

Newcastle Disease (Chapter 2.1.15)

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Summary

Newcastle disease (ND) is caused by specified viruses of the avian paramyxovirus type I (APMV-1) serotype of the genus Avulavirus belonging to the family Paramyxoviridae. There are nine serotypes of avian paramyxoviruses designated APMV-I to APMV-9.

Strains of ND virus vary widely in the severity of the disease they may produce in birds. The less pathogenic strains may induce severe disease when exacerbated by the presence of other organisms or by adverse environmental conditions. The preferred method of diagnosis is virus isolation and subsequent characterisation.

Identification of the agent: Suspensions in an antibiotic solution prepared from tracheal and cloacal swabs (or faeces) obtained from live birds, or of faeces and pooled organ samples taken from dead birds, are inoculated into the allantoic cavity of 9-11-day-old embryonating fowl eggs. The eggs are incubated at 37°C for 4-7 days. The allantoic fluid of any egg containing dead or dying embryos, as they arise, and all eggs at the end of the incubation period are tested for haemagglutinating activity.

Any haemagglutinating agents should be tested for specific inhibition with a monospecific antiserum to ND virus. ND virus (APMV-1) may show some antigenic cross-relationship with some of the other avian paramyxovirus serotypes, particularly APMV-3 and APMV-7.

The pathogenicity of any newly isolated virus can be assessed by determining the mean death time in chicken embryos, the intracerebral pathogenicity index in 1-day-old chicks or by the intravenous pathogenicity index in 6-week-old chickens. In some countries, variations of these standard techniques are used. The pathogenicity of isolates can also be evaluated using molecular techniques, i.e. reverse-transcription polymerase chain reaction and sequencing. Isolation and characterisation of suspected pathogenic strains of the virus should be conducted in a virus-secure laboratory.

Serological tests: The haemagglutination inhibition test is used most widely in ND virus serology, its usefulness in diagnosis depends on the vaccinal immune status of the birds to be tested and on prevailing disease conditions.

Requirements for vaccines and diagnostic biologicals: Live viruses of low virulence (lentogenic) or of moderate virulence (mesogenic) are used for the vaccination of poultry depending on the disease situation. Inactivated vaccines are also used.

Live vaccines may be administered to poultry by various routes. They are usually produced by harvesting the infective allantoic/amniotic fluids from inoculated embryonating fowl eggs; some are prepared from infective cell cultures. The final product should be derived from the expansion of master and working seeds.

Inactivated vaccines are given intramuscularly or subcutaneously. They are usually produced by the addition of formaldehyde to infective virus preparations, or by treatment with beta-propiolactone. Most inactivated vaccines are prepared for use by emulsification with a mineral or vegetable oil.

If pathogenic forms of ND virus are used in the production of vaccine or in challenge studies, the facility should meet the OIE requirements for Containment Group 4 pathogens.



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INTRODUCTION

Newcastle disease (ND) is caused by specified viruses of the avian paramyxovirus type I (APMV-I) serotype of the genus Avulavirus belonging to the subfamily Paramyxovirinae, family Paramyxoviridae. The paramyxoviruses isolated from avian species have been classified by serological testing into nine serotypes designated APMV-1 to APMV-9; ND virus has been designated APMV-1 (4).

Since its recognition in 1926, ND is regarded as being endemic in many countries. Prophylactic vaccination is practised in all but a few of the countries that produce poultry on a commercial scale.

One of the most characteristic properties of different strains of ND virus has been their great variation in pathogenicity for chickens. Strains of ND virus have been grouped into five pathotypes on the basis of the clinical signs seen in infected chickens (13). These are:

1. Viscerotropic velogenic: a highly pathogenic form in which haemorrhagic intestinal lesions are frequently seen;
2. Neurotropic velogenic: a form that presents with high mortality, usually following respiratory and nervous signs;
3. Mesogenic: a form that presents with respiratory signs, occasional nervous signs, but low mortality;
4. Lentogenic or respiratory: a form that presents with mild or subclinical respiratory infection;
5. Asymptomatic enteric: a form that usually consists of a subclinical enteric infection.

Pathotype groupings are rarely clear-cut (6) and even in infections of specific pathogen free (SPF) birds, considerable overlapping may be seen. In addition, exacerbation of the clinical signs induced by the milder strains may occur when infections by other organisms are superimposed or when adverse environmental conditions are present.

