CHAPTER 3.3.2.

AVIAN INFECTIOUS BRONCHITIS

SUMMARY

Description and importance of the disease: *Avian infectious bronchitis* (*IB*) *is caused by the* Gammacoronavirus *infectious bronchitis virus* (*IBV*). *It has no zoonotic relevance. 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. Several strains of the virus are nephropathogenic and may 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 detection of the virus by virus isolation, antigen staining techniques 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 detection and serology findings. The occurrence of antigenic variant strains may overcome immunity induced by vaccination.*

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, or in a more chronic phase of the infection, kidney, oviduct or the caecal tonsils of the intestinal tract tissues may be better sources of virus depending on the pathogenesis of the disease. Reverse-transcriptase polymerase chain reaction* (*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 serotyping by haemagglutination inhibition* (*HI*) *or virus neutralisation* (*VN*) *tests. Supplementary tests include electron microscopy, the use of monoclonal antibodies, VN, immuno-histochemical or immunofluorescence tests, and immunisation–challenge trials in chickens. 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. Alternatively, VN or HI tests using specific antiserum may be used to identify the serotype.*

Serological tests: *Commercial enzyme-linked immunosorbent assays* (*ELISA*) *kits are often 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 and VN test testing cannot be used with a high degree of confidence for serotyping the infection.*

Requirements for vaccines: *Both live attenuated and oil emulsion inactivated vaccines are available. Live vaccines, usually attenuated by serial passage in chicken embryos, 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 significant protection unless preceded by one or more live IBV priming vaccinations.

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 (Britton & Cavanagh, 2007; Cavanagh *et al*., 2002); IBV-like viruses have been isolated from turkeys, pheasants and guinea fowl. 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 at a very young age can lead to permanent damage 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, especially in young birds. After apparent recovery, chronic nephritis can produce death at a later time. 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. For an in-depth review of IB, refer to Jackwood & de Wit, (2013). A detailed discussion of IBV antigen, genome and antibody detection assays prepared by de Wit (2000) is also available.

There have been no reports of human infection with IBV.

B. DIAGNOSTIC TECHNIQUES

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

Table 1. Test methods available for the diagnosis of infectious bronchitis virus and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent						
Virus isolation (embryos or TOCs)	+[a]	++[c]	–	+++	+	+[h]
Staining by immunohistochemistry	–	–	–	++	+	+[h]
Gene sequencing (virus identification)	–	–	–	–	++[f]	–
RT-PCR (detection of virus genome)	+[a]	++	++	++	+	+[h]

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Real-time RT-PCR	+[a]	++	++	++	+	+[h]
Haemagglutination test (virus identification)	–	–	–	–	+	–
VN (virus identification)	–	–	–	–	+	–
Detection of immune response						
VN (antibody detection)	–	–[d]	–	+[e]	–	+[e]
HIT (antibody detection)	–	–[d]	+	+[e]	+	++[e]
ELISA (antibody detection)	++[b]	++	++	++[e]	++[g]	++[e]

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
[a]Suitable for ensuring lack of infection during the past 10 days; [b]suitable for ensuring lack of infections dating back to more than 10 days; [c]suitable at the individual level only during excretion periods; [d]limited suitability for this purpose as it may be too specific of the serotype used as an antigen; [e]Suitable provided paired samples collected a few weeks apart can be analysed; [f]especially suitable for surveillance of a given or an emerging genotype; [g]especially suitable when IB surveillance is not focused on a given serotype; [h]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-transcriptase polymerase chain reaction; VN = virus neutralisation; HIT = haemagglutination inhibiton 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. For acute respiratory disease, swabs from the upper respiratory tract of live birds or tracheal and lung tissues from diseased birds should be harvested. For birds with nephritis or egg-production problems, samples from the kidneys or oviduct, respectively, should be collected in addition to respiratory specimens. Although virus isolation is a well established method, IBV identification by reverse-transcriptase polymerase chain reaction (RT-PCR) and subsequent sequencing or genotype-specific RT-PCRs will be the most used methods in the field. A positive RT-PCR result is not proof that IBV is present in the kidney cells and causing damage; it could also be caused by viraemia or contamination of the swabs by organs other than the airsac. In situations where IB-induced nephritis is suspected, kidney samples should also be selected from fresh carcases for histochemistry or immunofluorescense to demonstrate local replication. A high rate of virus recovery has been reported from the caecal tonsil or faeces. 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. Blood samples from acutely affected birds as well as convalescent chickens can also be submitted for serological testing.

