DEVELOPMENT AND EVALUATION OF A MULTIPLEX ASSAY TO MEASURE BOVINE IgG1 AND IgG2 USING MICROSPHERES AND FLOW CYTOMETRY

By

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ABSTRACT

Failure of passive transfer (FPT) is one of the main reasons for increased mortality rate in newborn calves and diagnosis is dependent on determination of serum IgG concentrations (diagnosis is based on < 1 g/dL of total IgG). Several qualitative assays are available, but the reference method, single radial immunodiffusion assay (SRID), albeit quantitative measures only one subclass at a time. We set out to develop a competitive multiplex microsphere flow cytometry assay to measure bovine IgG1 and IgG2 concentrations in 30 serum samples acquired from newborn Holstein calves prior to and 24 hours after ingestion of colostrum and to compare the values with SRID. A triplex bead assay was created by mixing three distinct sets of Quantum plex carboxylated fluorescent microspheres that were coated with purified bovine IgG1, IgG2 or albumin using a two step chemical reaction. The triplex protein coated beads were reacted with a cocktail of sheep anti-bovine IgG1 and IgG2. Evaluation of analytical specificity demonstrated cross reactivity between anti-bovine IgG2 and IgG1 coated beads that precluded determination of IgG2 > 0.5 g/dL. Cross reactivity between anti-IgG1 and IgG2 coated beads was minimal and did not affect IgG1 concentrations between 0.15 to 1.2 g/dL. A competitive linear decrease in the fluorescence intensity was observed in the triplex assay when 2-fold dilutions spanning a concentration range of 12 mg/dL – 100 mg/dL of either purified bovine IgG1 or IgG2 were included as a competitive inhibitor of the reaction. Precolostral serum samples from 29 calves were determined to be < 0.4 g/dL by SRID. Standard calibrants for the flow assay were prepared from two fold serial dilutions of purified bovine IgG (stock concentration 10 g/dL) using a precolostral calf serum pool as the diluent. The standard calibrants (IgG1 was 1.0- 0.16 g/dL and IgG2 was 3.4 – 0.22

g/dL) were used as the inhibitors in a triplex assay to develop a standard curve for unknown samples. Dilutions of bovine reference serum containing known amounts of IgG1 (1.2 – 0.15 g/dL) and IgG2 (1.6 – 0.2 g/dL) was used as positive control. The intra Intra-assay and inter-assay precision of the mutiplex assay was good (coefficient of variation < 10%). Since the IgG2 concentrations of post colostral samples were below detection limit, only IgG1 values were compared to the SRID. The agreement between triplex microsphere assay and SRID for IgG1 was poor with a mean bias of 0.743 g/dL towards triplex microsphere assay (95% confidence interval of 0.382 to 1.105 g/dL). Method comparison studies between total IgG determined by SRID and the gamma-globulin fraction determined by serum electrophoresis indicated that the SRID calculated higher values than the protein method (mean bias of -1.4 g/dL, 95% confidence interval was -1.8 to -1.05 g/dL). We hypothesized that the positive bias for the microsphere assay was explained in part by the use of dilution factors, use of standards that had a low analytical range, and erroneously high standards used in the SRID method.

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Review of Literature

Failure of passive transfer (FPT) and associated neonatal disease.

Calves are born with a naïve but competent fully developed peripheral immune system. Newborn calves are susceptible to environmental pathogens because there is a prolonged lag period and primary immune responses produce low amounts of antibody. Protective memory immune responses may not be optimal until after one month of age (Tizard & Schubot, 2005), therefore, immune protection from the dam is paramount at the time of birth. Ruminants differ from humans in that intrauterine transfer of maternal immunoglobulins does not occur. This phenomenon is due to the fact that maternal blood does not bathe the trophoblast and the chorionic epithelium is in direct contact with the uterine tissues, described as syndesymochorial placentation (Tizard, et al, 2005). As a consequence, the transplacental transfer of immunoglobulin (Ig) molecules is completely precluded, and newborn calves are dependent on receiving maternal antibodies by oral consumption of colostrum (Quigley et al., 2001;Bush & Staley, 1980). Adequate levels of gamma globulin (IgG) transfer are essential for health and survival of neonatal calves. Passive immunity is achieved by ingestion of an adequate mass of IgG in colostrum produced by the dam (Bush, et al, 1980;Quigley, et al, 2001;Besser & Gay, 1994). Failure of passive transfer (FPT) occurs when the plasma IgG concentration is below 1g/dL (Besser, et al, 1994;Quigley, et al, 2001) and predisposes the newborn calf to disease. Calves with IgG concentrations greater than 1g/dL have lower mortality rates from infectious enteritis and respiratory diseases (Besser, et al, 1994). The frequency of failure

of passive transfer in dairy calves is reported to be as high as 35% of the calves (Stott et al., 1979;Brignole & Stott, 1980), whereas it is less frequent in beef calves (Wilson et al., 1999).

Colostral immunoglobulin absorption.

The colostrums in cattle consist of the accumulated secretions of the mammary gland during the last weeks of pregnancy. Estrogens and progesterone influence the transfer of immunoglobulins and other proteins from the blood to the mammary gland. IgG is the major Ig present in the blood and it accounts for 65 – 90% of the total antibody content in the colostrum whereas IgA and IgM are 10 – 20% of the total (Tizard, et al, 2005). The subclass of IgG that predominates in bovine colostrum is IgG1. In fact, colostral IgG1 concentrations are five to ten times higher than maternal serum IgG1 (Besser, et al, 1994;Tizard, et al, 2005;Sasaki et al., 1976). Transfer of IgG1 from the blood across the mammary glad secretory epithelium is facilitated by receptors to IgG1 (Barrington et al., 1997a) and peaks 1-3 days before partiturition (Brandon et al., 1971). As lactation of the dam progress colostrum changes to milk and the glandular epithelial cells cease IgG1 receptors production under the influence of prolactin (Barrington et al., 1997b). Thereafter, most of the IgG and IgA are derived not from the blood but produced locally in the udder (Tizard, et al, 2005).

Selective transfer of maternal immunoglobulin occurs over the first few hours of birth by an apical tubular system in the intestinal absorptive cells. After 12 hours the absorptive capacity of the intestine decreases (Bush, et al, 1980;Stott, et al, 1979), and by

24 hours of birth passive transfer of maternal Ig stops. Maternal antibodies are detected in the lymphatics at 1 to 2 hours after birth (Bush, et al, 1980). Absorption of IgM is slower than IgG and IgA (Bush, et al, 1980) and gradual decreased absorption for each class is independent and observed at 16 hours for IgM, 22 hours for IgA, and 24 hours for IgG. Irrespective of when the calf is fed, absorption of proteins decreases spontaneously at 12 hours of age. Cessation of the nonselective absorption of proteins through intestinal epithelial cells is referred to as gut closure, and is thought to be a function of exhaustion of the pinocytic capability of the apical enterocytes, and due to enterocyte replacement by a more mature population of epithelial cells that may lack specific Fc receptors (Mayer et al., 2002). The basal cell membrane fails to release the pinocytosed products resulting in cessation of transport, hence closing the uptake by the tubular system. The amount of IgG consumed has a positive linear relationship to the concentration of the IgG in the serum within 24 hours (Bush, et al, 1980). Unlike other piglets or lambs (Lecce & Morgan, 1962), in cattle feeding or dietary regimens do not influence time to closure, however gut closure may be extended to 36 hours if feeding is delayed (Stott & Fellah, 1983).

Factors affecting passive transfer in calves.

Attainment of passive immunity in newborn calves is influenced by IgG concentration and the volume of the colostrum (Arthington et al., 2000;Besser, et al, 1994;Bush, et al, 1980;Quigley, et al, 2001;Stott, et al, 1979;Besser et al., 1991) , the age of calves at first feeding (Arthington, et al, 2000;Bush, et al, 1980;Stott, et al, 1979), birth

weight, (Bush, et al, 1980;Stott, et al, 1979), and absorption of IgG from intestine to blood (Besser, et al, 1994). In an early report, it was described that fermented colostrum had a lower pH and lower absorption of gamma-globulins (Bush, et al, 1980), however, lower pH did not affect the absorption of exogenous IgG spiked in colostrum supplements (Quigley, III et al., 2000). Furthermore, high rates of FPT occur in dairy calves that are allowed to suckle naturally. This observation was attributed to the fact that the IgG concentration in holstein cow colostrum is more dilute than beef cows. Dairy calves are unable to achieve adequate passive transfer unless they receive adequate IgG mass when fed 4 liters of colostrum (Besser, et al, 1991).

Methods for determining failure of passive transfer.

There are several traditional methods to diagnose FPT such as salt turbidity assays including zinc sulfate and sodium sulfite, total protein by refractometry, gluteraldehyde turbidity assay and immunological methods including SRID and immunoturbidity assays but none of them are entirely satisfactory.

Salt turbidity assays.

Salt turbidity assays that are commonly used to measure Ig in serum include the zinc sulfate assay and sodium sulfite assay. Both are considered qualitative assays and their principle is based on the fact that when a salt mixture is added to serum containing Igs, the immunoglobulins precipitate or salt out resulting in a turbid solution. In both

methods, the turbidity is proportional to the amount in milligrams of immunoglobulin using known standards to compare with the patient sample. Both the zinc sulfate and sodium sulfite assay are easy, inexpensive and less time consuming when compared to SRID. However, hemolysis caused a false increase in turbidity of the zinc sulfate test (Weaver et al., 2000). In addition the zinc sulfate turbidity assay overestimated the concentration of immunoglobulins compared to SRID (Pfeiffer et al., 1977). When a cut off of < 1g/dL of IgG1 was used to define FPT, the zinc sulfate test had a diagnostic sensitivity of 100% and specificity of 52% when compared with the SRID. The low specificity of the zinc sulfate test indicates that false positive results for FPT were frequent (48% of the time) and greater than when the sodium sulfite assay and serum total protein by refractometer were used to predict FPT (Tyler et al., 1996b)

In the sodium sulfite turbidity assay 14%, 16% and 18% sodium sulfite solutions are used to test serum samples for Ig. The highest salt concentrations (18%) induce turbidity at low concentrations of high molecular weight proteins, whereas low salt concentrations precipitate proteins of high concentrations. For example in a study of 242 calf serum samples, mean serum IgG1 concentration was found to be 2.9 g/dL (range 2.4 – 3.6 g/dL) by SRID when precipitation or turbidity was present in all three sodium sulfite solutions. Precipitation in the 16% and 18% sodium sulfite solutions was equal to a range of 1.0 – 4.3 g/dL for IgG1; whereas when precipitation occurred only in the 18% sodium sulfite solution the IgG1 concentration was equal to a range of 0.6 – 2.5 g/dL. Absence of turbidity in all three sodium sulfite solutions was considered a negative result and reflected a mean serum IgG1concentration of 0.6 g/dL, (range was 0 – 2.4 g/dL) (Tyler, et al, 1996b). When the performance of the sodium sulfite test was determined by

calculating the diagnostic sensitivity and specificity of the assay to correctly identify animals with FPT using SRID as the gold standard method, the diagnostic sensitivity (85%)and specificity (86%) was best when the highest salt solution was used (18%) (Tyler, et al, 1996b). The sodium sulfite turbidity test can be used to evaluate a broad range of IgG compared to zinc sulfate turbidity assay. However, the zinc sulfate test was shown to a high number of false positives (specificity was 52% and sensitivity was 100%) (Tyler, et al, 1996b). Although the results from this study indicated that the 18% salt solution provided the best diagnostic sensitivity and specificity, today commercially available kits provide only the 16% salt solution (Bova-S, VMRD, Pullman, WA). Based on previous findings, this assay would serve as only a screening test for FPT and would only identify samples that had < 1.0 g/dL IgG.

Total protein.

Total protein can be measured using a refractometer which measures the protein concentration based on change in refractive index caused by the solid components in the plasma (Stockham & Scott, 2002). Serum total protein concentrations of the 242 calf samples described previously were determined using a temperature-compensated refractometer and compared to IgG1 concentrations using SRID. A protein concentration of 5.2 g/dL is shown to correlate with a concentration of 1 g/dL of IgG1 (Tyler, et al, 1996b). When 5 g/dL of total protein was considered as the cutoff for FPT, the specificity was 96%, but sensitivity was 59%. In contrast, the sensitivity was 94% and specificity 74% when 5.5 g/dL was considered as the cutoff (Tyler, et al, 1996b).

The total protein by refractometer is considered excellent for herd monitoring, however, there are concerns regarding the effects of age and hydration status (Weaver, et al, 2000). Similarly, the biuret reaction using brom-cresol green dye is one of the most common spectrophotometric methods used to measure total protein. Copper in the biuret reagent binds to peptide bonds creating a blue-green colored complex. The color change is proportional to the amount of protein in the solution, but not all the polypeptide chains are available for the reaction (Stockham, et al, 2002)

Serum electrophoresis.

