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A comparison of globulins, laboratory and refractometer measured total proteins as indirect tests to diagnosis failure of passive transfer in newborn calves

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LIST OF ABBREVIATIONS

α	Alfa
Alb	Albumin
ATP	Adequate Passive Transfer
β	Beta
ELISA	Enzyme-Linked Immunosorbent Assay
FPT	Failure of Passive Transfer
γ	Gamma
g	Gram
Glob	Globulin
IgA/D/E/M	Immunoglobulin A/D/E/M
IgG	Immunoglobulin G
L	Litre
LOA	Limits of Agreement
mg/ml	milligram/millilitre
PCV	Packed Cell Volume
PFPT	Partial Failure of Passive Transfer
PTP	Plasma Total Protein
r	(Pearson's) Coefficient of Correlation
r^2	Coefficient of Determination
RID	Radial Immunodiffusion Assay
ROC	Receiver Operating Characteristic
SD	Standard Deviation
Se	Sensitivity
Sp	Specificity
STP	Serum Total Protein
STIGA	Split Trehalase IgG Assay
TIA	Turbidimetric Immunoassay
TP	Total Protein

1. INTRODUCTION

Immunoglobulins are produced by plasma cells and make up a percentage of the globulin fraction of proteins in the blood. Other parts of the globulin fraction are enzymes, carrier proteins, and complement, all of which are produced in the liver. Globulins can be separated into four fractions by electrophoresis, α_1-, α_2-, β-, and γ-globulins. Immunoglobulins are mainly in the γ-globulin fraction, with some in the β- and α_2-globulin fractions (Walker, 1990). They are glycoproteins composed of 2 heavy chains and 2 light chains held together by disulfide bonds (Etzel et al., 1997). They are denoted by a capital letter that refers to their heavy chains, IgG, IgA, IgM, IgE, and IgD (Walker, 1990). Immunoglobulin G (IgG) are a major class of proteins called antibodies which provide immunity and protection to the organism (Etzel et al., 1997).

Colostrum is the first mammary secretion produced, it contains immunity compounds such as immunoglobulins, white blood cells, and cytokines (Buczinski and Vandeweerd, 2016) and many nutrients such as vitamins, minerals, fats, proteins, carbohydrates, and growth factors (Stojic et al., 2017). The immunoglobulins are secreted into the colostrum during the last few weeks of pregnancy (Buczinski and Vandeweerd, 2016), and IgG is the most abundant in bovine colostrum, representing over 75% of the total concentration. Consequently, the concentration of IgG is used to access the quality of colostrum. Colostrum quality has been proven to be extremely variable, beef cows tend to produce greater quality colostrum than dairy cows (Gelsinger et al., 2015a; Dunn et al., 2018). The industry standard used to define the quality of colostrum is IgG >50 mg/ml = Good, and IgG <50 mg/ml = Poor. At least 4L of good quality colostrum should be ingested as soon as possible after birth (Gelsinger et al. 2015a; Buczinski and Vandeweerd, 2016; Drikic et al., 2018). Colostrum is an important source of immunity and nutrition for the neonate (Stojic et al., 2017), it is the sole source of initial acquired immunity for calves. After first milking the immunoglobulin and chemical quality of colostrum decreases (Csilla et al. 2016).

When calves are born they are agammaglobulinemic, they rely on passive transfer of IgG through absorption of colostrum to acquire immunoglobulins for immunity, this must be done within the first 24 hours of life (Elsohaby and Keefe, 2015; Cuttance et al., 2017; McCracken et al. 2017). Gut immunoglobulin absorption is time limited in the calf. The

permeability of their enterocytes and their ability to absorb macromolecules is highest at birth, by 6 hours it has reduced by 50%, and decreases rapidly by 12 hours, with mean gut closure time at 24 hours. This is largely due to the replacement of foetal-type enterocytes with adult type enterocytes. Mature cells populations do not allow macromolecule transfer, and their lysozyme activity increases, leading to enzymatic digestion of immunoglobulins. This results in gut closure and cessation of absorption of antibodies into the blood. Calves rely on these maternal antibodies for immunity until the calf is able to synthesize their own (McCracken et al., 2017; Marc et al., 2018). There are many other factors that also effect IgG absorption; the quality and quantity of colostrum fed, the time of the first colostrum feeding, bacterial contamination of colostrum, cow's lactation number, breed, season of calving, cow's dry period length, maternity microclimate, immunity status of the cow (Marc et al., 2018), birthing stress, cold temperatures, and herd management factors (Etzel et al., 1997).

Failure of passive transfer (FPT) occurs when calves have IgG <10 mg/ml circulating in their blood at 24 hours post-birth (McCracken et al., 2017; Stojic et al., 2017; Dunn et al., 2018). Those that have IgG ≥10 mg/ml are considered to have adequate passive transfer (APT) (McCracken et al., 2017). It is advised that calves receive a minimum of between 100-200 g of IgG within 6 hours of birth, this is usually present in 4L of good quality colostrum (Etzel et al., 1997; Buczinski and Vandeweerd, 2016). With FPT calves cannot benefit from immunization of the dam (Hogan et al. 2015). Estimation of the quantity of immunoglobulins absorbed from colostrum by calves is essential for monitoring the effectiveness of colostrum feeding practices on dairy farms (Chigerwe et al., 2015). In Ireland 56% of samples submitted by private veterinary practitioners and from dead calves returned values consistent with FPT (Dunn et al., 2018), and only 34% of samples submitted to the Department of Agriculture had APT (Hogan et al., 2015). In the USA the National Animal Health Monitoring System (2014) report stated that 14% of heifer calves had FPT and only 6.2% of dairy operations routinely measure passive transfer status of calves. This suggests that assessment of FPT is still not widely used on dairy operations (McCracken et al., 2017). In a survey of dairy calves in the USA the prevalence of FPT was between 19-38%. In New Zealand, the prevalence of FPT has been reported as approximately 33% in beef calves, and 45% in dairy calves (Cuttance et al. 2017). From this data it is clear that FPT is still a big problem in most countries.

Several studies have reported calves with FPT to be at greater risk of neonatal morbidity, pre-weaning morbidity and mortality compared to calves which achieved APT at 24 hours of age (Elsohaby and Keefe, 2015; Cuttance et al., 2017; Dunn et al., 2018; Marc et al., 2018). In Ireland mortality was seen to increase up to 10 weeks of age, and 39% of mortalities in dairy calves were attributed to FPT (Hogan et al., 2015). In the USA FPT contributed to up to 50% of mortality in dairy calves and increased the risk of death from diarrhoeal and respiratory diseases in the first 10–12 weeks of life (Chigerwe et al., 2015). Consequences for FPT are not only limited to increased morbidity and mortality, but it also contributes to, reduced weight gain, increased drug use which cause huge economic loss on dairy farms (Marc et al., 2018), reduced growth rates, reduced milk production and increased culling rates in their first lactation (Chigerwe et al., 2015; Cuttance et al., 2017), increased production costs, and reduced profitability (Elsohaby and Keefe, 2015). Monitoring of FTP rates can identify herd management deficiencies and ensure timely detection and medical interventions, this can help reduce the risk of associated diseases and the economic impact for dairy producers (Elsohaby and Keefe, 2015).

As FPT has been shown to be so important, there are a number of tests that are used to detect and diagnose it. Tests that directly measure IgG levels, and indirect tests that measure another variable that correlates with IgG concentrations in the blood. Radial Immunodiffusion assay (RID) (Tyler et al., 1996; Lee et al., 2008; Hogan et al., 2015), Enzyme-Linked Immunosorbent assay (ELISA) (Lee et al., 2008; Hogan et al., 2015), Turbidimetric Immunoassay (TIA) (Etzel et al. 1997; Elsohaby et al., 2015b) and Attenuated Total Reflectance Infrared Spectroscopy (Elsohaby et al., 2015b), are all direct methods of measuring IgG. To indirectly estimate IgG, tests that are used are; optical or digital refractometry, to measure total protein, %Brix or specific gravity in serum or plasma (Hogan et al., 2015; Cuttance et al., 2017), total protein measured by laboratory methods, total globulins (Hogan et al., 2015), sodium sulphite test, zinc sulphate turbidity assay, whole blood glutaraldehyde coagulation, γ-glutamyl transferase (Dunn et al., 2018), and Split Trehalase IgG Assay (STIGA) (Drikic et al., 2018). There are also SNAP-tests available that measure IgG, but only in a qualitative capacity, identifying particular IgG ranges (Stilwell and Carvalho, 2011; Elsohaby and Keefe., 2015c).