As signs of clinical disease in chickens vary widely and diagnosis may be complicated further by the different responses to infection by different hosts, clinical signs alone do not present a reliable basis for diagnosis of ND. However, the characteristic signs and lesions associated with the virulent pathotypes will give rise to strong suspicion of the disease.

DIAGNOSTIC TECHNIQUES

1. Identification of the agent

a) Samples for virus isolation

When investigations of ND are the result of severe disease and high mortality in poultry flocks, it is usual to attempt virus isolation from recently dead birds or moribund birds that have been killed humanely. Samples from dead birds should consist of oro-nasal swabs, as well as samples collected from lung, kidneys, intestine (including contents), spleen, brain, liver and heart tissues. These may be collected separately or as a pool, although intestinal samples are usually processed separately from other samples.

Samples from live birds should include both tracheal and cloacal swabs, the latter should be visibly coated with faecal material. Small delicate birds may be harmed by swabbing, but the collection of fresh faeces may serve as an adequate alternative.

Where opportunities for obtaining samples are limited, it is important that cloacal swabs (or faeces) and tracheal swabs (or tracheal tissue) be examined as well as organs or tissues that are grossly affected or associated with the clinical disease. Samples should be taken in the early stages of the disease.

The samples should be placed in isotonic phosphate buffered saline (PBS), pH 7.0-7.4, containing antibiotics. The antibiotics can be varied according to local conditions, but could be, for example, penicillin (2000 units/ml); streptomycin (2 mg/ml); gentamycin (50 µg/ml); and mycostatin (1000 units/ml) for tissues and tracheal swabs, but at five-fold higher concentrations for faeces and cloacal swabs. It is important to readjust the solution to pH 7.0-7.4 following the addition of the antibiotics. Faeces and finely minced tissues should be prepared as 10-20% (w/v) suspensions in the antibiotic solution. Suspensions should be processed as soon as possible after incubation for 1-2 hours at room temperature. When immediate processing is impracticable, samples may be stored at 4°C for up to 4 days.

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b) Virus culture

The supernatant fluids of faeces or tissue suspensions obtained through clarification by centrifugation at 1000 g for about 10 minutes at a temperature not exceeding 25°C are inoculated in 0.2 ml volumes into the allantoic cavity of each of at least five embryonated SPF fowl eggs of 9-11 days' incubation. After inoculation, these are incubated at 35-37°C for 4-7 days. Eggs containing dead or dying embryos as they arise, and all eggs remaining at the end of the incubation period, should first be chilled to 4°C and the allantoic fluids tested for haemagglutination (HA) activity. Fluids that give a negative reaction should be passaged into at least one further batch of eggs.

c) Virus identification

HA activity detected in bacteriologically sterile fluids harvested from inoculated eggs may be due to the presence of any of the 15 haemagglutinin subtypes of influenza A viruses or of the eight other paramyxovirus serotypes. (Nonsterile fluid could contain bacterial HA.) ND virus can be confirmed by the use of specific antiserum in a haemagglutination inhibition (HI) test. Usually chicken antiserum that has been prepared against one of the strains of ND virus is used.

Cross-reactions in HI tests between ND virus and some of the other APMVs, especially APMV-3 and APMV-7 serotype viruses may cause some problems that can be resolved by the use of suitable antigen and antiserum controls.

d) Pathogenicity indices

The extreme variation in virulence of different ND virus isolates and the widespread use of live vaccines means that the identification of an isolate as ND virus from birds showing clinical signs does not confirm a diagnosis of ND, so that an assessment of the virulence of the isolate is also required (see Section B.1.f. below 'Definition of Newcastle disease'). Several potential in-vitro tests for establishing virulence usually related to the molecular basis for pathogenicity (Section B.1.e. below) are being investigated by various groups around the world. At present, a definitive assessment of virus virulence is usually based on one or more of the following in-vivo tests, although the current OIE definition (Section B.1.f. below) allows molecular assessment of virulence:

Mean death time in eggs:

- i) Fresh, sterile, infective allantoic fluid is diluted in sterile saline to give a tenfold dilution series between 10⁻⁶ and 10⁻⁹.
- ii) For each dilution, 0.1 ml is inoculated into the allantoic cavity of each of five 9-10-day-old embryonated SPF fowl eggs, which are then incubated at 37°C.
- iii) The remaining virus dilutions are retained at 4°C and another five eggs are inoculated with 0.1 ml of each dilution 8 hours later and incubated at 37°C.
- iv) Each egg is examined twice daily for 7 days and the times of any embryo deaths are recorded.
- v) The minimum lethal dose is the highest virus dilution that causes all the embryos inoculated with that dilution to die.
- vi) The mean death time (MDT) is the mean time in hours for the minimum lethal dose to kill all the inoculated embryos.
- vii) The MDT has been used to classify ND virus strains into the following groups: velogenic (taking under 60 hours to kill); mesogenic (taking between 60 and 90 hours to kill); and lentogenic (taking more than 90 hours to kill).