Serum electrophoresis is used to determine the concentration of immunoglobulin due to different migration pattern of proteins in an electric field. The major disadvantage of this method is that it requires expensive equipment and it cannot be used to determine the concentrations of the subclasses of the gamma fraction (Pfeiffer, et al, 1977). The assay also requires knowledge of the total protein concentration because the percentage of the gamma fraction is then multiplied by the total protein to determine the concentration of the gamma fraction.

Gluteraldehyde coagulation test.

The molecular cross linking caused by a 10% gluteradehyde solution coagulates basic proteins such as immunoglobulins and fibrinogen. The amount of coagulation is thought to be dependent on IgG as it is the predominant immunoglobulin in postcolostral serum (Stockham, et al, 2002). The gluteraldehyde coagulation test is inexpensive and

easy to perform, but the sensitivity and specificity is inadequate for diagnostic use when compared with SRID. Diagnostic sensitivity was reported to be < 41% and specificity varied from 85% to 100% (Tyler et al., 1996a).

Single radial immunodiffusion assay (SRID).

Single radial immunodiffusion is a quantitative assay which can determine the concentrations of total bovine IgG and the subclasses IgG1 and IgG2 (Pfeiffer, et al, 1977). The assay is based on a precipitation reaction that occurs in an agarose gel between the immunoglobulin (Ig) in the bovine serum sample and specific antibody to bovine Ig incorporated into the gel at the time of gel preparation. The standards consist of bovine serum containing known concentrations of IgG and its subclasses or isotopes. In the early assay development, standards were initially prepared by precipitating immunoglobulin from adult bovine serum with ammonium sulfate (50 and 40%) then dialyzed and lyophilized. The protein concentration of the IgG product was determined by the Lowry method (Pfeiffer, et al, 1977).

Today commercially available standards to measure total bovine IgG by SRID are in the range of 400mg/dL to 3200mg/ dL. For IgG1 and IgG2 standards are 125 mg/dL to 1000 mg/dL and 94 mg/dL to 750 mg/dL, respectively. The diameters of the precipitin rings resulting from the antigen antibody reaction of the standards are used to create a linear standard curve. The diameters of the precipitin rings of the bovine serum samples are compared to the diameters produced by the standards. Concentrations of the unknown samples are determined by a linear standard curve created by plotting diameters

in millimeters vs. concentration on semilog paper. The SRID is considered the gold standard because of the specificity and quantitative properties of the method (Davis et al., 2005). In addition, there are several advantages to the SRID method compared to other methods. For example, SRID uses only 3 μL of serum and can be used to determine the concentrations of the subclasses of IgG. However, the disadvantage is that the SRID requires 18 to 20 hours of incubation. Since age of feeding is an important factor in treating FPT, this long incubation period is a major disadvantage of this method. Finally, SRID is very expensive compared to other methods.

SRID gave better results compared to other methods because it is more quantitative than either sodium sufite assay or zinc sulfate assay. SRID showed fewer false positive results when compared to zinc sulfate assay and correlated well with refractometric determination of total protein (Tyler, et al, 1996b). Most of these methods measure IgG based on the assumption that IgG is the most abundant protein in the serum whereas, SRID directly measures IgG and its subclasses by antigen antibody reaction.

Automated and transportable turboimmunometric assay.

Automated assays have been designed to detect human IgG, equine IgG, and bovine IgG. The are based on detection of agglutination reaction between IgG and specific antibody that results in light scatter and is measured spectrophotometrically by an automated instrument (Davis, et al, 2005;Etzel et al., 1997). This assay is not affected by hemolysis in serum and can be used for serum or plasma samples. The major advantage of this assay over SRID is the automation, shorter turnaround time (< 1 hour)

and elimination of human error in reading precipitation rings. Comparison studies between the turboimmunometric assay and SRID for equine and bovine IgG showed good correlation (Davis, et al, 2005;Etzel, et al, 1997).

A commercially available immunoassay using lateral-flow technology is now marketed for field testing. This assay produced by Midland BioProducts was evaluated for performance in predicting FTP by testing 204 male Holstein calves (ranging from 4 to 8 days old) and comparing the results to refractometry of total protein and zinc sulfate turbidity methods. The lateral-flow immunoassay values correlated well with the refractometry and zinc sulfate turbidity techniques, but the lateral flow immunoassay had the best diagnostic accuracy (95%) compared to refractometry (80%), and zinc sulfate (73%) methods (McVicker et al., 2002), but still cannot read IgG1 and IgG2 in the same tube.

Multiplex microsphere technology using flow cytometry.

Multiplex fluorescent microsphere bead assays are extensively used in human medicine. This is a novel technology that enables one to analyze multiple analytes in a single tube using a flow cytometer. These microspheres are internally dyed with fluorophores (combinations of red and orange dyes) of different intensities and act as solid base to which the analyte or protein can be covalently linked using various chemical or molecular techniques. The flow cytometer can differentiate amoung microspheres up to 100 different bead sets based on size, fluorescence intensity, and fluorescent wavelength. This technology can analyze numerous biomolecules such as nucleic acids,

viruses, ligand binding interactions, proteins such as cytokines and antibodies when proteins, nucleic acids or capture molecules are covalently linked to the microspheres and treated with specific reporter antibodies following incubation with the analyte of interest (Camilla et al., 2001;Dasso et al., 2002;Lal et al., 2004;Paul et al., 2005).

There are hurdles to using this novel technology as the cytometer and the associated software is very expensive. Microsphere bead technology is very sensitive, thus standardization of the assay and the reagents for the multiple components is very critical (Camilla, et al, 2001;Dasso, et al, 2002;Lal, et al, 2004). Minor variations in the coupling protocols will significantly alter the fluorescence intensity of the capture antibodies because of the variation in the density of the coupled capture antibodies (Dasso, et al, 2002).

Many studies have reported good correlation between microsphere bead assay and enzyme-linked immunosorbent assay (ELISA) (Camilla, et al, 2001;Dasso, et al, 2002;Lal, et al, 2004). However, studies that compare methods for agreement are lacking. Correlation coefficients used to determine correlation between methods identify associations, which are not unexpected if the methods measure the same analyte. However, there are better statistical models to determine the degree of agreement or bias between methods (Altman, 1991).

Using flow cytometry, the microsphere assay directly measures multiple Igs or their isotypes by antigen-antibody reaction, if the beads are covalently linked with the proper purified proteins and allowed to react with fluorochrome labeled specific reporter antibodies. The main advantage of using a microsphere assay to determine IgG and its subclass concentrations in the foreseeable future is the expected shorter turn around time

and multiplexing that would allow multiple Igs to be measured, thus facilitating the ability to identify animals with FPT or other immune deficiencies.

Introduction

Newborn calves are immunologically naïve at birth and require the passive transfer of maternal immunoglobulins (Ig) after birth to maintain health and immune protection against environmental pathogens. Unlike humans, transfer of Ig to calves and foals does not occur *in utero* due to the strict barriers of placentation in these species. Instead, maternal immunoglobulins are absorbed from colostrum (first fraction of milk) in a narrow window of time after birth (≤ 24 hours). Determination of adequate passive transfer of these critical life saving antibodies in neonatal animals is a common procedure requested by practitioners in the field. Several commercially available assays used to measure serum Ig concentrations in neonatal calves include refractometer determination of total serum protein concentration, sodium sulfite turbidity, zinc sulfate, and SRID. Previous studies indicate there is marked variation in the results and poor accuracy or lack of precision of the assays. Serum electrophoresis was found to be very accurate and quantitative (Pfeiffer, et al, 1977). The RID was not linear at high concentrations of serum immunoglobulin, whereas sodium sulfite turbidity assay is semi-quantitative and zinc sulfate is qualtitative (Tyler, et al, 1996b). Variables that contribute to variability from one study to another include the age of the calves that were sampled, the time of sampling, and venipuncture site (Chorfi et al., 2004).

New technology using immunological reactions, microspheres, and flow cytometry has become available that provides a multiplexing capability in which multiple analytes can be measured in a sample at one time. This technology uses polystyrene carboxylated microspheres that are internally dyed with fluorochromes of various intensities. The beads serve as a solid matrix in which multiple different analytes can be covalently

attached. Fluorescently conjugated reporter antibodies specific to the protein are then used to capture the analyte in the serum. Thereafter, the microspheres coupled with analyte and antibodies can be analyzed by flow cytometry. The main advantages of using the microsphere assay to determine IgG and its subclass concentrations in the foreseeable future are the expected shorter turn around time and the ability to measure multiple Igs at one time, thus facilitating the ability to identify animals with FPT or other immune deficiencies.

Many studies have reported good correlation between microsphere assays and enzyme-linked immunosorbent assays (ELISA) (Camilla, et al, 2001;Dasso, et al, 2002;Lal, et al, 2004). However, studies that compare methods for agreement are lacking. Correlation coefficients used to determine correlation between methods identify associations, which are not unexpected if the methods measure the same analyte (Bland & Altman, 1986b). There are better statistical models to determine the degree of agreement or bias between methods (Bland & Altman, 1986a;Altman, 1991).

The goals of this project were to develop a multiplex microsphere flow cytometry assay to measure bovine IgG subclasses and to compare the IgG1 and IgG2 results to that of the reference method (SRID) using serum samples obtained from calves prior to and 24 hours after ingestion of colostrum. Total IgG concentrations determined by SRID were also compared with the gamma-globulin fraction by serum electrophoresis.

Materials and Methods

Calf samples.

Serum samples from 30 newborn Holstein calves were collected at birth and 24 hours later, at which time all calves had received one to two liters of banked colostra. Venipuncture by jugular vein and animal handling protocols were followed using approved guidelines established by the Kansas State University Institutional Animal Care and Use Committee. A precolostral serum pool was prepared by pooling 1 mL serum aliquots from each of 29 calf samples. All twenty-nine samples were determined to be below the detection limit of the single radial immunodiffusion assay (SRID).

Quantitation of bovine IgG1 and IgG2 by single radial immunodiffusion.

Concentrations of total IgG in all calf samples (pre and postcolostral) were determined by SRID in duplicate using a commercially available assay (Immunocheck, SRID, VMRD, Pullman, WA) with an analytical range of 400 – 3200 mg/dL.

Quantitation of bovine IgG by combined biuret and serum protein electrophoresis.

The gamma fraction of calf samples was quantified independently by the clinical pathology laboratory at the College of Veterinary Medicine, Kansas State University using a combined cellulose acetate serum protein electrophoresis method and the biuret method for total protein (Hitachi 911, Boehringer Mannheim, Indianapolis, IN).

Conjugation of analyte to carboxylated microspheres.

Three distinct sets of carboxylated fluorescent microspheres (5.5 μm, Quantum Plex beads, Bangs Laboratories, Fishers, IN) were separately covalently linked with either purified bovine IgG1, IgG2 (Bethyl laboratories, Montgomery, TX) or globulin free bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) using a modification of a previously described method (Paul, et al, 2005). To prepare a stock of carboxylated beads for protein conjugation, three separate 1 mL aliquots of bead suspensions were washed twice with 2-[N-morpholino] ethane sulfonic acid buffer (Sigma, St. Louis, MO). Two mL of 1-ethyl-3-3-(3-dimethyl aminopropyl) carbodimide hydrochloride (stock was 10 mg/ml of EDC in deionized water, PIERCE, Rockford, IL) was brought up to 10 mL by adding 2-(N-morpholino)ethane sulfonic acid (MES) buffer, pH 7.4, then incubated with the stock beads for 15 minutes at room temperature. To open the amine group on the proteins, each protein (bovine IgGs and serum albumin) were treated with the EDC/MES mixture in a similar manner. The activated bovine proteins (IgG1, IgG2, and albumin, 50 mg/L) were added to the carboxylated activated microsphere sets for 3 hours and 30 minutes on a rocker platform at room temperature. This carbodiimide coupling procedure results in a water soluble intermediate product that creates a protein attachment to the beads by an amide linkage. After the incubation period, the beads were washed twice with 0.05% Tween in PBS, pH 7.4 (PBST). The conjugated microspheres were incubated with 30mM glycine at room temperature for 15 minutes to quench the charge and reduce nonspecific absorption. To block the remaining nonspecific binding sites, beads were incubated with 3% BSA for 1 hour on a

rocker platform at 37 °C. The bead suspension was washed twice with 0.05% PBST. Finally, an equivalent amount of conjugated beads were suspended in 0.05% PBST and stored at 4°C. All washing steps were at high speed (2000 x g) for 5 minutes (Beckman TJ6 centrifuge, Fullerton, CA).

