Laboratory test descriptions for bovine respiratory disease diagnosis and their strengths and weaknesses: Gold standards for diagnosis, do they exist?

Robert W. Fulton, Anthony W. Confer

Abstract — The diagnosis of bovine respiratory diseases (BRD) poses significant challenges to the clinician as there are numerous infectious etiologies, operating singly or most often in combination. Clinical signs alone may not be diagnostic and the diagnostic laboratory is often used to assist the clinician. Recently many molecular-based tests have been taken from the research laboratory to the veterinary diagnostic laboratory. This review describes the "traditional tests" and several "molecular tests" and discusses the benefits and limitations of the tests and their interpretation. Clinicians should consult with their diagnostic laboratory regarding the interpretation of the test results. The rate of development and use of molecular diagnostic tests have outpaced validation, standardization, and standards for interpretation relative to their use in BRD diagnostics.

Résumé — Description des tests de laboratoire pour le diagnostic des maladies respiratoires bovines et de leurs forces et faiblesses : les étalons or pour le diagnostic existent-ils? Le diagnostic des maladies respiratoires bovines (MRB) suscite des défis importants pour le clinicien, car il existe de nombreuses étiologies infectieuses, qui opèrent seules ou le plus souvent en combinaison. Les signes cliniques seuls peuvent ne pas permettre un diagnostic et le laboratoire de diagnostic est souvent utilisé pour assister le clinicien. Récemment, de nombreux tests moléculaires ont été empruntés au laboratoire de recherche pour utilisation dans le laboratoire de diagnostic vétérinaire. Cet article décrit les «tests traditionnels» et plusieurs «tests moléculaires» et analyse les avantages et les limitations des tests et de leur interprétation. Les cliniciens devraient consulter leur laboratoire de diagnostic concernant l’interprétation des résultats des tests. La vitesse de développement et l’utilisation des tests diagnostiques moléculaires ont dépassé la validation, la normalisation et les normes pour l’interprétation relativement à leur utilisation dans les diagnostics des maladies respiratoires bovines.


Introduction

There are several questions facing animal owners and veterinarians with respect to bovine respiratory disease (BRD) diagnostics: 1) Sick calves with BRD signs recover from illness. What caused the disease? 2) Calves dying after acute illness. What caused the disease? 3) Cattle dying after prolonged disease and treatment. What caused the disease initially, why did the treatment fail, and what was the cause of death? 4) Above cattle were “well-vaccinated”. What went wrong with the vaccinations? 5) Whenever an agent is found, is it an “infection looking for a disease”? 6) Experimental infections and disease? How to prove the infecting agents cause disease?

Bovine respiratory diseases (BRD) have a major impact on the feedlot industry in North America (1–3); economic losses are due to mortality, cost of therapy and prophylaxis, and reduced performance. Veterinarians and animal owners are faced with challenges of accurate and timely diagnosis of ill and dying cattle to implement intervention strategies to control or minimize BRD. The clinician and those managing the animals are the first line of defense in disease control. The observation of the animals to detect clinically affected animals is important. However, the clinically ill animal often does not exhibit signs or lesions which are diagnostic for a specific etiology.

In veterinary medicine, particularly food animal production, diagnostic testing is used for prevention and control measures. Four disease intervention areas are: 1) vaccination programs, 2) selection of antimicrobial treatment for infected animals, 3) attempts to remove the cause of disease, usually infectious, but metabolic and toxicity causes are often involved, and 4) implementation of a biosecurity plan. Veterinary medicine and the animal industry also have a definite role in animal health...
regulations, both federal and state, such as reporting potential foreign animal diseases, as well as those North American agents regulated for cattle movement.

Diagnostic support for the veterinarian and cattle production system has been by 3 areas: 1) state and provincial diagnostic laboratories, with most accredited by the American Association of Veterinary Laboratory Diagnosticians (AAVLD); 2) the Canadian Food Inspection Agency (CFIA) diagnostic laboratories and USDA National Veterinary Services Laboratory; and 3) a growing number of private laboratories offering a variety of tests.

