

Herd-Based Biological Testing for Metabolic Disorders

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■ Take Home Messages

- Herd-based testing can be used as part of an overall diagnostic scheme for solving herd problems.
- Biological test results do not stand alone, but must be corroborated by other herd data.
- Tests must be interpreted in light of the biology they evaluate; some are interpreted as the proportion of cows above or below a threshold, and others are interpreted as means.
- Minimum sample sizes are about 12 cows for proportional outcomes and 8 cows for mean outcomes.

■ Biological Testing Basics

Biological tests that I have found useful in herd nutritional investigations are ruminal pH, serum beta-hydroxybutyric acid (BHBA), plasma non-esterified fatty acids (NEFA), urinary pH, and milk or blood urea nitrogen (UN). Biological tests can be very useful in assessing problems found in nutritional management, ration formulation, or disease incidence. However, they are subject to error due to sample size, sample handling, time of collection relative to feeding, and laboratory error. Thus, biological test results generally do not stand alone, but should be supported by other data from the herd investigation.

Interpreting Test Results – Individual Animals or Groups?

The interpretation of herd-based nutritional tests is very different from interpreting laboratory results from individual cows. Interpretation of individual animal test results is straightforward – just compare the animal's lab value to a "normal" range established by the laboratory that did the testing (usually a 95%

confidence interval of test results from 100 or more clinically normal animals). Interpretation of group results requires a different mind-set. Normal ranges for individual animals as defined by laboratories are not necessarily pertinent. Rather, different standards for “normal” values in groups of animals must be defined by research done on groups of animals. Also, the appropriateness of the sample size must be considered (i.e., was the sample size large enough to give an adequate representation of the entire group?).

Interpreting Test Results – Proportions or Means?

The first question to ask in interpreting biological test results is whether we want to interpret the mean test result, or the proportion of animals above or below a certain cut-point. The biology of the disease we are trying to diagnose determines which interpretive approach is the most appropriate. Ruminal pH, BHBA, and NEFA are tests for diseases in which animals are affected only when they are above or below a certain biological threshold (cut-point). For example, ruminal pH ≤ 5.5 (for fluid collected by rumenocentesis) puts cows at risk for subacute ruminal acidosis (SARA) with subsequent rumenitis and other complications (Garrett and others, 1999). Ruminal pH values above 5.5 are considered “normal” in that they do not put animals at risk for SARA. So, we are not interested in interpreting mean ruminal pH values, but rather in interpreting the proportion of animals with ruminal pH below the 5.5 cut-point.

The BHBA and NEFA test results are likewise interpreted on a proportional basis. The BHBA test is used to detect subclinical ketosis. Research has identified 14.4 mg/dl (1400 μ moles/l) as the cut-point for significant subclinical ketosis (Duffield, 2000). So, we evaluate this test on the basis of the proportion of animals with BHBA values above 14.4 mg/dl. This herd-based cut-point is considerably higher than the upper end of the laboratory normal reference range for individual cows. The NEFA test is an indicator of negative energy balance (with subsequent risk for fatty liver, ketosis, displaced abomasum, retained placenta, and infertility) in pre-fresh cows. A threshold value of above 0.400 mEq/l in cows between 2 and 14 days before calving has been established as the appropriate cut-point. Again, we are not interested in the mean NEFA value from a group of pre-fresh cows, but rather in the proportion of cows above the cut-point.

Besides defining the appropriate cut-points for these tests, it is also necessary to determine the alarm level for the proportion of animals above (or below) the described cut-point. In any dairy herd, we expect a few individual animals to be above or below the cut-points. The alarm level is established from research results and/or clinical experience with these tests in herd settings. Suggested cut-points and alarm levels for ruminal pH, BHBA, and NEFA test results are listed in Table 1.

