

Dairy diagnostics 101

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Abstract

This paper will discuss the ever-changing world of diagnostics in dairy veterinary practice and point out a few things that veterinarians should know concerning test selection and test type. It will be focused primarily on diagnostics for herd-level problems of dairy cattle and will touch on mastitis, abortion and BVD. In addition, some time will be allocated to a brief overview on PCR and advances in culturing. The remaining content of this paper will focus on diagnostic sampling strategies for common dairy herd problems, some disease investigation, routine sampling and interpretation of results.

Key words: diagnostic, dairy, mastitis, abortion, bvd

Understanding diagnostic tests

While veterinarians use diagnostic testing terms almost daily, there is an assumed standardization by veterinarians that really doesn't exist. There is confusion by some around types of tests, what they are capable of and what they are used for. As examples, we'll briefly discuss PCR tests, ELISA tests and even culture techniques and methodologies.

Polymerase chain reaction (PCR) testing has been around for 20-30 years. It consists of cycles that comprise repeated steps of DNA denaturation, primer annealing and extension. The basic concept is amplification of a very small amount of DNA that represents a pathogen or genomic material that helps the practitioner solve disease outbreaks or determine parentage, etc. The scope of this discussion is not to understand each step of the PCR process, but understand the advantages and disadvantages of the test.

Quantitative PCR testing has become very popular in veterinary labs in the past decade due to the quickness of the test and its high sensitivity and specificity. In almost all cases samples are pooled to offset the cost of the test. Different labs use different pool numbers and pooling processes. The veterinarian should be certain of the number of samples pooled together and, specifically, what organisms are detected by the test.

The majority of PCR used in food safety and veterinary diagnostics is quantitative PCR (qPCR). The difference between traditional (qualitative) PCR and qPCR is the ability of qPCR to measure the amount of DNA found in the sample. Additionally, you'll hear the term RT-PCR. This is mistakenly referred to as "Real Time" PCR. RT-PCR involves an RNA step for the detection of viruses so it utilizes a reverse transcriptase (RT) step in the PCR process. Hence the name RT-PCR. It is important for the practitioner to understand which PCR test a particular lab is running to properly interpret the test result.

PCR tests are among the most sensitive of tests we have. These tests can find even the smallest fragment of target DNA. A large consideration for the practitioner is that qPCR cannot determine if the DNA is from a live or dead organism. Chronicity of the disease process along with sample type and tissue should be interpreted alongside of the PCR test result. Analytical specificity and sensitivity of a PCR test is based on statistical

calculations to determine what it finds and how much amplification is needed to call a sample positive. Standards within the scientific community for testing development essentially say that inclusivity (analytical specificity) should be determined on 20-50 well-defined (certified) strains of the target organism. A practitioner needs to keep in mind that the 20-50 strains used to determine positivity may or may not have included the disease-causing strain he or she is seeing in the field. Likewise, analytical sensitivity is determined by the lowest level a target may be identified at a specific level of confidence.

In today's qPCR reporting methods, much of the sensitivity is already interpreted using the stated CT (Cycle Threshold) value. qPCR goes through a cycle of DNA denaturation, primer annealing and extension, then repeats the process. The lower the number of cycles when detection occurs indicates a higher concentration of DNA in the sample. If qPCR reached its defined maximum number of cycles without detection, the sample is considered, loosely, as negative. More accurately it says that there is a 95% chance that the sample is negative.

Things to consider when using PCR tests are whether there may be similar organisms in the sample that are not detected by the target (e.g., *Mycoplasma bovis* vs *Mycoplasma californicum*), the cause for the test (e.g., screening vs active disease) and the CT value if reported.

ELISA testing is another widely used test that is usually easier to interpret when compared to qPCR. These tests can be highly sensitive and specific, however, there is a broader range in these values depending on the antigen of interest. Most will not have sensitivities as high as PCR, however, their costs are much lower, and samples tend to be screened individually allowing for less issue with background noise in the sample. The detection by these tests is typically a process of complexing antigens and antibodies to produce a measurable, usually photometric, result.