Diagnostics in veterinary medicine
In addition to the initial examination of live affected animals and obtaining a history, important areas for the veterinarian to gain information include: 1) assessment of involved organ system(s) by gross and microscopic lesions (histopathology); 2) identification of the etiologic agent(s); and 3) contributing factors such as metabolic defects and altered nutrition. An example of the contributing factor and the role of the diagnostic laboratory regarding the identification of the etiology/diagnosis might be the effect of mineral deficiencies interacting in the pathogenesis of disease. All 3 areas might require tests by the diagnostic laboratory for the veterinarian to provide prevention and control recommendations.

Diagnosis of BRD pathogens utilizes detection of lesions of involved organ systems plus detection of etiology. What are the “Gold Standards for diagnosis”? Usually there is a request to identify the infectious etiologic agents. These agents include numerous viruses [bovine herpesvirus-1 (BHV-1), parainfluenza-3 virus (PI-3V), bovine viral diarrhea viruses (BVDV), bovine respiratory syncytial virus (BRSV), bovine adenoviruses (BAV), and bovine coronavirus (BCV)], and several bacteria (*Mannheimia haemolytica, Pasteurella multocida, Histophilus somni,* and *Mycoplasma spp.*)(1–19).

It is important to address the infection versus disease issue. In the fall 2010, a post on the AAVLD LISTSERVE (August 13, 2010) was made by Dr. Dave Zeman, South Dakota State University concerning diagnostic tests and diagnostic criteria. He commented, “Gold standard relative to what diagnostic question you are attempting to answer? Clinical disease? Subclinical disease? Infection without disease? To what diagnostic question you are attempting to answer?” The PPV answers the question, “of the animals testing positive, how many actually do have the disease?” The NPV is the ratio of the number of test positives to the number of true positive tests. Negative predictive value (NPV) answers the question, “of the animals testing negative, how many actually do not have the disease?” The PPV is the ratio of the number of true negatives.

Prevalence. Prevalence is the number of cases that are present in a population at a given time (21). This is opposed to the often-misused term, incidence, which is a measure of the risk of developing disease and is a fraction of a population developing disease during a given period time, i.e., the rate of new cases.

Diagnostic tests
Serology. Laboratories providing antibody assays have mainly focused on viruses. 1) Virus neutralization tests (VNT) involve neutralization of a fixed amount of virus by the serum containing antibodies. The titer is the inverse of the highest dilution causing neutralization (endpoint). The term seroconversion for VNT is indicated when there is a 4-fold increase in VNT titers between the acute and convalescent sera, which may take 10 to 14 d to develop (20). 2) Enzyme-linked immunosorbent assays (ELISA) measure primary binding of antigen to antibody. Results of ELISA are often reported as the optical density of resulting reaction of the second antibody tagged with an enzyme reacting with its substrate. The second antibody in this case would be antibody to the Ig subclass reacting to the antigen. Potentially, reactions to the different Ig subclasses (IgM, IgA, and IgG) can be determined using appropriate reagents.

In addition to reporting results as optical density values, sometimes serum dilutions are made, and endpoint ELISA titers are reported. The laboratory may provide information on interpretation of titers relating to seroconversion. The ELISA
Table 1. Comparison of uses of diagnostic tests along with their strengths and weaknesses.

