

## Very Virulent Infectious Bursal Disease Virus

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### SUMMARY

Very virulent infectious bursal disease virus (vvIBDV) is a pathotypic variant of IBDV, which was first detected in chickens in the Netherlands in 1986. Since then it has spread to most countries with only North America, Papua New Guinea, Australia and New Zealand remaining free.

The primary feature of vvIBDV is the ability to induce high mortality. Mortality ranges from 5% – 25% in broilers and 30% – 70% in layers. Surviving birds are severely affected with a high condemnation rate. vvIBDV induces clinical signs similar to Gumboro disease, which is induced by classical virulent strains of IBDV, except that the disease is more pronounced and acute in individual birds and generalised in flocks. Lesions are also more pronounced with haemorrhages in the bursa of Fabricius and muscular tissue and rapid bursal and thymic regression. Microscopic lesions are similar to those seen with classical types of IBDV.

vvIBDVs are indistinguishable from classical virulent strains of IBDV in some aspects. They do not grow in cell culture unless propagated in cells such as chicken embryo fibroblasts for a number of passages to allow adaptation. This, however, results in loss of pathogenicity for chickens and genetic changes in the viral protein 2 (VP2). vvIBDVs are antigenically identical with other classical strains of serotype 1, and no antigenic differences can be detected by the available serological tests. Some of the recently generated monoclonal antibodies have, however, shown that minor antigenic differences exist between vvIBDVs and these have been used to differentiate strains.

vvIBDVs differ at the genetic level from other IBDV strains in the hypervariable region of the VP2 gene. All vvIBDV isolates from different countries are genetically almost identical and have three unique amino acid residues at position 222(Ala), 256(Ile) and 294(Ile). Detection of these three amino acid residues in a newly isolated virus is indicative of a very virulent phenotype.

### Status in Australia and New Zealand

Chickens in New Zealand are now believed to be free from all IBDV infection and commercial chicken flocks are also IBDV antibody negative. In Australia, an immunosuppressive form of IBDV is endemic, characterised by the absence of prominent clinical disease and mortality. Commercial breeder flocks are vaccinated against IBDV and hence most meat and layer breeder flocks are antibody positive. Broilers and commercial egg layers are not vaccinated; until 4 weeks of age they are antibody positive due to transfer of maternal antibodies from breeder hens

and may also become antibody positive thereafter due to either natural exposure to endemic field or vaccine strains of IBDV.

### Identification of the agent

Diagnosis of vvIBDV is based on clinical signs, ability of isolate to induce high mortality in SPF chickens, and genetic analysis. Clinical disease due to vvIBDV should be readily recognised in New Zealand. It is most likely that it would be seen in its typical acute form, with high mortality in chicks as young as 2 weeks of age. In Australia, clinical disease due to vvIBDV would be less pronounced and mortalities would be lower (10% – 25%), with chicks of between 4– 6 weeks of age most likely affected. For diagnosis, bursae collected in the acute phase of disease would be the sample of choice. For rapid diagnosis, bursal tissue (20% homogenate) should be used simultaneously in four assays: Real time polymerase chain reaction (PCR), conventional PCR followed by nucleotide sequencing, antigen capture ELISA and pathogenicity testing in antibody-free chicks. Real time PCR and antigen ELISA would give results within 24 h. These results would be confirmed by conventional PCR and nucleotide sequencing within 2-3 days. Pathogenicity testing, which should be done within a containment facility, should confirm the results of genetic analysis.