1.2. Culture

Samples must be placed in cold transport media containing penicillin (10,000 International Units [IU]/ml) and streptomycin (10 mg/ml) and kept on ice and be frozen as soon as possible. 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 used to infect 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 produce 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 and detection

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, and sequence analysis, are used commonly to identify an isolate as IBV. The presence of IBV in infective allantoic fluid or TOCs is usually detected by RT-PCR amplification. Other techniques may be used as well, for example cells present in the chorioallantoic membranes or allantoic fluid of infected eggs or TOCs may be tested for IBV antigen using fluorescent antibody tests, immunohistochemistry or an antigen- enzyme-linked immunosorbent assays (ELISA) using a group-specific monoclonal antibody (MAb) or polyclonal antiserum.

1.4. Serotype identification

Antigenic variation among IBV strains is common (Jackwood & de Wit, 2013), but at present there is no agreed definitive classification system (Valastro *et al.,* 2016). 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) (and virus neutralisation (VN) tests in chick embryos, TOCs and cell cultures. MAbs have been described and used in antigen-ELISAs or immunofluorescence tests for typing of IBV strains, but the number of suitable

MAbs is very limited (de Wit, 2000). Owing to the number of variants and the complexity of serotyping, serotyping is hardly used anymore.

1.5. Genotype identification

Development of improved techniques for nucleotide sequencing, the availability of IBV sequence data, especially relating to the S gene, in computer databases and the demonstration that even relatively short sequence lengths can allow meaningful results in phylogenetic analyses have led to an increase in such studies and 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 where it is believed that the epitopes to which neutralising antibodies bind are found. A high correlation with HI or VN results has not been seen, while different serotypes generally have large differences (20–50%) in the deduced amino acid sequences of the S1 subunit, other viruses that are clearly distinguishable in neutralisation tests show only 2–3% differences in amino acid sequences.

The primary advantages of genotyping methods are a rapid turnaround time, and the ability to detect a variety of genotypes, depending on the primer sets used. Genetic diversity between IBV isolates has been confirmed, but viruses sharing temporal, geographical, antigenic or epidemiological parameters tend to fall into specific lineages and this has proven valuable in assessing both the global epidemiology and local spread of IBV (de Wit *et al.*, 2011; Jackwood, 2012; Valastro *et al.*, 2016). Sequence analysis of the S gene provides the required information for determining the level of genetic homology and the relationship of an IBV S gene to other IBV isolates including vaccine strains.

It has been shown that coronaviruses isolated from turkeys, pheasants and guinea fowl 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. This is the same for the gammacoronaviruses that have been detected in wild birds (Muradrasoli *et al.*, 2010)

The major uses of RT-PCR tests are virus identification at the genetic level 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. Reverse-transcriptase polymerase chain reaction (RT-PCR)

The wider availability and increased speed of production of results obtained using commercially available kits for RT-PCR and automatic sequencers now means such studies are within the capabilities of many more diagnostic laboratories, providing more accurate and meaningful results. Tracheal or oropharyngeal swabs are often used as the specimens of choice because they are easy to obtain and process. Such samples usually contain little extraneous organic material that can interfere with RNA recovery and amplification by PCR. The system used for RNA extraction will also affect the success of RT-PCR on clinical specimens, and even with commercial kits care should be taken in selecting the most appropriate system validated for the samples to be analysed. The usual target region for IBV characterisation is the S1 subunit of the S glycoprotein gene.

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. Most diagnostic laboratories determine the genotype of IBV isolates using partial sequence analysis, usually about 700 nt long, of the S1 region of the S gene. Given the diverse nature of the IBV S1 sequence, the actual region sequenced may vary. Such analyses allow rapid epidemiological assessment of the origins and spread of the viruses responsible for IBV outbreaks and the detection of vaccine strains within flocks. It should be noted that for more refined epidemiological studies and for more accurate studies on the evolution of IBV, the phylogenetic studies need to use complete S gene and even whole genome sequences.

1.5.2. Real-time RT-PCR analysis

Rapid confirmation of IBV can be determined using a strategy that avoids post-amplification processing (sequence analysis). The most common method for IBV is real-time RT-PCR. The real-time RT-PCR assay uses specific probes against the target sequence, the use of fluorogenic hydrolysis probes or fluorescent dyes eliminate the requirement of any post-amplification processing step and results can be obtained in less than 3 hours.