<table>
<thead>
<tr>
<th>Test</th>
<th>Use</th>
<th>Positives</th>
<th>Negatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serology</td>
<td>Antibody detection</td>
<td>Detect vaccine responses and past infections.</td>
<td>Titters do not necessarily infer resistance and are not able to differentiate vaccine-induced antibodies from infection-acquired antibodies.</td>
</tr>
<tr>
<td>Culture — nasal, nasopharynx, trachea, BAL</td>
<td>Detect bacteria and viruses</td>
<td>Demonstrate the presence of colonization or active infection.</td>
<td>Positive culture does not necessarily mean lung infection or causative for disease. Times for results to be obtained are days to weeks.</td>
</tr>
<tr>
<td>Culture — lung lesion</td>
<td>Detect bacteria and viruses</td>
<td>Require active replication of the agent in the tissue at time of death, so isolation usually indicates that high concentrations are in tissue. Antimicrobial resistance can be determined.</td>
<td>Sensitivity is not great and may miss true positives due to concurrent infections and antimicrobial therapy. Times for results to be obtained are days to week.</td>
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<tr>
<td>Immunohistochemistry — lung lesion</td>
<td>Detects antigen in lung lesion</td>
<td>One can localize the infectious agent within the lesion. Strong evidence that infectious agent is related to disease.</td>
<td>Sensitivity and specificity depend on available monospecific immune serum or monoclonal antibodies to specific infectious agent.</td>
</tr>
<tr>
<td>In-situ hybridization — lung lesion</td>
<td>Detects region of genome of agent in lesion</td>
<td>One can localize the infectious agent within the lesion. Strong evidence that infectious agent is related to disease. Monospecific antiserum or monoclonal antibodies not needed.</td>
<td>Depends on known, pathogen-specific genomic region for development of specific oligonucleotide primers.</td>
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<tr>
<td>Single PCR — nasal, nasopharynx, trachea, BAL swabs or collection</td>
<td>Detects genetic material of agent in sample</td>
<td>Provides specific evidence that infectious agent is in or recently has been in a sample.</td>
<td>Cannot differentiate subclinical or incidental concurrent infection from natural exposure or vaccination. Does not always detect infectious material. Cannot determine antimicrobial resistance.</td>
</tr>
<tr>
<td>Single PCR — lung lesion from supernatant of tissue homogenate</td>
<td>Detects region of agent genome</td>
<td>Potential evidence of specific infectious agent is associated with disease.</td>
<td>May not represent causative infectious agent within diseased tissue or differentiate natural infection versus MLV vaccine.</td>
</tr>
<tr>
<td>Multiplex PCR — nasal, nasopharynx, tracheal, BAL swab or collection</td>
<td>Detects region of several agents’ genomes</td>
<td>With a single test, potential evidence of one or more infectious agent associated with disease can be determined. Test provides more information than single PCR.</td>
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for antibody determination can be reversed with assays detecting antigen, such as the antigen capture ELISA (ACE) for BVDV antigen detection and the ELISA for detection of human RSV antigen used by some diagnostic laboratories to detect BRSV. The VNT measures antibodies that interfere with viral infection and, therefore, provide a functional indication of antibody efficacy against the virus. The ELISA results, in contrast, provide only indication that antibodies bind to the virus, and those antibodies may bind portions of the virion that are not essential for viral invasion of host cells. Detection of antibodies to bacteria, including *Rickettsia* spp., *Mycoplasma* spp., and chlamydia, and to protozoa varies widely with the agent. The methods of detection may include ELISA, as described, complement fixation tests (CF), and/or agglutination tests. Often testing for shipment of semen, fertilized ova, and animals for international movement requires special testing, and must be performed by certain accredited laboratories. In addition to the assays described, toxin-neutralization assays can be used for certain bacteria that secrete toxins. Detection of antibodies to *Mannheimia haemolytica* leukotoxin in cattle is a good example of this.

Agglutination or enzyme-linked immunosorbent assay (ELISA) tests have been used to measure the antibody responses to bacterial somatic antigens, whereas leukotoxin-neutralization (LN) assays and ELISA have been reported for measuring antibody responses to the leukotoxin. As with VNT and ELISA for viral infections, an LN assay indicates a functional antibody that interferes with the toxin-induced cytolytic process. In contrast, anti-leukotoxin ELISA only measures binding of antibodies to all parts of the molecule. Although there is good statistical correlation between anti-LKT ELISA and LKT-neutralization assays when numerous sera are tested, LN may be a better indicator of possible protective immunity than would anti-LKT ELISA. Viral isolation. Viral isolation (VI) tests by diagnostic laboratories have utilized the attempt to grow virus in cell cultures from diagnostic samples (22). These have been used traditionally until recent use of molecular diagnostics (discussion follows). The VI tests use susceptible cell cultures for inoculation and incubation periods. During incubation, evidence of virus replication may be observed visually under the microscope as cytopathic effects (CP). However, not all viruses show cytopathology, such as noncytopathic BVDV, and other detection methods are used to confirm virus presence such as antigen detection by fluorescent antibody, ELISA, or immunohistochemistry (IHC). For VI, initial incubation usually is 7 d, and if no CP or antigen is detected, a second incubation is used.
A downside to VI in cell cultures is that 2 passages are used, taking at least 2 wk before a test is called negative. Secondly, there may be a lack of antibody reagents for identifying "new or re-emerging viruses." Thirdly, some bovine viruses may require a special cell line for the virus to be identified, such as bovine coronavirus-susceptible human rectal tumor cells (HRT), which was not readily available to many laboratories for several years after their susceptibility to bovine coronavirus was demonstrated. Thus, there is growing use of molecular diagnostic tests such as polymerase chain reaction (PCR).