There are four major types of ELISA tests

- Direct ELISA (antigen-coated plate; screening antibody)
- Indirect ELISA (antigen-coated plate; screening antigen/antibody)
- Sandwich ELISA (antibody-coated plate; screening antigen)
- Competitive ELISA (screening antibody)

For the veterinarian, there is less concern with which ELISA test to select since most often there isn't a choice. In general, Sandwich and Competitive ELISAs are more sensitive and can detect lower concentrations of antigen than Direct or Indirect ELISA tests.

Bacteriologic culture remains the gold standard for most bacterial diseases today. Thanks to advances in ancillary testing allowing identification of both genus and species of bacteria within 24 hours, culture now competes with qPCR for turnaround time. In years past, it took 1-2 days to grow bacteria from a sample. Accurate identification came from re-streaking the colonies onto fresh agar then performing different ancillary tests that would allow identification to the species level. This often takes another 2-3 days after the original growth occurred.

Today, more and more labs are using mass spectrometry to quickly speciate organisms after a pure growth is established. A specialized type of mass spectrometry called MALDI-ToF (Matrix-Assisted Laser Desorption/Ionization-Time of Flight) can speciate organisms in minutes, eliminating the time required for re-streaking the organisms and conducting more traditional microbiological testing.

When a choice exists for the use of different test types, some thought should be given to the goals of the sampling. Things like cost and turnaround time should always be considered, however, additional things such as sensitivity and pooling should also be considered. In many cases, a combination of testing modalities will often work better than picking one over another for herd surveillance or herd outbreak situations. Advantages and disadvantages should always be considered.

Mastitis

Mastitis alone is a topic that we spend hours, if not days, at a time discussing in continuing education forums. There are a lot of differing thoughts and approaches out there, so you are receiving my bias as a practitioner who has worked primarily with large dairies and provides diagnostics to dairies and others from the private sector.

Before any diagnostics occur, case definition of mastitis, prevalence or incidence of mastitis in the herd and the economics surrounding mastitis need to be considered.

The first thing required from a veterinarian is to determine if there is a problem. There can be a large variation in prevalence of mastitis between different dairy systems (i.g. freestall vs drylot) as well as detection systems within these dairies. The most common detection system would be forestripping so one can observe for clots and flakes as udder prep occurs. Examples of other detection systems that aid in mastitis detection would be the use of software programs that monitor electrical conductivity, activity and other parameters applied to the cow each time milk is harvested. Different detection systems result in different prevalence across herds.

Economics should always be considered with culture programs. In many cases the best outcome is derived from a combination or hybrid program where individual cultures, pen/tank

cultures and PCR for mycoplasma is combined. This allows for rapid turnaround in case of a mycoplasma positive sample, control of all contagious mastitis pathogens and the ability to utilize a Treatment by Culture (TbC) program.

TbC programs utilize results from mastitis samples to provide either no treatment (coliform or negative results), or first/second line mastitis therapies, and may also be customized to each dairy.

Ultimately, goals for mastitis rates should be based on achieving low bulk tank somatic cell counts alongside a low number of mastitis cases.

Mastitis diagnostics – the old and the new

Most diagnostics for mastitis are either applied for disease screening (subclinical infection) or identification of clinical cases. The major diagnostic tools for on-farm, subclinical detection would be somatic cell count (SCC) by California Mastitis Test (CMT) or enumeration of SCC from milk by near infrared (NIR) technology. Many inline systems to detect biomarkers of mastitis such as N-acetyl-β-D-glucosaminidase and lactate dehydrogenase have been developed and applied in some milking systems where measurements are taken daily. Robots milking cows are a good example of this. While these systems are continuing to improve, they still do not identify the mastitis-causing organism and only identify the animal as developing or having increased risk of mastitis. The take-home message for the veterinarian is that they need to equate the detection systems to clinical mastitis and then it's prevention.

Technologies such as PCR and mass spectrometry (MALDI-ToF) have greatly influenced the outcome of mastitis detection for the veterinarian. It is imperative that the veterinarian truly understands how these modalities detect organisms when translating results for control or infection back to the dairy.

Basic culture of milk (composite sample for screening and quarter testing for clinical disease) is still the gold standard and still used extensively. Culturing applications continue to evolve and are often supplemented with other technologies like MALDI-ToF. Interpreting a milk culture result today is much different than in years past. Culture techniques were more basic and standardized even 10 years ago than they are today, mostly due to the fact there were limited tools available back then.