A generic IBV real-time PCR that targets a conserved region in the IBV genome (e.g. the 5' UTR) can be used for the detection of IBV in a clinical sample (Callison *et al.*, 2006). Genotype-specific real-time PCRs targeting the S1 gene for genotypes that are known to circulate in the region may be used in conjunction with this generic IBV PCR. Genotype-specific primers and probes have been published for a number of genotypes such as Massachusetts (primer sets XCE3-(CAG-ATT-GCT-TAC-AAC-CAC-C) and MCE1+ (AAT-ACT-ACT-TTT-ACG-TTA-CAC), 793B primer sets XCE3-(CAG-ATT-GCT-TAC-AAC-CAC-C) and BCE1+ (AGT-AGT-TTT-GTG-TAT-AAA-CCA), Arkansas and others (Cavanagh *et al.,* 1999; Roh *et al.,* 2014). However, due to the high variability between and on-going evolution within genotypes (Valastro *et al*., 2016), continual updating of the genotype-specific primers and probes is needed. When the genotype-specific RT-PCRs are negative and the generic RT-PCR is positive, additional sequencing may be needed to identify the strain involved and to adapt the primers and probes used in the genotype specific PCRs accordingly.

2. Serological tests

A number of tests have been described. Those considered here include VN, agar gel immunodiffusion (AGID), HI and ELISA. Each test has advantages and disadvantages in terms of practicality, specificity, sensitivity and cost. In general, ELISAs are most suitable for routine serological testing and can detect antibodies caused by vaccination and field exposure. ELISA can detect antibody responses to all serotypes. AGID also detects IBV antibodies against all types of IBV but lacks sensitivity and is liable to yield inconsistent results as the presence and duration of precipitating antibodies may vary with individual birds. VN and HI tests are more serotype specific, especially in young birds that have not been exposed to different types of IBV (de Wit, 2000). VN tests are too expensive and impractical for use in routine conditions. VN and 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 that have been in contact with vaccines and the field strain of several serotypes contain antibodies that are highly cross-reactive against antigenically unrelated strains, serodiagnosis of suspected disease outbreaks of IB at the serotype level cannot be used with a high degree of confidence as the detected antibodies against a certain serotype might be induced by infections of other types of IBV.

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 or cells. Two- or four-fold dilutions of antiserum are reacted in equal volumes with a dilution of virus, usually fixed at 100 or 200 EID_{50} (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_{50}. 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. 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_{50}). 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. Most strains and isolates of IBV will agglutinate chicken red blood cells (RBCs) after neuraminidase treatment (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 or a validated higher temperature, such as 20°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. 1/2, 1/3, 1/5, 1/7 and 1/9.

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. Because chicken sera from older birds that have been in contact with several types of IBV contain antibodies that can be highly cross-reactive against antigenically unrelated strains, a cut-off of the HI test of 2^4 will be too low.

2.3. Enzyme-linked immunosorbent assay

Commercial kits for ELISAs are widely available and used. These ELISAs use different cut-offs and mathematical formulas to convert the ELISA result into a titre. This means that every ELISA has its own interpretation and the titre results of different ELISAs on the same serum will differ.

C. REQUIREMENTS FOR VACCINES

1. Background

Strains used in live virus vaccines generally require attenuation. At present, a minority of countries only permits the use of attenuated live vaccines of the Massachusetts type, such as the H120 strain. Most countries also have licensed vaccines against other serotypes depending on the local situation and needs. Commonly used administration routes for live vaccines are spray (course spray or aerosol) or in the drinking water (oral route). Administration by eye-drop, if carefully done, is a very good method but in many cases too expensive.

The grouping of IBV strains that confer cross-protective immunity is the most important typing system from a practical point of view because it provides direct information about the efficacy of a vaccine (de Wit, 2000). The number of cross-protective groups that exists is unknown, but cross-challenge experiments in chicken tend to identify a smaller number of groups compared with serotypes and genotypes, presumably because they are measuring the complete immune response and not just a part of it. In general, there is a higher chance of a good level of cross-protection between strains with a high level of genetic homology in the S-gene than between strains with a low homology. However, the vaccination-challenge experiments have shown that the relationship is not very strong (de Wit *et al*., 2011). Therefore, a cross-immunisation study has to be performed to be able to determine the cross-protective immunity of a strain. Many factors can influence the outcome of such studies and should be accounted for (de Wit & Cook, 2014).