**Electron microscopy (EM).** Electron microscopy is used primarily for identification of enteric viruses; however, viruses from respiratory swabs can also be identified. Samples, often from nasal swabs and fecal swabs, are prepared for EM for visualization for viral morphology in negatively stained samples (22). In addition, ultra thin sections from fixed tissue may be examined for viral morphology.

**Bacterial isolation.** Isolation of bacteria from cases of respiratory disease can be done from nasal, nasopharyngeal, or tracheal swabs; transtracheal wash or bronchoalveolar lavage fluids; or lungs at necropsy. These samples should be maintained cold but not frozen when submitted to the laboratory. Lung specimens should be large enough where the surface of the lung specimen can be seared without killing the bacteria within the tissue. Bacterial growth is usually attempted on blood agar plates with selection of colonies for further testing. If the specimen yields multiple colony types, which is the norm for nasal and nasopharyngeal specimens, a trained technician must select colonies that appear similar to those that are known to be from pathogenic bacteria. This selection is only as good as the technician doing the selection.

Use of nasal, nasopharyngeal, transtracheal, and bronchoalveolar samples to determine the cause of pneumonia in a living animal can provide data that can be helpful for the clinician. However, several considerations must be kept in mind. First, as described, if multiple bacteria grow on the plate, the selection and identification of the one(s) responsible for pneumonia can be missed by an inexperienced technician. Secondly, all bovine respiratory bacterial pathogens can be members of the normal upper respiratory flora, and identification of one or more of such bacteria does not necessarily indicate that an organism is the cause of the disease or lesion. A laboratory indication of large numbers of colonies of one or more of those bacteria does strongly infer a cause and effect relationship. Thirdly, one of the most frustrating aspects for a pathologist is when bacterial isolation from an obvious case of bacterial pneumonia is negative due to antimicrobial therapy.

Finally, use of nasal, nasopharyngeal, transtracheal, or BAL cultures in a living animal as a surrogate for lung culture is less than perfect. For example, when bacterial isolates from nasal and tracheal samples from cattle were compared, there was 96% correlation between the bacterial species recovered from the 2 sites (23). When isolates were genetically tested with ribotyping and antimicrobial sensitivity, only 70% of the nasal and tracheal samples were similar, indicating that multiple strains of individual bacterial species are carried in the respiratory tract of cattle.

In another study, *M. haemolytica* and *Mycoplasma bovis* were isolated from deep nasopharyngeal swabs collected prior to euthanasia and from the lungs collected by lung lavage or from tissue samples at necropsy and were compared genetically (24). There was 86% correlation between upper and lower respiratory isolates of *M. haemolytica* and 100% correlation for *M. bovis* isolates; however, this study only represents isolates from 10 calves.

