Diagnostic Techniques for Bluetongue Viruses

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The Biology of Bluetongue Virus Infections

Our current knowledge of livestock infections with bluetongue viruses has improved significantly with the introduction of modern technology, and with a better appreciation of issues that affect the reproducibility and quality control for diagnostic tests. In particular, we now have a more informed understanding of the interactions between bluetongue virus and different animal species; of animals' responses to infection; and of the persistence of virus in nature. These issues are extremely important for disease control and for the safe movement of animals between regions and countries.

The length of time during which a bluetongue virus is present in the blood, and during which antibodies are produced and persist, will vary, especially between cattle and sheep. In the early stages of bluetongue infection, up to the sixth or seventh day after an animal has been bitten by an insect, virus is only present in the bloodstream (ie. the animal has viraemia). The level of virus in the blood reaches a peak at about seven to ten days after infection and this may coincide with a fever. In some animals, antibodies to the virus first appear at about this time. However, the ability to detect antibodies in the early stages of infection depends on the type of test and the type of antigen to which the antibodies respond after the first week of infection. Viable virus and antibodies coexist in the bloodstream for a variable number of weeks, usually not more than about three to four weeks in sheep and four to eight weeks in cattle. Virus fragments, especially RNA, may be found in blood samples for much longer periods, as shown by polymerase chain reaction (PCR) testing. However, we do not believe these fragments are able to infect insects or animals. After the virus is completely gone, antibodies remain in the bloodstream, perhaps for a year and sometimes for life. The measured length of these antibody periods depend on the species of animal, the possibility of further infections with related viruses, and the type of test used.

Diagnostic tests can be directed towards the virus; towards group antibody (antibodies common to all bluetongue viruses); or towards serotype-specific antibody (antibodies directed against the antigens unique to viruses of a particular serotype).

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Tests for Virus, Antigen and Nucleic Acid

Compared to tests for antibody, most tests to detect bluetongue virus, antigen or nucleic acid take longer to obtain results or are more expensive. However, these techniques may be the only option during the very early stages of infection.

Animal inoculation

Animal (especially sheep) inoculation has often been used as a standard for the detection of viable bluetongue virus and generally has high sensitivity. This approach, which depends on the availability of susceptible animals, is usually expensive and is not suitable for processing large numbers of specimens. The method allows the inoculation of a large volume of specimen. Confirmation of the presence of bluetongue depends on serology, so a final result may not be available for up to four weeks after inoculation. Blood from viraemic animals from a natural transmission can be a valuable source of 'wild' virus (ie. virus that has never been passaged through cell culture, or mice or chicken embryos) for pathogenicity studies.

Virus isolation

Virus isolation generally involves the inoculation of chicken embryos and/or cell cultures. One method uses intravenous inoculation of embryonated chicken eggs, followed by passage first in mosquito cells and then in BHK21 tissue cultures: this method has a level of sensitivity similar to that of sheep inoculation. The presence of virus in a specimen is detected by the occurrence of cytopathic effects (CPE) in the indicator (usually BHK21) cells, but needs further confirmation by antigen detection methods (including neutralisation with specific antiserum). The advantage of this method is that viable virus is available for typing to current world standards, and its virulence may also be tested by using the source material.

Methods using the direct detection of antigen in chicken embryos are of equal sensitivity and reduce the time for screening for the presence or absence of bluetongue virus. Antigen detection by enzyme linked immunosorbent assay (ELISA) has high sensitivity, but nucleic acid probes, immunostaining and 'dot blot' techniques may also be useful. Direct inoculation of specimens into cell cultures, bypassing the chicken embryo amplification step, usually has a markedly lower sensitivity.

Antigen and nucleic acid detection

Methods for the direct detection of antigen or nucleic acid in animal tissues have been developed in research projects but generally have a lower sensitivity than virus isolation, and so have not been adapted for routine diagnostic use. Antigen detection ELISA appears to show some promise in this area.

