CHAPTER 3.3.12.

INFECTIOUS BURSAL DISEASE (GUMBORO DISEASE)

SUMMARY

Description of the disease: *Infectious bursal disease (IBD) virus (IBDV, genus* Avibirnavirus, *family* Birnaviridae) *infects chickens, turkeys, ducks, guinea fowl and ostriches, but causes clinical disease solely in young chickens. Severe acute disease, usually in 3- to 6-week-old birds, is associated with high mortality, but less acute or subclinical infections are common earlier in life. IBDV causes lymphoid depletion in the bursa of Fabricius. Significant depression of the humoral antibody responses may result, thus promoting secondary infections. Two serotypes of IBDV, designated serotypes 1 and 2, are recognised. Clinical disease has been associated only with serotype 1, against which all commercial vaccines are prepared. Some antigenic variants of serotype 1 IBDV may require special vaccines for maximum protection. Very virulent strains of serotype 1 IBDV are common worldwide and cause serious disease.*

Clinical IBD, also known as Gumboro disease, can be diagnosed by a combination of characteristic signs and post-mortem lesions. Subclinical IBD can be confirmed in the laboratory by demonstrating a humoral immune response in unvaccinated chickens, or by detecting viral antigens or viral genome in tissues. In the absence of such tests, histological examination of bursae may be helpful.

Detection of the agent: *IBDV isolation is seldom carried out in routine diagnosis. Specific antibody-negative (SAN) chickens, embryonated eggs from SAN sources, or cell cultures, may be used. It may be difficult to adapt IBDV to the latter two systems. The identity of the isolated virus should be confirmed by virus neutralisation (VN).*

Viral antigens can be detected in the bursa of Fabricius before anti-IBDV antibodies are elicited; this can be useful for early diagnosis. In the agar gel immunodiffusion (AGID) test, a bursal homogenate is used as an antigen against a known positive antiserum. Antigen-capture enzyme-linked immunosorbent assays (AC-ELISAs) using plates coated with IBDV-specific antibodies can also detect IBDV antigens in bursal homogenates. IBDV antigens may be evidenced by immunostaining of infected tissues, using an IBDV-specific chicken antiserum.

The reverse-transcription polymerase chain reaction (RT-PCR) may be used to detect viral RNA.

Strain characterisation: *IBDV strains can be characterised by pathotyping in SAN chickens, by antigenic typing in cross VN assays or in tests based on monoclonal antibodies, or by nucleotide sequencing of RT-PCR amplification products derived from both segments of IBDV genome. Tests should be performed by specialised laboratories and should include reference control strains.*

Serological tests: *AGID, VN or ELISA may be carried out. IBDV infection usually spreads rapidly within a flock: only a small percentage of the flock needs to be tested for antibodies. If positive reactions are found in unvaccinated birds, then the whole flock must be regarded as infected.*

Requirements for vaccines: *Live attenuated vaccines, inactivated (killed) vaccines, live recombinant vaccines expressing the capsid (VP2) antigen of IBDV or Immune-complex (Icx) vaccines are available. Live attenuated, recombinant or Icx vaccines are used to actively immunise young chickens. A complementary approach is to provide young chickens with passive protection by vaccinating the parents using a combination of live and killed vaccines. Effective vaccination of breeding stock is therefore of great importance.*

Live attenuated IBDV vaccines should be stable, with no tendency to revert to virulence. Live vaccines are referred to as mild, intermediate, or 'intermediate plus' ('hot' or 'invasive'), based on their

increasing ability i) to replicate and cause lymphocytic depletion in the bursa and ii) to overcome residual maternally derived antibodies (MDA). Mild vaccines are rarely used in broilers, but are used widely to prime broiler parents prior to inoculation with inactivated vaccine. When MDA are present at 1 day of age, vaccination with live vaccines should be delayed until MDA in most of the flock has waned. The best schedule can be established by serological testing to determine when MDA has fallen to a low level. Live vaccines are usually administered by spray or in drinking water.

Recombinant and Icx vaccines allow for automated administration by injection, either in ovo *at 18 days of incubation, or at 1-day old, even in the presence of MDA.*

Killed vaccines need to have a high antigen content to be effective. They are mostly used to stimulate high and uniform levels of antibody in parent chickens, and as a consequence in their progeny, but they can occasionally be used in young valuable birds with MDA. The killed vaccines are manufactured in oil emulsion adjuvant and given by injection. They must be used in birds already sensitised by either live vaccine or field virus. This can be checked serologically. High levels of MDA can be obtained in breeder birds by giving, for example, live vaccine at approximately 8 weeks of age, followed by inactivated vaccine at approximately 18 weeks of age.

A. INTRODUCTION

Infectious bursal disease (IBD), also known as Gumboro disease, is caused by a virus that is a member of the genus *Avibirnavirus* (family *Birnaviridae*). Although turkeys, ducks, guinea fowl, pheasants and ostriches may be infected, clinical disease occurs solely in chickens. Only chickens younger than 10 weeks are usually clinically affected. Older chickens usually show no clinical signs.

Severe acute disease of 3- to 6-week-old birds is associated with high mortality, and signs including prostration, diarrhoea, and sudden death. Post-mortem examinations of acute IBD cases reveal a combination of muscular and proventricular haemorrhages, nephritis and bursal inflammation, with bursal oedema or haemorrhages in the first 4 days, followed by bursal atrophy later in the course of the disease (see Section B.1 *Identification of the agent* for details). Differential diagnosis of acute IBD should take into account other diseases that can induce sudden death in young chickens, with either haemorrhages or nephritis or bursal lesions. This certainly includes infectious diseases such as Newcastle disease (ND), chicken infectious anaemia (CIA), and infections by infectious bronchitis viruses (IBV) with nephropathogenic tendencies. Bursal lesions in the early stages of the disease are critical in the differential identification of acute IBD.

A less acute or subclinical disease is common in 0- to 3-week-old birds. This can cause secondary problems due to the effect of the virus on the bursa of Fabricius. IBD virus (IBDV) causes lymphoid depletion of the bursa, and, especially if this occurs in the first 2 weeks of life, significant depression of the humoral antibody response may result. The only lesions associated with subclinical IBD may be bursal atrophy and lesions associated with secondary infections. The characterisation of histopathological changes associated with bursal atrophy will be of utmost importance in identifying subclinical IBD.

Two serotypes of infectious bursal disease virus (IBDV) are known to exist. Serotype 1 viruses replicate in the bursa of Fabricius and some serotype 1 viruses cause clinical disease in chickens. Antibodies or virus are sometimes found in other avian species, but no signs of infection are seen. Serotype 2 viruses have been detected from the respiratory tract of turkeys, cloacal swabs of ducks or in the bursae of Fabricius of chickens. Antibodies against serotype 2 viruses are very widespread in turkeys and are sometimes found in chickens and ducks. There is no report of clinical disease caused by infection with serotype 2 virus (Eterradossi & Saif, 2013).

IBD has not been reported to have any zoonotic potential (Eterradossi & Saif, 2013).

B. DIAGNOSTIC TECHNIQUES

Isolation and identification of the agent provide the most certain diagnosis of IBD, but are not usually attempted for routine diagnostic purposes as the virus may prove difficult to isolate. In practice, laboratory diagnosis of IBD depends on detection of specific antibodies to the virus, or on detection of the virus in tissues, using immunological or molecular methods. Several methods are available for diagnosis depending on the objectives (Table 1).

Table 1. Test methods available for IBD diagnosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Pathology and virus[a]						
Histopathological examination of bursae	+[b]	–	–	+++	+[b]	+[c]
Virus isolation	+[b]	–[d]	–	+[e]	+[e]	–
Virus characterisation (pathotyping, antigenicity, nucleotide sequencing)	+[f]	–	–	+++	+[f]	+[c]
Virus detection in the bursa by immunoassays (AGID, AC-ELISA, immunostaining)	+[b]	–[d]	–	+++	+	–
Virus detection by RT-PCR	+[b]	–[d]	+[b]	+++	–[g]	+
Detection of immune response						
AGID for antibody detection	++[b]	++	++	–	–	+
ELISA for antibody detection	+++[b]	+++	+++	–	–	+++
Virus neutralisation	+[h]	++[h]	–	–	–	++[h]

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
AGID = agar gel immunodiffusion assay; AC-ELISA = antigen capture enzyme-linked immunosorbent assay.
RT-PCR = reverse transcription - polymerase chain reaction.
[a]A combination of agent identification methods applied on the same clinical sample is recommended.
[b]If performed on a large scale and always negative in an area where no live vaccination is performed, or to check for subclinical IBD;
[c]Could be used post-vaccination to check replication of live vaccine in the bursa of Fabricius;
[d]Not suitable as could be negative if infection occurred several weeks before testing;
[e]Labour intensive and needs to be complemented with virus characterisation to differentiate between live vaccines and field isolates;
[f]Could be necessary if live vaccines are used in the investigated area;
[g]Not suitable as does not normally differentiate live vaccines from field isolates;
[h]Labour intensive, however reference method in non-poultry birds, or non-avian species, or when small number of chickens are investigated, or when it is critical to correlate the presence of detected antibody with protection.