**Fluorescent antibody tests.** The direct fluorescent antibody test (FA) is a laboratory test that uses fluorescent dye–tagged, agent-specific antibodies to detect the presence of infectious agents (22). Fresh/frozen tissue sections, cytology preparations, or tissue touch preparations are fixed with acetone or alcohol, and reacted with the fluorescently labeled antigen-specific antibody. If fluorescence is observed microscopically, this is a positive test for antigen. Another form of the FA test is the indirect FA test. Untagged antigen-specific serum is reacted with the tissue, and a second fluorescein-labeled anti-immunoglobulin is reacted as well. The direct FA test has been widely used by veterinary diagnostic laboratories since the days of hog cholera control programs. Most BRD viruses may be identified in tissues by FA testing. A drawback to the FA test is that samples cannot be stored or archived for long due to rapid decay of the dye activity. Therefore, one cannot send a slide of an FA test to another laboratory for consultation. This difficulty is overcome by using an immunohistochemistry test (description follows).

**Immunohistochemistry.** Immunohistochemistry (IHC) has become a much-used diagnostic tool in veterinary diagnostic laboratories. It is used when infected tissues embedded in paraffin are available as starter material (22). The potential use of IHC for identification of viruses in cells obtained from bovine BAL has been demonstrated (25). In the case of tissues, the paraffin block is sectioned and mounted on slides as done for routine histopathology preparations. The sections are deparaffinized, hydrolyzed, digested with proteinase, and incubated with monospecific immune serum or an antigen-specific monoclonal antibody. The sections are then reacted with an-immunoglobulin-specific antiseraum tagged with an enzyme such as horseradish peroxidase.

The enzyme reacts with a color substrate and the localized color reaction identifies the antigen in the tissue section. This permits detection of antigen within a lesion or within specific cells by light microscopy. The positive IHC test along with the presence of microscopic lesions offers the strongest evidence for an infectious agent being associated with lesions. The accuracy of the IHC test depends on the specificity of the monoclonal antibody or hyperimmune serum for the infectious agent. The limitation to this test is often the lack of reactive monoclonal antibodies to selected agents, especially newly recognized ones.

**Molecular amplification methods in the diagnostic laboratory.** Several molecular based assays are used in veterinary diagnostic laboratories to detect pathogen DNA or RNA (26). Users should consult with their laboratory for available tests and the interpretation of test results. There is often not a standard protocol, and thus each laboratory should provide information for the interpretation of results. The critical information required for this testing is the genetic sequence information for the agent so that oligonucleotide primers can be produced and used for testing.
These PCR-based tests are widely used in veterinary medicine; however, strict adherence to good laboratory practice must be observed to obtain consistent and credible results. Methods of reporting data and quality control standards can vary from laboratory-to-laboratory. Veir and Lappin (26) recently recommended to small animal veterinarians, but the same would apply to large animal veterinarians, that if a new molecular diagnostic test is published, the originating laboratory should be used, because they will be more familiar with the nuances and have more experience with the largest number of samples. Users should consult with the Web sites for the diagnostic laboratories using PCR and IHC.

Two Web sites have comprehensive lists of molecular tests available to veterinarians: http://ihc.sdstate.org and http://pcr.sdstate.org Descriptions of molecular tests follow (22).

One of the most common uses of molecular technology in microbiology is for identification of bacterial genera, species, and subspecies as well as viral genotyping. These technologies allow laboratories to rapidly identify bacteria without the requirements of additional time-consuming biochemical tests. In the case of P. multocida, capsular type of A, B, D, E, or F can be determined without cumbersome enzymatic digestion techniques. Similarly, molecular techniques are used for identifying BVDV types 1 and 2.

Conventional PCR detection. A known genetic region is amplified in a thermocycler using polymerase to produce an amplified segment of nucleic acid. Those products are then compared to known positive controls using gel electrophoresis or sequenced and compared to published sequence for the specific agent. For RNA viruses, a reverse transcriptase enzyme reaction is required. The PCR product is directly visualized using agarose gel electrophoresis with dyes such as ethidium bromide. These gel-based PCR assays are qualitative, indicating only presence or absence of visualized product of the amplification. Some tests use a “nested” format, wherein a second round of amplification is used with another set of primers. Most laboratories have moved away from gel-based PCR to the real-time PCR formats.

