

Diagnosing Viral Infections: Beyond The Paired Sera

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Introduction

One of the traditional methods of diagnosing viral infections is by use of paired sera. The testing of paired sera usually involves one or more tests performed on serum taken during the acute phase of disease and during the convalescent phase. A significant rise in titer to a particular virus during these time frames confirms the diagnosis of recent infection.

Serologic methods suffer from many limitations, the most serious of which may be that diagnostic information is not obtained within a clinically relevant time frame. That is, the disease episode may be long past before serologic titers are obtained, and the resulting information is useful only in a retrospective way. In addition, serologic testing suffers from variation in titers due to differing degrees of exposure of the animal to the agent(s); inherent differences between individual antibody responses; differences among the types of serologic tests employed; and the laboratories performing the tests. Serologic results are only indirect evidence of infection and thus require interpretation which is often subjective.

Usually much more definitive information is at hand if a viral agent is detected directly from a specimen or in a tissue from the affected animal. The knowledge that a particular virus is present permits assumptions to be made regarding the cause of the disease. Compared to serologic testing, virus detection is usually more easily interpreted as well. It is not subject to the high degree of variation of serologic titers, nor is it necessary to ascertain when the samples were taken relative to the antibody response of the animal. The detection of viruses or viral antigens in specimens has its own set of limitations, however, which may restrict its use in a variety of circumstances.

This paper addresses some of the inherent limitations of detecting viral infections in cattle. It also addresses new diagnostic tests that offer significant practical advantages as well as increased sensitivity of virus detection. The methods presented have been selected from among many new methods that have been recently published and are continuing to be published at an increasing rate. At this time it is best to remark that most of the newer methods have great potential, but have not been subjected to enough field situations or animal specimens to predict future usefulness. In order for development and application

of a truly useful test, the critical underlying assumptions necessary for successful diagnosis of bovine viral infections must be examined for their impact on diagnostic methods and diagnostic laboratories.

Some Underlying Assumptions Regarding the Success of Virus Diagnosis

Assumption 1: A terminally ill animal with a suspected viral disease can be appropriately sampled and a diagnostic lab can diagnose the viral etiology.

For most common bovine viral diseases, the virus initiates the disease (e.g. IBRV or BRSV in the respiratory tract), but complicating factors and secondary infections often lead to the eventual demise of the animal. Such an animal (or derived specimen) is one that is frequently singled out for submission to a diagnostic laboratory for viral diagnosis. However, viruses replicate in the animal for only a limited period of time. When sampled late in the course of disease, as the animal becomes more sick due to complicating factors, the virus is usually no longer detectable. It is important, therefore, to sample at the earliest opportunity an animal showing the initial or earliest signs of viral infection, regardless of whether the herd has animals suffering from more severe and/or late manifestations. No method, new or old, detects viral infection after shedding has ceased. Thus, the assumption that terminally ill animals can frequently be diagnosed by detection of virus is false: animals must be sampled early for successful virus detection.

Assumption 2. An animal located at a site distant from a diagnostic virology laboratory can be appropriately sampled and the laboratory can diagnose the viral infection.

Whether or not it is a traditional, new, or highly sensitive test, preservation of specimens for detection of viruses is necessary. Most of the common viruses infecting cattle are not stable after sampling; that is, the viruses lose viability with time, and particularly with elevated temperature. Thus, specific measures must be taken in order to preserve

virus viability and/or prevent antigen degradation so that a laboratory can detect them after transport to the laboratory. Fortunately, this is not difficult. It is readily accomplished by specific transport media available from most laboratories, or by transport of virus specimens, such as tissues, under refrigeration. When detection methods are used that rely only on the presence of viral antigens or viral nucleic acids, and not the viability of virus, specimens must still be preserved to prevent degradation of the viral components. Thus, the assumption that remote animals can be readily diagnosed is essentially true, but specific measures must be followed for success.

Assumption 3. The laboratory procedures will be rapid, economical, and sensitive for the detection of desired viruses in collected specimens.

