

Differences in Total Protein Concentration between Fresh and Frozen Serum and Plasma Samples Used to Assess Failure of Passive Transfer in Dairy Calves

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Abstract

The objectives of this study were to determine differences in total protein (TP) concentration between samples before and after freezing, and to determine the correlation between total protein in serum and plasma to evaluate passive transfer in calves. Blood was collected via jugular vein puncture from a total of 127 calves (34 Holstein and 93 Jersey) once between 1 and 3 days of age. Serum and plasma protein concentrations were measured via digital refractometer in fresh specimens; samples were then frozen for storage. After a variable period of time in storage (≤ 1 to 8 months), frozen samples were thawed and re-analyzed. Passing-Bablok regression showed that total protein concentration in plasma was consistently higher than in serum. Additionally, multivariate regression showed that Jersey calves had higher total protein concentration in serum than Holstein calves (0.429 ± 0.049 g/dL), and that each additional month of storage resulted in lower protein concentration in serum (-0.116 ± 0.017 g/dL). Electrophoresis showed that all protein fraction levels varied after long-term storage of serum, although albumin accounted for most of the variation. It is concluded that plasma samples can be used as a new and improved option to evaluate effectiveness of colostrum management protocols under field conditions because plasma samples are easier to handle in the field and they correlate well with fresh serum total protein concentration and are not significantly affected by frozen storage.

Keywords: Failure of Passive Transfer (FPT); Calves; Colostrum; Total protein (TP); Refractometer; Serum; Plasma; Fresh frozen

Abbreviations: FPT: Failure of Passive Transfer; IgG: Gamma Immunoglobulin; TP_{plasma}: Total Protein Measured in Plasma; TP_{serum}: Total Protein Measured in Serum

Introduction

Due to the unique placental interface between cow and calf, there is minimal transfer of immunoglobulins prior to parturition. The majority of the immunoglobulins are absorbed from maternal colostrum through endothelial cells lining the gut of the calf by active endocytosis and transport of macromolecules such as immunoglobulins [1]. Absorption is usually limited to the first 24 hours following birth [2,3]. These immunoglobulins enable the establishment of a targeted immune system within the calf that can protect it from infection until it is capable of producing endogenous immunoglobulins [4,5]. This is why adequate colostrum feeding and management is an important aspect of disease prevention in the neonatal calf.

The term "failure of passive transfer" (FPT) is used to identify calves that do not achieve a specific concentration of Immunoglobulin G (IgG) in serum, which is why it is also termed hypoglobulinemia. This target concentration varies significantly in the literature. The most commonly cited threshold for considering calves as having FPT is established at 1000 mg/dL [6], while others have set the limit at 800 mg/dL [7], 750 mg/dL [8], or even 350 mg/dL [9]. Whatever the threshold, most studies have shown that calves with hypoglobulinemia are more susceptible to disease than calves with adequate passive transfer under the same conditions. Therefore, evaluation of passive transfer as a monitoring system to evaluate adequate colostrum management on dairy farms is an important part of preventive herd health protocols.

Several methods have been reported to evaluate passive transfer status in neonatal calves including zinc sulfate and sodium sulfite turbidity tests [10,11], glutaraldehyde coagulation tests [12],

measurement of serum gamma-glutamyl transferase (GGT) activity [13,14], and direct measurement of IgG through single radial immune diffusion (SRID), which is considered the gold standard [11,13,15]. Serum total protein (TP_{serum}) concentration has been shown to correlate with serum IgG concentration [10,16]. This correlation allows fairly rapid assessment of passive transfer in the field with a refractometer using TP_{serum} concentration as a proxy for IgG concentration, avoiding the need for processing samples in a laboratory. However, this correlation is not perfect, which leads to great variability in the threshold of TP_{serum} concentration used to establish FPT throughout the literature. TP_{serum} concentrations between 5.0 g/dL and 5.5 g/dL have been reported corresponding to a serum IgG concentration of 1,000 mg/dL [16-18]. Measurement of TP_{serum} has been reported mostly by use of a manual refractometer [11,19], although the use of a digital refractometer has been recently validated [20].