Multiplex PCR formats. An advancement of PCR testing has been multiplex PCR, wherein multiple viruses and/or bacteria may be detected with one test. This greatly decreases costs to the veterinarian when compared to the one PCR assay/one pathogen approach described. It is also valuable for diseases such as BRD when multiple viral and bacterial pathogens can be involved. Primers producing different sized fragments permit the simultaneous detection of nucleic acids from various infectious agents based on the size of the product. These multiplex PCR tests are often used for human virus detection, especially for multiple human respiratory viruses in the differential diagnosis including influenza, coronaviruses, parainfluenza viruses, and respiratory syncytial virus.

These multiplex PCR tests have been attempted for veterinary medicine, mostly for research purposes including typing of Salmonella spp., P. multocida, Haemophilus parasuis, and toxigenic Escherichia coli from pigs and Clostridium spp. in meat. Recently, a multiplex PCR test for BHV-1, BVDV, and PI-3 viruses was described (27). Several veterinary diagnostic laboratories are now offering the multiplex PCR for bovine infectious agents including BRD agents.

Real-time PCR. Real-time PCR has largely taken over molecular diagnostics, including molecular tests used in veterinary diagnostic laboratories. The most common sequence-specific oligonucleotide probe format used in diagnostic real-time PCR is the dual-labeled TaqMan probe consisting of a fluorescent reporter dye coupled at the 5’- end and a quenching dye at the 3’-end (22). When the probe is intact, the close proximity of the quenching dye prevents the emission of the fluorescent dye. However, during PCR primer extension the DNA polymerase enzyme digests any bound TaqMan probe, separating the 2 dyes.

The reporter dye is no longer suppressed by the quencher dye and may now emit a fluorescent signal. The principle behind the quantitative real-time PCR (qPCR) is that during thermocycling PCR amplification will begin sooner in specimens containing a higher infectious agent nucleic acid load compared to a specimen with a lower infectious agent load. This will be observed as earlier generation of fluorescent signal [or earlier cycle threshold values (Ct)]. Conventionally, qPCR protocols are set for 40 cycles, which yield in theory, a trillion amplicons. Thus, the lower the Ct the more infectious agent. Another method for reporting results is copies per mL. The specificity of the primers and length of the amplified PCR product may alter the efficiency of the reaction and alter the Ct value. Therefore, one should be aware of each laboratory’s interpretation for positive or negative qPCR results.

Microarrays. In human medical diagnostics, broad microarrays are used for diagnosis of many pathogens from patients, and in veterinary medicine attempts are underway to develop broad-spectrum microarrays for diagnostic testing. This is a multiplex technology consisting of thousands of microscopic spots of DNA oligonucleotides for specific DNA sequences known as probes. Probe-specific hybridization is detected and quantitated by detection of a fluorophore, silver, or chemiluminescence to reveal abundance of nucleic acid sequences in the target (22). Microarrays have been used to study pathogenesis and host-pathogen interactions as well as to detect, and type various animal pathogens (28). Correctly conducted microarray analyses require strict adherence to proper controls and substantial experience on the part of those designing the arrays and conducting the assays.

In-situ hybridization. This is a relatively simple molecular probe assay that, unlike PCR, measures direct binding of a complementary nucleic acid probe to the nucleic acid of an infectious agent within a tissue specimen (26). The complementary probe is labeled with fluorescein or an enzyme for detection purposes and is a molecular-based counterpart to the FA and IHC tests. In-situ hybridization can potentially identify small numbers of organisms within a specific lesion, and the pathologist can visualize whether they are associated with foci of inflammation, within macrophages, or other lesion components.

Compared to conventional IHC, in-situ hybridization is potentially more sensitive and can identify low numbers of copies of the infectious agent nucleic acid. It is a test that can be developed and applied when specific antibodies are not available, but the gene sequence for the infectious agent is known.
Sensitivity of in-situ hybridization is not as great as for PCR-based tests, which amplify the copies of nucleic acid.

**Sequencing of infectious agent genomes.** A few veterinary diagnostic laboratories may provide sequencing of selected regions of infectious agents specific for determination of bacterial species, genotyping, subtyping, or identifying toxin or antimicrobial resistance genes. It is likely that, with new rapid sequencing regimens and lower costs for the technique, gene sequencing will be offered more in the future for diagnostic testing.