Considerations for the different levels of culturing are numerous, and most have a situation or two where they work. The biggest reason to not request a full culture is cost. Today we often combine testing modalities to create the most effective and economical program for a producer.

The Dairy Authority lab has three general culture levels for mastitis.

1. Full culture – detection of all organisms in milk including mycoplasma, yeast, and algae
2. Bacteria only culture – detection of all bacteria except mycoplasma
3. Contagious (Mycoplasma, Staph aureus, Strep ag., Prototheca(?))

Note that culture levels may be defined differently in different labs. The practitioner should always ask what is detected in the sample with different culturing levels.

| Test | Advantages | Disadvantages |
|---------|--|---|
| PCR | Highly sensitive/specific Rapid turnaround | \$\$\$\$ Pooled samples Organism specific Detection of viable and dead organisms |
| ELISA | Sensitive Rapid turnaround | \$\$ Individual samples Organism/molecule specific |
| Culture | Gold standard for bacteria (not virus) Rapid turnaround (not mycoplasma) Not organism specific | \$ Individual Samples Slow turnaround for mycoplasma/virus |

Full microbiological culture determines all organisms in a milk sample including mycoplasma, yeast and algae. This approach good for overall mastitis containment and in our lab is still where most dairies spend their dollars. The advantage of the full culture is that it detects any organism in the milk even if it's an atypical pathogen (e.g. *Salmonella* spp, *Pasteurella* spp, etc.) is present. Additionally, treatment/cull programs can be easily administered from the same sample result. Cost is a disadvantage when compared to other types of cultures utilizing reduced screening (i.e. a contagious screen) however, price is an advantage to culturing when compared to PCR.

Many laboratories will utilize little to no ancillary testing when reporting a result. The veterinarian interpreting the results should know the level of ancillary testing used to determine the sample result. Minimally, all labs should be utilizing ancillary testing (ie: CAMP or Coagulase test) for contagious organisms or employ selective media. This type of ancillary test, while warranted, can lead to increased levels of false negative and false positive results in the hands of inexperienced technicians. Many of the larger labs or labs certified for payment testing and reporting will typically use a variety of ancillary tests to verify the observations of bacteria from the petri dish before they are reported. The use of MALDI-ToF mass spectrometry is becoming more and more widespread. This technology replaces most of the microbiological ancillary tests required to accurately identify bacteria. The precision is much higher, and the application is much faster, allowing for a 24-hour turnaround of a bacteria result. Results where MALDI-ToF was utilized are much more dependable than traditional ancillary tests.

Bacteria Only cultures are used when herds are convinced or have a history of not having mycoplasma in the herd. Often, mycoplasma is still monitored by pen or tank samples instead of individual mastitis sampling. These types of samples still allow the producer to monitor almost all bacteria causing mastitis in their herd except mycoplasma so the producer may still utilize TbC programs where cows negative on culture or shown to have a case of coliform mastitis are not administered any antibiotics. With the diminished availability of intramammary antibiotics more producers should be utilizing TbC programs.

Culturing only for the contagious organisms is common as part of a mastitis control program in non-clinical cows such as fresh cow cultures or in herds that blanket treat all cows with mastitis. While it does allow for control of contagious mastitis, it does not allow the use of TbC programs since only contagious organisms are reported.

PCR

PCR tests for mastitis have become much more common in the past 10 years. Much like culture, PCR testing has its place in the veterinarian's repertoire for mastitis detection. Earlier PCR tests also suffered from lack of standardization, and to some degree, still do today, so it is important for the practitioner to understand, exactly what the PCR test is identifying. Broadly, the veterinarian needs to understand the concept of PCR compared to culture. A culture will find any viable organism in the milk sample. A PCR test has 2 dimensions when it comes to pathogen identification. The first is that it only finds the target bacteria it's been programmed to find. If a PCR test only has targets for *Staph aureus*, *Mycoplasma* spp and *Strep ag.* and the sample contains *Strep* spp., the PCR test will call the milk sample negative or (more likely today) no pathogens detected. If the practitioner doesn't understand that *Strep* spp was in not included in

the PCR test's pool of targets, they may come to the wrong conclusion. The second dimension is that of viability. A culture will only grow the bacteria if it is alive and well. PCR, on the other hand, only needs to find a fragment of the bacterial DNA to be positive. This DNA fragment could have come from a white blood cell that had destroyed the bacteria previously. It gives new meaning to terms like spontaneous cure. We can often see "increased prevalence" when using tests like PCR, but still have the same bulk tank SCC and mastitis prevalence.

