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A Method for Differentiating Fetal Bovine Serum from Newborn Calf Serum

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Abstract

The continued use of animal serum as an important component in biotechnology manufacturing processes has raised questions regarding both the reliability of geographic origin and possible adulteration of product. The International Serum Industry Association (ISIA) has implemented a traceability certification program designed to demonstrate traceability from slaughterhouse or abattoir to the end-user. This is based on an audit performed by an independent, approved third-party auditor according to an approved audit plan, using a detailed audit checklist. Recent advances have led to the development of a complementary testing program to determine geographic origin of material. The methodology described in this paper differentiates fetal bovine serum from newborn calf serum on the basis of biochemical composition.

Serum and its Role in Cell Culture

Despite significant efforts to avoid the use of animal-source products, serum and other animal-derived products continue to play critical roles as culture medium supplements in the biomedical and biopharmaceutical arenas. Animal blood is a by-product of the meat industry and, as such, is sourced from animals fit for human consumption. Bovine-sourced products are the most commonly used. Serum, plasma, and other animal-derived materials from a large variety of animal sources are also used by the biopharmaceutical industry in daily applications including:

- Biomedical research
- Cell culture-based safety testing of pharmaceuticals and cosmetics
- Cell culture-based production of human and veterinary vaccines as well as therapeutic proteins
- Cell therapies

Serum Use

Serum has been used extensively as a growth medium supplement in cell culture for many years, and its use for this purpose has greatly contributed to the battle against disease, both human and veterinary. One of the most varied factors in culture systems is the growth medium, which provides the nutrients and/or the growth factors required for cell growth and product yield. Growth media are most often supplemented at concentrations of 5–20% serum by volume.

The choice of which serum is most appropriate for use in any application can be a difficult one, and the selection of the serum supplier can be important to ensure quality product is available. FBS provides the proper cell growth environment for many applications, but is not yet fully characterized. NBCS presents a less expensive alternative to FBS but is also appropriate for use in some applications. This difference in value, which is based on availability, could provide an incentive for misrepresentation. The ability to differentiate between these serum types would therefore be important, and would, among other aspects, help to ensure reproducibility of results. This capability has not been available in the past. ISIA has promulgated an industry-approved list of serum definitions.

ISIA Definitions of FBS and NBCS:

- **Fetal Bovine Serum** is obtained from the blood of fetuses of healthy, pre-partum bovine dams that have been deemed fit for human consumption through ante- and/or post-mortem veterinary inspection. It is collected in abattoirs inspected by the competent authority in the country of origin. There are no deletions or additions (including preservatives) allowed.
- **Newborn Calf Serum** is the liquid fraction of clotted blood derived from healthy, slaughtered bovine calves aged less than 20 days, deemed fit for human consumption through ante- and/or post-mortem inspection. It is collected in abattoirs inspected by the competent authority of the country of origin. There are no deletions or additions (including preservatives) allowed.

One of the goals of this study was to determine a biochemical fingerprint that could be used to individually identify these two types of bovine serum. A major difference between FBS and NBCS is the levels of circulating antibodies. This is due to the fact that colostrum, the first feed from mother to calf, is rich in antibodies which are essential for the establishment of the immune system of the calf. It has long been known that higher levels of immunoglobulin (IgG) are found in NBCS.[1] It is also known in other species that the birthing process can result in increased levels of other marker molecules.[2,3,4]

One of the simplest methods for assessing animal health

status is to run a general veterinary panel of tests on a serum sample. This panel will give an indication of the general health and function of various organs. A list of the tests usually performed is shown in **Table 1**. Such a panel is of particular interest because it provides useful markers for distinguishing between FBS and NBCS.

TABLE 1. Veterinary panel tests and focus.

