Developing new orbivirus diagnostic platforms

B.T. Eaton & J.R. White

Australian Animal Health Laboratory, CSIRO Division of Livestock Industries, 5 Portarlington Road, Geelong, VIC 3220, Australia

Summary

Traditionally, successful orbivirus identification and characterisation has been dependent upon the development and application of techniques for virus isolation. In recent years however polymerase chain reaction (PCR)-based molecular detection systems have revolutionised medical infectious disease diagnosis and in some instances have removed the requirement to isolate pathogens for confirmation of clinical diagnoses. In multiplexing formats, PCR-based methods also have the capacity to detect novel pathogens and variants of existing pathogens. Detection and characterisation of veterinary pathogens such as bluetongue virus will follow the same evolutionary path. Work is underway in a number of laboratories to develop the infrastructure and databases required to permit the use of DNA-based molecular systems for orbivirus detection and characterisation. Novel multiplexed protein analysis platforms also offer opportunities to not only enhance the speed and sensitivity of serological assays but also permit the development of serological procedures that a few years ago were not technically feasible.

Keywords

Array technologies – Bluetongue virus – Diagnosis – Embryonated chicken eggs – *Orbivirus* – Polymerase chain reaction – Multiplex platforms – Virus isolation.

Virus isolation

A number of methods for the isolation of bluetongue virus (BTV) have been developed over the past fifty years in an attempt to increase the efficiency with which virus in field material can be amplified to facilitate identification. Favoured methods include replication in embryonated chicken eggs (ECE), sheep, suckling mouse brain and a wide variety of cultured cells (7, 12, 13, 17, 33) . A number of the more popular approaches will be summarised briefly, prior to discussing the impact of polymerase chain reaction (PCR) and multiplexing technologies on the continuing need for virus isolation. The potential application of multiplexed platforms for other aspects of orbivirus diagnosis will also be discussed.

Embryonated chicken eggs

In a letter to *Nature* in 1940, Mason, Coles and Alexander first reported the growth of BTV in chicken embryos following inoculation into the yolk sac of ECE (25). Over a quarter of a century later, Goldsmit and Barzilai (14) and Foster and Luedke (11) showed that intravenous inoculation of ECE was 100-1 000 times more sensitive than yolk-sac administration. Since then, intravenous inoculation of 10-13-day-old ECE has been widely used as the method of choice in the isolation of BTV from clinical samples. A detailed procedure is described by Clavijo *et al.* (7). The preferred tissues for isolation include washed, unseparated blood cells, spleen, lung and lymph nodes (28). Preparation of washed blood cells for inoculation into ECE is straightforward, whereas tissues must be homogenised by grinding with sand in a mortar and pestle, in tissue grinders or more recently in 'BeadBeaters' with zirconia/silica beads. The number of ECE inoculated per sample varies but is usually 10, the incubation temperature 33-34°C and the inoculum dose 0.01 ml. Although dead embryos are usually the source of virus for the next step in the isolation process, embryo deaths are neither an indication of BTV replication nor are surviving embryos indicative of virus absence. Following death of the embryos (only a proportion of embryos may die even at the highest sample concentrations), the tissues are emulsified and the virus subjected to a second passage in either cultured cells or in ECE via the yolk sac or intravenous routes of administration.

Sheep inoculation

Sheep have been variously described to be as efficient as ECE (11, 15), less efficient than ECE (4) and more efficient than ECE (23, 27). The latter authors suggested that the larger sample volume that can be administered to sheep might account for the enhanced efficiency of isolation compared with ECE. However, sheep inoculation is often an impracticable option for many laboratories because of the requirement to maintain the sheep in holding facilities for at least 30 days after inoculation to permit development of the antibody response that provides evidence of virus infection.

Cultured cells

The first successful attempt to grow BTV in cultured cells was in 1956. BTV adapted for growth in eggs by serial passage in ECE was shown to replicate in primary lamb kidneys (18). This observation was reproduced several years later and a variety of continuous cell lines were shown to support the replication of BTV (10, 22, 29). The first successful isolation in tissue culture of wild-type non-egg adapted virus from the blood of infected sheep was in 1959 (10). Shortly thereafter Livingston and Moore (22) and Pini *et al.* (29) confirmed the feasibility of direct isolation in cultured cells.

With the development of an increasing number of continuous cell lines from vertebrates and invertebrates, many investigators sought to identify cultured cells with increased susceptibility to BTV. Methods to enhance virus adsorption by modifying either the sample, increasing the efficiency of the infection process or altering the method used to detect infected cells were investigated. Among the large number of mammalian cell lines that have been evaluated for their sensitivity to BTV and the maximal titres of virus generated, baby hamster kidney (BHK), African green monkey (Vero) and calf pulmonary artery endothelium (CPAE) are most frequently used or cited (28). Of two continuous invertebrate cell lines examined, the C6/36 cloned line derived from *Aedes albopictus* (20) manifests greater sensitivity than the cells derived from the primary insect vector of BTV in the United States, *Culicoides variipennis* (34). It is worth noting that whereas mammalian cells exhibit a cytopathic effect (CPE) after BTV infection, invertebrate cells routinely become persistently infected after peak production of virus in the absence of a CPE.