Polymerase chain reaction

The polymerase chain reaction (PCR) is the newest and most rapid method currently available to confirm early bluetongue infections by detecting viral nucleic acid. The presence of bluetongue virus in blood or tissue specimens can be proven within 36 to 48 hours. Although the technique is generally as sensitive as virus isolation, it is technically difficult, requires staff training, stringent quality control and laboratory discipline, and expensive equipment and reagents. Another disadvantage is that PCR does not distinguish between intact viable virus and RNA fragments. This is particularly important with vector studies, as the virus may be contained within the remains of a blood meal and not infective. Finding bluetongue RNA in an insect does not prove that it is a vector of that virus as the virus may not have multiplied, and residual virus may persist at low levels. The PCR technique may also be used to serotype some bluetongue viruses.

Tests for Antibodies

The requirements of satisfactory tests for antibodies are that:

- the tests should be sensitive, specific, highly reproducible, able to be standardised and evaluated internationally, and inexpensive; and
- reagents should be readily available, preferably based on non-infectious antigens, and stable after transport over long distances at variable temperatures.

Preferred tests are those that indicate the correct status of an animal soon after infection and over a long period of time. Available tests include complement fixation (CF), agar gel immunodiffusion (AGID), the competitive ELISA (cELISA) and the virus neutralisation (VN) test. The CF, AGID and cELISA tests are bluetongue group tests while the VN test is serotype-specific. The advantages and uses of these tests are as follows:

Group antibody

The *CF test* is technically complex and frequently has problems with unsuitable or anti-complementary sera. Antibodies may not be detected in this test for a relatively long time after infection (up to 45 days) and antibody is usually short lived (4–12 months). This test also has problems with a lack of specificity and many false positives are detected, especially in cattle or sheep in regions where neither bluetongue nor related viruses exist. The CF test is no longer recommended for bluetongue diagnosis, and is not routinely used in Southeast Asia, being applied only to imported animals.

The *AGID test* is cheap, simple to perform, requires minimal laboratory facilities, and can be used with poor quality sera. It detects antibodies to all viruses in the bluetongue group but, to a variable extent, also detects cross-reacting antibodies to viruses in related orbivirus groups. The reading of test results is subjective so weak positives may be missed. Antibodies develop very early and may be detected from eight days after infection, with animals almost always positive after 14 days. Antibody usually persists for at least one year in cattle after a single infection and longer in sheep. While AGID is not as sensitive as the cELISA, it is very useful for testing sentinel animal sera for the first appearance of antibodies to indicate that virus may be found in a blood sample.

The *cELISA test* uses monoclonal antibodies against a specific component of the bluetongue virion which is common to all bluetongue viruses. As these monoclonal antibodies do not react with other viruses, the cELISA test is bluetongue-specific. It is also more sensitive: antibodies may be detected in some cases from 7 or 8 days after infection and per-

sist for a very long time (perhaps many years). While cELISA is quicker than AGID, it needs specific laboratory equipment: however, as the latter is generally available in most diagnostic laboratories, it allows the test to be semi-automated and eliminates subjectivity during reading. The cELISA has been standardised and evaluated internationally and is the preferred test for bluetongue group antibodies. Kits of test reagents are available commercially.

Serotype-specific tests

Animal protection tests are the oldest serotype-specific test. They depend on the availability of susceptible sheep and the ability to reproduce disease under experimental conditions. These tests take more than one month to complete and require specific controls.

Virus neutralisation tests are based on the detection of neutralising antibodies, which are usually detected from 8 to 18 days after infection and usually persist for at least one year. Individual tests are required for each serotype: tests have been developed for all 24 serotypes of bluetongue viruses. The tests depend on cell culture, and require good laboratory facilities and good quality samples. Virus neutralisation tests generally detect antibody that is specific to a particular serotype but there can be cross-reactions between some serotypes. As there are minor variations even in viruses of the same serotype, for optimal results VN tests must be standardised for each country. However, since these tests rely on an active biological system, standardisation is more difficult than for cELISA and AGID and may become more subjective. This means that VN testing should be used for confirmatory and serotyping after the use of a group screening test such as cELISA. Nevertheless, a VN test is especially useful when applied to diagnostic sera and sera from sentinel animals where seroconversion on samples taken two to four weeks apart can identify the serotype: this kind of test is often referred to as a serum neutralisation test.