Group	Measurements	Function
Electrolyte, Fluid, pH Balance	• Sodium • Anion Gap • BiCarb (HCO_3) • Chloride • Potassium	Indicators of dehydration, electrolyte, and acid/base balance. They are the main intracellular ions, and are responsible for the maintenance of osmotic pressure.
Vitamins and Minerals	• Phosphorus • Calcium • Iron • Magnesium	Important for synthesis of hemoglobin and activation of numerous enzymes and hormones.
Renal and Pancreas Function, Injury	• Creatinine • Creatine Kinase (CK) • Glucose	Abnormal values indicate skeletal muscle damage. Includes assessment of kidney function.
Lipid	• Cholesterol • Triglycerides	Critical role in the function of the cell membrane. Lipids are precursors to hormones and aid in the absorption of nutrients from food.
Liver	• Aspartate Aminotransferase (AST) • Alkaline Phosphatase (ALP) • Gamma-Glutamyl Transferase (GGT) • Bilirubin, Total	Enzymes and protein that indicate soft tissue, liver, and bile duct damage.
Proteins	• Protein, Total • Albumin • Globulin • Immunoglobulin G	Used to identify serum protein disorders. Albumin provides 80% of osmotic activity in plasma and serves as a transport protein in metabolic processes. Changes in alpha globulin indicate acute phase of inflammation. Beta fractions of globulin are part of complement, transferrin, c-reactive protein, and some immunoglobulin. IgG is a type of antibody that is produced by the immune system.[5]

Methods

Biochemical Profile of FBS and NBCS

Each unique FBS or NBCS sample was the result of the standard, multi-animal pool manufacturing process. Where indicated, samples were either filtered, finished product or unfiltered, unprocessed raw serum, as processing did not appear to affect the results obtained. Statistical analysis was performed using GraphPad InStat Version 3.1 for Microsoft Windows. A *P*-value less than 0.05 was considered statistically significant.

Thirty-nine FBS samples and 32 NBCS sample were obtained from five ISIA-certified traceable suppliers. Of these, 55 samples were submitted to a third party to be aliquoted, blinded, and directly submitted to the testing facility while

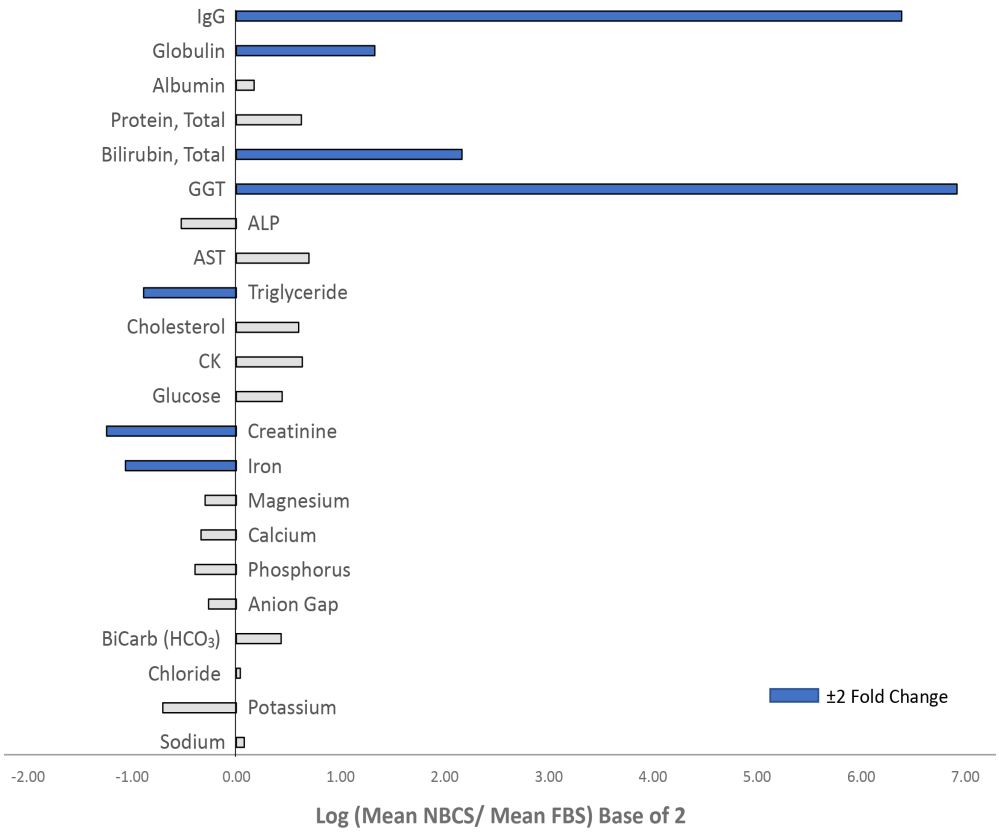

FIGURE 1. Biomarker comparison to analyze distinction between NBCS and FBS. For this comparison, the mean values from Table 1 are compared to show fold differences.