## Introduction

Very virulent (vv) infectious bursal disease virus (IBDV) has not been detected in either New Zealand or Australia. New Zealand is now thought to be free from IBDV infection.<sup>1,2</sup> Vaccination is no longer practised in New Zealand, and antibodies to IBDV have not been detected in commercial flocks. IBDV strains endemic in Australia are characterised by an inability to cause marked signs and lesions, or death. Strain 002/73 isolated in 1973 from a flock with mild disease,<sup>3</sup> is still the typical representative of IBDV strains prevalent in Australia. Infectious bursal disease as seen in the original cases described as Gumboro disease,<sup>4</sup> has not been seen in Australia. In Australia meat and layer breeders are vaccinated at 6 – 8 and 16 – 18 weeks of age with a single dose of an attenuated live and inactivated IBDV vaccine, respectively (TM Grimes, Lewisham NSW, personal communication). Consequently, antibodies are detected in most breeder flocks and their progeny. The antibody levels are monitored in many of these flocks, aiming to maintain high and uniform antibody levels, particularly in meat breeders and their progeny. In Australia broilers and commercial egg layers are not vaccinated against IBDV. Maternal antibodies in these chicks decline to undetectable levels by about 4 weeks of age. Antibodies to IBDV are detected in older chickens after natural exposure to endemic field IBDV or vaccination with IBDV.

IBDV strains of serotype 1 have been classified according to their virulence into classical virulent, classical immunosuppressive, variant and very virulent strains. Only some of the classical virulent strains, such as F52/70, Cu-1, IM and STC, induce disease initially described as Gumboro disease with mortality of less than 25% in specific pathogen-free (SPF) chickens. Immunosuppressive and variant strains cause impaired growth, lack of immunological responses following vaccination and

increased susceptibility to secondary infections, which may be accompanied with low mortalities. These strains do not kill SPF chickens. Vaccines manufactured using serotype 1 strains of IBDV retain some pathogenicity, which is seen as microscopic lesions in the bursa of Fabricius, Serotype 2 strains are avirulent and cause neither mortality nor bursal lesions in SPF chicks.

## Aetiology

### *Causal agent*

Physical, chemical and structural characteristics, as well as the replication strategy of vvIBDV are identical to other types of IBDV. The virus belongs to the family Birnaviridae, being 55 – 65 nm in diameter, non-enveloped virus with a single capsid structure of icosahedral symmetry. The viral genome consists of two segments of double-stranded RNA (segments A and B) which encode five viral proteins (VP): VP1, VP2, VP3, VP4 and VP5.

### *Genetic characteristics*

Isolates of vvIBDV from different countries are genetically almost identical. Comparison of segments A and B of vvIBDV isolates with other less pathogenic types of IBDV, have indicated only limited genetic differences typical for vvIBDV. Most differences occur in a region of the VP2 gene known as the hypervariable region, which consists of 390 nucleotides or 130 amino acids.<sup>5</sup> In this region, all vvIBDV have three unique amino acid residues at position 222(Ala), 256(Ile) and 294(Ile).<sup>6,7</sup> Detection of these three amino acid residues in a newly isolated virus is indicative of a very virulent phenotype. It has not been demonstrated with certainty, however, that these three amino acids are responsible for a strain's ability to induce high mortality.<sup>8</sup> vvIBDVs have additional genetic changes when compared with Australian IBDV strains,<sup>9</sup> but these are not typical for

vvIBDV and also occur in other overseas IBDV strains. Hence it is important that a comparison at the genetic level of a suspected vvIBDV is made against classical overseas strains such as F52/70.

### ***Antigenic characteristics***

vvIBDVs are considered antigenically identical with other classical strains of serotype 1, since no antigenic differences can be detected by the available serological tests such as virus neutralisation, antibody ELISA and antigen ELISA. However, there is evidence that vvIBDVs have undergone minor antigenic changes. Firstly, vvIBDV can infect chicks with higher levels of antibodies in comparison to other classical strains.<sup>10</sup> Secondly, two monoclonal antibodies have recently been obtained that react in an antigen capture (Ac) ELISA with classical IBDV strains, whereas they do not react with vvIBDV strains, thereby indicating the loss of at least two antigenic epitopes on vvIBDV which otherwise exist in classical strains.<sup>11</sup> Thirdly, a chicken recombinant antibody that reacts only with vvIBDV strains has been obtained, further indicating that vvIBDV strains have undergone some degree of antigenic change.<sup>12</sup>

