godden et al., 2009; Chigerwe et al., 2012; Laestander, 2016). In contrast to this assertion, Kaske et al. (2005) state that the esophageal tube is better, but in this study the volume of colostrum was different (nipple bottle = 2 liters, esophageal tube = 4 liters).

4.2 Pasteurization

Unpasteurized colostrum is a potential mean of exposure to microbial pathogens for the newborn calf, where contamination may occur during milking, storage or feeding of the newborn (McGuirk and Collins, 2004). These pathogens can also be spilled directly from an infected gland (Stewart et al. 2005). One way to control the transfer of these pathogens through colostrum is the heat treatment (Godden et al., 2003; Johnson et al., 2007). Pasteurization is a method of exposing liquids at elevated temperatures and thereafter the decrease in temperature over a period of time as a means of reducing bacterial contamination of the product (Godden et al., 2003). This method was developed by Louis Pasteur in 1864, when he developed this method to reduce the transmission of diseases to people by contaminated wine (Quigley, 2003).

Table 2. Least squares means for bacterial load of bovine colostrum after heat treatment at 3 different temperatures for 0, 30, 60, or 90 minutes[1] (Elizondo-Salazar et al., 2010)

		Bacterial count[2] (log_{10} cfu/mL)						
Temperature (ºC)	**Time** (minutes)	**SPC**	**ES**	**SAG**	**CNS**	**SA**	**CC**	**NC**
Ambient	0	4.60^{a}	4.23^{a}	1.33^{a}	4.31^{a}	4.31^{a}	4.32^{a}	4.58^{a}
	30	3.85^{b}	3.86^{bc}	-0.52^{b}	3.85^{b}	1.15^{b}	3.87^{b}	3.85^{b}
57	60	4.02^{b}	4.01^{b}	0.00^{b}	3.84^{b}	0.48^{b}	3.84^{b}	3.53^{bc}
	90	3.58^{b}	3.53^{bc}	0.00^{b}	3.53^{c}	0.00^{b}	1.46^{c}	0.00^{c}
	30	3.63^{bcd}	3.85^{bc}	0.00^{b}	3.54^{c}	0.00^{b}	0.48^{c}	0.00^{c}
60	60	3.55^{bc}	3.63^{bc}	0.00^{b}	1.60^{c}	0.00^{b}	0.00^{c}	0.00^{c}
	90	2.63^{de}	3.53^{bc}	0.00^{b}	0.90^{c}	0.00^{b}	0.00^{c}	0.00^{c}
	30	2.83^{cde}	3.53^{bc}	0.00^{b}	1.46^{c}	0.00^{b}	0.00^{c}	0.00^{c}
63	60	2.31^{e}	3.52^{bc}	0.00^{b}	0.00^{c}	0.00^{b}	0.00^{c}	0.00^{c}
	90	0.30^{e}	0.70^{c}	0.00^{b}	0.00^{c}	0.00^{b}	0.00^{c}	0.00^{c}

Legends:[a–e] Means with different superscripts within a column indicate a significant difference ($P < 0.05$);[1] n = 3/sample point;[2] SPC = standard plate count; ES = *environmental streptococci*; SAG = *Streptococcus agalactiae*; CNS = Coagulase - Negative *staphylococci*; SA = *Staphylococcus aureus*; CC = coliform count; NC = noncoliformcount.

There are various uses of temperatures and times, but the most common in the use are high temperature and longtime pasteurization, which consists in heating the milk to 63°C for 30 minutes and thereafter the milk is rapidly cooled (Quigley, 2003). Exposing colostrum to a

60°C heat treatment for 60 minutes does not adversely affect IgG and TP concentrations but decreases or eliminates of pathogens such as *Mycoplasma bovis*, *Listeria monocytogenes*, *E. coli*, *Salmonella enteritidis* and *Mycobacterium avium subsp. paratuberculosis* (Godden et al., 2006; Johnson et al., 2007; Donahue et al., 2012). Elizondo-Salazar et al. (2010) showed that the temperature of 60°C for 60 minutes (or 30 minutes) is the ideal thermal treatment of colostrum, without significantly altering the concentration of Ig. These researchers also analyzed the concentration of bacteria present in colostrum from different thermal treatments (Table 3). The concentration of bacteria declined over time and increase in temperature compared to bacterial counts in untreated colostrum. A decline ($P < 0.05$) in SPC was observed at the shortest time and temperature combination (57°C for 30 minutes). Treatment at 57°C for 90 minutes or 60°C for 30 or 60 minutes resulted in almost 1 $\log_{10}$ reduction in SPC, and treatment at 60°C for 90 minutes or 63°C for 60 minutes resulted in approximately 2 reductions $\log_{10}$ in SPC. Fresh colostrum supplied to calves should contain concentrations less than 100,000 CFU/mL of total bacteria and 10,000 CFU/mL of total coliforms (McGuirk and Collins, 2004).

