Estimate of serum immunoglobulin G concentration using refractometry with or without caprylic acid fractionation

K. M. Morrill,^{*1,2} J. Polo,[†] A. Lago,^{†3} J. Campbell,[†] J. Quigley,^{*†4} and H. Tyler^{*}

^{*}Department of Animal Science, Iowa State University, Ames 50014

[†]APC Inc., Ankeny, IA 50021

ABSTRACT

Objectives of this study were to develop a rapid calf-side test to determine serum IgG concentrations using caprylic acid (CA) fractionation, followed by refractometry of the IgG-rich supernatant and compare the accuracy of this method with results obtained using refractometry using raw serum. Serum samples ($n = 200$) were obtained from 1-d-old calves, frozen (-20°C), and shipped to the laboratory. Samples were allowed to thaw for 1 h at room temperature. Fractionation with CA was conducted by adding 1 mL of serum to a tube containing 45, 60, or 75 μL of CA and 0.5, 1.0, or 1.5 mL of 0.06 *M* acetic acid. The tube contents were mixed well, allowed to react for 1 min, and then centrifuged at $3,300 \times g$ for 0, 10, or 20 min at 25°C . The %Brix and refractive index of the fractionated supernatant were determined using a digital refractometer. Nonfractionated serum was analyzed for %Brix (BRn), refractive index (nDn), and IgG concentration by radial immunodiffusion. The mean serum IgG concentration was 19.0 mg/mL [standard deviation (SD) = 9.7], with a range of 3.5 to 47.0 mg/mL. The mean serum BRn was 8.6 (SD = 0.91), with a range of 6.8 to 11.0. The mean serum nDn was 1.34566 (SD = 0.00140), with a range of 1.34300 to 1.34930. Serum nDn was positively correlated with IgG concentration (correlation coefficient = 0.86; $n = 185$). Fractionated samples treated with 1 mL 0.6 *M* acetic acid and 60 μL of CA and not centrifuged before analysis resulted in a strong relationship between the refractive index of the fractionated supernatant and IgG (correlation coefficient = 0.80; $n = 45$). Regression was used to determine cut points indicative of 10, 12, and 14 mg of IgG/mL to determine the sensitivity and specificity of refractometry to identify failure of passive transfer (serum IgG <10 mg/mL at 24 h old). The nDn

were 1.34414, 1.34448, and 1.34480 to predict 10, 12, and 14 mg of IgG/mL of serum, respectively. The BRn cut points were 7.6, 7.8, and 8.0, respectively. The nDn cut points of 1.34448 and 1.34480 resulted in similar specificities (82.9%), whereas the 1.34414 cut point had a specificity of 60.0%. The BRn cut point of 7.6 and 7.8%Brix resulted in a similar percentage of correctly classified samples (89.7 and 90.8%, respectively); however, the 7.8% Brix cut point resulted in fewer false positives. These results suggest that Brix refractometry of nonfractionated calf serum provides a strong estimate of IgG concentration and 7.8% Brix may be used as the cut point to identify failure of passive transfer in 1-d-old calves.

Key words: passive transfer, immunoglobulin, refractometer, calf

INTRODUCTION

Newborn calves are born agammaglobulinemic, without circulating IgG or IgM. The newborn calf derives passive immunity by absorbing IgG from maternal colostrum (MC) provided within the first hours of life (Smith et al., 1964). In the calf, passively acquired immunity is important to the health of the calf until it is able to synthesize its own antibodies (Smith, 1948). Rapid postnatal growth of the intestine results in the replacement of fetal-type enterocytes by adult-type enterocytes (Smith, 1985), contributing to gut closure or cessation of macromolecule absorption from gut to blood. Neonates that obtain transfer of antibodies postpartum have short closure times, with cessation of macromolecule transport increasing after 12 h, with a mean closure time of approximately 24 h after birth (Stott et al., 1979a; Bush and Staley, 1980).