### ***Biological characteristics***

***In vivo.*** The rate of vvIBDV replication in bursal tissue (virus titres) and pathogenicity (microscopic lesions) do not differ from classical IBDV strains.<sup>13,14</sup> vvIBDV, as well as classical virulent IBDV, are able to infect tissues such as caecal tonsils, thymus and spleen. Although IBDV antigen is present in these tissues during the acute phase for both type of strains, only vvIBDV induce microscopic lesions in these tissues.<sup>13</sup> vvIBDV also induce rapid atrophy of the thymus and pale bone marrow, which are not as prominent in infections caused by other IBDV. None of these characteristics have been used for differential diagnosis.

***In vitro.*** Neither vvIBDV nor classical strains of IBDV grow in cell monolayers. To achieve growth in cell culture, extensive passaging of vvIBDV is required resulting in genetic changes in the hypervariable region of VP2 and loss of pathogenicity for chicks.<sup>15</sup>

***In ovo:*** vvIBDV grow more readily in embryonating chickens eggs than do classical IBDV strains. This is particularly evident when viruses are inoculated via the allantoic route, as low doses of vvIBDV induce embryo mortality whereas classical virulent strains do so only at very high doses. Extensive passaging in embryonating eggs, either via chorio-allantoic membrane or allantoic route, results in adaptation and changes in the genetic make up of the virus and loss of pathogenicity.

### ***Epidemiology***

vvIBDV was detected for the first time in Holland in 1986. This virus, strain DV86, is believed to have spread to the UK where it was first detected in 1988.<sup>10</sup> Soon after it was detected in Belgium and Japan.<sup>16,17</sup> Since then it has been detected in many countries in Europe, Asia and Africa where it has become endemic.<sup>18,19,20</sup> vvIBDV has also been detected recently in South America, testifying to the ease of its spread and the threat that it poses to poultry industries of countries that are still vvIBDV free.<sup>21</sup> The prevailing opinion is that all vvIBDVs are of a single origin, probably European, from where they have spread to other countries. There is, however, a report of occurrence of vvIBDV in 1985, in the Ivory Coast.<sup>6</sup> The Ivory Coast isolates are genetically different from the 'European' vvIBDV and form a separate 'African' phylogenetic branch of vvIBDV. This would suggest that there were two independent cases of emergence of vvIBDV. However, most vvIBDV strains that have been

isolated in Africa are the 'European', rather than 'African' type.

The incubation period following infection with vvIBDV is only 2 – 3 days, being the same as for other types of IBDV. Natural infection is by ingestion of contaminated material such as faeces, food and water. vvIBDV spreads only horizontally, as do other IBDV. Infected birds excrete virus in faeces from 48 h to about 16 days after infection. Mechanical transmission is the only means of transmission.

### Clinical Signs

As is the case with classical pathogenic strains, clinical disease following infection with vvIBDV occurs only in chicks of between 3 – 6 weeks of age. Clinical signs induced by vvIBDV are similar to those induced by classical virulent strains of IBDV, such as F52/70, except that the disease is more pronounced, acute and accompanied with high mortality. Onset of the acute phase is faster and the acute phase is more generalised. Characteristically the course of disease is very short, with the sudden onset of depression for a day or two, then prostration and reluctance to move, with ruffled feathers and frequently watery or white diarrhoea. Onset of mortality is sudden and duration is short often referred to as 'spiking mortality', with birds dying within the first 4 days following signs of clinical disease. Recovery of surviving birds is rapid and likely to occur within 5-7 days from the onset of the first case of disease.

### Pathology

Post mortem findings do not differ markedly between infections with vvIBDV and classical virulent strains, except that haemorrhaging in the bursae and other tissues might be more extensive and frequent. Lesions induced by Australian IBDV strains are, in comparison, less prominent.<sup>22</sup> On necropsy the

bursae of Fabricius is turgid, oedematous and haemorrhagic, and in surviving birds is atrophied by 5 days after the onset of clinical disease. Kidneys may be dehydrated and swollen, sometimes nephrotic. Haemorrhages in skeletal muscles and in the mucosa of the proventriculus are seen in most chicks. Surviving birds are emaciated.

