AVIAN INFECTIONOUS LARYNGOTRACHEITIS

SUMMARY

Avian infectious laryngotracheitis (ILT) is a respiratory disease caused by Herpesviridae alphaherpesvirinae gallid herpesvirus 1. It is principally a disease of chickens, although it can also affect pheasants, partridges and peafowl. The clinical signs and pathological reactions may vary from extremely severe, with some birds dying due to 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 may be of value.

Identification of the agent: Virus isolation may be done by inoculation of suspected material on to the dropped chorioallantoic membrane of embryonated eggs, or into avian embryonic cell cultures. These methods are time-consuming but sensitive. Rapid methods include direct electron microscopy on tracheal exudate, immunofluorescence on tracheal exudate or frozen sections, agar gel immunodiffusion (AGID) to detect viral antigens in tracheal samples or infected egg material, 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.

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 and diagnostic biologicals: Vaccines against ILT are usually prepared from attenuated live virus. Those available at present afford some degree of protection, but are not completely satisfactory. There have been recent promising studies on the efficacy of a genetically engineered vaccine.

A. INTRODUCTION

Avian infectious laryngotracheitis (ILT) is a respiratory disease of chickens caused by an alphaherpesvirus. 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 antigens or products (9, 24, 29) or specific antibodies in the serum (1, 20).

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. 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 (12). 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 ILTV strains in their tropism for trachea or conjunctiva and those with affinity for the latter site can severely affect weight gain (18).

**B. DIAGNOSTIC TECHNIQUES**

1. **Identification of the agent**

The virus may be isolated in chick embryo liver (19), chicken embryo kidney (6) or in chicken kidney (26) cell cultures. Of these, monolayers of chicken embryo liver cells have been found to be the most sensitive (11). The virus can also be grown on the dropped chorioallantoic membrane (CAM) of 10–12-day-old specific pathogen free embryonated chicken eggs (14).

The causative herpesvirus may be demonstrated directly in tracheal exudate by electron microscopy (26). Viral antigens may be detected by immunofluorescence (4, 28), agar gel immunodiffusion (AGID) (15), or enzyme-linked immunosorbent assay (ELISA), using tracheal mucosal scrapings (30). Histopathological examination of the trachea for typical herpesvirus intranuclear inclusions may also be helpful (3, 23). Methods of detecting ILTV (ILTV) using polymerase chain reaction (PCR) have been described and PCR has been reported to be generally more sensitive than virus isolation (2, 16, 19, 29).

a) **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.

b) **Electron microscopy**
To demonstrate the presence of virus by electron microscopy, tracheal exudate or epithelial scrapings from the trachea are smeared on to a microscope slide and mixed with a few drops of distilled water. One drop of suspension is placed on a carbon and formvar-coated grid and left for 2 minutes, after which excess moisture is removed using filter paper. One drop of 4% phosphotungstic acid, pH 6.4, is added and the excess removed after a further 3 minutes. The grid is allowed to dry thoroughly and examined using the electron microscope at a magnification of ×30–45,000 for typical herpesvirus particles, measuring 100 nm diameter with icosahedral symmetry.

c) 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.

d) Agar gel immunodiffusion

ILT viral antigens may be demonstrated by AGID tests on tracheal exudate, infected CAMs and infected cell culture material using hyperimmune ILTV antiserum. The gel is made with Noble agar (1.5%) containing sodium chloride (8%) and sodium azide (0.02%) – as preservative – in distilled water. The ingredients are autoclaved at 15 lb/sq. inch (2.4 bar) for 15 minutes; 5 ml of the molten agar is poured into a 5 cm diameter Petri dish. When the agar has set, a pattern of wells is punched in the agar, consisting of a central well and six surrounding wells. The wells are usually 8 mm in diameter and 4 mm apart. The hyperimmune serum is pipetted into the central well, while the surrounding wells are filled with suspect virus samples under test, but with at least one well containing positive viral antigen. Dishes are incubated in a humid atmosphere at room temperature or at 37°C, and examined 24–48 hours later by oblique illumination for lines of precipitation (reactions of identity). Tests should include uninfected material as negative antigen and known negative antiserum as controls. For economy of materials, the test can be done on a microscale – the agar being poured in a thin layer on to a microscope slide and holes punched of 4 mm diameter and 2 mm apart.

e) Enzyme-linked immunosorbent assay

When the monoclonal antibody (MAb) ELISA is used for detecting viral antigens (19), 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.

f) Histopathology

Tracheas for histopathological examination should be placed in formol saline immediately after removal from the birds and embedded in paraffin wax. Eyelids and lung are sometimes examined. Intranuclear inclusions may be seen in the epithelial cells of the trachea in longitudinal sections after staining by haematoxylin and eosin. They 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.

g) Molecular methods

Several molecular methods for identifying ILTV DNA in clinical samples have been reported, but the PCR has proved it 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 (16, 17). Humberd and others (13), 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 (29). Alexander & Nagy (2) 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.