The biggest advantage of using PCR for mastitis sampling is time. This advantage is primarily for the detection of mycoplasma. Cultures still require up to 10 days in the incubator in most labs to be called negative. PCR testing can be complete in as little as 6 hours, start to finish. This usually translates into a 24 hour turnaround time for the person waiting on the result. The discussion then centers on non-mycoplasma bacteriology. As mentioned previously, milk samples may be plated for non-mycoplasma bacteriology and read the next day. If the lab utilized MALDI-ToF, the finalized sample can be sent out as quick as a PCR test unless a same day result is requested by the producer. Same day results mean lab personnel move the sample to the top of the priority list. There is usually a substantial cost for this, so almost all PCR results are sent out by a lab at 24-48 hours.

In many herds we deal with, we use a hybrid system. Such a program would be comprised of fresh cow cultures using a contagious screen, a complete culture on pen/tank samples used weekly or monthly for an additional safety net for contagious organisms and for monitoring environmental bacteria and a complete culture including mycoplasma on mastitis samples. If mycoplasma has been a problem historically, conducting a complete bacteriologic culture without myco then utilizing Myco Only PCR in conjunction with the culture to find mycoplasma cows results in a much quicker turnaround time.

BVD control programs

BVD surveillance and control in dairy herds continues to be controversial to some extent. Many producers do not see the need until they experience BVD personally in their own herd. It is then they recognize the value of such testing.

The reasons herds start BVD testing are;

1. General sub-par dairy performance (low fertility, increased disease rates, etc.)
2. Compliance to voluntary programs (milksheds, genetics, etc.)
3. BVD positives in pneumonia cases or aborted fetuses (not very common)

Prevalence of BVD in the U.S. ranged from .25% to 0.75% in 2018 depending on the research group. Practically, a veterinarian will see a few herds throughout their career that tend to struggle with BVD and will also see a positive BVD calf show up randomly in herds that test but don't struggle with BVD control. BVD tends to occur in larger herds and sporadically at that if a herd is well-vaccinated for BVD. Some herds can test negative for 2-3 years, then have a positive calf born. Usually around the birth of this calf will be 3-5 others born also positive to BVD. Most of these are chalked up to a transient BVD infection in the herd and experienced by the dam that had a conceptus that was less than 125 days of age in utero. Occasionally, these will get traced back and the dams checked positive only to find the animal missed their BVD test as a youngster.

The goal for BVD control programs is to get all animals tested on the facility. Ideally, this would be by individual sampling on an ELISA test or a pooled PCR test that has been validated for pooling numbers. Keep in mind that the dam will be negative if the calf is negative, so testing the calf also tests the dam. This is a 2-for-1 deal in herds that are beginning a BVD control program. If a calf tests positive, be sure to test the dam. To quickly evaluate the lactating herd, pooled milk samples may be collected and run for BVD using qPCR. Don't forget about dry cows. Pens and tanks should be checked for BVD at least twice a year, and better if checked monthly. The goal would be no more than 400 cows in comingled milk sample and fewer would be better. There is no formal research that defines the number set at 400 cows. It is a number our lab has derived by collecting a comingled milk sample when the entire group was tested individually and finding it 90% of the time in the pool.

The test of choice if using ELISA would be a Sandwich ELISA test. There are a few manufacturers who have these kits available to labs for their use. As mentioned earlier, a practitioner really won't have a choice in the test any lab is using, but they should be aware of what technology is being employed on individual samples to aid in the interpretation of the results. There are also other tests not yet mentioned that would have good sensitivity and specificity such as immunohistochemistry (IHC). Unfortunately, the ear notch needs to be stored in formalin for this test and when shipping, the weight of the formalin adds to the shipping cost. This can be substantial when shipping hundreds of notches at a time. This test also needs to be read by a lab technician as opposed to an instrument, sometimes adding significant differences in sample interpretation. Nevertheless, it is a good test when in the hands of a skilled technician.

