

## CHAPTER 2.3.2.

# AVIAN INFECTIOUS BRONCHITIS

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### SUMMARY

*Avian infectious bronchitis (IB) is caused by the gammacoronavirus infectious bronchitis virus (IBV). The virus causes infections mainly in chickens and is a significant pathogen of commercial meat and egg type birds. IB is an acute, contagious disease characterised primarily by respiratory signs in growing chickens. In hens, decreased egg production and quality are often observed. Some strains of the virus are nephropathogenic and produce interstitial nephritis and mortality. The severity of IBV-induced respiratory disease is enhanced by the presence of other pathogens, including bacteria, leading to chronic complicated airsacculitis. Diagnosis of IB requires virus isolation or demonstration of viral nucleic acid from diseased flocks. Demonstration of a rising serum antibody response may also be useful. The widespread use of live and inactivated vaccines may complicate both the interpretation of virus isolation and serology findings. The occurrence of antigenic variant strains may overcome immunity induced by vaccination.*

*Diagnosis requires laboratory testing. Virus detection and identification is preferred. Reverse-transcription polymerase chain reaction (RT-PCR) techniques are commonly used to identify the IBV genotype. Haemagglutination inhibition (HI) tests to determine serotype (appropriate in young birds) and enzyme-linked immunosorbent assays (ELISA) are often used for sero-diagnosis and/or monitoring. Supplementary tests include electron microscopy, the use of monoclonal antibodies, virus neutralisation (VN), immunohistochemical or immunofluorescence tests, and immunisation-challenge trials in chickens.*

**Identification of the agent:** *For the common respiratory form, IBV is most successfully isolated from tracheal mucosa and lung several days to one week following infection. For other forms of IB, kidney, oviduct, the caecal tonsils of the intestinal tract or proventriculus tissues are better sources of virus depending on the pathogenesis of the disease.*

*Specific pathogen free chicken embryonated eggs or chicken tracheal organ cultures (TOCs) from embryos may be used for virus isolation. Following inoculation of the allantoic cavity, IBV produces embryo stunting, curling, clubbing of the down, or urate deposits in the mesonephros of the kidney, often within three serial passages. Isolation in TOCs has the advantage that IBV produces stasis of the tracheal cilia on initial inoculation. RT-PCR is increasingly being used to identify the spike (S) glycoprotein genotype of IBV field strains. Genotyping using primers specific for the S1 subunit of the S gene or sequencing of the same gene generally provides similar but not always identical findings to HI or VN serotyping. Alternatively, VN or HI tests using specific antiserum may be used to identify the serotype.*

**Serological tests:** *Commercial ELISA kits may be used for monitoring serum antibody responses. The antigens used in the kits are broadly cross-reactive among serotypes and allow for general serological monitoring of vaccinal responses and field challenges. The HI test is used for identifying serotype-specific responses to vaccination and field challenges especially in young growing chickens. Because of multiple infections and vaccinations, the sera of breeders and layers contain cross-reactive antibodies and the results of HI testing cannot be used with a high degree of confidence.*

**Requirements for vaccines:** *Both live attenuated and oil emulsion inactivated vaccines are available. Live vaccines, attenuated by serial passage in chicken embryos or by thermal heat treatment, confer better local immunity of the respiratory tract than inactivated vaccines. The use of live vaccines carries a risk of residual pathogenicity associated with vaccine back-passage in flocks. However, proper mass application will generally result in safe application of live vaccines.*

*Inactivated vaccines are injected and a single inoculation does not confer protection unless preceded by one or more live IBV priming vaccinations. Both types of vaccines are available in combination with Newcastle disease vaccine; in some countries inactivated multivalent vaccines are available that include two to three IBV antigens or Newcastle disease, infectious bursal disease, reovirus and egg-drop syndrome 76 viral antigens.*

## A. INTRODUCTION

Avian infectious bronchitis (IB) was first described in the United States of America (USA) in the 1930s as an acute respiratory disease mainly of young chickens. A viral aetiology was established, and the agent was termed avian infectious bronchitis virus (IBV). The virus is a member of the genus *Gammacoronavirus*, subfamily *Coronavirinae*, family *Coronaviridae*, in the order *Nidovirales*. IBV and other avian coronaviruses of turkeys and pheasants are classified as *Gammacoronaviruses*, with mammalian coronaviruses comprising *Alpha* and *Betacoronaviruses*. Novel related coronaviruses have been discovered in wild birds and pigs and have been designated *Deltacoronaviruses* (Woo *et al.*, 2012), interestingly the avian *Deltacoronaviruses* have a different genomic order and show no close relationship to the *Gammacoronaviruses*. Coronaviruses have a non-segmented, positive-sense, single-stranded RNA genome.

IB affects chickens of all ages, which, apart from pheasants (Britton & Cavanagh, 2007; Cavanagh *et al.*, 2002) are the only species reported to be naturally affected. The disease is transmitted by the air-borne route, direct chicken to-chicken contact and indirectly through mechanical spread (contaminated poultry equipment or egg-packing materials, manure used as fertiliser, farm visits, etc.). IB occurs world-wide and assumes a variety of clinical forms, the principal one being respiratory disease that develops after infection of the respiratory tract tissues following inhalation or ingestion. Infection of the oviduct can lead to permanent damage in immature birds and, in hens, can lead to cessation of egg-laying or production of thin-walled and misshapen shells with loss of shell pigmentation. IB can be nephropathogenic causing acute nephritis, urolithiasis and mortality (Cavanagh & Gelb, 2008). After apparent recovery, chronic nephritis can produce death at a later time. IBV has also been reported to produce disease of the proventriculus (Yu *et al.*, 2001). Vaccine and field strains of IBV may persist in the caecal tonsils of the intestinal tract and be excreted in faeces for weeks or longer in clinically normal chickens (Alexander *et al.*, 1978). For an in-depth review of IB, refer to Cavanagh & Gelb, 2008. A detailed discussion of IBV antigen, genome and antibody detection assays prepared by De Witt (2000) is also available.