A problem with the PCR for ILTV was that initially it was not able to differentiate between field and vaccine strains. However, 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 (5). Several reports have shown that while some field strains are closely related to and likely to be derived from vaccines viruses, others are true ‘wild types’ (21). Genes commonly examined by different international authors include ICP4, TK (thymidine kinase), glycoprotein G (gG), glycoprotein E (gE) and UL47. Oldoni & Garcia (22) used 36 restriction enzymes, while others have used as few as four.

i) **PCR protocol**

In a typical PCR protocol for ILTV, viral DNA is extracted from clinical samples (swabs, tissues pieces), 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. Amplifications are made using Taq DNA polymerase. Typical amplification reactions use an initial denaturing step of 94°C for 1 minute followed by 35 amplification cycles of 94°C for 1 minute with annealing temperatures ranging from 54–60°C for 30 seconds. Extension may be performed at 68°C, with extension times varying according to the size of the target region amplified and a final extension at 68°C for 7 minutes. The PCR products are separated by electrophoresis in 1% agarose gels, stained with ethidium bromide and exposed to UV light for visualisation.

ii) **Real-time PCR**

Recently a real-time PCR assay had been described for ILTV (7). This has the advantage that, including amplification and melt-curve analysis, it can be conducted in less than 2 hours. It therefore provides a very rapid method of ILT diagnosis in comparison with traditional virus isolation, or even the standard PCR followed by gel electrophoresis.

iii) **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 (thymidine kinase), UL15, UL47 glycoprotein G and ORF-BTK genes. Amplification products are digested separately with 10U RE for 3 hours. Digestion fragments are separated in 15% polyacrylamide gels. Fragments are observed after DNA silver staining and analysed under a light box. 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 (7, 10, 21, 26).

2. **Serological tests**

Antibodies to ILTV in chicken serum can be detected by virus neutralisation (VN), AGID, indirect immunofluorescence tests and ELISA (1).

a) **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 due to 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 (EID_{50}) for egg inoculations, or 100 median tissue culture infectious doses (TCID_{50}) 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 onto 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 TCID_{50} 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.

b) **Agar gel immunodiffusion**
For AGID tests, antigen is prepared from virus-infected CAMs or infected cell cultures. For the former, at least $10^4$ TCID$_{50}$ of ILTV is inoculated into the allantoic cavity of a batch of 10-day-old embryonating specific pathogen free (SPF) chicken eggs. The CAMs are harvested after 4 days’ incubation, and those with large pools are homogenised and sonicated in a small amount of PBS, pH 7.1. Alternatively, heavily infected monolayers of chicken embryo liver or kidney, or chicken kidney cells are inoculated at 37°C until the CPE is maximal. Any remaining attached cells are scraped from the culture vessel into the medium. Total culture harvests may be concentrated up to 100-fold by dialysis against polyethylene glycol (PEG 20,000 or PEG 30,000). For the test, the agar is prepared as described previously for antigen detection, but this time the CAM or cell culture antigen is placed in the central well with test sera in the surrounding wells. Known positive and negative antisera are incorporated in the test, which is read after 24–48 hours’ incubation at room temperature or at 37°C. AGID tests are simple, economical to perform, and useful for flock screening, although they are less sensitive than the other methods.

c) Indirect fluorescent antibody test

For indirect fluorescent antibody tests, the antigen consists of ILT-virus-infected cell culture monolayers grown on teflon-coated multispot slides. When CPE is beginning to develop, the cultures are fixed in acetone for 10 minutes. Dilutions of test sera prepared in PBS are applied to each spot culture and the slides are incubated at 37°C for 1 hour. The slides are washed in PBS as described previously, drained and treated with an appropriate dilution of a commercial FITC-labelled rabbit anti-chicken IgG. After incubation at 37°C for 1 hour, the slides are re-washed and coverslips are applied over a non-fade mountant. They are examined by epifluorescence with ultraviolet illumination, and end-point titres are read as the highest serum dilutions giving specific fluorescent staining. This test is more sensitive than AGID, but the interpretation of results may be subjective.

d) Enzyme-linked immunosorbent assay

The antigen for ELISA is obtained by sonication of heavily infected cell cultures at the time of maximum CPE, which is then 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. Several laryngotracheitis antibody ELISA kits are available commercially.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

