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Bluetongue virus antigen and antibody detection, and the application of laboratory diagnostic techniques

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Summary

Despite the significant advances that have been made in molecular techniques, the traditional approach using biology-based test procedures is still the mainstay for the laboratory confirmation of clinical diagnoses. The serological and virological techniques available for the detection and identification of bluetongue virus and antibody fall into two categories; those that are serotype-specific and those that are serogroup-specific. Although several assay methodologies have been described and used, thought should always be given to their use in different epidemiological situations and to the interpretation of results obtained therein.

Keywords

Bluetongue – Diagnosis – Enzyme-linked immunosorbent assay – Neutralisation – Serological test – Serogroup – Serotype – Virological test – Virus.

Introduction

Identification of bluetongue virus (BTV) and antibody is an essential part of the laboratory confirmation of BTV infection. This may be achieved in three different ways, as follows:

a) identification of antibody by serological assay
b) identification of the virus antigen by virological assay
c) identification of the specificity of nucleic acids by reverse transcriptase-polymerase chain reaction (RT-PCR) and sequence analysis (44), which are discussed elsewhere. Both serological and virological assays rely on the fact that BTV-specific antibody 'complexes' or 'binds' with BTV.

Pearson gave a clear and concise overview of some the most frequently used antibody detection tests at the Second International Symposium on bluetongue (35). Details of some of the traditional virological assays currently used are outlined in the OIE *Manual of standards for diagnostic tests and vaccines* (33). This paper discusses the application of some of these frequently used techniques, in different epidemiological situations.

Serological techniques

BTV has a 10-segmented genome and each segment encodes for a different viral protein, seven of which are structural and three non-structural. The outer capsid, structural viral proteins VP2 and VP5 are the serotype determinants and are responsible for generation of serotype-specific neutralising antibody. Serogroup specificity on the other hand is generally considered to be a measurement of the immunodominant VP7, a major, structural core viral protein, although antibody against other structural and non-structural viral proteins undoubtedly contribute (38).

It is essential that diagnostic laboratories use the most appropriate test methods available to achieve the desired result and therefore they must have a clear understanding of the test uses and limitations. Testing sera for the presence of BTV antibody may be required for the following purposes:

a) to facilitate safe international trade in live animals, animal products and germplasm
b) for serological surveillance
c) for monitoring vaccination campaigns
d) for serotype identification of field strains.

The OIE *Manual* (33) outlines two prescribed tests for international trade, namely, the agar gel immunodiffusion (AGID) (34) and competitive enzyme-linked immunosorbent assay (c-ELISA) (24). Both of these tests are serogroup-specific. Other methodologies employed to measure serogroup-specific antibody include the complement fixation test (CFT) (6), the indirect or blocking ELISA (2, 25), fluorescent antibody (37) and dot immunoblotting (13). Although use of the haemagglutination inhibition (HI) test (42) has been reported for serotype identification, serum neutralisation (18, 28) is probably the most frequently used type-specific assay.

Agar gel immunodiffusion (AGID): group-specific test

The AGID test (34) is well documented as a serogroup-specific test for the detection of BTV antibody. The 2000 edition of the OIE *Manual* includes the AGID test as a prescribed test for international trade but goes on to say that the c-ELISA is the preferred test. Although the AGID test may still be used in some laboratories, the lack of sensitivity (14, 35) and documented cross-reactions that can occur with other *Orbivirus* serogroups, particularly epizootic haemorrhagic disease virus (35), makes the continued use of this assay questionable when more rapid, sensitive and specific tests are readily available.