There have been no reports of human infection with IBV.

## B. DIAGNOSTIC TECHNIQUES

Confirmation of diagnosis is based on virus isolation, often assisted by serology. Extensive use is made of live and inactivated vaccinations, which may complicate diagnosis by serological methods as antibodies to vaccination and field infections can not always be distinguished. Persistence of live vaccines may also confuse attempts at recovering and/or identifying the causative field strain of IBV.

**Table 1.** Infectious bronchitis virus (IBV) test methods available and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom of infection	Efficiency of eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
<b>Agent identification<sup>1</sup></b>						
Virus isolation (embryos or TOCs)	+	++	–	+++	+	+
Staining by immunohistochemistry	–	–	–	++	+	+

<sup>1</sup> A combination of agent identification methods applied on the same clinical sample is recommended.

Method	Purpose					
	Population freedom from infection	Individual animal freedom of infection	Efficiency of eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of virus genome (RT-PCR)	<sup>a</sup> +	++	++	++	+	<sup>h</sup> +
Virus identification (haemagglutination test)	–	–	–	–	+	–
Virus identification (VN)	–	–	–	–	+	–
Virus identification (gene sequencing)	–	–	–	–	<sup>f</sup> ++	–
Detection of immune response						
Antibody detection (AGID)	<sup>b</sup> +	+	–	<sup>e</sup> +	+	–
Antibody detection (VN)	–	<sup>d</sup> –	–	<sup>e</sup> +	–	<sup>e</sup> ++
Antibody detection (HIT)	–	<sup>d</sup> –	+	<sup>e</sup> +	+	<sup>e</sup> ++
Antibody detection (ELISA)	<sup>b</sup> ++	++	++	<sup>e</sup> ++	<sup>g</sup> ++	<sup>e</sup> ++

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose. Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

<sup>a</sup>Suitable for ensuring lack of infection during the past 10 days; <sup>b</sup>suitable for ensuring lack of infections dating back to more than 10 days; <sup>c</sup>suitable at the individual level only during excretion periods; <sup>d</sup>limited suitability for this purpose as it may be too specific of the serotype used as an antigen; <sup>e</sup>Suitable provided paired samples collected a few weeks apart can be analysed; <sup>f</sup>especially suitable for surveillance of a given or an emerging genotype; <sup>g</sup>especially suitable when IB surveillance is not focused on a given serotype; <sup>h</sup>sometimes used in the evaluation of vaccines to assess protection against viral excretion, but can be positive even when good clinical protection is achieved. TOC = tracheal organ culture; RT-PCR = reverse-transcription polymerase chain reaction; VN = virus neutralisation; AGID = agar gel immunodiffusion; HIT = haemagglutination inhibition test; ELISA = enzyme-linked immunosorbent assay.

## 1. Identification of the agent

### 1.1. Sampling

Samples appropriate to the form of IB observed must be obtained as soon as signs of clinical disease are evident. Samples must be placed in cold transport media and be frozen as soon as possible. The cold chain from bird to laboratory should be maintained. For acute respiratory disease, swabs from the upper respiratory tract of live birds or tracheal and lung tissues from diseased birds should be harvested, placed in transport medium containing penicillin (10,000 International Units [IU]/ml) and streptomycin (10 mg/ml) and kept on ice and then frozen. For birds with nephritis or egg-production problems, samples from the kidneys or oviduct, respectively, should be collected in addition to respiratory specimens. In some cases, IBV identification by reverse-transcription polymerase chain reaction (RT-PCR) may be desirable without virus isolation. In this case, swabbings from the respiratory tract or cloaca may also be submitted alone, without being placed in liquid transport media (Cavanagh *et al.*, 1999). In situations where IB-induced nephritis is suspected, kidney samples should also be selected from fresh carcasses for histopathological examination as well as virus isolation. Blood samples from acutely affected birds as well as convalescent chickens should be submitted for serological testing. A high rate of virus recovery has been reported from the caecal tonsil or faeces (Alexander *et al.*, 1978). However, isolates from the intestinal tract may have no relevance to the latest

infection or clinical disease. IBV isolation may be facilitated using sentinel specific pathogen free (SPF) chickens placed at one or more times in contact with commercial poultry (Gelb *et al.*, 1989).

## 1.2. Culture

Suspensions of tissues (10–20% w/v) are prepared in sterile phosphate buffered saline (PBS) or nutrient broth for egg inoculation, or in tissue culture medium for chicken tracheal organ culture (TOC) inoculation (Cook *et al.*, 1976). The suspensions are clarified by low-speed centrifugation and filtration through bacteriological filters (0.2 µ) before inoculation of SPF embryonated chicken eggs or TOCs.

SPF embryonated chicken eggs and/or TOCs are used for primary isolation of IBV. Cell cultures are not recommended for primary isolation as it is often necessary to adapt IBV isolates to growth in chicken embryos before cytopathic effect (CPE) is produced in chick embryo kidney cells.

Embryonated eggs used for virus isolation should originate preferably from SPF chickens or from breeder sources that have been neither infected nor vaccinated with IBV. Most commonly, 0.1–0.2 ml of sample supernatant is inoculated into the allantoic cavity of 9–11-day-old embryos. Eggs are candled daily for 7 days with mortality within the first 24 hours being considered nonspecific. The initial inoculation usually has limited macroscopic effects on the embryo unless the strain is derived from a vaccine and is already egg adapted. Normally, the allantoic fluids of all eggs are pooled after harvesting 3–6 days after infection; this pool is diluted 1/5 or 1/10 in antibiotic broth and further passaged into another set of eggs for up to a total of three to four passages. Typically, a field strain will induce observable embryonic changes consisting of stunted and curled embryos with feather dystrophy (clubbing) and urate deposits in the mesonephros on the second to fourth passage. Embryo mortality in later passages may occur as the strain becomes more egg adapted. Other viruses, notably adenoviruses that are common to the respiratory tract, also produce embryo lesions indistinguishable from IBV. The IBV-laden allantoic fluid should not agglutinate red blood cells and isolation of IBV must be confirmed by serotyping or genotyping. Infective allantoic fluids are kept at –20°C or below for short-term storage, –60°C for long-term storage or at 4°C after lyophilisation.

