

CHAPTER 2.3.3.

AVIAN INFECTIOUS LARYNGOTRACHEITIS

SUMMARY

Avian infectious laryngotracheitis (ILT) is a respiratory disease caused by gallid herpesvirus 1 an alphaherpesvirus. It is principally a disease of chickens, although it can also affect pheasants, partridges and peafowl. The clinical signs and observed pathology reactions may vary from extremely severe, with some birds dying from asphyxiation, to very mild, indistinguishable from other mild respiratory diseases of chickens. The principal lesion is tracheitis. In infected birds, the virus can become latent and re-excreted at a later date without clinical signs.

Laboratory diagnosis depends on isolation of the virus, demonstration of the presence of the virus or viral antigens, and detection of specific antibodies in the serum. Histopathological examination of the trachea for characteristic intranuclear inclusions and syncytial cell formations are of diagnostic value.

Identification of the agent: *Virus isolation may be done by inoculation of suspected material on to the dropped chorioallantoic membrane of embryonated hens' eggs, or into avian embryonic cell cultures. These methods are time-consuming but sensitive. Rapid methods include immunofluorescence on tracheal exudate or frozen sections, and an enzyme-linked immunosorbent assay (ELISA) to demonstrate viral antigen in mucosal scrapings. Polymerase chain reaction (PCR) methodology has been shown to be more sensitive than virus isolation for the examination of clinical material and is now widely used. Virus characterisation and differentiation of vaccine and wild-type viruses are possible using PCR followed by restriction fragment length polymorphism or sequencing.*

Serological tests: *Antibodies to ILT virus (ILTV) can be detected by virus neutralisation (VN) tests conducted in eggs or in cell cultures, or by AGID reactions, indirect immunofluorescence, or ELISA. The latter is preferred for screening flocks.*

Requirements for vaccines: *Vaccines against ILT are usually prepared from attenuated live virus. Those available at present afford some degree of protection, but are not ideal. A number of recombinant vaccines are also available commercially with various degrees of protective efficacy.*

A. INTRODUCTION

Avian infectious laryngotracheitis (ILT) is a respiratory disease of chickens caused by an alphaherpesvirus, *gallid herpesvirus 1*. It can also affect pheasants, partridges and peafowl. In the virulent form, the history, clinical signs and very severe tracheal lesions are highly characteristic of the disease, but the mild form may be indistinguishable from other mild respiratory diseases. Laboratory diagnosis depends on the demonstration of the presence of the virus or viral components (Guy & Bagust, 2003; Scholz *et al.*, 1994; Williams *et al.*, 1994) or specific antibodies in the serum (Adair *et al.*, 1985; Meulemans & Halen, 1978).

Clinically, the disease may appear in three forms, namely peracute, subacute, and chronic or mild. In the peracute form, onset of disease is sudden with a rapid spread. The morbidity is high and mortality may exceed 50%. Some birds may die in good body condition before the appearance of signs, which are characteristic and comprise difficulty in breathing with extension of the neck and gasping in an attempt to inhale. There is also gurgling, rattling and coughing when birds try to expel obstructions in the trachea. Conjunctivitis may also be observed. Clots of blood may be coughed up and can be found on the floor and walls of the house. Post-mortem changes are confined to the upper respiratory tract and are also characteristic, consisting of haemorrhagic tracheitis with blood clots, mucoid rhinitis, and blood-stained mucus along the length of the trachea.

In the subacute form, the onset of illness is slower and respiratory signs may extend over some days before deaths are seen. The morbidity is high but the mortality is lower than in the peracute form, between 10% and 30%. Post-mortem findings are less severe and consist of mucoid exudate with or without blood in the trachea. Yellow caseous diphtheritic membranes may be found adherent to the larynx and upper tracheal mucosa.

Chronic or mild ILT may be seen among survivors of either of the above forms of the disease, although some outbreaks themselves may be entirely mild. Incidence of chronic ILT within a flock may be only 1–2%, with most affected birds dying of suffocation. Signs include spasms of coughing and gasping, with nasal and oral discharge and reduced egg production. Infection is acquired via the upper respiratory tract and transmission occurs most readily from acutely infected birds, but clinically inapparent infection can persist for long periods with intermittent re-excretion of the virus, and these recovered carrier birds are also a potential means of transmission of the disease (Hughes *et al.*, 1987). On post-mortem examination, diphtheritic and caseous necrotic plaques and plugs are found in the trachea, larynx and mouth. Outbreaks of mild ILT may affect large numbers of birds simultaneously, in which case gross lesions may consist only of conjunctivitis, sinusitis and mucoid tracheitis. Given that transmission of ILT takes place by close contact, transmission is slower in cage houses than where birds are loose-housed, and the path of infection through a cage house may be apparent. Recent work has confirmed considerable variation among ILT virus (ILTV) strains in their tropism for trachea or conjunctiva and those with affinity for the latter site can severely affect weight gain (Kirkpatrick *et al.*, 2006a).