In an investigation carried out by Johnson et al. (2007) the concentration of IgG and TP in the serum of calves fed different treatments were evaluated. The result of this research was that newborns fed pasteurized colostrum had a greater success in passive transfer (IgG = 22.3 mg/mL; TP = 6.3 mg/dL) when compared to natural colostrum's (IgG = 18.1 mg/mL; TP = 5.9 mg/dL).

Table 3. Effect of heat treatment of colostrum and milk on STP levels, mortality and morbidity rates, and cause of illness and death in 21-d-old Holstein calves fed with raw (NP group) and pasteurized (P group) colostrums (Armengol and Fraile, 2016)

	Treatment group		
Parameter	**NP group** (n = 287)	**P group** (n = 300)	***P*-value**
STP[1] (g/dL)	7.27[a] (5.8 - 9.2)	7.34[a] (5.8 - 9.0)	0.12
Mortality (%)	6.5[a]	2.8[b]	<0.001

Legends:[a,b] Values within a row with different superscript letters are significantly different;[1] STP = serum total protein. Reported values reflect mean and range in parentheses.

It is observed that the supply of pasteurized colostrum and milk significantly reduces morbidity and mortality during the first 3 weeks of life due to the concentration of TP that is not significantly altered by the heating of colostrum and also by the reduction of bacteria. In

this work, the unpasteurized group was fed with frozen colostrum (- 20°C) (6-8 liters during the first 12 hours of life) which was preheated to 40°C (4°C) of the bulk tank which was also reheated to 40°C (1.8 liters each 12 hours). The pasteurized group was also fed colostrum and milk, but both were pasteurized before freezing (pasteurization = 60°C for 60 minutes).

4.3 Storage

Storing high quality colostrum is a good management practice (Heinrichs and Jones, 2003). Colostrum can be preserved frozen without any loss of immune value and is a good strategy to ensure that good quality colostrum is always available (Wattiaux, 1996). Yet it should be collected and stored properly, otherwise dangerous microbial contamination may occur in acquired colostrum (La Belle Colostrum, 2012). Heinrichs and Jones (2003) made several recommendations:

- Not to let the colostrum rest at room temperature; even half an hour at room temperature during the summer may allow bacterial populations to fold, taking the same care after the frozen colostrum is thawed;
- Refrigeration (at 1.5°C) can preserve colostrum quality for only about 24 hours before bacterial growth reaches unacceptable levels;
- For colostrum long-lasting storage, freezing is the best alternative. Colostrum can be frozen (at -20 °C) for up to one year without antibody decomposition;
- Freezers without freezing are not ideal for long-term storage of colostrum as they undergo freeze-thaw cycles that can allow the colostrum to melt;
- Repeated freezing and thawing cycles markedly reduce colostrum storage life.

Therefore, management strategies to prevent bacterial proliferation in stocked colostrum may include freezing, refrigeration, and the use of preservatives such as potassium sorbate in fresh frozen colostrum (Bittar et al., 2006).

5. Filter

The majority of clinical blood tests are performed in serum or plasma to prevent hemoglobin and red blood cells from interfering in biochemical detection, where isolation is commonly achieved by centrifugal methods (Wu et al., 2011). In human medicine, in order to maximize yield or reduce processing time, a filter has been developed which features a

transverse flow microfilter device to isolate plasma from the total blood (Crowley et al., 2005) (Figure 3). The microfilters fractionate the blood in a controlled manner with cellular lysis (Crowley et al., 2005). Another filter model was developed by Wang et al. (2012) (figure 3).