TOCs prepared from 19- to 20-day-old embryos can be used to isolate IBV directly from field material (Cook *et al.*, 1976). An automatic tissue-chopper is desirable for the large-scale production of suitable transverse sections or rings of the trachea for this technique (Darbyshire *et al.*, 1978). The rings are about 0.5–1.0 mm thick, and are maintained in a medium consisting of Eagle's N-2-hydroxyethylpiperazine N'-2-ethanesulphonic acid (HEPES) in roller drums (15 rev/hour) at 37°C. Infection of tracheal organ cultures usually produces ciliostasis within 24–48 hours. Ciliostasis may be produced by other viruses and suspect IBV cases must be confirmed by serotyping or genotyping methods.

## 1.3. Methods for identification

The initial tests performed on IBV isolates are directed at eliminating other viruses from diagnostic consideration. Chorioallantoic membranes from infected eggs are collected, homogenised, and tested for avian adenovirus group 1 by immunodiffusion or PCR. Group 1 avian adenovirus infections of commercial chickens are common, and the virus often produces stunted embryos indistinguishable from IBV-infected embryos. Furthermore, harvested allantoic fluids do not hemagglutinate (HA) chick red blood cells. Genetic based tests (RT-PCR or RT-PCR-RFLP [restriction fragment length polymorphism]) are used commonly to identify an isolate as IBV. Other techniques may be used, for example cells present in the allantoic fluid of infected eggs may be tested for IBV antigen using fluorescent antibody tests (Clarke *et al.*, 1972) and direct negative-contrast electron microscopy will reveal particles with typical coronavirus morphology in allantoic fluid or TOC fluid concentrates. The presence of IBV in infective allantoic fluid may be detected by RT-PCR amplification and use of a DNA probe in a dot-hybridisation assay (Jackwood *et al.*, 1992). Direct immunofluorescence staining of infected TOCs for the rapid detection of the presence of IBV has been described (Bhattacharjee *et al.*, 1994). Immuno-histochemistry, with a group-specific monoclonal antibody (MAb), can be used to identify IBV in infected chorioallantoic membranes (Naqi, 1990).

## 1.4. Serotype identification

Antigenic variation among IBV strains is common (Cavanagh & Gelb, 2008; Cook, 1984; Dawson & Gough, 1971; Hofstad, 1958; Ignjatovic & Sapats, 2000), but at present there is no agreed definitive classification system. Nevertheless, antigenic relationships and differences among strains are important, as vaccines based on one particular serotype may show little or no protection against viruses of a different antigenic group. As a result of the regular emergence of antigenic variants, the viruses, and hence the disease situation and vaccines used, may be quite different in different

geographical locations. Ongoing assessment of the viruses present in the field is necessary to produce vaccines that will be efficacious in the face of antigenic variants that arise. Serotyping of IBV isolates and strains has been done using haemagglutination inhibition (HI) (Alexander *et al.*, 1983; King & Hopkins, 1984) and virus neutralisation (VN) tests in chick embryos (Dawson & Gough, 1971), TOCs (Darbyshire *et al.*, 1979) and cell cultures (Hopkins, 1974). Neutralisation of fluorescent foci has also been applied to strain differentiation (Csermelyi *et al.*, 1988).

MAbs, usually employed in enzyme-linked immunosorbent assays (ELISA), have proven useful in grouping and differentiating strains of IBV (Ignjatovic *et al.*, 1991; Koch *et al.*, 1986). The limitations of MAb analysis for IB serotype definition are the lack of availability of MAbs or hybridomas and the need to produce new MAbs with appropriate specificity to keep pace with the ever-growing number of emerging IB-variant serotypes (Karaca *et al.*, 1992).

### 1.5. Genotype identification

RT-PCR genotyping methods have largely replaced HI and VN serotyping for determining the identity of a field strain. The molecular basis of antigenic variation has been investigated, usually by nucleotide sequencing of the gene coding for the spike (S) protein or, more specifically, nucleotide sequencing of the gene coding for the S1 subunit of the S protein (Cavanagh, 1991; Kusters *et al.*, 1989) where most of the epitopes to which neutralising antibodies bind are found (Koch *et al.*, 1992). An exact correlation with HI or VN results has not been seen, in that while different serotypes generally have large differences (20–50%) in the deduced amino acid sequences of the S1 subunit (Kusters *et al.*, 1989), other viruses that are clearly distinguishable in neutralisation tests show only 2–3% differences in amino acid sequences (Cavanagh, 1991). However, there is, in general, good agreement between data represented by the S1 sequence and the VN serotype, and it may eventually be possible to select vaccine strains on the basis of sequence data.

The primary advantages of genotyping methods are a rapid turnaround time, and the ability to detect a variety of genotypes, depending on the tests used. RFLP RT-PCR differentiates IBV serotypes based on unique electrophoresis banding patterns of restriction enzyme-digested fragments of S1 following amplification of the gene by RT-PCR (Jackwood *et al.*, 1997; Kwon *et al.*, 1993). The RFLP RT-PCR procedure may be used in conjunction with a biotin-labelled DNA probe to first detect IBV in egg fluids harvested following the inoculation of eggs with clinical samples (Jackwood *et al.*, 1992). The RFLP RT-PCR test is sometimes used to identify the different serotypes of IBV as well as variant viruses, however nucleotide sequencing is now preferred (see below).