There is no known risk of human infection with ILTV. Biocontainment measures should be determined by risk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of avian infectious laryngotracheitis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribution to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent identification¹						
Virus isolation	–	–	–	++	–	–
Immuno-fluorescence for antigen	–	+	–	++	–	–
ELISA – antigen detection	+	++	+	+++	+	–
PCR	++	+++	++	+++	++	–
Histopathology	–	–	–	++	–	–
Detection of immune response						
VN	+	–	+	–	–	+
ELISA – antibody detection	++	+	+++	+	+++	+++

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.

ELISA = Enzyme-linked immunosorbent assay; PCR = polymerase chain reaction; VN = virus neutralisation.

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

1. Identification of the agent

The virus may be isolated in chick embryo liver (McNulty *et al.*, 1985), chicken embryo kidney (Chang *et al.*, 1960) or in chicken kidney (Van Kammen & Spadbrow, 1976) cell cultures. Of these, monolayers of chicken embryo liver cells have been found to be the most sensitive (Hughes & Jones, 1988). The virus can also be grown on the dropped chorioallantoic membrane (CAM) of 10- to 12-day-old specific pathogen free (SPF) embryonated chicken eggs (Jordan, 1964).

The causative herpesvirus may be demonstrated directly in tracheal exudate by electron microscopy (Van Kammen & Spadbrow, 1976). Viral antigens may be detected by immunofluorescence (Braune & Gentry, 1985; Wilks & Kogan, 1979), or enzyme-linked immunosorbent assay (ELISA), using tracheal mucosal scrapings (York & Fahey, 1988). Histopathological examination of the trachea for typical herpesvirus intranuclear inclusions and syncytial cell formation may also be helpful (Armstrong, 1959; Pirozok *et al.*, 1957). Methods of detecting ILTV using polymerase chain reaction (PCR) have been described and PCR has been reported to be generally more sensitive than virus isolation (Alexander & Nagy, 1997; Keam *et al.*, 1991; McNulty *et al.*, 1985; Williams *et al.*, 1994).

1.1. Virus isolation

When samples are taken from live birds for virus isolation, tracheal swabs are superior to oropharyngeal or conjunctival swabs. These are placed in transport medium containing antibiotics. When selecting material for virus isolation from chronic outbreaks, it is more productive to cull a bird in the early stages of the infection, rather than to attempt to isolate virus from a bird that has died of asphyxiation after a long illness. The quality of sample is further improved if the bird is killed by barbiturate or other injection rather than by cervical dislocation. The whole head and neck from dead birds may be submitted, or only the trachea and larynx after their removal with minimal contamination. Tracheas should be transported in antibiotic broth for virus isolation, but wrapped in moist tissue paper if destined for electron microscopy. Any prolonged storage of infected tissues should be at -70°C or below to minimise loss of virus titre. Repeated freezing and thawing must be avoided as this reduces virus infectivity.

Exudate and epithelial cells are scraped from the tracheas, diluted approximately 1/5 in nutrient broth containing penicillin and streptomycin, and agitated vigorously. The resulting suspension is centrifuged at low speed to remove debris, and 0.1 ml of the supernatant fluid is inoculated on to the dropped CAM of at least three embryonated chicken eggs of 10–12 days' incubation. The eggs are sealed with paraffin wax and incubated at 37°C for up to 7 days. They are candled daily and the CAMs of dead embryos or of those surviving for 7 days are examined for typical pocks. Alternatively, at least two confluent chick embryo liver or chicken embryo kidney cell monolayers, with their medium removed, are inoculated and allowed to adsorb for 1–2 hours. Cultures are overlaid with fresh medium, incubated for up to 7 days and examined daily under the microscope for evidence of a typical syncytial cell cytopathic effect (CPE).