1. Detection of the agent

Clinical IBD has clearly characteristic signs and post-mortem lesions. A flock will show very high morbidity with severe depression in most birds lasting for 5–7 days. Mortality rises sharply for 2 days then declines rapidly over the next 2–3 days. Usually between 5% and 10% of birds die, but mortality can reach 30–40% or more with very virulent IBDV (vvIBDV). The main clinical signs are watery diarrhoea, ruffled feathers, reluctance to move, anorexia,

trembling and prostration. Post-mortem lesions include dehydration of the muscles with numerous ecchymotic haemorrhages, enlargement and discoloration of the kidneys, with urates in the tubules. The bursa of Fabricius shows the main diagnostic lesions. In birds that die at the peak of the disease outbreak, the bursa is enlarged and turgid with a pale yellow discoloration. Intrafollicular haemorrhages may be present and, in some cases, the bursa may be completely haemorrhagic giving the appearance of a black cherry. Peribursal straw-coloured oedema will be present in many bursae. Confirmation of clinical disease or detection of subclinical disease is best done by using immunological methods as IBDV is difficult to isolate. For virus isolation, the methods described below should be followed. Differentiation between serotypes 1 and 2 or between serotype 1 subtypes or pathotypes should be undertaken by a specialised laboratory (e.g. the WOAH Reference Laboratories for infectious bursal disease[1]).

1.1. Sample preparation

Remove the bursae of Fabricius aseptically from approximately five affected chickens in the early stages of the disease. Chop the bursae using two scalpels, add a small amount of peptone broth containing penicillin and streptomycin (1000 µg/ml each), and homogenise in a tissue blender. Centrifuge the homogenate at 3000 ***g*** for 10 minutes. Harvest the supernatant fluid for use in the investigations described below. Filtration through a 0.22 µm filter may prove necessary to further control bacterial contamination, although this may cause a reduction in virus titre.

1.2. Identification by the agar gel immunodiffusion test

A protocol for the agar gel immunodiffusion (AGID) test is described in Section B.2.1. For detection of antigen in the bursa of Fabricius by AGID, the bursae should be removed aseptically from about ten chickens at the acute stage of infection. The bursae are minced using two scalpels in scissor movement, then small pieces are placed in the wells of the AGID plate against known positive serum. Freeze–thaw cycles of the minced tissue may improve the release of IBDV antigens from the infected bursal tissue, and the freeze–thaw exudate may be used to fill the wells.

1.3. Identification by immunofluorescence

Sections of bursa are prepared using a microtome cryostat, dried at room temperature and then fixed in cold acetone. Fluorescent-labelled IBDV-specific antisera are applied to the sections, which are then incubated at 37°C for 1 hour in a humid atmosphere. At the end of the incubation period, they are washed for 30 minutes using phosphate-buffered saline (PBS), pH 7.2, then rinsed in distilled water. The sections are mounted using buffered glycerol, pH 7.6, and examined by UV microscopy for IBDV-specific fluorescence (Meulemans *et al*., 1977).

1.4. Identification by antigen-capture enzyme-linked immunosorbent assay (AC-ELISA)

Since the first protocol was described by Snyder *et al.* (1988) for the detection of serotype 1 IBDV using an antigen-capture enzyme-linked immunosorbent assay (AC-ELISA), many other assays have been developed (Eterradossi & Saif, 2013). Briefly, ELISA plates are coated with IBDV-specific antibodies. Depending on the chosen AC-ELISA protocol, the capture antibody may be a mouse anti-IBDV monoclonal antibody (MAb), or a mix of such MAbs, or a chicken post-infectious anti-IBDV polyclonal serum. It has been suggested that AC-ELISAs using polyclonal antibodies may have a higher sensitivity. Samples of bursal homogenates (see above) diluted 1/10 to 1/25 (w/v) in a suitable dilution buffer are incubated in the coated wells. Unbound antigens are discarded at the end of the incubation period by washing with a suitable washing buffer (e.g. PBS, pH 7.2 + 0.2% Tween 20). The captured antigens are then revealed, as in an indirect ELISA, with a detection antibody (which must have been developed from a different animal species than the capture antibody), followed by an enzyme conjugate that binds to the detection antibody only (in some protocols the detection antibody may be directly conjugated to the enzyme), followed by the enzyme substrate. Finally, optical densities, which parallel the amount of captured IBDV antigens, are read with an ELISA reader.

AC-ELISA is based on the use of samples possibly containing live virus and should be performed only in suitable containment facilities such as a class II safety cabinet. All liquid (washing buffers) and solid

1 For details see the list on line at: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

wastes should be considered to be contaminated by IBDV and decontaminated accordingly before disposal.

Critical steps in the implementation or assessment of AC-ELISA are i) the need to perform extensive washings between each step of the reaction to keep background reactions low, ii) the requirement for known positive and negative samples to be included in each assay as controls, and iii) the need for both the capture and detection antibodies to positively react with all serotype 1 IBDV strains (i.e. neither capture nor detection should critically depend on IBDV antigenic variation that occurs among serotype 1 strains).

1.5. Identification by molecular techniques

Molecular virological techniques have been developed that allow IBDV to be identified more quickly than by virus isolation. The most frequently used molecular method is the detection of IBDV genome by the reverse-transcription polymerase chain reaction (RT-PCR) (Lin *et al*., 1993; Wu *et al*., 1992). This method can detect the genome of viruses that do not replicate in cell culture, because it is not necessary to grow the virus before amplification.

RT-PCR is performed in three steps: extraction of nucleic acids from the studied sample, reverse transcription (RT) of IBDV RNA into cDNA, and amplification of the resulting cDNA by PCR. The two latter steps require that the user selects oligonucleotidic primers that are short sequences complementary to the virus-specific nucleotidic sequence. Different areas of the genome will be amplified depending on the location from which the primers have been selected. The example below allows the amplification of the middle third of the gene encoding the outer capsid protein VP2 (Eterradossi *et al*., 1998) or the partial amplification of the 5' extremity of the VP1 gene in IBDV segment B (Le Nouen *et al*., 2006).

1.5.1. Extraction of nucleic acids

Unlike single-stranded RNA, the IBDV double-stranded RNA (dsRNA) genome resists degradation by RNases. However, infected cells also contain IBDV-derived positive-sense single-stranded RNA species that can be used as a template at the RT step and may contribute to improving the sensitivity of the assay. It is thus important that RNA extraction be performed using gloves and RNase-free reagents and labware.

IBDV RNA can be extracted from infected tissues using some kits available from commercial suppliers of molecular biology reagents. Alternatively, IBDV RNA can be extracted by adding 1% (weight/volume final concentration) sodium dodecyl sulphate and 1 mg/ml proteinase K to 700 µl of virus suspension (e.g. bursal homogenate). Incubate for 60 minutes at 37°C. Nucleic acids are obtained using a standard protocol for phenol/chloroform extraction (caution: phenol is toxic and should be handled and disposed accordingly). Nucleic acids are harvested from the final aqueous phase by ethanol precipitation and are resuspended in RNase-free distilled water or a suitable buffer. Water-diluted RNA should be kept frozen at a temperature below –20°C until use.

1.5.2. Reverse transcription

A variety of reverse transcriptases are commercially available. Follow the supplier's instructions to prepare the RT reaction mix. Use the 'lower' PCR primer (complementary to the positive strand of IBDV genome, see below) for reverse transcription, as this allows the synthesis of cDNA both from the positive strand of IBDV dsRNA genome and from IBDV-derived positive-sense single-stranded RNAs previously contained in infected cells. Alternatively, random primers (hexanucleotides) can be used to prime cDNA synthesis.

The IBDV RNA matrix must be denaturated before transfer to the RT reaction mix. Add one part (by volume) molecular biology grade dimethylsulfoxide to four parts the unfrozen solution of IBDV RNA. Heat for 3 minutes at 92°C and chill on ice; an alternative method is to heat for 5 minutes and immediately incubate the mixture in liquid nitrogen. Transfer the relevant volume of denaturated matrix to the reaction mix. Incubate according to the instructions of the enzyme supplier.