Most studies reporting TP_{serum} concentration as a measurement to evaluate adequate colostrum intake have used fresh serum samples. However, in some situations, fresh serum samples may not be available. Instead, only frozen serum or plasma samples may be available, usually collected for other reasons, but they could be used to assess passive transfer status if it is known how to interpret the results. Therefore,

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the correlation between TP_{serum} concentration in fresh serum samples and frozen serum or plasma samples needs to be established, so that appropriate limits can be used for determining adequate colostrum intake in neonatal calves.

The objectives of this study were (1) to determine if TP concentrations differ in fresh and frozen/thawed samples; and (2) to determine the correlation between serum and plasma samples.

Materials and Methods

Sample population

Heifer calves at five commercial dairy farms were enrolled in the study over a period of 18 months, between December 2008 and June 2010. Animals eligible for inclusion in this study were Holstein and Jersey heifer calves between 1 and 3 days of age that appeared physically healthy, and were not visibly dehydrated.

Experimental design

There were two sampling periods. In 2008 and 2009, only serum samples were obtained as the original question was to measure the difference between fresh and frozen/thawed serum samples. During this time a new question arose as to the correlation between serum and plasma TP concentration. Thus, in 2010 plasma samples were added to the protocol.

Blood samples were obtained from each calf once via jugular vein puncture and collected into a serum-separator tube (BD Vacutainer® SST, BD Diagnostics) to obtain the serum samples and into an EDTA tube (BD Vacutainer® EDTA, BD Diagnostics) to obtain the plasma samples. A unique sampling ID number was assigned to each calf. All samples were processed within four hours of collection. The EDTA and serum separator tubes were centrifuged at 3,000 RPM for 10 minutes to allow removal of plasma and serum, respectively. At this time, fresh sample TP was determined using a digital refractometer (Model 300027, Sper Scientific Ltd.) for both serum and plasma samples [20]. The refractometer was calibrated every 10 samples using distilled water. After measurement of fresh TP concentrations, serum and plasma samples were transferred into 2 mL cryopreservation vials (ML5355, Market lab, Inc.) for storage in a frost-free freezer (-20°C). Frozen/thawed samples collected between December 2008 and August 2009 were analyzed in September 2009, and those collected during 2010 were analyzed in October 2010. Therefore, samples were stored in the freezer for varying lengths of time before being analyzed, and the effect of length of time stored was evaluated. Measurement of TP in frozen/thawed samples was performed in the same manner and with the same equipment as prior to freezing, after allowing samples to warm overnight to room temperature, and being vortexed.

As the study evolved and realizing that a difference in serum samples was being observed, a new question arose as to what proteic fraction was responsible for the difference. To assess this, new samples were collected in a serum-separator tube from 20 heifer calves (10 Holstein and 10 Jersey) to perform electrophoresis. The limited number of samples was due to the high expense of the electrophoresis test and budget limitations. Serum samples of all 20 calves were collected on the same day and processed in the same manner and with the same equipment as described above. Then each serum sample was separated into three aliquots. The first aliquot was submitted immediately (overnight shipping) to the Veterinary Diagnostic Laboratory at Cornell University for electrophoresis testing. The remaining two aliquots were frozen in the same freezer as the other samples and

submitted for electrophoresis testing two weeks and four months after the initial blood draw, respectively, to evaluate changes at different times of frozen storage.

Statistical analyses

Correlation between TP concentration in fresh and frozen/thawed samples, as well as between serum and plasma samples was evaluated using Passing and Bablok regression, with frozen/thawed TP_{serum} and TP_{plasma} being the respective dependent variables at a level of significance of 5%. Multivariate linear regression was also performed to determine if breed (dummy variably, Jersey=yes/no) and length of storage of the samples (random variable, months) influenced TP concentration in frozen/thawed samples.

