CHAPTER 2.3.12.
INFECTIOUS BURSAL DISEASE
(Gumboro disease)

SUMMARY

Infectious bursal disease (IBD) is caused by a virus that is a member of the genus Avibirnavirus of the family Birnaviridae. Although turkeys, ducks, guinea fowl and ostriches may be infected, clinical disease occurs solely in chickens. Only young birds are clinically affected. Severe acute disease of 3–6-week-old birds is associated with high mortality, but a less acute or subclinical disease is common in 0–3-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 if this occurs in the first 2 weeks of life, significant depression of the humoral antibody response may result. Two serotypes of IBDV are recognised. These are designated serotypes 1 and 2. Both serotypes can be differentiated by cross-neutralisation assays. Clinical disease has been associated with only serotype 1 and all commercial vaccines are prepared against this serotype. Antigenic variants of IBD serotype 1 have been described and these may require special vaccines for maximum protection. Very virulent strains of classical serotype 1 are now common and are causing serious disease in many countries.

Clinical disease due to infection with the IBDV, also known as Gumboro disease, can usually be diagnosed by a combination of characteristic signs and post-mortem lesions. Laboratory confirmation of disease, or detection of subclinical infection, can be carried out by demonstration of a humoral immune response in unvaccinated chickens or by detecting the presence of viral antigen or viral genome in tissues. In the absence of such tests, histological examination of bursae may be helpful.

Identification of the agent:
Isolation of IBDV is not usually carried out as a routine diagnostic procedure. Specific antibody-negative chickens may be used for this purpose, as may cell cultures or embryonating eggs from specific antibody-negative sources. However, some difficulty may be experienced if using the latter two systems as the virus does not readily adapt to them. If successful, the identity of the virus can be confirmed by the virus neutralisation (VN) test.

The agar gel immunodiffusion (AGID) test can be used to detect viral antigen in the bursa of Fabricius. A portion of the bursa is removed, homogenised, and used as antigen in a test against known positive antiserum. This is particularly useful in the early stages of the infection, before the development of an antibody response. An immunofluorescence test using IBDV-specific chicken antiserum can also be used to detect antigen in bursal tissue. Antigen-capture enzyme-linked immunosorbent assays (ELISAs) based on plates coated with IBDV-specific antibodies have also been described for the demonstration of IBDV antigens in bursal homogenates. The reverse-transcription polymerase chain reaction (RT-PCR) with specific primers may be used to detect viral genomic RNA in the bursa of Fabricius.

Strain characterisation: IBDV strains can be further identified by testing their pathogenicity in specific antibody-negative chickens, by investigating their antigenic reactivity in cross VN assays or in tests based on monoclonal antibodies, 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 different 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.
Serological tests: An AGID, VN or ELISA may be carried out on serum samples. The infection usually spreads rapidly within a flock of birds. Because of this, only a small percentage of the flock needs to be tested to detect the presence of antibodies. If positive reactions are found in unvaccinated birds then the whole flock must be regarded as infected.

Requirements for vaccines and diagnostic biologicals: Both live attenuated and inactivated (killed) vaccines are available to control the disease. A live recombinant vaccine expressing the VP2 antigen of IBDV has also been licensed recently. It is important that live vaccines be stable, with no tendency to revert to virulence on passage. To be effective, the inactivated vaccines need to have a high antigen content.

Live vaccines are used to produce an active immunity in young chickens. A complementary approach to this is to provide 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 vaccines: Attenuated strains of IBD viruses are used. These are referred to as either mild, intermediate, or ‘intermediate plus’ (‘hot’) vaccines. The mild vaccines cause limited bursal damage, while the intermediate and intermediate plus vaccines cause some lymphocytic depletion in the bursa of Fabricius. Usually none of the vaccine types causes immunosuppression when used in birds over 14 days old that have been hatched from IBD immune parents.

Mild vaccines are rarely used in broilers, but are used widely to prime broiler parents prior to inoculation with inactivated vaccine. Intermediate and ‘hot’ vaccines are more capable of overcoming low levels of maternally derived antibodies (MDA). Live vaccines are usually administered by spray or in drinking water. In the absence of MDA, mild vaccines are given at 1-day old. When MDA are present at 1 day of age, vaccination should be delayed until MDA in most of the flock has waned. The best schedule can be determined by serological testing of the birds to detect the time at which MDA has fallen to a low level. More recently, vaccines have been developed that can be administered in ovo at 18 days of incubation.