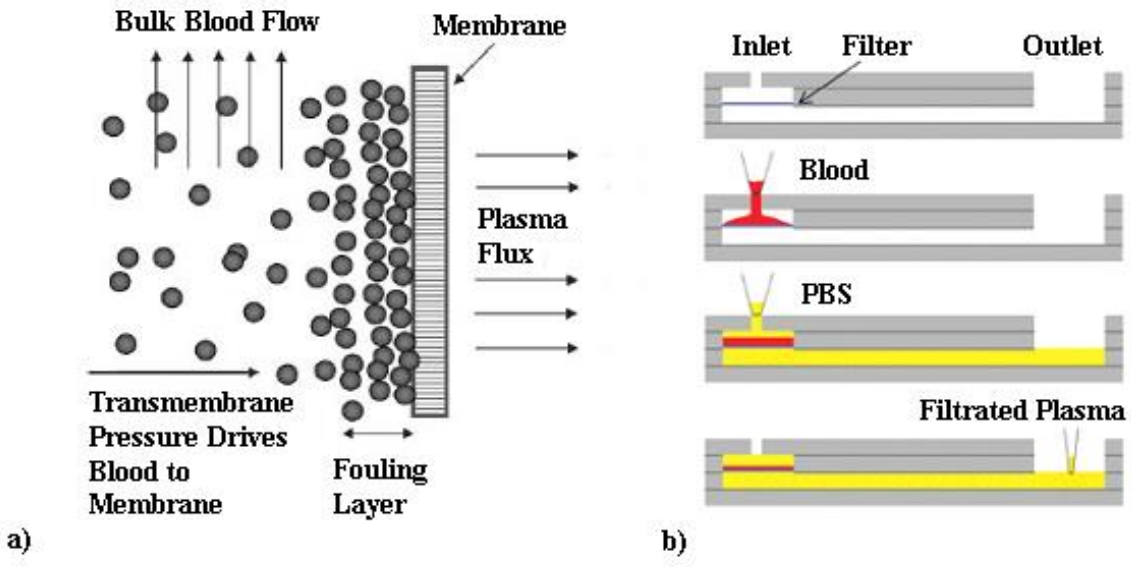

Figure 3. Systems developed in human medicine for blood filtration; a) published by Crowley et al. (2005); b) published by Wang et al. (2012).

Both teams concluded that their filter models are efficient at separating plasma, not interfering with cells, making the apparatus very efficient. In addition, they emphasize the practicality of the apparatuses by being portable and with a rapid obtaining of the sample. The filter developed for veterinary medicine (Figure 4), in which it is being tested, performs the same function as the filters developed to human medicine.

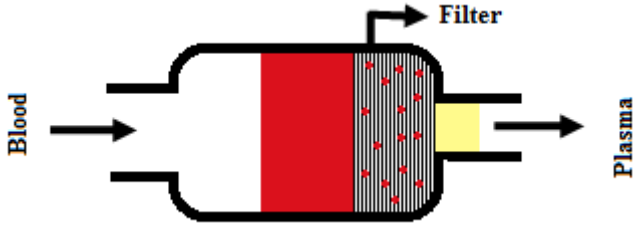

Figure 4. Filter device developed for veterinary medicine.

II – EXPERIMENTAL WORK

1. Material and Methods

The experimental procedures reported here in were conducted with the approval of the Institutional Animal Care and Use Committee of the Freie Universität Berlin.

1.1. Samples

Holstein calves from 4 commercial dairy herds in the Northeast Germany were sampled between August and December 2017.Whole blood was collected from 227 calves (127 female and 100 male) 1 to 7 days 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, Belliver Industrial) and the other contained Lithium-Heparin (9 mL, Greiner Bio-One GmbH). Samples were stored on ice for transportation to the Freie Universität Berlin.

Within 4 to 6 hours of collection, serum and heparinized plasma was separated by centrifugation at 1,500 × g for 6 min at ~ 20°C or by filtration. Aliquots of serum and plasma were collected for further analysis using a standard optical refractometer (Handheld refractometer, Euromex Holland, Arnhem, Netherlands). One aliquot of each serum sample was shipped on ice to the Department of Veterinary Sciences, Chair of Animal Welfare, Ethology, Animal Hygiene and Animal Husbandry (Faculty of Veterinary Medicine, LMU Munich, Germany) for IgG testing.