Passive transfer of IgG can be determined from blood collected from the calf after 24 h of age. Neonatal calves are classified as having failure of passive transfer (FPT) if the serum IgG concentration is <10 mg/mL after 24 h of age (Godden, 2008). Failure of passive transfer increases risk of mortality (Tyler et al., 1999) and morbidity due to increased susceptibility to pathogens and subsequent disease (Boyd, 1972; McGuire et al., 1976).

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¹Corresponding author: kmm434@cornell.edu

²Current address: Cornell Cooperative Extension, Ithaca, NY 14853.

³Current address: Dairy Experts, Tulare, CA 93274.

⁴Current address: Provimi North America, Brookville, OH 45309.

The most recent National Animal Health Monitoring System (NAHMS) report stated that 19.2% of heifer calves had FPT and only 2.1% of US dairy operations routinely measure passive transfer status of calves (NAHMS, 2007). Data from Ontario (Trotz-Williams et al., 2008) reported FPT rates of 8.4% ($n = 933$) on a calf-level study and 37.1% ($n = 407$) on a herd-level study. Although these results vary, one thing is in common: changes in management practices could potentially reduce occurrences of FPT in calves but, more importantly, producers need tools to rapidly and accurately identify potential calves with FPT.

Radial immunodiffusion (RID) is a direct measurement of IgG concentration and is considered the gold standard to determine IgG concentration in bovine serum. Unfortunately, RID assays require a relatively long incubation time (~ 24 h), which precludes their use for rapid on-farm screening. Additionally, discrepancies between RID kits exist and may be due to inaccuracies in the internal standards (Ameri and Wilkerson, 2008).

Short-chain FA have long been recognized to be powerful plasma protein precipitants (Chanutin and Churnish, 1960). Caprylic acid has been used to precipitate non-IgG proteins from a solution, leaving an IgG-rich supernatant (Bernard et al., 1996). This technique has been adapted to determine the IgG content of colostrum (Morrill et al., 2012), mammary secretions from nonlactating dairy cattle (Guidry and O'Brien, 1996), and for the purification of serum for therapeutic uses in humans (Perosa et al., 1990; Rojas et al., 1994; Parkkinen et al., 2006; Bergmann-Leitner et al., 2008) and horses (Mpandi et al., 2007). In the neonatal calf, IgG constitutes a large proportion of the protein in serum, suggesting that fractionation with caprylic acid and analysis of supernatants by refractometer could improve estimates of serum IgG concentrations in calves less than 24 h old compared with measuring total protein (TP).

Protein solutions refract light, and refractometers use this property to estimate TP in a solution (Chavatte et al., 1998; George, 2001). Previous studies have reported serum values as TP or grams per deciliter (Calloway et al., 2002; Wallace et al., 2006; Trotz-Williams et al., 2008). The TP value (g/dL) is calculated from the refractive index (nD); however, depending on the company producing the refractometer, the conversion factor used to calculate TP can be different, thus resulting in different TP values between refractometers (George, 2001).

Refractometers, digital and optical, can be used to evaluate colostrum quality (Bielmann et al., 2010; Morrill et al., 2012) or determine adequate passive transfer in calves (Calloway et al., 2002; Moore et al., 2009). Currently, producers need to purchase 2 refractometers.

Serum needs to be evaluated on a TP scale (g/dL) and before data released in the summer of 2012 (Morrill et al., 2012), MC was evaluated on a %Brix scale. Objectives of this study were to evaluate the use of caprylic acid fractionation to improve estimates of serum IgG concentrations measured by refractometry and to demonstrate that if measured on the true refractive index scale, the same refractometer can be used to evaluate both serum and colostrum.

MATERIALS AND METHODS

Serum samples (5 mL) were obtained from 1-d-old Holstein calves on a California calf ranch. Blood was collected from calves on arrival at the ranch by jugular venipuncture into evacuated tubes and allowed to clot. Serum was separated by centrifugation at $3,300 \times g$ for 20 min at 25°C and frozen (-20°C). Frozen samples were packed on ice and shipped to Iowa State University and stored (-20°C) until analyzed. Samples were allowed to thaw at room temperature before analysis.