2. LITERATURE REVIEW

2.1 DIRECT METHODS TO MEASURE IgG's

2.1.1 Radial Immunodiffusion Assay (RID)

Radial Immunodiffusion assay is considered the gold standard for testing IgG concentration in calf serum, it provides quantitative results for IgG concentrations (Alley et al., 2012; McCracken et al. 2017). It is the reference standard method for determining passive immunoglobulin transfer in calves (Elsohaby et al., 2015a). It was described by Mancini over 50 years ago. Drawbacks for its routine use for testing cattle herds for FPT are its long diffusion time (24-72 hours), it is labour intensive for large sample numbers (Etzel et al., 1997), it is expensive and technically demanding (Hogan et al., 2015), and it is also not amenable to automation (Elsohaby et al., 2015a). However, it is used as the reference method in research settings (Chigerwe et al., 2015). Although it is the gold standard and most used reference method, it has demonstrated inaccuracies due to inconsistencies in internal standards (Elsohaby et al., 2015a; McCracken et al., 2017). A cut-off value of <10 mg/ml IgG has been established to diagnose FPT (Gelsinger et al., 2015a; Hogan et al., 2015; Buczinski and Vandeweerd, 2016; Drikic et al., 2018).

A study was carried out by Ameri and Wilkerson (2008) to investigate the consistency between kits after noticing discrepancies between two RID kits commonly used in their lab. It was found that there was poor agreement between the two test kits. The VET-RID kit (Bethyl Laboratories, Montgomery, TX) showed good approximation to the expected IgG concentrations in the reference serum, an average of 102% recovery. While the SRID kit (VMRD Inc., Pullman, WA) continuously overestimated the concentration, an average of 144% recovery, it was thought that this was due to inaccuracies in the internal standards of the SRID kit.

Lee et al. (2008) also investigated the level of agreement between two different RID kits, VET-RID Kit (Bethyl Laboratories, Montgomery, TX) and Immunocheck (VMRD, Pullman, WA). They tested 115 dairy calves aged 0-10 days old. The agreement between methods was calculated by the Bland-Altman method and Interclass Correlation coefficients. They concluded that calculating the degree of agreement would give a clearer picture of the interchangeability of two methods than a correlation analysis. They calculated the mean differences, the standard deviation of the differences, and the limits of

agreement of the differences, between the IgG measurements produced by the different kits. They looked at 3 different ranges of IgG's: <5 mg/ml (FPT), 5-10 mg/ml (partial FPT), and >10 mg/ml (APT). The values can be seen in Table 1. The agreement between both RID kits at placing the samples within these ranges was 97%. The mean bias all tend towards the negative, this suggests that the Immunocheck VMRD kit measured higher values of IgG's than the VET-RID kit. The SD and hence the limits of agreement increased as the amount of IgG measured increased.

Table 1: Lee et al. (2008) – comparison of RID and RID.

IgG (mg/ml)	Mean Bias (mg/ml)	SD (mg/ml)	LOA (mg/ml)	Range of LOA (mg/ml)
<5	-0.07	1.01	-2.04 – 1.91	3.95
5 – 10	-1.08	1.76	-4.52 – 2.37	6.89
>10	-1.67	2.84	-7.25 – 3.91	11.16

Davis and Giguère (2005) compared two RID kits (Equine RID kit – VMRD Inc, Pullman, WA, and Equine Test Kit – Kent Lab/Triple J Farms, Bellingham, WA) for the amount of IgG's in foals. Serum samples were taken and tested from 65 foals with various medical conditions admitted to the Neonatal Intensive Care unit, and from 35 clinically healthy foals <24 hours after birth. They compared the two kits at 3 different ranges: IgG <4 mg/ml (FPT), IgG 4-8 mg/ml (partial FPT), and IgG >8 mg/ml (APT). The results of which can be seen in Table 2. In the lowest range (<4 mg/ml) agreement was good between kits, there was a small mean bias and a small standard deviation producing narrow limits of agreement. With increasing IgG concentrations, the mean bias did not change significantly however the standard deviation and hence the limits of agreement increased significantly. Although there were increasing limits of agreement as concentrations of IgG's increased, at the set cut-off values there was 100% agreement for identifying FPT and 92% agreement in identifying partial FPT between the two kits. The mean bias at the 3 ranges mainly tended towards the negative, suggesting that the Triple J Farms kit was measuring overall higher values of IgG's than the VMRD kit.

Table 2: Davis and Giguère (2005) – comparison of RID and RID.

IgG (mg/ml)	Mean Bias (mg/ml)	SD (mg/ml)	LOA (mg/ml)	Range of LOA (mg/ml)
<4	0.73	0.91	-1.01 – 2.51	3.52
4 – 8	-1.37	2.39	-6.05 – 3.32	9.37
>8	-0.62	7.48	-15.28 – 14.05	29.33

Both Ameri and Wilkerson (2008) and Lee et al. (2008) compared RID kits from Bethyl Laboratories, Montgomery, TX and VMRD Inc., Pullman, WA. Both reported that the RID kit from VMRD Inc. consistently produced higher IgG values than the RID kit from Bethyl Laboratories. When tested on reference serum the Bethyl Laboratories kit produced the best recovery of 102%. In Davis and Giguère (2005) VMRD Inc., Pullman, WA was compared to Triple J Farms, Bellingham, WA. Triple J Farms was seen to produce higher vales than VMRD Inc, however these were testing for foal IgG's rather than calf IgG's like the other studies.

In Lee et al. (2008) and Davis and Giguère (2005) a similar trend can be seen, as IgG levels increase the discrepancies between the test kits also increase. A 2-fold increase between each range can be seen in Lee et al. (2008) and a 3-fold increase in Davis and Giguère (2005). This means that at lower IgG concentrations the kits have much better agreement than at higher levels of IgG's. It would be better to have a consistent level of difference across all IgG concentration, however for the diagnosis of FPT it is the lower levels of IgG's that we are most concerned with, so it is good that at lower IgG concentrations the test kits do have a good agreement, and an agreement of 90-100% on the diagnosis of FPT.

From this study it is important to note that although RID is the gold standard and is extremely accurate, it is dependent on the quality of the test kit used.

2.1.2 Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-Linked Immunosorbent Assay (ELISA) is a more recent direct measurement of IgG in calf serum (Gelsinger et al. 2015a), it is a commercially available test kit (Hogan et al. 2015). Advantages it has over RID are, it has a much higher throughput, is less expensive and analysis can be completed in a much shorter time (Dunn et al. (2018). As far as I am aware there has been no studies that have used ELISA to establish cut-off values for FPT, the current established value of <10 mg/ml was determined using RID. There have however been a few studies that have compared RID and ELISA for consistency.

Dunn et al. (2018) compared RID (Triple J Farms, Bellingham, WA, USA) with ELISA (Bio-X Diagnostics, Jemelle, Belgium), to access the agreement between the two methods, noting that limited research has be conducted to appraise the IgG concentrations measured by both methods and that standardization needed to be carried out to properly compare the

two methods within the literature. The RID kit was found to consistently produce higher values of IgG's than the ELISA kit, on average it was 1.8 times higher. However, there was a strong correlation between the kits for each of the samples tested $r^2 = 0.97$. A reference serum was used to test the accuracy of each kit. For RID it was applied at 24 mg/ml and it recovered 103.5%, and for ELISA it was diluted to 0.048 mg/ml and it recovered 114.3%. Both kits provided consistent reproducible results over repeated analysis.

Gelsinger et al (2015a) found that there was a weak correlation between plasma IgG levels measured by RID (Triple J Farms, Bellingham, WA.) and ELISA (Bethyl Laboratories, Montgomery, TX.). They tested 104 bulls 48 hours after birth. IgG measurements produced by ELISA were plotted against those measured by RID and $r = 0.59$, this is a moderate correlation. This study did not measure the accuracy of either method, simply the agreement or disagreement between the two. They also looked at the number of times a sample had to be re-tested, this accessed how consistent a kit was. There were very specific standards set for constancy for each sample before it would be accepted as a valid result for inclusion. There had to be agreement with a coefficient of variation <10.5% within a plate and <15% between plates to be accepted. Based on this the ELISA showed much more variation in results than the RID which proved to be much more consistent. One possibility put forward for this was that there were extensive dilution requirements for the plasma due to the narrow range they wanted to maintain. IgG values were consistently higher when measured with RID than ELISA. Their recommendation was that direct comparison of RID and ELISA should not be done. If the cut-off value of <10 mg/ml established with RID is used as the cut-off value for ELISA to diagnosis FPT it could result in the misdiagnosis of some calves.

Gelsinger et al. (2015b) looked at the effects of heat treatment and bacterial population on IgG absorption. As a secondary objective they looked at comparing the IgG's measured with RID (Triple J Farms, Bellingham, WA) and ELISA (Bethyl Laboratories, Montgomery, TX). Onehundred-three bull calves were tested at birth and at 48 hours of age. RID IgG values were consistently higher than ELISA, however both were correlated. On average the RID was 1.63 time higher than ELISA. They also looked at whether the same conclusions for diagnosing FPT would have been reached regardless of which method for measuring IgG's was used. It was found that conclusions would have been

similar with both methods. I have looked at the values based on the unheated colostrum as this is the colostrum most similar to that used in the other studies I am comparing.