### Histopathology

Bursa is the preferred tissue to assess microscopic changes. There are, however, no marked differences between lesions induced by vvIBDV and other types of IBDV. Lesions become visible 24 h after infection and until day 3 are characterised by an increase in follicular lymphoid necrosis and acute inflammation. At day 3, all lymphoid follicles are affected with fewer lymphoid cells, more macrophages, bursal oedema and hyperaemia. Lymphoid depletion continues and, from day 5, cystic cavities filled with tissue debris develop in the medullary areas. In chicks infected with classical IBDV strains of low pathogenicity, regeneration of the bursa seen as repopulation with lymphocytes begins 8–21 days after infection. In the bursae of chicks infected with vvIBDV, as in those infected with more virulent classical IBDV strains, there is no recovery phase and chronic lesions develop from 3 weeks after infection. These lesions include scattered and irregularly repopulated lymphoid follicles separated by fibroblastic, interfollicular connective tissue. The bursal epithelium has a proliferative and mucin-containing glandular structure. Identification of the type of IBDV strain involved is not possible based on microscopic examination alone.

vvIBDV, unlike other types of IBDV, induce microscopic lesions in tissues such as the thymus, caecal tonsil, spleen and bone marrow. The presence of these lesions has been described in experimental infections only and the lesions have not been used for

differential diagnosis. In the thymus mild cortical atrophy is seen between 2 and 7 days. In the caecal tonsils, lymphoid cell necrosis is seen in all germinal centres from 2 to 5 days after infection. In the spleen there are many fewer lymphocytes in the germinal centres and more macrophages and heterophils. In the bone marrow there are fewer haemopoietic cells from 2 – 7 days after infection.<sup>13</sup>

## Mortality

The ability of an IBDV isolate to induce high mortality in SPF chicks is the only conclusive evidence of a strain's very virulent phenotype. The exact cause of clinical disease and death is still unclear. Mortality rates differ in field and experimental infections, and virus dose and breed and age of chickens influence both. In experimental infections vvIBDV induce high mortality only following inoculation of  $10^5$  or  $10^6$  median egg (EID<sub>50</sub>) or chicken infective (CID<sub>50</sub>) doses. Bursae from field cases might not be collected at an optimum time and therefore might not contain sufficient virus to assess pathogenicity.

Genetic breed influences mortality levels.<sup>23</sup> Mortality in broiler chickens (range 10% – 25%) is lower than in layer chickens (range 30% – 70%). In contrast, the range of mortality in SPF chickens, which are all White Leghorn, a layer breed, is 25% – 100%. For this reason, it is recommended to use a known pathogenic strain of IBDV, such as F52/70, as a standard when assessing the pathogenicity of a vvIBDV isolate. Strain F52/70, when given at high dose to three-week-old SPF chicks, will induce up to 25% mortality. A vvIBDV strain is expected to cause at least double the mortality rate of that induced by the F52/70 strain.<sup>10</sup> Establishment of a median lethal dose for standardisation of vvIBDV is not recommended.

The age of chickens is the most important factor for the development of clinical signs and mortality.

Clinical disease typical of IBDV and mortality are induced only in chicks of between 3 and 6 weeks of age, either commercial or SPF. Infected chicks younger than two weeks of age do not die. For example, mortality rates in SPF chicks infected with vvIBDV strain VB849 at 7, 14, 21 and 28 days of age were 1%, 55%, 73% and 100%, respectively.<sup>24</sup> If chicks are infected before two weeks of age, less acute or subclinical disease with severe immunosuppression occurs. The reason for this narrow age range of susceptibility to clinical disease and mortality has not been explained. The absence of clinical signs or mortality in chicks younger than 2 weeks of age is not related to a lack of target cells, since virus replication and destruction of lymphoid cells (microscopic lesions) takes place to the same degree in younger and older chicks.<sup>25</sup>

Antibody status plays a significant role in field infections. In flocks that are antibody free, mortality rates are high. In the early years following the emergence of vvIBDV, mortality rates in broilers were in the order of 20% – 30%, and in layers in the order of 60% – 70%. Such high mortality rates are now unusual because managers try to ensure that young chicks have adequate maternal antibody levels. As a result, the mortality rates observed in broilers and layers, which have maternal antibody and are not vaccinated, are in the order of 5% – 10% and 20% – 30%, respectively. The presence of some poultry pathogens such as adenovirus, reovirus, or chicken anaemia virus will contribute to increased mortality rates.