Caprylic Acid Test Fractionation

One milliliter of serum was added to a tube containing 1 of 3 acid treatments (Table 1), mixed for 10 s, and allowed to react for 60 s. Samples were centrifuged at $3,300 \times g$ for 0, 10, or 20 min at 25°C. A sample of the supernatant was analyzed using the digital refractometer (model 300034; Sper Scientific, Scottsdale, AZ). The nD of nonfractionated serum (nDn), nD of fractionated samples (nDf), and %Brix (sugar content) of both nonfractionated (BRn) and fractionated (BRf) serum samples were recorded. The nD is the refractive index of a solution measured at the wavelength of the sodium D line (589.3 nm) at 20°C. The %Brix value can be obtained from the polynomial fit to the International Commission for Uniform Methods of Sugar Analysis (ICUMSA, 2009) table: $\text{Brix} = \{[(11,758.74 \times \text{nD} - 88,885.21) \times \text{nD} + 270,177.93] \times \text{nD} - 413,145.80\} \times \text{nD} + 318,417.95\} \times \text{nD} - 99,127.45$. The refractometer was calibrated with distilled water at the start of each set of analyses.

RID Analysis

Five microliters of raw serum was added to each well of a bovine IgG RID test plate (Triple-J Farm, Bellingham, WA). Radial immunodiffusion plates were incubated for 24 h and the diameter of the precipitin ring was measured. The diameter of the precipitin ring was compared with a standard curve created by the internal test standards to determine IgG concentration.

Table 1. Caprylic acid and acetic acid concentrations evaluated to determine the relationship between refractive index and IgG determined by radial immunodiffusion assay¹

Item	TRT A	TRT B	TRT C
Serum (mL)	1.0	1.0	1.0
0.6 M acetic acid (mL)	0.5	1.0	1.5
Caprylic acid (μL)	45	60	75

¹TRT = treatment.

Statistical Analysis

The SAS PROC CORR (SAS 9.2; SAS Institute, 2008) was used to determine the relationship between IgG concentration and the BRn, BRf, nDn, or nDf value for each treatment. Significance was declared at $P \leq 0.05$. The SAS PROC MEANS was used to determine the variable means.

Each refractometer measurement (nDn, nDf, BRn, and BRf) was regressed (PROC REG of SAS) on IgG measured by RID and cut points of 10, 12, and 14 mg of IgG/mL of serum calculated. Epidemiological diagnostic test characteristics (sensitivity, specificity, and predictive values) for each cut point were subsequently calculated using PROC FREQ of SAS. Sensitivity was defined as the probability of a test result (nDf, nDn, BRn, or BRf) indicative of FPT for a sample with IgG >10 mg/mL, as determined by means of RID. Specificity was defined as the probability of a test result (nDf, nDn, BRn, or BRf) indicative of adequate passive transfer for a sample with IgG <10 mg/mL, as determined by means of RID. Positive predictive values (PPV) and negative predictive values were calculated using the prevalence of samples with an RID IgG of greater than or less than 10 mg/mL and a refractometer reading above or below the cut point, respectively.

RESULTS AND DISCUSSION

Two hundred serum samples were analyzed; 15 samples had IgG concentrations <3.43 mg/mL (lowest standard of RID kit) and were not included in the analysis. Descriptive statistics of serum sample measurements are in Table 2. Mean IgG concentration was 19.0 mg/mL (SD = 9.7), with a range from 3.5 to 47.0 mg/mL. A total of 150 samples had IgG concentrations

greater than 10 mg/mL indicating adequate passive transfer, whereas 50 samples had IgG concentrations less than 10 mg/mL, indicating FPT. The percentage of calves used in this study with FPT (25%) was greater than the 8.4% reported by Trotz-Williams et al. (2008) in a calf-level study, greater than the 19.2% estimated by Beam et al. (2009) from the NAHMS (2007) report, identical to that reported in Ontario dairy calves (Wallace et al., 2006), and less than the 37.1% reported by Trotz-Williams et al. (2008) in a herd-level study. The mean BRn was 8.6% (SD = 0.9), with a range from 6.8 to 11.0%. The mean nDn was 1.34566 (SD = 0.00140), with a range from 1.34300 to 1.34930.

