Supplemental testing on milk recording samples

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Summary

Milk samples taken from cows by milk recording organizations on periodic test dates are an underutilized resource for both dairy producers and recording organizations. While analysis of fat, protein, and solids continue to be the mainstay of the milk testing laboratory, there are a growing number of supplemental assays that deliver additional information and provide a greater return on investment in the milk recording process. Enzyme-linked immunosorbent assays (ELISA) and polymerase chain reaction (PCR) are two widely used procedures in veterinary research and medicine that have direct applicability to milk recording samples. Compared to traditional blood testing programs for widespread diseases such as Johne's disease, the utilization of the milk recording platform offers dairy producers, veterinarians and animal health regulatory agencies an attractive alternative that greatly reduces the cost and inconvenience of control and surveillance programs. Milk testing programs developed by Antel BioSystems for Johne's, leukosis, bovine viral diarrhea, and progesterone and successfully implemented by NorthStar DHI are described.

***Key words**: Milk testing, Johne's, Leukosis, Bovine viral diarrhea, Progesterone.*

Introduction

Traditionally, milk has been viewed as the saleable product of the dairy industry, providing valuable nutrition to consumers and important ingredients to other sectors of the agricultural industry. Even in the milk recording industry, individual cow milk samples obtained on a periodic basis are analyzed for fat, protein, and other solids as they relate to the commercial value of milk and cow performance. As technological advances bring these traditional analyses closer to the farm, the role, or even the existence, of the milk recording industry could be threatened. To answer this threat, supplemental milk testing should be explored to recover more of the inherent value of milk recording by extracting and delivering additional information from milk samples.

Milk, as blood, is an accessible window to the health and metabolic state of the dairy cow and, except for the presence of red blood cells, its composition is basically comparable. Therefore, while blood is typically the sample of choice for diagnostic testing and metabolic analysis, milk samples can satisfy many of the requirements for comprehensive analyses. In the dairy industry, and specifically for milk recording organizations, the major advantage for utilizing milk samples instead of blood samples for additional analysis is that the milk samples are already routinely collected and transported to centralized laboratories. In other words, milk recording organizations invest a considerable portion of their budget on the acquisition and analysis of individual cow samples. The extraction, sale and delivery of additional information from supplemental milk testing can greatly improve the return on this investment.

Johne's disease

Johne's disease (JD) is a wide-spread, chronic digestive disorder in ruminants caused by Mycobacteria paratuberculosis. Transmitted primarily to calves through fecal-oral routes, the bacteria colonize leukocytes in intestinal mucosal and lymph tissue, inducing varied immune responses that ultimately interfere with nutrient absorption. Clinically, infected animals are unable to sustain most productive functions and ultimately succumb to malnutrition. An association between bovine JD and human Crohn's disease has led to the development of specific, nationally-sponsored control and eradication programs in many countries.

In the absence of effective treatments and vaccines, Johne's disease is best controlled through a combination of sanitary animal husbandry practices and diagnostic testing to identify and manage infected animals. Current diagnostic tests focus on either the detection of the organism (manure, milk, tissue) or the detection of antibodies (blood, milk). The slow and variable progression of JD dictates serial testing to effectively identify infected animals in a timely fashion, thus milk recording offers a convenient platform to regularly screen dairy animals for infection.

JD milk testing

An enzyme-linked immunosorbent assay (ELISA) was developed to detect the presence of JD antibodies in milk samples. The ELISA was validated against commercially available and USDA-approved testing procedures in herds of varying breed, size and incidence of JD (Figure 1).

Of the individual milk samples from 688 animals in herds considered free of JD, only 4 were found to be positive in the JD milk ELISA. The apparent specificity for milk sample testing was 99.4% and was not different from serum testing where 1/688 (0.15%) samples were positive. In herds suspected of having JD, there were 799 samples with both fecal culture and milk ELISA results. There were 183 samples (22.9%) that were fecal culture positive and 128 (16.0%) that were milk ELISA positive. Of these 183 fecal culture positive animals, 90 (49.2%) were milk ELISA positive and 98 (53.6%) were serum ELISA positive (88% agreement). Clearly, antibodies for JD are in milk and their detection by ELISA is a reliable diagnostic.