Enzyme-linked immunosorbent assay (ELISA): group-specific test

The ELISA has been used for approximately 40 years (43) and has provided a valuable means of studying numerous antigens and their antibodies. Several different formats have since been applied to the detection of BTV antibody that include the indirect (25), the antibody blocking (2, 29), the competitive (1, 14) and IgM capture ELISA (45). Of significance is the fact that these ELISAs are all serogroup-specific, identifying primarily the highly conserved BTV VP7 of all 24 known serotypes. The OIE *Manual* (33) describes the competitive c-ELISA as a prescribed test for international trade and as a result the c-ELISA is probably the most widely used and validated method (24). Provided the test has been fully validated and fulfils the requirements in terms of sensitivity and specificity, some modification to the procedure and/or reagents used e.g. cell extracted, yeast (24) and baculovirus (20) expressed antigens, seem to have little effect. More important is when, where and how the assays are applied. For example, the slope of the antibody curve can vary considerably depending when (after infection or vaccination) the sera were collected. Using the c-ELISA as a spot test will only provide a qualitative measurement of positivity.

International trade

Diagnostic laboratories are frequently required to assay sera from apparently normal animals, for example, the import/export of llama, alpaca and deer, and sheep used to raise therapeutic antibodies such as anti-rattlesnake venom. Usually these types of samples are expected to be negative and therefore testing at a single dilution using a prescribed c-ELISA is recommended. Regardless of the modifications to the test protocol used in different laboratories, this rapid assay has proven to be sensitive, specific and reliable.

If a positive reaction is recorded in the spot test then a second blood sample may be requested and both sera can be titrated by c-ELISA. A significant rise in antibody titre recorded in sequential samples would indicate a recent infection and therefore present a potential risk to the importing country.

Incursion of virus in the absence of vaccination

As the ELISA is rapid and reliable, it is ideally suited for confirmation of exposure to a single BTV serotype and thereafter for serological surveillance to help determine the transmission and spread of BTV, particularly in the absence of disease. If the virus incursion involves more than one serotype and infection is prevalent, testing young and/or sentinel animals rapidly by ELISA may provide information on the presence and distribution of BTV and allow virus isolation to be attempted at an early stage of the infection.

Epizootic/enzootic and vaccinated countries/regions

The c-ELISA cannot differentiate between infection and vaccination with a live-attenuated vaccine, but being more rapid and economic, it is the preferred method of testing animals (non-vaccinated cattle and sheep) to help determine the distribution of BTV in a country/area, and for monitoring the efficiency of a vaccination campaign in non-infected sheep. If, as expected, inactivated and/or recombinant BTV vaccines become commercially available then new and improved ELISA methodologies that will differentiate between infection and vaccination can be devised, validated and introduced into routine use. Previously developed assays for the detection of BTV NS1 (3) and African horse sickness virus (AHSV) NS3 (27) have shown this to be feasibile.

Serum neutralisation tests: type-specific test

Serologists often refer to the method used for identifying antibody to type as a serum neutralisation

test (SNT). This is to help differentiate this method from the virus neutralisation test (VNT) which, as the name implies, is used to identify a virus to type. The SNT is serotype-specific and can be used to differentiate between the antibody produced against each of the 24 antigenically distinct serotypes of BTV. The methodologies used can vary considerably but the principles remain the same; that is, test sera are reacted separately with a constant amount of each BTV serotype. The amount of neutralisation of virus, relative to a homologous virus control in the absence of any serum, is measured biologically using mammalian cells as an indicator of virus infectivity.

The SNT is considered to be highly sensitive and specific in that it does not cross-react with other *Orbivirus* serogroups. The assay is not usually used for routine testing because it is time-consuming, expensive of reagents and the quality of the test sera may affect the cells. Other techniques such as the ELISA are often more rapid and reliable.

Incursion of virus in the absence of vaccination

Following infection by a single BTV serotype, animals develop homologous, neutralising antibody to the infecting agent and in some cases lower levels of heterologous antibody (23). In the absence of disease and/or isolation of virus, the SNT can be used to identify the dominant antibody serotype and thereby help in the selection of the most appropriate vaccine at the commencement of an outbreak. As these assays are relatively expensive in terms of time and reagents, the advantages and disadvantages should be considered before they are used for routine surveillance.