S1 genotype-specific RT PCR may be used to identify specific IBV serotypes (Keeler *et al.*, 1998). S1 gene primers specific, for example, for serotypes Massachusetts (Mass), Connecticut, Arkansas, and JMK may be used in conjunction with a universal primer set that amplifies all IBV serotypes. Primers for the DE/072/92 and California serotypes have also been developed, and other primer sets may be used, based on the contemporary IBV serotypes circulating in a region. Other variant serotypes may be determined to be IBV using the general primers, but the specific serotype cannot be identified. Infections caused by multiple IBV serotypes may be identified.

Nucleotide sequencing of a diagnostically relevant fragment of the S1 gene is the most useful technique for the differentiation of IBV strains and has become the genotyping method of choice in many laboratories. Nucleotide sequencing has also produced evidence that recombination between IB strains occurs often (Cavanagh *et al.*, 1992; Zwaagstra *et al.*, 1992). RT-PCR product cycle sequencing of the hypervariable amino terminus region of S1 may be used diagnostically to identify previously recognised field isolates and variants (Kingham *et al.*, 2000). Comparison and analysis of sequences of unknown field isolates and variants with reference strains for establishing potential relatedness are significant advantages of sequencing.

Recently, it has been shown that coronaviruses isolated from turkeys and pheasants are genetically similar to IBV, having approximately 90% nucleotide identity in the highly conserved region II of the 3' untranslated region (UTR) of the IBV genome (Cavanagh *et al.*, 2001; 2002). The potential role of these coronaviruses in IBV infections has not been determined.

The major uses of RT-PCR tests are virus identification and its application in the understanding of epidemiological investigations during IBV outbreaks. The RT-PCR tests, as they now exist however, do not provide information on viral pathogenicity.

### 1.5.1. RT-PCR test procedure

#### i) *Extraction of viral RNA*

Any RNA extraction method can be used. There are many protocols available in journals, books and on the web. All extracted RNA should be stored between –20°C and –80°C until tested. It is advised that for long-term storage, RNA be kept at –80°C.

#### ii) *Custom oligos*

Custom oligos can be purchased through any commercial supplier. The target gene for IBV characterisation is the S1 subunit of the spike glycoprotein gene. A commonly used primer pair for amplification of genotypically diverse IBV strains is oligo S15' mod (forward): 5'-TGA-AAA-CTG-AAC-AAA-AGA-3' and CK2 (reverse): 5'-CNG-TRT-TRT-AYT-GRC-A-3' (Gelb *et al.*, 2005). The oligo S15' mod/CK2 amplicon is approximately 700 bp in length beginning from the start of the S1 gene spanning two hypervariable regions used for genotyping.

#### iii) *Reverse-transcription polymerase chain reaction*

Many one and two-step RT-PCR kits are commercially available from manufacturers claiming superior enzyme sensitivity and fidelity. Reverse transcription is performed according to the manufacturer's instructions. RT priming is accomplished with the use of random hexamers (supplied with the kit) or with the reverse PCR primer, in this case CK2 (Keeler *et al.*, 1998). One cycle of RT is performed with the following parameters: 25°C for 10 minutes, 42°C for 25 minutes, 95°C for 5 minutes, hold at 4°C. The full RT reaction volume is added to the PCR sample master mix. PCR is performed using the following parameters: 95°C for 2 minutes, 45 cycles of 95°C for 30 seconds, 52°C for 30 seconds, 68°C for 30 seconds, final extension of 68°C for 12 minutes, hold at 4°C. Samples are resuspended in 6 µl of loading buffer prior to electrophoresis on a 1.8% agarose gel containing nucleic acid stain. Gels are visualised with a UV light box. Bands are compared to a commercially available 100 bp ladder and an IBV positive control.

#### iv) *S1 gene sequencing*

Bands visualised in the agarose gel that are of similar size to the positive control are excised from the gel. The PCR product is isolated from the agarose gel using a commercial gel extraction kit. Purified PCR products are run on a second 1.8% agarose nucleic acid stain gel to determine the quantity of product present. Approximately 20 µl (10 ng/µl) of PCR product is required for sequencing. Sequencing can be performed at a university or commercial sequencing facility. Sequence chromatograms are edited using suitable analysis software. Edited sequences of IBV isolates are characterised using BLASTn for nucleotide or BLASTp for protein analysis (<http://www.ncbi.nlm.nih.gov/BLAST/>).

## 2. Serological tests

A number of tests have been described. Those considered here include VN (Dawson & Gough, 1971), agar gel immunodiffusion (AGID) (Witter, 1962), HI (Alexander *et al.*, 1983) and ELISA (Mockett & Darbyshire, 1981). Each test has advantages and disadvantages in terms of practicality, specificity, sensitivity and cost. In general, for routine serological testing, the VN tests are too expensive and impractical, and AGID tests lack sensitivity. ELISA and HI tests are most suitable for routine serology. ELISAs are useful for general monitoring of IBV exposure and can detect antibody responses to all serotypes. HI when used on serial sera from young growing chickens such as pullets and broilers can give information on the serotype-specific antibody status of a flock. Regular monitoring of sera from flocks for IB antibody titres may help to indicate the level of vaccine or field challenge responses. Because chicken sera from older birds contain antibodies that are highly cross-reactive against antigenically unrelated strains, serodiagnosis of suspected disease outbreaks of IB cannot be used with a high degree of confidence.

### 2.1. Virus neutralisation

In VN tests, all sera should first be heated to 56°C for 30 minutes. Virus is mixed with serum and incubated for 30–60 minutes at 37°C or room temperature. Chicken embryos are most often employed, but antibodies can be measured using TOC or cell culture systems. Two methods have been used to estimate neutralising antibodies. One employs a constant serum concentration reacted with varying dilutions of virus (the alpha method) and the other employs a constant amount of virus and varying dilutions of serum (the beta method).

