

Calf Notes.com

Calf Note #135 – On methods of IgG analysis

Introduction

I recently had an interesting conversation with a colleague and friend regarding variation in test results from a colostrum feeding study that he conducted. In this study, my colleague fed calves two different amounts of colostrum (2 L and 4 L) to which was added a compound (0 or 1 gram per feeding) that was expected to improve the apparent efficiency of absorption of IgG in the calves. The exact nature of the compound isn't important here, since it's not available commercially and it didn't work anyway.

My colleague (we'll call him John) sent his colostrum and blood samples to an outside lab to measure the IgG concentration of the colostrum and serum samples from the calves. Well, the lab completed their tests and sent the results back. When John saw the results, he didn't believe them, since the serum results seemed a little lower than expected and the colostrum samples were far more variable (some high, some low) than he expected. So, John sent a set of samples to a second lab for confirmation. The results that came back only made matters worse. Serum samples from the second lab were consistently lower than the first lab and colostrum samples were much more consistent, but were all higher than the first lab! Well, as you might imagine, John was confounded and upset at the variability in the results. He expected that the results of the testing should all be the same – and they weren't.

So, what's going on? Before we get into the specifics of these two labs and what they did, let's discuss a little bit about testing for immunoglobulins and the nature of the tests that we currently use.

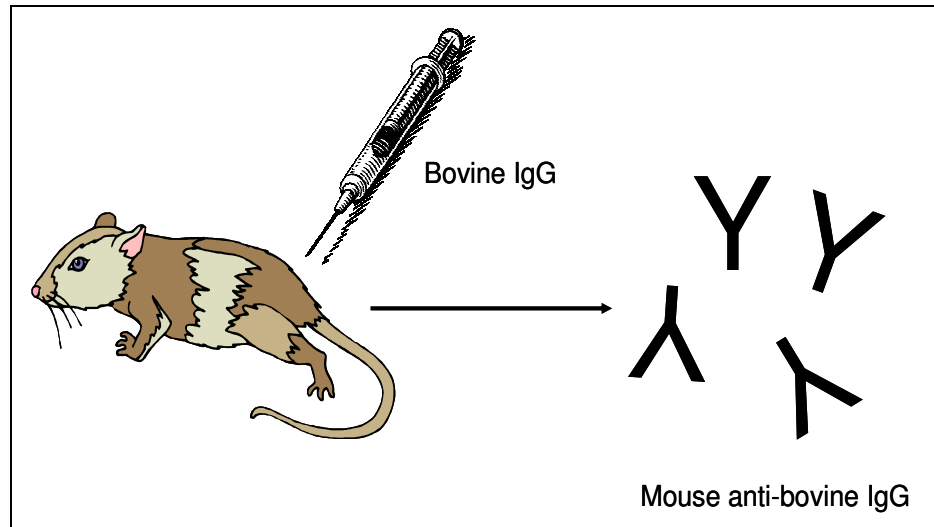
Immunoglobulin testing

There are a number of methods used to measure the IgG content of various substances. The most commonly used procedure is called radial immunodiffusion (**RID**). Other popular methods include turbidimetric immunoassay (**TIA**) and enzyme linked immunosorbent assay (**ELISA**).

All of these studies are based on a similar concept. That is, it is possible to raise antibodies to antigens and use these raised antibodies for diagnostic purposes. Let's have a look to see what I mean. In figure 1, we start with a healthy animal – in this case a mouse. Goats, rats, mice and horses are commonly used animals. Rats and mice are cheap, easy to maintain and can give you very specific anti-IgG whereas goats and horses can provide lots of serum at a low cost. These animals are injected with purified preparations of antigens from *another species* – in our case, antigens from cows. So, if we inject the mouse with bovine IgG, after a period of time, the mouse will develop an immune response (i.e., will produce antibodies) to the injected IgG. The serum of the mouse will then contain *mouse anti-bovine IgG*. We can then collect the serum and use the mouse anti-bovine IgG in our assays.

When we mix bovine IgG from serum of our newborn calves along with mouse anti-bovine IgG, the two antibodies should react with each other. And, depending on the test, we can use this interaction to determine how much bovine IgG is in our calves.

The RID test is relatively straightforward, where anti-bovine IgG is mixed with agar (a kind of gelatin derived from seaweed) and poured into plates. Small holes are then cut into the plates and the liquid sample is then placed into the holes.



Over the next several hours (an RID to measure IgG will take about 24 hours), the calf serum will diffuse through the agar. As the bovine IgG interacts with mouse anti-bovine IgG, it will form a precipitate which appears as a cloudy white circle around the hole.

A depiction of the RID technique is at: <http://www.lib.mcg.edu/edu/esimmuno/ch4/radial.htm>. We can then read the size of the circles and calculate the amount of bovine IgG in the sample.

The TIA procedure uses a similar theory (bovine IgG interacting with anti-bovine IgG), but in a liquid medium rather than using agar. This gives the TIA technique the advantage of being much faster and it can be automated. Rather than reading ring diameters (as is done with RID), the turbidity (cloudiness) of the solution can be measured by machine, which can dramatically increase the number of samples that can be done in a day.