Correlations

Raw serum nDf was positively correlated with IgG ($r = 0.86$, $P \leq 0.0001$; Table 3). This is similar to the relationship between serum TP (determined by TP refractometry) and IgG ($r = 0.85$; McBeath et al., 1971). Calloway et al. (2002) evaluated 3 refractometers for detection of FPT in calves and reported the relationship between IgG and serum TP to be slightly weaker ($r = 0.77$, 0.77 , and 0.74 , respectively). Similarly, correlation between BRn and serum IgG ($r = 0.87$) in the current study indicates that a refractometer calibrated to measure %Brix may provide similar estimates of IgG compared with a refractometer calibrated to estimate TP. Regressions of IgG and BRn (Figure 1) and that of IgG and nDn (Figure 2) indicate linear relationships throughout the range of the data tested.

Correlation coefficients for nDf and IgG were positive, but had weaker relationships compared with nDn and IgG or BRn and IgG (Table 3). Correlation between nDf and IgG was greatest for samples analyzed with treatment (TRT) A that were centrifuged for 20 min, and TRT B samples with centrifugation times of 0 and 10 min.

The strongest correlation between nDf and IgG was achieved by adding 1 mL of serum to a tube containing 1 mL of 0.06 M acetic acid and 60 μL of caprylic acid, shaking the sample for 10 s, and allowing the sample to incubate for 1 min before analysis of the supernatant (TRT B, 0; Table 3). The strongest correlation between BRf and IgG was also observed with TRT B, but following 10-min centrifugation.

Table 2. Descriptive statistics of whole serum data used in analyses

Item ¹	n	Mean	SD	Minimum	Maximum
IgG (mg/mL)	185	19.0	9.7	3.5	47.0
Brix (%)	185	8.6	0.9	6.8	11.0
nDn	185	1.34566	0.00140	1.34300	1.34930

¹IgG = IgG concentration as determined by radial immunodiffusion; nDn = refractive index of whole serum.

Table 3. Correlation coefficients between serum IgG concentration and %Brix or refractive index (nD)

Item	n	IgG × %Brix		IgG × nD	
		r	P-value	r	P-value
Whole serum	185	0.87	<0.0001	0.86	<0.0001
Caprylic acid test variable ¹					
TRT A, 0	41	0.61	<0.0001	0.61	<0.0001
TRT A, 10	40	0.72	<0.0001	0.72	<0.0001
TRT A, 20	39	0.76	<0.0001	0.77	<0.0001
TRT B, 0	45	0.77	<0.0001	0.80	<0.0001
TRT B, 10	41	0.78	<0.0001	0.77	<0.0001
TRT B, 20	40	0.72	<0.0001	0.52	<0.0001
TRT C, 0	53	0.56	<0.0001	0.59	<0.0001
TRT C, 10	51	0.69	<0.0001	0.69	<0.0001
TRT C, 20	35	0.35	<0.0001	0.75	<0.0001

¹Treatment (TRT) followed by the number of minutes allowed to incubate before centrifugation at $3,300 \times g$ at 25°C, where 0 = no centrifugation, TRT A = 0.5 mL of acetic acid and 45 μ L of caprylic acid, TRT B = 1 mL of acetic acid and 60 μ L of caprylic acid, and TRT C = 1.5 mL of acetic acid and 75 μ L of caprylic acid.