In each instance, up to three passages of material may be necessary before a specimen is considered to be negative. A virus isolate can be confirmed as ILTV by a neutralisation test in eggs or cell culture using hyperimmune antiserum to ILTV. Alternatively, virus particles may be identified rapidly in cell culture fluid or in pocks on CAMs by electron microscopy, viral antigens by immunofluorescence in acetone-fixed ILT-virus-infected cell cultures or in frozen sections of CAM and viral nucleic acid by PCR.

1.2. Immunofluorescence

In immunofluorescence tests for viral antigens, epithelial cell scrapings from the trachea are smeared on to a glass slide. Alternatively, 5 μm thick cryostat sections of trachea, snap-frozen in liquid nitrogen may be used. The preparations are fixed in acetone at room temperature for 10 minutes. These can be stained directly by applying chicken anti-ILTV immunoglobulin labelled with fluorescein isothiocyanate (FITC) for 1 hour, followed by rinsing for 15 minutes in a bath of phosphate-buffered saline (PBS), pH 7.2, agitated with a magnetic stirrer. Otherwise, they can be stained indirectly by applying an appropriate dilution of chicken anti-ILT serum for 1 hour. The slide is rinsed thoroughly with PBS for 15 minutes as above, and an FITC-labelled anti-chicken immunoglobulin is applied for 30 minutes. After a final rinse, cover-slips are applied over non-fade mountant. The preparations are examined for specific intranuclear fluorescence in the epithelial cells using a microscope with epifluorescent ultraviolet illumination. Suitable controls include the use of known uninfected specimens and, for the indirect method, the application of nonimmune chicken serum. Particular care should be taken in the reading of indirect immunofluorescence preparations, as endogenous chicken IgG in the trachea may cause unwanted attachment of FITC-labelled anti-chicken IgG.

1.3. Enzyme-linked immunosorbent assay

A number of ELISA kits are available commercially, and the assay procedure may vary depending on the kit; the manufacturer's instructions should always be followed. When the monoclonal antibody (MAb) ELISA is used for detecting viral antigens (McNulty *et al.*, 1985), tracheal exudate is mixed with an equal volume of PBS containing 1% (v/v) of a detergent, such as Nonidet P40 (BDH Chemicals, Poole, United Kingdom), then vortexed for 30 seconds and centrifuged at 10 *g* for 1 minute. The supernatant fluid is dropped in 50 µl volumes in wells of microtitre plates previously coated with rabbit IgG against ILTV, diluted 1/200 in 0.05 M carbonate/bicarbonate buffer, pH 9.0, and incubated for 1 hour. Next, 50 µl of MAb against major glycoproteins of ILTV, diluted 1/50 in PBS, is added to each well, followed by 50 µl of a 1/1000 dilution of affinity-purified goat anti-mouse IgG conjugated to horseradish peroxidase. The substrate, 5-aminosalicylic acid (6.5 mM), is added to the wells in 100 µl volumes. After 30 minutes, the plates are read at 450 nm on a spectrophotometer and the absorbance reading for each well is corrected by subtracting the reading obtained for wells containing diluting buffer instead of tracheal exudate. The positive/negative cut-off point is taken as the mean absorbance value for several negative (i.e. tracheal material without ILTV) samples plus 3 standard deviations.

1.4. Histopathology

Birds selected for post mortem examination should be in the acute phase of disease. Euthanasia should be by intravenous injection of barbiturates or exposure to halothane, to avoid damaging the trachea. Tracheas for histopathological examination should be placed in 10% neutral buffered formalin or Bouin fixative (preferable for detection of intranuclear inclusion bodies) immediately after removal from the birds and, after fixation, embedded in paraffin wax. Eyelid and lung are sometimes examined. Intranuclear inclusions may be seen in the epithelial cells of the trachea after staining with haematoxylin and eosin. Syncytial cell formations are often present within the exudates and commonly contain intranuclear inclusion bodies. Inclusion bodies are the classical Cowdry type A inclusions of herpesviruses, but they may be present for only 3–5 days after infection. In severe cases where most infected cells have detached from the tracheal lining, inclusions may be seen in intact cells among the cellular debris in the lumen of the trachea. Longitudinal rather than transverse sections of trachea permit examination of the whole length of the organ.

1.5. Molecular methods

Several molecular methods for identifying ILTV DNA in clinical samples have been reported, but the PCR has proved the most useful. Dot-blot hybridisation assays and cloned virus DNA fragments were shown to be highly sensitive for detecting virus when isolation and ELISA were negative (Keam *et al.*, 1991; Key *et al.*, 1994). Humberd *et al.* (2002), using a nested PCR, showed that ILTV DNA could be detected in formalin-fixed, paraffin-embedded tissues independently of the presence of syncytial cells, intranuclear inclusions or both.