Determination of optimal concentrations of fluorescin labeled secondary antibody.

Using a monoplex assay for each IgG subclass coated bead set, a cocktail of secondary antibodies was serially diluted ten fold (final concentrations 0.1g/L to 0.01 mg/L) and incubated with IgG1 or IgG2 coated microspheres for 30 minutes on a rocker at room temperature and assayed on flow cytometer.

Comparison of monoplex and biplex assays.

To determine whether or not there was interference in the antibody-antigen reaction when multiple bead sets were introduced, the mean fluorescence intensity values for each monoplex assay generated by reacting a single set of protein conjugated microspheres with a single antibody was compared with values determined in a biplex assay. For the biplex assay, a cocktail of beads coated with IgG1 and IgG2 were incubated with a cocktail of anti-bovine IgG1 and IgG2. The antibodies were tested in ten fold dilutions. A cocktail of conjugated beads were made by adding 50 μL of each conjugated bead set to a tube (1.5mLEpendorf tube). The microsphere cocktail was mixed with 10 μL of a bovine standard or serum sample diluted 1:4 in PBS.

Immediately, 100 μL of a cocktail of two FITC- labeled affinity purified sheep antibodies to bovine IgG1 and IgG2 (4 μg/mL stock in 0.01M PBS, Bethyl laboratories, Montgomery, TX) was added to the bead mixture and incubated for 30 minutes on a rocker platform at room temperature. The bead suspension was suspended in 300 μL of PBS prior to acquisition by flow cytometry. Mean fluorescence intensities (MF) generated from monoplex assay for both IgG1 and IgG2 were compared with the biplex assay.

Development of triplex assay to measure bovine IgG1 and IgG2.

The characteristics of the Quantum plex microspheres used to covalently link purified bovine IgG1, IgG2 and BSA are illustrated in Fig. 1A prior to reacting with FITC-labeled anti-bovine antibodies. The different bead sets of 5.5μm diameter can be detected by far red emission (690 nm) after excitation with the argon laser (Fig. 1.B). Bovine serum albumin (BSA) was conjugated to the microsphere set (designated as pink beads) with the lowest fluorescent intensity detected by the FL3 detector (10 to 20 mean fluorescent channels). Bovine IgG2 was conjugated to the microsphere set (designated as blue beads) with fluorescent intensity between 100 and 200 mean fluorescent channels of the FL3 detector. Bovine IgG1 was conjugated to the microsphere set (designated as green beads) with fluorescent intensity between 700 and 800 mean fluorescent channels of the FL3 detector. To confirm specific protein coating of the beads, beads were incubated with a cocktail of fluorescein isothiocynate (FITC) sheep anti-bovine IgG1 and IgG2 (Bethyl Laboratories, Inc., Montgomery, TX). An

arbitrary cut-off for background fluorescence intensity was established based on the shift in fluorescent intensity of the negative control bead set coated with BSA or background fluorescence intensity established for IgG1 and IgG2 conjugated beads in cross reactivity studies. Two fold dilutions of standard serum calibrants diluted in precolostral serum were used as the inhibitor of the assay to generate a standard curve. The decrease in mean fluorescence intensity that occurred with inclusion of inhibitor was proportional to the concentration of the inhibitor protein.

Determination of analytical specificity of microsphere assay,

To determine the cross reactivity of the IgG1 or IgG2 bovine antibodies to the bovine subclass immunoglobulin molecules coating the microspheres, beads conjugated with IgG1 were reacted with sheep anti-bovine IgG2, and beads conjugated with IgG2 were reacted with sheep anti-bovine IgG1. The mean fluorescence intensities of these reactions were compared to the mean fluorescence intensity values of the isotype specific reactions (i.e. anti-bovine IgG1 with IgG1 and anti-bovine IgG2 with IgG2).

To determine if the cross reactivity of the sheep anti-bovine antibodies to the IgG subclasses altered the decrease in mean fluorescence intensity obtained with specific IgG subclass inhibitors, a triplex assay was performed using one inhibitor at a time. In separate tubes either purified IgG1 or IgG2 (serial dilutions starting at 1 mg/mL) was added to the mixture of beads coated with IgG1, IgG2 and BSA. Two fold serial dilutions of purified protein were tested in separate tubes. Immediately, the cocktail of

antibodies (sheep anti-bovine IgG1+anti-bovine IgG2) was added and incubated for 30 minutes on rocker at room temperature and assayed on flow cytometer.

Stability of bead protein conjugates.

To determine the stability of the proteins covalently linked to the beads, the triplex assay was performed weekly for four consecutive weeks using the same stock of beads and cocktail of secondary antibodies.

Preparing the standards and creating a standard curve.

Standard serum calibrants were prepared by two fold serial dilutions of purified bovine IgG (10 g/dL, Sigma Co, St. Louis, MO.) using precolostral calf serum pool as the diluent. The total concentration of bovine IgG in the commercial product was confirmed by serum protein electrophoresis using cellulose acetate and biuret reactions (Hitachi 911, Boehringer, Mannheim). This assay was performed by the clinical pathology laboratory at Kansas State University. Concentrations of IgG1 and IgG2 in the standard calibrants were determined by the SRID (VMRD, Pullman, WA). Controls consisted of bovine reference containing known amounts of IgG1 and IgG2 (Bethyl laboratories, Montgomery, TX). The concentrations of the serum calibrants were converted to natural log and plotted on the X-axis vs. mean fluorescence (Y-axis). A first order polynomial equation [Y = slope(X) + intercept] was generated from the standard curve. The equation was solved for the X value and the natural log value was

converted using the exponent. Serial dilutions of the bovine reference serum were run as positive controls between ranges of 2.8 to 0.35 g/dL for total IgG (IgG1 was 1.2 - 0.15 g/dL and IgG2 was 1.6 – 0.2 g/dL). A set of beads conjugated with BSA was used as a negative control.

Flow cytometry

A total of 1000 gated bead events for each bead set were collected by a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA) and the data were analyzed using Cell Quest software (Becton Dickinson, San Jose, CA). Three gates were placed on each microsphere set using two color dot plots. Distinction of Quantum plex bead sets that reacted with FITC sheep anti-bovine IgG antibodies was visualized by displaying the beads in on FL3 (y-axis) vs. FL1 (x-axis) (Fig. 1). The mean fluorescence intensity of the binding of FITC conjugated antibody (peak emission 535 nm) to each bead set, (BSA, IgG1, and IgG2) was recorded by the FL1 detector.

Assay reproducibility and analytical accuracy.

Intra-assay and inter-assay precision or reproducibility was determined by assaying standard serum calibrants and four fold dilutions of the bovine reference serum on the same day (intra-assay) or over several days (inter-assay) using a stock of conjugated beads. Results were obtained from each set and coefficient of variation was calculated (standard deviation divided by the mean) and expressed as a percentage.

The analytical accuracy of the assay was determined by assaying the bovine reference serum with known concentrations of 0.175 – 1.2 g/dL determined by SRID and ELISA (communication with Bethyl labs, Montgomery, TX). Percentage of recovery was calculated by dividing the observed values determined by the microsphere assay by the expected values and multiplying the result by 100.

Method comparisons.

IgG1 concentrations of 30 postcolostral serum samples were determined in duplicates by SRID (VMRD, Pullman, WA) and by the triplex micrsophere assay. The average of each replicate sample was determined for each assay. To identify constant and proportional bias the average concentrations of each assay were plotted by Deming regression analysis (Analyse-it software for Microsoft excel, version 1.71). For NCCLS (National Committee for Clinical Laboratories) bias plots, the differences between the methods were plotted against the mean concentration determined for the reference method (NCCLS, now Clinical Laboratory Standards Institute). The SRID method was designated as the reference method, whereas the microsphere assay was designated as the new or comparative method. Total IgG concentrations determined by SRID were compared to the total serum gamma fraction by protein serum electrophoresis using cellulose acetate combined with the biuret reaction for total protein. In each method comparison, the average values for the reference method (SRID) were subtracted from the average values of the comparison method to determine the differences.

Statistical analysis

Data was analyzed for normality using Kolmorgorov-Smirnov (K-S) and replicates were tested for differences in the median by the Mann-Whitney Rank Sum test. Association between the methods that determined IgG1 concentrations (microsphere assay and SRID and protein serum electrophoresis and SRID) was determined using the Spearman Correlation coefficient (RS).

Results

SRID assay results of pre and post colostral samples.

Concentrations of total IgG for 29 calves in precolostral sera were below detection limits of the SRID assay (0.4 g/dL). One calf had 1.6 g/dL. Total IgG, IgG1 and IgG2 concentrations in post colostral sera determined by SRID were listed in Table 1. The median concentrations for total IgG were 3.2 g/dL, whereas IgG1 and IgG2 were between 2.0 - 2.05 and 0.11 – 0.115 g/dL, respectively. The proportion of IgG1 of the total bovine IgG determined by SRID was 65%, whereas IgG2 was 3%. There was no statistically significant difference between the replicates (Mann-Whitney Rank Sum test, P =0.865).

Analytical specificity of the microsphere assay,

Specificity of the assay was evaluated to identify any cross reactivity of the FITC-labeled sheep anti-bovine antibodies to the protein coated microspheres or to soluble protein inhibitor. In the first experiment, secondary antibodies were tested for binding to IgG1 or IgG2 coated beads without serum inhibitor (Fig. 2). Slight binding of IgG1 coated beads occurred with anti-bovine IgG2 (Fig. 2A, mean fluorescence intensity was 33), whereas anti-bovine IgG1 reacted with IgG2 coated beads resulting in a greater shift in fluorescence intensity (up to 10^2) (Fig 2B.) indicating cross reactivity of anti-bovine IgG2 with IgG1 and anti-bovine IgG1 with bovine IgG2. However, the shift in fluorescence intensity that occurred with specific isotype reactions for IgG1 and IgG2 were substantially higher, 2-logs for IgG1 (Fig. 2C), and 1-log for IgG2 (Fig. 2D).

In the second experiment when purified IgG1 was added alone in increasing concentrations to the FITC-labeled anti-bovine antibody cocktail, the mean fluorescence intensity for the reaction between antibody and IgG1 coated beads decreased in a linear manner, while the mean fluorescence intensity of IgG2 and BSA coated beads remained essentially unaltered (Fig. 3). Similarly, when IgG2 was added without IgG1 inhibitor, only the IgG2 coated beads showed a decrease in mean fluorescence intensity (Fig 4) indicating specific competition with soluble IgG2. There was a slight drop in mean fluorescence intensity for IgG1 and IgG2 when the opposite IgG class inhibitor was added at the low inhibitor concentrations. These results indicated the cross reactivity of the sheep antibodies did not affect the triplex assay when purified inhibitor was used.

Comparison studies between monoplex and biplex assay.

Interference between the bead sets was investigated by comparing mean fluorescence intensities generated by the monoplex assay with those generated by the biplex assays. In Figure 5, the mean fluorescence intensity of the monoplex and biplex assay were similar and demonstrated linearity over the dilutional range for both IgG1 (Fig. 5) and IgG2 (Fig. 6). There was a slight decrease in the mean fluorescence intensities for IgG2 when the biplex was compared to the monoplex assay. In conclusion, the biplex assay having two sets of beads did not substantially alter the fluorescence intensities detected by the binding of FITC cocktail antibodies to either IgG1 or IgG2 coated beads.

Assay stability and optimization of report antibody concentrations.

Stability of the beads after protein coating was analyzed by comparing mean fluorescence intensity values generated from the triplex assay over time. The mean fluorescence intensity for each set of microspheres of the triplex assay did not change up to four weeks, thereafter the protein coated beads deteriorated causing an increase in scatter properties and a decrease in the mean fluorescence intensity. The optimal concentration of FITC-labeled sheep anti-bovine IgG1 and IgG2 was determined to be 1:250 because this concentration did not cause BSA-coated beads to shift in fluorescent intensity and gave the highest mean fluorescence intensities compared to further dilutions of antibody.

IgG subclass concentrations of bovine IgG standards and reference sera.

The IgG concentrations of the Sigma serum standard and reference serum were compared in Table 2. Protein electrophoresis and the biuret method confirmed the concentrations of the Sigma serum product after dilution with the precolostral sera pool. The total amount of IgG determined by serum protein electrophoresis approximated the sum of the two subclasses of each product. Concentrations of the IgG subclasses of the two products were comparable with IgG2 being consistently higher than IgG1 in both serum products.

Development of a standard curve for the triplex assay.