The cDNA solution obtained after the RT step should be kept frozen at a temperature below –20°C. Delaying the PCR step for several weeks after the cDNA synthesis may cause false-negative PCR results.

1.5.3 Polymerase chain reaction

A variety of DNA polymerases suitable for PCR are commercially available. Follow the manufacturer's instructions to prepare the PCR reaction mix. Protocols for the amplification and molecular typing of IBDV have been reviewed recently (Wu *et al*., 2007). As an example, the U3/L3 and +290/–861 pairs of PCR primers shown below can be suggested and have been found useful for amplifying the middle third of the VP2 gene in segment A of serotype 1 IBDV strains (Eterradossi *et al*., 1998), and a region at the 5' extremity of IBDV segment B (Le Nouen *et al*., 2006), respectively. Both regions have been shown to be suitable for molecular epidemiology studies (Le Nouen *et al*., 2006), and the amplified region in segment B encompasses the B-marker subsequently confirmed to reliably represent the phylogenetic information derived from full B-segment (Alfonso-Morales *et al.,* 2015). Although a significant number of IBDV strains have two nucleotide changes at position 35 (G–A) and 38 (T–C) of the U3 primer (including isolates from Japan [OKYM], Hong Kong [HK46], UK [UK661], Nigeria [N4]), it has been shown that the U3-L3 primer pair successfully amplifies some of these viruses that exhibit both mutations. This is probably because the 3' extremity of U3 is highly conserved. However, as with most PCR assays, IBDV strains may exist with nucleotide changes at the annealing positions of the primers, thus requiring the use of other primers for optimised RT-PCR detection.

The combination of segment A- and segment B-targeted RT-PCR protocols enhances the probability that, if present, serotype 1 IBDV will indeed be detected; it also allows a thorough genetic characterisation of the IBDV strains detected.

Nucleotide sequence of the U3 and L3 IBDV-specific PCR primers (specific for Segment A, VP2 gene):

Upper U3: 5'-**TGT-AAA-ACG-ACG-GCC-AGT**-GCA-TGC-*GGT-ATG-TGA-GGC-TTG-GTG-AC*-3'

Lower L3: 5'-**CAG-GAA-ACA-GCT-ATG-ACC**-GAA-TTC-*GAT-CCT-GTT-GCC-ACT-CTT-TC*-3'

Nucleotide sequence of the +226 and –793 IBDV-specific PCR primers (specific for Segment B, VP1 gene):

Upper +290: 5'-**TGT-AAA-ACG-ACG-GCC-AGT-**GAA-TTC-*AGA-TTC-TGC-AGC-CAC-GGT-CTC-T*-3'

Lower -861: 5'-**CAG-GAA-ACA-GCT-ATG-ACC**-CTG-CAG-*TTG-ATG-ACT-TGA-GGT-TGA-TTT-TG*-3'

The U3 and L3 primers are both 44 nucleotides long, whereas primers +290 and –861 are 46 and 47 nucleotides long, respectively. The four primers include an IBDV-specific 3' extremity (in italics in the sequence shown above) corresponding to nucleotide positions 657–676 and 1193–1212 of IBDV segment A in primers U3 and L3, respectively (numbering as in segment A of strain P2, Acc No X84034), and to nucleotide positions 290–311 and 861–883 of IBDV segment B in primers +290 and –861, respectively (numbering as in segment B of strain D6948, Acc No AF240687). The IBDV-specific extremity is coupled to a non-IBDV 5' extremity (bold type in the sequence above) corresponding to the M13 and RM13 universal primers in the upper and lower primers, respectively. The M13 and RM13 universal primers are commonly used as primers in DNA sequencing reactions, so that purified PCR products resulting from amplification with the U3/L3 and +290/–861 primer pairs can be easily sequenced in both directions. Finally, restriction sites (underlined in the above sequence) are included for the following restriction endonucleases: *Sph*I (in primer U3), *Eco*RI (in primers L3 and +290), and *Pst* I (in primer –861). These restriction sites are positioned so that the PCR products resulting from amplification with the U3/L3 or +290/–861 pairs can be cloned if required. The U3/L3 pair generates a 604 base pair (bp) product, 516 bp of which are specific of the amplified IBDV sequence and encompass the region encoding the hyper-variable region of the VP2 protein. The +290/–861 pair generates a 642 bp product, 549 bp of which are specific of the amplified IBDV sequence. Both products are derived from genomic regions that are suitable for phylogenetic analysis (Eterradossi *et al*., 1998; Le Nouen *et al*., 2006).

Perform an initial denaturation step as recommended by the DNA polymerase supplier, followed by 35 cycles, each including one denaturation, one annealing and one elongation step. In such cycles, denaturation at 95°C for 30 seconds and annealing at 64°C for 45 seconds may be used with both the U3/L3 and +290/–861 primer pairs (the annealing temperature should be adapted if other primers are used). The parameters for the elongation step should be set according to the supplier's recommendations.

Revelation may be performed by electrophoresis with the PCR products and DNA molecular weight markers in a 1% agarose gel stained with ethidium bromide (caution: ethidium bromide is toxic and carcinogenic. It should be handled and disposed accordingly).

Three PCR reactions should be performed for each cDNA sample (pure, 10- and 100-fold diluted cDNA) to avoid false-negative results due to PCR inhibition in mixes containing high amounts of the cDNA preparation.

Each PCR should include negative and positive control reactions. Protocols that include an internal control to test for the presence of PCR inhibitors have been developed (Smiley *et al*., 1999).

Delaying the PCR for several weeks after the RT step may cause false-negative PCR results.

One step RT-PCR may also be used for IBD diagnosis with both conventional and real-time methods.

1.6. Isolation of virus in cell culture

Inoculate 0.5 ml of sample into each of four freshly confluent chicken embryo fibroblast (CEF) cultures (from a specific pathogen free [SPF] source) in 25 cm^2 flasks. Adsorb at 37°C for 30–60 minutes, wash twice with Earle's balanced salt solution and add maintenance medium to each flask. Incubate the cultures at 37°C, observing daily for evidence of cytopathic effect (CPE). This is characterised by small round refractive cells. If no CPE is observed after 6 days, discard the medium, then freeze–thaw the cultures and inoculate the resulting lysate into fresh cultures. This procedure may need to be repeated at least three times. If CPE is observed, the virus should be tested against monospecific IBDV antiserum in a tissue culture virus neutralisation (VN) test (see Section B.2.2 *Virus neutralisation tests*). The more pathogenic IBDV strains usually cannot be adapted to grow in CEF unless the virus has first been submitted to extensive serial passage in embryos (see below).

1.7. Isolation of virus in embryos

Inoculate 0.2 ml of sample into the yolk sac of five 6- to 8-day-old specific antibody negative (SAN) chicken embryos and on to the chorioallantoic membrane (American Association of Avian Pathologists, 2008) of five 9- to 11-day-old SAN chicken embryos. SAN embryos are derived from flocks shown to be serologically negative to IBDV. Candle daily and discard dead embryos up to 48 hours post-inoculation. Embryos that die after this time are examined for lesions. Serotype 1 IBD produces dwarfing of the embryo, subcutaneous oedema, congestion and subcutaneous or intracranial haemorrhages. The liver is usually swollen, with patchy congestion producing a mottled effect. In later deaths, the liver may be swollen and greenish, with areas of necrosis. The spleen is enlarged and the kidneys are swollen and congested, with a mottled effect. If lesions are observed, the virus should then be tested against a monospecific anti-IBDV serum in an embryo-revealed virus neutralisation assay.

Serotype 1 IBDV usually causes death in at least some of the embryos on primary isolation.

Serotype 2 IBDV does not induce subcutaneous oedema or haemorrhages in the infected embryos, but embryos are of a smaller size with a pale yellowish discolouration.

For the preparation of embryo-propagated stock virus or for subsequent passaging, embryos with lesions or embryos suspected to be infected, respectively, are harvested aseptically. Their head and limbs are discarded and the main body is minced as described in Section B.1.1 *Sample preparation* for the preparation a virus suspension.

1.8. Isolation of virus in chickens

This method has been used in the past but is no longer recommended due to animal welfare concerns. Five susceptible and five IBD-immune chickens (3–7 weeks of age) are inoculated by the eye-drop route with 0.05 ml of sample. Humanely euthanise the chickens 72–80 hours after inoculation, and examine their bursae of Fabricius. The bursae of chickens infected with virulent serotype 1 IBDV appear yellowish (sometimes haemorrhagic) and turgid, with prominent striations. Peribursal oedema is sometimes present, and plugs of caseous material are occasionally found. The plicae are petechiated.