Since endemic Australian IBDV strains do not kill SPF chickens, and IBDV is not present in New Zealand, mortality in field cases in either country, particularly if above 25% and associated with clinical signs and lesions previously described, would be indicative of vvIBDV.

## Diagnosis

Until recently no simple method for differentiation of vvIBDV from other types of IBDV has been available. Diagnosis of vvIBD and IBD relied on the presence of clinical disease, sudden onset of morbidity, and high and 'spiking' mortality in chicks of 4 to 6 weeks of age. Virus isolation and pathotyping by inoculation of SPF chicks were not routinely performed. In countries where vvIBDVs are not endemic and only sporadic outbreaks occur, diagnosis of vvIBD has been made entirely on clinical signs alone.

It is now current practice in most countries with vvIBDV to vaccinate broilers routinely during the first two weeks of life using IBDV vaccines of high residual pathogenicity. Consequently, it is now difficult to differentiate IBD caused by vvIBDV from that attributed to an adverse vaccine reaction. For this reason, alternative approaches to the differentiation of vvIBDV and IBDV were needed and now virus isolation and genetic analysis are used. Since pathogenicity assessment and genetic analysis are time-consuming and require a specialised laboratory, simpler methods for differentiation such as the antigen capture (Ac) ELISA have been developed.

## Diagnostic Tests

### General methods

#### *Clinical signs*

Since poultry in New Zealand are believed to be free from IBDV, disease caused by vvIBDV would be readily recognised if occurring in chicks 2–6 weeks of age because of clinical signs and high mortality (>25%). If infection occurred in chicks younger than 2 weeks of age, disease would be less obvious, or

absent, and mortality would be low. In Australia where chicks have IBDV antibodies, disease might be less severe, but still possible to distinguish from IBD because of the previous absence of IBDV-induced mortality. Mortalities could be in the order of 10%–20% in chickens 4–6 weeks old.

#### *Virus isolation*

For positive diagnosis, isolation of virus is necessary. vvIBDVs, as other IBDV strains, are best isolated from the bursa of chicks in the acute phase of the disease. A 20% bursal homogenate is made in phosphate buffered saline (PBS) from one or several pooled bursae. Bursal homogenate is best treated with solvents such as chloroform, which remove unwanted microorganisms such as enveloped viruses and bacteria, and additionally produce a homogenate that is suitable for nucleic acid extraction. Isolation of vvIBDV in cell culture for differential diagnosis is not recommended. vvIBDV will infect embryonating chicken eggs, as will other IBDV strains, following inoculation onto the chorio-allantoic membrane. Inoculation by the allantoic route is also possible but may be less sensitive, about 10 times in the case of vvIBDV. Specific deaths will begin at day 3 after infection with haemorrhages and oedema in the embryos that are typical of all IBDV strains.<sup>22</sup> It is possible to isolate vvIBDV from infected embryos or cell monolayers but changes in virus pathogenicity and genome sequence occur during adaptation to both embryos and cells. Therefore, vvIBDV isolation is best done by inoculation of undiluted bursal homogenate to SPF chickens and recovery of virus from bursal tissue 3 days after inoculation. This virus suspension should then be titrated by inoculation onto the chorioallantoic membranes of 10-day-old embryonated eggs and estimating the endpoint titres by specific deaths. The titre of vvIBDV obtained by chorioallantoic membrane inoculation is very similar to that obtained when titration is performed in

chickens in which titres are no more than ten-fold greater.