Increasing concentrations of the standard bovine IgG (Sigma, CO, St. Louis, MO) diluted with the precolostral sera pool was used as the inhibitor in a triplex assay to develop a standard curve for unknown samples (illustrated in Fig. 7 dot plots). The bead sets coated with IgG1 (green dots) and IgG2 (blue dots) shifted to the left or decreased in fluorescence intensity as the inhibitor concentrations increased (Fig. 7B – F) compared to precolostral serum without inhibitor (Fig. 7A). The highest concentration of standard IgG1 (1.0 g/dL) did not shift into the fluorescence region in which anti-IgG2 cross reacted with IgG1 coated beads (Fig. 7F compared to Fig. 2A). Several concentrations of standard IgG2 (3.4 – 0.8 g/dL) overlapped with the

fluorescence shift that occur when anti-IgG1 cross reacted with IgG2 (Fig. 7D-F compared to Fig. 2B).

Because the IgG2 concentration of the post colostral sera from all calves was < 0.2 g/dL by SRID, further quantification of IgG2 by the microsphere assay was not performed. To create a standard curve and a first order polynomial equation to solve for unknowns, the standard IgG1 concentrations were plotted against the mean fluorescence intensity of the standards (Fig. 8A). However, this plot produced a nonlinear curve, therefore, the x-axis data (standard concentrations) were converted to natural log (Fig. 8B) and the equation for the line was created.

Assay reproducibility and analytical accuracy for IgG1.

The imprecision data for the microsphere assay for IgG1 is presented in Tables 3 and 4. The within assay reproducibility (intra-assay precision) was good (CV < 10%) for all concentrations of standards and reference serum (Table 3), however, reference sera at 0.3 g/dL concentration had the highest CV (8%). The inter-assay precision was also good (CV < 10%) for each concentration of standard or reference sera (Table 3). The standard curves for the intra-assay and inter-assay reproducibility experiments were linear with similar values for the slope and intercept (Fig 9A and B).

The accuracy of the microsphere assay was near 100% (± 8%) for all concentrations of bovine reference sera (0.35 g/dl – 1.2 g/dL) except for the lowest concentration (0.175 g/dL), in which the percent recovery was 183%.

Method comparison of SRID and microsphere assays.

The amount of agreement between the triplex microsphere assay and the reference method SRID was determined by comparing the results of all the 30 pre and post colostral serum values performed in duplicate. Since IgG2 concentrations were less than 0.3 g/dL in both assays, statistical comparisons were not performed. The mean IgG1 concentrations determined by the flow cytometry microsphere assay and SRID were compared using a Deming regression plot. Figure 10 shows the presence of an extreme outlier that caused the regression line to cross the line of identity. The outlier was due to a post colostral sample that measured 7.5 g/dL (mean) by the RID and 2.3 g/dL by the microsphere assay. This outlier was removed from the database because of the extreme discrepancy between the two assays and the data from the methods were reanalyzed for comparison (Fig. 11). In the new plot the data points were spread out and based on the position of the new regression line, there was a notable constant and proportion bias. The NCCLS bias plot (Fig. 12) showed a mean positive bias of 0.743 g/dL (95% confidence interval was 0.382 to 1.105 g/dL) over the analytical range. The RS value was low (0.31) indicating a poor correlation between the two assays. Since the microsphere assay for IgG1 had poor agreement with the SRID assay, total IgG concentrations of the post colostral sera samples were compared with a protein electrophoresis method using cellulose acetate to determine the gamma globulin fraction and biuret method to determine the total protein of the samples. Although the RS value was fairly high (0.78) indicating a good correlation between the gamma globulin concentrations and the total IgG determined by SRID, the Deming regression

plot showed poor agreement (Fig. 13). There was a negative mean bias of -1.4 g/dL, (95% confidence interval was -1.8 to -1.05 g/dL) for the protein electrophoresis method compared to the SRID indicating the SRID had higher values (NCCLS bias plot, Fig. 14). Because of this observation, the serum calibrants that were used as standards for the microsphere assay and verified by protein electrophoresis were sent to VMRD for quantitation by SRID using the total IgG SRID plate. The results indicated that the SRID assay produced higher IgG concentrations than the protein electrophoresis method (Table 6).

Discussion

In this report, we developed a competitive multiplex microsphere flow cytometry assay to determine bovine IgG1 and IgG2 concentrations in 30 serum samples from newborn Holstein calves acquired prior to and 24 hours after ingestion of one to two liters of banked colostra, and compared the values with SRID. We choose to measure bovine IgG1 and IgG2 because previous studies have shown that there is selective absorption of IgG during the first few hours of life and that adequate concentrations of IgG (predominantly IgG1) in the serum of newborn calves decrease the mortality rate.

To accomplish this goal, we obtained serum samples from 30 newborn Holstein calves before and 24 hours after ingestion of colostrum. In this manner, the age of the calves was controlled. Using SRID to measure total IgG concentrations, the precolostral serum samples contained less than 0.4 g/dL for 29 calves. One calf had 1.6 g/dL of IgG indicating that sampling of this calf occured after feeding colostrum. The total concentration of IgG in post colostral sera from 30 calves ranged from 0.8 to 7.2 g/dL

with a median value of 3.2 g/dL (interquartile range = 2.25 to 4 g/dL). According to the SRID only two calves had IgG concentrations < 1.0 g/dL. The IgG1 concentrations had a similar range as the total IgG, but the median concentration and interquartile range was lower (2.0 g/dl; 1.4 – 2.5 g/dL) constituting about 60 – 65% of the total IgG. IgG2 determined by SRID using a specific IgG2 plate revealed very low concentrations of IgG2 (interquartile range = 0.11 – 0.15 g/dL). Unexpectedly, the IgG1 and IgG2 subclasses contributed to approximately 65- 75% of the total IgG. Possible explanations for this finding are that other IgG classes contribute to the total IgG in post colostral sera, a suggestion that has not been reported before, or that the SRID assay that measures total IgG is overestimating the total IgG. We support the latter hypothesis, for additional studies by others demonstrate the SRID overestimates IgG compared to cellulose acetate protein electrophoresis assays which measure the gamma globulin fraction (R.Di Terlizzi, 2005).

The multiplex microsphere bead assay developed in this study uses the flow cytometry to differentiate microsphere beads based on size and fluorescence intensity. The multiplexing property of this assay enables the quantitation of the IgG subclasses in a single sample. This simultaneous analysis reduces sample volumes, time and labor. The triplex bead assay was developed based on the covalent linking of proteins to carboxylated microspheres by chemical reactions and the microsphere assay is reproducible and has shown good correlation with ELISA (Camilla, et al, 2001;Lal, et al, 2004;Dasso, et al, 2002).

In contrast to many multiplexing formats that use a capture antibody and reporter antibody conjugated to a fluorochrome, the principle of the assay that we developed was

based on competition for a reporter antibody between soluble Ig and Ig bound to microspheres. Therefore, antigenic determinants on bovine IgGs present in the serum competed with purified bovine IgG coating the microspheres for the FITC-labeled antibodies. As a result, as serum IgG concentrations increase the fluorescence intensity of the beads reacting with antibody decreases proportionally to the amount of soluble serum Ig.

We found that one of the limitations of the microsphere assay was the observation that the FITC-labeled sheep anti-bovine IgG1 and IgG2 antibodies cross reacted with IgG2 and IgG1 coated microspheres in the absence of serum Ig inhibitors. Although the isotype specific reaction was one to two log higher than the nonspecific reaction, the cross reactivity between IgG1 antibodies and IgG2 coated beads impaired the ability to determine accurate concentrations of IgG2 between 0.8 – 3.4 g/dL. Although the sheep anti-bovine isotype specific antibodies used in this study were affinity purified and preabsorbed with opposite IgG subclasses, some degree of cross reactivity is expected (personal communication, Bob Wilds, Technical support, VMRD). When purified bovine IgG1 or IgG2 at low concentrations (up to 0.1 g/dL) was added as an inhibitor to the assay the decrease in fluorescence intensity was linear indicating that the cross reactivity properties of the antibodies did not significantly alter the change in fluorescence intensity determined for each IgG subclass in a low analytical range. However, the assay would not be able to accurately determine IgG2 at concentrations > 0.5 g/dL.

To test the interference for the microsphere assay, the biplex assay was compared with monoplex assay. The biplex assay did not significantly alter the

fluorescence values detected by the binding of FITC cocktail antibodies to either IgG1 or IgG2 coated beads (monoplex). This can also be considered as a test for cross reactivity because the fluorescence in the biplex assay was not different from the monoplex assay. We predicted that the mean fluorescence intensity for detection of IgG2 in the biplex assay would be greater than the monoplex assay because of the cross reactivity of anti-IgG1 against IgG2 coated beads that we demonstrated previously. However, the mean fluorescence intensity for IgG2 in the biplex assay was actually less than the monoplex assay.

The optimization of the triplex assay required a two step carbodimide chemical reaction and similar incubation techniques for all the three activated proteins and microspheres. It was observed that variation in the conjugation steps affected the reproducibility of the assay and the standard curve (data not shown); therefore large stocks of protein coated beads were prepared to analyze the entire set of serum samples. Previous studies to test the process of coupling antibodies to microsphere beads has shown that slight variations in the coupling procedure affect the reproducibility of microsphere assay when new stock beads are prepared each time, there was change in CVs up to 25% (Dasso, et al, 2002).

The triplex microsphere assay demonstrated linearity over the two fold dilutions of standard serum calibrants which included the cutoff range for FPT (< 1 g/dL), but the triplex assay was designed to determine the concentrations of IgG1 and IgG2 not just detect FPT. The mean fluorescence intensity generated by using a known amount of bovine IgG1 and IgG2 as the inhibitors in a triplex assay was used to develop a standard curve for unknown samples. Bovine reference serum containing known amounts of IgG1

(1.2 - 0.15 g/dL) and IgG2 (1.6 – 0.2 g/dL) was used to test the linearity of the curve and as a control. The triplex microsphere bead assay for IgG1 was reproducible. The intra-assay and inter-assay precision was good with CV less than 10% for all concentrations of standards and reference serum with similar slope for the first order polynomial equation. The analytical accuracy of the IgG1 assay approached 100% for bovine reference serum concentrations between 0.35 to 1.2 g/dL, however was poor at the lowest concentration (0.175 g/dL). Possibilities for this observation included nonlinearity of the standard curve at the lowest concentration, lack of sensitivity of the assay, or errors in sample preparation.

In our studies we measured the IgG1 and IgG2 concentrations of all the 30 precolostral and postcolostral serum samples from Holstein calves in duplicates. The Deming method comparison of IgG1 from the flow assay was done using SRID as the reference. The presence of an outlier caused the regression line to cross the identity line, When this outlier was removed there was constant and proportion bias. The concentration of IgG1 determined by the microsphere assay was higher than SRID and method comparison studies showed poor agreement between the microsphere assay and SRID assay. The positive bias for the flow cytometry assay can be explained in part by the use of dilution factors and use of standards with a low analytical range. Moreover, independent studies using protein electrophoresis to determine the gamma globulin fraction demonstrated that the Ig standards used to test total IgG by SRID were erroneously high (Table 6). Based on these findings we propose that the SRID has several limitations and should not be considered the gold standard method. The limitations include the variations that occur between individuals when measuring the

diameter of the precipitation ring, which is dependent on having a crisp diffusion ring and the accuracy of using a millimeter ruler. Furthermore, samples may diffuse into neighboring wells. The error rate is reported to be high in SRID if the serum concentrations of immunoglobulins are higher than 3g/dL due to the lack of linearity of the standard curve and the amplification of errors when concentrations are corrected for the dilution factor (Pfeiffer, et al, 1977). In fact, this group reported a negative bias of 0.3 g/dL between the serum protein electrophoresis method and total IgG determination by SRID. Similar problems occur with the microsphere assay because the postcolostral serum samples that have IgG1 concentrations greater than 1g/dL must be diluted due to low analytical range of the bovine IgG1 and IgG2 standards. Most of commercial preparations of purified bovine IgG1 have higher IgG2 concentrations than those of newborn calves.

Although the triplex assay is potentially more sensitive than SRID, because of the sensitivity of fluorescence intensity measured by a flow cytometer, further studies must be performed to optimize the multiplex assay to measure bovine IgG subclasses. Future experiments that must be done include using a monoclonal anti-bovine reporter antibodies to minimize the cross reactivity, preparing bovine standards that cover a wide analytical range, independent quantitation of IgG1 and IgG2 standards other than by SRID, and recovery studies that involve spiking bovine serum with known concentrations of IgG1 and IgG2 to determine the analytical sensitivity of the assay.