PCR has been found to be more sensitive than virus isolation for clinical samples, especially when other contaminant viruses such as adenoviruses are present (Williams *et al.*, 1994). Alexander & Nagy (1997) found that during the middle to the end of the infection phase, PCR and virus isolation were similar in sensitivity, but PCR was superior in the recovery phase.

The combination of PCR with restriction fragment length polymorphism (RFLP) analysis of single and multiple viral genes and genome regions has enabled the characterisation of different strains within a country or region (Chang *et al.*, 1997). Several reports have shown that while some field strains are closely related to and likely to be derived from vaccine viruses, others are true 'wild types' (Ojkic *et al.*, 2006). Genes commonly examined by different authors include ICP4, TK (thymidine kinase), glycoprotein G (gG), glycoprotein E (gE) and UL47. Oldoni & Garcia (2007) used 36 restriction enzymes, while others have used as few as four. There are as yet no universally accepted molecular tests that enable differentiation between field and vaccine strains. A good history of vaccination in the flock may assist with interpretation of results although it should be noted that field strain infection can occur in vaccinated birds. Vaccine strains can also occasionally be isolated from unvaccinated birds,

1.5.1. Test procedures

i) Conventional PCR

In a typical PCR protocol for ILTV, viral DNA is extracted from clinical samples (swabs, tissues), chorioallantoic membrane plaques, cell culture supernatants or vaccines using DNA extraction kits. Primers used can be obtained from previously published work or designed using ILTV sequences on the Genbank international database.

The following protocol is routinely used in a number of veterinary diagnostic laboratories for detection of ILTV in clinical specimens and has potential for the preliminary typing of the virus by restriction fragment length polymorphism of the PCR amplicons. Full details of this PCR are provided in Kirkpatrick *et al.* (2006b). In brief, an approximately 2.24 kbp region of the ILTV thymidine kinase gene is amplified using a pair of forward (5'-CTG-GGC-TAA-ATC-ATC-CAA-GAC-ATC-A-3') and reverse (5'-GCT-CTC-TCG-AGT-AAG-AAT-GAG-TAC-A-3') primers and the resultant amplicons are separated by electrophoresis through 0.8% agarose gels, stained with an appropriate nucleic acid stain and exposed to UV light for visualisation. The 50 µl amplification reaction contains 200 µM each of dATP, dCTP, dGTP and dTTP, 1 mM MgCl₂, 250 µM each of the primers, 1 µl (2.5 units) Taq DNA polymerase, 5 µl of 10 × Taq DNA polymerase buffer and 5 µl extracted DNA as template. The reaction mixture should be incubated at 94°C for 3 minutes, then subjected to 35 cycles of 94°C for 15 seconds, 60°C for 45 seconds and 72°C for 150 seconds, and finally incubated at 72°C for 3 minutes. In each series of PCRs, a control tube containing sterile distilled H₂O, instead of extracted DNA, should be included as a negative control.

Another protocol that has been used in veterinary diagnostic laboratories around the world for the detection and preliminary typing of the virus involves the amplification of two regions of the ICP4 gene. Primers ICP4-1F (5'-ACT-GAT-AGC-TTT-TCG-TAC-AGC-ACG-3') and ICP4-1R (5'-CAT-CGG-GAC-ATT-CTC-CAG-GTA-GCA-3') amplify a 688 bp fragment at position 181 to 869²; ICP4-2F (5'-CTT-CAG-ACT-CCA-GCT-CAT-CTG-3') and ICP4-2R (5'-AGT-CAT-GCG-TCT-ATG-GCG-TTG-AC-3') amplify a 635 bp fragment at position 3804 to 4440³. Full details of these PCRs are provided by Chacon & Ferreira (2009).