The presence of lesions in the bursae of susceptible chickens along with the absence of lesions in immune chickens is diagnostic of IBD. The bursae from both groups may be used as antigen in an AGID test against known positive IBD antiserum (see Section B.1.2 *Identification by the agar gel immunodiffusion test*).

The extent of bursal damage may vary considerably with the pathogenicity of the studied IBDV strain. However, as the samples submitted for virus isolation may vary in virus content, the extent of bursal damage observed in susceptible chickens at the isolation stage gives only a limited indication on strain pathogenicity.

The bursae of chickens infected with serotype 2 IBDV do not exhibit any gross lesions.

1.9. Strain differentiation

IBDV strains can be further identified by testing their pathogenicity in SAN chickens, by investigating their antigenic reactivity in cross VN tests or using MAbs, by determining the nucleotide sequence of RT-PCR amplification products derived from IBDV genome, or by studying the number and size of the restriction fragments obtained following digestion of such RT-PCR products with restriction endonucleases. Several protocols have been described for each of these approaches. Tests should be performed by specialised laboratories and should include a panel of reference strains as controls. Although the molecular basis for antigenic variation is now better understood, no validated virulence marker has been described yet.

1.9.1. Pathogenicity testing

Studies to compare the pathogenicity of IBDV strains must be carried out in secure biocontainment facilities to avoid the dissemination of the studied virus (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). SAN birds with a known microbial status (ideally SPF chickens) must be used to avoid interference by contaminating agents.

The main variables when comparing the results of pathogenicity trials are the breed, age and immune status of the challenged chickens, the dose and route of inoculation of the challenge virus, and the possible presence of contaminating agents in the inoculum. Light layer breeds have been reported to be more susceptible than heavy broilers (Van den Berg & Meulemans, 1991). Differences in susceptibility may also occur between different SPF chicken lines. The highest susceptibility to acute IBD occurs in chickens between 3 and 6 weeks of age (Eterradossi & Saif, 2013). (The influence of the immune status is described in Section C.) A high dose of challenge virus, such as that recommended in Section C.1.3 *Live recombinant vector vaccines: methods of use,* is necessary so that all inoculated chickens become infected at once without requiring bird-to-bird transmission of the inoculated virus. Finally, the presence in the inoculum of contaminating agents, such as adenovirus or chicken infectious anaemia virus, may modify the severity of IBD and signs observed after challenge (Rosenberger *et al*., 1975).

The terms 'variant', 'classical' and 'very virulent' have been used to describe IBDV strains that exhibit differences in pathogenicity. Based on the signs and lesions observed in two lines of White Leghorn SPF chickens during acute experimental IBD following a 10^5 50% embryo infective dose (EID_{50}) challenge, North American 'variant' IBDVs induce little if any clinical signs and no mortality but marked bursal lesions, 'classical' IBDVs induce approximately 10–50% mortality with typical signs and lesions whereas 'very virulent' IBDVs induce approximately 50–100% mortality with typical signs and lesions (Eterradossi *et al.,* personal observation).

1.9.2 Antigenicity testing

Antigenic relatedness among IBDV strains may be assayed in cross VN tests, which correlate best with cross protection. Such tests have to be performed in SAN embryonated eggs when the studied viruses do not grow in CEF (e.g. vvIBDV). Differences in cross VN results among serotype 1 IBDV strains have led to the definition of serotype 1 'subtypes', some of which include the antigenically 'variant' North American IBDV isolates (Jackwood & Saif, 1987).

Another approach to the study of genetic relatedness is the use of mouse MAbs that bind to IBDV neutralising epitopes. Several panels of MAbs exist world-wide for use in AC-ELISA (Eterradossi *et al*., 1999; Snyder *et al*., 1992). Some of the MAbs have been included in commercially available kits, but no unified MAb panel as yet been proposed. All neutralising epitopes of IBDV characterised to date have been mapped into a major immunogenic domain in the middle third (amino acid positions 200 to 340) of the VP2 capsid protein (Eterradossi *et al*., 1998; Schnitzler *et al*., 1993; Vakharia *et al*., 1994). This region is termed 'VP2 variable domain' because most amino acid changes observed among IBDV strains are clustered in it. Within vVP2, four amino acid stretches are of critical importance to antigenicity and are referred to as vVP2 hydrophilic peaks. These are amino acid positions 210 to 225 (major peak A), 249 to 252 (minor peak 1), 281 to 292 (minor peak 2) and 313 to 324 (major peak B) (Van den Berg *et al*., 1996). According to the crystal structure of the VP2 protein and IBDV particles, the amino acid stretches previously known as "VP2 hydrophilic peaks" correspond to the most exposed amino acid loops in the projection domain of the VP2 protein (Coulibaly *et al.,* 2005). Both North American 'variants' and 'very virulent' IBDV exhibit in these areas amino acid changes that correlate with epitope variation (Eterradossi *et al*., 1998; Vakharia *et al*., 1994). To date, no antigenic marker has been shown to correlate strictly with IBDV pathogenicity.

1.9.3. Molecular identification

Most efforts at molecular identification have focused on the characterisation of the larger segment of IBDV (segment A) and especially of the vVP2 encoding region. Efforts were made initially to characterise RT-PCR products using restriction endonucleases (Lin *et al.*, 1993). These approaches are known as RT-PCR/RE or RT-PCR-RFLP (restriction fragment length polymorphism). The usefulness of the information they provide depends on the identification of enzymes that cut in restriction sites that are phenotypically relevant. Several RE or RFLP protocols resulted in defining a high number of profiles, which may prove confusing to use in molecular epidemiology studies and difficult to correlate with antigenicity or pathogenicity. Nucleotide sequencing of RT-PCR products provides an approach to assessing more precisely the genetic relatedness among IBDV strains. Using a reverse genetics approach, it was demonstrated that cell culture adaptation of IBDV strains critically depends on VP2 amino acid pairs 279 N–284 T or 253 H–284 T (Mundt, 1999). In most very virulent viruses, four typical amino acids are present (222 A, 256 I, 294 I and 299 S) (Brown *et al*., 1994; Lin *et al*., 1993). Several recent studies indicated that although VP2 is an important virulence determinant, segment B also appears to be important (Boot *et al.*, 2000; Escaffre *et al.*, 2013; Jackwood *et al*., 2011). It has been reported that segment A and B of IBDV mostly co-evolve (i.e. most significant IBDV clusters, such as vvIBDV-related strains, may be identified by analysis of both genome segments). However, some potentially reassortant viruses have been identified. The pathogenicity of putative reassortant IBDV is often modified, as compared with what would have been expected from the characterisation of their segment A alone (Le Nouen *et al.*, 2006; Jackwood *et al.*, 2011; Wei *et al.*, 2008). Molecular identification of IBDV isolates based on the sequencing of both genome segments is therefore highly recommended.

2. Serological tests

Blood samples should be taken early in the course of the disease, and repeat samples should be taken 3 weeks later. As the virus spreads rapidly, only a small proportion of the flock needs to be sampled. Usually 20 blood samples are enough.

2.1. Agar gel immunodiffusion test

The AGID test is the most simple of the serological tests for the detection of specific antibodies in serum.

2.1.1. Preparation of positive control antigen

Inoculate 3- to 5-week-old susceptible chickens, by eye-drop, with a clarified 10% (w/v) bursal homogenate known to contain viable IBDV [2]. Humanely euthanise the birds 3 days post-inoculation, and harvest the bursae aseptically. Discard haemorrhagic bursae and pool the remainder, weigh and add an equivalent volume of cold distilled water (or of a suitable buffer such as PBS or tryptose phosphate broth) and an equivalent volume of undiluted methylene chloride. (Caution: methylene chloride is toxic and possibly carcinogenic. It should be handled and disposed accordingly. A possible alternative to avoid health hazards caused by methylene chloride is to use trichlorotrifluoroethane, which is however an environmental hazard and should be handled and disposed accordingly). Thoroughly homogenise the mixture in a tissue blender and centrifuge at 2000 ***g*** for 30 minutes. Harvest the supernatant fluid and dispense into aliquots for storage at –40°C. The antigen contains live virus and should be handled only in suitable containment facilities such as a class II safety cabinet. If required, the antigen can be inactivated prior to dispensing: add 0.3% (v/v) ß-propiolactone to the harvested supernatant, then further incubate at 37°C for 2 hours. It is important that incubation takes place on an orbital shaker or a mechanical rocker, so that any inner part of the vial that has been in contact with live virus indeed gets into contact with ß-propiolactone. Dispense and store as above. Check the efficacy of the inactivation process by attempting IBDV isolation from the inactivated antigen, with three serial passages on SAN embryonated eggs (see Section B.1.7 *Isolation of virus in embryos*).