Hogan et al. (2015) compared several different FPT tests to RID (Triple J farms Bovine IgG kit). Onehundred-twenty-six serum samples were tested. One of the tests evaluated was ELISA (Bovine IgG Elisa Kit, Alpha Diagnostic International). The cut-off point used for ELISA was <10 mg/ml on the bases that it was also a direct measure of IgG's so the same cut-off value should be used as was established by RID. A ROC curve was used to determine the cut-off value which produced the best Se + Sp combination, this found that <8 mg/ml IgG was a more accurate cut-off value for the ELISA test. At <10 mg/ml Se=97%, Sp=98%, and at <8 mg/ml Se=97% and Sp=100%, so sensitivity stayed the same but specificity improved.

Lee et al. (2008) compared VET-RID Kit, (Bethyl Laboratories, Montgomery, TX) and an ELISA kit, Maxisorp (Nalgene Nunc International, Rochester, NY). The reference serum used was the same for both kits. They calculated the mean differences, the standard deviation of the differences, and the limits of agreement of the differences, between the IgG measurements produced by the different kits. The results of which can be seen in Table 3. The agreement between both kits at placing the samples within the ranges (<5 mg/ml, 5-10 mg/ml, and >10 mg/ml) was 94%. The mean bias were positive, suggests that the ELISA kit measured higher values of IgG's than the VET-RID kit. The standard deviation of the differences and hence the limits of agreement of the differences increased as levels of IgG's increased, however not dramatically until after concentrations were >10 mg/ml, at which point they increase by 3-fold. Se + Sp were marginally better at the cut-off of <5 mg/ml than at <10 mg/ml, however both were good.

Table 3: Lee et al. (2008) – comparison of RID and ELISA.

IgG (mg/ml)	**Mean Bias (mg/ml)**	**SD (mg/ml)**	**LOA (mg/ml)**	**Range of LOA (mg/ml)**	**ELISA Se + Sp**	
<5	0.92	0.71	-0.48 – 2.33	2.81	100%	92%
5 – 10	1.43	1.41	-1.33 – 4.19	5.52	98%	91%
>10	1.48	4.46	-7.26 – 10.21	17.47	-	-

All showed good correlation between RID and ELISA, except Gelsinger et al (2015a & b) which showed moderate correlation. All the studies except Dunn et al. (2018) reported ELISA consistently measuring lower levels of IgG's than RID. Dunn et al. (2018) found RID measured higher levels of IgG than ELISA. In the reference serum RID recovery was 103.5% and ELISA recovered 114.3% suggesting that RID kit was more accurate and

maybe these unexpected results produced by the ELISA kit were the result of this ELISA kit being less accurate than the others on the market. Where sensitivity, specificity and accuracy were accessed for diagnosis of FPT all were above 90%. From a review of these studies, it appears that the inconsistencies between RID and ELISA are not that different to the differences seen in the above studies comparing different RID kit and that they could be used as an alternative for measuring IgG's instead of RID. The only alteration that these studies do suggest is that a lower cut-off point should be used to diagnose FPT. More research needs to be done to confirm a specific cut-off value, but the one suggested by Hogan et al (2015) is <8 mg/ml which was calculated using a ROC curve.

2.1.3 Turbidimetric Immunoassay (TIA)

An automated Turbidimetric Immunoassay (TIA) was developed by Etzel et al. (1997), which directly measured IgG concentrations in approximately 10-minutes. The test was lab based and allowed high throughput as it used biochemical analysers and commercially available reagents (Etzel et al., 1997). It was cheaper than RID, but still relatively expensive (Cuttance et al. 2017). There is now a commercially available TIA that can produce exact IgG concentrations in 10-15 minutes, it is designed to run on a small portable laboratory instrument (which can also be used with other commercially available kits to measure serum calcium, magnesium, and phosphorus), which means it can be used out of a laboratory setting (Alley et al., 2012).

Etzel et al. (1997) developed an automated turbidimetric immunoassay for measurement of bovine IgG. After developing the method, they compared 24 bovine serum samples with both RID and TIA. The IgG measurements produced by TIA were plotted against those produced by RID and r = 0.996 showing nearly perfect correlation between the two methods of analysis.

Alley et al. (2012) looked at serum from 100 Holstein calves between 1-5 days old. They were analysed by RID (as described in Chelack et al., 1993) and a rapid TIA (Immunoassay: bovine serum/plasma IgG; Midland BioProducts Corp., Boone, IA, [individual tubes cost $7] and a portable analyser: MBC QTII; Midland BioProducts Corp.). The range of IgG's, the mean and standard deviation were almost identical, and 83% of all samples had IgG values within 1 mg/ml of each other. The results can be seen in Table 4. When the results of TIA were plotted against RID, r = 0.988 and with linear regression analysis $r^2 = 0.98$, showing nearly perfect correlation and agreement.

Table 4: Alley et al. (2012) – comparison of RID and TIA.

Tests	Range of IgG's (mg/ml)	Mean IgG's (mg/ml)	SD (mg/ml)
RID	4.60 – 36.40	15.15	0.71
TIA	4.02 – 35.86	14.73	0.70

Dawes et al. (2002) tested serum samples from 119 beef and dairy calves less than 2 weeks of age. TIA Immunoassay (Quick test calf IgG kit, Midland Bio-Products, Boone, Iowa) was compared to RID (as described in Fahey et al. 1965). TIA was found to have a Se=93%, Sp=88% for diagnosing FPT.

There are not many studies looking at TIA, but the correlation and consistency shown in the studies that have been carried out suggest it as a very good, quick and accurate alternative to RID.

2.2 INDIRECT METHODS TO MEASURE IgG's

2.2.1 Total Protein

Total protein can be measured in a number of ways. By autoanalyser (Hogan et al., 2015), by the Biuret method (Teixeira et al., 2012; Cuttance et al., 2017), or by refractometer (Elsohaby et al., 2015a; McCracken et al., 2017).

A refractometer can be optical or digital and can measure a number of different quantities in a fluid. Total protein, %Brix, specific gravity, and refractive index can all be measured by refractometer (Hogan et al., 2015), of these, total protein and then %Brix are the most commonly used as indirect tests for FPT (Elsohaby et al. 2015a; Buczinski and Vandeweerd, 2016; Cuttance et al., 2017; McCracken et al., 2017; Stojic et al., 2017). Proteins in fluids refract light (Stojic et al., 2017) and 1% Brix is equivalent to the refractive index of 1 g of sucrose in 100 g of solution (Cuttance et al., 2017). Indirect tests are used because they are cheaper and more practical in the field than the direct measurements of IgG's, they can also be used on blood samples that have or have not been centrifuged. The choice of unit depends on the refractometer used as when measured on the same refractometer there is no difference between TP and %Brix (Cuttance et al., 2017). A drawback of this method that has been suggested by many authors that it is not suitable for use in sick, dehydrated or moribund calves (Alley et al., 2012; Hogan et al., 2015). Another issue that has been raised about this method for diagnosis of FPT is it has been documented to be highly variable and lacking specificity for IgG (Etzel et al., 1997).

2.2.2 Globulins

Globulins can be easily measured in a lab, either by calculation (TP-Albumin), or by electrophoresis. Variation in albumin levels can create variation in TP measurements (Hogan et al., 2015), and Fecteau et al. (2013) have shown that in sick animals, fibrinogen concentration can have a significate effect of TP measurements. Globulin levels used to diagnose FPT has not been widely explored in the literature but measuring globulin levels would help remove the variability caused by other proteins and help obtain a more accurate estimation of IgG's than TP measurements.

2.2.3 Studies

Total protein and globulin measurements are both carried out on blood plasma or serum. Serum is the fluid left after plasma has clotted, this removes fibrinogen and most clotting factors. The main protein fractions left in serum, are albumin and globulins. Albumin is produced in the liver and is the major protein in the blood, making up over 50%. It provides oncotic pressure, and it is a transporter for substances such as hormones, bilirubin, drugs, vitamins and minerals. The globulin fraction is made up of enzymes, carrier proteins, complement, and immunoglobulins. Immunoglobulins are mainly in the γ-globulin fraction, there are a number of different immunoglobulins, IgG, IgA, IgM, IgE, and IgD (Walker, 1990).

Burton et al. (1989) measured IgG, IgA, and IgM levels in calf serum at birth and at 24 hours of age and calculated what percentage each immunoglobulin made up. The results can be seen in Table 5. Even though there was high calf to calf variability, it still shows at this stage in life IgG's are the most significant immunoglobulin in calf serum, and that IgG is the immunoglobulin to significantly increase in the first 24 hours of life.

Table 5: Burton et al. (1989) – Immunoglobulin concentration in calf serum.