There are also at least 2 qPCR tests available for BVD testing of individuals. The process here is a little different. The lab would typically pool according to a validated test kit, meaning that if validated using 20 individual notches, the lab running the test should not put 25 individuals in the PCR sample. Also keep in mind that if a PCR pool is positive, the individual samples need to be tested alone. Some labs will take the pooled samples and run them using the ELISA tests, and yet other labs may run them all on individual PCR tests. Both are good for sensitivity and identification of disease, however, keep in mind that PCR tests cost 10-20 times more to perform, so individual samples getting tested by PCR will be much more expensive.

Overall BVD Control Program

1. Notch all calves individually
2. Sample pen or tank monthly for at least a year when starting a new herd
3. Collect notches from aborted fetuses
4. Positive calves should also have their dams checked.
5. Positive calves should be euthanized

Both Sandwich ELISA and validated pooled qPCR tests are good for individual animals. Using a qPCR test is also good for evaluating the lactating herd, especially when starting a BVD control program and the status of the adult herd is unknown.

Abortion

Abortion, like all other herd level disease, should have a case definition. This has been rigorous over the years and hasn't changed. Case definition is defined as loss of conceptus between day 42 and 260 of gestation.

Counting abortions in a herd can be difficult. Days of gestation at first pregnancy check, frequency of pregnancy checks and even the method of pregnancy detection all influence the abortion rate and should be considered. When examining aborted cows out of software, abortion rates of 5 to 15% are not uncommon. Keep in mind that many of the abortions recorded will be a result of early embryonic death (EED) in conjunction with early pregnancy diagnosis. For example, a herd that uses ultrasonography at 28 days post-breeding will find a higher rate of pregnancy than a herd that's palpated at 35 days post-breeding but also experience a higher pregnancy loss due to EED. These EED losses are often recorded as an abortion in the dairy management software. Monitoring abortion rates (as well as other disease rates) monthly would be advantageous on dairy for which the veterinarian will have any long-term relationship. This provides a baseline for abortion rates within the herd since there may be a large amount of variation between herds.

Most dairy management software has a way to count pregnancies and abortions by cohort groups. DC305 is one of these. If the command BREDSUM\E is used, the software will provide a report that lists pregnancies, abortions and several other items. This can be used to calculate abortion rates and, in addition, see the effect of seasonality (heat stress) on pregnancy and abortion rates using 21-day cohorts. Don't be surprised if manual calculations are different than software reports. There is often back-end programming that adjusts these numbers of which the user is unaware.

The two most important facets of abortion investigation are a complete herd workup followed by diagnostics. A complete herd workup should include at least a basic epidemiological investigation looking at the cohort of cows at risk and period of risk (which cows were affected and when did abortions start?). A veterinarian needs to keep in mind that there may be as many non-infectious cause of abortions as there are infectious cause of abortions. Diagnostics will not replace a thorough herd investigation, however greatly enhance identifying the causative agent. Because there are so many different sample types, there is no single diagnostic test that can be used to determine abortion causation. The diagnostic lab will be best suited to use the tests they have for the samples submitted.

Once an abortion problem is established, it's important to quickly gather samples. In many instances, there is not a clear indication as to whether the insult is infectious or environmental. In these cases, review of the herd and some idea as to causative agent greatly enhances the outcome of a positive diagnosis. Research done previously showed that when tissue and sera submitted to a diagnostic lab it greatly increased the likelihood of a diagnosis.

A basic abortion screen should include paired sera taken 2-3 weeks apart, fresh tissue for culture, fixed tissue for histopathology, placenta for culture and histopathology and, of course, a complete history including vaccination history should be taken. Submission of a basic abortion screen increases the chances of a successful diagnosis by 5 times (Dr. Mark Kinsel, Agricultural Information Management, Inc.).

Many university diagnostic labs offer excellent websites that lay out samples to collect, how they should be stored or transported, etc. The table on the following pages is the abortion panel as listed on the Washington State Diagnostic Lab website. It is among some of the best seen and lists sample type, how to submit, test type and the agents detected. It can be used as a guide.