2.1.2. Preparation of positive control antiserum

Inoculate 4–5-week-old susceptible chickens, by eye-drop, with 0.05 ml of a clarified 10% (w/v) bursal homogenate known to contain viable IBDV (see footnote 2). Exsanguinate 28 days post-inoculation. Pool and store serum in aliquots at –20°C.

2.1.3. Preparation of agar

Dissolve sodium chloride (80 g) and phenol (5 g) in distilled water (1 litre) (caution: phenol is toxic and should be handled and disposed of accordingly). Add agar (12.5 g) and steam until the agar has dissolved. To avoid the health and environmental hazards caused by the use of phenol, another suitable recipe for the preparation of agar is as follows: sodium chloride (80 g), kalium dihydrogenophosphate (0.45 g), sodium hydrogenophosphate dihydrate (1.19 g), agar (10 g) and distilled water to a final volume of 1 litre (final pH 7.1 at 20–25°C). This second recipe can be homogenised by heating up to 90°C under agitation. While the mixture is still very hot, filter it through a pad of cellulose wadding covered with a few layers of muslin and dispense the medium in 20 ml volumes into glass bottles. The medium without phenol can further be sterilised by autoclaving at (at most) 115°C for 15 minutes. Store the bottles at 4°C until required for use.

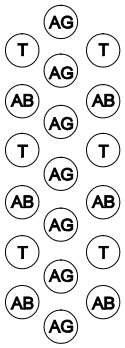
2.1.4. Test procedure

i) Prepare plates from 24 hours to 7 days before use. Dissolve the agar by placing in a steamer or boiling water bath. Take care to prevent water entering the bottles.

ii) Pour the contents of one bottle into each of the required number of 9 cm plastic Petri dishes laid on a level surface. (Some laboratories prefer to pour the gel on 25 × 75 mm glass slides, 3 mm deep.)

iii) Cover the plates and allow the agar to set, and then store the plates at 4°C. Poured plates may be stored for up to 7 days at 4°C. (If the plates are to be used the same day that they are poured, dry them by placing them opened but inverted at 37°C for from 30 minutes to 1 hour.)

2 A suitable classical strain of IBDV (serotype 1, classical pathotype) is strain 52/70, obtainable from one of the WOAH Reference Laboratories (https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).



AG = Positive antigen

AB = Positive antiserum

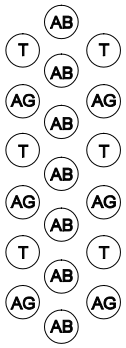


Fig. 1. Protocol for tests for antibody.

T = test sera

Fig. 2. Protocol for tests for antigen.

T = test tissues

Notes:

1. The linear pattern of wells is preferred although a hexagonal pattern may be used. Each test serum or test bursa (T in Figs 1 and 2 above) should be placed adjacent to a positive control antibody (AB) or antigen (AG), respectively.
2. Wells, 3 mm deep, 6 mm in diameter, and 3 mm apart (or wells of any other size previously shown to be effective), are used.

iv) Cut three vertical rows of wells 6 mm in diameter and 3 mm apart, using a template and tubular cutter.

v) Remove the agar from the wells by aspiration or remove using a pen and nib, taking care not to damage the walls of the wells.

vi) Using a pipette, dispense 50 µl of the test sera into the wells as shown in Figure 1.

Or, for the detection of IBDV antigens in bursae:

Dispense small pieces of finely minced test bursae by means of curved fine-pointed forceps into the wells, as shown in Figure 2, to just fill the wells. Alternatively, the freeze–thaw exudate of minced tissues can be used to fill the wells.

vii) Dispense 50 µl of the positive and negative control reagents into the relevant wells.

viii) Incubate the plates at between 22°C and 37°C for up to 48 hours in a humid chamber to avoid drying the agar.

ix) Examine the plates against a dark background with an oblique light source after 24 and 48 hours.

2.1.5. Quantitative agar gel immunodiffusion tests

The AGID test can also be used to measure antibody levels by using dilutions of serum in the test wells and taking the titre as the highest dilution to produce a precipitin line (Cullen & Wyeth, 1975). This can be useful for measuring maternal or vaccinal antibodies and for deciding on the best time for vaccination; however, this AGID quantitative determination has now been largely replaced by the ELISA.

2.2. Virus neutralisation tests

VN tests are carried out in cell culture. The test is more laborious and expensive than the AGID test, but is more sensitive for detecting antibody. This sensitivity is not required for routine diagnostic purposes, but may be useful for evaluating vaccine responses or for differentiating between IBDV 1 and 2 serotypes. The test uses either SPF chicken embryo fibroblast cells, or a suitable continuous cell line (such as QT-35, BGM-70, MA-104, Vero or DF1), in conjunction with an adapted strain of IBDV.

First, 0.05 ml of virus diluted in tissue culture medium to contain 100 $TCID_{50}$ (50% tissue culture infective doses) per 0.05 ml is placed in each well of a tissue-culture grade microtitre plate (See American Association of Avian Pathologists, 2008, for virus titration methods). The test sera are heat-inactivated at 56°C for 30 minutes. Serial doubling dilutions of the sera are made in the diluted virus. After 30 minutes

at room temperature, 0.2 ml of cell suspension, with a cell density allowing confluent layers to be obtained after 24 hours of incubation, is dispensed into each well. Plates are sealed and incubated at 37°C for 4–5 days, after which the monolayers are observed microscopically for typical CPE. The end-point (serum titre) is expressed as the reciprocal of the highest serum dilution that did not show CPE. To reduce test-to-test and operator-to-operator variation, a standard reference antiserum may be included with each batch of tests [3] and the titre of the virus suspension must be reassessed in each new experiment using a sufficient number of repeats (wells) per virus dilution.

2.3. Enzyme-linked immunosorbent assay

ELISAs are in use for the detection of antibodies to IBD. Coating the plates requires a purified, or at least semipurified, preparation of virus, necessitating special skills and techniques. Methods for preparation of reagents and application of the assay were described by Marquardt *et al.* (1980). Commercial kits are available.

The test sera are diluted according to the established protocol or kit instructions and each is dispensed into the requisite number of wells. After incubation under the appropriate conditions, the sera are discarded from the plates, and the wells are washed thoroughly. Anti-chicken immunoglobulins conjugated to an enzyme are dispensed into the wells, and the plates are again incubated as appropriate. The plates are emptied and rewashed before substrate containing a chromogen that gives a colour change in the presence of the enzyme used is added to the plate. After a final incubation step, the substrate/chromogen reaction is stopped by addition of a suitable stopping solution and the colour reactions are quantified by measuring the optical density of each well. The Sample to Positive (S/P) ratio for each test sample is calculated.

2.4. Interpretation of results

The AGID test is surprisingly sensitive, though not as sensitive as the VN test; the latter will often give a titre when the AGID test is negative. Positive reactions indicate infection in unvaccinated birds without maternal antibodies. As a guide, a positive AGID reaction in a vaccinated bird or young bird with maternal antibody indicates a protective level of antibody. ELISA gives more rapid results than VN or AGID and is less costly in terms of labour, although the reagents are more expensive. VN and AGID titres correlate well, but as VN is more sensitive, AGID titres are proportionally lower. Correlation between ELISA and VN and between ELISA and AGID is more variable depending on the source of the ELISA reagents, however it should be kept in mind that both VN and ELISAs are highly sensitive and subject to both intra- and inter-laboratory variations. It is therefore highly advisable that a positive sentinel serum with a known titre be introduced in every test in laboratories that perform IBDV ELISA or VN routinely (De Wit *et al.*, 2007; Kreider *et al.*, 1991). When testing for the decay of maternally derived antibodies (MDA), it is not uncommon to find residual VN antibodies at an age when ELISA results are already negative. Formulae have been devised that allow ELISA titres to be used to calculate the optimal age for vaccination, which will vary depending on the vaccine used (Block *et al.*, 2007). Nonspecific positive reactions may occur with most ELISAs because they are usually designed for monitoring vaccine responses, in which case sensitivity is regarded as more important than specificity. This should be taken into account when the ELISA is used for diagnosis. In commercial chicken flocks or experimentally infected chickens, a serotype 1 ELISA antigen also detects antibodies induced by serotype 2 IBDV (Ashraf *et al.*, 2006), however this cross reactivity has not yet been demonstrated to interfere with serological monitoring programmes of IBD based on the ELISA.