1.2. Animals

The first phase of the study involved a questionnaire about general management practices on farms. During the sampling period, 2 dairy herds separated the calf immediately after birth. The protocol for feeding colostrum aimed at providing neonatal calves with 4 liters of colostrum within 2 hours after birth. Tube feeding of colostrum was used on 2 farms. The other 2 farms were using a nipple bottle. One farm used a colostrometer (1459 colostrometer, Albert Kerbl GmbH, Buchbach, Germany) and another farm used a digital refractometer (HI 96801 Digital Refractometer, Hanna Instruments, Woonsocket, USA) to assess colostrum quality, respectively. Threshold to define high quality colostrum using the digital BRIX refractometer was 22% BRIX.

1.3. Samples Analysis

Two blood samples from the jugular vein of each calf inscription were collected in a common vacutainer tube:

- Samples in a vacutainer tube without anticoagulant: The samples were centrifuged and the serum was obtained from the separation with the red blood cells. Then, with the aid of a pipette and a tip, a small serum sample was placed on the prism of the digital and optical refractometer (Figure 5) for reading TP concentration. Two types of digital refractometers were used.

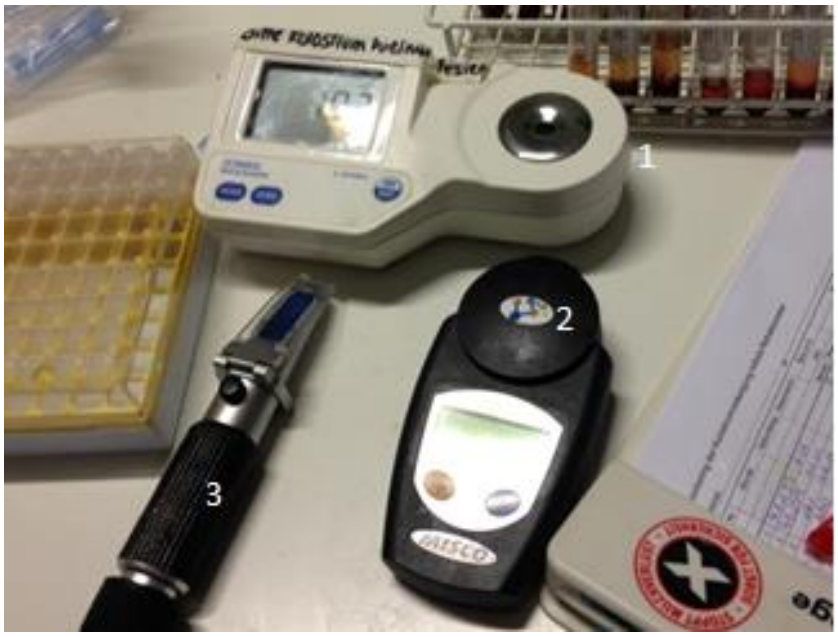

Figure 5. Devices used in the study; 1) Device 1: Digital refractometer Hanna HI 96801; 2) Device 2: Digital refractometer Misco PA201; 3) Device 3: Optical refractometer.

- Samples in a vacutainer tube with heparin: With the aid of a syringe and a needle, a blood sample was withdrawn from the tube and transferred to a filter device. Two plasma drops (< 0.5 mL) were obtained from the filter (Figure 6), where they were placed directly in the prism of the digital (device 2; Figure 5) and optical refractometers for reading TP concentration. Subsequently, the same samples were placed in a centrifuge to obtain the plasma and the same reading process was performed in digital and optical refractometers.

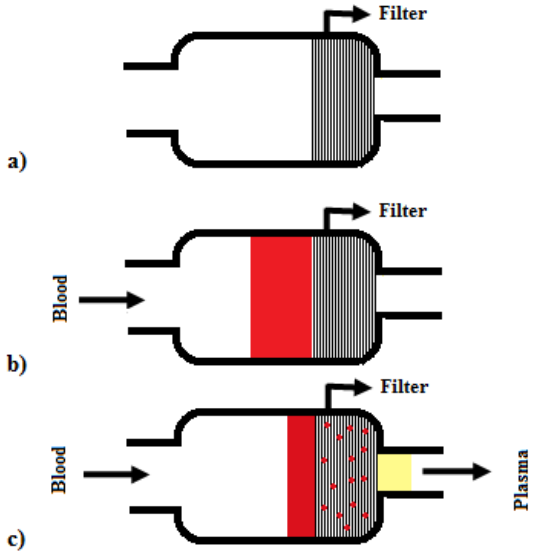

Figure 6. Illustration of the blood filtration process to obtain the plasma; a) with the aid of a syringe, the blood is injected into the apparatus; b) with the pressure that the syringe makes, the sample passes through the filter; c) plasma sample is filtered.