ILT is usually controlled with live vaccines, although inactivated vaccines have also been used for safety reasons. There has been some recent work with genetically engineered vaccines and the results of the initial studies look promising (8, 27). The live virus seed is a suitably attenuated or naturally avirulent strain of ILTV. Vaccines may be administered by eye-drop, spray or in the drinking water. If administered by spray and a small droplet size is produced and inhaled, clinical disease may be precipitated. Young chickens may require vaccinating in endemic areas, but show more severe reactions to the vaccine. Repeated doses may be required to afford good protection. The level of virulence of the vaccine virus is critical. Strains of low virulence may not be effective, and those of higher virulence may cause severe disease. The spray route of administration requires care over droplet size and uniformity of application. It can be more effective with low virulence strains, but may be more dangerous with high virulence strains. At present, the available vaccines attempt to make a compromise between lack of efficacy and poor safety. Because of persistence of virulent vaccine virus on a site, 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.

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. ref. 25).

1. Seed management

a) Characteristics of the seed
The master seed virus (MSV) is selected and can be propagated in SPF chicken embryos or tissue cultures derived from such embryos. The MSV is tested in chicken embryos or chickens for the following: 1) purity, 2) *Mycoplasma* spp., 3) *Salmonella* spp., 4) avian leukemia virus, 5) haemagglutinating viruses, 6) virus identity, and 7) extraneous pathogens. Additionally, initial tests are performed to demonstrate the safety and efficacy of the chosen master seed. The safety test on the MSV must include tests to show lack of reversion to virulence on serial passage and also safety in birds. Evidence of shed and spread is also required. The MSV is stored in aliquots at –70°C. The MSV should not cause mortality or a severe respiratory reaction in chickens following ocular instillation, although pheasants are more susceptible. Administration by spray is convenient but may cause quite severe respiratory disease in some flocks.

b) **Method of culture**

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.

c) **Validation as a vaccine**

A test must be carried out to establish the efficacy of the vaccine in birds of the minimum age for which the product is destined and also for each avian species. This is repeated in further batches of chickens for each of the recommended routes of administration and/or age of bird. Three weeks later (or 10–14 days in the USA), the birds, together with ten controls of the same age and source, are challenged intratracheally or in the orbital sinus with a strain of ILTV of known high virulence. To be satisfactory, only 5% of the vaccinated birds should die or show severe signs of ILT. No more than four should show mild signs of ILT. At least eight of the controls should die or show severe signs of ILT.

2. **Method of manufacture**

The vaccine is made by inoculation of the production seed virus into 9–11-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. 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 and 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.

3. **In-process control**

The infected tissue or tissue culture homogenate may be tested for purity, potency, and virus content, mixed with a stabiliser (usually beef peptone and sucrose) and then lyophilised and stored at 4°C.

4. **Batch control**

a) **Sterility**

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

b) **Safety**

Using the recommended route of administration, each batch of vaccine is tested in ten SPF chickens, or ten birds of other target species, using ten doses per bird. The birds are observed for at least 21 days for adverse effects attributable to the vaccine.

c) **Potency**

Once the *in-vivo* efficacy of the vaccine has been established, the batch potency may be determined by measuring the virus content. Serial dilutions of the vaccine are inoculated on to the dropped CAM of 9–11-day-old SPF chicken embryos, using at least seven eggs per dilution, in a volume of 100 µl. The eggs are incubated for 5 days and the virus titre is calculated by observing characteristic lesions on the CAMs. The virus content should be at or above a release value and above and expiration titre during dating of the product. Both the release and expiration titres are based on the minimum protective dose described above.

d) **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.

e) **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.

f) Preservatives
Preservatives may not be required, but some antibiotics may be added to the tissue harvest or at serial assembly during manufacture. For products licensed in the USA, any antibiotics added are listed on the label.

g) Precautions (hazards)
Care should be taken over diluting and administering the vaccine, and over the proper disposal of unused vaccine.

5. Tests on the final product

a) Safety
In the USA, 25 susceptible chickens 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, tests of virus content are carried out. The virus titre shall normally be no higher than one-tenth of the dose at which the vaccine has been shown to be safe.

b) Potency
The test of virus content (see above) may also be used as a measure of potency. It must be no lower than the agreed minimum release titre. Each serial or subserial shall have a virus titre of $10^7$ greater than the minimum protective dose, but not less than $10^{25} \text{EID}_{50}$ (or TCID$_{50}$ for tissue culture prepared product)/dose.

c) Tests of final product
The lack of chicken pathogens should be confirmed in embryos or chickens. It should also be confirmed by testing for *Mycoplasma* spp., *Salmonella* spp., avian leukosis virus, and haemagglutinating viruses.

REFERENCES