This assumption is quite a broad one, and frequently presumed to be true. In fact, individual tests vary highly with respect to how rapidly they are done, cost, and sensitivity. Some tests are quite rapid, but insensitive. Others are time consuming, but highly sensitive. In our laboratory, virus isolation for BVDV may take 2-4 weeks, but this is ten-times more sensitive than detection of the virus in tissues with immunofluorescence, which takes a few hours. The assumption is influenced not only by the type of test performed, but also by the specimen collected. While the laboratory may know the best test to select, test choice may be limited because the specimen collected and/or submitted precludes performing the test of choice. Thus, for assumption 3 to be correct, knowledge of the different possible tests, and each test's sensitivity to the suspected virus, must be taken into account for the specific specimen collected. This is often accomplished by prior consultation with a laboratory, particularly in the case of some of the new test methods.

Assumption 4. The laboratory results will be accurate.

This may be the most common assumption by far! It is an important assumption, however, not because laboratory procedures are accurate, but because they are occasionally inaccurate. Even if a test is 95% sensitive (an excellent level of sensitivity, often not achieved), there will still be 5% inaccurate tests, mostly negative results which are falsely negative. The more important consideration may be whether a test is falsely negative however, but whether it is falsely positive: that is, whether it reports the presence of a virus agent when it is not really there. Most laboratories will make every effort not to use tests that give false positive results; that is, have poor specificity. However, using only tests that are highly specific often involves a sacrifice of sensitivity. This assumption then, that laboratory results

are accurate, must always be qualified by some knowledge of the sensitivity and specificity of each test. A laboratory should readily communicate information about accuracy of the test results.

Development of New Tests

The last assumption (that laboratory results are always accurate) is at the core of development of new and useful diagnostic virology tests. One reason for the existence of diagnostic laboratories is to help veterinarians to make sound, valid, scientifically-based decisions about animal health, including control, diagnosis, and appropriate treatment of disease. For this, diagnostic testing must be accurate above all else. It is easy to say that any new test must be accurate. The question that must be asked, however, is accurate compared to what? It is very helpful when a new test is more sensitive than ones currently offered, but it is also necessary to show that the increased sensitivity is reliable. Increased sensitivity must not be claimed when the increase in positive results cannot be verified and may represent results that are not truly positive, i.e. false positive. This is a central problem in the development and application of new diagnostic tests. It is the major reason that we have so many new tests with good potential, but which are slow to be utilized. They must first be rigorously tested in field or laboratory conditions that represent routine applications to naturally occurring diagnostic cases. Finally, new tests should also offer improved practical features, such that the test is easier to do, more rapid, or offers advantages in particularly difficult diagnostic situations. With these considerations in mind, the following sections review some new and practical viral diagnostic tests which have been shown to be useful in specific diagnostic situations.

Antigen Detection: Immunoperoxidase and Immunofluorescence

The use of antigen detection for virus diagnosis has become an important, rapid, and widespread approach to bovine virus diseases. Traditionally, fluorescent antibody (FA) tests have provided rapid results on frozen sections derived from freshly obtained tissues (1-4). These FA tests have good sensitivity when the tissues are fresh, taken early in the course of the infection from appropriate sites, and appropriately transported to the laboratory.

Recently, immunoperoxidase (IP) tests have been developed for the detection of several bovine viruses (5-12) and these tests offer several advantages. First, several IP tests have been developed for the detection of antigen in formalin-fixed tissue sections (5-7,9,11). This is a distinct advantage of IP over FA because application of the test procedure lends itself to routine histopathologic specimens, whereas FA tests usually must be performed with cryostat sections of frozen tissue. Thus, collection of specimens can be simplified by collecting a single tissue sample

for both histopathology and virus detection, instead of collecting and fixing one specimen for histopathology and preserving one fresh specimen for virus detection. With IP, the presence of the virus can be correlated with the lesion(s) in the single tissue specimen, simultaneously in the laboratory. A good example of this is provided by use of IP to detect IBRV in aborted fetus tissues (figure 1a) where multifocal lesions of necrosis were shown to contain virus antigen (5,11). Another useful IP test is the detection of BRSV in adult lung tissue which has demonstrated a close correlation with histopathologic lesions (5,13). By obtaining a clear visualization of the antigen in a lesion, the amount of information derived from routine histopathologic examination is increased.