3 A suitable reference antiserum may be obtained from the WOAH Reference Laboratories (https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

C. REQUIREMENTS FOR VACCINES

1. Background

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principle of veterinary vaccine production*. The guidelines given below and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

IBDV vaccines have been reviewed recently (Muller *et al*., 2012). Four major types of vaccines are available for the control of IBD, these are: i) live attenuated vaccines; ii) immune-complex vaccines; iii) live recombinant vectored vaccines expressing IBDV antigens; and iv) inactivated oil-emulsion adjuvanted vaccines.

To date, IBD vaccines have been made with serotype 1 IBDV only, although a serotype 2 virus has been detected in poultry. The serotype 2 virus has not been associated with disease, but its presence will stimulate antibodies. Serotype 2 antibodies do not confer protection against serotype 1 infection, neither do they interfere with the response to type 1 vaccine. There have been numerous descriptions of antigenic variants of serotype 1 virus (Rosenberger & Cloud, 1986). Cross-protection studies have shown that inactivated vaccines prepared from 'classical' serotype 1 virus require a high antigenic content to provide good protection against some of these variants. IBD vaccines that contain both classical and variant IBD serotype 1 viruses have been authorised. vvIBDV strains with limited antigenic changes as compared with 'classical' serotype 1 viruses have emerged since 1986. Active immunisation with a 'classical' serotype 1 virus or vaccine provides a good protection against the vvIBDVs, however the latter viruses are less susceptible to neutralisation by MDA than 'classical' pathogenic viruses (Van den Berg & Meulemans, 1991).

1.1. Live vaccines: methods of use

Live IBD vaccines are produced from fully or partially attenuated strains of virus, known as 'mild', 'intermediate', or 'intermediate plus' ('hot'), respectively.

Mild or intermediate vaccines are used in parent chickens to produce a primary response prior to vaccination near to point-of-lay using inactivated vaccine. They are susceptible to the effect of MDA so should be administered only after all MDA has waned. Application is by means of intramuscular injection, spray or in the drinking water, usually at 8 weeks of age (Skeeles *et al*., 1979).

Intermediate or intermediate plus vaccines are used to elicit protection in broiler chickens and commercial layer replacements. Some of these vaccines are also used in young parent chickens if there is a high risk of natural infection with virulent IBD. Although intermediate vaccines are susceptible to the presence of MDA, they are sometimes administered at 1-day old, as a coarse spray, to protect any chickens in the flock that may have no or only minimal levels of MDA. This also establishes a reservoir of vaccine virus within the flock that allows lateral transmission to other chickens when their MDA decay. Second and third applications are usually administered, especially when there is a high risk of exposure to virulent forms of the disease or when the vaccinated chicks exhibit uneven MDA levels. The timing of additional applications will depend on the antibody titres of the parent birds at the time the eggs were laid. As a guide, the second dose is usually given at 10–14 days of age when about 10% of the flock is susceptible to IBD, and the third dose 7–10 days later. The route of administration is by means of spray or in the drinking water. Intramuscular injection or eye-drop is used rarely. If the vaccine is given in the drinking water, clean water with a neutral pH must be used that is free from odour or taste of chlorine or metals. Skimmed milk powder may be added at a rate of 2 g per litre. Care must be taken to ensure that all birds receive their dose of vaccine. To this end, all water should be removed (cut off) for 2–3 hours before the vaccine is made available and care must be taken that no residual water remains in the pipes or in the drinkers. It is possible to divide the medicated water into two parts, giving the second part 30 minutes after the first.

Live IBD vaccines are generally regarded as compatible with other avian vaccines. However, it is possible that live IBD vaccines that cause bursal damage could interfere with the response to other vaccines. Only healthy birds should be vaccinated. The vials of vaccine should be kept at temperatures between 2°C and 8°C up to the time of use.

1.2. Immune complex vaccines: methods of use

To make an immune complex IBD vaccines a live infectious IBDV vaccine virus is blended with IBDV-specific antibodies. Such vaccines may be administered in the hatchery by *in-ovo* injection at 18 days of incubation. The eggs go on to hatch and the vaccine virus is supposedly released when the chicks are about 7–14 days of age. In this way, the problem of maternally derived IBD antibody is overcome and the chicks are effectively immunised (Haddad *et al*., 1997). The immune complex vaccine can also be injected subcutaneously at 1-day old in the hatchery (Ivan *et al*., 2005).

1.3. Live recombinant vector vaccines: methods of use

Live recombinant vaccines that use a viral vector (herpes virus of turkeys) to express the VP2 antigen of IBDV in chickens have been developed for *in-ovo* or day-old use and are currently authorised in many countries worldwide. Activity in the face of maternally derived IBD antibody, and compatibility with other Marek's disease vaccines have been documented (Le Gros *et al*., 2009, Lemiere *et al*., 2011). The anti-IBDV antibody response elicited by live recombinant IBDV vaccines expressing the VP2 protein will contain antibodies directed against VP2 only (as opposed to antibodies against all IBDV proteins, primarily VP2 and VP3, following infection by live IBDV). While neutralising antibodies against the VP2 protein will be readily detected in the standard VN test, detection of a VP2-specific antibody response in ELISA may require specific kits with an extended sensitivity. Antibodies against VP3 being absent in birds receiving the live recombinant IBDV vaccine, but present in birds infected with live IBDV, the combined use of ELISAs specific for anti-VP2 or anti-VP3 antibodies would allow implementation of a DIVA (detection of infection in vaccinated animals) strategy in birds vaccinated with such recombinant vaccines (Muller *et al*., 2012).

1.4 Inactivated vaccines: method of use

Inactivated IBD vaccines are mostly used to produce high, long-lasting and uniform levels of antibodies in breeding hens that have previously been primed by live vaccine or by natural exposure to field virus during rearing (Muller *et al*., 2012). The usual programme is to administer the live vaccine at about 8 weeks of age. This is followed by the inactivated vaccine at 16–20 weeks of age. Occasionally, inactivated vaccines may be used in programmes combining inactivated and live vaccines, in young valuable birds with high MDA levels reared in areas with high risk of exposure to virulent IBDV. The inactivated vaccine is manufactured as a water-in-oil emulsion, and has to be injected into each bird. The preferred routes are intramuscular into the leg muscle, avoiding proximity to joints, tendons or major blood vessels or the subcutaneous route. A multidose syringe may be used. All equipment should be cleaned and sterilised between flocks, and vaccination teams should exercise strict hygiene when going from one flock to another. Vaccine should be stored at between 2°C and 8°C. It should not be frozen or exposed to bright light or high temperature.

Only healthy birds, known to be sensitised by previous exposure to IBDV, should be vaccinated. Used in this way the vaccine should produce such a good antibody response that chickens hatched from those parents will have passive protection against IBD for up to about 30 days of age (Wyeth & Cullen, 1979). This covers the period of greatest susceptibility to the disease and prevents bursal damage at the time when this could cause immunosuppression. It has been shown that bursal damage occurring after about 15 days of age has little effect on immunocompetence as by that time the immunocompetent cells have migrated into the peripheral lymphoid tissues. However, if there is a threat of exposure to infection with very virulent IBDV, live vaccines should be applied as described above. The precise level and duration of immunity conferred by inactivated IBD vaccines will depend mainly on the concentration of antigen present per dose. The manufacturing objective should be to obtain a high antigen concentration and hence a highly potent vaccine.

Subunit vaccines, in which the inactivated whole IBDV antigen used in the inactivated vaccines is replaced by recombinant VP2 expressed either in the baculovirus system, or in *Escherichia coli*, or in the yeast *Pichia pastoris* (Pitcovski *et al.,* 2003), have been described. Similar to inactivated vaccines, they also require to be injected and result in a better immunisation when i) their antigen content is high and ii) they are administered as a booster in birds previously primed with a live vaccine (Muller *et al*., 2012).

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

See also Chapter 1.1.8 *Principles of veterinary vaccine production* and Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*.

2.1.1. Biological characteristics of the master seed

i) Live vaccines

Virus strains used in live IBD vaccines are sometimes referred to as "mild", "intermediate" and "intermediate plus"/"invasive"/"hot" depending on their ability to replicate in the face of increasing amounts of residual maternally derived anti-IBDV antibodies. Consistently with the increasing replication ability of the least attenuated vaccine strains, these strains usually induce more severe vaccine-induced bursal lesions (microscopic lesions and reduced size) and may exhibit some levels of residual immunosuppressive properties (see Section C.2.1.3 *Validation as a vaccine strain*).

ii) Inactivated vaccines

Subtypes have been reported among serotype 1 IBDV, and it has been demonstrated that protection against a given subtype using an inactivated vaccine requires either an homologous antigen or a high antigenic content. As a result, information relating to the subtype of the strain used as an antigen in the inactivated vaccine may prove helpful.