### ***Assessment of pathogenicity***

Mortality induced in SPF chickens remains the definitive method for typing of vvIBDV. The type of infection protocol used (breed or age of chickens, virus dose, route of inoculation) is critical (see section on Mortality). SPF chickens aged 3–6 weeks should be used. The 20% bursal homogenate recovered from field cases can be used to determine the level of mortality. Care must be taken, however, to ensure that sufficient infectious virus is present. This is particularly important if mortality occurred in field cases and the bursal homogenate does not kill SPF chicks. In such cases, the bursal homogenates from field cases should be titrated in embryonated eggs by the chorioallantoic route. If titres of  $10^5$  or  $10^6$  median egg infective doses/0.1 mL are obtained, the homogenate is appropriate for direct inoculation into SPF chicks; usually 0.2 mL intranasally and intra-ocularly/chick. Cumulative mortality at 4 or 5 days after inoculation is expected to be in the order of 25% – 75%. Bursal homogenates from field cases suspected to contain vvIBDV should be handled within a containment facility. All material coming into contact with infected materials should be sterilised by autoclaving.

### ***Histopathology***

Examination of microscopic lesions may confirm a diagnosis of IBD but cannot differentiate IBDV and vvIBDV.

### **Molecular diagnostic tests**

#### ***Nucleotide sequencing***

The most accurate and accepted method for the identification of vvIBDV is genetic analysis, which is done by nucleotide sequencing a portion of the VP2

gene, in conjunction with pathogenicity testing in chickens. This is the recommended method for initial identification of vvIBDV in Australia and New Zealand. Sequencing is performed in specialised laboratories. It is done, in brief, in the following manner: Viral RNA is extracted from 0.5 mL of clarified 20% bursal homogenate. The RNA is then used for reverse transcription-polymerase chain reaction (RT-PCR) using a set of primers that are expected to amplify the hypervariable region of VP2 of all IBDV strains between nucleotides 762 – 1151.<sup>5</sup> Several independent PCR clones are usually generated for each sample in order to avoid uncertainty due to possible external contamination during PCR. The mixture obtained in the PCR reaction is then subjected to gel electrophoresis in which the amplified product of approximately 390 base pairs are recovered from the gel and their DNA sequence determined. The sequences are translated into amino acids and aligned with the amino acid sequences of reference strains including very virulent, classical virulent, variant and Australian IBDV strains, using computer-assisted programs. Sequences of reference strains are obtained from GenBank and are identified by accession numbers, for example Z25480 for UK661, the prototype of vvIBDV strains. Alignment of the amino acid shows to which IBDV strain the diagnostic sample is most similar and whether it contains the three amino acids [222(Ala), 256(Ile) and 294(Ile)] characteristic of vvIBDV. Phylogenetic analysis is performed using the nucleotide sequences allowing confirmation of the type of IBDV isolated. A result would be obtained in 2 – 3 days in laboratories set up for PCR of IBDV and automated DNA sequencing.

#### ***RT-PCR/ Restriction enzyme analysis***

This method is also able to differentiate vvIBDV from other-IBDV strains. The method is faster than DNA sequencing, but in my opinion, the method is



less reliable, particularly for initial differentiation. It is based on the presence of a specific DNA sequence within the VP2 gene of vvIBDV, which is recognised by the restriction enzyme *SspI*. Positive and negative virus controls should be included such as Australian 002/73 and a reference vvIBDV strain such as UK661 or CS89. The PCR product that is obtained, 743 base pairs in size, is incubated with *SspI* enzyme and then separated on an agarose gel. If a vvIBDV strain is present, two bands of 468 and 275 base pairs will be visible whereas only one band of 743 base pairs will be visible with Australian strains such as 002/73. In recent times some strains of IBDV, including one Australian strain, have been identified that have an *SspI* site, although located at different position in the VP2 gene. In these cases, *SspI* will generate two fragments but of different sizes to that obtained with vvIBDV.