A real-time PCR protocol described by Mahmoudian *et al.* (2011) can also be used as a conventional PCR and the final products can be examined by electrophoresis through 2% agarose gels.

ii) Real-time PCR

Real-time PCR assays have been described for ILTV (Creelan *et al.*, 2006; Mahmoudian *et al.*, 2011). These have the advantage that they can be conducted in less than 2 hours and therefore provide a very rapid method of ILT diagnosis in comparison with traditional virus isolation, or even the standard PCR followed by gel electrophoresis. The following protocol can be used for rapid detection of ILTV in clinical specimens. Full details of the methodology are provided in Callison *et al.* (2007). In brief, two oligonucleotide primers, ILTVgCU771 (5'-CCT-TGC-GTT-TGA-ATT-TTT-CTG-T-3') and ILTVgCL873 (5'-TTC-GTG-GGT-TAG-AGG-TCT-GT-3'), are used to amplify a 103-bp product from the ILTV gC gene. A Taqman dual-labelled probe, ILTV probe817 (5'-FAM-CAG-CTC-GGT-GAC-CCC-ATT-CTA-BHQ1-3'), is also used. The reaction is conducted with appropriate PCR kits, for example, using 25 µl total volume containing 10 µl of the 2x master mix, 0.5 µM of forward and reverse primers, 0.1 µM of the probe, 1 µl of HK-UNG, and 5 µl of DNA template. Incubation and data acquisition are performed using a real-time thermocycler although PCR protocols will need to be optimised for the machine used. Reactions are incubated for 2 minutes at 50°C, 15 minutes at 95°C, then 40 cycles of 15 seconds at 94°C and 1 minute at 60°C. For each assay, the threshold cycle number (CT value) is the PCR cycle number at which the fluorescence of the reaction exceeds 30 units of fluorescence.

iii) Nucleotide sequence analysis

A number of ILTV genes can be targeted for PCR followed by nucleotide sequence analysis of the resultant amplicons for strain identification purposes. For example, ICP4 may be amplified by PCR using the primers described by Chacon & Ferreira, (2009) and the resultant amplicons purified using a disposable mini column method and submitted to bi-directional DNA sequencing using the PCR primers as sequencing primers. Various software programs including clustal W may be used for analysis of the sequences and comparison with the existing sequences in GenBank. It should be noted that sequence analysis of multiple genes may be required for proper identification of the ILTV strains.

2 Genbank accession number NC 006623
3 Genbank accession number NC 006623

iv) Restriction fragment length polymorphism (RFLP)

A range of restriction endonucleases (RE) have been described for RFLP analysis of ILTV PCR products and several genes have been targeted for digestion. They include ICP4, TK, UL15, UL47 glycoprotein G and ORF-BTK genes. Detailed description of the methodology can be found in Creelan *et al.* (2006), Han & Sim (2001), Kirkpatrick *et al.* (2006a), Ojkic *et al.*, (2006) and Oldoni & Garcia (2007). A brief description of methodology for TK, ICP4, ICP18.5 and ORFB-TK PCR RFLP is provided below from Kirkpatrick *et al.* (2006a) with some modifications.

For amplification of TK, PCR is performed in a 50- μ l reaction mixture containing 200 μ M each dATP, dCTP, dGTP, and dTTP, 1 mM MgCl₂, 250 μ M of each primer (TK forward: CTG-GGC-TAA-ATC-ATC-CAA-GAC-ATC-A; and TK reverse: GCT-CTC-TCG-AGT-AAG-AAT-GAG-TAC-A), 1.25 U of Taq DNA polymerase (Promega), 5 μ l of 10 \times Taq DNA polymerase buffer, and 2 μ l of extracted DNA. The reaction mixture is incubated at 94°C for 3 minutes, then subjected to 35 cycles of 94°C for 15 seconds, 60°C for 45 seconds and 72°C for 60 seconds, and finally incubated at 72°C for 3 minutes.

PCR amplification of ICP4, ICP18.5 and ORFB-TK, is performed in separate tubes using primers ICP4 forward: AAA-CCT-GTA-GAG-ACA-GTA-CCG-TGA-C and ICP4 reverse: ATT-ACT-ACG-TGA-CCT-ACA-TTG-AGC-C; ICP18.5 forward: TCG-CTT-GCA-AGG-TCT-TCT-GAT-GG and ICP18.5 reverse: AGA-AGA-TGT-TAA-TTC-ACA-CGG-ACA-C; and ORFB-TK forward: TCT-GCG-ATC-TTC-GCA-GTG-GTC-AG and ORFB-TK reverse: TGA-CGA-GGA-GAG-CGA-ACT-TTA-ATC-C. A 50- μ l reaction mixture contains 200 μ M each dATP, dCTP, dGTP, and dTTP, 2 mM MgSO₄, 250 μ M each primer, 1 U of Platinum Taq DNA Polymerase high fidelity (Invitrogen), 5 μ l of 10 \times Platinum Taq DNA polymerase buffer, and 2 μ l of extracted DNA. The reaction mixture is incubated at 94°C for 1 minute, then subjected to 35 cycles of 94°C for 1 minute, 68°C for 7 minutes, and finally incubated at 68°C for 10 minutes.