When an incursion of BTV involves more than one serotype, the interpretation of results can be more difficult; however, if the outbreaks are sporadic then it is possible to identify and map the location and spread of different virus serotypes (Table I).

Incursion of virus and after vaccination

The currently available BTV vaccines are live and contain attenuated strains of the virus and the antibodies they induce are indistinguishable by SNT from those produced after natural infection. However, provided only sheep are vaccinated, as is the case in some countries, it is possible to derive important epidemiological information about the circulating virus serotypes by testing cattle. The SNT also has value for monitoring the efficiency of sheep vaccination campaigns in buffer zones.

Table I
Identification and/or surveillance of bluetongue virus serotypes using the serum neutralisation test in different locations in Greece following incursions of more than one virus type

Origin	Species	BTV serotype 4	9	16
Mainland				
Drama	Cattle	–	120*	–
Rodopi	Cattle	–	160	–
Islands				
Rhodes	Sheep	–	20	–
Rhodes	Cattle	–	–	480
Rhodes	Goat	–	–	640
Kos	Sheep	–	–	320
Kos	Sheep	–	–	480
Larisa	Sheep	–	30	–
Evia	Sheep	–	30	–
Lesbos	Sheep	–	<60	30
Samos	Goat	–	–	320
Samos	Goat	–	–	120
Samos	Cattle	–	80	–
Samos	Cattle	–	240	–

* Reciprocal, arithmetic antibody titre

Sera supplied by Olga Mangana and Kiki Nomikou, Ministry of Agriculture, Athens

Multiply infected epizootic/enzootic/vaccinated regions

In 1986, Jeggo and others demonstrated experimentally that sheep serially inoculated with two or more different BTV serotypes over a period of time developed a broad, heterotypic antibody response against multiple BTV serotypes (22, 23). If this is indeed the case with only two BTV serotypes, then the situation will be considerably more complex in BTV epizootic and enzootic regions where multiple serotypes are circulating (41). Testing sera from older animals with an unknown history from these areas is of limited value but testing non-vaccinated susceptible animals between the age of six months, after waning of maternal antibody, and one year of age by SNT can provide valuable information about the BTV serotypes currently circulating (12). This was achieved using a technique often referred to as 'clustering'. By testing several younger animals against each of the known BTV serotypes it became apparent that certain serotypes predominated in antibody titre and occurrence. High frequencies (clusters) of antibody against specific BTV serotypes are believed to correspond to the BTV serotypes that are currently circulating in an area. From the results, these authors (12) suggested that at least three different BTV serotypes, namely: BTV-6, BTV-14

and BTV-17 were likely to have been circulating in some of the Caribbean and South American countries between 1981 and 1982.

Virological techniques

Isolation of virus and identification of serotype are the preferred and most certain methods of determining a BTV infection but the entire process of virus isolation, adaptation, amplification and finally identification can be expensive, particularly in terms of time. Application of a fully validated, serotype-specific PCR, together with relevant sequence data (44) would be considerably faster and will be a welcome and valuable addition to the bank of assays currently available.

As mentioned earlier, the serotype determinants of BTV, VP2 and VP5, are located on the outer capsid. Although the 24 BTV serotypes are antigenically distinct, they all belong to the same *Orbivirus* serogroup and are therefore related. The degree of relatedness between 23 of the 24 BTV serotypes as determined by plaque reduction was originally reported by Erasmus (11). Figure 1 includes the results obtained at the Institute for Animal Health (IAH) in Pirbright by SNT for the 24th BTV serotype. Remarkably the phylogenetic sequence analysis of genome segment 2 of the 24 BTVs shows a very similar relatedness between the serotypes (30).