#### ***Real time RT-PCR***

Real time RT-PCR is a new method available for differentiation of vvIBDV. It is the result of a particular development in molecular biology: the use of Real time PCR and automated robotic PCR machines. RT-PCR represents a significant improvement on conventional PCR technology both in terms of time and number of samples that can be tested simultaneously. Results of the tests are available within 4 h. The test requires selection of a set of primers and probes that are specific for vvIBDV and also another set of primers and probes that are specific for other IBDV strains. Real time RT-PCR for identification and differentiation of vvIBDV has been recently developed and is now available at the CSIRO Australian Animal Health Laboratory, Geelong (H. Heine, CSIRO AAHL, personal communication).

#### **Antigen detection tests**

Antigen capture (Ac) ELISA has become increasingly popular for differentiation of vvIBDV, as it provides a result within 24 h with a good degree of confidence, can be performed in any diagnostic laboratory and is suitable for testing large numbers of samples. Two Ac ELISAs have recently been developed.

#### ***Monoclonal antibody (Mab) Ac ELISA***

This Ac ELISA is based on a panel of seven mouse Mabs and has been in use at the French Food Safety Agency, Ploufragan, France, an OIE reference laboratory for IBDV.<sup>11</sup> This ELISA is performed in two steps: the amount of test antigen is standardised, and then an ELISA measures the binding of seven Mabs for the test antigen. For Ac ELISA wells of a microtitre plate are coated with chicken anti-F52/70 sera. After blocking with 2% skimmed milk, the test antigen (20% bursal homogenate) is added at the predetermined dilution (antigen in excess); bursal homogenate of one classical strain of IBDV, such as F52/70, is also included as a control antigen. Following incubation, the panel of seven Mabs designated 1, 3, 4, 6, 7, 8 and 9 are added. Binding of Mabs is detected by addition of anti-mouse IgG-HRP and substrate. If a classical strain such as F52/70 is present in the test sample, all seven Mabs will react giving a positive signal. If vvIBDV is present in a test sample, Mabs 2 and 3 will not bind and colour will not develop. The remaining five Mabs will react with vvIBDV giving a positive signal. This two stage ELISA takes two days to complete.

#### ***Chicken recombinant antibody (CRAb) Ac ELISA***

CRAb Ac ELISA uses two CRAbs of which CRAb154 reacts with all known IBDV strains, and CRAb88 reacts only with vvIBDV strains. This

ELISA has been developed and is available at CSIRO Australian Animal Health Laboratory, Geelong. It is performed in the following way: wells of a microtitre plate are coated with polyclonal rabbit or chicken anti-IBDV sera. Test antigens, usually 20% homogenate of bursae collected from field cases, undiluted or diluted 1/10, are added to six wells. In addition two positive control antigens, one classical Australian strain 002/73 and one inactivated vvIBDV CS88 antigen, (bursal homogenates diluted 1/10) are added, each to six wells. After incubation at room temperature, CRAb154 and CRAb88 are added, each to two wells. To the two remaining wells, PBS is added. Binding of CRAbs to IBDV antigen is detected indirectly by addition first of a mouse Mab, designated anti-E-tag, followed by the addition of anti-mouse Ig-G HRP and substrate, such as aminosalicyclic acid or AZINO-bis 3-ethylbenz 2, 2'-thiazoline-6-sulfonic acid. If vvIBDV is present in a test sample, both CRAb88 and CRAb154 will react giving a positive reaction in ELISA. If a classical strain is present, CRAb154 will react, whereas CRAb88 will not. The assay takes 6 h to complete, and the results are thus available within 24 h from submission of samples.

## Serological tests

### ***Antibody ELISA***

Infection by vvIBDV and by other IBDV strains cannot be differentiated by serological tests, which can only detect seroconversion to IBDV, in either field or experimental infections. In a field infection, detection of seroconversion might not be possible if infection occurred within 10 days of the slaughter of the flock, which is insufficient time for birds to develop detectable antibody. Convalescent sera collected 4 weeks after inoculation of vvIBDV to SPF chicken should be tested for antibodies to other

avian pathogens and in particular to reovirus, adenovirus and chicken anaemia virus.