Live vaccines confer better local immunity in the respiratory tract and suitable combinations of vaccines of different serotypes also may protect against a wider antigenic spectrum of field strains (Cook *et al*., 1999; de Wit *et al*, 2013). Live vaccines carry a risk of residual pathogenicity associated with vaccine back-passage in flocks. However, proper application of vaccine can achieve uniform distribution of the vaccine in the flock and avoid back-passage. Furthermore, the use of vaccines at the manufacturer's recommended dosages will also help avoid back-passage reversion that may be caused by fractional dose application. For long-living birds, vaccination with live vaccines only during the rearing period will often not be sufficient to induce a long-lasting protection against challenge in the laying period. Boosting with inactivated vaccines of a good quality can be very efficient in increasing the level of protection against challenge in the laying period. 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.

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), but the progress for live vaccines is slow compared with other poultry diseases.

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 (European Pharmacopoeia [2017a; 2017b]; USDA, 2017).

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 (Mass) type has been used for challenge tests of both live and inactivated vaccines of the Mass serotype. Although this type is still common, it is not the only or the dominant type in any country and many countries allow the use of vaccines from other serotypes of IBV. Establishing criteria for validating the challenge virus may be more difficult for non-Massachusetts types, because of their varying virulence. Inactivated vaccines are often expected to protect against drops in egg production. The traditional M41 challenge should cause a drop of at least 67% in the unvaccinated controls, which was considered by many IB specialists as being excessive and also too dependent on the chicken genetic line and on particular challenge parameters. For other types of IBV, 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% in non-vaccinated birds, provided that the drop is 'commensurate with the documented evidence' (European Pharmacopoeia). However, under laboratory conditions, in many cases even a 35% drop in egg production by M41 is hard to achieve. It can be recommended that an egg drop of at least 15% in non-vaccinated birds should be the minimal drop produced by any IB challenge virus. Appropriate statistical analyses should also be included in any vaccination-challenge study. Inactivated and live vaccines may also claim protection against other clinical signs such as respiratory signs.

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 for sterility and freedom from contamination of biological materials for veterinary use*.

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. The master seed virus is the backbone of the vaccine. A few samples of the master seed are used to produce a working seed. This working seed is used for production batches. The master seed should be of such a size that it is sufficient for the life span of a product.

For live virus vaccines, a minority of countries permit only strains of the Massachusetts type. Many countries allow other strains as well, 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 may arise throughout the IBV genome some of which lead to attenuation of the virus, or minor populations present in the inoculum may be selected during passaging, leading to attenuation. 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 are that once the virus is used to inoculate chickens the mutations within the attenuated vaccine viruses may back-mutate or be re-selected 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. Therefore, it is recommended that the number of passages from the master seed to the working seeds is preferably not more than five. Results from efficacy studies using experimental batches at the highest passage claimed for production should be taken into account when setting the upper limit of passage allowed for the vaccine. To test the worst-case scenario, it is recommended to test the efficacy on the working seed that is used to produce the final product that will be used in the field. The master seed has to comply satisfactorily with the required tests for extraneous agents and safety. The working seeds must be grown in SPF chicken eggs to minimise the risk of introduction of potential pathogens.

ii) Inactivated vaccines

The IBV strains, for inactivated vaccines, are grown in SPF eggs, non-SPF eggs from healthy flocks (where allowed by the regulatory authority) or cell culture and are chemically inactivated usually by an agent that binds to and destroys the genomic RNA. Batches of inactivated virus suspension 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 reticuloendotheliosis 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 or other cells that are genetically susceptible for leukosis virus subgroups A, B, and J but not to endogenous avian leukosis virus. 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 reticuloendotheliosis 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.

2.1.2. Validation as a vaccine strain

The vaccine virus shall be shown to be satisfactory with respect to safety and efficacy for the chickens for which it is intended. Tests on vaccine 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.

Efficacy should be demonstrated using a batch vaccine at the highest passage level intended to be registered.