Diagnostic Test Characteristics

Diagnostic test characteristics for determining FPT based on BRn are in Table 4. The best combination of sensitivity and specificity was at 7.8% Brix (equivalent to 12 mg of IgG/mL, using the regression equation $Y = 9.12846 \times \%Brix - 59.2122$), where Y = serum IgG concentration (mg/mL). Diagnostic test characteristics to determine FPT based on nDn cut points are in Table 5. The cut point 1.34448 (equivalent to 12 mg of IgG/mL, based on the regression equation $Y = 5,941.39299 \times nD - 7,976.08747$) provided the best combination of sensitivity and specificity for nonfractionated serum samples. For nonfractionated serum analysis, cut points 7.8 BRn and 1.34448 nDn should be used to identify FPT.

Brix refractometers have been evaluated for estimating IgG in MC (Morrill et al., 2012); however, evaluation of adequate passive transfer is typically conducted

using a refractometer specifically calibrated for TP. The refractometer used in this study was the same refractometer used in the study to determine the relationship between IgG and refractive index of MC (Morrill et al., 2012). Thus, it is possible to use the same refractometer to evaluate both MC and serum when samples are evaluated on a %Brix or nD scale.

Diagnostic test characteristics for samples fractionated with TRT B and not centrifuged are in Table 6. The cut point 1.33895 (equivalent to 10 mg/mL, using the regression equation $Y = 7,449.22185 \times nD - 9,64.10430$) provided the greatest sensitivity and PPV; however the cut point 1.33950 resulted in a greater specificity and PPV. Both cut points resulted in similar percentages of samples being correctly classified.

Many advantages exist of using refractometry of serum to determine IgG over other currently available methods to measure IgG: RID, ELISA, turbidimetric immunoassay, and sodium sulfite or zinc sulfite turbidity tests. Radial immunodiffusion has a long incubation

Figure 1. Serum IgG concentration, determined by radial immunodiffusion (RID), compared with %Brix of whole calf serum ($n = 185$; $r = 0.87$). Color version available in the online PDF.

Figure 2. Serum IgG concentration, determined by radial immunodiffusion (RID), compared with the refractive index (nD) of whole calf serum ($n = 185$; $r = 0.86$). Color version available in the online PDF.

Table 4. Diagnostic test characteristics for the digital refractometer (%Brix of nonfractionated serum; BRn) measuring whole serum samples compared with IgG determined by radial immunodiffusion assay

Cut point	Test characteristic ¹ (%)					Test high/low ²
	Sensitivity	Specificity	PPV	NPV	Correctly classified ³	
7.6	93.30	74.30	94.00	72.20	89.70	149/36
7.8	90.00	94.29	98.54	68.75	90.81	137/48
8.0	84.67	100	100	60.34	87.57	127/58

¹PPV = positive predictive value; NPV = negative predictive value.

²Number of samples declared high (>10 mg of IgG/mL) or low (<10 mg/mL) by Brix refractometry. Analysis by radial immunodiffusion indicated that 150 and 35 samples were high and low, respectively.

³Percentage of samples correctly classified as adequate or failure of passive transfer.

time (18 to 24 h), preventing identification of FPT calves before gut closure. Refractometry takes only the time necessary to obtain a blood sample and separate serum, and less than 15 s for the refractometer to produce a digital reading. Refractometry does not require internal standards that may introduce error in determining the IgG concentration of serum (Ameri and Wilkerson, 2008). Digital refractometers often contain a temperature-compensating device, thus reducing the confounding factor of temperature that can affect the accuracy of other methods to determine IgG concentration in serum (Aba-Adulugba et al., 1989; Calloway et al., 2002). The simplicity of the refractometer to determine the IgG concentration of serum compared with RID, ELISA, zinc sulfate turbidity test, and sodium sulfate precipitation test is a great advantage and will easily allow for on-farm adaptation.