The Figure 6 shows how blood filtration works. Separation of liquids is initiated by inserting 2.0 mL of blood into the filter device. The sample will pass through the filtration system through a small pressure placed by the syringe. The red blood cells are held in the filter and the plasma is obtained. The result of this procedure is two drops of plasma.

Each calf was categorized as having or not FPT according to each threshold determined by the statistical program. The following diagram (Figure 7) summarizes what was done for total protein analysis.

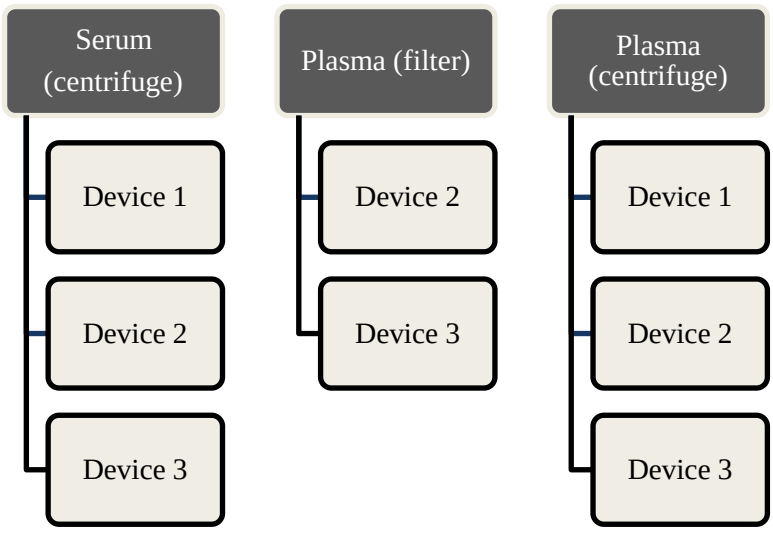

Figure 7. Summary scheme of total protein analysis.

1.4. Statistical Analysis

The TP and Brix refractometer results, in grams per deciliter and percentage points Brix, respectively, from centrifuged plasma or filtered plasma were plotted against centrifuged serum. From these distribution plots, correlation coefficients (r-values) were determined. These comparisons were performed only for the optical refractometer and the digital BRIX device 2. Test characteristics (sensitivity and specificity) were calculated using MedCalc software (version 15.6.1, MedCalc, Mariakerke, Belgium) 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. A receiver operating characteristic curve was created to plot the true positive rate against the false positive rate at 0.1 g/dL and 0.1% Brix intervals, respectively.

2. Results and Discussion

Results of Holstein calves (n = 227) tested for IgG (ELISA), STP, and percentage points Brix, were normally distributed (P > 0.05, respectively). Mean and standard deviation (SD) data are provided (Table 4).

Table 4. Descriptive statistics of serum and plasma samples from two different populations of Holstein calves.

Device	Method	n	Mean	SD	Minimum	Maximum
IgG (mg/mL)		227	18	11.6	1.1	55.5
Device 1[1] (%)	Centrifuged plasma	227	9.6	0.80	7.7	12.4
	Centrifuged serum	227	9.0	0.85	6.9	11.6
Device 2[2] (%)	Filtered plasma	227	9.8	0.86	7.6	12.5
	Centrifuged plasma	227	9.6	0.82	7.7	12.5
	Centrifuged serum	227	9.0	0.87	7.0	11.6
Device 3[3] (g/dL)	Filtered plasma	227	6.5	0.68	4.9	8.8
	Centrifuged plasma	227	6.4	0.66	4.8	8.6
	Centrifuged serum	227	5.9	0.68	4.4	8.0

[1]Digital refractometer Hanna HI 96801; [2]Digital refractometer Misco PA201; [3]Optical refractometer.

Sixty-seven (30%) calves had serum IgG concentrations ≤ 10 mg/mL and 160 (70%) calves had serum IgG concentrations ≥10 mg/mL. 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). Hernandez et al. (2016) found only 1.3% of FPT, but here 12 mg/mL was used as a threshold to define FPT. It was not the objective of our study to determine a representative prevalence of FPT because the dairy farms enrolled constitute a convenience sample. It is difficult to conclude if the participating dairies are representative for all dairies of the given region. In fact, the selection of herds might have been biased by an underlying interest to participate stimulated by a previous history with the condition at hand (i.e. diarrhea in calves).