In the alpha method, tenfold dilutions of egg-adapted virus are reacted with a fixed dilution (usually 1/5) of antiserum, and the mixtures are inoculated into groups of from five to ten eggs. The virus alone is titrated in parallel. End-points are calculated by the Kärber or the Reed and Muench methods. The results are expressed as a neutralisation index (NI) that represents the  $\log_{10}$  difference in the titres of the virus alone and that of the virus/antiserum mixtures. The NI values may reach 4.5–7.0 in the case of homologous virus/serum mixtures; values of <1.5 are not specific, but a heterologous virus will give a value as low as 1.5.

The beta method is the more widely used neutralisation test for antibody assay with chicken embryos. Two- or four-fold dilutions of antiserum are reacted in equal volumes with a dilution of virus, usually fixed at 100 or 200 EID<sub>50</sub> (median embryo-infective doses) per 0.05 ml and 0.1 ml of each mixture inoculated into the allantoic cavity of each of from five to ten embryonated eggs. A control titration of the virus is performed simultaneously to confirm that the fixed virus dilution in the virus/serum mixtures was between  $10^{1.5}$  and  $10^{2.5}$  EID<sub>50</sub>. End-points of the serum titres are determined by the Kärber or Reed and Muench method as before, but here are expressed as reciprocals of  $\log_2$  dilutions. This fixed-virus/varying-serum method is also employed for neutralisation tests in tracheal organ cultures using five tubes per serum dilution, as is conventional with other viruses (Darbyshire *et al.*, 1979). The results are calculated according to Reed and Muench, and the virus titres are expressed as median ciliostatic doses per unit volume ( $\log_{10}$  CD<sub>50</sub>). Serum titres are again expressed as  $\log_2$  dilution reciprocals. This test is more sensitive than others, but technical logistics hamper its more widespread adoption.

## 2.2. Haemagglutination inhibition

A standard protocol for a HI test for IBV has been described (Alexander *et al.*, 1983), and the test procedure detailed below is based on that standard. Strains and isolates of IBV will agglutinate chicken red blood cells (RBCs) after neuraminidase treatment (Ruano *et al.*, 2000; Schultze *et al.*, 1992). The strain selected to produce antigen may be varied, depending on the requirements of diagnosis. The antigen for the HI test is prepared from IBV-laden allantoic fluids.

For HA and HI tests, procedures are carried out at 4°C.

### 2.2.1. Haemagglutination test

- i) Dispense 0.025 ml of PBS, pH 7.0–7.4, into each well of a plastic U or V-bottom microtitre plate.
- ii) Place 0.025 ml of virus antigen in the first well. For accurate determination of the HA content, this should be done from a close range of an initial series of dilutions, i.e. 1/3, 1/4, 1/5, 1/6, etc.
- iii) Make twofold dilutions of 0.025 ml volumes of the virus antigen across the plate.
- iv) Dispense a further 0.025 ml of PBS into each well.
- v) Dispense 0.025 ml of 1% (v/v) chicken RBCs to each well.
- vi) Mix by tapping the plate gently and allow the RBCs to settle for 40–60 minutes at 4°C, when control RBCs should be settled to a distinct button.
- vii) HA is more easily determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. The titration should be read to the highest dilution giving complete HA in which there is no sedimentation or streaming; this is 100% HA and represents 1 HA unit (HAU) and can be calculated accurately from the initial range of dilutions.

### 2.2.2. Haemagglutination-inhibition test

The HI test is used in the diagnosis and routine flock monitoring of vaccine responses.

- i) Dispense 0.025 ml of PBS into each well of a plastic U or V-bottom microtitre plate.
- ii) Place 0.025 ml of serum into the first well of the plate.
- iii) Make twofold dilutions of 0.025 ml volumes of the serum across the plate.
- iv) Add 4 HAU of virus antigen in 0.025 ml to each well and leave for 30 minutes.

- v) Add 0.025 ml of 1% (v/v) chicken RBCs to each well and, after gentle mixing, allow the RBCs to settle for 40–60 minutes when control RBCs should be settled to a distinct button.
- vi) The HI titre is the highest dilution of serum causing complete inhibition of 4 HAU of antigen. The agglutination is assessed more exactly by tilting the plates. Only those wells in which the RBCs 'stream' at the same rate as the control wells (containing 0.025 ml RBC and 0.05 ml PBS only) should be considered to show inhibition.
- vii) The validity of results should be assessed against a negative control serum, which should not give a titre  $>2^2$ , and a positive control serum, for which the titre should be within one dilution of the known titre.
- viii) Sera are usually regarded as positive if they have a titre of  $2^4$  or more. However, it should be noted that even in SPF flocks, a very small proportion of birds may show a nonspecific titre of  $2^4$ , but usually in birds over 1 year of age.

### 2.3. Enzyme-linked immunosorbent assay

The ELISA technique is a sensitive serological method and gives earlier reactions and higher antibody titres than other tests (Mockett & Darbyshire, 1981). It lacks type or strain specificity, but is valuable for monitoring vaccination responses under field conditions. Commercial kits for ELISAs are available – these are based on several different strategies for the detection of IBV antibodies. Usually, such tests have been evaluated and validated by the manufacturer, and it is therefore important that the instructions specified for their use be followed carefully. The ELISA is widely used to identify IBV-infected flocks (broilers) based on high antibody titres. If IB reoccurs in the next flock on the farm, virus isolation attempts are performed and the virus is genotyped by RFLP or S1 sequencing.

### 2.4. Agar gel immunodiffusion

AGID can be used in diagnosis (Witter, 1962). The antigen is prepared from a homogenate of the chorioallantoic membranes of infected chicken embryos. The Beaudette embryo-lethal strain is often employed to produce antigen. The test lacks sensitivity and is liable to yield inconsistent results as the presence and duration of precipitating antibodies may vary with individual birds.