16 samples were directly submitted (without blinding) for testing. The geographic origin of source material included United States (US), Mexico, Australia, New Zealand, and Costa Rica. Unless otherwise indicated, serum biochemical determination was performed by Colorado State University (CSU) Diagnostic Laboratory (Fort Collins, Colorado USA) using the Roche cobas c 501 Analyzer. Serum IgG determination by enzyme-linked immunosorbent assay (ELISA) was performed by ZeptoMetrix Corporation (Buffalo, New York USA). The panel of 22 analytes included electrolytes, pH, vitamins, minerals, indicators of renal and pancreatic function, lipids, liver function, and proteins, as outlined in **Table 1**. Mean, standard deviation (SD), and *P*-value (as calculated by the Mann-Whitney test) for each analyte are summarized in **Table 2**.

In order to identify which of the biochemical properties of NBCS were distinct from FBS, a comparison of the difference in the mean value of the results was performed. To determine the increase or decrease in level of analytes, the mean value for NBCS was divided by the mean value for FBS for each analyte, and transformed data (logarithm to base 2) were plotted as shown in **Figure 1** (previous page).

Analytes exhibiting a fold change ≥ 2 are indicated in blue. Differences to the right of zero and ≥ 2 indicate that the NBCS value was greater than that for FBS (*i.e.*, GGT, IgG, total bilirubin, and globulin). Changes to the left of zero and ≤ 2 indicate that the NBCS result was less than that for FBS (*i.e.*, triglyceride, creatinine, and iron). The fold difference was statistically significant for GGT at 121.3 and IgG at 83.9. The maximum GGT value of the sample population for FBS was 5 IU/L. In contrast, the minimum GGT value of the sample population for NBCS was 157 IU/L. GGT was the analyte with the highest difference seen and became the focus of further experiments.

High GGT and IgG were found to have statistically significant associations with NBCS. A GGT of >100 was associated with NBCS while that for FBS was <10. FBS is characterized as having a significantly lower IgG when compared to NBCS but showed a wider range of values when compared to GGT ranges. Although a

TABLE 2. Comparison of veterinary panel results for FBS and NBCS. "*N*" indicates the number of samples tested.