Sensitivity of detection may be increased as well with IP. With both of the examples cited above, IP provided an improved sensitivity over other commonly used methods (7,13). In a three way comparison of IP, FA, and VI, on aborted fetuses for the detection of IBR virus, IP had a sensitivity of 94%, FA of 67% and VI of 6% (7). Infectious bovine rhinotracheitis virus is difficult to recover from aborted fetuses usually because autolysis has rendered the virus non-viable; similar problems with autolysis can arise upon FA testing. With IP, the problems with autolysis are not as frequent (7,8,10). Bovine respiratory syncytial virus infection is also difficult to diagnose. It is especially difficult to recover the virus due to its fragility and its low amount of shedding. The application of IP to BRSV diagnosis has also improved sensitivity of diagnosis over FA (13,14).

The increased sensitivity of IP over FA may largely be due to amplification systems which are often used after the application of the primary antibody. Among the amplifica-

tion systems used are the indirect method, the peroxidase anti-peroxidase method, and the avidin-biotin peroxidase complex (ABC) method (15). The ABC method has the advantage of sensitivity and ease of use. Although it is a more complex, multistep procedure, and therefore a little more difficult to perform, increased sensitivity is gained because higher amounts of the detecting reagent, the peroxidase enzyme, are bound, and in an exquisitely specific manner. This is accomplished through the binding of avidin-linked enzyme to biotin-linked antibody which provides a specific binding reaction with a very high binding coefficient. With this amplification system, more peroxidase molecules are bound and they are bound more tightly; in comparison, FA, which is accomplished by direct conjugation of the fluorescent dye to the antibody molecule, there is no amplification, and the binding depends only on the strength of the antibody-antigen bond. The disadvantage of having to perform more steps with IP and at a somewhat higher cost are far outweighed by the increase in sensitivity, application to formalin fixed tissues, and with the correlation of tissue lesions on histologic examination.

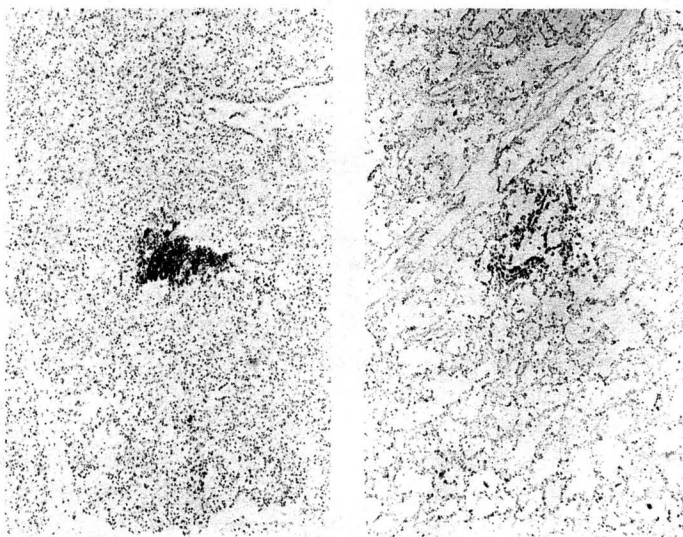
The application of IP to material obtained from live animals, such as nasal epithelial cells, lung lavage samples, or peripheral blood lymphocytes, has not been reported in a diagnostic setting. However, fluorescent antibody tests have been successfully applied in these circumstances, and include studies performed with BRSV, IBR and PI3 on nasal epithelial cells (3,4), with BRSV on lung lavage cells (17), and on peripheral blood lymphocytes for BVDV (16). The advantage has been the rapidity with which the FA test could be performed. It would seem beneficial to apply some of the more sensitive IP techniques on these specimens as well.

Antigen Detection: Antigen Capture ELISA

The enzyme-linked immunosorbent assay (ELISA) has revolutionized diagnostic testing. With respect to detecting viruses, the capture ELISA has been by far the most useful of all the newly developed ELISA techniques. With this test, an antibody, usually attached to some sort of plastic solid phase, is utilized to capture the virus out of some type of clinical specimen. The "captured" virus is then detected with a second, enzyme-linked antibody which acts as the detecting reagent, converting substrate into a visible end product. At this step various amplification methods may also be applied. Several examples of antigen capture ELISAs exist which have been successfully used in diagnosing bovine viral infections.