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Table 1. Post colostral IgG concentrations (g/dL) measured by RID

	SRID (Total IgG)		SRID (IgG1)		SRID (IgG2)	
Calf no	**Rep. 1.**	**Rep. 2.**	**Rep. 1.**	**Rep. 2.**	**Rep. 1.**	**Rep. 2**
3421	3.2	3.2	2.1	2.1	0.04	0.04
3429	2.3	2.3	1.3	1.3	0.07	0.07
3430	1.6	1.6	1.4	1.3	0.07	0.07
3431	1.7	1.7	0.7	0.7	0.12	0.15
3432	2.9	2.9	1.5	1.3	0.19	0.19
3436	4.0	4.2	1.4	1.4	0.19	0.19
3437	4.0	4.0	1.3	1.3	0.12	0.12
3438	1.5	1.5	2.1	2.1	0.10	0.10
3439	3.2	3.2	2.0	2.0	0.09	0.09
3440	7.2	6.8	7.5	8.0	0.20	0.20
3441	4.8	4.8	2.1	2.1	0.09	0.09
3444	3.2	3.2	1.9	1.9	0.09	0.09
3446	5.0	5.0	2.7	2.7	0.09	0.09
3448	0.8	0.8	0.7	0.7	0.04	0.04
3451	1.2	1.2	1.5	1.5	0.12	0.12
3454	0.9	0.9	0.7	0.9	0.05	0.05
3418A	5.0	5.0	2.6	2.6	0.12	0.12
3419A	3.2	3.2	2.7	2.7	0.09	0.09
3420A	3.2	3.2	2.0	2.0	0.09	0.09
3421A	3.2	3.2	2.1	2.1	0.14	0.14
3422A	2.4	2.4	2.2	2.2	0.11	0.11
3423A	2.3	2.3	1.7	1.7	0.10	0.11
3426A	2.4	2.5	2.3	2.3	0.15	0.15
3427A	4.8	4.8	3.0	3.0	0.18	0.16
3431A	3.2	3.2	2.5	2.5	0.13	0.13
3433A	5.0	5.0	1.2	1.2	0.11	0.11
3435A	3.8	3.8	3.2	3.2	0.16	0.16
3437A	1.5	1.5	1.2	1.5	0.5	0.05
3438A	3.0	3.0	2.0	2.4	0.27	0.27
3440A	4.0	4.0	2.6	2.6	0.94	0.09
Median	3.2	3.2	2	2.1	0.12	0.11
25%	2.3	2.3	1.4	1.4	0.09	0.09
75%	4.0	4.0	2.5	2.5	0.16	0.15

Table 2. Concentrations of bovine IgG subclasses in serum calibrants and reference sera

Sigma bovine IgG (PE)*	Sigma bovine IgG1 by SRID	Sigma bovine IgG IgG2 by SRID	Bovine Ref. Sera IgG1	Bovine Ref. Sera IgG2
5.0 g/dL	1.0 g/dL	~3.4 g/dL	1.2 g/dL	1.6 g/dL
2.5 g/dL	0.5 g/dL	1.7 g/dL	0.6 g/dL	0.8 g/dL
1.3 g/dL	0.25 g/dL	0.85 g/dL	0.3 g/dL	0.4 g/dL
0.6 g/dL	0.16 g/dL	0.45 g/dL	0.15 g/dL	0.2 g/dL
0.3 g/dL	Too small	0.225 g/dL	--	--

*****PE – protein electrophoresis by cellulose acetate

Table 3. Intraassay reproducibility.

A.

Sigma bovine IgG1 Concentration g/dL	SD	MF	CV
1	16.0	291.3	5%
0.5	26.8	427.5	6%
0.25	22.3	579.3	4%
0.16	20.5	824.8	2%
too small	28.7	1001.8	3%

B.

Ref-serum IgG1 Concentration g/dL	SD	MF	CV
1.2	17.0	286.3	6%
0.6	14.0	407.3	3%
0.3	42.0	530.5	8%
0.1	30.3	607.0	5%
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Table 4. Interassay reproducibility.

A.

Sigma Bovine IgG1 by flow	SD	MF	CV
1g/dL	14.8	270.3	8%
0.5 g/dL	17.1	418.3	8%
0.25 g/dL	41.8	616.8	9%
0.16 g/dL	26.1	788.8	1%
too small	36.2	953.5	--

B.

Ref-serum IgG1 by flow	SD	MF	CV
1.2 g/dL	21.5	279.5	8%
0.6 g/dL	30.1	364	8%
0.3 g/dL	44.7	500.5	9%
0.1 g/dL	7.4	571.3	1%
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Table 5. Analytical accuracy of IgG1 in bovine reference serum

Expected concentration of IgG1	Observed concentration of IgG1	Percentage recovery (observed/expected)
1.2 g/dL	1.3 g/dL	108%
0.7 g/dL	0.70 g/dL	100%
0.35 g/dL	0.37 g/dL	106%
0.175 g/dL	0.32 g/dL	183%

Table 6. Comparison of IgG concentrations of Serum calibrants by Protein electrophoresis and SRID methods.

Protein electrophoresis	SRID for total IgG
5.0 g/dL	Too large
2.5 g/dL	3.4 g/dL
1.3 g/dL	1.7 g/dL
0.6 g/dL	0.9 g/dL
0.3 g/dL	0.4 g/dL

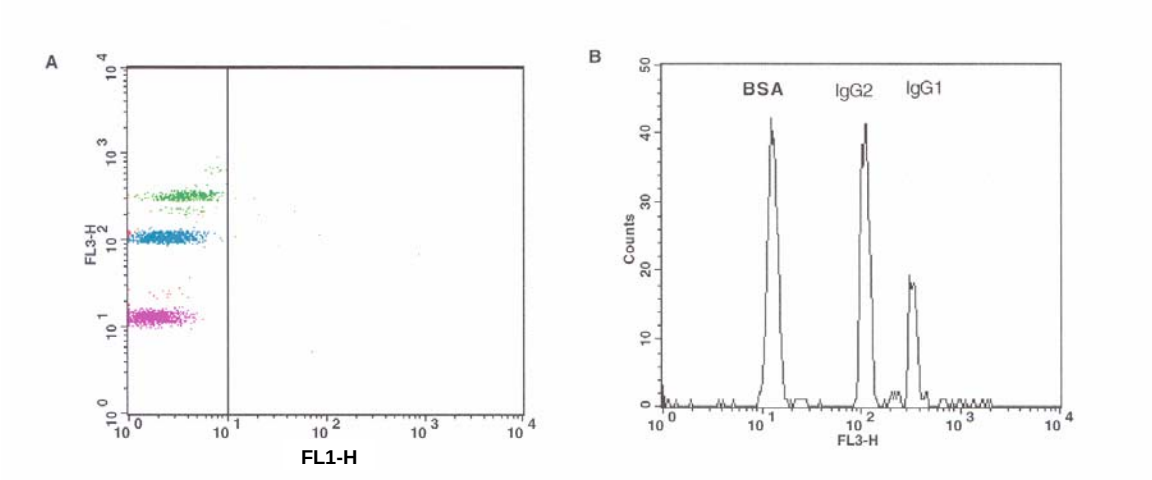


Figure 1. Flow cytometry dot plot (A) and histogram (B) of Quantum Plex beads illustrates the different red fluorescent properties of the beads that were coated with BSA (pink dots), IgG2 (blue dots), and IgG1 (green dots) as the microsphere sets are distinguished from one another by a two color dot plot using the FL3 detector and FL1 detector (A) and plotted as a histogram on a log scale using the number of bead events (counts) vs. the height of fluorescent channels.

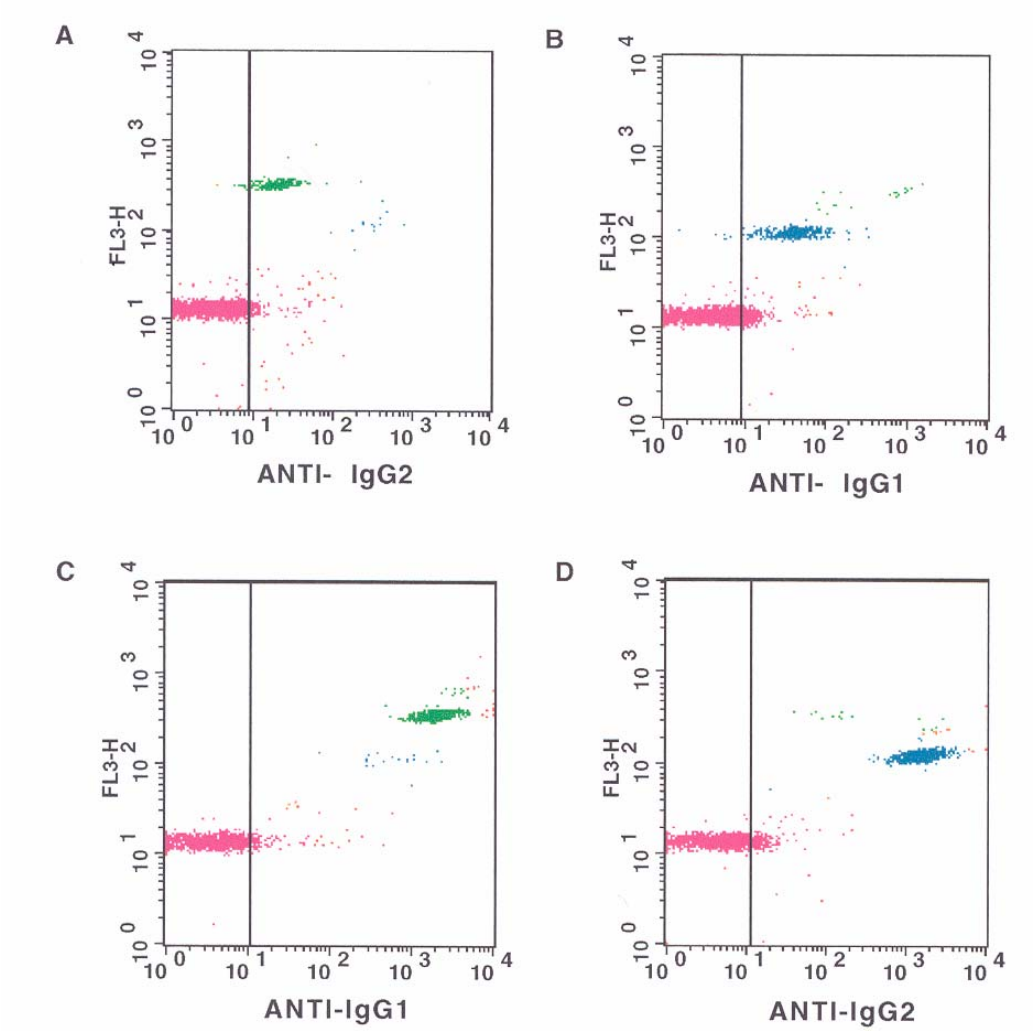


Figure 2. Flow cytometry dot plots (FL3-H vs. FL1-H) illustrating the binding of FITC sheep anti-bovine Igs to a combination of IgG1 and IgG2 coated beads. Cross reactive binding of FITC-labeled sheep anti-bovine IgG2 occurred with IgG1 coated microspheres, green dots (A) and FITC-labeled sheep anti-bovine IgG1 with IgG2 coated beads, blue dots (B). Isotypes specific reactions demonstrate a greater shift in fluorescence intensity for bovine IgG1 coated beads treated with anti-bovine IgG1(C) and bovine IgG2 coated beads treated with anti-bovine IgG2 (D). No changes were observed in BSA, red dots.