Separation of IgG from other serum proteins with caprylic acid did not improve the overall predictability of refractometry. Absorption of protein from the intestine of neonatal calves before gut closure is thought to be nonspecific (Stott et al., 1979b) and IgG are protected from degradation by the presence of neonatal Fc receptors in the endothelium (Kacskovics et al., 2006). Thus, the change in TP from birth to 24 h of age in neonatal calves is due primarily to the increasing concentration

of IgG absorbed from the gut. Non-IgG proteins are rapidly metabolized or excreted (Pierce, 1961).

It is also possible that conditions selected for evaluation were not optimal for complete separation of IgG from non-IgG serum proteins. Processing conditions (amount of caprylic acid and acetic acid, and times of processing) were based on data from Morrill et al. (2012). However, others used different processing parameters to isolate IgG from serum. For example, Rojas et al. (1994) reported that varying caprylic acid concentrations from 4 to 7% affected the purity of IgG derived from horse plasma. Those authors also reported that stirring and pH of the solution affected the yield and purity of the resulting IgG. Further research appears necessary to potentially optimize conditions for isolating IgG from serum using caprylic acid.

The primary disadvantage of refractometry to determine FPT in calves is that it requires the use of serum. Very few dairy farms own a centrifuge; 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). This suggests that producers could take a blood sample from calves, allow

Table 5. Diagnostic test characteristics for the digital refractometer (refractive index; nD) measuring whole serum samples compared with IgG determined by radial immunodiffusion

Cut point	Test characteristic ¹ (%)					Test high/low ²
	Sensitivity	Specificity	PPV	NPV	Correctly classified ³	
1.34414	96.00	60.00	91.20	77.70	89.20	158/27
1.34448	90.00	82.86	95.70	82.70	88.60	141/44
1.34480	86.70	82.90	95.60	59.20	81.10	136/49

¹PPV = positive predictive value; NPV = negative predictive value.

²Number of samples declared high (≥ 10 mg of IgG/mL) or low (<10 mg/mL) by nD. Analysis by radial immunodiffusion indicated that 150 and 35 samples were high and low, respectively.

³Percentage of samples correctly classified as adequate or failure of passive transfer.

Table 6. Diagnostic test characteristics for the digital refractometer (refractive index; nD) measuring supernatant from the caprylic acid test compared with IgG determined by radial immunodiffusion

Cut point	Test characteristic ¹ (%)					Test high/low ²
	Sensitivity	Specificity	PPV	NPV	Correctly classified ³	
1.33895	97.14	50.00	87.20	83.30	86.70	39/6
1.33920	91.40	50.00	86.50	62.50	82.20	37/8
1.33950	85.71	100	100	66.70	88.90	30/15

¹PPV = positive predictive value, NPV = negative predictive value.

²Number of samples declared high (≥ 10 mg of IgG/mL) or low (< 10 mg/mL) by nD. Analysis by radial immunodiffusion indicated that 35 and 10 samples were high and low, respectively.

³Percentage of samples correctly classified as adequate or failure of passive transfer.

it to clot without centrifugation, and use a transfer pipette to remove a small amount of the serum for analysis by refractometer.

The goal of any calf-monitoring program is to monitor the success of passive transfer on a farm and provide additional support to calves with FPT. If producers have access to simple tools that rapidly and accurately estimate IgG concentration, calves at risk of FPT could be identified and provided additional MC, or preventative care.

CONCLUSIONS

The objectives of this study were to determine if caprylic acid fractionation of serum could improve estimates of IgG measured. Adding 1 mL of serum to a tube containing 1 mL of 0.06 M acetic acid and 60 μ L of caprylic acid, shaking the sample for 10 s, and allowing the sample to incubate for 1 min before analysis of supernatant resulted in a strong correlation between nDf and RID. However, a stronger relationship existed between IgG and nDn of calf serum. A cut point of 7.8% Brix or 1.34448 nD was optimal for predicting FPT in this study. We conclude that the nDn provides a rapid and accurate estimate of serum IgG concentration and the same refractometer can be used to determine IgG concentration of MC and calf serum.

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