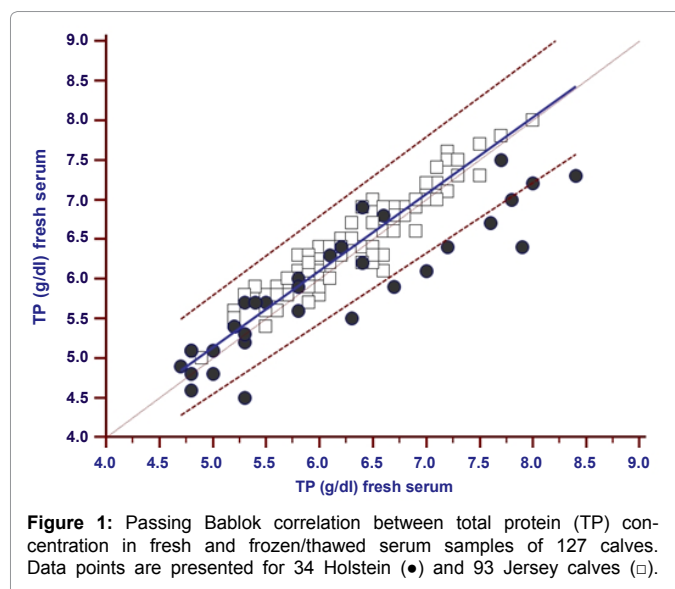
Differences between concentrations of proteic fractions measured by electrophoresis in the tested aliquots (fresh, 2 weeks and 4 months of freezing) were analyzed by paired t-test at a level of significance of 10% due to the small sample size. Specialized statistical software was used for all analyses (MedCalc Software v. 11.2).

Results

A total of 127 heifer calves 1-3 days old were enrolled in the study: 34 Holstein and 93 Jersey.

Fresh vs. Frozen Serum

The simple correlation coefficient between fresh and frozen TP_{serum} was $r = 0.896$ ($P < 0.001$). The Passing Bablok correlation between fresh TP_{serum} values (x) and frozen/thawed TP_{serum} values (y) of the same serum sample was represented by the equation ($y = 0.30 + 0.97x$). The 95% confidence interval (CI) for the intercept was (0.10 to 0.79) and the 95% CI for the slope was (0.889 to 1.000) indicating a systematic difference between fresh and frozen TP_{serum} but no significant deviation from linearity ($P < 0.680$), indicating that the correlation was equal for all values. Additionally, the relationship between fresh and frozen/thawed TP_{serum} was evaluated using a multivariate linear regression. Both breed (Figure 1) and length of time the samples were frozen (Figure 2) influenced the relationship between fresh and frozen/thawed TP_{serum} concentration. Jersey calves had on average 0.429 ± 0.049 g/dL higher TP_{serum} values than Holstein calves ($P < 0.001$). For each



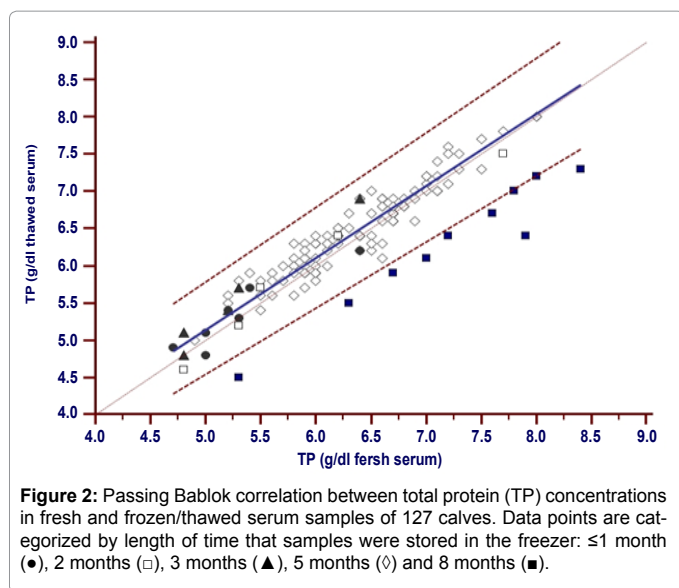


Figure 2: Passing Bablok correlation between total protein (TP) concentrations in fresh and frozen/thawed serum samples of 127 calves. Data points are categorized by length of time that samples were stored in the freezer: ≤ 1 month (●), 2 months (□), 3 months (▲), 5 months (◇) and 8 months (■).

additional month that the samples were stored in the freezer, TP_{serum} concentration in frozen/thawed samples was 0.116 ± 0.017 g/dL lower than the concentration measured in the fresh sample ($P < 0.001$). The resulting multiple correlation coefficient was 0.895.

Electrophoresis performed on a sample of 10 Holstein and 10 Jersey calves to determine whether specific protein fractions differed between fresh and frozen/thawed serum samples showed a larger decrease in albumin concentration as length of storage increased (Table 1). However, there was large variability between calves in all proteic fractions limiting the reliability of the results.