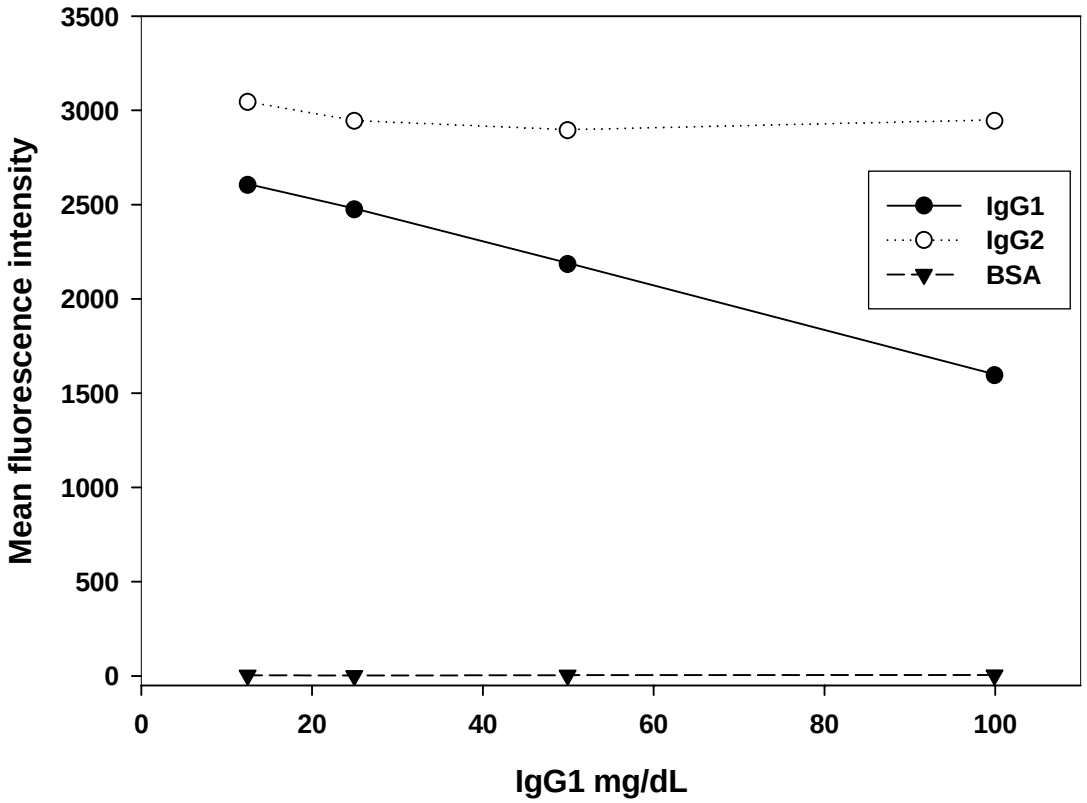


Figure 3. Triplex bead assay illustrating a specific linear decrease in fluorescence intensity from IgG1 coated beads resulting from competitive inhibition of FITC sheep anti-bovine IgG1 caused by purified bovine IgG1(solid circles). There is slight drop in fluorescence intensity from IgG2 coated beads (empty circles). Fluorescence from BSA coated beads was unaltered (solid triangles).

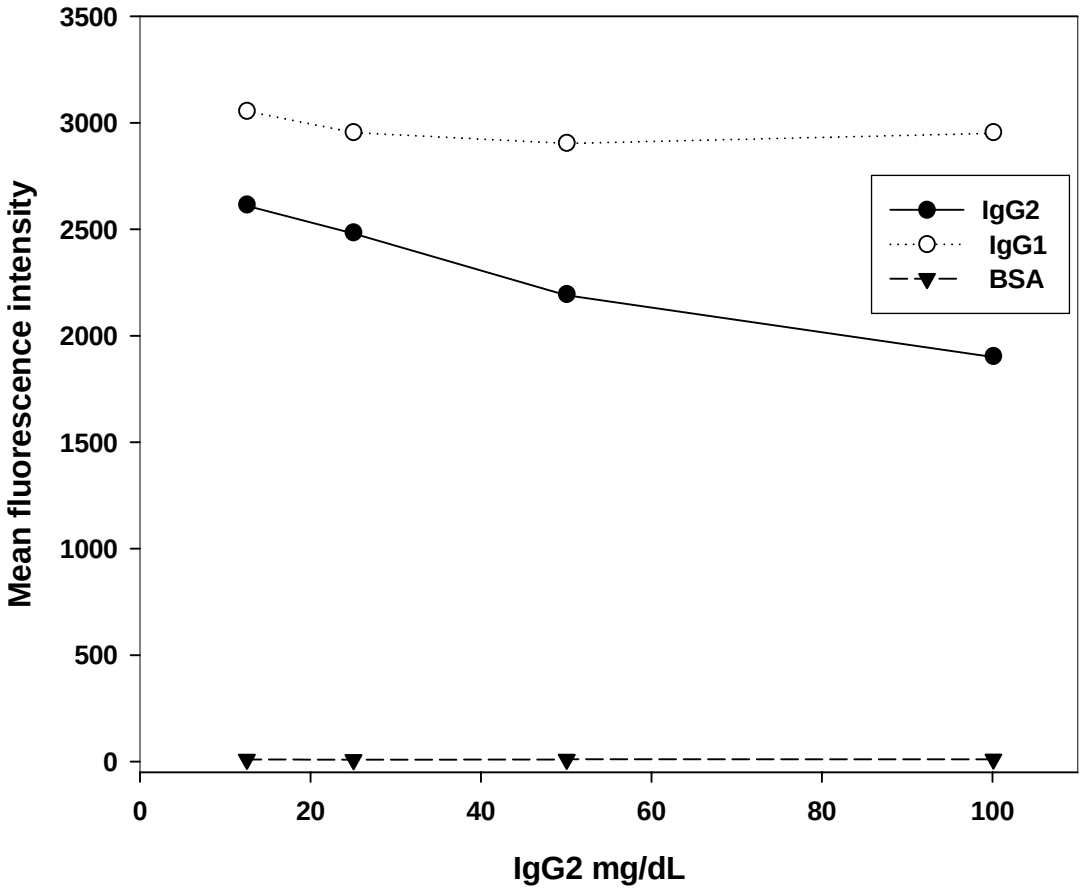


Figure 4. Triplex bead assay illustrating a specific linear decrease in fluorescence intensity from IgG2 coated beads resulting from competitive inhibition of FITC sheep anti-bovine IgG2 caused by purified bovine IgG2(solid circles) (except for highest concentration of IgG2). There is slight drop in fluorescence intensity from IgG1 coated beads (empty circles). Fluorescence intensity from BSA coated beads was unaltered (solid triangles).

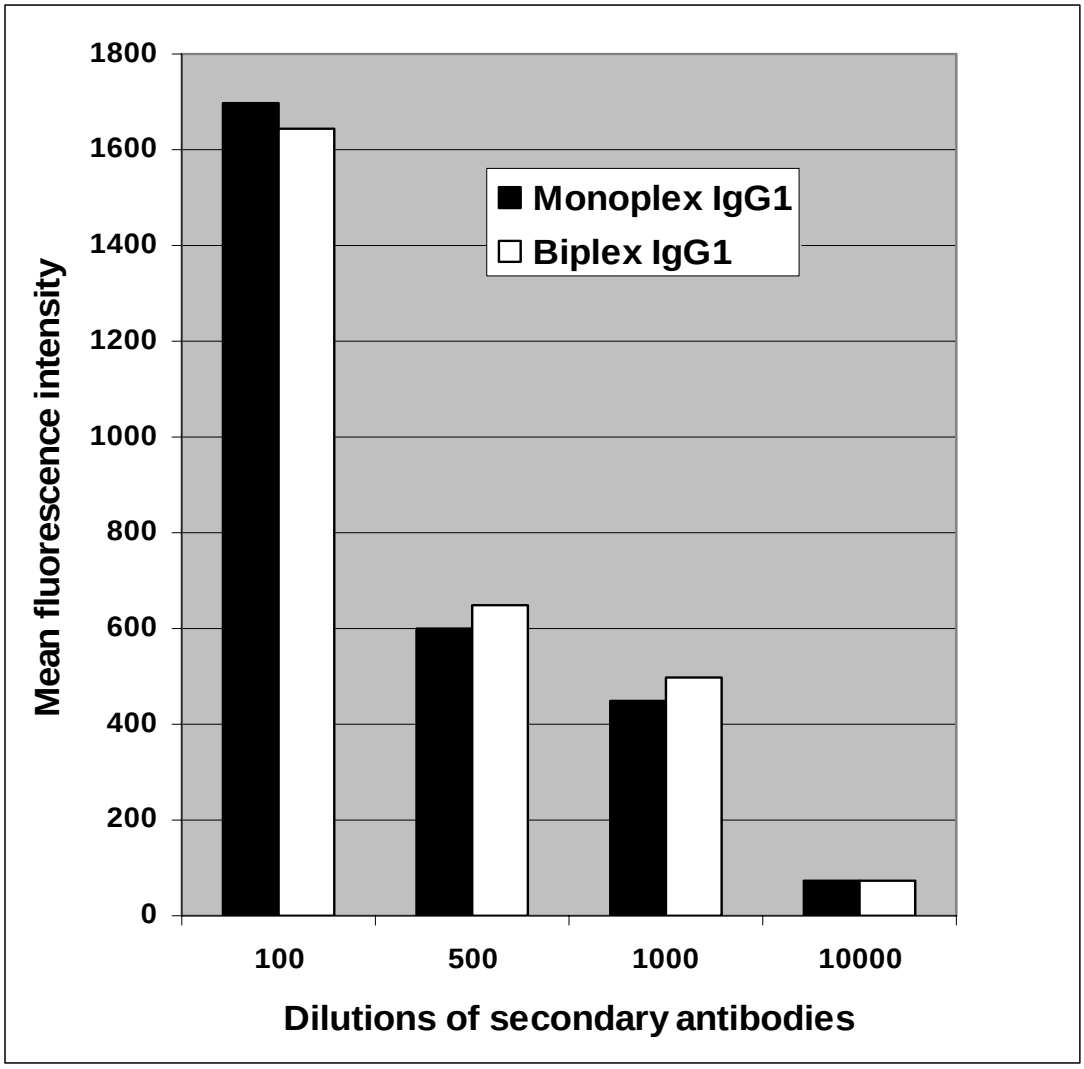


Figure 5. Bar chart comparing monoplex IgG1(black bars) and biplex IgG1(white bars) microsphere assay over the dilution range of sheep anti-bovine IgG1 and IgG2, the mean fluorescence intensity of the monoplex IgG1 and biplex IgG1are similar as there is no significant interference in biplex for IgG1.

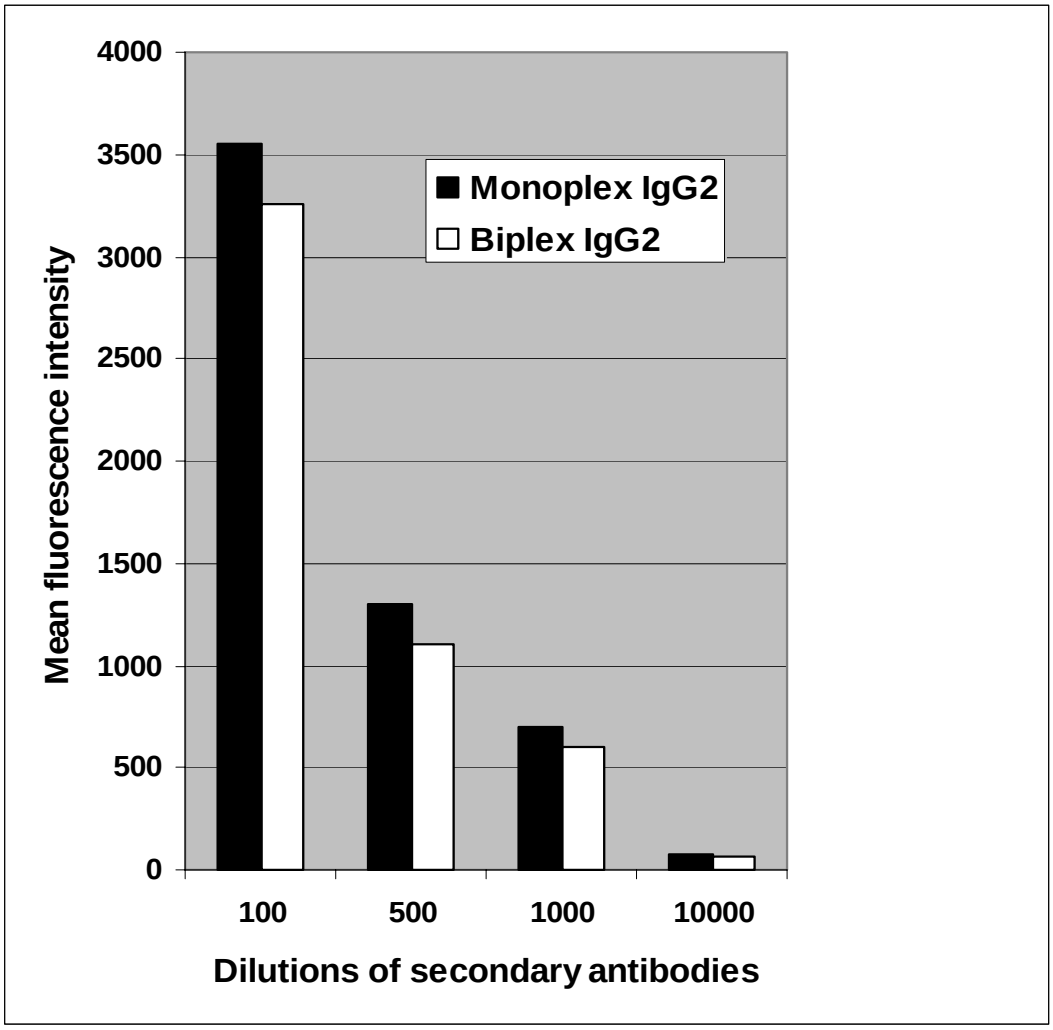


Figure 6. Bar chart comparing monoplex IgG2 (black bars) and biplex IgG2 (white bars) microsphere assays over the dilution range of sheep antibovine IgG1 and IgG2 antibodies, there is slight decrease in fluorescence intensity for the biplex IgG2 on comparision with the monoplex IgG2.