ELISA techniques are more flexible and complex. There are many different approaches to the ELISA technique. It has many advantages compared to other methods, including speed, accuracy and being able to measure very small quantities of IgG. It is a more complex method and requires greater technical skill and equipment compared to the other techniques. Therefore, it tends to be less widely used in measure total IgG concentration in calf serum studies.

The research

So, back to the research. Why was there so much difference between the two labs in both colostrum and serum IgG concentrations reported to John? How did he reconcile these differences?

Let's look at the colostrum results first. As you may recall, the first lab reported lower and less consistent results compared to the second lab. Bovine colostrum contains lots of "stuff" that can affect how the bovine IgG migrates through the agar in an RID assay. Large amounts of fat, casein

and other compounds can affect the IgG/anti-IgG complex, also. Also, when a sample is received at the lab, how should it be processed to make sure the analyses are correct? Should the fat be removed prior to the analysis to keep it from interfering with the assay? Should it be included to better reflect the content of IgG in the whole colostrum? Well, this was the crux of the difference between the two labs. The first lab received the frozen colostrum samples, allowed them to thaw at room temperature, mixed them (blending the fat and protein fractions of the colostrum) and placed a sample onto the RID plate. The second lab received the sample, thawed it and took a sample of the liquid from under the fat layer. This effectively eliminated the fat from the analysis. Therefore, the IgG assay values were higher, but were based on the fat-free content of colostrum, not on the whole colostrum. To compare these values precisely, John would have to know the amount of fat in the colostrum samples and calculate the IgG in the whole colostrum.

In 1981, Fleenor and Stott reported considerable differences in results in IgG from colostrum samples that were processed using different methods. Their recommendation was that whole colostrum (not fat-free or processed) should be used. If samples have to be diluted (and samples of colostrum with IgG > 50 g/L usually have to be diluted), they should be diluted using a sample of milk that is free of IgG. Serum samples should be diluted in serum also free of IgG.

The point of the differences between labs is that there is no single agreed on standard method of measuring IgG in colostrum by RID or TIA. Some labs have more consistent when they remove potentially interfering substances, whereas other labs like to keep the assay as simple as possible.

Serum IgG samples also varied by lab. John wanted to compare the results from the two labs and prepared Figure 2. Note that the results were highly correlated – i.e., as the results from one lab increased, so did the results from the other lab. The degree of relationship, called the r^2 , was 0.96, which indicates that 96% of the variation in one can be explained by the other. This is excellent and indicates – in reality – that either of these tests can accurately predict the IgG concentration in serum samples.

The equation in the graph indicates that $Y = 1.1025X$. This means that the RID measurement is consistently 10.25% higher than the results estimated by the TIA method. For example, if a TIA measurement was 10 g/L in the serum of the calf, the RID assay would be $10 \times 1.1025 = 11.025$ g/L. (NOTE: for full disclosure to you statistical purists, I did force the intercept through the origin, which did reduce the r^2 very slightly).

Why the difference in results? It's important to remember that the RID and TIA methods differ in important ways. The assays used by the labs differed not only by the technique, but also by the antibody used (one used goat the other, horse). The buffer system used in the TIA was (of course) different from the agar matrix of the RID, also.

So, which one of these assays is “right” – i.e., is the serum IgG 10 g/L or 11? Or, put another way, is there enough difference between these assays to worry about? Well, if you're interested in

Table 1. Bovine IgG in five lots of frozen, pooled colostrum.

Sample	Lot	Lab 1	Lab 2
1	1	51.3	67.5
2	1	33.4	62.7
3	2	17.5	46.5
4	2	45.5	50.2
5	3	102.7	110.5
6	3	98.5	114.0
7	4	33.0	45.8
8	4	30.4	49.5
9	5	66.1	70.4
10	5	71.5	72.6

Samples 1 and 2, 3 and 4, etc., were from the same pool of colostrum.

declaring whether or not calves have “FPT” – i.e., 10 g/L or higher, then the difference between the method could be significant. If we calculate FPT from John’s study, using the RID results declared that 35% of the calves had FPT, whereas 47% of the calves had FPT is we used the TIA results. On the other hand, as a producer, the difference between 10 and 11 g of IgG in the serum of a calf at 24 hours of age may not be terribly significant.

Is there a “best” test? In my opinion, no. Any of the methods – RID, TIA, ELISA (and some others) can be very accurate and precise. Although RID has long been considered the “gold standard for testing” there are several problems with RID and it may not be optimal under all situations (e.g., colostrum testing). Other methods – particularly TIA – has the advantage of high throughput with low cost, which makes large scale measurement of IgG reasonable. Several researchers have reported that TIA is as accurate as RID in measuring serum IgG concentrations (Davis et al., 2005; Etzel et al., 1997; McCue, 2007).