Killed vaccines: These are usually used to stimulate high and uniform levels of antibody in parent chickens so that the progeny will have high and uniform levels of MDA. The killed vaccines may 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 primary exposure, either to live vaccine or to 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) is caused by a virus that is a member of the genus *Avibirnavirus* of the family *Birnaviridae*. Although turkeys, ducks, guinea fowl and ostriches may be infected, clinical disease occurs solely in chickens. Only young birds are clinically affected. Severe acute disease of 3–6-week-old birds is associated with high mortality, but a less acute or subclinical disease is common in 0–3-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 if this occurs in the first 2 weeks of life, significant depression of the humoral antibody response may result. Two distinct serotypes of infectious bursal disease virus (IBDV) are known to exist. Serotype 1 virus causes clinical disease in chickens younger than 10 weeks. Older chickens usually show no clinical signs. Antibodies are sometimes found in other avian species, but no signs of infection are seen. Serotype 2 antibodies are very widespread in turkeys and are sometimes found in chickens and ducks. There are no reports of clinical disease caused by infection with Serotype 2 virus (20).

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 (25). 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.
1. Identification 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%. 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. Infrafollcular 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 the 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 OIE Reference Laboratories for infectious bursal disease [see Table given in Part 3 of this Terrestrial Manual]).

a) 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 µ filter may prove necessary to further control bacterial contamination, although this may cause a reduction in virus titre.

b) Identification by the agar gel immunodiffusion test

A protocol for the AGID test is described in Section B.2.a. 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.

c) 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 (27).

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

Different protocols have been described for the detection of serotype 1 IBDV using an antigen-capture enzyme-linked immunosorbent assay (AC-ELISA) (12, 19, 36). 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.

The 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 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).

e) Identification by molecular techniques

Molecular virological techniques have been developed that allow IBDV to be identified more quickly than by virus isolation (8, 16, 43). The most frequently used molecular method is the detection of IBDV genome by the reverse-transcription polymerase chain reaction (RT-PCR) (24, 43). This method can detect the genome of IBDV, which is unable to grow 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 (8, 10).

o Extraction of nucleic acids

Single-stranded RNA is extremely susceptible to degradation by RNases. 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.

o 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 denatured before transfer to the RT reaction mix. Add one part (by volume) molecular biology grade dimethylsulphoxide 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 denatured 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.

o 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 (44). 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 (9, 10), and a region at the 5’ extremity of IBDV segment B (21), respectively. Both regions have been shown to be suitable for molecular epidemiology studies (22). 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-ATC-GGTT-GCC-ATT-CTC-TG-5'

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-AGT-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: SphI (in primer U3), EcoRI (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 primer 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 (9, 10, 21, 22).

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 (35).

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

f) 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 cm² 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 and 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 IBDV antiserum in a tissue culture virus neutralisation (VN) test (see below). 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).

g) Isolation of virus in embryos

Inoculate 0.2 ml of sample into the yolk sac of five 6–8-day-old specific antibody negative (SAN) chicken embryos and on to the chorioallantoic membrane (1) of five 9–11-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
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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 discoloration.

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.a for the preparation a virus suspension.

h) 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. Kill 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 agar gel immunodiffusion (AGID) test against known positive IBD antiserum (see Section B.1.b).

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 a limited indication on strain pathogenicity.

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

i) 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.

o 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 1.1.2 Biosafety and biosecurity in the veterinary microbiological 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 (42). 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 (25). (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.c 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 (32).

The terms ‘variant’, ‘classical’ and ‘very virulent’ have been used to describe IBDV strains that exhibit a different 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).
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. very virulent IBDV [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 (15).

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 (12, 13, 37). 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 outer capsid protein (10, 33, 40). 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) (2, 41). Both North American ‘variants’ and ‘very virulent’ IBDV exhibit in these areas amino acid changes that correlate with epitope variation (9, 40). To date, no antigenic marker has been shown to correlate strictly with IBDV pathogenicity.

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. Several protocols have been published on characterisation using restriction endonucleases of RT-PCR products. These approaches are known as RT-PCR/RE or RT-PCR-RFLP (restriction fragment length polymorphism) (17, 24, 46). The usefulness of the information they provide depends on the identification of enzymes that cut in restriction sites that are phenotypically relevant. Some sites involved in antigenicity have already been identified (see above), however, restriction sites reliably related to virulence still need to be defined and validated. Nucleotide sequencing of RT-PCR products, although more expensive than restriction analysis, provides an approach to assessing more precisely the genetic relatedness among IBDV strains. Markers have been demonstrated experimentally, using a reverse genetics approach, for cell culture-adapted strains, which exhibit amino acid pairs 279 N–284 T (23) or 253 H–284 T (28). In most very virulent viruses, four typical amino acids are present (222 A, 256 I, 294 I and 299 S) (3, 9, 24). However, it is not yet known whether these amino acids play a role in virulence or if they are merely an indication of the clonal origin of most vvIBDV isolates. Several recent studies indicate that although VP2 is an important virulence determinant, it may not be the only one (4). 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 (21).