Function	Analyte	Serum	*N*	Mean	SD	*P*-Value
Electrolyte, Fluid, pH Balance	Sodium mEQ/L	FBS	39	132.03	14.68	0.1353
		NBCS	32	139.19	24.25	
	Potassium mEQ/L	FBS	39	11.84	1.81	< 0.0001
		NBCS	32	7.26	2.50	
	Chloride mEQ/L	FBS	39	93.37	11.75	0.3010
		NBCS	32	95.88	18.97	
	BiCarb (HCO_3) mEQ/L	FBS	39	16.84	6.21	0.0004
		NBCS	32	22.70	6.25	
	Anion Gap mmol/L	FBS	39	33.72	6.26	0.0164
		NBCS	32	28.03	10.53	
Vitamins and Minerals	Phosphorus mg/dL	FBS	39	10.19	2.97	< 0.0001
		NBCS	32	7.74	2.06	
	Calcium mg/dL	FBS	39	13.22	1.76	< 0.0001
		NBCS	32	10.45	3.12	
	Magnesium mg/dL	FBS	39	3.09	0.44	0.0006
		NBCS	32	2.51	0.96	
	Iron µg/dL	FBS	39	179.77	33.59	< 0.0001
		NBCS	32	85.91	14.32	
Renal and Pancreas Function, Injury	Creatinine mg/dL	FBS	39	2.49	0.46	< 0.0001
		NBCS	32	1.05	0.34	
	Glucose mg/dL	FBS	39	102.77	29.35	0.9080
		NBCS	32	139.43	111.56	
	CK IU/L	FBS	39	178.74	75.32	0.0006
		NBCS	32	277.34	138.47	
Lipid	Cholesterol mg/dL	FBS	39	28.33	7.07	< 0.0001
		NBCS	32	42.91	7.66	
	Triglyceride mg/dL	FBS	39	66.85	11.35	< 0.0001
		NBCS	32	36.06	16.17	
Liver	AST IU/L	FBS	39	32.10	14.21	< 0.0001
		NBCS	32	52.06	23.31	
	ALP IU/L	FBS	31	213.65	65.17	< 0.0001
		NBCS	24	148.04	34.35	
	GGT IU/L	FBS	39	3.64	1.14	< 0.0001
		NBCS	32	441.56	188.57	
	Bilirubin, Total mg/dL	FBS	39	0.14	0.06	< 0.0001
		NBCS	32	0.62	0.28	
Proteins	Protein, Total g/dL	FBS	39	3.52	0.45	< 0.0001
		NBCS	32	5.42	0.80	
	Albumin g/dL	FBS	39	2.47	0.33	< 0.0001
		NBCS	32	2.78	0.44	
	Globulin g/dL	FBS	39	1.05	0.16	< 0.0001
		NBCS	32	2.63	0.44	
	IgG µg/mL	FBS	39	183.86	135.65	< 0.0001
		NBCS	32	15425.81	8854.20	

specification level for IgG has not yet been set by ISIA, it is more commonly reported on manufacturer's FBS certificates of analysis (COA). This analysis shows that in instances where IgG is >320 µg/mL (+1 SD from the mean), then an evaluation of GGT would be a determining factor for characterizing serum.

Variation

Five FBS and three NBCS samples of US origin were submitted to nine separate facilities for GGT analysis. Samples were transported on dry ice and stored frozen until the initial analysis was performed. Samples were then refrigerated until a re-evaluation could be performed 24 hours later.

In **Figure 2**, *N* was adjusted for instances where analysis was not performed 24 hours after initial evaluation. Analytical facilities included: CSU Diagnostic Lab, Cornell University Animal Health Diagnostic Center (Ithaca, New York USA), University of Illinois at Urbana-Champaign College of Veterinary Medicine (Urbana, Illinois USA), University of Missouri College of Veterinary Medicine (Columbia, Missouri USA), Oregon State University Diagnostic Laboratory (Corvallis, Oregon USA), Rocky Mountain Biologicals (Missoula, Montana USA), University of California Davis (UC Davis) Veterinary Medical Teaching Hospital (Davis, California USA), Utah State University Veterinary Diagnostic Laboratory (Logan, Utah USA), and Virginia Tech Animal Laboratory Services (Blacksburg, Virginia USA). Chemical analysis was performed using six analyzers: Beckman AU400, AU480, and AU680; Roche Modular P and cobas c 501; and Siemens Dimension Xpand.

GGT results obtained by the different analyzers are summarized in **Figure 2A** for FBS and **Figure 2B** for NBCS. A statistically significant difference was observed for FBS using the Siemens Dimension Xpand when compared to the Beckman and Roche analyzers. Maximum FBS GGT for the Beckman and Roche analyzers was 7 IU/L. No statistical significance with NBCS was observed for all the analyzers. Analysis was performed using Kruskal-Wallis test (nonparametric one-way analysis of variance [ANOVA]). There was a statistically higher amount of FBS GGT, as determined