Serum vs. Plasma

The simple correlation coefficient between TP_{serum} and TP_{plasma} was $r = 0.912$ ($P < 0.001$). Plasma values were only available for 58 calves sampled in 2010 (6 Holstein and 93 Jersey). The Passing Bablok regression model obtained for the correlation between fresh TP_{serum} values and fresh TP_{plasma} values of the same calf was explained by the equation ($y = 0.60 + 1.00x$). The 95% CI for the intercept (0.059 to 1.543) was different than zero, indicating that TP_{plasma} concentration was consistently higher than TP_{serum} concentration. The 95% CI for the slope (0.857 to 1.091) did include one, indicating that there was no proportional difference as concentration of TP_{serum} increased (Figure 3). The cusum test for linearity showed no significant deviation from linearity ($P = 0.79$). Breed did not influence this relationship.

Fresh vs. Frozen Plasma

The simple correlation coefficient between fresh and frozen TP_{plasma} was $r = 0.959$ ($P < 0.001$). The Passing Bablok regression model obtained

for the correlation between fresh TP_{plasma} values (x) and frozen TP_{plasma} values (y) was explained by the equation ($y = 0.10 + 1.00x$). The 95% CI for the intercept (0.100 to 0.600) did not include zero, indicating a systematic difference between fresh and frozen/thawed samples. The slope (0.923 to 1.000) included one, indicating that there was no proportional difference between fresh and frozen/thawed plasma samples. However, there was a significant deviation from linearity indicating that the differences were not consistent throughout concentration (Figure 4). Breed had no influence in this relationship, and the length of time the samples were frozen was the same, and thus did not allow evaluating any differences.

Discussion

To our knowledge, this is the first study that compares TP concentrations in samples before and after freezing. Our data showed a lack of a direct linear relationship between fresh and frozen/thawed TP_{serum} concentration in the same samples, meaning the relationship was inconsistent throughout the range of TP concentration, mostly due to the presence of some outliers that had a significantly reduced TP concentration after freezing. Multiple samples, dropped below 4 g/dL after frozen storage, which is the minimum recorded in fresh serum samples from calves that have not received colostrum [2,21]. Additionally, we saw a correlation between how long the sample had been frozen and the degree of variation between TP_{serum} concentrations before and after freezing. Samples frozen for a shorter period of time appeared to have less difference than those samples frozen for longer periods. These results show that freezing can detrimentally affect TP concentrations, especially during longer freezing periods, as other studies have shown in different species [22] or with other blood analytes [23].

To determine which protein fractions were affected during the freezing/thawing process that could explain our results, electrophoresis was performed in another 20 samples. Data showed that all protein fractions changed after freezing, but these changes were not consistent. Some fractions increased, at one point and decreased at another. The only consistent change was a decrease in albumin concentration, which represents the largest portion of TP in serum. Most of the variability was due to changes in the concentration of albumin and IgG. The concentration of the different protein fractions in this study were similar to those found by others [21], showing external validity.

The effect of storage time could not be tested for plasma samples because all plasma samples had the same length of freezing time (5 months), which was similar to the second longest time used for serum samples. Data in this study showed a similar lack of a direct linear relationship between fresh and frozen/thawed TP_{plasma} concentration as with serum samples, but the correlation was higher than for serum samples. In contrast to abnormally low TP_{serum} samples, there were no samples with TP_{plasma} concentrations below minimum recorded in neonates before receiving colostrum (4.0 g/dL) [21]. All results together

	Fresh serum g/dL	Frozen (2 weeks) g/dL	Frozen (4 months) g/dL
Total protein	5.92 ± 0.82^a	5.89 ± 0.84^b	5.77 ± 0.78^c
Albumin	2.45 ± 0.17^a	2.40 ± 0.19^b	2.31 ± 0.19^c
Alpha	1.16 ± 0.12^a	1.19 ± 0.13^b	1.17 ± 0.12^b
Beta 1	0.99 ± 0.16^a	1.01 ± 0.15^b	0.98 ± 0.15^a
Beta 2	0.44 ± 0.15	0.46 ± 0.16	0.46 ± 0.14
Gamma	0.88 ± 0.29^a	0.83 ± 0.29^b	$0.85 \pm 0.26^{a,b}$

^{a, b, c} Values within the same row with different superscripts differ at a level of significance of 10%.