Age	IgG	IgA	IgM
Birth	61%	23%	16%
24 hours	87%	5%	8%

Tóthová et al. (2016) and Marc et al. (2018) looked at the change in protein fractions in calf serum from birth through the first few days of life. They both showed that albumin stays relatively constant, and that the main increase in serum total protein was caused by a rise in the γ-globulin fraction, 72% and 80% respectively. They also showed that globulin

levels peak about 24 hours after birth. Tóthová et al (2016) sampled the calves at days 1, 2, 7, 14, and 30, and showed that globulin levels consistently decline by about half from day 2 to day 30. The γ-globulin fraction specifically reduces by about two thirds.

Teixeira et al. (2012) looked at 13 Criollo Lageano and 10 Black and White Holstein calves. Serum was collected between 24 – 36 hours and then 15, 30, 60, 90, 120, 150, and 180 days. Total protein was measured in a lab using the Biuret method, the protein fractions were separated using electrophoresis. Albumin stayed very consistent. Total protein peaked at day 1 and slowly declined to day 60 and then stayed quite constant. globulins and the γ-globulins fraction both peaked on day 1 and decreased sharply by day 15, by about a third and a half, respectively. They then continued to decrease by just less than a further quarter to day 60, before slowly increasing again. This can be seen in Table 6.

Table 6: Teixeira et al. (2012) – % at different days.

Tests	**Day1**	**Day 15**	**Day 60**	**After**
STP	Peaked	13% ↓	further 14% ↓	Stays constant
Globulins	Peaked	30% ↓	further 22% ↓	Slowly ↑
γ-globulins	Peaked	49% ↓	further 23% ↓	Slowly ↑

As the most significant fraction to rise in serum total protein in the first 24 hours of life is the γ-globulin fraction, which are mainly immunoglobulins, it would be expected that there would be good correlation between serum globulins and IgG's. Globulins can be easily calculated by subtracting measured albumin from measured total protein. These three values are usually part of a liver function test profile. As such it is one of the more recent indirect tests being investigated to measure FPT in newborn calves.

Serum total protein has been used as an indirect measurement of IgG's for a long time. As seen in Tóthová et al. (2016) and Marc et al. (2018) the main increase in STP in the first 24 hours of life is caused by a rise in the γ-globulin fraction, which is mainly composed of IgG's (Walker, 1990). Using established cut-off points it can be used to diagnosis FPT.

Elsohaby et al. (2015a) compared an optical refractometer and digital %Brix with RID. They tested 200 Holstein calves from 1 – 11 days of age. Their mean values for IgG's, STP and %Brix were lower than those in the current literature and their range of values was wider. Optical refractometer and %Brix were both positively correlated with RID, with r = 0.74 and r = 0.79, respectively. Optical refractometer and %Brix were positively correlated, with r = 0.73. To evaluate the ability of the two refractometers at diagnosing

FPT several previously recommended standard cut-offs were accessed for both STP=50, 52, 55, 57 mg/ml and %Brix=7.8, 8.0, 8.2, 8.4%. A ROC curve was used to establish the best cut-off values to obtain the best combination of Se + Sp, these results can be seen in Table 7. STP=55 mg/ml produced the best Se + Sp, but 52 mg/ml produced the best accuracy for diagnosing FPT. %Brix of 8.3% produced the best Se + Sp and accuracy. The agreement between the two refractometers was high, at 85%, and the McNemar's test showed no significant difference between proportion of calves classified with or without FPT, concluding that both preformed similarly for diagnosing FPT in newborn calves.

Table 7: Elsohaby et al. (2015a) – STP and %Brix cut-off values.

Tests	**Cut-off**	**Se**	**Sp**	**Accuracy**
STP	55 mg/ml 52 mg/ml	80%, 69.1%	80.7% 89%	80.5% 83.5%
% Brix	8.3 %	85.5%	82.8%	83.5%

Dawes et al. (2002) tested serum from 119 beef and dairy calves less than 2 weeks of age. Optical refractometry was compared to RID (as described in Fahey et al. 1965). Using a cut-off value of 52 mg/ml Se=71%, Sp=83%, and accuracy of 82%.

Lee et al. (2008) compared STP measured by optical refractometer to IgG's measured by RID (Bethyl Laboratories, Montgomery, TX). Using a ROC curve, they calculated cut-off values that produced the best Se + Sp for two different values of IgG's, see Table 8.

Table 8: Lee et al. (2008) – STP cut-off values.

IgG (mg/ml)	**STP Cut-off (mg/ml)**	**Se**	**Sp**
<5 *(FPT)*	≤54	86%	89%
<10 *(PFPT)*	≤58	73%	78%

Harvey (2003) tested 38 Holstein and Brown Swiss calves at birth and at 48 hours of age and compared IgG's measured with RID (Triple J Farms, Bellingham, WA) to optical refractometry measurements of STP, <50 mg/ml was used as the cut-off value. A linear regression curve showed $r^2 = 0.78$. Accuracy=96.8% to diagnose FPT.

Tyler et al. (1996) took serum samples from 242 calves, 1 – 8 days old. IgG's were tested using RID (as described in Besser et al. 1965). STP was measured using an optical refractometer, cut-off points from 40 – 80 mg/ml at 5 mg/ml intervals were accessed for Se and Sp. The highest number of calves correctly classified were found at the cut-off values of 50 mg/ml and 55 mg/ml, the results for these can be seen in Table 9. Using linear

regression analysis gave $r^2 = 0.76$ and a regression equation was developed to calculate an optimum cut-off of 52 mg/ml, which was equivalent to 10 mg/ml IgG.

Table 9: Tyler et al. (1996) – STP cut-off values.

STP cut-off (mg/ml)	Se	Sp	Accuracy
50	59%	96%	83%
55	94%	76%	82%

MacFarlane et al. (2014) investigated agreement between optical refractometer measurements of TP in plasma and serum, as plasma is rarely used. Blood was taken from 61 calves from 1 – 7 days of age. A linear regression and Bland-Altman plot was used to access agreement between the two sets of measurements. The STP range was lower than the PTP range, mean STP=57 mg/ml, SD=8 mg/ml, mean PTP=61 mg/ml, SD=8 mg/ml, but there was very good agreement shown by the regression and Bland-Altman plots, $r^2 = 0.965$. This suggests that either serum or plasma can be used to measure TP by refractometer once the cut-off value is adjusted. Using the regression equation, it was calculated that if a cut-off of 52 mg/ml is used for STP, then a cut-off of 56 mg/ml can be used for PTP.

McCracken et al. (2017) tested 97 serum samples from 1 – 3 day old Jersey calves. The serum was analysed for Total Protein and %Brix, both by digital refractometer and compared to IgG's measured by RID (Triple-J Farm, Bellingham, WA). Results for both were plotted against RID to access correlation. Total protein and %Brix were both moderately correlated with IgG's, $r = 0.79$ and $r = 0.77$, respectively. Total protein was evaluated at cut-off points of 46, 50, 55 mg/ml: 50 mg/ml gave the best Se + Sp, but 46 mg/ml classified the most calves correctly as having FPT or APT. %Brix was evaluated at cut-off points of 7.1, 7.3, 7.6, 7.8%: 7.8% gave the best Se + Sp, for all others Se was in the 90% but Sp was low 22-66%. However, 7.6% classified the most calves correctly as having FPT or APT. These can be seen in Table 10.

Table 10: McCracken et al. (2017) – STP and %Brix cut-off values.

Tests	Cut-off	Se	Sp	Accuracy
STP	50 mg/ml	86.4%	77.8%	85.6%
	46 mg/ml	95.5%	55.6%	91.8%
% Brix	7.8 %	86.4%	96%	84.5%
	7.6 %	94.3%	66.7%	91.8%

Deelen et al. (2014) sampled 397 3 – 6 day old Holstein dairy calves from 3 different herds. STP and %Brix were measured with a digital refractometer on serum, they were

then compared with RID (as described in Chelack et al., 1993). The results were plotted against RID to produce correlation coefficients for STP-IgG, r = 0.93, %Brix-IgG, r = 0.93, and % Brix-STP, r = 1.0. Several previous suggested cut-off points were accessed for STP=52, 55, 57 mg/ml and %Brix=7.8, 8.0, 8.3%: 55mg/ml had the best combination of Se + Sp (Se=76.3%, Sp=94.4) for STP and a ROC curve was produced for %Brix to produce a cut-off value that provide the best combination of Se + Sp: %Brix=8.4% (Se=88.9%, Sp=88.9%). This study shows the same correlation between STP and %Brix, this suggests that if %Brix is used to estimate FPT instead of STP, then the same refractometer could be used on farm to screen for FPT in calves and access colostrum quality.