10- μ l volumes of TK, ICP4, ICP18.5 and ORFB-TK PCR products are digested separately with the restriction endonucleases MspI, HaeIII, HaeIII, and FokI respectively, at 37°C for 1 hour. After digestion, the resultant DNA fragments are separated in a 15% polyacrylamide gel and restriction DNA fragments visualised by an appropriate nucleic acid stain and exposed to UV light for visualisation. Pattern differences are recorded for each enzyme and results can be developed into dendrograms. The combination of PCR and RFLP has enabled field strains of ILTV to be distinguished from vaccine strains (Creelan *et al.*, 2006; Han & Sim, 2001; Kirkpatrick *et al.*, 2006a; Ojkic *et al.*, 2006; Oldoni & Garcia 2007).

2. Serological tests

The method of choice for detection of antibodies to ILTV in chicken serum is ELISA. Virus neutralisation (VN) can also be used (Adair *et al.*, 1985). AGID and indirect immunofluorescence tests are little used nowadays.

2.1. Enzyme-linked immunosorbent assay

A number of ELISA kits are available commercially, and the assay procedure may vary depending on the kit; the manufacturer's instructions should always be followed. The antigen for ELISA is obtained by sonication of heavily infected cell cultures at the time of maximum CPE, which is then centrifuged and the supernatant absorbed on to the wells of microtitre plates. A negative antigen is provided by uninfected cell culture material treated in the same way. The test consists essentially of the addition of 0.1 ml of 1/10 dilutions of test sera to duplicate wells coated with positive or negative antigen. After incubation at 37°C for 2 hours, the plates are washed four times and a 1/4000 dilution of a rabbit anti-chicken IgG conjugated with peroxidase is added. After incubation at 37°C for 1 hour, the plates are washed again four times. Finally a substrate consisting of 5-aminosalicylic acid is added to each well followed by hydrogen peroxide to a final concentration of 0.0005%, and the absorbance of the fluid in each well is read at 450 nm on a spectrophotometer. The result for each serum is expressed as the difference between the mean absorbance produced with the positive and negative antigens. The positive/negative cut-off point is taken as the mean absorbance value for numerous negative sera plus 3 standard deviations. The test is very sensitive and possibly the best available for surveillance purposes. Antibody responses as measured by ELISA are detectable 7–10 days after infection and peak at about 2 weeks. The response to ILT vaccines may be variable and testing is not worthwhile before 14 days post-vaccination.

2.2. Virus neutralisation

VN tests may be conducted on the dropped CAMs of embryonating chicken eggs that have been incubated for 9–11 days, where antibody specifically neutralises pock formation caused by ILTV. Alternatively, the tests can be performed in cell cultures, where antibody specifically neutralises the ILTV thus preventing CPE. Doubling dilutions of serum are added to equal volumes of a constant concentration of virus. This concentration may either be 100 median egg infectious doses (EID₅₀) for egg inoculations, or 100 median tissue culture infectious doses (TCID₅₀) for the inoculation of cultures. The mixtures are incubated at 37°C for 1 hour to allow any neutralisation to occur.

When the test is performed in eggs, the virus/serum mixtures are inoculated on to the dropped CAMs, using at least five eggs per dilution. Eggs are sealed and incubated at 37°C for 6–7 days. The end-point is recorded as the highest dilution of the serum where no pocks are present on the CAMs. When the tests are done in cell cultures, serum dilutions are prepared in 96-well microculture plates and virus is then added. After the period allowed for neutralisation, freshly prepared chicken embryo liver or kidney cells are added to each well. The plates are incubated at 37°C in an atmosphere of 5% CO₂ and examined daily for CPE; 50% end-points are read after approximately 4 days when the virus control titre indicates that 30–300 TCID₅₀ of virus have been used in the test. For the cell culture method of testing, virus neutralisation at 1/8 (initial dilution) or greater is considered positive.