Intracerebral pathogenicity index:

- i) Fresh infective allantoic fluid with a HA titre >24 (>1/16) is diluted 1/10 in sterile isotonic saline with no additives, such as antibiotics.
- ii) 0.05 ml of the diluted virus is injected intracerebrally into each of ten chicks hatched from eggs from an SPF flock. These chicks must be over 24-hours and under 40-hours old at the time of inoculation.
- iii) The birds are examined every 24 hours for 8 days.
- iv) At each observation, the birds are scored: 0 if normal, 1 if sick, and 2 if dead. (Dead individuals must be scored as 2 at each of the remaining daily observations after death.)
- v) The intracerebral pathogenicity index (ICPI) is the mean score per bird per observation over the 8-day period.

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The most virulent viruses will give indices that approach the maximum score of 2.0, whereas lentogenic strains will give values close to 0.0.

Intravenous pathogenicity index:

- i) Freshly collected infective allantoic fluid (which should be no older than 24-48 hours and should have tested negative for bacterial contamination) with a HA titre of >24 (>1/16) is diluted 1/10 in sterile isotonic saline.
- ii) 0.1 ml of the diluted virus is injected intravenously into each of ten 6-week-old SPF chickens.
- iii) Birds are examined at 24-hour intervals for 10 days and scored at each observation: 0 if normal, 1 if sick, 2 if paralysed or showing other nervous signs, and 3 if dead. (Dead individuals must be scored as 3 at each of the remaining daily observations after death.)
- iv) The intravenous pathogenicity index (IVPI) is the mean score per bird per observation over the 10-day period.

Lentogenic strains and some mesogenic strains will have IVPI values of 0, whereas the indices for virulent strains will approach 3.0.

Some variations have been recommended in these tests. Swabbing of the cloaca and conjunctiva of 8-week-old chickens with undiluted allantoic fluid has been substituted for the IVPI test (23). The intention is to distinguish between viscerotropic velogenic and neurotropic velogenic viruses.

Interpretation of pathogenicity indices:

Interpretation of the pathogenicity indices obtained with a view to imposing trade or movement restrictions, or other policies, is not straightforward. The objective is to control strains significantly more virulent than lentogenic strains, such as Hitchner-B1 or La Sota. As viruses capable of producing quite severe disease may have IVPI values of 0, the ICPI test is used most often for such assessments. However, as different strains show a range of values from 0.00 to 2.00 in this test, it is clear that any value used for definition must be governed by practicality.

e) Molecular basis for pathogenicity

During replication, ND virus particles are produced with a precursor glycoprotein, F0, which has to be cleaved to F1 and F2 for the virus particles to be infectious. This post-translation cleavage is mediated by host-cell proteases. Trypsin is capable of cleaving F0 for all ND virus strains.

It would appear that the F0 molecules of viruses virulent for chickens can be cleaved by a host protease or proteases found in a wide range of cells and tissues, and thus spread throughout the host damaging vital organs, but F0 molecules in viruses of low virulence are restricted in their cleavability to certain host proteases resulting in restriction of these viruses to growth only in certain host-cell types.

Most ND viruses that are pathogenic for chickens have the sequence 112R/K-R-Q-K/R-R116 at the C-terminus of the F2 protein and F (phenylalanine) at residue 117, the N-terminus of the F1 protein, whereas the viruses of low virulence have sequences in the same region of 112G/E-K/R-Q-G/E-R116 and L (leucine) at residue 117. Some of the pigeon variant viruses (PPMV-1) examined have the sequence 112G-R-Q-K-R-F117, but give high ICPI values. Thus there appears to be the requirement of at least one pair of basic amino acids at residues 116 and 115 plus a phenylalanine at residue 117 and a basic amino acid (R) at 113 if the virus is to show virulence for chickens.

Several studies have been done using molecular techniques to determine the F0 cleavage site sequence by reverse-transcription polymerase chain reaction (RT-PCR), either on the isolated virus or on tissues and faeces from infected birds, followed by analysis of the product by restriction enzyme analysis, probe hybridisation or nucleotide sequencing with a view to establishing a routine *in vitro* test for virulence (for a review see ref. 2). Determination of the F0 cleavage sequence may give a clear indication of the virulence of the virus, and this has been incorporated in to the definition of ND (see Section B.1.f).