Fecteau et al. (2013) tested 78 critically ill Holstein bull calves between 1 – 13 days of age. They wanted to access what effect age, clinical covariates and laboratory covariates might have on the cut-off values of indirect tests for FPT, one of the tests they accessed was STP measured with a refractometer. RID (Immunoglobulins Bovine IgG Kit; ICN Biomedicals, Costa Mesa, California, USA) was used to evaluated IgG's. 86% of the calves had FPT, this is very high, however as they were all clinically ill calves this supports the argument for the importance of APT, in the prevention on illness. Hydration status was seen to have a significant and similar effect on both STP and PTP. They found that clinical dehydration was related to a decrease in serum IgG's, it was suggested this could be because of poor suckling. However, unexpectedly PCV, which is usually an indicator of hydration status, showed to be a poor predictor in a simple linear regression model and was not included in a predictor model for adjusting cut-off values for indirect measurements. It was found that fibrinogen concentration was a significant predictor of IgG concentration in the models when serum and plasma protein was measured by refractometer. It was suggested that they are probably a good indicator for the presence of non-immunoglobulin proteins. Including it in the model increased the r^2 by 7% in serum and 16% in plasma. There were 10 different models produced, comprised of different indirect tests and different covariates, for these the increase in r^2 ranged from 34 – 62%. This shows that although about half the changes in IgG concentration could be explained, half of the changes were still unknown.

Cuttance et al. (2017) tested 469 serum from Jersey, Jersey-Friesian cross and Ayrshire cross dairy calves between 1 – 7 days of age. IgG's were measured using TIA (Midland Bio, Boone, Iowa, USA) and serum total protein using the Biuret method. Samples (n=138) were tested with a digital %Brix refractometer. They looked at what effect the age of the calf had. IgG's peaked on day 1 and then concentration of IgG's decreased as the

age of the calf increased. The results of the indirect tests were plotted against TIA to access correlation, and good correlation was found for both STP and %Brix. A ROC curve was produced to establish the cut-off points that produced the best combination of Se + Sp, these can be seen in Table 11.

Table 11: Cuttance et al. (2017) – STP and %Brix compared to TIA.

Tests	Cut-off	r	Se	Sp	Accuracy	LOA (mg/ml)
STP	52 mg/ml	0.97	92%	97%	95%	±6.1
%Brix	8.8 %	0.92	78%	98%	92%	±10.7

Hogan et al. (2015) tested 126 Friesian calves, serum samples were taken just after birth and at 48 hours of age. STP and albumin were measured using an autoanalyser (Rx Daytona), globulins can then be calculated, STP-Alb=Glob. It is cheap, only several cents per sample, quick, taking only about 15 min., and 1 – 40 samples can be tested at a time. They were compared with RID (Triple J farms Bovine IgG kit) to measure IgG's. Their ability to diagnosis FPT was then analysed at standard cut-off values, and a ROC curve was produced to establish cut-off values that would give the best Se + Sp, and cut-offs that will produce Se=100% or Sp=100%, these can be seen in Table 12.

Table 12: Hogan et al. (2015) – STP and Globulin cut-off values.

Tests		mg/ml	Se	Sp	Accuracy
STP	Standard cut-off	52	100%	57%	82%
	ROC	45	97%	74%	87%
	For 100% Se	48.9	100%	69.8%	-
	For 100% Sp	25.4	21.9%	100%	-
Globulins	Standard cut-off	20	100%	85%	94%
	ROC	16	99%	89%	94%
	For 100% Se	19.11	100%	84.9%	-
	For 100% Sp	12.65	86.3%	100%	-

Based on correlation and regression analysis optical refractometers had the least correlation, followed by digital refractometers and then lab-based tests, however all showed good correlation, see Table 13. Any of the studies that compared refractometer with %Brix got very similar results from both. Deelen et al. (2014) directly compared TP with %Brix on a large sample of calves using the same digital refractometer to measure both and obtained a very high correlation coefficients and found perfect correlation between the TP and %Brix. This suggests that if an on-farm test is to be used it may be more economical to use a %Brix to access FPT, as then the same instrument can also be used to access colostrum quality. The main drawback of using STP or %Brix from the data

above is deciding on an appropriate cut-off value for diagnosing FPT. The suggested cut-off values range from 45 – 58 mg/ml, and from analysis of the studies above there is no one value that seems to dominate, however 50, 52, and 55 mg/ml, seem to be the most common, see Table 13. The same situation appears with %Brix. A possible solution to this for a farm that wanted to use a STP or %Brix as a screening method for FPT would be to carry out a RID analysis on a sample of their herd and create a ROC curve to establish the best cut-off value to use. Another interesting observation is that at the cut-off value that produces the best combination of Se + Sp, it does not always produce the best accuracy for successfully classifying FPT or APT in calves, however they are usually quite close, see Table 13. In the studies here that accessed for both, increased accuracy always increased Sp at the cost of decreasing Se. When deciding on an appropriate cut-off value to use it is probably wise to decide what is the cost of misdiagnosis in the herd, you are accessing. In dairy herds or show cattle where the financial cost of a sick, weak, or dead calf will be a lot more, then it is more important not to miss calves with FPT. In this case you would rather over treat than under treat and so it is better to pick a cut-off value that achieves a higher sensitivity at the cost of a lower specificity. If over treating is not economical based on the value of the calf, in beef herds for example, then picking a cut-off value that favours specificity at the cost of sensitivity is the better option. Beef cattle are also less prone to FPT (Dunn et al., 2018).

Table 13: Studies summery (^{op}=optical refractometer, ^{d}=digital refractometer, ^{l}=Lab)

Study	TP cut-off (mg/ml)	r	r^2	Se	Sp	Accuracy
Elsohaby et al. (2015a)	52^{op}	0.74	-	69.1%	89%	83.5%
	55^{op}		-	80%	80.7%	80.5%
Dawes et al. (2002)	52^{op}	-	-	71%	83%	82%
Lee et al. (2008)	58^{op}	-	-	73%	78%	-
Harvey (2003)	50^{op}	-	0.78	-	-	96.8%
Tyler et al. (1996)	50^{op}	-	0.76	59%	96%	83%
	55^{op}	-		94%	76%	82%
McCracken et al. (2017)	46^{d}	0.79	-	95.5%	55.6%	91.8%
	50^{d}		-	86.4%	77.8%	85.6%
Deelen et al. (2014)	55^{d}	0.93	-	76.3%	94.4%	-
Cuttance et al. (2017)	52^{l}	0.97	-	92%	97%	95%
Hogan et al. (2015)	45^{l}	-	-	97%	74%	87%

3. AIMS

The first aim of this experiment was to compare globulin concentrations, total proteins measured in a laboratory, and total proteins measured with refractometer, to immunoglobulin G (IgG) concentrations measured by Enzyme-Linked Immunosorbent Assay (ELISA).

The second aim of this experiment was to determine the true prevalence of failure of passive transfer in the calves sampled.

The third aim of this experiment was to access the ability of globulin concentrations, total proteins measured in a laboratory, and total proteins measured with refractometer to be indirect tests for diagnosis of failure of passive transfer in newborn calves.

4. MATERIALS & METHODS

4.1 Calf Feeding and Sampling

Fifty-seven samples were collected from Holstein-Friesian dairy calves in a Hungarian dairy farm (CLAESSENS Általános Mezőgazdasági Kft, Somogyszob) Calves received frozen and thawed high quality colostrum. Colostrum quality was checked with a %Brix refractometer. Four litres were fed at 38 – 40ºC, via gastric tube within 12 hours of life. After 12 hours, pooled colostrum was provided from a bucket in the calf farm, and they were removed from the dam immediately after birth.

Whole blood samples were taken by venipuncture using a 20-gauge, 1-inch hypodermic needle from each calf, into a S-Monovette 4 ml serum vacutainer tube (Sarstedt, Budapest) at >24 h of age. The blood samples were centrifuged within 1 h after sampling, and serum samples were frozen until measurements. All serum samples were transported to the Laboratory of Food Animal Medicine Clinic at the University of Veterinary Medicine Budapest for analysis.

4.2 ELISA Analysis

The serum samples were tested for IgG concentration using an ELISA test (Bovine IgG ELISA Quantitation Set Cat. no. E10-118 Lot no. E10-118-33, Bethyl Laboratories, Inc., USA.). The samples were carried out according to the manufacturer's instructions.

4.3 Laboratory Total Protein, Albumin and Globulin Analysis

Testing for total protein and albumin was carried out using an autoanalyzer (Beckman Coulter AU Clinical Chemistry Analyzer) using reagent (Beckman Coulter Total Protein OSR6132). Globulin concentration was determined by calculation, TP – Albumin = Globulins.

4.4 Refractometer Analysis

Total protein was measured using an optical refractometer. Approximately one drop of serum was placed on the refractometer prism and the sample cover lowered, the

refractometer was then held up to the light and the total protein was read from the scale and recorded. The refractometer was calibrated with distilled water between each sample.