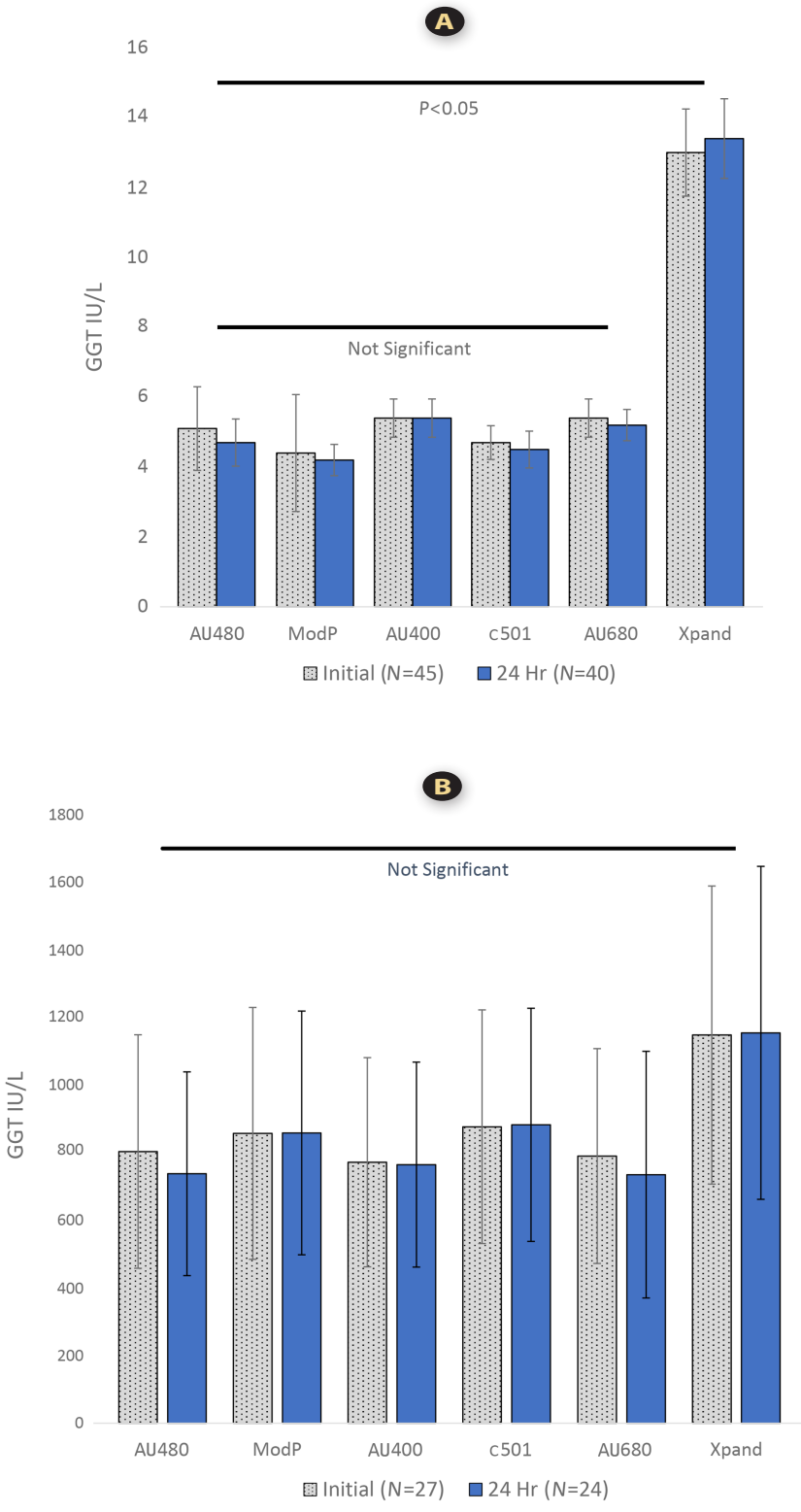

FIGURE 2. Comparison of GGT results from six different analyzers for: **(A)** FBS; and **(B)** NBCS.

by the Xpand analyzer. Because this observable significant difference could not be attributed to the method or time, results from the Xpand were excluded from future experiments.