Recently, a more sensitive JD milk ELISA has been tested for use in bulk tank samples. As part of the National Animal Health Monitoring Survey (NAHMS) in 2007, the JD bulk milk ELISA was compared to cultured environmental fecal samples to detect the presence of JD at the herd level. Results presented in figure 2 show that the bulk milk ELISA is capable of detecting the majority of infected farms with sufficient specificity to screen herds efficiently and cost effectively.

		Serum Analysis		
		Positive	Negative	Total
Milk Analysis	Positive	0	4	**4**
	Negative	1	683	**684**
	Total	**1**	**687**	**688**

		Fecal Analysis		
		Positive	Negative	Total
Milk Analysis	Positive	90	38	**128**
	Negative	93	578	**671**
	Total	**183**	**616**	**799**

		Serum Analysis		
		Positive	Negative	Total
Milk Analysis	Positive	83	7	**90**
	Negative	15	78	**93**
	Total	**98**	**85**	**183**

Figure 1. Performance of milk ELISA testing for Johne's Disease in individual animals compared to serum and fecal testing.

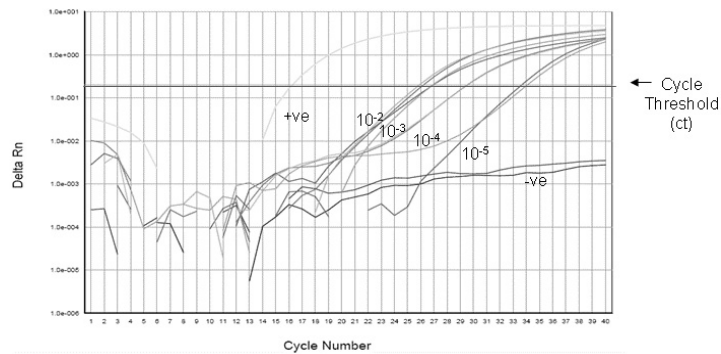
		HEYENV#		
		POS	NEG	Total
	POS	199	8	207
Milk-ELISA	NEG	185	123	308
	Total	384	131	515
Relative Sensitivity		51.80%	SE= 0.025	
Relative Specificity		93.90%	SE= 0.02	

Figure 2. Performance of bulk milk ELISA testing compared to Environmental fecal cultures (HEYENV#).

An organism-based bulk milk assay has also been developed using real-time PCR to target the IS900 genetic element as another method of detecting herd-level presence of JD. Figure 3 shows that as few as 50 cells of *M. paratuberculosis* can be quantitatively detected by real-time PCR analysis of spiked milk samples. When applied to the NAHMS 2007 milk samples, the assay was able to detect 40% of the infected farms with similar specificity as the JD bulk milk ELISA (Figure 4). More importantly, the data suggests that 40% of US dairy farms have detectable levels of *M. paratuberculosis* in their bulk tanks, dramatically emphasizing the necessity of effective control and surveillance programs for JD.

Enzootic bovine leukosis

Bovine leukosis is caused by a blood-borne retrovirus (BLV) that permanently infects and interferes with the immune system. Transmission occurs through transfer of infected white blood cells from blood or milk to susceptible cattle of any age. Clinical disease is relatively rare however, and the economic impact of BLV is questionable even in herds with prevalence rates greater that seventy percent. In rare cases, infection can result in bovine lymphosarcoma with variable affects dependent on



Dilution in Milk	10^{-2}	10^{-3}	10^{-4}	10^{-5}
No. of cells	50,000	5,000	500	50
Av. ct value	26.0	26.9	29.5	33.8

Figure 3. Real-time PCR analysis (IS900) of milk spiked with Mycobacteria paratuberculosis.