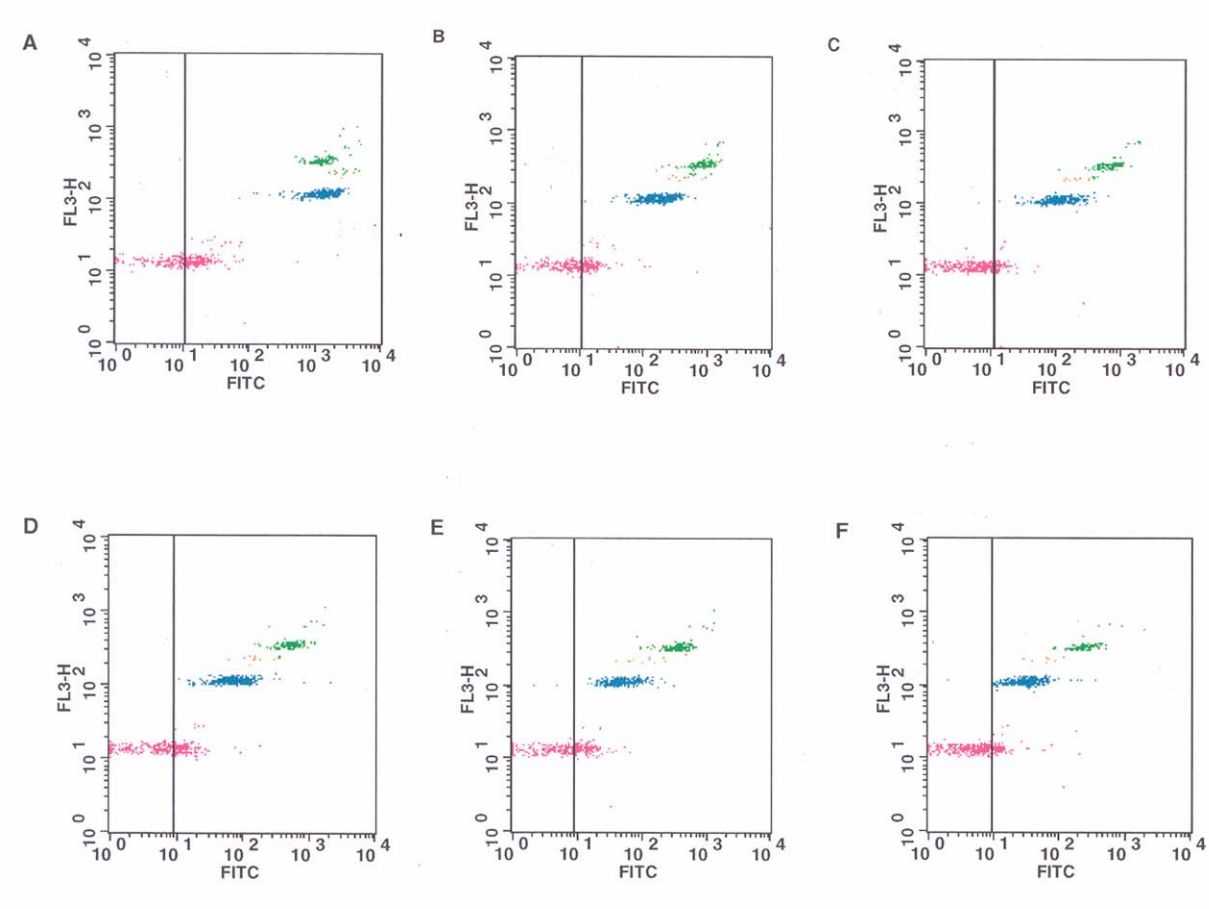
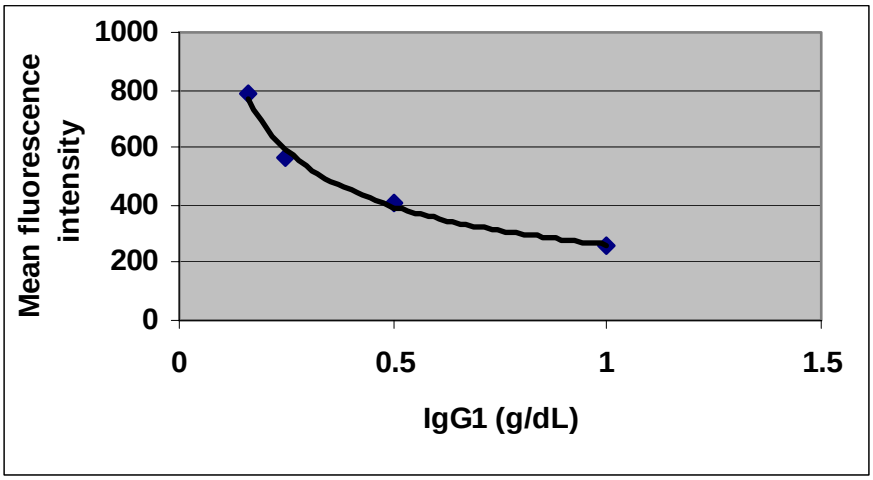


Figure 7. Flow cytometry dot plots (FL3-H vs. FL1-H) illustrating decrease in fluorescence intensity for IgG1 (green dots) and IgG2 (blue dots) coated beads with increasing concentration of inhibitor. No changes were observed in BSA (red dots). (A) Precolostral serum-no inhibitor (B) IgG1 too small to detect by SRID, IgG2-0.22 g/dL, (C) IgG1-0.16 g/dL, IgG2-0.45 g/dL (D) IgG1-0.25 g/dL, IgG2-0.85g/dL, (E) IgG1-0.5 g/dL,IgG2-1.4 g/dL, (F). IgG1-1g/dL, IgG2 ~3.4 g/dL.

A



B

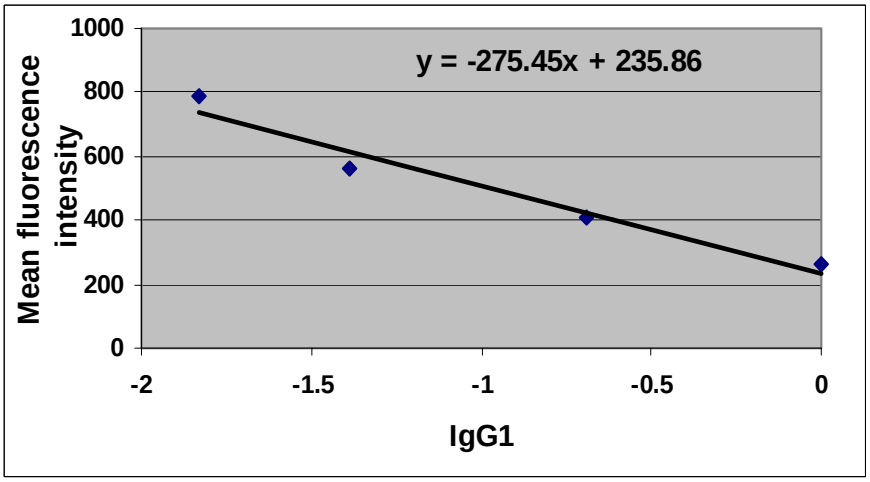
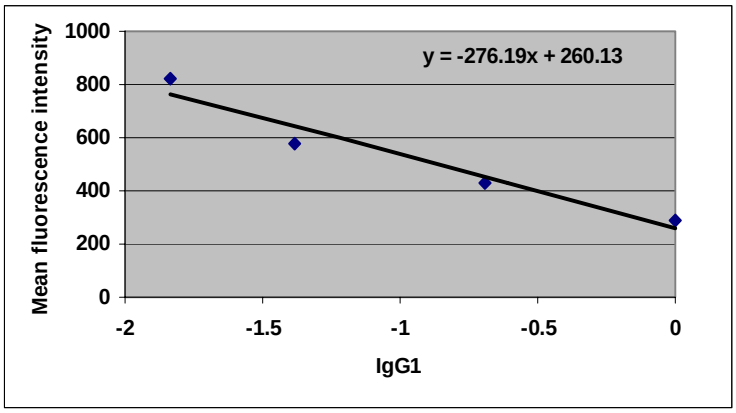


Figure 8. The standard curve and first order polynomial equation created by plotting IgG1 standard serum calibrants against mean fluorescence intensity of the IgG1 standards. (A) A nonlinear curve is observed when concentrations of IgG1 in g/dL plotted against mean fluorescence intensity. (B) Linear curve resulted when concentrations of IgG1 in x-axis were converted to natural log and the polynomial equation for the curve was created

A



B

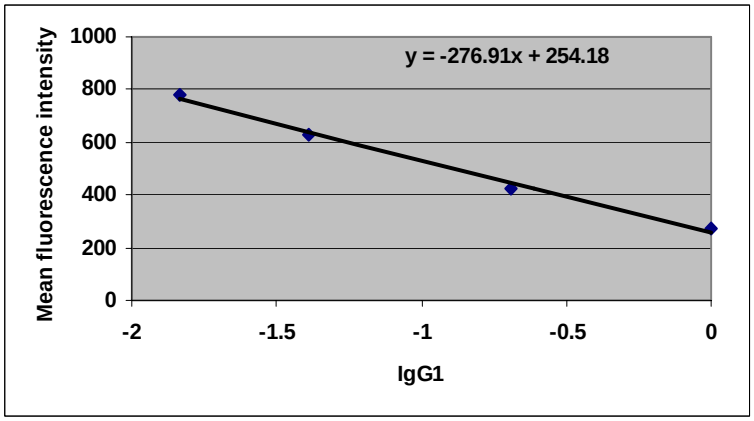


Figure 9. The standard curve and first order polynomial equation created by plotting concentrations of IgG1 standard serum calibrants in natural log in x-axis against mean fluorescence intensity of the IgG1 standards. The standard curves for the intraassay (A) and interassay (B) reproducibility experiments were linear with similar values for the slope.

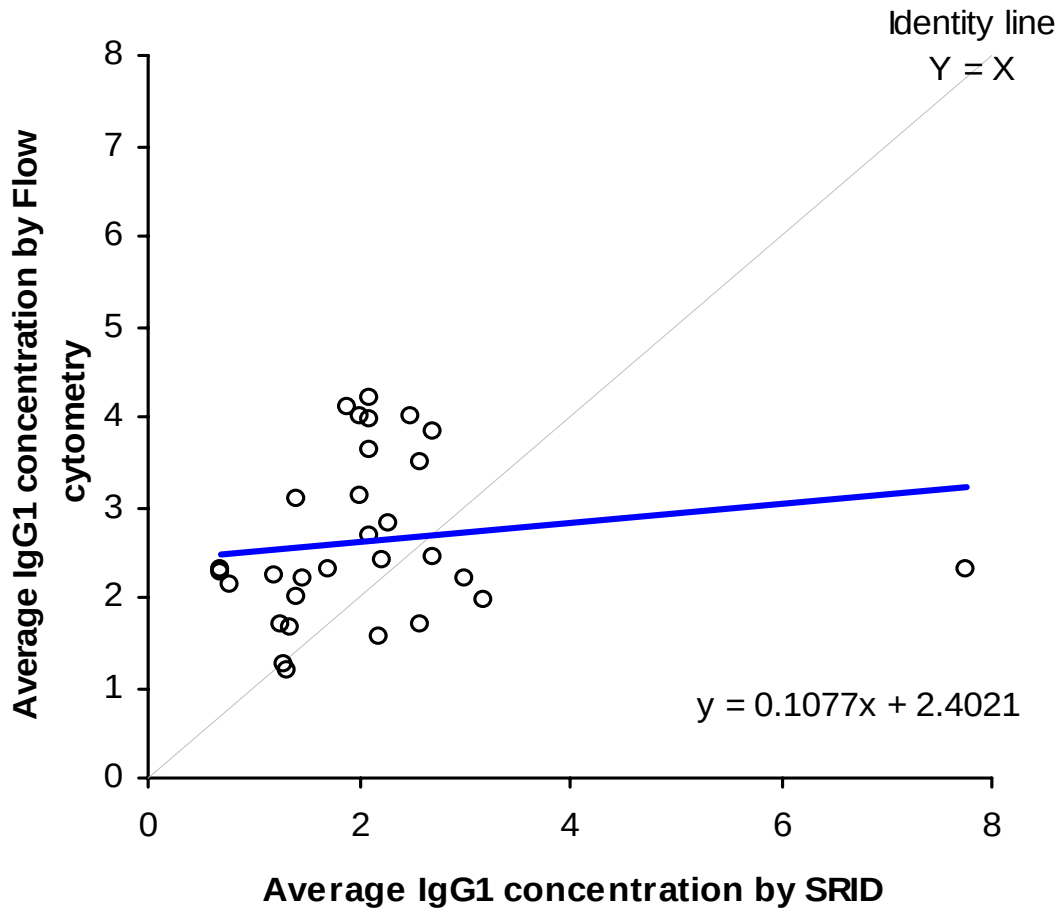


Figure 10. The average concentrations of duplicate samples for IgG1 determined by flow and SRID were plotted using Deming method comparison. The faint line represents the line of identity if the samples had the same concentrations. The dark line represents the best fit line, which is extremely altered because of the presence of an outlier.