Variation in Results

FBS GGT data obtained using the Roche cobas c 501 analyzer are summarized in **Table 3**. The data set includes five samples analyzed at two locations, CSU and UC Davis, over a period of 24 hours. There was no significant difference in the mean results for GGT tested at these diagnostic facilities ($P>0.05$) when analyzed using nonparametric ANOVA and Dunn's multiple comparison. No significant difference was observed in GGT ($P>0.999$, unpaired *t*-test) when samples were re-analyzed within a 24-hour period. There was no significant variation in GGT results based on testing facility, time of analysis, or the Beckman and Roche analyzers.

The Siemens Dimension Xpand analyzer gave statistically significant differences in measurements of GGT in FBS, but not for NBCS. Variations between Beckman and Roche analyzers were not statistically significant for FBS or NBCS. GGT values were reproducible regardless of the testing facility and not susceptible to variation, suggesting that this might be the most useful marker for differentiating NBCS from FBS. It should be stressed that if FBS GGT is analyzed using the Siemens Dimension Xpand, values over 10 IU/L will be reported.

Detecting NBCS in FBS

Three NBCS samples were diluted into FBS from 5–20% (in increments of 5%) by volume. Diluted samples were submitted to the CSU diagnostic facility for GGT determination by the cobas c 501 analyzer.

Figure 3A shows the results of the mixing experiments. The average GGT level detected was plotted against the percentage of NBCS dilution. This suggested a strong linear relationship between the percent dilution of NBCS and the corresponding GGT measurement ($R^2=0.9994$). In order to further analyze statistical variance, the sum of the squares of the deviations from the mean (SDM) was calculated to be 9.113, indicating little variance. The predicted GGT value of the various levels of mixtures was determined

TABLE 3. Summary of GGT results obtained by two facilities over 24 hours using the Roche cobas c 501 analyzer.

c 501 Analyzer	Initial		24 hr		Mean
	CSU	UC Davis	CSU	UC Davis	
Sample 1	4.00	4.00	5.00	4.00	4.25
Sample 2	5.00	5.00	5.00	5.00	5.00
Sample 3	5.00	5.00	5.00	5.00	5.00
Sample 4	5.00	4.00	4.00	4.00	4.25
Sample 5	4.00	5.00	5.00	4.00	4.50
CSU vs. UC Davis	$P>0.05$	$P>0.05$	$P>0.05$	$P>0.05$	Dunn's*
Initial vs. 24 hr	$P>0.999$		$P>0.999$		*t*-test**

*Non-parametric ANOVA with Dunn's post-test. **Unpaired *t*-test.