For live vaccines, a minimum of ten SPF chickens that are not older than the minimum age to be recommended for vaccination are vaccinated by the route intended for field use (e.g. intranasally or by eyedrop) at 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 or other time interval in line with the desired claim for onset or duration of immunity, with $10^{3.0}$–$10^{5.0}$ EID_{50} of reference challenge virus, the optimal challenge dose may depend on the challenge strain. 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. Failure to resist challenge is indicated by an extensive loss of ciliary motility. The live vaccine is suitable for use if at least 80% of the challenge vaccinated birds show no evidence of IBV in their trachea, while 80% 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 after the live priming vaccination according to the vaccination schedule to be recommended. 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 or in similar conditions. Individual egg production is monitored and once it is regular, all birds are challenged, egg production being recorded for a further 3–4 weeks. The challenge should be sufficient to ensure loss of production during the 3 weeks after challenge. The loss in the non-vaccinated control group should be at least 35% where challenge has been made with a Massachusetts-type strain unless justified. 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 not less than 15% unless justified; the group that received primary live virus vaccine followed by inactivated vaccine should not significantly drop in production compared with 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_{50} (50% chick infective dose) or other dose depending on the strain of virulent virus, killed 4–7 days later, and tracheal sections are examined for ciliary motility, or tracheal swabs evaluated for challenge virus recovery. 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. Tracheal swabs from at least 90% of the vaccinated birds should be negative for virus isolation, while tracheal swabs from at least 90% of the control birds should be positive for virus isolation.

Both live and inactivated multivalent vaccines containing other fractions such as Newcastle disease, infectious bursal disease, avian metapneumo-, reo- and EDS76 viruses are available. The efficacy of the different fractions of these vaccines should each be established; this should also be evaluated in the combination vaccine to assess possible interference between the different vaccine components.

2.2. Method of manufacture

2.2.1. Procedure

All virus strains destined for live attenuated 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 (where permitted by the regulatory authority) or suitable cell cultures may be used. The pooled fluid is clarified and then titrated for infectivity. For live vaccines this fluid is lyophilised or frozen in vials, tablets or other forms, and for inactivated vaccines it is blended with e.g. high-grade mineral oil or other suitable adjuvant to form an emulsion to which a preservative can be 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 have a titre not less than the minimum dose, which has been proven to be efficacious in the laboratory studies. For inactivated vaccines, which are produced on hens' eggs from healthy non-SPF flocks, validated methods must be in place to exclude the presence of potential contaminants in the eggs. Otherwise, the inactivating agent and inactivation procedure must be shown under manufacture not only to be effective on IBV, but also on other 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.

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). For vaccines administered via drinking water, spray or skin scarification one non-pathogenic micro-organism per dose is acceptable.

ii) Safety (target animal batch safety test)

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- to 28-day-old chickens from an SPF flock. Observe the chickens for 21 days. Ascertain that no abnormal local or systemic reaction occurs.

Safety tests in target animals are not required by many regulatory authorities for the release of each batch. Where required, standard procedures are generally conducted using fewer animals than are used in the safety tests required for the relevant regulatory approval.

iii) Batch potency

The potency test is developed from the results of efficacy tests on the furthest passage from the master seed virus. Live vaccines are tested for potency by titration of infectivity, and inactivated vaccines by measuring antibody production or alternative methods. An example of a potency test for inactivated vaccine consists of vaccinating 10 SPF chickens, at least 2 weeks of age, and showing that their mean HI titre 4 weeks later is not significantly less than those obtained for a batch that has shown satisfactory efficacy.

iv) Stability

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. Information shall be provided from three consecutive vaccine batches to demonstrate consistency of production.

2.3.2. Safety requirements

Additional testing required for live IB vaccines and precautions:

i) 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 and non-target animals in case of genetically modified vaccines). The vaccine complies when there is no indication of an increase in virulence of virus recovered from the final bird passage compared with the original vaccine that was used for the first passage. It is recommended to use at least five sequential passages over birds by natural spreading or eye-drop application of a suspension of fresh mucosal tissue. Validated alternative methods may be used as well.

ii) 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. 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.

2.3.3. 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); Efficacy should be demonstrated using a batch vaccine at the highest passage level intended to be registered. Each batch of live vaccine should contain sufficient live virus per dose per bird to last until the expiry date, indicated as the minimum dose that has been proven to be efficacious in laboratory studies.

Vaccine efficacy (protection) is estimated in vaccinated animals directly by evaluating their resistance to challenge. Vaccine efficacy should be established for each serotype of IBV against which protection is claimed.

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

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NB: At the time of publication (2018) there were no WOAH Reference Laboratories for avian infectious bronchitis (adenomatosis) (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2018.