2.1.2 Quality criteria

i) Purity

The seed virus must be shown to be free from extraneous viruses, bacteria, mycoplasma and fungi, particularly avian pathogens. This includes freedom from contamination with other strains of IBDV.

ii) Lack of reversion to virulence of live vaccines

For vaccine strains that claim to be attenuated and with limited immunosuppressive properties, the seed virus must be shown to be stable, with no tendency to revert to virulence. This can be confirmed by carrying out sequential passage through five groups of SPF chickens, at 3- to 4-day intervals using bursal suspension as inoculum, in SPF chickens of the minimum age recommended for vaccination. It must be shown that the virus was transmitted: if the passage virus was not found at a passage level, the passage should be repeated by administration to a group of 10 chickens. A histological comparison is made to show that there is no difference between bursae from birds inoculated with the initial and the final passage material. Bursal scoring (Muskett *et al.,* 1979) and imaging techniques have been developed.

2.1.3. Validation as a vaccine strain

i) Live vaccine

Validation of an IBDV strain as a live vaccine requires the evaluation of its innocuity, immunosuppressive potential, lack of reverting potential and immunogenicity.

Innocuity may be tested in a number of ways. Some countries recommend vaccinating SPF chickens of the youngest recommended age for vaccination using a high dose (usually tenfold) of the vaccine at its least attenuated passage level, then checking the lack of signs and usually moderate and transient bursal lesions after this vaccination. There is no report documenting the innocuity of IBDV vaccines in non-target species.

The immunosuppressive potential is an important characteristic to assess, indeed the vaccine virus should not produce damage to the bursa of Fabricius such that it causes immunosuppression in susceptible birds. Live vaccines of the 'intermediate' or 'intermediate plus' type may be authorised even though they may be capable of causing immunosuppression. A possible protocol for the experimental assessment of

immunosuppression is the following: the IBD vaccine is administered by injection or eye-drop, one field dose per bird, to each of 10 SPF chickens, at 1-day old. Two further groups of 10 birds of the same age and source are housed separately as controls. At 2 weeks of age, each bird in both the IBDV-vaccinated group and in one of the control groups is given one field dose of live ND vaccine by eye-drop. Alternatively, the IBDV vaccine may be administered at the minimum age recommended for vaccination, and the ND vaccine at the time when bursal lesions induced by the IBDV vaccine are maximal. The haemagglutination inhibition (HI) response of each bird to ND vaccine is measured 2 weeks after the administration of the ND vaccine, and the protection is measured against challenge with $10^{5.0}$ to $10^{6.5}$ ELD_{50} (50% embryo lethal doses) Herts 33/56 strain (or similar) of ND virus (NDV) (the second control group, that was kept without IBDV- or NDV-vaccine, is used at this stage to validate the severity of the NDV challenge). The IBD vaccine fails the test if the HI response and protection afforded by ND vaccine is significantly less in the group given IBD vaccine than in the control group. In countries where NDV is exotic, an alternative is to use sheep erythrocytes or Brucella abortus-killed antigen as the test antigen, measuring the response using the haemagglutination or serum agglutination test, respectively. However, another live vaccine is a preferable test system because it also evaluates cell-mediated immunity.

Lack of reverting potential of the vaccine strain can be evaluated as described (see Section C.2.1.2.ii *Lack of reversion to virulence of live vaccines*).

a) Immunogenicity

The vaccine should be administered to birds in the way in which it will be used in the field. Live vaccine can be given to young birds and the response measured serologically and by resistance to experimental challenge: administer one vaccine dose of the minimum recommended titre to each of 20 SPF chickens of the minimum age of vaccination. Inoculate separate groups for each of the recommended routes of application. Leave 20 chickens from the same hatch as uninoculated controls. After 14 days, challenge each of the chickens by eye-drop with approximately 100 CID_{50} (50% chicken infective dose) of a virulent strain of IBDV as recommended by one of the WOAH Reference Laboratories for IBD[4]. Observe the chickens daily for 10 days. Register the number of birds that die or exhibit IBD signs. Perform a histological examination of the bursa in chickens that survive at day 10. The vaccine fails the test unless at least 90% of the vaccinated chickens survive without showing either clinical signs or severe lesions in the bursae of Fabricius at the end of the observation period. If more than half the controls do not show IBD signs, or one or more control chicken does not exhibit severe lesions of the bursa of Fabricius, or control or inoculated birds die from causes not attributable to the test, the test is invalid. Lesions are considered to be severe if at least 90% of follicles show greater than 75% depletion of lymphocytes, or if at least 51% of the bursal follicles exhibit a histopathological score of 3 or more according to the European Pharmacopoeia (2014).

ii) Inactivated vaccine

Validation of an IBDV inactivated vaccine requires the evaluation of its innocuity and immunogenicity.

Safety of the inactivated vaccine should be tested for all recommended administration routes and with a batch of vaccine whose activity is at least the maximal activity of future commercial batches. One dose, or a double dose to ensure maximal activity, of vaccine is administered to SAN or SPF chickens. Clinical signs in vaccinated chickens are checked daily and for 14 days. The vaccine passes the test if no signs are observed and no death can be attributed to the vaccine. The test is invalid if nonspecific death occurs.

Efficacy of IBD inactivated vaccines should be evaluated in older birds that go on to lay, using the recommended vaccination schedule, so that their progeny can be challenged to determine resistance due to MDA at the beginning and end of lay.

At least 20 unprimed SPF birds are given one dose of vaccine at the recommended age (near to point-of-lay) and by at least one of the recommended routes; an alternative

4 See footnote 1.

recommended procedure is to test one dose of vaccine in the recommended routes listed on the label, using 20 unprimed SPF birds for each route. The antibody response is measured between 4 and 6 weeks after vaccination by serum neutralisation with reference to a standard antiserum[5].

Eggs are collected for hatching 5–7 weeks after vaccination, and 25 progeny chickens are then challenged at 3 weeks of age by eye-drop with approximately 100 CID_{50} of a recognised virulent strain of IBDV. Ten control chickens of the same breed but from unvaccinated parents are also challenged. Protection is assessed 3–4 days after challenge by removing the bursa of Fabricius from each bird; each bursa is then subjected to histological examination or tested for the presence of IBD antigen by the agar gel precipitin test. Not more than three of the chickens from vaccinated parents should show evidence of IBD infection, whereas all those from unvaccinated parents should be affected.

These procedures may be repeated towards the end of the period of lay when the vaccinated birds are at least 60 weeks of age, but, on this occasion challenge of the progeny should be undertaken when they are 15 days old.

If the inactivated vaccine is intended to be used as a booster after a priming vaccination, the efficacy test should be repeated on primed birds vaccinated by the recommended schedule. The final dose of killed vaccine is given at the earliest recommended age. Chickens hatched from fertile eggs collected at the beginning and the end of lay are tested for protection against challenge as described above.

2.2. Methods of manufacture

2.2.1. Procedure

Seed virus may be propagated in various culture systems, such as SPF chicken embryo fibroblasts, or chicken embryos. In some cases, propagation in the bursa may be used. The bulk is distributed in aliquots and freeze-dried in sealed containers. There have been claims that bursal origin vaccines are better immunogens than tissue culture vaccines. In controlled studies, it was concluded that both types of virus, when included with a similar antigenic mass in inactivated vaccines, elicited similar immune responses; standardisation of the antigenic mass in inactivated vaccines would therefore appear to be desirable (Maas *et al.*, 2004).

The vaccine must be manufactured in suitable clean and secure accommodation, well separated from diagnostic facilities or commercial poultry.

Production of the vaccine should be on a seed-lot system using a suitable strain of virus of known origin and passage history. Live vaccines are made by growth in eggs or cell cultures. Inactivated IBD vaccines may be made using virulent virus grown in the bursae of young birds, or using attenuated, laboratory-adapted strains of IBDV grown in cell culture or embryonated eggs. A high virus concentration is required. Inactivated vaccines can be prepared as different types of emulsions. A typical water-in-oil formulation is to use 80% mineral oil to 20% suspension of bursal material in water, with suitable emulsifying agents, however vaccines prepared as double or micro-emulsions also exist.

2.2.2 Requirements for ingredients

i) Ingredients of animal origin

All ingredients of animal origin, including serum and cells, must be checked for the presence of viable bacteria, viruses, fungi or mycoplasma. Ingredients of animal origin should be sourced from a country with negligible risk for transmissible spongiform encephalopathies (TSEs).

SPF eggs must be used for all materials employed in propagation and testing of the vaccine.