		HEYENV#		
		POS	NEG	Total
Milk-PCR	POS	153	8	161
	NEG	231	123	354
	Total	384	131	515
Relative Sensitivity		39.80%	SE= 0.024	
Relative Specificity		93.90%	SE= 0.02	

Figure 4. Performance of Real-time PCR analysis of bulk milk compared to environmental fecal cultures (HEYENV#).

the anatomical site of tumor formation. The greatest concern for BLV stems from the fact that several countries will not import cattle or germplasm from BLV-infected areas.

There is no treatment or vaccine for BLV. Unlike JD, diagnostic signs of BLV are more readily detectable however, and antibody detection assays have been used successfully to eradicate BLV in several countries. The immune response to BLV infection is rapid with persistent production of high antibody titers to dominant antigens that can be easily used in ELISA format. Once detected, infected animals can be physically isolated, sorted or culled to control transmission. Milk samples obtained through milk recording provide an excellent platform for regular detection and management of BLV.

BLV milk testing

There are several commercial assays available for the detection of antibodies to BLV in milk samples. The sensitivity and specificity of these assays are generally greater than 95% when compared to the serum-based assays (Figure 5), and their utility in control and surveillance programs is unquestionable. There is one problem that must be accounted for when using these assays on milk recording samples; carryover contamination. Because of the large antibody response to BLV infection,

		Serum ELISA		
		Positive	Negative	**Total**
BLV Milk ELISA	Positive	89	0	**89**
	Negative	0	87	**87**
	Total	**89**	**87**	**176**

Figure 5. Performance of milk ELISA testing for bovine leukosis in individual animals compared to serum testing.

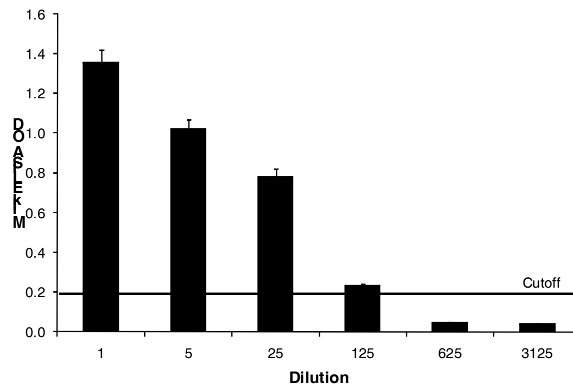


Figure 6. Effect of dilution on the leukosis milk ELISA.

the mixing of milk samples from subsequent cows that occurs during milk collection (through metering devices) and component analysis in the laboratory can lead to false positives. Figure 6 shows positive milk samples can be diluted by as much as 1:125 before the resultant signal is reduced to undetectable levels. Therefore, any carryover contamination beyond 1% will lead to a reduction in the specificity of the assay. This likelihood is negated by minimizing carryover contamination during collection and including a “Suspect” category for milk samples with low antibody titers that would be indicative of a potential carryover effect.

Bovine Viral Diarrhea (BVD)

Another prominent viral infection in cattle is BVD. Infection occurs in many epithelia tissues; its predilection to the respiratory system accounts for aerosol-based transmission, its presence in the reproductive system leads to a variety of congenital disorders, and its effects on the digestive system results in death in extreme cases. Thus, the clinical manifestation of BVD infection has a large and consequential economic impact on dairy operations.

The reservoir for the BVD virus is persistently infected animals. When exposed during early gestation, the developing immune system of the fetus may become immunotolerant to the virus, unable to mount an immune response and postnatal, the animal remains persistently infected throughout life. Persistently infected animals act as incubators for new, mutant BVD strains that are not represented in the present generation of vaccines, resulting in new infections and outbreaks even in properly vaccinated herds.

Testing for BVD is a component of control programs for unvaccinated and vaccinated herds. In unvaccinated herds, the presence of antibody titers suggests previous exposure to and thus, circulating BVD virus. Most BVD infections are acute however, and the immune system effectively clears the virus without complication and thus, diminishes the utility of antibody detection by ELISA. On the other hand, persistently infected animals are unlikely to produce antibody titers and their detection requires antigen (virus) detect tests whether or not vaccination is included in the control program. Since BVD virus is shed in milk, milk recording samples can be used in BVD screening programs for the milking herd; calves are typically screened with blood samples or ear notches in antigen detection assays.