2.1. Correlation Coefficients and Linear Regression

The correlation coefficients and the coefficients of determination are summarized in Table 5 and Figure 9. The readings from the optical and the 2 digital refractometers were positively correlated with IgG using centrifuged serum, centrifuged plasma, or filtered plasma.

The correlation between TP and IgG determined by ELISA in our study was lower ($r = 0.57$) than previously reported in other studies (Deelen et al., 2014; Elsohaby et al., 2015; Hernandez et al., 2016; Cuttance et al., 2017). All reported studies, however, used RID or TIA for IgG analysis instead of an ELISA test. The correlation between percentage points BRIX and IgG determined by ELISA in our study was lower (device 1: $r = 0.50$; device 2: $r = 0.55$) than previously reported in other studies (Morril 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 of calves and found only weak to moderate correlations (Gelsinger et al., 2015; Dunn et al., 2018). They emphasize that a new threshold should be attributed for ELISA testing to determine FPT in newborn calves. While this might be a limitation of our study when we want to compare with other studies using RID or TIA, it is not limiting our ability to compare different devices and media using the same gold standard to assess FPT.

The primary disadvantage of refractometry to determine FPT in calves is that it requires the use of serum and very few dairy farms own a centrifuge to obtain the samples (Morril et al., 2013). However, Canadian researchers reported that serum collected from blood tubes allowed to clot had a serum TP content (as determined by refractometer) that was highly correlated ($r^2 = 0.95$; $n = 234$) to serum TP content of a duplicate sample that was centrifuged before serum collection (Wallace et al., 2006).

Table 5. Calculated correlation coefficients (r), coefficients of determination (r^2), and results of linear regression between serum IgG determined by ELISA, total protein by optical refractometer and percentage points Brix (% Brix) in Holstein calves

	n	**r**	**r^2**	**Y-intercept**	**Regression coefficient**	***P***
Device 1[1]serum (%) x IgG (mg/mL)	227	0.50	0.25	0.036	8.280	<0.001
Device 1 plasma (%) x IgG (mg/mL)	227	0.43	0.18	0.029	9.056	<0.001
Device 2[2] serum (%) x IgG (mg/mL)	227	0.55	0.30	0.040	8.239	<0.001
Device 2 plasma (%) x IgG (mg/mL)	227	0.49	0.24	0.034	8.983	<0.001
Device 2 filter (%) x IgG (mg/mL)	227	0.46	0.21	0.033	9.194	<0.001
Device 3[3] serum (g/dL) x IgG (mg/mL)	227	0.57	0.33	0.033	5.314	<0.001
Device 3 plasma (g/dL) x IgG (mg/mL)	227	0.51	0.26	0.028	5.873	<0.001
Device 3 filter (g/dL) x IgG (mg/mL)	227	0.47	0.22	0.027	5.998	<0.001

[1]Digital refractometer Hanna HI 96801; [2]Digital refractometer Misco PA201; [3]Optical refractometer.