5 See footnote 1.

ii) Preservatives

A preservative may be required for vaccine in multidose containers. The concentration of the preservative in the final vaccine and its efficacy until the end of the shelf life should be checked. A suitable preservative already established for such purposes should be used.

2.2.3. In-process control

i) Antigen content

Having grown the virus to high concentration, its titre should be assayed by use of cell cultures, embryos or chickens as appropriate to the strain of virus being used. The antigen content required to produce satisfactory batches of vaccine should be based on determinations made on test vaccine that has been shown to be safe and effective in laboratory and field trials.

ii) Inactivation of inactivated vaccines

This is often done with either ß-propiolactone or formalin. The inactivating agent and the inactivation procedure must be shown under the conditions of vaccine manufacture to inactivate the vaccine virus and any potential contaminants, e.g. bacteria, that may arise from the starting materials.

Prior to inactivation, care should be taken to ensure a homogeneous suspension is free from particles that may not be penetrated by the inactivating agent. A test for inactivation of the vaccine should be carried out on each batch of both the bulk harvest after inactivation and the final product. An alternative approach is to test inactivation of the final or bulk harvest, but not both. The test selected should be appropriate to the vaccine virus being used and should consist of at least two passages in susceptible cell cultures, embryos or chickens, with ten replicates per passage. No evidence of the presence of any live virus or microorganism should be observed.

iii) Sterility of inactivated vaccines

Oil used in the vaccine must be sterilised by heating at 160°C for 1 hour, or by filtration, and the procedure must be shown to be effective. Tests appropriate to oil-emulsion vaccines are carried out on each batch of final vaccine as described, for example, in the European Pharmacopoeia (2014) or in title 9, *Code of Federal Regulations* (*9-CFR*), *part 113.26*.

2.2.4. Final product batch test

i) Identity

The identity of a live IBD vaccine can be confirmed at batch level by incubating an appropriate dilution of the vaccine with a monospecific anti-IBDV antiserum neutralising serotype 1 IBDV, then inoculating the mix to susceptible SAN or SPF eggs or susceptible cell cultures. The neutralised vaccine should not exhibit any infectivity.

The identity of inactivated IBD vaccine can be confirmed at batch level by administrating the vaccine to SAN or SPF chickens, and demonstrating that the vaccine does induce antibodies that neutralise serotype 1 IBDV. In some instances, this test can be combined with the potency test in order to reduce the number of animals used in the experiments.

ii) Sterility and absence of extraneous agents

Tests for sterility and freedom from contamination of biological materials by bacteria, fungi, mycoplasma and extraneous agents are described in Chapter 2.3.4 *Minimum requirements for the production and quality control of vaccines*.

iii) Safety

a) Live vaccine safety test

Ten field doses of vaccine are administered by eye-drop to each of 15 SPF chickens of the minimum age recommended for vaccination and not older than 2 weeks. The chickens are observed for 21 days. If more than two chickens die due to causes not related to the vaccine, the test must be repeated. The vaccine fails the test if any chickens die or show signs of

disease attributable to the vaccine. This test is performed on each batch of final vaccine, unless controls at earlier production stages complemented by implementation of GMP advocate for the safety of the overall process. Alternative safety tests may be used as described in *9-CFR* 113.212(d)(1) and 113.331(d)(2).

b) Extraneous agents in inactivated vaccines

Ten to 21 SPF birds, 14–28 days of age, are inoculated by the recommended routes with the recommended dose or twice the field dose. The birds are observed for 3 weeks. No abnormal local or systemic reaction should develop. No antibodies against any avian pathogen but the vaccine antigen should develop. The test is performed on each batch of final vaccine, unless controls at earlier production stages complemented by implementation of good manufacturing practices advocate for the safety of the overall process.

iv) Residual live vaccine in inactivated vaccines

The process described in Section C.2.2.3 *In process controls* is may be performed on each batch of final product.

v) Potency

a) Live vaccine potency test

A potency test (virus titration) in eggs or cell cultures must be carried out on each serial (batch) of vaccine produced.

In addition, the method described in Section C.2.1.3.i.a *Immunogenicity* must be used and yield satisfactory results on one batch representative of all the batches prepared from the same seed lot.

b) Inactivated vaccine potency test

Ten SPF chickens, approximately 4 weeks of age, are each vaccinated with one dose of vaccine given by the recommended route. An additional ten control birds of the same source and age are housed together with the vaccinates. The antibody response of each bird is determined 4–6 weeks after vaccination in a VN test with reference to a standard antiserum. The mean antibody level of the vaccinated birds should not be significantly less than the level recorded in the test for protection (see Section C.2.1.3.ii.a *Immunogenicity*). No antibody should be detected in the control birds. This test must be carried out on each batch of final vaccine. Alternatively a vaccination-challenge potency test may be used (*9-CFR* 113.212(d)(2)).

2.3. Requirements for authorisation/registration/licensing

2.3.1. Manufacturing process

For registration of vaccine, all relevant details concerning the manufacture of the vaccine and quality control testing (see Section C.2.1 *Characteristics of the seed* and C.2.2 *Methods of manufacture*) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

2.3.2. Safety requirements

i) Target and non-target animal safety

Live attenuated IBD vaccines with the highest replication ability and the potential to induce lymphoid depletion in the bursa are usually authorised for use in animals with high titres of maternally derived anti-IBDV antibodies and in premises characterised by a high infectious pressure of highly pathogenic viruses. This information should be indicated when relevant in the instructions for use of the vaccine.

No interaction of live IBD vaccines with non-target avian species has been documented so far. Any information regarding a negative effect in a non-target animal species should be provided in the vaccine instructions for use.

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

It is critical that the potential of live attenuated IBD vaccines to revert to virulence is assessed prior to regulatory approval (See Section C.2.1.2.ii above).

Environmental considerations to be taken into account in the regulatory approval process include the knowledge of the IBDV strains that circulate in the area where the approved vaccine will be used, as this knowledge may help i) in selecting the vaccines suitable to control these strains and ii) in deciding whether it is justified or not to introduce a live attenuated IBDV vaccine strain possibly significantly different from the local IBDV strains.

iii) Precautions (hazards)

Oil-emulsion vaccines cause serious injury to the vaccinator if accidentally injected into the hand or other tissues. In the event of such an accident, the person should go at once to a hospital, taking the vaccine package with them. Each vaccine bottle and package should be clearly marked with a warning of the serious consequences of accidental self-injury. Such wounds should be treated by the casualty doctor as a 'grease gun injury'.

2.3.3. Efficacy requirements

The tests, challenge models and criteria used to assess the efficacy of IBD vaccines are described under Sections C.2.1.3.i *Live vaccine* and C.2.1.3.ii *Inactivated vaccine*. When assessing efficacy in an IBDV challenge model, it is advisable that the selected challenge virus be representative of contemporary IBDV strains that circulate in the area where the authorised vaccine will be used.

2.3.4. Vaccines permitting a DIVA strategy

Among the currently commercially available vaccines, live recombinant vectored vaccines expressing the VP2 protein of IBDV, and subunit vaccines containing the VP2 protein as the sole IBDV antigen, have the potential to be used in a DIVA strategy. Indeed, chickens vaccinated with such vaccines will develop anti-VP2 antibodies only, whereas birds infected by IBDV will present a broader antibody response directed at all IBDV antigens, including the VP3 protein (IBDV ribonucleoprotein). Based on the presence of anti-VP2 antibodies only, or of both anti-VP2 and anti-VP3 antibodies, it would therefore be theoretically possible to differentiate the birds that received only such vaccines, from the infected birds. However, implementation of the DIVA strategy would require ELISAs allowing the differential study of these two types of antibody responses. Although commercial ELISAs may exhibit different sensitivity to these different types of antibodies, the validation of the commercial assays for such a purpose has not been reported in the scientific literature.

2.3.5 Duration of immunity

As explained above (see Section C.2.1.3.ii *Inactivated vaccine*), repeating the evaluation of the efficacy of inactivated vaccines in breeder birds, both early after point of lay and later-on at the end of the laying period, may help in assessing whether the prolonged protection of progeny requires the implementation of a booster vaccination during the laying period.

2.3.6. Stability

Evidence should be provided on three batches of vaccine to show that the vaccine passes the batch potency test at the requested shelf life or as an alternative at 3 months beyond.

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NB: There are WOAH Reference Laboratories for Infectious bursal disease (Gumboro disease) (see WOAH Web site for the most up-to-date list: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for infectious bursal disease (Gumboro disease)

NB: FIRST ADOPTED IN 1990; MOST RECENT UPDATES ADOPTED IN 2016.