Table 1. Cut-Points and Alarm Levels for Herd-Based Tests

Test	Cut-Point	Alarm Level Proportion	Associated Risk
Ruminal pH	≤ 5.5	> 25%	Subacute ruminal acidosis
BHBA	> 14.4 mg/dl	> ~10%	Subclinical ketosis
NEFA	>0.400 mEq/l if -2 to -14 days	> ~10%	Pre-partum negative energy balance, fatty liver

Urinary pH and UN concentrations are also useful tests in herd nutritional investigations. For these tests, and evaluation of the mean value is most appropriate. There is no single biological threshold for these tests. Instead, there is an “optimal,” mid-range value. Either exceeding or falling short of this optimal range has adverse consequences. So, the mean test result from a group of animals is the most appropriate method of interpretation. Optimal mean urinary pH values for groups of pre-fresh cows fed anionic diets is about 6.0 to 6.5, and optimal mean UN values for groups of lactating cows are about 12 to 16 mg/dl. The normal range for UN concentrations in groups of cows is considerably narrower than the normal reference range for individual cows.

Appropriate Sample Sizes for Biological Tests.

We must sample enough cows in each group of eligible animals in order to have reasonable confidence that our results (either a proportion or a mean) truly represents the entire population of eligible animals on that farm. We do not, however, need to sample as many animals as a researcher would sample in order to achieve a 95% confidence ($P < .05$) in the results. Rather, a 75% confidence is acceptable under most herd testing conditions. Thus, the large sample sizes typically used by researchers are generally not necessary for clinical herd investigations. Certainly larger sample sizes are desirable and will increase your confidence in the result; however, practicality and cost constraints may dictate that you choose an “optimal” sample size that is smaller.

When solving herd nutritional problems, there is never the option to make “no decision.” Leaving things the way they are is just as active a decision as implementing a nutritional change. So, we have to make decisions on less than perfect information.

As a general rule, a minimum of about 12 eligible cows should be sampled for tests with proportional outcomes (ruminal pH, BHBA, and NEFA) and a minimum of about 8 total animals should be sampled for tests with mean outcomes (urinary pH and UN). Larger sample sizes are always required when evaluating tests with proportional outcomes compared to mean outcomes – this is a statistical “fact of life.”

Sample sizes larger than about 12 animals are suggested when the results of a proportional outcome are very close to the cut-point. For example, if 2/12 (16.7%) of cows tested for ruminal pH had pH <5.5, then it would be reasonable to test additional cows. The upper end of a confidence interval of this value would be above the cut-point of 25%. In contrast, if 5/12 (41.7%) of the cows tested had ruminal pH <5.5, then additional testing would not be warranted because the lower end of a reasonable confidence interval of the value would still be above the cut-point.

Cows to be sampled for these tests need to come from the appropriate “eligible” or “at risk” group. It is of no clinical value to test cows for a condition which they have no risk for because of their current stage of lactation. Appropriate eligible groups for herd-based nutritional tests are listed in Table 2.

Table 2. Appropriate Groups of Cows Eligible for Different Herd-Based Nutritional Tests

Test	Eligible Group
Ruminal pH	Lactating cows, about 5 to 150 days in milk
BHBA	Lactating cows, about 5 to 50 days in milk
NEFA	Pre-fresh cows, 2 to 14 days from actual calving
Urinary pH	Pre-fresh cows on an anionic diet for >24 hours
UN	Lactating cows or sub-groups, any days in milk

The size of the eligible group for testing has some, but limited influence on the appropriate sample size. In larger herds, there is little statistical value in testing more animals. The same sample size will yield almost the exact same information about the group average even when the group is large. In smaller herds, it may be possible to test the entire eligible group and still not have an adequate sample size. For example, only the pre-fresh cows (from three weeks prior to expected calving up to calving time) are eligible for urinary pH and NEFA testing. If there are only four cows in the group, then all four should be tested. However, a sample size of four cows is too small to be conclusive. So, additional cows should be tested as they move into the eligible group, and the group results interpreted only after about eight or more test results have been accumulated. If cows are repeatedly tested for NEFA or urinary pH as they approach calving, only the last test result before actual calving for that cow should be used when interpreting the test results (i.e., do not use multiple test results from the same cow to achieve your sample size goal).

■ Ruminal pH Testing for Subacute Ruminal Acidosis

Ruminal pH is the definitive test for ruminal acidosis. However, ruminal pH may vary from day to day and time of day within a herd. Thus, single samplings of a group of cows are vulnerable to error.