4.5 Statistical Analysis

Statistical analysis was carried out using Microsoft Excel + Analyse-it®. All the units from each test were converted to mg/ml for ease of comparison. Descriptive statistics for the results of each of the tests were calculated: frequency distribution, data range, mean, and standard distribution. The results from globulins, TP-Lab, and TP-Refractometer were all plotted against the IgG measurements from the ELISA test. From these plots Pearson's correlation coefficients (r) were calculated. Linear regression plots were also made for each, and the coefficient of determination (r^2) was calculated along with the equation of the line. Epidemiology diagnostic test characteristics: sensitivity (Se), specificity (Sp), and accuracy were calculated to evaluate the ability of globulins, TP-Lab, and TP-Refractometer as diagnostic tests for FPT. A positive test result was considered the identification of FPT and a negative test result was considered the identification of APT. The previously recommended cut-off value for FPT is IgG <10 mg/ml (McCracken et al., 2017; Stojic et al., 2017; Dunn et al., 2018), this however was established using RID. In this study ELISA is used to provide the IgG concentration in the samples. After a review of the literature, it is suggested that a lower value of IgG be used as a cut-off values for the diagnosis of FPT when using ELSIA as the reference method, as ELISA has consistently shown to produce higher IgG readings when compared to RID (Gelsinger et al, 2015a; Hogan et al., 2015; Lee et al., 2008). Hogan et al. (2015) suggested a cut-off value of IgG <8 mg/ml based on the results of a ROC curve. Based on this both IgG <10 mg/ml and IgG <8 mg/ml were evaluated. The equation of the line established during the linear regression plot was used to calculate the corresponding globulin, TP-Lab, and TP-Refractometer values for IgG values of 8 mg/ml and 10mg/ml. These calculated values are theoretically the cut-off values for these indirect tests that would represent 8 mg/ml and 10 mg/ml of IgG. The Se, Sp, and accuracy of the globulins, TP-Lab, and TP-Refractometer tests were evaluated at a number of previously recommended cut-off values (Tyler et al., 1996; Dawes et al., 2002; Deelen et al., 2014; Elsohaby et al., 2015a; Hogan et al., 2015; Cuttance et al., 2017), and at the calculated values established using the equations of the regression lines.

5. RESULTS

5.1 Descriptive Analysis

The frequency distribution of the ELISA IgG, the globulins, the Tp-Lab, and the TP-Refractometer all had approximately normal distribution with a slight skewness to the left, i.e. the tails were a little longer to the left with the curve leaning to the right. The frequency distribution of albumin was also approximately normal distribution, but slightly skewed to the right (Figure 1a-e). The range, mean and standard deviation for ELISA IgG, globulins, TP-Lab, TP-Refractometer, and albumin can be seen in Table 14.

Table 14: Range, mean and standard deviation of the tests.

Tests	Range (mg/ml)	Mean (mg/ml)	SD (mg/ml)
IgG	11 – 37 (26)	24.4	4.7
Globulins	26.1 – 48 (21.9)	36.7	4.9
TP Lab	46.9 – 73.3 (26.4)	59.4	5.6
TP Refractometer	48 – 68 (20)	57.9	4.6
Albumin	19.5 – 27.3 (7.8)	22.7	1.8

Zero of the 57 samples tested by ELISA had IgG concentrations less than the cut-off value of 10 mg/ml (and hence 8 mg/ml), that indicates FPT. Therefore all 57 calves tested had APT, and zero calves had FPT, giving a true FPT prevalence of 0%.

5.2 Correlation Coefficients, Coefficients of Determination and Equations

Scatter plots were produced for each of the indirect tests being assessed against ELISA IgG values (Figure 2a-c). The correlation coefficient (r) was calculated for each to show the strength of the association between the two variables, and a regression line with a coefficient of determination (r^2) and equation of the line was produced to access the best fit line to the data. These can be seen in Table 15.

Table 15: Indirect test, r, r^2, and equation of regression lines.

IgG – Indirect tests	r	r^2	Equation of Regression Line
Globulins	0.91	0.78	$\hat{y} = 0.9166 x + 14.26$
TP Lab	0.87	0.68	$\hat{y} = 0.9696 x + 35.69$
TP Refractometer	0.78	0.51	$\hat{y} = 0.7031 x + 40.75$

All 3 tests show good positive correlation, meaning as IgG concentrations increased so did the indirect test measurements at a similar rate. Globulins showed the best correlation, followed by TP-Lab, then TP-Refractometer which showed good to moderate correlation. The best fit regression lines showed a good correlation for globulins and moderate correlation for TP-Lab and TP-Refractometer.