## C. REQUIREMENTS FOR VACCINES

### 1. Background

All live and inactivated commercial vaccines must be licensed. Strains used in live virus vaccines generally require attenuation. At present, many countries only permit live vaccines of the Massachusetts type, such as the H120. Some countries may also have licensed vaccines to other live strains such as Connecticut, Arkansas, or Delaware 072 (USA) or the 4/91 strain (United Kingdom). Live vaccines may be given as aerosols, in the drinking water, or by the intraocular route (eyedrop).

The efficacy of inactivated vaccines depends heavily on proper priming with a live vaccine(s). Inactivated vaccines must be administered to birds individually, by intramuscular or subcutaneous injection. Variant strains may be used to prepare inactivated autogenous vaccines for controlling IB in layers and breeders, subject to local legislative requirements.

Live vaccines confer better local immunity in the respiratory tract and also may protect against a wider antigenic spectrum of field strains (Cook *et al.*, 1999). However, vaccination with live vaccines may not protect layer flocks against variant serotype challenge especially common on farms with flocks of multiple ages where production drops as early as 40 weeks of age are not uncommon (Gelb *et al.*, 1991). Live vaccines carry a risk of residual pathogenicity associated with vaccine back-passage in flocks. However, proper mass application techniques (e.g. spray or drinking water) can achieve uniform distribution of the vaccine in the flock and avoid back-passage. Furthermore, the use of vaccines at manufacturer's recommended dosages will also help avoid back-passage reversion that may be caused by fractional dose application.

There are prospects for genetically engineered vaccines (Armesto *et al.*, 2011; Casais *et al.*, 2003), and *in-ovo* vaccination (Tarpey *et al.*, 2006; Wakenell *et al.*, 1995).

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. National and international standards that apply in the country in which IB vaccines are manufactured must be complied with.



The licensing authority should provide information and guidance on requirements. These are now often presented in general terms, as applying to all vaccines – avian and mammalian, live and inactivated, or viral and bacterial vaccines. There may also be specific requirements applying to IB vaccines, live and inactivated. As examples, references are given to the European and USA regulations (Commission of the European Communities [Interim Publication Nov. 1993]; European Pharmacopoeia [2010a; 2010b]; USDA Code of Federal Regulations 113.327).

The list of extraneous agents that must be shown to be absent continues to grow. Manufacturers must be familiar with those that currently apply in their country. Recent additions are avian nephritis virus and avian pneumovirus.

For IB vaccines, important differences among countries may arise regarding the challenge virus to be used for potency tests, and its validation. Traditionally, the virulent M41 (Mass 41) strain of the Massachusetts type has been used for challenge tests of both live and inactivated vaccines. Although this type is still common, it is often not the only or the dominant type in many countries and it may be advisable to prepare vaccines from other types. It is logical for challenges to be made by the same type as present in the vaccine. Establishing criteria for validating the challenge virus may be more difficult for non-Massachusetts types, because of their lower virulence in general. Inactivated vaccines are usually expected to protect against drops in egg production. The traditional M-41 challenge should cause a drop of at least 67% in the unvaccinated controls, which was considered by some IB specialists as being excessive as too dependent on the chicken genetic line and on particular challenge parameters. When using other types much lower drops in egg production may be regarded as satisfactory, depending on published evidence of the effects of these strains in the field. It therefore seems necessary to relax the criteria for Massachusetts type challenges, and the European Pharmacopoeia now defines a satisfactory drop in egg production for Massachusetts types to be at least 35%, and for non-Massachusetts types to be at least 15%, provided that the drop is 'commensurate with the documented evidence' (European Pharmacopoeia).

## **2. Outline of production and minimum requirements for vaccines**

### **2.1. Characteristics of the seed**

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production* and Chapter 1.1.9 *Tests of biological materials for sterility and freedom from contamination*.

The seed-lot (master seed) system should be employed for whatever type of vaccine is produced. Each virus must be designated as to strain and origin and must be free from contamination with other strains of IBV and extraneous agents. Separate storage facilities should be provided between the strains of virus intended for vaccines or for challenge.

For live virus vaccines, many countries permit only strains of the Massachusetts type. Some countries allow other strains, usually on the basis that those strains are already present in their national flocks. The antigenic type incorporated in both live and inactivated vaccines requires justification if there is doubt as to its existence in a country.

#### **2.1.1. Biological characteristics of the master seed**

##### **i) Live vaccines**

Currently live IBV vaccines are normally attenuated by multiple repeat passage of a virulent virus in specific pathogen free (SPF) embryonated chicken eggs (Cavanagh, 2003). Spontaneous mutations arise throughout the IBV genome some of which lead to attenuation of the virus. However, as a consequence of this method the attenuated viruses produced by this approach have only a few mutations that are responsible for loss of virulence and these will differ between vaccine strains. Two major drawbacks of this method is that once the virus is used to inoculate chickens the mutations within the attenuated vaccine viruses may back-mutate resulting in virulent virus, an undesirable consequence, or that as a consequence of multiple passage the immunogenicity of the attenuated virus will not result in adequate protection. Given the nature of these live attenuated vaccines, further passage beyond the master seed stock must be kept to a minimum to prevent potential loss of immunogenicity. The stocks must be grown in SPF chicken eggs to prevent the introduction of other potential pathogens.

##### **ii) Inactivated vaccines**

IBV inactivated vaccines are also used to combat IBV usually in layer hens and used as a boost vaccination or to induce some protection against other IBV strains. Inactivated IBV vaccines have poor efficacy unless the chickens have previously been primed by vaccination with a live virus vaccine. The IBVs, for inactivated vaccines, are grown in non-

SPF eggs and are chemically inactivated usually by destroying the genomic RNA. Batches of inactivated vaccines must be tested for residual infectivity using embryonated eggs.

Every seed lot must be free from bacterial, fungal, mycoplasmal and viral contamination.