Another potential source of error in ruminal pH measurements is the accuracy and correct calibration of the pH meter. Use a high-quality pH meter for this purpose – pH paper is not accurate enough and is influenced by the green color of the ruminal fluid. Any pH meter may not work well when operated at cold temperatures. Therefore, in cold weather I bring all the ruminal fluid samples (in capped syringes with the air excluded) into a warm parlor or office to run the pH determinations. Also, pH electrodes dry out while they are not being used. I typically add buffer to the electrode before I start collecting the ruminal fluid samples. When all the samples are collected, I calibrate the meter twice before actually running my samples. After the last calibration, I check the readings with pH 7 and 4 buffers back on the electrode to verify the calibration.

Research has established a cut-point of 5.5 for ruminal pH (for fluid collected by rumenocentesis) and an alarm level of >25% of animals below this cut point. Necessary sample sizes for herd-based ruminal pH evaluation have been described in detail (Garrett and others, 1999). A practical sample size for most herds is 12 animals per diet. If 4 or more of the 12 cows tested have a ruminal pH ≤ 5.5 , then the group is considered to be at high risk for SARA and the diet should be modified to reduce the risk for SARA. This testing scheme works very well for herds with high (>30%) or low (<15%) prevalences of cows with low ruminal pH. Example guidelines for interpreting ruminal pH test results are listed in Table 3.

Table 3. Interpretation of Ruminal pH Test Results
(Group Size = 100; Sample Size = 12; CI = 75%; Alarm Level = 25%)

Outcome	Percentage	Interpretation
0/12	0%	Negative
1/12	8.3%	negative
2/12	16.7%	borderline
3/12	25.0%	borderline
4/12	33.3%	borderline
5/12	41.7%	positive
6/12	50.0%	positive

The intended purpose of the rumenocentesis test is to identify herds with high prevalences of cows with low ruminal pH. These herds require immediate dietary corrections. Herds with intermediate prevalences (16.7 to 33.3%) of low

ruminal pH may require a greater sample size to be more likely to be classified correctly, or may require that other diagnostic indicators of SARA be more carefully considered. Immediate dietary intervention is not critical in herds with intermediate prevalences, so it is not unreasonable to take some additional time to gather more information.

The effect of time relative to feeding on ruminal pH is great. The purpose of this test is to identify cows with low ruminal pH, so sampling should be done around the time of the expected lowest point (nadir). In component-fed herds, the nadir in ruminal pH occurs about 2 to 4 hours post-feeding. In TMR-fed herds, the nadir in ruminal pH occurs about 6 to 8 hours post-feeding.

■ BHBA Testing for Subclinical Ketosis

The “gold standard” test for subclinical ketosis is blood BHBA. This ketone body is more stable in blood than acetone or acetoacetate. The cut-point for subclinical ketosis is 1400 μ moles/l (14.4 mg/dl) BHBA. Above this cut-point, cows are at increased risk for displaced abomasum, clinical ketosis, and decreased milk production. Clinical ketosis generally involves much higher levels of BHBA (26 mg/dl or more) (Duffield, 2000).

The alarm level for the proportion of cows above the cut-point of 14.4 mg/dl BHBA has not been well defined. Studies show an average prevalence of about 15% subclinical ketosis in early lactation cows. My clinical impression is that we should tolerate no more than about 10% subclinical ketosis in early lactation cows. Most herds I test have a 0 to 8% prevalence of subclinical ketosis. An example interpretation guide for BHBA testing is presented in Table 4.

Table 4. Interpretation of BHBA Test Results
(Group Size = 50; Sample Size = 12; CI = 75%; Alarm Level = 10%)

Outcome	Percentage	Interpretation
0/12	0%	negative
1/12	8.3%	borderline
2/12	16.7%	borderline
3/12	25.0%	positive
4/12	33.3%	positive
5/12	41.7%	positive
6/12	50.0%	positive

The BHBA test can be performed on serum samples, and there are no special sample handling requirements. Marshfield Laboratories and the Michigan State Animal Health Diagnostic Laboratory perform this test. Serum BHBA

concentrations typically increase after feeding (Eicher and others, 1998). Consistent sampling at 4 to 5 hours after the start of feeding has been suggested in order to capture peak BHBA concentrations (Eicher and others, 1998). The post-feeding peak in serum BHBA concentrations is due to ruminal production of butyric acid. Excess amounts of butyric acid (either from ruminal production or from silage) are easily converted to BHBA in the wall of the rumen.