Probably the most notable examples are the antigen capture ELISA kits available for detecting rotavirus in feces from scouring calves. There are now many commercially available rotavirus kits and several are being used in veterinary diagnostic laboratories (18-20). For a variety of

Figure 1. Immunoperoxidase staining (a) and nucleic acid hybridization probe reaction (b) of IBRV-infected fetal lung lesions.



reasons having to do with cost, shelf-life, and number of samples, these kits have not been widely accepted by veterinarians. However, they are sensitive, specific, easy to perform, require minimal equipment, and can be used to screen large numbers of specimens in a relatively short time. Equally sensitive are latex agglutination kits for detecting rotavirus in feces (21), and these kits have similar advantages. Despite advantages, these antibody-based kits do not provide greater sensitivity over electron microscopic (EM) tests; sensitivity is between 82-87% of that achieved with EM with animal specimens (19), and around 90% with human rotavirus specimens.

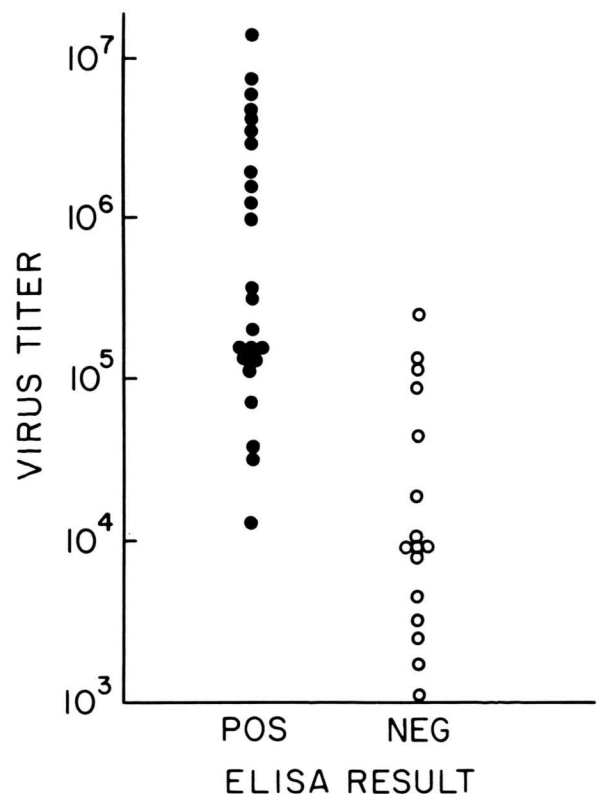
It must also be considered that an antigen capture ELISA detects only one agent. Thus, in the case of a scouring calf, where other agents besides rotavirus must be considered, such as coronavirus, K99 *E. coli*, and/or cryptosporidia, a negative ELISA test for rotavirus, while relatively convenient to perform, leaves at least three other potential causes of diarrhea that must be confirmed or ruled out. There are reports of newly developed ELISA tests for coronavirus (22,23) and kits that are available for K99 *E. coli*, but considering the time to perform the tests, the increasing cost as additional tests might be selected, and the decreasing usefulness of the information obtained, the utility of the rotavirus ELISA for veterinarians becomes low. Without an "easy to run" panel of ELISA test which covers all of the potential agents, most of the newly developed ELISA tests will be performed in referral laboratories, as they become available for each individual agent.

Other antigen capture ELISA tests that have been developed and tested with infectious diseases of cattle include ELISAs for bovine respiratory syncytial virus (24), for IBR virus (25,26), for bluetongue virus (27) and for *Chlamydia psittaci* (28). The *C. psittaci* ELISAs have been evaluated for detection of reproductive tract infection in sheep (28), where sensitivity has been found to be lacking. However, this ELISA is rapid and easily performed, and applicable to other species such as cattle infected with *C. psittaci*. The bluetongue antigen capture ELISA has been applied to detection of the virus in insects which transmit the agent to cattle, but it may have applicability to detection of the virus in blood from bluetongue virus infected cattle.