For the detection of extraneous viruses, the seed is first treated with a high-titred monospecific antiserum prepared against the strain under examination or against one of identical type. This mixture is cultured in a variety of ways, designed to confirm the absence of any viruses considered from past experience to be potential contaminants. The antiserum must not contain antibodies to adenovirus, avian encephalomyelitis virus, avian rotavirus, chicken anaemia virus, fowlpox virus, infectious laryngotracheitis virus, influenza A virus, Newcastle disease virus, infectious bursal disease virus, leukosis virus, reovirus, Marek's disease virus, turkey herpesvirus, adeno-associated virus, egg-drop syndrome 76 (EDS76) virus, avian nephritis virus, avian pneumovirus or reticulo-endotheliosis virus. The inoculum given to each unit of the culture system used should contain a quantity of the neutralised IBV component under test that had an initial infectivity of at least ten times the minimum field dose. These systems include:

1. SPF chicken embryos, incubated for 9–11 days, inoculated via both allantoic sac and chorioallantoic membrane (two passages);
2. Chicken embryo fibroblast cultures, for leukosis virus subgroups A and B. The COFAL test (test for avian leukosis using complement fixation) or double-antibody sandwich ELISA for group-specific leukosis antigen is performed on cell extracts harvested at 14 days. An immunofluorescence test for reticulo-endotheliosis virus is done on cover-slip cultures after two passages.
3. SPF chicken kidney cultures that are examined for CPEs, cell inclusions and haemadsorbing agents passaged at intervals of no fewer than 5 days for up to 20 days' total incubation.
4. SPF chickens of minimum vaccination age inoculated intramuscularly with 100 field doses, and on to the conjunctiva with ten field doses; this is repeated 3 weeks later when the chickens are also inoculated both into the foot pad and intranasally with ten field doses. Observations are made for 6 weeks overall, and serum is collected for tests for avian encephalomyelitis, infectious bursal disease, Marek's disease, Newcastle disease and *Salmonella pullorum* infection.

Vaccines intended to protect against loss of egg production should be tested for duration of antibody response. Mean HI titres should be  $>6 \log_2$  up to at least 60 weeks of age. Serological tests should be done at intervals frequent enough to show that titres have not been boosted by extraneous IBV infection.

Vaccines intended for protection of broiler chickens or rearing chickens against the respiratory form of the disease should be similarly tested for duration of antibody responses; in the case of broilers this would be up to the normal age for slaughtering, and in the case of pullets up to the age when a booster vaccination would be administered (often at 16–18 weeks of age).

### 2.1.2. Validation as a vaccine strain

Tests on seed virus should include a test for any potential ability to revert to virulence. Live and inactivated vaccine seed must be tested for safety as in Section C.2.2.4.

To demonstrate efficacy, a trial vaccine must be made from the master seed and the working seed at five passages from the master seed and subjected to tests that demonstrate their protective effect.

For live vaccines, a minimum of ten SPF chickens aged 3–4 weeks are vaccinated intranasally or by eyedrop with the recommended dose. Ten unvaccinated control birds from the same age and source are retained separately. All birds of both groups are challenge inoculated either intranasally or by eyedrop 3–4 weeks later, with  $10^{3.0}$ – $10^{3.5}$  EID<sub>50</sub> of reference challenge virus, representative of the vaccine virus antigenic group. A swab of the trachea is taken from each bird 4–5 days after challenge and placed in 3 ml of antibiotic broth. Each fluid is tested for IBV by the inoculation (0.2 ml) of five embryonated eggs, 9–11 days of age. An alternative test to that of taking swabs is to kill birds at 4–6 days after challenge and examine microscopically the tracheal rings for ciliary activity (Darbyshire, 1985). Failure to resist challenge is indicated by an extensive loss of ciliary motility. The live vaccine is suitable for use if at least 90% of the

challenge vaccinated birds show no evidence of IBV in their trachea, while 90% or more of the control birds should have evidence of the presence of the virus.

To assess an inactivated vaccine intended to protect laying birds, 30 or more SPF chickens are vaccinated as recommended at the earliest permitted age. If a primary vaccination with live vaccine is first undertaken, an additional group of birds is given only the primary vaccination. In both cases, these primary vaccinations should be done at no later than 3 weeks of age. The inactivated vaccine is given 4–6 weeks after the live priming vaccination. A further group of 30 control birds are left unvaccinated. All groups are housed separately until 4 weeks before peak egg production, and then are housed together. Individual egg production is monitored and once it is regular, all birds are challenged, egg production being recorded for a further 4 weeks. The challenge should be sufficient to ensure loss of production during the 3 weeks after challenge. The loss in the control group should be at least 35% where challenge has been made with a Massachusetts-type strain. Where it is necessary to carry out a challenge with a strain of another serotype for which there is documented evidence that the strain will not cause a 35% drop in egg production, the challenge must produce a drop in egg production commensurate with the documented evidence and in any case not less than 15%; the group that received primary live virus vaccine followed by inactivated vaccine should remain at the previous level, and the group given only a primary vaccination should show an intermediate drop in production. The vaccine complies with the test if egg production or quality is significantly better in the group having received the inactivated vaccine than in any control group. Sera are collected from all birds at vaccination, 4 weeks later, and at challenge; there should be no response in the control birds.

To assess an inactivated vaccine intended to protect birds against respiratory disease, 20 SPF chickens aged 4 weeks are vaccinated as recommended. An additional 20 control birds of the same age and origin are housed with this first group. Antibody responses are determined 4 weeks later; there should be no response in the control birds. All birds are then challenged with  $10^3$  CID<sub>50</sub> (50% chick infective dose) of virulent virus, killed 4–7 days later, and tracheal sections are examined for ciliary motility. At least 80% of the unvaccinated controls should display complete ciliostasis, whereas the tracheal cilia of a similar percentage of the vaccinated birds should remain unaffected.