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In the diagnosis of ND it is important to understand that the demonstration of the presence of virus with multiple basic amino acids at the F0 cleavage site confirms the presence of virulent or potentially virulent virus, but that failure to detect virus or detection of ND virus without multiple basic amino acids at the F0 cleavage site using molecular techniques does not confirm the absence of virulent virus. Primer mismatch, or the possibility of a mixed population of virulent and avirulent viruses mean that virus isolation and an in vivo assessment of virulence will still be required.

Recent analyses of viruses isolated in Ireland in 1990 and during the 1998–2000 outbreaks of ND in Australia have given strong evidence that virulent viruses may arise from progenitor viruses of low virulence (5, 39). Virulent ND virus has also been generated experimentally from low virulence virus by passage in chickens (34).

f) Definition of Newcastle disease

It seems likely that the vast majority of birds are susceptible to infection with ND viruses of both high and low virulence for chickens, although the clinical signs seen in birds infected with ND virus vary widely and are dependent on factors such as: the virus, host species, age of host, infection with other organisms, environmental stress and immune status. In some circumstances infection with the extremely virulent viruses may result in sudden high mortality with comparatively few clinical signs. Thus the clinical signs are variable and influenced by other factors so that none can be regarded as pathognomonic.

Even for susceptible hosts, such as chickens, ND viruses show a considerable range of virulence. Generally, variation consists of clusters around the two extremes in tests used to assess virulence, but, for a variety of reasons, some viruses may show intermediate virulence.

The enormous variation in virulence and clinical signs means it is necessary to define carefully what constitutes ND for the purposes of trade, control measures and policies. The definition of ND currently in use in all member states of the European Union is defined in Directive 92/66/EEC (17).

The OIE definition for reporting an outbreak of ND is:

'Newcastle disease is defined as an infection of birds caused by a virus of avian paramyxovirus serotype 1 (APMV-1) that meets one of the following criteria for virulence:

*a) The virus has an intracerebral pathogenicity index (ICPI) in day-old chicks (*Gallus gallus*) of 0.7 or greater.*

OR

b) Multiple basic amino acids have been demonstrated in the virus (either directly or by deduction) at the C-terminus of the F2 protein and phenylalanine at residue 117, which is the N-terminus of the F1 protein. The term 'multiple basic amino acids' refers to at least three arginine or lysine residues between residues 113 and 116. Failure to demonstrate the characteristic pattern of amino acid residues as described above would require characterisation of the isolated virus by an ICPI test.'

In this definition, amino acid residues are numbered from the N-terminus of the amino acid sequence deduced from the nucleotide sequence of the F0 gene, 113–116 corresponds to residues -4 to -1 from the cleavage site.'

g) Monoclonal antibodies

Mouse monoclonal antibodies (MAbs) directed against strains of ND virus have been used in HI tests to allow rapid identification of ND virus without the possible cross-reactions with other APMV serotypes that may occur with polyclonal sera. MAbs have been produced that give reactions in HI tests that are specific for particular strains or variant ND virus isolates (4, 9).

Panels of MAbs have been used to establish antigenic profiles of ND virus isolates based on whether or not they react with the viruses. This has proven to be a valuable method for grouping and differentiating isolates of ND virus, and has been particularly valuable to the understanding of the epidemiology of outbreaks (9).

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h) Phylogenetic studies

Development of improved techniques for nucleotide sequencing, the availability of sequence data of more ND viruses in computer databases and the demonstration that even relatively short sequence lengths could give meaningful results in phylogenetic analyses have led to a considerable increase in such studies in recent years. Considerable genetic diversity has been detected, but viruses sharing temporal, geographical, antigenic or epidemiological parameters tend to fall into specific lineages or clades and this has proven valuable in assessing both the global epidemiology and local spread of ND (3, 8, 16, 24, 27, 28, 33, 36-38).

Although in the past phylogenetic studies have been impracticable as a routine tool, the greater availability and increased speed of production of results obtained using sophisticated, commercially available kits for RT-PCR and automatic sequencers now means such studies are within the capabilities of many more diagnostic laboratories and can give meaningful results that are contemporaneous rather than retrospective (2).

i) Molecular techniques in diagnosis

In addition to the use of RT-PCR and other similar techniques for the determination of the virulence of ND viruses (see Section B.1.e) or for phylogenetic studies (see Section B.1.h), there have been several reports of the use of such molecular techniques to detect ND virus in clinical specimens, the advantage being the extremely rapid demonstration of the presence of virus and even its virulence if primers covering the part of the genome coding for the F0 cleavage site are used (12, 20, 21). Care should be taken in the selection of clinical samples as some studies have demonstrated lack of sensitivity in detecting virus in some organs and particularly in faeces (20, 21, 25). As with virulence determination, it is important that such techniques alone are not used to record a negative result in investigations of suspected ND.