**Benefits and limitations to diagnostic tests**

**Serology.** Serotesting for naïve animals (susceptible and without prior exposure) and absence of maternally derived antibodies is often performed using acute (early as possible after exposure) and convalescent (collected 3 to 4 wk later) serum samples. Seroconversion could follow either vaccinal or natural exposure (with or without disease) to the test antigen or to another antigen with similar binding properties (20). Seroconversion suggests exposure and is not diagnostic for disease.

Potentially, use of gene-deletion mutants in vaccines or highly purified antigens can permit differentiation of types of exposure, such as selected vaccines versus natural infection. Serology is best used to monitor for active infections in a group of cattle, rather than specific disease in a single animal. There are published studies where there were seroconversions to BVDV, PI3V, BRSV, and/or bovine coronavirus (BCV) in animals without clinical signs of BRD and no history of vaccinations against these viruses (6,7,29,30). However, there were clinically ill animals seroconverting to these agents as well. Those studies support the use of serology to detect active infections on a group or herd basis.

Some may try to equate magnitude of antibody levels as means to differentiate vaccine-induced antibodies from those naturally induced. Yet there are studies, using BVDV antibody levels, where this concept was not confirmed (10,31). Variations in antibody responses can be great with some animals being high antibody responders and some low responders, and there are variations in vaccine potency. It is also noted that an animal with active immunity induced by an MLV BVDV vaccine, may have a high response upon exposure to a persistently infected BVDV animal (10,31).

Practitioners may want to know what is the “protective titer” that a vaccine must induce. Unfortunately, that is often not known. Under experimental conditions, a protective titer may be determined for a specific vaccine and pathogen; however, under field conditions with multiple infectious agents, stressed cattle from various sources and genetic pools, and pathogens with varying virulence, the statistically calculated “protective titer” may not hold true. Antibodies represent one of many adaptive immune effector mechanisms. It would also not be possible to determine a protective antibody titer even under experimental conditions. In addition, individual animal variation with respect to intensity of immune responses, specificity of immune response and susceptibility to pathogens come into play. We have experienced the occasional individual cattle that have high antibodies to viral or bacterial agents and yet are highly susceptible to experimental BRD challenge. Conversely, individual cattle with low antibody responses can occasionally be highly resistant to challenge.

**Molecular-based tests.** Nucleic acid-based assays are usually not broad spectrum as compared to culture of viruses and bacteria. The adage, “You only find what you are looking for” applies to PCR and other nucleic acid-based tests (32). Molecular tests identify nucleic acid sequences specific for the agent. A positive result may indicate either infectious or noninfectious material. For instance, it has been shown that DNA from inactivated organisms injected into the bloodstream of laboratory animals can be detected using PCR assay for more than a week after injection (26). This demonstrates the high sensitivity of the test and indicates that demonstration of the nucleic acid does not guarantee that infection is occurring. In addition, subtle genetic variations in infectious agent strains may cause the agent to be not detected by a highly specific molecular technique. The design of PCR probes may target sequences that are highly conserved among a broad based agent such as viral family, genus, or species, yet it is also possible to include multiple sequences for a specific agent. Thus the design of the probes may enhance the testing.

Nucleic acid-based tests offer rapid turnaround time with high throughput, permitting large number of tests to be performed in hours compared to culture requiring several days to weeks. Improper controls, variation among RNA/DNA extraction methods, and contamination can confound test results. Other issues include the lack of standardized criteria among laboratories for interpretation of positive or negative. Each laboratory has the criteria that they use for reporting results.

Another critical issue is whether a positive nucleic acid-based test equates in all instances to the detection of infectious virus. A case in point is reflected in a recent paper on brucellosis (33). Inoculation of embryonated chicken eggs has been the standard method for the titration of infectious BTV. Egg inoculation for detection of BTV was compared to real-time PCR in blood samples collected from experimentally infected sheep. There was positive correlation for the first 28 d post-infection, but not thereafter as there were positive PCR results in later samples. Thus the implication is that PCR positive results may be found in samples in which infectious virus may no longer be detected.