These ELISAs have as their main advantage the speed with which the tests can be completed, which is usually on the order of a few hours. With sensitivity of detection ranging from 60-80% with most of these tests, an approach has been to collect multiple specimens from a number of suspect animals and perform the ELISA test on all of them in order to obtain a herd diagnosis. With IBR, such an approach has been successful (25). This approach overcomes the fact that with ELISA, a wide range of results are often obtained, and the results do not always correlate with the amount of infectious agent present. Figure

2 demonstrates the kind of data usually obtained when antigen capture ELISA data are compared to more sensitive diagnostic procedures, in this case virus isolation of IBRV. Lower level detection limits with ELISA are usually in the range of 10^3 to 10^4 infectious units of agent (24,25, 27, 29). Thus, it is only in those situations where the agent is shed and/or accessible in high levels from some secretion or tissue that the antigen capture ELISA is really going to be useful. As discussed above this may include enteric agents such as rotavirus in feces, respiratory agents such as BRSV and IBR in respiratory secretions, and perhaps bluetongue virus in blood. The development of an accurate ELISA for the detection of bluetongue in cattle blood would be of great benefit, in that it would replace a very laborious and expensive virus isolation procedure using embryonating chicken eggs.

Figure 2. Amount of IBR virus recovered in nasal swab specimens that were positive or negative for IBR antigen when assayed by ELISA.



Although the antigen capture ELISA has some disadvantages, including lack of exquisite sensitivity, and the availability of tests for only a few agents, the continued development of these types of diagnostic kits will certainly enhance detection of viruses in the future. As additional technical advances are made in sensitivity, and as panel kits become available which cover several agents, and which are uncomplicated to use in the field, the acceptance and use of this type of diagnostic method will increase.

Nucleic acid hybridization probes

A current trend in the development of new diagnostic techniques is the use of recombinant DNA technology to develop specific molecular probes for the direct detection of gene sequences in specimens. These are indeed powerful tools which may enhance the sensitivity and specificity of detection of infectious agents (30-32). DNA probes are usually single-stranded pieces of nucleic acid labeled in some way which allows detection (radioactive isotope, enzyme, etc.) and that will hybridize with a complementary sequence of nucleic acid in a specimen. A great variety of methods are available for formatting a hybridization procedure to detect (or probe for) a specific sequence, including filter hybridization (33-39), solution hybridization (40), in situ hybridization (40-43), and others. In all cases probing with nucleic acid sequences involves disrupting and denaturing all or some of the specimen in order to convert the nucleic acids in the specimen to a single stranded state so that hybridization with the probe can take place. In this process, the infectivity of any live agent and most antigens are destroyed.

The potential advantages of nucleic acid hybridization probes are several. First, as with antigen detection, probes offer the potential for culture-independent detection of agents directly in the specimen. Thus, specimens in which the live agent has been inactivated may still be very useful when analyzed with probes. Detection of nucleic acid sequences may also avoid the problem with antigen detection which results from antibody masking of the antigen in animals that have generated an immune response. Second, probe reactions may take much less time than other methods, particularly isolation methods. For those agents that are difficult to isolate, or that grow slowly, probes could greatly increase efficiency. Third, through detection of unique sequences, probes may offer a high degree of specificity for detecting a particular agent, without as much concern for cross-reactivity that is often present when antisera are used in antigen detection.

Although probes have many potential advantages, their performance is often less than desired when applied to clinical specimens. The detection of a few organisms in a tissue or a specimen of blood may push the capability of probing to the limits, as it does antigen detection. Considering the nature of a specimen, where the amount of material from the animal source may greatly eclipse the amount of infectious agent, the ascertainment of its presence may exceed technical capabilities. Many studies tout high theoretical limits of detection, which are achieved under experimental conditions of tissue culture, but may never be achieved with chemically and morphologically complex animal specimens. This is supported by recent evaluations of diagnostic hybridization probes with clinical specimens (32).