ND, as defined in Section B.1.f. of this chapter, is subject to official control and the virus has a high risk of spread from the laboratory; consequently, a risk assessment should be carried out to determine the level of biosecurity needed for the diagnosis and characterisation of the virus.

The facility should meet the requirements for the appropriate Containment Group as determined by the risk assessment and as outlined in Appendix I.1.6.1 of Chapter I.1.6. of this Terrestrial Manual. Countries lacking access to such a specialised national or regional laboratory should send specimens to an OIE Reference Laboratory.

2. Serological tests

ND virus may be employed as an antigen in a wide variety of serological tests, enabling neutralisation or enzyme-linked immunosorbent assays (ELISA) to be used for diagnosis. At present, the HI test is most widely used. Chicken sera rarely give nonspecific positive reactions in this test and any pretreatment of the sera is unnecessary. Sera from species other than chickens may sometimes cause agglutination of chicken red blood cells (RBCs), so this property should first be determined and then removed by adsorption of the serum with chicken RBCs. This is done by adding 0.025 ml of packed chicken RBCs to each 0.5 ml of antisera, shaking gently and leaving for at least 30 minutes; the RBCs are then pelleted by centrifugation at 800 g for 2-5 minutes and the adsorbed sera are decanted.

Variations in the procedures for HA and HI tests are practised in different laboratories. The following recommended examples apply in the use of V-bottomed microwell plastic plates in which the final volume for both types of test is 0.075 ml. The reagents required for these tests are isotonic PBS (0.1 M), pH 7.0-7.2, and RBC taken from a minimum of three SPF chickens and pooled in an equal volume of Alsever's solution. (If SPF chickens are not available, blood may be taken from unvaccinated birds monitored regularly and shown to be free from antibodies to ND virus.) Cells should be washed three times in PBS before use as a 1% (packed cell v/v) suspension. Positive and negative control antigens and antisera should be run with each test, as appropriate.

a) Haemagglutination and haemagglutination inhibition tests

Haemagglutination test:

- i) 0.025 ml of PBS is dispensed into each well of a plastic V-bottomed microtitre plate.
- ii) 0.025 ml of the virus suspension (i.e. infective allantoic fluid) is placed in the first well. For accurate determination of the HA content, this should be done from a close range of an initial series of dilutions, i.e. 1/3, 1/5, 1/7, etc.

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- iii) Twofold dilutions of 0.025 ml volumes of the virus suspension are made across the plate.
- iv) A further 0.025 ml of PBS is dispensed to each well.
- v) 0.025 ml of 1% (v/v) chicken RBCs is dispensed to each well.
- vi) The solution is mixed by tapping the plate gently. The RBCs are allowed to settle for about 40 minutes at room temperature, i.e. about 20°C, or for 60 minutes at 4°C if ambient temperatures are high, when control RBCs should be settled to a distinct button.
- vii) HA is 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 (no streaming); this represents 1 HA unit (HAU) and can be calculated accurately from the initial range of dilutions.

Haemagglutination inhibition test:

- i) 0.025 ml of PBS is dispensed into each well of a plastic V-bottomed microtitre plate.
- ii) 0.025 ml of serum is placed into the first well of the plate.
- iii) Twofold dilutions of 0.025 ml volumes of the serum are made across the plate.
- iv) 4 HAU virus/antigen in 0.025 ml is added to each well and the plate is left for a minimum of 30 minutes at room temperature, i.e. about 20°C, or 60 minutes at 4°C.
- v) 0.025 ml of 1% (v/v) chicken RBCs is added to each well and, after gentle mixing, the RBCs are allowed to settle for about 40 minutes at room temperature, i.e. about 20°C, or for about 60 minutes at 4°C if ambient temperatures are high, 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 by tilting the plates. Only those wells in which the RBCs stream at the same rate as the control wells (containing 0.025 ml RBCs 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 $>1/4$ (>22 or $>\log_2 2$ when expressed as the reciprocal), and a positive control serum for which the titre should be within one dilution of the known titre.

