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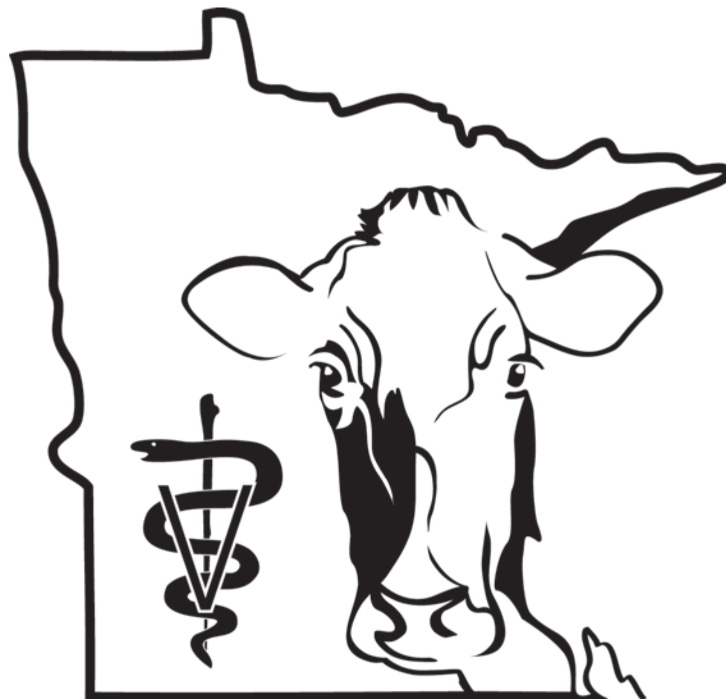

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Molecular Based Diagnostic Testing: Strengths and Pitfalls

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Introduction

Today comprehensive molecular testing services are offered by only a few diagnostic laboratories in the United States. In the near future, most AAVLD accredited laboratories will offer this service. For many laboratories, it will be the most complex addition of new services since the addition of cell culture techniques over 35 years ago. Practitioners should be aware of this new technology and understand the pitfalls associated with it. A good understanding of quality control and assurance issues for this technology will enable practitioners to select laboratories that are best prepared to give valid results.

In general, molecular based testing can be thought of as three distinct steps. The first being isolation of nucleic acid (DNA or RNA) from a diagnostic sample (blood, serum, swabs, tissues, urine, feces, semen etc.) The second is the enzymatic amplification of the target nucleic acid. Finally, the amplified nucleic acid products (amplicons) are analyzed with specialized equipment and reagents. The most common method used to amplify DNA is called the polymerase chain reaction (PCR). Other technologies have been developed but in general lack the universal applications of PCR. Initially, PCR based tests will be used in four distinct situations at diagnostic laboratories. The first case being replacement of existing tests that for whatever reason lacks satisfactory performance. A good example of this is Leptospirosis. Leptospirosis is an important bacterial disease that affects livestock, companion animals and humans. Antibody based tests for Leptospirosis suffers from a high number of false negatives and tissue tests have both a high numbers of false negatives and false positives. The second situation is where speed of diagnosis is essential. For example, a veterinarian is dealing with a herd outbreak of respiratory disease and diarrhea where bovine viral diarrhea virus (BVDV) is suspected. In general, virus isolation and paired serology testing (acute and convalescent samples) can take up to 2-3 weeks before a definitive diagnosis can be made. PCR tests can be completed in 2-3 days. The third situation is molecular finger printing. For example, is the virus or bacteria that caused disease in herd A the same strain that caused disease in herds B and C? This type of analysis was done in 1996 on BVDV isolates from six different Wisconsin herds where several animals had died from BVDV. The fourth situation is genetics testing.

However, there are advantages of existing tests over PCR-based testing. For example, PCR will not discover infectious agents from diagnostic samples that are not suspected by the laboratory or the practicing veterinarian. Bacterial and viral isolation is capable of doing this. Also in some cases, existing diagnostic tests are more sensitive than PCR. This is particularly true for small RNA viruses such as BVDV. In addition, PCR cannot distinguish between live and dead organisms. This can make clinical interpretation

difficult particularly for infectious agents where non-viable organisms can be detected for several weeks or months after the animal has become immune to re-infection.

What is PCR?

The polymerase chain reaction or PCR test became possible when a thermal stable (not destroyed at high temperatures) DNA polymerase was isolated from a hot springs bacterium found in Yellowstone National Park. The bacterium is known as *Thermophilus aquaticus* and its DNA polymerase is known as Taq polymerase. DNA polymerase is the eukaryotic and prokaryotic enzyme responsible for making a DNA copy from the complementary DNA strand when the double helix unwinds during cellular division. The Taq polymerase made it possible to automate the process of DNA replication in a machine called a thermal cycler. Prior to discovering the Taq enzyme, the relatively high temperature required to "melt" the double stranded DNA for DNA replication destroyed the DNA polymerase after just one cycle making automation impossible.