BVD Milk Testing

Pooled samples such as bulk tanks and group samples can be originally screened for BVD virus by PCR to reduce the overall testing requirements and cost to find persistently infected cows. The PCR results presented in figure 7 show the amplification products from the 5′ UTR (untranslated region) of the BVD virus and an internal control (β-actin) in the analysis of naturally infected milk samples at various dilutions. While detection is clearly seen at 1:1000, conservatively pool sizes are typically limited to 1:400.

The major advantage of pooling milk recording samples for PCR analysis is that the remaining sample can be stored for immediate, individual sample analysis if required upon a positive pool test. In contrast, if line samples are used for pooled analysis, cow movement and trafficking while waiting on pooled test results can confound individual animal testing if required. For example, if the persistently infected cow is transferred to another group before the test results are returned, individual animal testing in the indicated group would not result in the identification of the persistently infected cow. By using milk recording samples, the individual samples within groups are fixed and easily tracked and accounted for.

A more economical antigen detection test is required for individual milk sample testing within positive pool samples. Figure 8 compares the results of a BVD milk ELISA to traditional testing using serum and/or ear notch samples. There was 100% agreement between the BVD milk ELISA and traditional analysis, indicating that viral shedding in milk is consistent and indicative of persistent BVD infection.

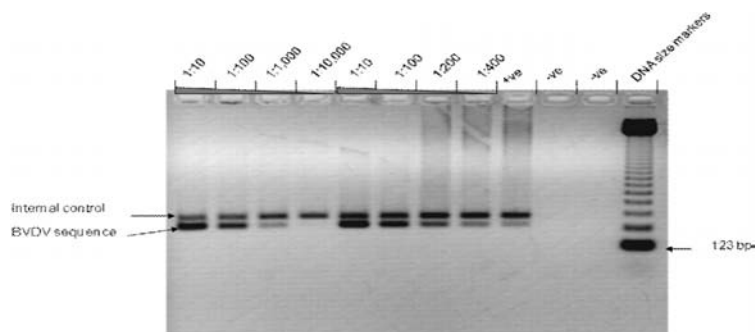


Figure 7. Effect of dilution on PCR analysis of milk for BVD virus.

		Serum / Earnotch ELISA		
		Positive	Negative	**Total**
BVD Milk ELISA	Positive	18	0	**18**
	Negative	0	386	**386**
	Total	**18**	**386**	**404**

Figure 8. Performance of milk ELISA testing for BVD virus in individual animals compared to serum or earnotch testing.

The advantages of the milk testing program described above are best illustrated in Figure 9, which outlines the testing protocol and costs for a 1 000-head dairy. Without a pooling strategy, the cost to screen this herd by individual BVD milk ELISA at US$6.00 a test would be US$6 000. By pooling individual samples and using PCR analysis to narrow the scope of testing, the total cost to the dairy producer is reduced to US$810, including a charge of US$0.15 per sample for pooling. More significantly however, the dairy producer did not have to incur the cost or effort of drawing blood samples or ear notches from individual cows.

Progesterone

Theriogenologists have analyzed progesterone for many years to research and develop breeding programs for dairy herds. Progesterone is a hormone produced and released into the blood by the corpus luteum (CL) on the ovary. The CL is formed after the follicle has ovulated (estrus) and is maintained for the nine months of gestation if the cow becomes pregnant. If conception fails, the CL regresses after 18 days and progesterone concentration falls, allowing the initiation of another cycle. Thus, progesterone is at low levels for non-cycling cows, and during the 6 days that surround estrus (d 20 to d 4) until the CL is producing sufficient levels of progesterone, which can be measured in both blood and milk.