C. REQUIREMENTS FOR VACCINES

1. Background

ILT is usually controlled with live vaccines. Vaccines may be used in response to disease outbreaks, or may be used routinely in endemic areas. Repeated doses may be required to afford good protection. Live attenuated ILT vaccines produced in cell cultures or embryonated hens' eggs are most commonly used. In recent years, live recombinant (vectored) vaccines have been produced using herpesvirus of turkeys or fowlpox virus to express ILTV glycoproteins. There has also been some recent work with genetically engineered deletion-mutant vaccines and the results of these initial studies look promising (Coppo *et al.*, 2013). For attenuated ILT vaccines, the live virus seed is a suitably attenuated or naturally avirulent strain of ILTV. Live attenuated ILT vaccines may be administered by eyedrop, spray or in the drinking water. Recombinant vaccines may be delivered by wing-web puncture, subcutaneous injection or *in-ovo* inoculation. There are advantages and disadvantages associated with each of the different types of vaccine and the different methods of delivery (Coppo *et al.*, 2013). For example, vectored vaccines may only be partially protective and live attenuated ILT vaccines may have residual virulence that can cause clinical disease, especially if administered by spray and a small droplet size is produced and inhaled. Live attenuated ILT vaccines may also revert to higher levels of virulence following bird-to-bird passage and persist in the field (Coppo *et al.*, 2013). For this reason, it may be difficult to discontinue vaccination once it has been started. Subclinical mixed infections of vaccine and field virus, in vaccinated birds, can cause severe disease in unvaccinated in-contacts. Natural recombination between attenuated vaccine strains of ILTV to produce virulent viruses has been reported and is also a risk for other live vaccines. The use of multiple different ILTV vaccines in the same populations should therefore be avoided (Coppo *et al.*, 2013).

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements (e.g. Code of Federal Regulations of the United States of America [USA], 2000; European Pharmacopoeia, 2010).

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics of the master seed

The virus seed for attenuated live ILT vaccines is a suitably attenuated or naturally avirulent strain of ILTV. Attenuation is achieved by serial passage of virus in embryonated eggs or tissue cultures. Recombinant vectored vaccines are created using recombinant DNA methodologies. The master seed virus (MSV) can be propagated in SPF chicken embryos or tissue cultures derived from such embryos. Initial tests are performed to demonstrate the safety and efficacy of the chosen master seed and the master seed must be tested for purity, virus identity and the presence of extraneous pathogens. The MSV is stored in aliquots at –70°C. The viruses used in manufacture should be no more than five passages from the MSV.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Details on the origin of the virus seed, including passage history and any genetic manipulations should be recorded. The MSV is tested in chicken embryos or chickens for sterility and freedom from extraneous agents (see chapter 1.1.9). Virus content is determined by virus titration in chicken embryos or cell culture.

2.1.3. Validation as a vaccine strain

ILT vaccines cross-protect against different strains of ILTV. Infection of non-target species with vaccine strains of ILTV is not a major concern because of the narrow host range of ILTV.

2.2. Method of manufacture

2.2.1. Procedure

In large-scale vaccine production, the virus is propagated in SPF chicken embryos or tissue culture derived from such embryos, up to the fifth passage from the MSV. The acceptable passage level is supported experimentally by the passage level used to prepare the experimental product used in the efficacy study. The vaccine is made by inoculation of the production seed virus into 9- to 11-day-old chicken embryos or tissue culture prepared from chicken embryos derived from SPF flocks. Eggs are inoculated through a hole in the shell, on to the dropped CAM or into the allantoic sac. They are sealed and incubated at 37°C for 4–6 days. All eggs are candled before harvest and only those with living embryos are used. To harvest the virus, the eggs are chilled, then cleansed and opened aseptically. The CAMs or allantoic fluids are pooled in sterile, cooled containers. The CAMs should show the thick grey plaques typical of ILTV growth. Tissue culture-derived product would be prepared from virus-bearing cell culture fluids, which would also be subsequently pooled and tested.

2.2.2. Requirements for ingredients

Bovine serum used in vaccine production should be sourced from a country with negligible risk for transmissible spongiform encephalopathies [TSEs]. The origin of animal pancreatic trypsin used for cell culture should be considered. See chapter 1.1.8 with a special focus on products of biological origin originating from a country with negligible risk for TSEs.

2.2.3. In-process controls

The infected tissue or tissue culture homogenate may be tested for purity, potency, virus content and safety.