The search for the optimal cell line for routine isolation of BTV is made difficult by the large number of serotypes that need to be tested and compounded by additional isolates that defy unambiguous classification into one of the 24 currently defined serotypes. Thus few studies have examined more than the serotypes available in a specific country or geographic area and more often than not, ECE were not included in the comparison and the viruses examined were tissue culture- or ECE-adapted rather than wild-type virus isolates. The fact that tissue culture-adapted and non-adapted viruses have different biological properties is now recognised. Consequently, there is no standard procedure with international credentials for the isolation of all BTV serotypes in cell culture. However, a generalisation can be made. Primary isolation of BTV in tissue culture, even in 'susceptible' BHK or Vero cells, is significantly less efficient than in ECE (12). Although a number of techniques have been developed to identify BTV in ECE (6), primary isolation in ECE and subsequent passage in tissue culture results in the selection of a virus population amenable to investigation using traditional virus identification processes in cultured cells. These include immunofluorescence and immunoperoxidase assays using BTV-specific monoclonal antibodies (17). Neutralisation assays with serotype specific antisera are also conducted in cultured cells but it must be said that one of the major drawbacks of the current orbivirus diagnostic procedures is the time it takes – potentially a minimum of three weeks – to isolate a virus in ECE and provide a serotype diagnosis following virus neutralisation assays.

Virus isolation and identification or virus identification and isolation?

Traditionally, orbiviruses have been isolated in cultured cells prior to their identification and biochemical, antigenic and biological characterisation. However, in the past decade, traditional procedures for virus characterisation, such as enzyme-linked immunosorbent assay (ELISA) and serum neutralisation with serotype-specific antisera, have been supplemented by PCR and sequencing. In another paper in this section, Zientara *et al.* summarise current PCR technologies and their importance in differentiating wild-type and vaccine strains during the current European outbreaks (36). Hamblin discusses the application of the serological procedures currently used to detect BTV antigens and antibody (19). He also describes some of the practical problems that diagnostic laboratories face when using these tests in different epidemiological situations.

When the diagnostic technologies discussed at the Second International Symposium on bluetongue, African horse sickness and related orbiviruses in Paris in 1991 are compared with those in operation

around the world in 2003, not surprisingly there have been major advances, especially in the area of PCR-based diagnosis. I would like to look forward and consider the direction that improvements in orbivirus diagnostics might take in the next decade. In recent years, nucleic acid-based technologies have revolutionised medical infectious disease diagnosis and epidemiology and significantly decreased the time taken to confirm the presence of, or identify, pathogens in human clinical material. Molecular systems have, in many instances, removed the requirement to isolate pathogens as a mandatory step towards pathogen identification and for the confirmation of human clinical diagnoses. Detection and characterisation of veterinary pathogens will follow the same evolutionary path. Work is underway in a number of laboratories, particularly in the laboratory of Peter Mertens in Pirbright, to develop the infrastructure and databases required to underpin the use of DNA-based molecular systems for the detection and characterisation of orbiviruses.

Multiplexing platforms

In addition to PCR, the other generic technology platform that reduces the need to isolate virus prior to identification, is 'multiplexing', the ability to quantify multiple reactions occurring simultaneously in a single reaction vessel. There are a number of technologies that make multiplexing possible. Some, such as macro- and micro-arrays, can be used for the simultaneous screening of amplified DNA against multiple targets and will be an integral part of nucleic acid-based diagnostic platforms. For orbiviruses, such systems based on RNA 2 and perhaps RNA 5 will provide information on virus serotype and those based on other segments such as RNA 3, 6 and 7 will simultaneously provide geographic and topotypic data and identify the genetic origin of RNA segments in naturally occurring reassortants. Application of PCR-based methods in multiplexing formats also have the capacity to detect novel pathogens and variants of existing pathogens.

Array technologies are not the only multiplexing systems under investigation. Other approaches, for example particle-based flow cytometric assays like those developed by the Luminex Corporation, can be used for screening against 100, and potentially a greater number, of DNA and protein targets. Whilst multiplexed DNA array detection systems are becoming widespread in many areas of biological science where there is a need to look at 'whole organism' transcriptional responses, the concept of multiplexed protein-based assays is relatively new and offers the capacity to quantify the final products of the transcription and translation processes.

I would like to look at how the next generation of protein-based multiplexing assays may impact on the evolution of new diagnostic technologies for orbiviruses using Luminex© as the technology platform (www.luminex.com). As mentioned below, this technology can also be used as a DNA-based detection system, and in fact may be a more readily accessible platform for this purpose than macro- or micro-array techniques.