- 1000-Head Herd ($0.15/sample to pool)
  - 4 PCR X $40 = $160

250 Samples	250 Samples	250 Samples	250 Samples

  - 5 PCR X $40 = $200

50 Samples	50 Samples	50 Samples	50 Samples	50 Samples

  - 50 ELISA X $6 = $300

50 Samples

  - Total $810/1000 = $0.81/Cow ($0.50-$2.50)

PI Cow

Figure 9. Milk testing protocol for BVD virus using milk recording samples.

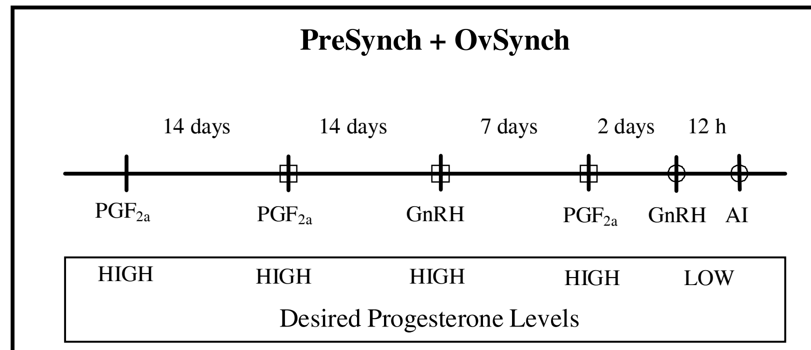


Figure 10. Predicted progesterone levels at critical hormone injection points during estrus synchronization using the PreSynch + OvySynch protocol.

Progesterone testing, along with other factors, can be used as an evaluation tool to assess a herd's breeding program, especially in herd's using synchronization programs for artificial insemination. As an example, Figure 10 details the PreSynch + OvSynch program.

In any synchronization program, cows need to be cycling for the hormone injections to be effective in predicting or timing estrus for AI. Milk samples can be checked for progesterone levels at critical hormone injection points (¡%) when the CL is expected to be present on the ovary. At these points, progesterone levels are expected to be high (>5 ng/mL); low or undetectable levels indicate animals in which the synchronization program is failing, either because they are not cycling or they received improper hormone injections. Likewise, milk samples can be checked for progesterone during predicted estrus (Ë%), when the CL is expected to be absent. At these points progesterone levels should be low (<5 ng/mL); high levels indicate an active CL and very little chance of an ovulation to coincide with the scheduled AI.

Progesterone milk testing

Blood as been the traditional sample for progesterone analysis and is the method of choice for most herds. However, a progesterone milk ELISA has been developed as a convenient alternative to use with milk recording samples. Based on the number of cows required for a worthwhile analysis, the milk testing option is best suited for larger herds that may be inseminating 20-30 cows in any given week. To use milk analysis, a dairy producer identifies cows at critical points (based on their synchronization program) on each milk recording test date for progesterone analysis. A continuous analysis of progesterone data for compliance in each of three lactation groups on successive test dates is shown in figure 11. While noncompliance rates generally average between 20-30% in aggressive yet successful breeding programs, upward trends in noncompliance as shown for the 1st lactation heifers indicate poor responsiveness to the synchronization protocol. As a diagnostic tool, the information is useful for troubleshooting and correcting poor reproductive performance.

Progesterone Non-Compliance

Figure 11. Noncompliance analysis of milk progesterone levels during estrus synchronization.

Future opportunities

Thus far, supplemental milk assays for JD, leukosis, BVD and progesterone have proven to be valid and useful tools in the dairy industry. The utility of these testing programs offered through milk recording organizations are attracting the attention of the entire animal health industry, including government regulatory agencies and diagnostic assay manufactures, which will stimulate the development of additional assays for milk samples in the future. Future assays will not only address other pathogens, hormones and metabolites, but will combine multiple assays in single, high throughput testing procedures (multiplex). How fast these possibilities become reality for milk recording organizations depends on the perceived market. At first glance, the market for diagnosing animal health and metabolism through milk recording might appear limited, but with recent advances in genomic analysis and marker assisted selection, one has to wonder about the future value of additional phenotypes.