Serological tests

a) Agar gel immunodiffusion test

The AGID test is the most useful of the serological tests for the detection of specific antibodies in serum, or for detecting viral antigen or antibodies in bursal tissue.

Blood samples should be taken early in the course of the disease, and repeat samples should be taken 3 weeks later. Because the virus spreads rapidly, only a small proportion of the flock needs to be sampled. Usually 20 blood samples are enough. For detection of antigen in the bursa of Fabricius, the bursae should be removed aseptically from about ten chickens at the acute stage of infection. The bursae are minced using two scalpels in a 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. A possible alternative to avoid health hazards caused by methylene chloride is to use trichlorotrifluoroethane). 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

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1 A suitable classical strain of IBDV (serotype 1, classical pathotype) is strain 52/70, obtainable from one of the OIE Reference Laboratories (see Table given in Part 3 of this Terrestrial Manual).
class II safety cabinet. If required, the antigen can be inactivated prior to dispensing: add 0.3% (v/v) β-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 β-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.g).

- **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 IBDV1. Exsanguinate 28 days post-inoculation. Pool and store serum in aliquots at −20°C.

- **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.

- **Test procedure**

  i) Prepare plates from 24 hours to 7 days before use. Dip 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.)

    ![Fig. 1. Protocol for tests for antibody.](image1)

    ![Fig. 2. Protocol for tests for antigen.](image2)

    T = test sera

    T = test tissues

    **Notes:**

    1. The linear pattern of wells is preferred although a hexagonal pattern may be used. Each test serum or bursa 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.

o **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 (5). This can be very 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.

b) **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.

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 (Spearman–Kärber [1] or the Reed & Muench [30]). 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 SPF chicken embryo fibroblast 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$^2$ and the titre of the virus suspension must be reassessed in each new experiment using a sufficient number of repeats (wells) per virus dilution.

c) **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. in 1980 (26). 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 andrewashed 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.

d) **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. 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. A formula has been devised that allows ELISA titres to be used to calculate the optimal age for vaccination (18), which will vary depending on the vaccine used. Nonspecific positive reactions may occur with most ELISAs because they are usually designed for monitoring vaccine

\[2 A suitable reference antiserum may be obtained from the OIE Reference Laboratories (see Table given in Part 3 of this Terrestrial Manual).\]
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, the possibility that a serotype 1 ELISA antigen also detects antibodies induced by a natural infection with serotype 2 IBDV cannot be ruled out, however this possible cross reactivity has not yet been demonstrated to interfere with serological monitoring programmes of IBD based on the ELISA.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Two types of vaccine are mostly available for the control of IBD. These are live attenuated vaccines, or inactivated oil-emulsion adjuvanted vaccines (39). A live recombinant vaccine expressing IBDV antigens has also been licensed (7).

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

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 (31). 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 are now licensed. 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 (16), however the latter viruses are less susceptible to neutralisation by maternally derived antibodies than 'classical' pathogenic viruses (42).

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 (34).

Intermediate or intermediate plus vaccines are used to protect 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 smell 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 medicated water is made available and care must be taken that no residual water remains in the water adduction 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.

Recently, technology has been developed to deliver live vaccine into eggs during the incubation period. Live vaccine virus is blended with IBD antibody and the complex is injected in ovo at 18 days of incubation. The eggs go on to hatch and the vaccine virus is released when the chicks are about 7 days of age. In this way, the problem of maternally derived IBD antibody is overcome and the chicks are effectively immunised (14).

A live recombinant vaccine that uses a viral vector (herpes virus of turkeys) to express the VP2 antigen of IBDV in chickens has been licensed recently in Europe. There is limited information available on the use of this vaccine.

Live IBD vaccines are generally regarded as compatible with other avian vaccines. However, it is possible that IBD vaccines that cause bursal damage could interfere with the response to other vaccines. Only healthy birds
Chapter 2.3.12. - Infectious bursal disease (Gumboro disease)

should be vaccinated. The vials of lyophilised vaccine should be kept at temperatures between 2°C and 8°C up to the time of use.