An evaluation of early lactation cows for subclinical ketosis requires testing most or all of the eligible cows in small to medium-sized herds. In larger herds, a suitable sample size may be obtained on a single herd visit.

I have found it relatively difficult to predict whether a herd will have a high or low prevalence of subclinical ketosis. Clinical signs of this disease are non-specific and subtle. They include depressed dry matter intake, impaired milk production, increased risk for displaced abomasum, and increased risk for clinical ketosis. Many dairy herds now are completely unaware that they have a clinical ketosis problem. Unfortunately, it is very difficult to diagnose clinical ketosis in free-stall herds because individual cow feed intakes are not typically monitored. Thus, I have discovered many individual cows with extremely high blood BHBA concentrations (above 30 mg/dl) that should have been identified as being clinically ketotic. In such cases, the herd has not only a subclinical ketosis problem, but also a problem of inadequate disease recognition in early lactation cows.

Common causes of subclinical ketosis include high protein / low energy diets in early lactation, pre-existing fatty liver due to negative energy balance late in the pre-fresh period, and ingestion of excessive butyric acid in very wet silages due to clostridial fermentation. Problems with nutrient balance in early lactation tend to cause elevated BHBA concentrations that are evenly distributed by days in milk in early lactation. Subclinical ketosis caused by pre-existing fatty liver late in the pre-fresh period tends to cause elevated BHBA concentrations in the first 5 to 15 days in milk. These cows often have other manifestations of fatty liver, including immune suppression and lack of response to ketosis treatment.

■ NEFA Testing for Pre-Partum Negative Energy Balance

The NEFA test is used to evaluate the presence of negative energy balance prior to calving. Cows should stay in positive energy balance up until the last 24 to 48 hours prior to calving. Negative energy balance is expected in milking cows, so the NEFA test is harder to interpret and is not typically evaluated after calving. Elevated NEFA concentrations in pre-fresh cows are associated with high risk for fatty liver, ketosis, and other peri-parturient diseases (Kaneene and others, 1997). Elevated NEFA concentrations in pre-fresh cows are also

associated with increased risk for displaced abomasum after calving (Cameron and others, 1998).

Based on this physiological understanding of NEFA, it is best positioned as a secondary test in a herd already known to have a high incidence of subclinical ketosis. The NEFA testing helps determine whether the post-partum ketosis is due to pre-calving negative energy balance and fatty liver. There is little value in conducting NEFA testing in herds with a low incidence of subclinical ketosis, since ketosis is key result of high NEFA prior to calving.

Michigan workers have described a NEFA cut-point of 0.400 mEq/l in pre-fresh cows between 2 and 14 days from calving. NEFA concentrations normally rise in the last 48 hours prior to calving, so results from cows that calve this soon after the sample was collected are difficult to interpret and should either be discarded or interpreted with caution.

The alarm level for the proportion of cows with elevated NEFA concentrations within a group has not been precisely defined. Michigan workers have suggested that up to three of seven cows tested may “normally” have elevated NEFA concentrations. My experience with this test (often in herds with serious pre-fresh diet problems) is that no more than 10% of the cows tested should have elevated NEFA concentrations. Negative energy balance prior to calving is not a condition that we should be tolerating.

In small dairy herds, the number of pre-fresh cows eligible for NEFA testing is small, so all eligible cows will need to be tested. Samples may need to be frozen and submitted as a group when about twelve or more samples have been accumulated. It makes sense to store the frozen plasma samples over time, and to wait until all of the calving dates for the cows are known and an adequate sample size has been accumulated before submitting the samples to the lab.

In large dairy herds, the pre-fresh group may be sub-sampled for NEFA screening. In this case, select the cows that appear to be the closest to calving, but avoid those cows in which calving appears to be imminent. I have found it extremely useful in some investigations to collect some plasma samples from cows in the maternity pen as well as the pre-fresh pen (even if the maternity pen cows appear to be very close to calving). Many of these cows will not calve for several more days, which indicates both a management error and puts them at risk for elevated NEFA concentrations. A good pre-fresh nutritional management program can be ruined if a cow is forced to spend several days in a maternity pen without adequate access to feed, water, and resting space.