Hybridization probes have been applied to the detec-

tion of several viral pathogens of cattle (33-41,43). One instance where the application of probe technology to viral diagnosis may enhance detection is with cattle persistently infected with BVDV. Currently virus isolation is routinely employed, and an increase in either sensitivity, rapidity of testing, or the number of specimens that could be easily handled, would greatly improve diagnosis. The identification of a few persistently infected cattle which serve as virus reservoirs in large herds would be highly beneficial, as would the rapid identification of BVDV sequences present in collected semen. Our laboratory has recently investigated a probe for detection of BVDV in peripheral blood lymphocytes from cattle suspected to be persistently infected with the virus (39). The study involved 400 cattle and for comparison purposes, virus isolation was performed simultaneously with blot hybridization of the nucleic acids extracted from the lymphocytes. Because cattle with BVDV develop a significant number of infected lymphocytes, it was feasible that hybridization probes would readily detect the viral sequences. The results of the comparison of hybridization to virus isolation were in agreement for 92% of cases (table 1). However, the sensitivity of hybridization when compared to virus isolation was only 59.5%, because some specimens displayed live virus that were not positive by hybridization. The reason for this was that a positive hybridization reaction required the equivalent nucleic acid sequences of about 10^4 viruses. The inverse was also true, namely that some specimens were positive by hybridization, but no virus was recovered. The explanation for this was that most of the virus present in the specimen was no longer alive, but the sequences could still be detected by hybridization. The principal result of this study was that different techniques work best under different circumstances. No single or new technique is going to be the answer to diagnostic problems, and even highly sensitive and specific hybridization probes have limitations when applied to variable clinical specimens. This is not to say that hybridization probes or techniques for the detection of BVDV may not be improved beyond this single study and prove superior in the future.

Table 1. Comparison of hybridization and virus isolation results of detection of BVDV in lymphocyte specimens from cattle suspected of BVDV infection.

	Virus Isolation Results ¹		
		Pos	Neg
Hybridization Results	Pos	22	18
	Neg	15	344

¹Sensitivity and specificity of hybridization compared to VI: 59.5%, 95%, respectively.

Recent studies using blot hybridization with probes for *Anaplasma marginale* in blood specimens (37) or bovine coronavirus in fecal specimens (38) have been quite sensitive. These are examples of infections where utilization of hybridization probe technology may be highly valuable. Both of these agents are ones where current detection techniques are very insensitive - light and electron microscopy, respectively. Isolation of the agent in culture is almost never attempted, and no other tests such as ELISA, are available. The disadvantage is cost (in development of probes) and the complex nature of the techniques. With further research and development, these should change dramatically, making widespread adoption of the hybridization techniques possible.

Direct detection of bluetongue virus using hybridization probes on specimens from cattle would be of great benefit (36, 44). Currently, bluetongue probes are highly sensitive for typing bluetongue virus isolates (36), and for detection in cell culture or chick embryo isolation systems (44), but they have not been utilized for routine detection in tissue, blood, or semen specimens from cattle.

An example of the use of hybridization probes with a different format is the detection of IBRV in aborted fetal tissue through in situ hybridization (43). This technique offers the advantage of visualization of the virus sequences in the specimen in a histopathologic format (figure 1b), similar to immunoperoxidase discussed earlier. In a comparison of in situ hybridization to immunoperoxidase using the same specimens, probing gave differing results: some tissue specimens, such as fetal lung, worked well; others, such as fetal liver, were not amenable to hybridization and gave poor results when compared to immunoperoxidase. In this study, the use of hybridization probes was much more technically complex than the use of immunoperoxidase, since sections had to be denatured at high temperatures, treated with enzymes, and hybridized for lengthy periods of time. Again, as with the above cited study with BVDV, the use of hybridization probes offered some advantages and some disadvantages, which must be taken into account with the particular application and set of specimens that is under investigation.

Undoubtedly more use will be made in the future of nucleic acid hybridization probes for diagnosis of infections of cattle and other species of veterinary importance. As each possible application arises, issues of the need for a new technique, the possible implications of false negative or false positive test results, cost, comparisons to standard techniques, as well as other concerns related to the control of disease will be raised. It is important that research proceed in this area, that veterinarians take advantage of the research in a knowledgeable and discerning way, and that new and truly useful diagnostic probe techniques be adopted.

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