Table 1: Electrophoresis results of serum samples from 10 Holstein and 10 Jersey calves at different times of storage under freezing conditions

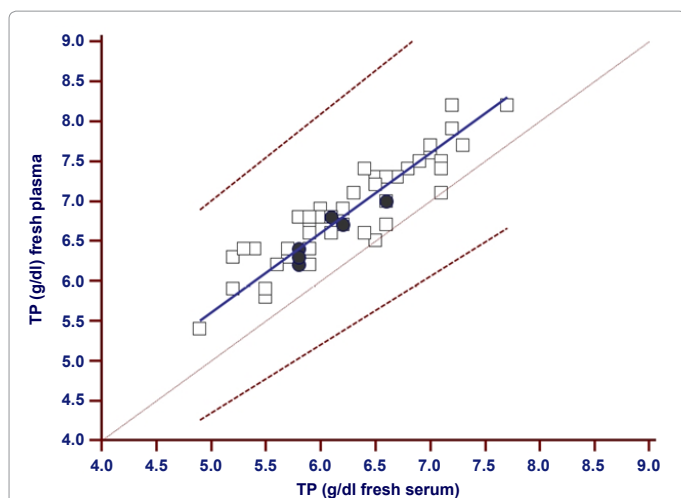


Figure 3: Correlation between total protein (TP) concentrations in fresh serum and plasma samples of 58 calves. Data points are presented for 6 Holstein (●) and 52 Jersey calves (□).

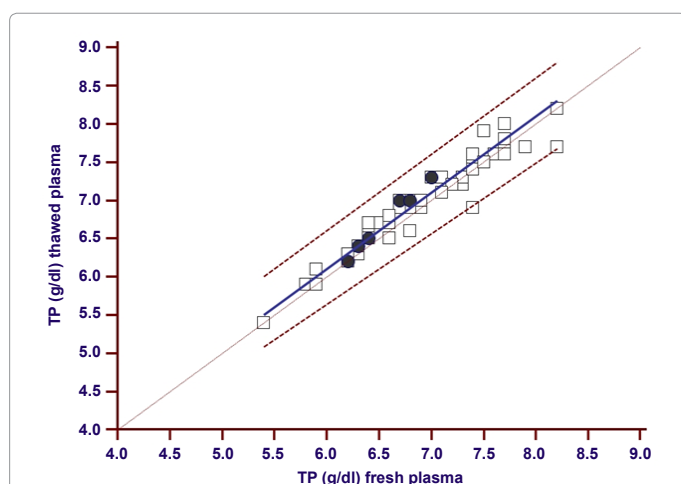


Figure 4 : Passing Bablok correlation between total protein (TP) concentration in fresh and frozen/thawed plasma samples of 58 calves. Data points are presented for 6 Holstein (●) and 52 Jersey calves (□).

may suggest that plasma samples are more stable under freezing conditions than serum samples. This is an important finding that can help in the monitoring of appropriate colostrum management in dairy calves, as plasma samples could be stored for longer periods of time without the apparent risk of altering TP concentrations. Therefore, the next question would be how TP_{plasma} concentrations correlate to TP_{serum} concentrations, given that most studies on passive transfer evaluation in dairy calves have been performed using serum samples.

Our data indicate that TP_{plasma} concentrations were consistently higher than TP_{serum} concentrations from the same calf. This is likely because plasma still contains fibrinogen and other coagulation proteins which are lost when processing serum. The significance of this finding is that when measuring TP_{plasma} to determine the effectiveness of colostrum management, the commonly used cut-off values for determination of FPT should be increased (by ~0.60 g/dL) compared to what would be expected in a serum sample. Further study is needed to show the correlation between TP_{plasma} and IgG concentration. Otherwise, most calves will be categorized as having adequate passive

transfer of immunoglobulins, when in fact they may be marginal or even deficient.

Conclusion

Plasma sample allow for easier monitoring of colostrum management when testing is performed in the field compared to serum samples, because they can be centrifuged immediately as opposed to serum samples, which have to wait for initial clotting before being centrifuged. Additionally, clotting time for serum samples depends on ambient temperature and can be significantly delayed in cold weather, making field management more difficult. The use of plasma will save time when testing TP in the field, although we realize this may not be possible in all situations. The apparent higher stability of plasma samples compared to serum samples during long periods of frozen storage provide a new option for veterinarians and producers, along with commonly used fresh serum samples, to determine the efficacy of colostrum management protocols established on farms.

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