2.2.4. Final product batch tests

i) Sterility

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

ii) Identity

The presence of ILTV may be confirmed by mixing the vaccine with ILTV antiserum and demonstrating that it is no longer able to infect embryonated hens' eggs from an SPF flock, or susceptible cell lines into which it is inoculated.

iii) Safety

Each batch of vaccine is delivered by eyedrop (10 doses per bird) or by intratracheal injection to SPF chickens, or other target species. The birds are observed for 14–21 days for adverse effects attributable to the vaccine.

iv) Batch potency

Once the *in-vivo* efficacy of the vaccine has been established, the batch potency may be determined by measuring the virus content. This is done by virus titration using serial dilutions of vaccine inoculated onto the dropped CAM of embryonated hens' eggs, or inoculation of suitable cell cultures. The virus content (tested at any time within the expiration period) should be at or above the minimum titre stated on the label. The release and expiration titres are based on the minimum protective dose of the vaccine. Although efficacy testing of each vaccine batch may not be required, efficacy testing of a

representative batch (using a vaccinating dose containing not more than the minimum virus titre stated on the label) may be needed.

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 Sections C.2.1 and C.2.2) 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

In the USA, 25 susceptible chickens, 3- to 4-weeks old, are injected intratracheally and observed for 14 days. Deaths are counted as failures. Four or fewer failures are allowed for satisfactory serials. In the European Union (EU), initial safety tests use the vaccine virus at the least attenuated passage level between the MSV and a batch of the vaccine. Twenty SPF chickens are inoculated with 10 times the vaccine dose and observed for 21 days. Each route and method of administration that is recommended for vaccination should be tested in chickens at the youngest age recommended for vaccination. No notable clinical signs or deaths attributable to ILT are allowed. Batch tests for safety in the EU require eye-drop inoculation of 10 SPF chickens, at the youngest age recommended for vaccination, with 10 times the vaccine dose. Chickens are observed for 21 days. No notable clinical signs or deaths attributable to ILT are allowed.

i) Reversion to virulence

The EU requires five *in-vivo* sequential passages of vaccine virus. Each passage is performed in groups of five SPF chickens. Sequential passage may be achieved by natural spreading, or by collecting and pooling infectious material from the respiratory tract of one group of birds and using this material to infect the subsequent group of birds via eye-drop inoculation. Passaged virus recovered after five sequential passages (or earlier if passage cannot be maintained) is compared with the unpassaged virus using an index of respiratory virulence test. The maximally passaged virus must show no increase in virulence. The index of respiratory virulence tests involves intratracheal inoculation of 0.2 ml of vaccine at three different doses, starting with stock that has titre of 10^5 EID₅₀ or 10^5 CCID₅₀ per 0.2 ml (or maximal obtainable titre) and then two serial tenfold dilutions of that stock. At least 20 SPF chickens are inoculated per dose. Chickens are observed for 10 days and deaths are recorded. The index of respiratory virulence is the total number of deaths observed in the three groups divided by the total number of chickens

ii) Precautions (hazards)

Care should be taken over diluting and administering the vaccine, and over the proper disposal of unused vaccine.

2.3.3. Efficacy requirements

A test must be carried out to establish the efficacy of the vaccine in groups of birds of the minimum age for which the product is destined and also for each avian species. Each route and method of administration recommended for vaccination must be tested. Twenty (USA) or 30 (EU) chickens are vaccinated with the most attenuated passage level of vaccine virus. Ten additional chickens are held as controls. After 10–14 days (USA) or 21 days (EU) vaccinated chickens and controls are challenged with virulent ILTV intratracheally or in the orbital sinus. At least 80% (USA) or 90% (EU) of unvaccinated controls must die or show severe clinical signs of ILT, or show notable macroscopic lesions. To be satisfactory, only 5% or 10% of the vaccinated birds should die or show severe clinical signs of ILT, or show notable macroscopic lesions.

2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

The advent of recombinant (vectored) ILT vaccines has brought the potential to differentiate serologically between infected and vaccinated birds and the potential to use DIVA control strategies. Many of the gene-deleted ILT vaccines that are currently under development offer the same potential. A number of studies have reported the use of serological screening tools to detect antibodies against specific ILTV glycoproteins (Coppo *et al.*, 2013).

2.3.5. Duration of immunity

The results of vaccination will depend on many factors, including dose schedule and route of administration. Some degree of protection should be given, over a period of several months.

2.3.6. Stability

Stability is tested by taking samples of correctly stored vaccine at intervals and measuring virus content. Tests should be carried out on at least six batches of the vaccine or until a statistically valid number of serials have been evaluated and be continued for 3 months after the claimed shelf-life.

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