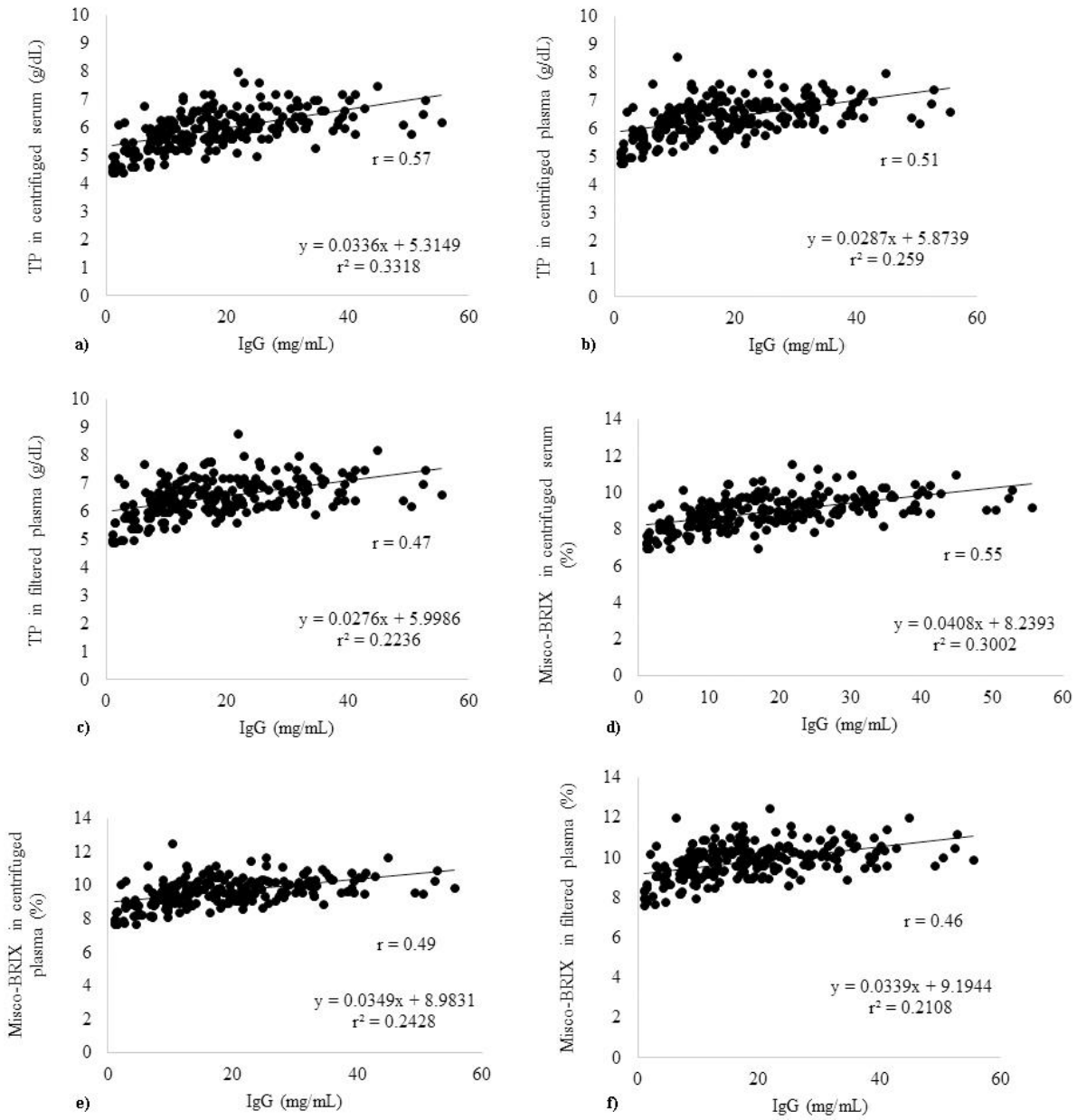

Figure 8. Correlation coefficients and linear regression graphs; a) **S**erum IgG concentration, determined by ELISA, compared with TP of calves in centrifuged serum (n = 227; r = 0.57); b) **S**erum IgG concentration, determined by ELISA, compared with TP of calves in centrifuged plasma (n = 227; r = 0.51); c) **S**erum IgG concentration, determined by ELISA, compared with TP of calves in filtered plasma (n = 227; r = 0.47); d) **S**erum IgG concentration, determined by ELISA, compared with percentage points BRIX of calves in centrifuged serum using the misco BRIX (n = 227; r = 0.55); e) **S**erum IgG concentration, determined by ELISA, compared with percentage points BRIX of calves in centrifuged plasma using the misco BRIX (n = 227; r = 0.49); f) **S**erum IgG concentration, determined by ELISA, compared with percentage points BRIX of calves in filtered plasma using the misco BRIX (n = 227;

r = 0.46); g) **S**erum IgG concentration, determined by ELISA, compared with percentage points BRIX of calves in centrifuged serum using the HANNA-BRIX (n = 227; r = 0.50); **S**erum IgG concentration, determined by ELISA, compared with percentage points BRIX of calves in centrifuged plasma using the HANNA-BRIX (n = 227; r = 0.43).

An alternative to obtain samples for analysis of the passive transfer, in a fast way, is the use of the filter. According to the results of the table 5, serum and plasma filter samples had a moderate correlation; r = 0.64 (device 3 serum); r = 0.62 (device 3 filter), indicating that both can be used.