Another consideration is that a PCR test may be applied to a situation for which it was not developed and validated. In a recent exchange of letters in *Applied and Environmental Microbiology* (2011;77:1923–1924), a reader questioned the authors of an article in which a specific test for *Mycobacterium avium* subsp. *paratuberculosis* that is valid for detection of the bacterial infection in cattle detected the bacterial DNA in 81% of drinking water sampled by PCR. The letter’s authors logically attacked the use of that assay under conditions for which it has not been validated and argued strongly that the results of the manuscript under attack do not make biological sense.
In the case of BRD pathogens, therefore, we must diligently fight the urge to ask a diagnostic laboratory to test water or feed samples for pathogens using their PCR tests designed for use on tissue or excretions, because it would likely be using that test under conditions for which it has not been validated, and therefore, finding of positive results would be invalid and meaningless. Another disadvantage of identification of pathogenic bacteria in specimens using molecular techniques is that antimicrobial susceptibility and resistance information is not readily ascertained. Once a bacterium is identified, resistance to some antimicrobials may be subsequently tested using molecular probes for certain resistance genes. For example a microarray was used to test *E. coli* DNA for several tetracycline resistance and β-lactamase genes (28). Antimicrobial resistance, however, is not always linked to 1 specific gene and may involve multiple genes, gene complexes and plasmid-based genes. Therefore, for now, antimicrobial resistance and susceptibility are best determined on isolates.

In some instances, the availability of more tests complicates the diagnostic process (34). Lungs were examined for *H. somni* from 65 cases of BRD pneumonia using conventional and molecular tests. The bacterium was cultured from 10 cases, identified by IHC in 17, and found by *in-situ* hybridization in 19 cases. Using PCR, they found 21 positives using material washed from the lung culture plates, 29 positive from lung swabs from lung cut surface and 30 positive from bronchial swabs. The authors recommended that, due to its rapidity and sensitivity, PCR should be considered for use as a supplemental tool for diagnosis of specific pathogens in bovine lungs, but they acknowledged that IHC and *in-situ* hybridization may give the most accurate and useful results.

It is our opinion that direct culture, IHC and *in-situ* hybridization can be more readily interpretable with respect to cause and effect of a specific pathogen in BRD. The PCR tests could be detecting only small amounts of bacterial DNA inhaled from nasal or tracheal normal flora, residual DNA from bacteria or viruses killed by host immunity, or incidental secondary agents.

An important issue facing those receiving diagnostic laboratory results from molecular testing is the interpretation of positive results in the absence of lesions or appropriate clinical signs confirmed by VI, FA, or IHC. One must ask, “Does PCR positive carry the day for the causative agent without other criteria?” Many times submitted tissues are unsuitable for traditional testing, yet a tissue homogenate can be tested by nucleic acid tests, and a positive result reported without recovery of the infectious agent.

There are differences in professional opinions among diagnosticians and clinicians as to the etiologic agent when infectious material is not isolated or agent specific lesions are not observed, yet a molecular test is positive. For example, there is a report of an experimental challenge study of heifers exposed to BHV-1, in which abortions occurred post-exposure with a small group of fetuses that had no histologic lesions, yet were VI positive (35). Studies need to be performed using known experimental challenge experiments and a complete battery of tests available including VI, FA, IHC, and PCR. Some of these are in progress, or at least archived tissues need to be examined.

### Conclusions

In recent years, a battery of diagnostic tests has become available; the extent of which was unthinkable 25 years ago. With modern molecular tests, the presence of infectious agents or their nucleic acids can be rapidly identified in samples from BRD cases. Unfortunately, the high sensitivity of molecular tests is such that positive results must be carefully considered with respect to their validity. The rate of development and use of molecular diagnostic tests has outpaced validation, standardization, and standards for interpretation relative to their use in BRD diagnostics. Finally, each veterinarian upon receipt of molecular test results should ask the question, “Does the result make biologic sense?”

### References