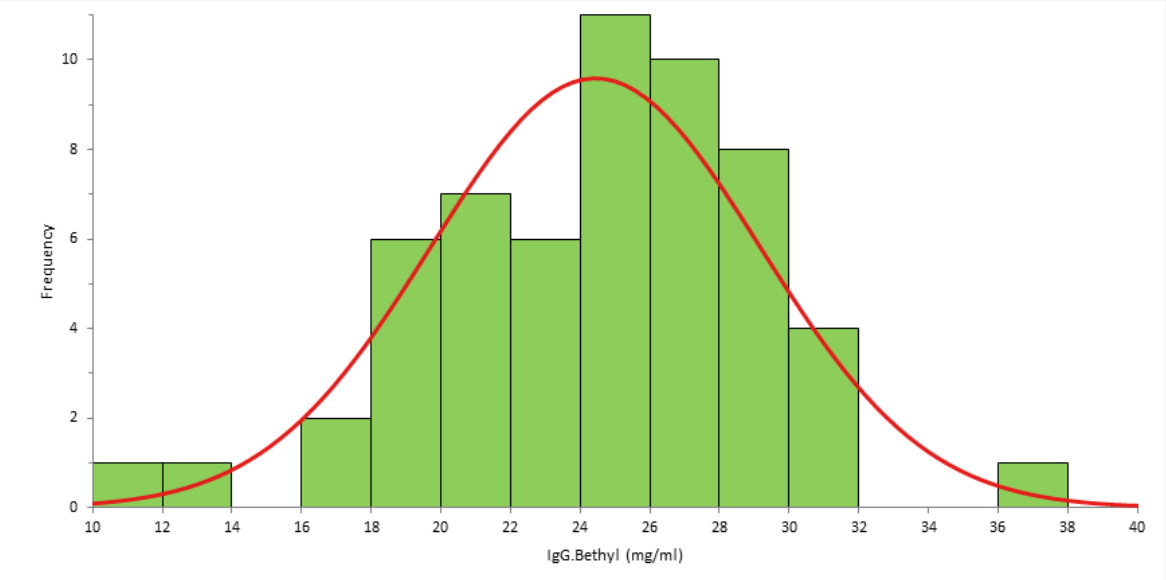

Figure 1a: ELISA

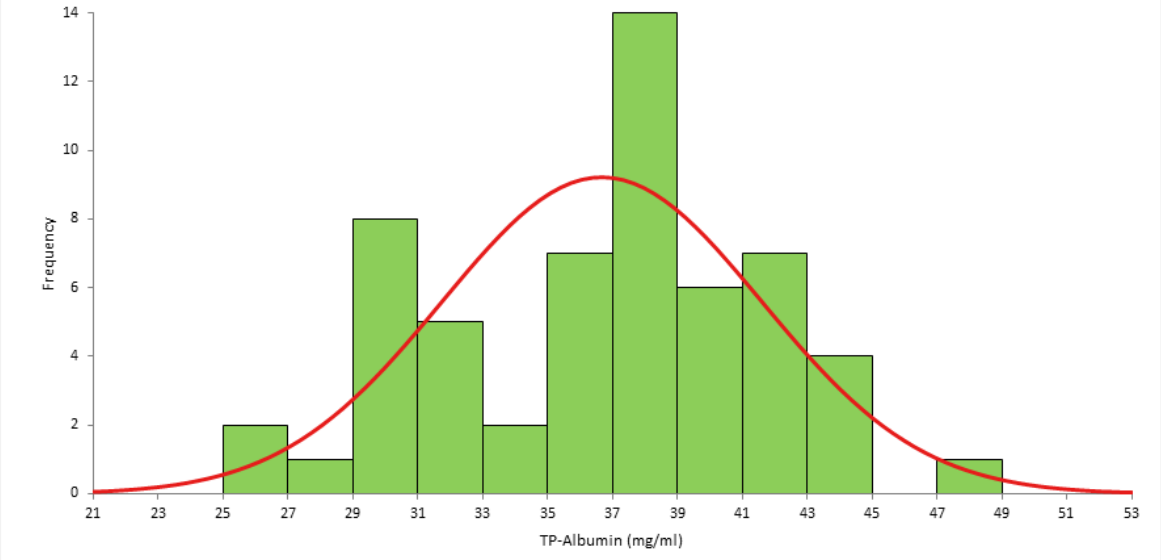

Figure 1b: Globulins

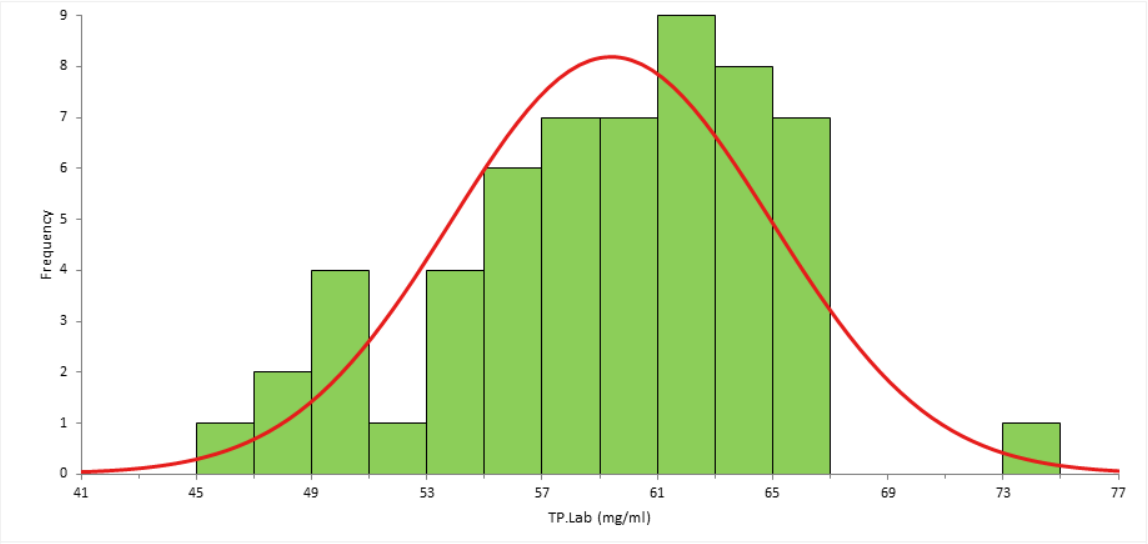

Figure 1c: TP-Lab

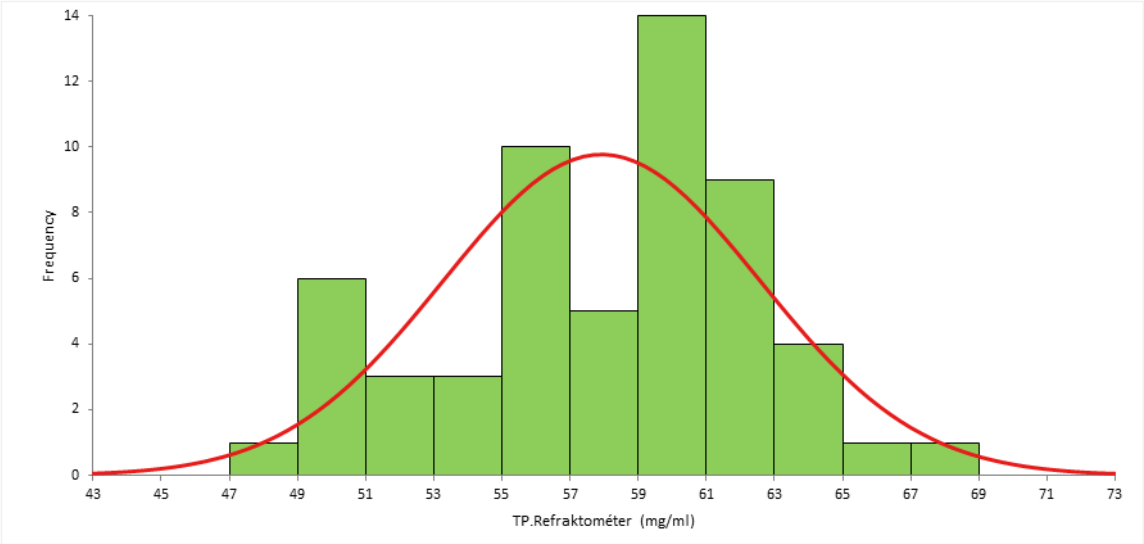

Figure 1d: TP-Refractometer

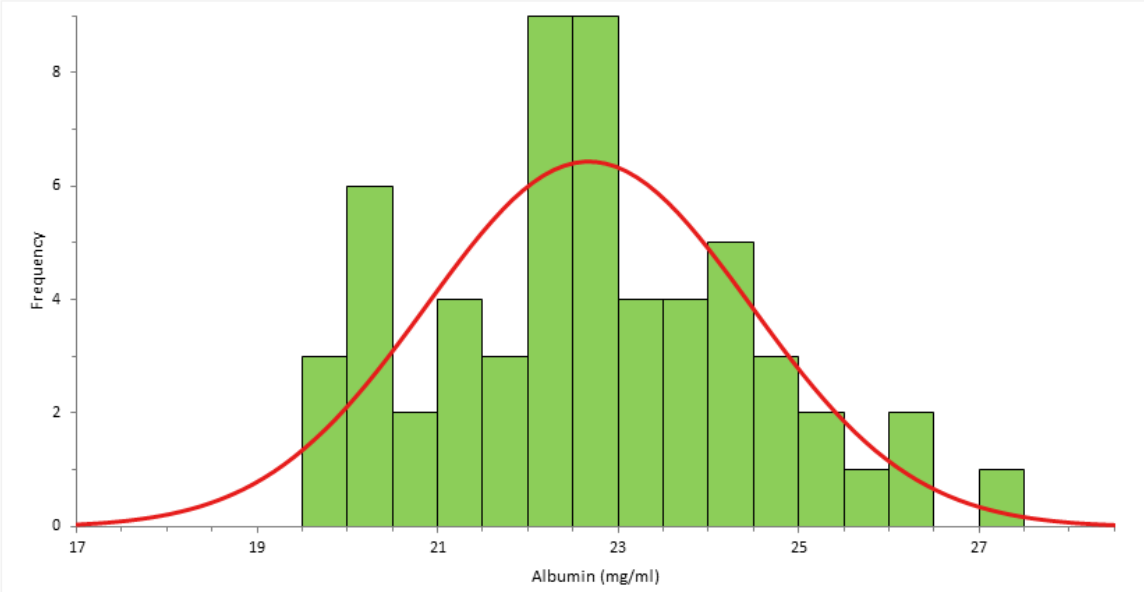

Figure 1e: Albumin

Figure 1a-e: Frequency distribution curves [green], with normal distribution curve [red].

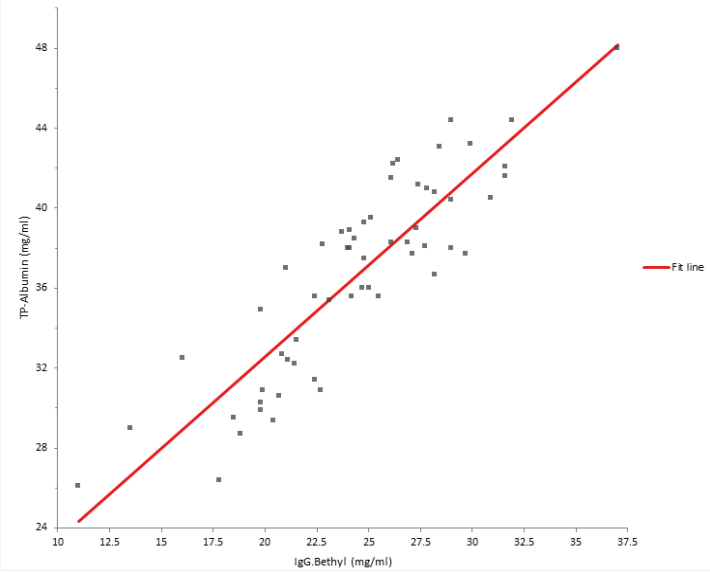

Figure 2a: Globulins – IgG

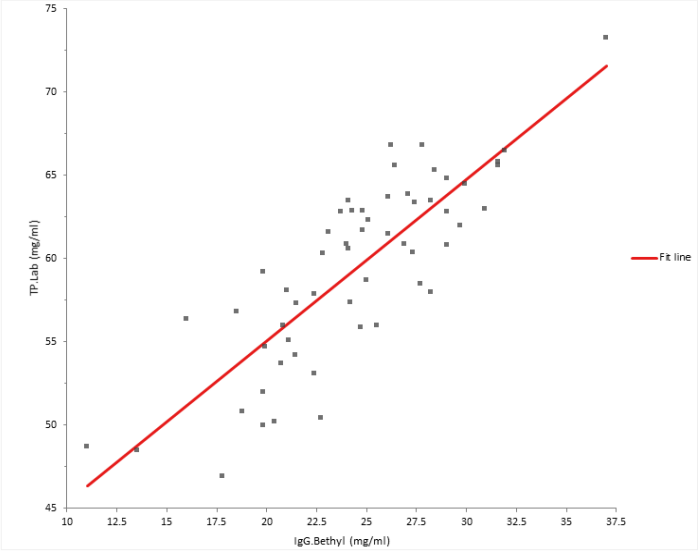

Figure 2b: TP-Lab – IgG

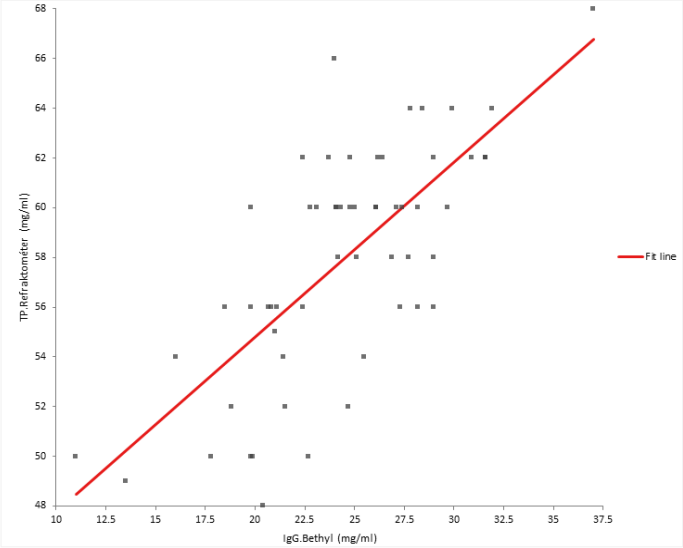

Figure 2c: TP-Refractometer – IgG

Figure 2a-c: Scatter plots with best fit linear regression lines [red].