The value of serology in diagnosis is clearly related to the expected immune status of the affected birds. HI titres may be regarded as being positive if there is inhibition at a serum dilution of 1/16 (24 or $\log_2 4$ when expressed as the reciprocal) or more against 4 HAU of antigen. Some laboratories prefer to use 8 HAU in HI tests. While this is permissible, it affects the interpretation of results so that a positive titre is 1/8 (23 or $\log_2 3$) or more. Back titration of antigen should be included in all tests to verify the number of HAU used.

HI titres may be used to assess the immune status of a flock. In vaccinated flocks that are being monitored serologically, it may be possible to identify anamnestic responses as the result of a challenge infection with field virus (11), but great care should be exercised as variations may occur from other causes. For example, it has been demonstrated that APMV-3 virus infections of ND-virus-vaccinated turkeys will result in substantially increased titres to ND virus (7).

There are a variety of commercial ELISA kits available and these are based on several different strategies for the detection of ND virus antibodies, including indirect, sandwich and blocking or competitive ELISAs using MAbs. At least one kit uses a subunit antigen. Usually such tests have been evaluated and validated by the manufacturer, and it is therefore important that the instructions specified for their use be followed carefully.

REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

A detailed account of all aspects of ND virus vaccines, including their production and use, has been published (11) and should be referred to for details of the procedures outlined here. Guidelines for the production of veterinary vaccines are given in Chapter I.1.7. Principles of veterinary vaccine production. The guidelines given here and in Chapter I.1.7 are intended to be general in nature and may be supplemented by national and regional requirements.

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In this section, conventional live and inactivated vaccines will be considered, as these are still used universally. However, it should be remembered that there has been much recent work on the application of molecular biology techniques to the production of new vaccines, and success has been reported in obtaining protective immunity with recombinant fowlpox virus, vaccinia virus, pigeonpox virus, turkey herpesvirus and avian cells in which the HN gene, the F gene, or both, of ND virus are expressed. Several of these recombinant viruses have been licensed for use in certain countries.

ND virus strains used in commercial live virus vaccines fall into two groups: lentogenic vaccines, such as Hitchner-B1, La Sota, V4, NDW, I2 and F, and mesogenic vaccines, such as Roakin, Mukteswar and Komarov. Strains from both these groups have been subjected to selection and cloning to fulfil different criteria in their production and application. The mesogenic vaccine viruses all have two pairs of basic amino acids at the F0 cleavage site and ICPI values of around 1.4. This means that infections of birds with these viruses would fall within the intended definition of ND (Section B.1.f.), but as these vaccines are primarily used in countries where ND is endemic this may not necessarily preclude their use.

The vaccine production facility should operate under the appropriate biosecurity procedures and practices. If ND, as defined in Section B.1.f. of this chapter, is used for vaccine production or for vaccine-challenge studies, that part of the facility where this work is done should meet the requirements for Containment Group 4 pathogens as outlined in Appendix I.1.6.1 of Chapter I.1.6 of this *Terrestrial Manual*.

Most live virus vaccines are grown in the allantoic cavity of embryonated fowl eggs but some, notably some mesogenic strains, have been adapted to a variety of tissue culture systems.

Live virus vaccines may be administered to birds by incorporation in the drinking water, delivered as a coarse spray, or by intranasal or conjunctival instillation. Some mesogenic strains are given by wing-web intradermal inoculation. Vaccines have been constructed to give optimum results through application by specific routes. In general, the more immunogenic live vaccines are more virulent, and are therefore more likely to cause adverse side-effects. For example, vaccination with the La Sota strain will cause considerably greater problems in young susceptible birds than the Hitchner-B1 strain, although La Sota induces a stronger immune response.

Inactivated vaccines are considerably more expensive than live vaccines, and their use entails handling and injecting individual birds. They are prepared from allantoic fluid that

has had its infectivity inactivated by the addition of formaldehyde or beta-propiolactone. This is incorporated into an emulsion with mineral oil, and is administered intramuscularly or subcutaneously. Individual birds thus receive a standard dose. There is no subsequent spread of virus or adverse respiratory reactions. Both virulent and avirulent strains are used as seed virus although, from the aspect of safety control, the use of the latter appears more suitable. As no virus multiplication takes place after administration, a much larger amount of antigen is required for immunisation than for live virus vaccination. A high yield of virus to produce a potent vaccine is important, and the Ulster 2C strain is very suitable for this purpose.