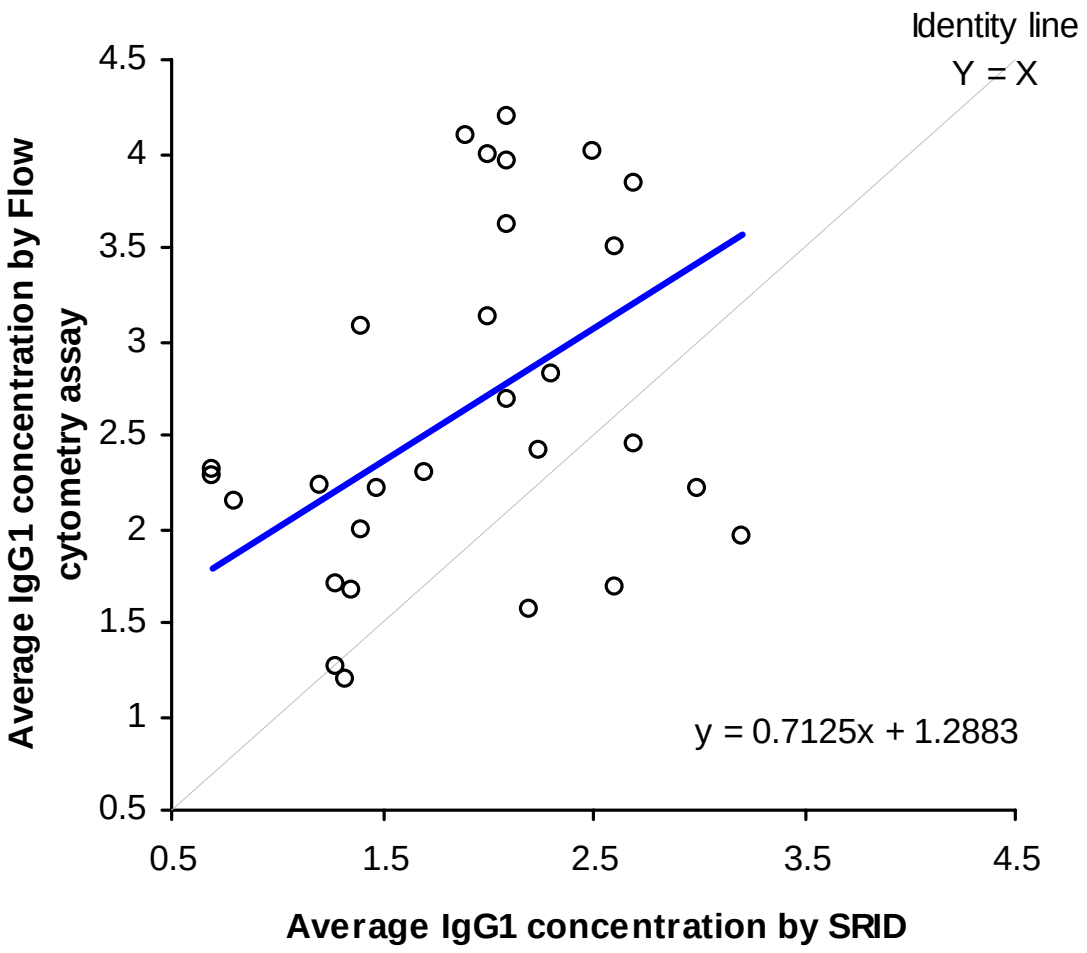


Figure 11. Average concentrations of duplicate samples for IgG1 determined by flow and SRID were plotted using Deming method comparison without outlier. The faint line represents the line of identity if the samples had the same concentrations. The dark line represents the best fit line, which shows a positive bias, constant and proportional bias. The mean fluorescence intensity of the duplicate measurements of SRID and flow is used for Deming method. The concentration of IgG1 determined by flow is higher than SRID.

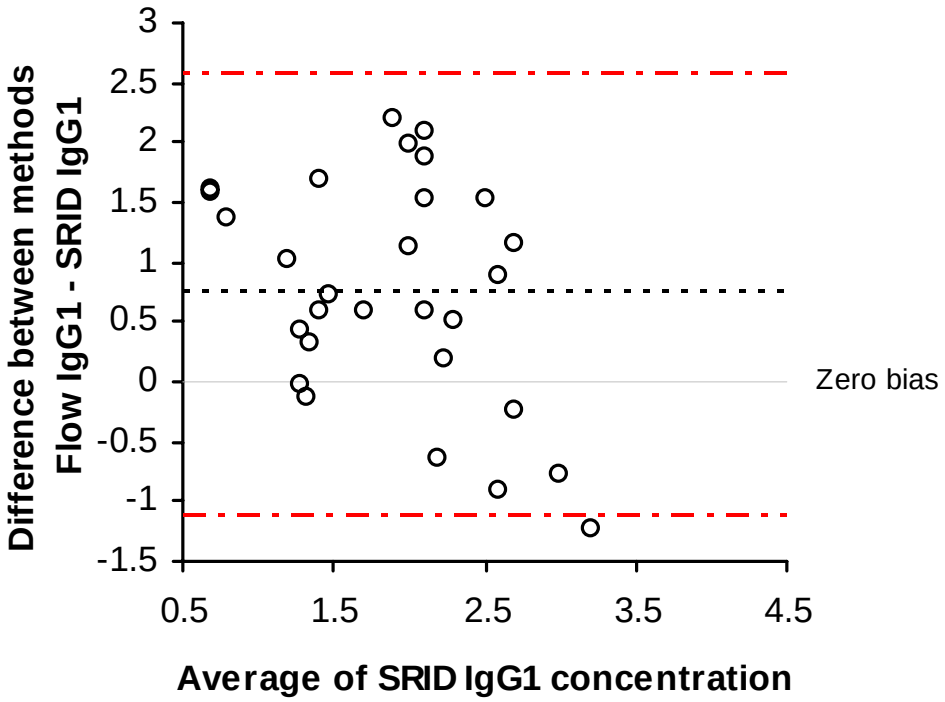


Figure 12. NCCLS Bias plot represents difference between methods compared to SRID. Differences were determined by subtracting the average of duplicate samples of the SRID from the average of duplicate samples from the flow cytometry assay. The short dashes represent positive bias of 0.743 g/dL. The short and long dashes represent 95% confidence interval of the bias (0.382 to 1.105 g/dL). One outlier was observed.

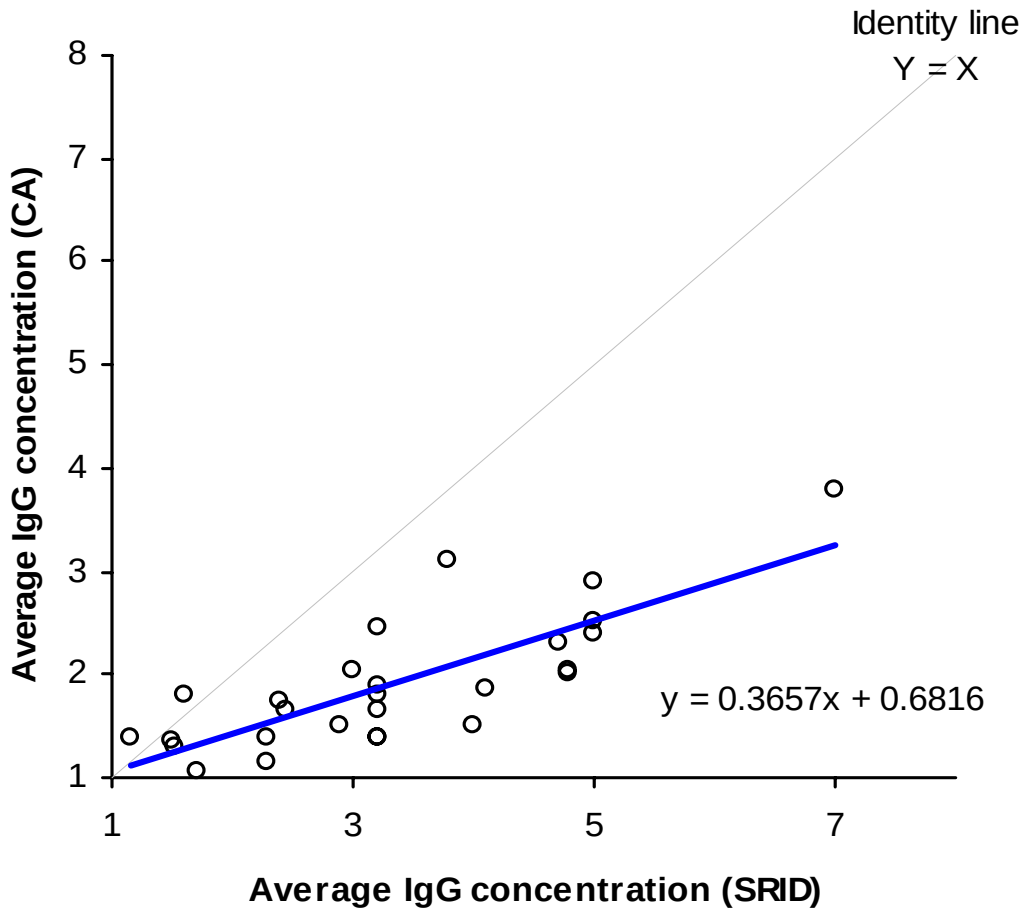


Figure 13. Average concentrations of duplicate samples for total IgG determined by cellulose acetate method and SRID were plotted using Deming method comparison. The faint line represents the line of identity if the samples had the same concentrations. The dark line represents the best fit line, which shows a negative bias, constant and proportional bias. The concentration of total IgG determined by SRID is higher than cellulose acetate method.

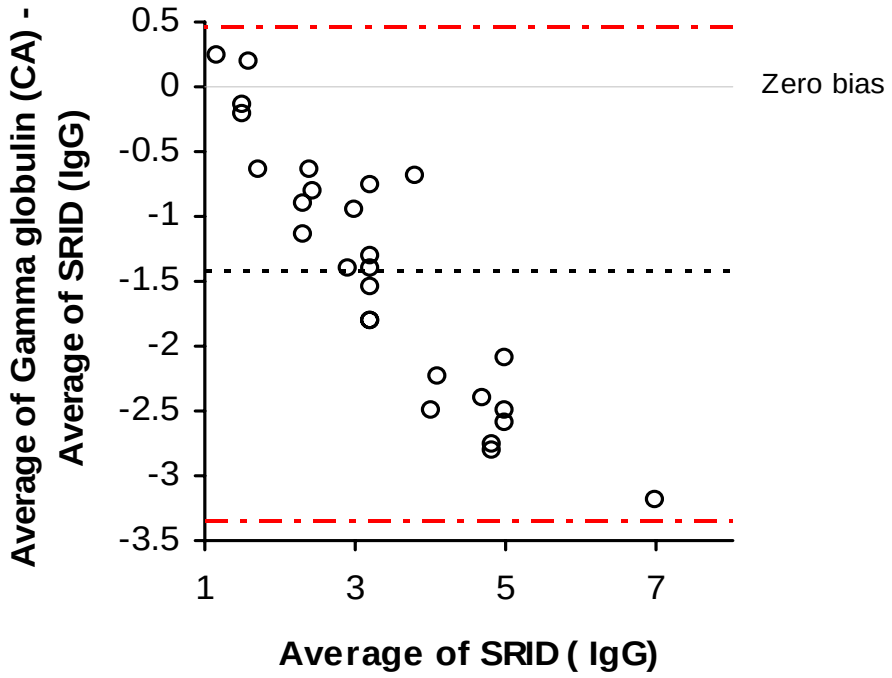


Figure 14. NCCLS bias plot represents the difference between methods compared to SRID. Differences were determined by subtracting the average of duplicate samples of the protein electrophoresis determined by cellulose acetate (CA) from the mean of duplicate samples from the flow cytometry assay. The short dashes represent negative bias of 1.4 g/dL. The short and long dashes represent 95% confidence interval of the bias (-1.8 to -1.05 g/dL).