### ***Virus neutralisation***

This is a laborious test that will not yield much useful information, since vvIBDV are serologically indistinguishable from other classical types of IBDV. If desired, such a test should be performed in SPF eggs using a constant amount of virus and varying dilutions of serum, starting dilution of sera 1/10. A cross-neutralisation approach is required in such a case by the inclusion of a classical type of IBDV, such as F52/70 and chicken sera against both the test virus and F52/70 strain.

## **Conclusions**

In Australia and New Zealand, the following approach for handling samples from suspected field cases of vvIBD is recommended:

### ***Samples & processing***

Only bursal tissue, collected during the acute phase of disease should be used for differential diagnosis. Bursae should be transported to the diagnostic laboratory frozen on dry ice. Bursal samples, either individually, or pooled, should be homogenized (20% tissue suspension), treated with chloroform or trichlorotrifluoroethane, and frozen/thawed twice.

### ***Initial diagnosis***

A portion of bursal homogenate should be used for Real time RT-PCR and conventional PCR, which will be followed by nucleotide sequencing and sequence comparisons. The CRAb Ac ELISA can also be performed on the same bursal homogenate.

### ***Subsequent diagnostic samples***

If vvIBDV is detected, it is likely that the poultry industry will attempt control and eradication. This

will require testing of a large number of samples. In such a case the CRAB Ac ELISA would be the test of choice.

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## Appendices

### Appendix 1

#### Preparation of bursal homogenate

1. Bursa should be collected from clinically affected live or recently dead chicks during the acute phase of infection: 2 – 4 days after onset of clinical signs or administration of virus and transported without delay frozen on ice bricks or dry ice.
2. A single, or pool of several bursa are cut into several segments and weighed. Four volumes of PBS are added to make a 20% w/v tissue homogenate. An equal volume of trichlorotrifluoroethane or chloroform can also be added at this time.
3. Homogenize tissues in a closed container at high speed for 1 min approximately.
4. Freeze samples at  $-70^{\circ}\text{C}$  and thaw. Repeat freeze/thaw cycle at least once.
5. Centrifuge samples at  $4^{\circ}\text{C}$  at 3000 g for 30 min and collect upper (water) phase into a new tube. Distribute into small volumes and store at  $-70^{\circ}\text{C}$ . Use this preparation in CRAb Ac ELISA, for inoculation into SPF eggs, inoculation to SPF chicks for virus isolation, and for genetic analysis such as Real time PCR, RT-PCR and nucleotide sequencing.

## Appendix 2

## Evaluation of mortality rates in SPF chickens

1. Strict measures for bio-containment must be in place in order to prevent spread of the virus within and from the experimental facility. All material coming into contact with infected material should be sterilised by autoclaving.
2. Place two groups of SPF chicks, 20 chicks/group, 3 – 6 weeks old, into separate isolation units. One group serves as controls.
3. Inoculate bursal homogenate, 0.2 mL/chick to one group of 20 chicks using a 1 mL syringe without needle; inoculate 100 µL containing  $10^5$  or  $10^6$   $CID_{50}$  of virus intra-ocularly and 100 µL intra-nasally.
4. Where high containment facilities are available, one additional group of 20 chicks can be inoculated with 0.2 mL of bursal homogenate of F52/70 strain containing  $10^5$  or  $10^6$   $CID_{50}$ /0.1 mL.
5. Observe chicks daily. If vvIBDV is present, the first clinical signs will be visible on day 1 in approximately 20% of chicks and on day 2 in up to 80% of chicks. First mortality will most likely occur at days 3 and 4. Chicks will recover by day 5 and no clinical signs or mortality occurs thereafter.
6. If vvIBDV is present, mortality may vary between 25% and 70% (at least double that of mortality induced by F52/70). Mortality in the group inoculated with F52/70 should be between 10% and 25%.