The duration of immunity depends on the vaccination programme chosen. One of the most important considerations affecting vaccination programmes is the level of maternal immunity in young chickens, which may vary considerably from farm to farm, batch to batch, and among individual chickens. For this reason, one of several strategies is employed. Either the birds are not vaccinated until 2-4 weeks of age when most of them will be susceptible, or 1-day-old birds are vaccinated by conjunctival instillation or by the application of a coarse spray. This will establish active infection in some birds that will persist until maternal immunity has waned. Revaccination is then carried out 3-4 weeks later. It has been demonstrated that inactivated vaccines may also be usefully employed to vaccinate 1-day-old chicks that have a degree of maternal immunity (15), and the best results of all were obtained when 1-day-old maternally immune chicks were given a combination of live and inactivated vaccines, compared with live or inactivated vaccines given alone (14). Vaccination of fully susceptible 1-day-old birds, even with the most mild of live vaccines, may result in respiratory disease, especially if common pathogenic bacteria are present in significant numbers.

Vaccination after 3 weeks of age is normally practised only in breeding hens and hens laying table eggs. This should be done at sufficiently frequent intervals to maintain an adequate immunity. Vaccination programmes often employ slightly more pathogenic live virus vaccines to boost immunity than those used initially. These more pathogenic live vaccines may also be used following initial vaccination with oil emulsion inactivated vaccines.

When devising a vaccination programme, consideration should be given to the type of vaccine used, the immune and disease status of the birds to be vaccinated, and the level of protection required in relation to any possibility of infection with field virus under local conditions (11). Two examples of vaccination programmes that may be used in different disease circumstances are listed here. For the first example,

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when the disease is mild and sporadic, it is suggested that the following order of vaccination be adopted: live Hitchner-B1 by conjunctival or spray administration at 1 day of age; live Hitchner-B1 or La Sota at 18-21 days of age in the drinking water; live La Sota in the drinking water at 10 weeks of age, and an inactivated oil emulsion vaccine at point of lay. For the second example, when the disease is severe and more widespread, the same protocol as above is adopted up to 21 days of age, and this is followed by revaccination at 35-42 days of age with live La Sota in the drinking water or as an aerosol; this revaccination is repeated at 10 weeks of age with an inactivated vaccine (or a mesogenic live vaccine) and again repeated at point of lay (11).

1. Seed management

a) Characteristics of the seed

The first principle to consider when selecting a strain for a live ND virus vaccine is whether it is to be used as a primary or a secondary vaccine, the main consideration being its pathogenicity. The methods of application and frequency of use are valid considerations. The use of MAbs has demonstrated considerable variation in the antigenicity of different strains (9). This may indicate a need to tailor vaccines more carefully to relate antigenically to any prevalent field virus.

A live vaccine based on ND virus strain V4, selected for heat stability, has been introduced to combat the specific problems associated with village chicken rearing in developing countries. The intention is that this vaccine could be coated on food fed to scavenging chickens. To date, trials in different countries have produced mixed results; it may well be that local factors are extremely important in affecting the success of this strategy (35). More recently the thermostable I2 vaccine has been developed specifically for vaccinating village chickens; it is currently recommended that this vaccine be given by eye drop (1).

Use of live vaccines may be restricted by legislation. For example, Commission Decision 93/152/EEC (18) restricts the use of vaccines in member states of the European Union from 1 January 1995 to those for which the master seed has been tested and shown to have an ICPI of <0.4 if no fewer than 107 mean egg infectious doses (EID₅₀) are administered to each bird, or <0.5 if no fewer than 108 EID₅₀ are administered to each bird.

The OIE Standards Commission has similarly recommended that while in principle vaccines should have an ICPI < 0.7 in order to account for interassay and interlaboratory variability a safety margin should be allowed so that vaccine master seed virus strains should not have an ICPI exceeding 0.4 (30).

The most important consideration in selecting a seed for the preparation of inactivated vaccine is the amount of antigen produced when grown in embryonated eggs; it is rarely cost-effective to concentrate virus. Both virulent and lentogenic strains have been used as inactivated vaccines, but the former offer an unnecessary risk because the manipulation of large quantities of virulent virus is involved, as well as the dangers of inadequate inactivation and possible subsequent contamination. This risk is reflected in Commission Decision 93/152/EEC (18), which restricts the use of viruses used for inactivated vaccine in member states of the European Union from 1 January 1995 to those for which the master seed has been tested and shown to have an ICPI of <0.7 if no fewer than 108 EID₅₀ are administered to each bird. Some lentogenic strains grow to very high titres in eggs. Exceptionally high titres can be obtained by the Ulster 2C strain, which has been recommended as a seed for inactivated vaccine (22). However, successful commercial inactivated vaccines are produced when the Hitchner B1, La Sota or F strains are used as seeds.