**o 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 (6). 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 4°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 (45). 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 very virulent IBDV, live vaccines should be applied as described above. The precise time the immunocompetent cells have migrated into the peripheral lymphoid tissues.

**1. Seed management**

**a) Characteristics of the seed**

- **Live vaccine**

  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. For vaccine strains that claim to be attenuated and nonimmunosuppressive, the seed virus must be shown to be stable, with no tendency to revert to virulence. This can be confirmed by carrying out at least five consecutive chicken-to-chicken passages at 3–4-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. A histological comparison is then made to show that there is no difference between bursae from birds inoculated with the initial and the final passage material. Bursal scoring (29) and imaging techniques have been developed.

  **Test for immunosuppression:** An important characteristic is that the virus should not produce such damage to the bursa of Fabricius that it causes immunosuppression in susceptible birds. Live vaccines of the ‘intermediate’ or ‘intermediate plus’ type may be licensed even though they may be capable of causing immunosuppression. A possible protocol for the experimental assessment of immunosuppression is the following: The vaccine is administered by injection or eye-drop, one field dose per bird, to each of 20 SPF chickens, at 1-day old. A further group of birds of the same age and source are housed separately as controls. At 2 weeks of age, each bird in both groups is given one field dose of live Newcastle disease vaccine by eye-drop. Alternatively, the IBDV vaccine may be administered at the minimum age recommended for vaccination, and the Newcastle disease vaccine at the time when bursal lesions induced by the IBDV vaccine are maximal. The haemagglutination inhibition (HI) response of each bird to Newcastle disease vaccine is measured 2 weeks after the administration of the Newcastle Disease vaccine, and the protection is measured against challenge with 10⁵.⁰ to 10⁶.⁵ ELD₅₀ (50% embryo lethal doses) Herts 33/56 strain (or similar) of Newcastle disease virus. The IBD vaccine fails the test if the HI response and protection afforded by Newcastle disease vaccine is significantly less (<0.01) in the group given IBD vaccine than in the control group. In countries where Newcastle disease virus 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.

- **Killed vaccine**

  For killed vaccines, the most important characteristics are high yield and good antigenicity. Both virulent and attenuated strains have been used. The seed virus must be shown to be free from extraneous viruses, bacteria, mycoplasma and fungi, particularly avian pathogens (38).
b) Method of culture

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 vaccines, when containing similar antigenic mass, elicited similar immune responses.

c) Validation as a vaccine

Data on efficacy should be obtained before bulk manufacture of vaccine begins. 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. In the case of killed vaccines, a test must be carried out 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.

- Live vaccine

**Efficacy test:** 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 OIE Reference Laboratories for IBD (see Table given in Part 3 of this *Terrestrial Manual*). 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. Providing results are satisfactory, this test need be carried out on only one batch of all those batches prepared from the same seed lot.

- Killed vaccine

**Efficacy test:** At least 20 unprimed SPF birds are given one dose of vaccine at the recommended age (near to point-of-lay) at least one of the recommended routes; an alternative 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.$^3$

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 should 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.

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. These tests need to be performed once only using a typical batch of vaccine.

2. Method of manufacture

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

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3 See footnote 2
Production of the vaccine should be on a seed-lot system using a suitable strain of virus of known origin and passage history. SPF eggs must be used for all materials employed in propagation and testing of the vaccine. 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. These vaccines are made as water-in-oil emulsions. A typical formulation is to use 80% mineral oil to 20% suspension of bursal material in water, with suitable emulsifying agents.

3. In-process control

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 effective in laboratory and field trials.

Inactivation of killed 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 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.

Sterility of killed 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.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

b) Safety

o 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.

o Killed vaccine safety test

Ten 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. The test is performed on each batch of final vaccine.

c) Potency

o 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.1.c ‘Live vaccine (efficacy test)’ must be used on one batch representative of all the batches prepared from the same seed lot.

o Killed 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. No antibody should be detected in the control birds. This test must be carried out on each batch of final vaccine.
d) 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.

e) Preservatives
A preservative is normally required for vaccine in multidose containers. The concentration of the preservative in the final vaccine and its persistence throughout shelf life should be checked. A suitable preservative already established for such purposes should be used.

f) 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’.

5. Tests on the final product

a) Safety
See Section C.4.b.

b) Potency
See Section C.4.c.

REFERENCES


**RECENT REVIEWS**


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**NB:** There are OIE Reference Laboratories for Infectious bursal disease (Gumboro disease) (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).