The PCR reaction starts when a small tube of reagents is placed in a thermal cycler. Typically the thermal cycler contains anywhere from 24 to 96 individual wells. The tube usually contains the following reagents. The PCR mix which contains buffer, magnesium chloride and deoxynucleotide triphosphates which are the building blocks of the DNA, the thermostable DNA polymerase, test sample nucleic acid and oligonucleotide primers. Primers are synthetic oligonucleotides, generally 15-30 nucleotides in length, which are complementary to a particular sequence of DNA found in the bacteria, virus or protozoa being tested. It is important to remember that the sequence of the primers confers specificity to the DNA amplification since the other reagents are common to all PCR reactions.

Thermal cycling profile

Each cycle of the PCR reaction consists of three distinct phases. A denaturing step at 94-95 °C (30–60 seconds) which causes the double stranded DNA template to melt or become single stranded, followed by an annealing step at 55 °C for 1-2 minutes where oligonucleotide primers hybridize to complementary sequences of target DNA. The hybridization step is followed by extension step at 72 °C for 1 to 5 minutes, depending on the length of the amplified product, where the DNA polymerase replicates a complementary copy of the template strand, extending from the primers. Primers are obligatory for DNA polymerase activity. Then the entire cycle is repeated (denaturing, annealing, extension) for 25 to 35 cycles. The theoretical amount of DNA amplification that can occur is $2^{25\text{-}35}$ which is literally millions of copies of double stranded DNA.

What are diagnostic laboratories offering for PCR tests?

In general, most of the large veterinary diagnostic laboratories in the U.S. and several smaller ones are offering PCR tests for animal disease diagnosis. The number of tests offered by different laboratories varies considerably but the repertoire is expanding and will continue to expand considerably in the next 1-3 years. For example, Minnesota, Illinois, Iowa, Michigan and Indiana all offer PCR based tests particularly for fastidious organisms or major pathogens such as porcine reproductive and respiratory syndrome (PRRS virus) in pigs.

There are a number of veterinary diagnostic laboratories doing PCR tests that lack sufficient safe guards (controls) to ensure the results are accurate (personal communication Steve Bolin, NADC, Ames, Iowa, August, 1999). In fact, I have received PCR results from veterinary diagnostic laboratories located in other states that contained a number of false positive results. To explain this comment, it will be necessary to briefly explain what the majority of veterinary diagnostic laboratories are doing for PCR testing and explain the pitfalls of their assays.

Sample cross-contamination

It is well established that the ability to amplify small amounts of DNA into millions of copies is both the blessing and the curse of PCR. The ability to amplify small quantities of DNA gives the technique its exquisite sensitivity but it also leads to the problem of inadvertently introducing small amounts of amplified DNA (amplicons) into a negative test sample before DNA amplification. It is virtually impossible to run the same diagnostic test every week without some level of cross contamination. While room segregation, the use of ultraviolet light (UV), bleach and alcohol to decontaminate work surfaces as well as the use of disposable gloves, clean labcoats etc., reduces the chances of cross-contamination it does not eliminate it. It is not feasible to run enough negative controls in the assay to detect low levels of sample cross-contamination. Statistically, the number of negative controls used by other laboratories cannot detect cross-contamination if fewer than 75-100% of the diagnostic samples are contaminated (Table 1). That is why it is obligatory that a single tube assay is used or steps taken to destroy or render chemically inert amplified DNA product (amplicons). Single tube assays (ex Tachman) only require the test sample nucleic acid be added to the PCR mix just prior to placing the tubes in the thermal cycler. The tube is never opened again thus virtually eliminating the risk of amplicon cross-contamination. While there are other potential sources of sample cross-contamination; amplicon contamination is by far the most important. Very few laboratories use specific methods to destroy amplicons and thus prevent sample cross-contamination and are unable to detect contamination unless virtually all the samples are contaminated.

False negatives

Most laboratories use well-characterized positive controls (example, tissue culture supernatant for BVDV) for their PCR assays. There are no other positive controls used in the test. Often the positive control is quite different from the type of samples diagnostic laboratories receive for testing. This is not adequate. It is essential to have internal controls in every tube of the assay to determine if amplifiable nucleic acid is present and there are no inhibitors present that will interfere with the PCR test. The PCR literature has indicated that up to 10% of fresh diagnostic samples contain natural inhibitors of the PCR test. Inhibitors are substances found in tissues, blood, feces etc. that inhibit the DNA polymerase enzyme making it incapable of amplifying the target DNA to detectable amounts thus leading to a false negative test results. False negatives cannot be identified unless internal controls are used in every tube of the assay to monitor DNA polymerase activity.