Some recommendations on understanding assays and reporting assay

Generally, it’ll suffice to know if the lab is reputable and follows a standard procedure for RID, TIA or ELISA methods. Working with one lab consistently can help you minimize the variation that can occur between labs and test methodologies. Talk to their technical people to make sure they have enough experience doing the assays and they have appropriate standards and internal control methods in place. If you’re really curious about the repeatability of their serum IgG tests, you can do this – collect serum from several calves (or a cow) so that you have 50-100 ml. Mix it all together and place it in separate tubes containing 2-3 ml of serum. Freeze them. From time to time, send in one of these tubes in with your test samples. These results (called an internal control) should be reasonably consistent (within 10-15%) every time. If these results vary, then it’s time to contact the lab.

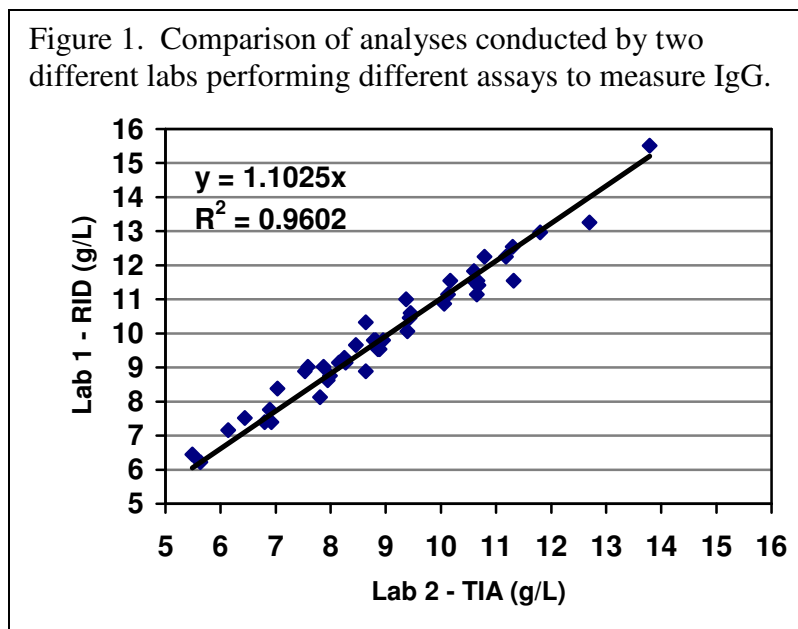
If you’re interested in more details, here are some of the things that you’ll need to know if you really want to understand IgG results from a laboratory conducting these assays.

Sample handling and preparation

1. What happens to the samples after they arrive?
2. How long are they stored?
3. How are they thawed?
4. What type of pre-assay processing takes place?

Antibody selection

1. In what animal are the anti-bovine IgG raised?
2. What is the specificity of the antibody?
3. Does the antibody recognize few or multiple epitopes?
4. Is the antibody specific for IgG1, IgG2 or both?



IgG assay

1. What type of assay was done (ELISA, TIA, RID)?
2. What were the conditions (buffers, diluents, times, temperatures)?
3. How were the concentrations read and calculated?
4. What were the standards used and in what concentration?
5. What was the inter-assay CV and intra-assay CV?

Bottom Line

The take home message from my conversation with my colleague was that assays to measure immune parameters are not as simple as many other analytical techniques. There's still some art to the development of a good immunoassay and there can be some variation from lab to lab and assay to assay. If we try to hard to parse small differences among assays, we may be disappointed by the variation that is typical with assays and laboratories. I recommended to John that he take the TIA results and RID results and average them as the "real" results. This would incorporate both assays into one number.

In a production setting, differences among assays (assuming they're done properly) is unlikely to be important from the calf's health standpoint. My recommendation is to find a lab that is economical, willing to work and talk with you, provides good turn around service and is consistent. Discuss your needs and don't get too bogged down on small differences. As a producer, you're really after those calves that are at greater risk of disease due to poor colostrum management. Generally, these are the calves that will have very low concentrations of IgG.

References

- Davis, D. G., D.M.W. Schaefer, K. W., Hinchcliff, M. L. Wellman, V. E. Willet, and J. M. Fletcher. 2005. Measurement of serum IgG in foals by radial immunodiffusion and automated turbidimetric immunoassay. *J. of Vet. Internal Med.* 19:93-96.
- Etzel L.R., R. E. Strohbehn and J. K. McVicker JK. 1997. Development of an automated turbidimetric immunoassay for quantification of bovine serum immunoglobulin G. *Am. J. Vet. Res.* 58:1201-1205.
- Fleenor, W. A., and G. H. Stott. 1981. Single radial immunodiffusion analysis for quantitation of colostrum immunoglobulin concentration. *J. Dairy Sci.* 64:740-747.
- McCue, P.M. 2007. Evaluation of a turbidimetric immunoassay for measurement of plasma IgG concentration in foals. *Am. J. Vet. Res.* 68:1005-1009.

Written by Dr. Jim Quigley (13 July 2008)
© 2008 by Dr. Jim Quigley
Calf Notes.com (<http://www.calfnotes.com>)