Concentrations of NEFA reach their nadir about 4 to 5 hours post-feeding (Eicher and others, 1998) and peak just prior to the next feeding. The best

approach, therefore, is to sample just prior to feeding in order to capture the peak value. The difference between peak and nadir values is probably influenced by the availability of feed throughout the day and the relative size of the meals consumed by the cows. Sampling at a consistent time relative to feeding is the most useful approach when monitoring pre-fresh cow NEFA concentrations in a herd over time. It is important to keep the plasma samples cool or frozen from the time they are collected from the cow until the time they are received at the laboratory for analysis.

If a high proportion of elevated NEFA concentrations are detected, then attention should be focused on increasing total energy intake in the pre-fresh group. This may require increasing the energy density of the pre-fresh diet, increasing pre-fresh dietary NFC content, improving diet palatability, increasing bunk space, increasing feeding frequency, and/or increasing daily feed refusals.

■ Urinary pH Testing for Dosing Anions

Dietary acidification is an effective method of preventing both clinical and subclinical hypocalcemia. The degree of dietary acidification is related to urinary pH (Vagnoni and Oetzel, 1998). Several studies suggest that dietary cation-anion difference (DCAD) and milk fever prevention are optimal at urinary pH values of about 6.0 to 6.5.

In small dairy herds, it makes sense to check all eligible pre-fresh cows for urinary pH. Results from about eight cows or more should be accumulated before calculating a mean value, interpreting the result, and possibly adjusting the diet. This will generally require that the herdsman, on the farm, do the urinary pH testing so that an adequate sample size can be accumulated. Urinary pH can be determined satisfactorily with pH paper – a calibrated pH meter is not required.

The effect of time post-feeding on urinary pH is small when access to feed is good throughout the day (Goff and Horst, 1998). If feed access is not good throughout the day for pre-fresh cows, then you have identified a problem that is much bigger than any concerns about dietary acidification and urinary pH.

Mean urinary pH values above about 6.5 (when acidifying diets are supposedly being fed) indicate that the acidification is not optimal. A common culprit is undetected increased potassium content of one of the forages. Either more anionic salts need to be fed, or less of the high DCAD forage should be fed. A small change in actual DCAD consumed by the cows could dramatically move the urinary pH values into the optimal range.

Mean urinary pH values below about 6.0 are uncommon and indicate over-acidification of the diet. A common culprit in these situations is over-zealous supplementation of anionic salts in the face of a milk fever outbreak. An undetected high chloride content in one of the forages could also contribute to the low urinary pH. In either case, the dose of anions added to the pre-fresh diet should be reduced; otherwise, dry matter intake of the pre-fresh group may be unnecessarily suppressed.

■ Urea Nitrogen Testing to Evaluate Protein and Energy Nutrition

Blood UN (BUN) or milk UN (MUN) are indirect measures of protein and energy nutrition in lactating cows. High UN's may be caused by either high dietary crude protein (especially soluble protein) and/or low dietary NFC. High UN's are a risk factor for infertility and body condition score loss due to the energy cost of detoxifying excessive ruminal ammonia into urea by the liver.

The effect of time relative to feeding on UN concentrations is great, particularly if the protein is fed as a separate component of the diet two or three times a day. Lack of control of the time of UN sampling relative to feeding has greatly hindered the effectiveness of this test in the past. Sampling at about 3 hours after a major protein feeding should assist in determining peak daily UN concentrations. Consistent time of sampling relative to feeding is necessary when monitoring a herd over time.

Milk UN concentrations are closely related to BUN concentrations. Therefore, either BUN or MUN samples are acceptable for evaluating herd UN. Bulk tank MUN is particularly attractive because it provides a mean value for a large group of lactating cows with a single test, without concerns of getting an adequate sample size. Wet chemistry procedures for MUN are preferred over NIRS tests (e.g., MUN testing provided through DHI) because of they are more accurate. Because bulk tank MUN testing is inexpensive and accurate (as long as a wet chemistry analysis is used), and because UN is evaluated on a basis of the group mean, bulk tank MUN screening it is a reasonable procedure to conduct on a routine basis. Individual cows (or milking strings) could then be evaluated for UN if the bulk tank MUN value falls outside the normal range for a group of animals.

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