2.2. Test Characteristics

Test characteristics for the optical and digital refractometers to identify calves with FPT using three different media were determined by ROC curve analyses and summarized in Table 6. In general, optimal thresholds to define calves with FPT using plasma were greater compared with serum irrespective of centrifugation. The 3 different devices had comparable AUC irrespective of the medium used.

Using the AUC as an overall indicator of test characteristics irrespective of the device or the medium used the AUC ranged from 0.80 to 0.85 in our study. This is comparable to other studies using an 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 identification of calves with FPT using TP in centrifuged serum was 5.6 g/dL. 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 a ROC curve analysis to determine the best threshold. Sensitivity (Se) and specificity (Sp) was 70.1% and 80.0%, respectively, using 5.6 g/dL. This is comparable with a recent meta-analysis (Buczinski et al., 2018), where Se and Sp was 88.2% and 77.9%, respectively, using 5.5 g/dL as a threshold in a population of calves below the age of 14 days. There were only studies included in the meta-analysis where calves with FPT were identified either with radial RID or TIA.

The 2 different digital BRIX refractometers we tested in our study were comparable to identify calves with FPT. The optimum threshold in our study for identification of calves with FPT using these 2 devices and centrifuged serum was 8.7% and 8.9% for device 1 and 2,

respectively. This is in agreement with other studies (Elsohaby et al., 2015; Hernandez et al., 2016; Cuttance et al., 2017) using serum IgG concentration as a gold standard to define FPT and a ROC curve analysis to determine the best threshold. Reported test characteristics for the 2 devices are comparable with other recent studies (Elsohaby et al., 2015; Cuttance et al., 2017). These 3 studies 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 = 0.83 device 1; AUC = 0.81 device 2). The reason for this difference remains speculative.

Using the AUC as an overall indicator of test quality, the results of the 3 different devices and media are comparable because the 95% CI for the AUC overlaps. Nevertheless, the different thresholds estimated by the ROC curve analysis show that one needs to use specific thresholds for serum and plasma. In general, threshold for plasma were higher compared with serum. This was somehow expected as fibrinogen represents a part of the protein fraction and this is captured during clotting (Lumeij and Maclean, 1996).

Table 6. Test characteristics for serum total protein (g/dL) and percentage points Brix for identification of calves with failure of passive transfer (FPT; IgG ≤ 10.0 mg/mL) aged from 1 to 7days using 3 different media

Device	Medium	n	Threshold[1]	AUC (95% CI)	*P*-value	Sensitivity	Specificity
Device 1[2]	Centrifuged plasma	227	9.4	0.80 (0.74 – 0.85)	0.001	76.1	73.7
	Centrifuged serum	227	8.9	0.81 (0.75 – 0.86)	0.001	82.1	63.8
Device 2[3]	Filtered plasma	227	9.2	0.80 (0.74 – 0.85)	0.001	58.2	87.5
	Centrifuged plasma	227	9.5	0.83 (0.77 – 0.88)	0.001	80.6	70.6
	Centrifuged serum	227	8.7	0.83 (0.78 – 0.88)	0.001	74.6	76.2
Device 3[4]	Filtered plasma	227	6.0	0.80 (0.74 – 0.85)	0.001	56.7	90.0
	Centrifuged plasma	227	6.3	0.84 (0.79 – 0.89)	0.001	82.1	68.1
	Centrifuged serum	227	5.6	0.85 (0.80 – 0.89)	0.001	70.1	80.0

[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]Digital refractometer Hanna HI 96801; [3]Digital refractometer Misco PA201;[4] Optical refractometer.

3. Conclusion

From the study, it can be concluded that:

- A filter system can be used to facilitate FPT assessment as a point of care analysis in calves without the need to centrifuge serum;
- All the devices showed a correlation with similar IgG, indicating that all tests may indicate FPT. With this, the filter is an alternative since the IgG test is expensive;
- The device 1 was not used because the amount of filtered plasma was not enough for the reading area of the apparatus. That is, perhaps the size of the filter could be larger to obtain more plasma and thus increasing the possibility of analysis;
- The objective of the study was not to evaluate the prevalence of FPT, but a high percentage of FPT was presented, indicating a reassessment of farm management;
- The best test for this study is ROC, since many samples presented false positive.

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