Table 1: Bovine abortion tissue panel: Washington State Diagnostic Lab

| Samples | Preservation | Test | Agents detected |
|---|---|---|--|
| Entire fetus & placenta | Fresh chilled | Necropsy (NA) | Rare presumptive causes may be detected grossly. |
| Dixed tissue: Minimum: Placenta*, liver, spleen, thymus, thyroid, kidney, lung, heart, lymph node, tongue, diaphragm, brain. Affected skin if suspect fungal causes. | Fixed in 10% buffered neutral formalin at 10:1 (formalin:tissue) ratio. Max. 1 cm sample thickness. | Histopathology (H) | Presumptive causes may be detected histologically (e.g. IBR, fungi, etc.). |
| Fresh (unfixed) tissue: <input type="checkbox"/> Pooled (liver, kidney, lung, & spleen) | Fresh chilled | Aerobic culture (B) | Bacterial causes (does NOT include Mycoplasma or Ureaplasma). |
| <input type="checkbox"/> Fetal stomach content | Fresh (unfixed) chilled | PCR (MD) Aerobic and Campylobacter culture (B) | BVDV Bacterial placentitis |
| <input type="checkbox"/> Fresh placenta* | Fresh (unfixed) chilled | Bacteriology (B) | Campylobacter |
| <input type="checkbox"/> Fresh liver | Fresh chilled | Selenium (T) | Selenium |

As can be seen, many different sample types may be required to reach a diagnosis.

In summary, abortion diagnosis has a well-defined case definition of pregnancy loss between 42 and 260 days of gestation. Sorting out true abortion numbers on any dairy may be difficult and provide variable results. One or 2 metrics are best to baseline abortions in the individual herd and should be tracked routinely on the dairy. A complete herd work up followed by diagnostics are the 2 principal components for achieving a successful diagnosis. Submitting a complete abortion screen and requesting the diagnostics for each type increases the chances of an abortion diagnosis by 5-fold.

Table 1, 2 and 3 may be found at https://waddl.vetmed.wsu.edu/docs/librariesprovider10/abortion-panels/bovineabortionpanel.pdf?sfvrsn=b53c753b_12

Table 2: Bovine abortion serologic profile: Washington State diagnostic lab

| Sample | Preservation | Tests included | Agents detected |
|--|---------------|----------------|--|
| Blood clotted or blood serum separator tube from dam acute & convalescent preferred. | Fresh chilled | Serology (S) | Neospora, IBR, Brucella abortus, BVDV, Leptospira (6 serovars) |

Table 3: Additional tests available: Washington State diagnostic lab

| Samples | Preservation | Test name | Agents detected |
|---|------------------------------------|--------------------------|---|
| Unfixed fetal stomach contents | Fresh chilled | Mycoplasma Culture (B) | Mycoplasma spp. |
| Unfixed fetal stomach contents | Fresh chilled | Fungal Culture (B) | Fungi |
| Pooled unfixed fetal liver, kidney, lung, & spleen | Fresh chilled | Fungal Culture (B) | Fungi |
| | | Virus Isolation (V) | e.g. herpesviruses |
| Unfixed fetal liver OR kidney | Fresh chilled | Trace Mineral Screen (T) | Arsenic, barium, cadmium, chromium, cobalt, copper, iron, lead, manganese, molybdenum, selenium, zinc |
| Blood clotted or blood serum separator tube from dam | Fresh chilled | Trace Element Screen (T) | Calcium, copper, iron, magnesium, phosphorus, zinc |
| Must be recommended by pathologist pending pathology exam | Fixed or fresh | PCR | Neospora caninum, Coxiella burnetii*, IBR, Ureaplasma, Bluetongue/Epizootic Hemorrhagic Disease Virus, Leptospira |
| Fetal thoracic fluid, must be recommended by pathologist pending pathology exam | Fresh chilled fetal thoracic fluid | Sendout (Hold) | Pine needle abortion (isocupressic acid) |
| Must be recommended by pathologist pending pathology exam | Fixed tissues | Immunohistochemistry | IBR, BVDV, Listeria, Coxiella burnetii, Leptospira |