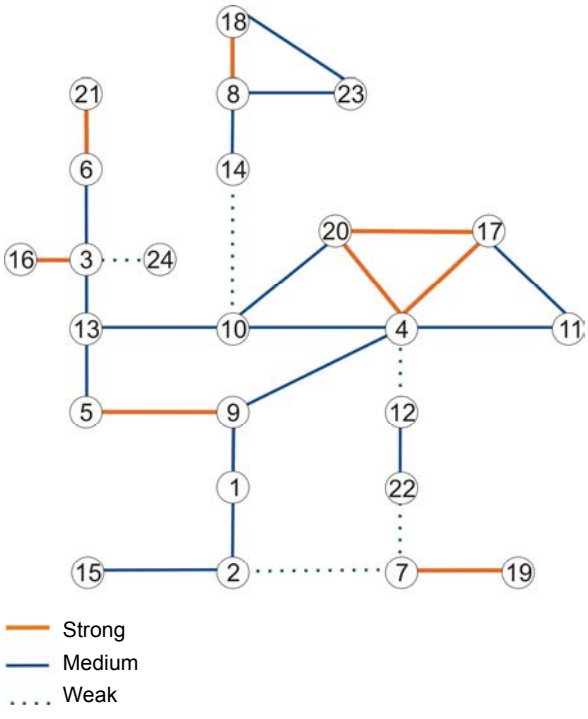

Figure 1
Schematic representation of the relatedness of each of the 24 bluetongue virus serotypes
Modified from Erasmus (11)

In the face of an outbreak of disease, it is of paramount importance to provide a rapid identification of BTV type so that appropriate control measures can be implemented without delay. Thereafter confirmatory serotyping of isolates should be made at intervals throughout the outbreak to ensure that incursions by new serotypes would be detected. Whole blood, animal tissue and, occasionally, field isolates are the most common samples submitted to the diagnostic laboratory for confirmation of a clinical diagnosis. Cultured isolates may be received although these are often for retrospective BTV serotyping.

Several virus/antibody-based methodologies for the identification of BTV have been described, which include fluorescence (5, 26, 36), immunoperoxidase (7), ELISA (10, 15), electron microscopy (21, 32), and plaque (9, 19), and virus neutralisation (33) techniques. As with the serological assays, they fall into two categories, being either serotype- or serogroup-specific. Not surprisingly, the serological and virological tests within each category complement one another and therefore the principles of the different assays are very similar. This paper discusses some of the applications of the two assays which are used at the IAH in Pirbright viz. the serotype-specific VNT and the serogroup-specific indirect ELISA (i-ELISA), also known as the antigen-capture or sandwich ELISA.

Enzyme-linked immunosorbent assay: serogroup-specific test

Mortality following infection with BTV can be as high as 70% in individual sheep flocks but it is usually much less than this. Studies have shown the i-ELISA to be capable of detecting approximately 3.0 $\log_{10}$TCID$_{50}$/ml of infectious virus (15, 40). Based on the work of Crafford (8), this equates to approximately 9 ng/ml of purified BTV antigen. Although infectious BTV can be isolated from the blood of some sheep up to at least 47 days after infection, and virus titres above 7.0 $\log_{10}$TCID$_{50}$/ml have been recorded at the peak of viraemia (16), the efficiency of ELISA for detecting BTV in blood remains questionable (17, 31, 39). The ELISA cannot therefore be relied upon as a diagnostic tool for assaying blood directly.

Most animals that die do so from sequelae after a chronic infection. Data recorded in studies performed on tissues collected from sheep killed sequentially after infection show that although infectious virus may persist in certain tissues for at least 12 days, the amount of antigen necessary to ensure a positive ELISA reaction does not (Table II). This may be due to clearance of antigen from the

Table II
Direct detection of bluetongue virus and viral antigen in sheep samples by ELISA and following egg inoculation