Both live and inactivated vaccines containing Newcastle disease, infectious bursal disease, reovirus and EDS76 viruses are available in some countries. The efficacy of the different components of these vaccines must each be established independently and then as a combination in case interference between different antigens exists.

## **2.2. Method of manufacture**

### **2.2.1. Procedure**

All virus strains destined for live vaccines are cultured in the allantoic sac of SPF chicken embryos or in suitable cell cultures. For inactivated vaccines, hens' eggs from healthy non-SPF flocks may be used. The pooled fluid is clarified and then titrated for infectivity. For live vaccines this fluid is lyophilised in vials, and for inactivated vaccines it is blended with high-grade mineral oil to form an emulsion to which a preservative is added.

### **2.2.2. Requirements for ingredients**

See chapter 1.1.8 with special focus on products of biological origin (POBs) originating from a country with negligible risk for transmissible spongiform encephalopathies (TSEs).

### **2.2.3. In-process controls**

The required antigen content is based on initial test batches of vaccine of proven efficacy in laboratory and field trials. Infectivity titrations are done in chicken embryos.

Live vaccine should contain not less than  $10^{3.5}$  EID<sub>50</sub> per dose per bird until the expiry date indicated, and not less than  $10^{2.5}$  EID<sub>50</sub> per dose per bird after incubation at 37°C for 7 days at the time of issue. For inactivated vaccine, the inactivating agent and inactivation procedure must be shown under manufacture to be effective on both IBV and potential contaminants. With the use of beta-propiolactone or formalin, any live leukosis viruses and *Salmonella* species must be eliminated; and with other inactivating agents, the complete range of potential contaminants must be rendered ineffective. Before inactivation procedures, it is important to

ensure homogeneity of suspensions, and a test of inactivation should be conducted on each batch of both bulk harvest after inactivation and the final product.

Tests of inactivation should be appropriate to the vaccine concerned and should consist of two passages in cell cultures, embryos or chickens, using inoculations of 0.2 ml and ten replicates per passage.

#### **2.2.4. Final product batch tests**

##### **i) Sterility**

Every batch of live vaccine should be tested for the absence of extraneous agents as for the seed virus (see chapter 1.1.9).

##### **ii) Safety**

###### **a) For live attenuated vaccines**

Use no fewer than ten chickens from an SPF flock that are of the minimum age stated on the label for vaccination. Administer by eyedrop to each chicken ten doses of the vaccine reconstituted so as to obtain a concentration suitable for the test. Observe the chickens for 21 days. For vaccines intended for chickens that are 2 weeks old or more, use the chickens inoculated in the 'test for extraneous agents using chickens' (see Section C.2.1.1 point 4). If during the period of observation, more than two chickens die from causes not attributable to the vaccine, repeat the test. The vaccine complies with the test if no chicken shows serious clinical signs, in particular respiratory signs, and no chicken dies from causes attributable to the vaccine.

###### **b) For inactivated vaccines**

Inject a double dose of vaccine by the recommended route into each of ten 14–28-day-old chickens from an SPF flock. Observe the chickens for 21 days. Ascertain that no abnormal local or systemic reaction occurs.

##### **iii) Batch potency**

The potency test is developed from the results of efficacy tests on the master seed virus. Live vaccines are tested for potency by titration of infectivity, and inactivated vaccines by measuring antibody production. The potency test for a batch of inactivated vaccine consists of vaccinating 20 SPF chickens, 4 weeks of age, and showing that their mean HI titre 4 weeks later is not less than 6 log<sub>2</sub>.

Vaccine must be shown to have the required potency to achieve the claimed duration of immunity at the end of the claimed shelf life.

At least three batches should be tested for stability and must give satisfactory results for 3 months beyond the claimed shelf life. The stability of a live vaccine must be measured by maintenance of an adequate infectivity titre. The stability of an inactivated vaccine is measured at intervals by batch potency tests. The concentration of preservative and persistence through the shelf life should be assessed. There should be no physical change in the vaccine and it should regain its former emulsion state after one quick shake.

There are maximum level requirements for the use of antibiotics, preservatives and residual inactivating agents.

### **2.3. Requirements for authorisation/registration/licensing**

#### **2.3.1. Manufacturing process**

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Section C.2.1 and 2) 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

A safety test must be carried out on each batch of final product, as in Section C.2.2.4. A potency test must be carried out on each batch of final product, as in Section C.2.2.4, at manufacture and at the end of the stated shelf life.

- i) Target and non-target animal safety (depending of the different status of the animals: young, pregnant animals, etc.)
- ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations (dissemination and spread of live vaccines and their potential to cause problems for non-vaccinated animals)
- iii) Precautions (hazards)

IBV itself is not known to present any danger to staff employed in vaccine manufacture or testing. Extraneous agents may be harmful, however, and the initial stages of handling a new seed virus should be carried out in a safety cabinet. It is a wise precaution with all vaccine production to take steps to minimise exposure of staff to aerosols of foreign proteins. Persons allergic to egg materials must never be employed in this work. All Vaccines should be identified as harmless or pathogenic for vaccinators. Manufacturers should provide adequate warnings that medical advice should be sought in the case of self-injection (including for adjuvants, oil-emulsion vaccine, preservatives, etc.) with warnings included on the product label/leaflet so that the vaccinator is aware of any danger.

### 2.3.4. Efficacy requirements

To register a commercial vaccine, a batch or batches produced according to the standard method and containing the minimum amount of antigen or potency value shall prove its efficacy (protection); each future commercial batch shall be tested before release to ensure it has the same potency value demonstrated by the batch(es) used for the efficacy test(s).

Usually vaccine efficacy (protection) is estimated in vaccinated animals directly by evaluating their resistance to live pathogen challenge.

In the case of pathogens with several serotypes, vaccine efficacy should be established for each serotype.

The challenge models for determining efficacy are as out lined in Section C.2.2.2.

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