In Luminex©, as in other bead technologies, the reactions take place on the surface of microspheres. Each bead has a unique colour-code generated by the relative content of red and orange fluorescent dyes. Protein (antigen, antibody, receptor, etc) or nucleic acid molecules are conjugated to the surface of specific beads, via a number of different chemistries, with the bead colour-code being used to identify the reagent on the surface of the bead. Once coupled, microspheres coated with individual viral antigens for example can be pooled to create a bead set that can be used to determine if an individual animal has been exposed to one or more of the viral antigens in the set. Serum is added to the bead set and secondary antibody or protein-A labelled with a fluorescent reporter dye such as phycoerythrin, measures reactions occurring on the surface of individual beads. Beads are aligned in single file by a microfluidics system and individually interrogated by two lasers. One laser illuminates the colours inside the bead thereby identifying the reagent on the bead surface. The second laser illuminates reporter molecules and provides information on the extent of the reaction on the bead surface. The optical signals for each bead are captured and digital signal processing translates them into real-time, quantitative data.

Multiplex reactions save on labour and consumables and the multi-analyte format supports faster decision-making than multi-stage screens. Current data on test sensitivity indicate that the fluorescent read-out is more direct, stable and sensitive than the colorimetric readout of the ELISA. As the ELISA requires enzyme amplification, it is prone to variability. Furthermore, reducing the number of beads per test can enhance sensitivity by increasing the number of fluorescent signals per bead. A single instrument can be used to assay nucleic acids, antigen-antibody binding, enzymes, and receptor-ligands interactions. The rapid reaction kinetics and the homogeneous format reduce incubation times and the throughput of 20 000 microspheres per second shortens analysis time. The technology has found increasing application for the simultaneous measurement of antibodies to multiple pathogens (3, 26), cytokines (9, 31), immunoglobulins (8, 16) and antibodies to multiple variable regions of the same

protein (21). Suspension arrays have also been used for multiplexed detection of viral nucleic acids (30) and high throughput multiplexed single nucleotide polymorphism genotyping (2, 5).

In addition to the use of microsphere bead sets in the detection and discrimination of orbivirus nucleic acids, as described above for array technology, I can envisage a number of additional ways in which such a system might be used to enhance orbivirus diagnostics.

Competitive ELISA

Although the current competitive ELISA (c-ELISA) assays utilise monoclonal antibodies that target the same region of the BTV core protein VP7 (32), they do not detect antibodies to all BTV serotypes with equal efficiency (1, 24) . This may be due to antigenic variation in some virus serotypes that lead to the removal or modification of the epitope defined by the monoclonal antibody used in the test (or of neighbouring epitopes). Antibodies generated in response to such serotypes may not react efficiently with the test epitope and thus may not prevent monoclonal antibody binding and may therefore not be detected in the test. The Luminex© platform enables the combination of a number of c-ELISA assays, thereby significantly decreasing the possibility of obtaining false negative results. This could be done by coupling the monoclonal antibodies in each c-ELISA to different beads and using a baculovirus-expressed biotin-labelled VP7 antigen to which the monoclonal antibodies bind with approximately equal efficiency. The capacity of antibodies in test serum to block binding of biotin-labelled VP7 to the beads can be measured using streptavidin-phycoerythrin. Because the beads used for each c-ELISA in the multiplex reaction can be differentiated, a single test run will simultaneously provide the results of different c-ELISA formats for the one serum.

Identification of antibody specificity

This can be a challenging and time-consuming task using currently available technology. In this application of the Luminex© platform, the bead set is composed of microspheres containing purified viruses and/or a range of expressed portions of each VP2 molecule representing the serotypes of interest. Addition of test serum to the bead set will result in the preferential binding of antibodies to epitopes from specific serotypes and closely related serotypes. Antibody binding may be detected using a fluorescent-labelled secondary antibody or labelled protein-A. The conformational and antigenic structure of VP2 is complex, as shown by the ability of monoclonal antibodies that neutralise one serotype to bind to, but not neutralise, other serotypes (35). However, it is the capacity to simultaneously determine the extent of antibody binding to a large number of different viruses, expressed proteins or peptides and the generation of patterns of reactivity that provide the possibility of identifying the virus against which the antibody response was mounted. In individual geographic areas, bead sets may be generated that contain a representative collection of all the viruses in the area.

Virus detection

Virus in ECE lysates, tissue culture medium and potentially blood may be trapped by polyclonal antiserum (or a mixture of sera) coupled to a single bead type and bound virus detected using biotinylated polyclonal anti-viral antibodies and streptavidin-phycoerythrin. This application does not make use of the multiplex capacity of Luminex© technology but the increased sensitivity and speed of the assay over current ELISA approaches may make this a viable alternative.

New platforms notwithstanding

Many reasons to isolate pathogenic and non-pathogenic BTVs remain. While rapid detection and identification of virus RNA in clinical or surveillance samples and characterisation of the virus to family, genus, serotype and topotype level on the basis of nucleic acid sequence are very powerful adjuncts to clinical and epidemiological studies, we do not yet know enough about the molecular basis of pathogenesis and virulence, or the molecular foundations of the complex antigenic basis of serotype definition to warrant relying on nucleic acid sequences solely for virus characterisation. There are still many situations where it will be necessary to use live virus in cell culture, animal or vaccine studies. In addition, the speed and multiplexing capacity of PCR-based technologies must not blind us to the fact that these techniques do not detect live virus *per se*. While nucleic acid and protein-based multiplexed procedures will clearly have a significant impact, for the foreseeable future, virus isolation will remain an important component of BTV diagnosis and research.

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