Sample	Bluetongue virus			
	ELISA	ELD_{50}	ELISA	ELD_{50}
Euthanised	7 dpi		12 dpi	
Blood[a]	NT	6.5	NT	5.7
Buccal salivary gland[b]	+	2.7	–	NVD
Parotid salivary gland	+	2.6	–	NVD
Mandibular salivary gland	+	2.3	–	NVD
Parotid lymph node	+	3.6	–	2.5
Retro pharyngeal lymph node	+	3.2	–	2.3
Axillary lymph node	+	3.7	–	2.4
Mediastinal lymph node	+	3.2	–	NVD
Mesenteric lymph node	+	3.2	–	2.9
Lung	+	4.4	–	NVD
Heart	+	NVD	–	NVD
Spleen	+	5.0	+	3.6

a) blood: log_{10} titre/ml
b) tissues: log_{10} titre/g
NT not tested
ELD_{50} egg lethal dose at 50% end point
dpi days post infection
NVD no virus detected

animal but is more likely to be as a direct result of interference from developing antibodies, particularly against the highly conserved VP7.

A similar observation was noted during the AHSV serotype 7 outbreak that occurred in the surveillance zone of the Western Cape Province of South Africa in 1999 (4). The i-ELISA proved very efficient at identifying AHSV in tissues during the early stages of the outbreak (A.J. Guthrie and P.G. Howell, personal communication). The decision to initiate vaccination was made prior to the confirmation of the infecting serotype. Polyvalent vaccine against seven of the nine AHSV serotypes is available in two vials, each vial being inoculated three weeks apart. Unfortunately the first vaccines inoculated did not contain the correct AHSV serotype. Shortly after administering this vaccine, the efficiency of the ELISA started to decrease. It is believed that this decrease was primarily due to the heterologous VP7 binding to (but not the neutralisation of) the infecting virus. Thus samples from susceptible animals that were infected and became sick and died after administration of the initial vaccine were recorded negative by i-ELISA.

Virus neutralisation tests: serotype-specific test

Like the SNT, the VNT is a serotype-specific test, which can be used to identify the 24 antigenically distinct serotypes of BTV. Knowledge of the spatial and temporal distribution of the different BTV serotypes can be an advantage to a diagnostician and with experience can obviate the need to use all 24 serotypes (33). The sensitivity of the assay used at Pirbright is dependent on the titre of virus in the test sample being sufficiently high to give a 2 log_{10} or greater reduction against the homologous serum when compared to the virus control in the absence of any BTV antisera.

Two problems may be encountered when attempting to serotype a BTV. First, the apparent relatedness and/or cross-reactivity that may be observed with some BTV isolates could make it difficult to confirm the actual virus designation. This may relate to the specificity of the antisera and/or the assay being used. Ideally, the diagnostician should know the reactivity of each of the 24 virus serotypes against each of the 24 currently used typing antisera in his/her laboratory because individual animals used to raise specific antibody may exhibit a slightly different antibody profile in different assays (9). Obviously this is a laborious task and such information is probably not available in most laboratories. Fortunately, many of the BTV serotypes can be excluded after the initial screening of a virus because they will not show any cross-reactivity. The information obtained each time a new BTV is serotyped can form the basis for a comprehensive database of the BTV cross-reactivities. Sometimes it may be sufficient to either test another virus isolate from the same outbreak or to use typing antisera produced in different animals.

Secondly, some isolates/serotypes that are readily isolated in eggs do not easily adapt to cell culture, therefore making it impossible to perform a 'normal' VNT. However, by using eggs as the indicator of virus neutralisation, rather than mammalian cells, it is possible to obtain a clean and reliable typing without trying to adapt the virus.

Finally, although the methods described by the OIE for the micro-VNT (33) suggest that test plates should be incubated at 37°C for between 3 and 7 days, it is possible to make a clear typing after as little as 24 h, providing the cells have grown adequately and the virus has replicated to a sufficiently high titre.

Conclusion

Regardless of the development and application of new and improved molecular-based diagnostic techniques there will, at least for the foreseeable future, be a requirement to demonstrate the presence of actively growing virus before full control measures (vaccination and slaughter) are introduced. Modifications and adaptations to the existing techniques continue to be made and reported in an attempt to develop super-sensitive tests that retain a high degree of specificity. In general, the traditional tests have withstood this barrage and have proven to satisfy the requirements for international trade, laboratory diagnosis and epidemiological investigation.

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