Failure to adequately confirm positive test results

Most laboratories do the following procedure to analyze PCR amplified product (s). The amplified samples are placed in individual wells of an agarose gel. In addition, positive and negative controls and molecular weight markers are added to the agarose gel as well. The test samples, controls and molecular weight markers are subjected to agarose gel electrophoresis for approximately 90 minutes. Nucleic acids are negatively charged and will migrate towards the positive charge or cathode of the gel apparatus. The smaller the size of the nucleic acid product, the farther it will migrate in the gel. After approximately 90 minutes, the gels are stained with ethidium bromide and viewed with an ultra violet (UV) light box. Ethidium bromide intercalates with the double stranded DNA and the amplified DNA can be visualized after excitation with UV light. The approximate molecular weight of the amplified product is determined by reference to the molecular weight markers. This type of analysis only tells the laboratory technician or microbiologist the approximate molecular weight of the amplified product. It provides no information about the genetic code of the amplified product. Thus this type of analysis should only be used as a screening or presumptive test that must be confirmed by a more definitive confirmation test.

Basically there are three methods to determine if the amplified product has the correct genetic code. Each amplicon can be sequenced and the genetic code determined. This is the most precise method but for practical reasons it is not feasible other than for research purposes. The second method is to do restriction fragment length polymorphism (RFLP) analysis. RFLP uses restriction enzymes (type 2 endonucleases) that cut amplified DNA at specific recognition sites creating smaller pieces of DNA. Each DNA product has a characteristic fingerprint (different numbers and sizes of DNA) that is analyzed by agarose gel electrophoresis. The third method is to use a technique known as nucleic acid hybridization. For nucleic acid hybridization, a short piece of nucleic acid (25 to 40 bases in length) which is also known as a DNA probe is used. The probe is complementary to internal sequences of the amplified DNA and will hybridize or bind to

it. The DNA probe has enzymes, haptens or fluorochromes covalently attached to it, which are used in the assay with other reagents to visualize or detect the amplified product. This is analogous to an ELISA assay or fluorescent antibody test. If the hybridization test is positive, it confirms that the amplified product contains the correct genetic code and is a true positive.

Quality Control and Assurance Measures for PCR Testing

a) Strict adherence to aseptic technique with the judicious use of disposable gloves, clean lab coats, bleaches, 70% isopropyl alcohol and UV light to minimize nucleic acid build up in the PCR laboratory.

b) Use of commercially available DNA/RNA nucleic extraction kits. This minimizes the risk of in-house reagents becoming contaminated with extraneous nucleic acid.

c) Segregation of tasks in separate rooms. A minimum of 3 different rooms for the PCR procedure should be used.

d) Single tube PCR tests should be used to minimize the chances of amplicon cross-contamination whenever possible.

e) Multiple negative and positive controls must be used for each test.

f) Internal controls should be added to each sample to monitor for PCR inhibition and the use of house keeping genes (DNA common to all nucleated cells) to determine if the nucleic acid extracted from each sample is amplifiable.

g) Hybridization tests should be done on every sample to verify the genetic code or identity of the amplified product.

h) Positive displacement pipettors, disposable RNAse-free barrier pipette tips and reagents should be used for all PCR tasks.

i) All practical measures should be used during assay development and optimization to improve the stringency (less false positive amplification) of the DNA targets. This may include using "hot start" PCR, polmerases with proof reading capability and other methods to improve the fidelity of the PCR reaction.

Concluding Remarks

PCR testing is now commonly used in human diagnostic and forensic testing. It is just starting to be used as a routine diagnostic test in veterinary medicine. It is difficult to predict the number of tests that will be performed in the next 2-5 years since many of the tests will be requested by customers in response to market forces, regulations and a

general acceptance of the technology. However, it is important that practitioners understand the technology and know the proper questions to ask to determine if the testing laboratory has sufficient quality control and assurance procedures to report accurate results.

Table: 1

	Number of Negative Controls Required to Detect Contamination at 99% C.I.						
Number of Tests	50%	40%	30%	20%	10%	5%	1%
10	5	6	7	10	10	10	10
20	6	8	10	15	18	20	20
30	6	8	11	15	23	30	30
40	7	8	11	16	27	36	40
50	7	9	12	17	29	42	50

*Number of negative controls required to detect contaminated samples.

Table adapted from Livestock Disease Surveys: A Field Manual for Veterinarians

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