5.3 Diagnostic Test Characteristics

The test characteristics of the 3 indirect tests for diagnosing FPT were accessed at various cut-off values that have been previously described in the literature, and at the cut-off values calculated by the regression line equations. These are shown in Table 16. Calculating sensitivity was not possible as there were no true positive test results. Although specificity and accuracy could be calculated, without any true positives or false negatives these are not really complete calculations.

Table 16: Epidemiology test characteristics. Calculated cut-off values are in italics.

Tests	Cut-off Values (mg/ml)	FPT #	APT #	Se	Sp	Accuracy
ELISA	<8	0	57	-	-	-
	<10	0	57	-	-	-
Globulins	<16	0	57	-	100%	100%
	<21.5 (8 mg/ml)	*0*	*57*	-	*100%*	*100%*
	<23.5 (10 mg/ml)	*0*	*57*	-	*100%*	*100%*
TP-Lab	*<43.5 (8 mg/ml)*	*0*	*57*	-	*100%*	*100%*
	<45.5 (10 mg/ml)	*0*	*57*	-	*100%*	*100%*
	<50	3	54	-	95%	95%
	<52	6	51	-	89%	89%
	<55	12	45	-	79%	79%
TP Refractometer	*<46.5 (8 mg/ml)*	*0*	*57*	-	*100%*	*100%*
	<48 (10 mg/ml)	*0*	*57*	-	*100%*	*100%*
	<50	2	55	-	96%	96%
	<52	7	50	-	88%	88%
	<55	13	44	-	77%	77%

For the globulins test all cut-off values gave 100% accuracy. For the TP-Lab test of the standard cut-off values, 50 mg/ml gave the best accuracy (95%), both calculated cut-off values gave an accuracy of 100%. For TP-Refractometer test the best standard cut-off value was also <50 mg/ml, and both the calculated cut-off values gave an accuracy of 100%.

6. DISCUSSION

None of the calves sampled in this study had IgG concentrations lower than the cut-off value (8 mg/ml or 10 mg/ml) for FPT, this is a true prevalence of 0% This is inconsistent with the prevalence reported in other studies (Tyler et al., 1996; Hogan et al., 2015; Elsohaby et al., 2015a; Cuttance et al. 2017), which ranged from about 20 – 55%, depending on country and study. Of the literature I reviewed no other study has reported 0% prevalence. This could be because most of the studies published on this topic are accessing different tests, and to get good results and carry out thorough analysis it is necessary to have positive and negative test results. From this point of view, it is unlikely samples with 0% prevalence would be published. Related specifically to the farm tested here however it does suggest very good colostrum feeding and husbandry practices, to obtain such good IgG levels in their calves. From a farm management and calve health point of view this is an excellent result. From a study to access the performance of specific testing methods it is not the ideal situation to have.

The range of values for albumin is narrow (7.8 mg/ml) in comparison to the range of IgG (26 mg/ml), TP-Lab (21.9 mg/ml) and TP-Refractometer (26.4 mg/ml) (Table 14), that means albumin is about 35% of the range of the other three. This shows agreement with other studies that albumin is more consistent than other proteins in the blood but does still show some variation between calves (Tóthová et al., 2016; Marc et al., 2018).

Based on the correlation coefficient globulin measurements performed very good in correlation with IgG concentrations, r = 0.91 (Table 15). As discussed above because at this age γ-globulins are the largest increase in serum total proteins (Burton et al., 1989; Tóthová et al., 2016; Marc et al., 2018), it was expected that this indirect test would perform the best. Hogan et al. (2015) also found very good correlation between globulins and IgG measurements. The coefficient of determination, r^2 = 0.78 (Table 15) showed good correlation, which would suggest that the cut-off values determined with the equation of the regression line would be good. Although, like with any theoretically calculated value it is important to use it as a guide, and with r^2 = 0.78 the calculated values of <21.5 mg/ml (8 mg/ml IgG) and <23.5 mg/ml (10 mg/ml IgG) (Table 16) would definitely be a good starting point for future analysis for determining cut-off values for FPT. They are however

higher than the cut-off value of <16mg/ml determined by Hogan et al. (2015) using a ROC curve.

TP-Lab performed good, and TP-Refractometer preformed moderate in relation to correlation coefficient, r = 0.87 and r = 0.78, respectively. There were not many studies I found with TP measured in the lab, Cuttance et al. (2017) got an excellent correlation with r = 0.97. The correlation coefficient for TP-Refractometer is consistent with that achieved by Elsohaby et al. (2015a) and McCracken et al. (2017), r = 0.74 and r = 0.79, respectively. The coefficient of determination for both were moderate, with TP-Lab doing better than TP-Refractometer, $r^2 = 0.68$ and $r^2 = 0.51$, respectively. Neither of these values would give me great confidence in the calculated cut-off values that would be produced by the equation of their regression lines, especially not the TP-Refractometer.

Conclusions that can be drawn from the diagnostic test characteristics are limited, as unfortunately none of the calves in these samples tested positive for the disease diagnosed, which does limit the completeness of any of the calculations. However, what can be seen is that as the cut-off values increase the number of false positives also increases, reducing the specificity. This suggests that if we are looking to reduce over treating animals, so limiting the number of calves diagnosed with FPT that do not have it, it is best to choose the lower cut-off values. As we do not have sensitivity values it is not clear at what cost increasing the specificity will have on reducing sensitivity, so the amount of calves we will miss, that do require treatment.

In conclusion the globulin tests as an indirect test for FPT shows the most promise out of the indirect tests I accessed. It is easy and cheap and accurate to test with the regular availability of automated machines in many vet practices. The r and r^2 values showed it to have very good correlation with actual IgG concentration, and the results of the albumin further suggest the benefit for having a measurement which removes albumin from the assessment. TP-Lab appears to be a better choice over TP-Refractometer, however if we are measuring TP in an automated machine in a lab then it is just as easy to use globulins, which is the better choice over TP.

7. ABSTRACT

In this study we compared globulin concentrations, total proteins measured in a laboratory, and total proteins measured with optical refractometer, to immunoglobulin G (IgG) concentrations measured by Enzyme-Linked Immunosorbent Assay (ELISA). Linear regression was used to determine their correlation coefficient (r): globulins r = 0.91, TP-Lab r = 0.87, and TP-Refractometer r = 0.78, their coefficient of determination (r^2): globulins r^2 = 0.78, TP-Lab r^2 = 0.68, and TP-Refractometer r^2 = 0.51, and using the equation of the regression line the theoretical corresponding value of each of the above measurements was determined for a IgG concentration of 8 mg/ml and 10 mg/ml: globulins = <21.5 mg/ml and <23.5 mg/ml, respectively, TP-Lab = <43.5 mg/ml and <45.5 mg/ml, respectively, and TP-Refractometer = <46.5 mg/ml and 48 mg/ml, respectively. Globulins had very good correlation with IgG levels and preformed the best in this study, followed by TP-Lab, and then TP-Refractometer.

The prevalence of FPT in this sample of calves was found to be 0%, determined using ELISA to measure IgG's concentrations. This limited the ability to thoroughly analysis the performance of the three indirect tests: globulin concentrations, TP-Lab and TP-Refractometer, to diagnosis FPT. However, what can be seen is that as the cut-off values increase the number of false positives also increases, reducing the specificity.

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Appendix 4. Supervisor counter-signature form

I hereby confirm that I am familiar with the content of the thesis entitled:

A comparison of globulins, laboratory and refractometer measured total proteins as indirect tests to diagnosis failure of passive transfer in newborn calves

written by **Sarah Moore** (student name)

which I deem suitable for submission and defence.

Date: Budapest, March 31, 2011.

Dr. Ottó Szenci

.....

Supervisor name and signature

University of Veterinary Medicine Budapest

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Department of Obstetrics and Food Animal Medicine Clinic

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