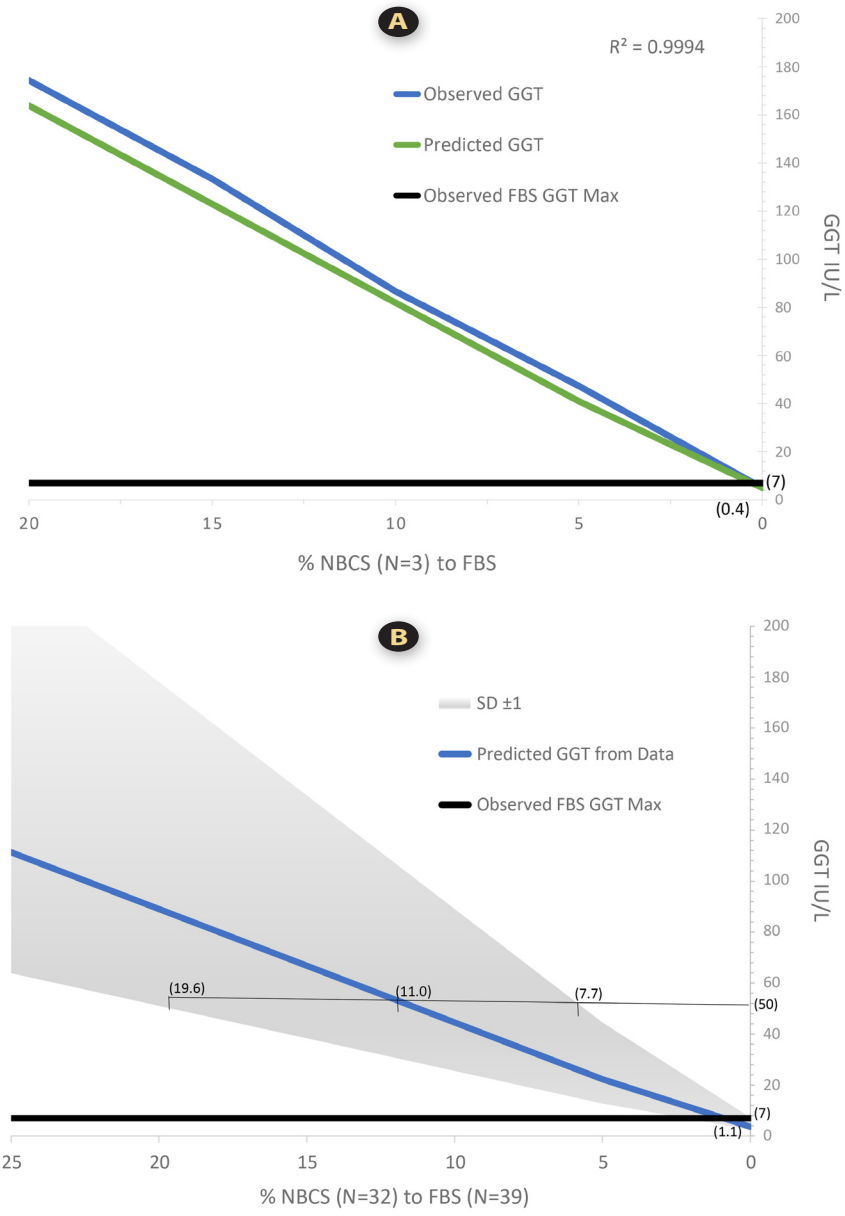

FIGURE 3. Determination of GGT levels in FBS containing up to 20% NCBS: **(A)** Observable GGT; and **(B)** Predicted GGT.

by adding the mean GGT value of 100% NBCS (814.33 IU/L) and FBS (5 IU/L) and multiplying by the dilution factors. Analysis confirmed that there was excellent agreement between the predicted and the observed GGT values. The maximum observed value of all the FBS samples analyzed was 7 IU/L, and was calculated excluding data generated by the Siemens Dimension Xpand. In these experiments, 7 IU/L would equate to a NBCS concentration of 0.4092%.

To determine if the percentage of NBCS in a mixture could be calculated from reported GGT values, GGT results at various percentages were calculated based on the average sample results for NBCS (441.56 IU/L) and FBS (3.64 IU/L) (**Table 2**). The slope was 4.342, the Y intercept was 2.145, and there was an R^2 value of 0.9993. The percent of NBCS diluted in FBS can be calculated using reported values of GGT, and the slope and Y intercept from the average sample results. For example, a GGT result of 50 IU/L would intercept this standard curve at 11.02%. A reasonable assumption would therefore seem to be that a GGT result of 50 IU/L could contain 8–20% NBCS (+/– 1 SD). In **Figure 3B** (previous page), the maximum observed value for GGT of 7 IU/L is shown for reference and intercepts at 1.15%.

In this study, we categorized FBS as having GGT values of < 10 IU/L. Reported GGT values over 10 IU/L in FBS would strongly suggest that the material is not entirely derived from fetal origin.

Conclusions

For many years, low levels of IgG were thought to be a suitable marker for FBS.[3,6] This has been questionable, based on the wide range of levels observed, and also the potential for removal of IgG from serum using protein A chromatography. In fact, commercially available ultra-low IgG FBS can be obtained from several suppliers.

Earlier work[3,4] indicated the presence of GGT in bovine serum. GGT was a potential indicator for passive transfer of immunity and might be an indicator of neonatal vs. fetal origin. The work discussed here strongly indicates that GGT can be used to distinguish NBCS from FBS. The presence of NBCS in FBS may be detected using this method, based on our analysis showing higher than expected GGT levels. Therefore, the method may be useful for detecting the adulteration of FBS by dilution in NBCS. ISIA will be working in the very near future to recommend specifications for IgG and GGT levels for FBS based on the results of this analytical study.

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