b) Method of culture

A master seed is established, and from this a working seed. If the strain has been cloned by limiting dilution or plaque selection, the establishment of a master culture may only involve producing a large volume of infective allantoic fluid (minimum 100 ml), which can be stored as lyophilised aliquots (0.5 ml).

c) Validation as a vaccine

Seed viruses of unknown pedigree should be passaged through SPF eggs and cloned before producing the master seed. Some passage through SPF chickens may also be desirable (11). In either case, the master seed should be checked after preparation for sterility, safety, potency and extraneous agents.

2. Method of manufacture

For vaccine production, a working seed, from which batches of vaccine are produced, is first established by expansion

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of an aliquot of master seed to a sufficient volume to allow vaccine production for 12-18 months. It is best to store the working seed in liquid form at -60°C or lower as lyophilised virus does not always multiply to high titre on subsequent first passage (11).

Most ND vaccines are produced in embryonated fowl eggs, and live virus vaccines should be produced in SPF eggs. The method of production is large-scale aseptic propagation of the virus; all procedures are performed under sterile conditions.

It is usual to dilute the working seed in sterile PBS, pH 7.2, so that about 103-104 EID₅₀/0.1 ml is inoculated into the allantoic cavity of 9- or 10-day-old embryonated SPF fowl eggs. These are then incubated at 37°C. Eggs containing embryos that die within 24 hours should be discarded. The incubation time will depend on the virus strain being used and will be predetermined to ensure maximum yield with the minimum number of embryo deaths.

The infected eggs should be chilled at 4°C before being harvested. The tops of the eggs are removed and the allantoic fluids aspirated after depression of the embryo. The inclusion of any yolk material and albumin should be avoided. All fluids should be stored immediately at 4°C and tested for bacterial contamination before large pools are made for lyophilisation or inactivation. Live vaccines are usually lyophilised. The methodology depends on the machinery used and the expertise of the manufacturers, but this is a very important step as inadequate lyophilisation results in both loss of titre and a reduced shelf life.

In the manufacture of inactivated vaccines, the harvested allantoic fluid is treated with either formaldehyde (a typical final concentration is 1/1000) or beta-propiolactone (a typical final concentration is 1/2000-1/4000). The time required must be sufficient to ensure freedom from live virus. Most inactivated vaccines are not concentrated; the inactivated allantoic fluid is usually emulsified with mineral or vegetable oil. The exact formulations are generally commercial secrets.

Generally oil-based inactivated vaccines are prepared as primary emulsions of water-in-oil. The oil phase usually consists of nine volumes of highly refined mineral oil, such as Marcol 52, Drakeol 6VR and BayolF, plus one volume of emulsifying agent, such as Arlacel A, Montanide 80 and Montanide 888 (31). The aqueous phase is the inactivated virus to which a non-ionic emulsifier such as Tween 80 has been added. The oil phase to aqueous phase ratio is usually 1:1 to 1:4. Manufacturers strive to reach a balance between adjuvant effect, viscosity and stability. Too high viscosity

and the vaccine is difficult to inject; too low viscosity and the vaccine is unstable.

3. In-process control

Each batch of live virus vaccine should be tested for viability and potency. For those produced in eggs, the most important process control is testing for bacterial and fungal contamination. This is necessary because of the occasional occurrence of putrefying eggs, which may remain undetected at the time of harvest.

For inactivated vaccines, the efficacy of the process of inactivation should be tested in embryonated eggs, taking 25 aliquots (0.2 ml) from each batch and passaging each three times through SPF embryos (11).

4. Batch control

Most countries have published specifications for the control of production and testing of ND virus vaccines (e.g. ref. 29), which include the definition of the obligatory tests on vaccines during and after manufacture.

It is necessary to test the infectivity of live virus vaccines to enable adequate levels of virus to be administered. The virus is usually titrated in embryonated fowl eggs to give the EID₅₀. This involves making tenfold dilutions of virus; 0.1 ml of each dilution is inoculated into between five and seven 9-10-day-old embryonated fowl eggs. After 5-7 days' incubation at 37°C, the eggs are chilled and tested for the presence of haemagglutinin activity, which is an indication of the presence of live virus. The EID₅₀ end-point is calculated using a standard formula such as Spearman-Kärber (10).

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter I.1.5.

b) Safety

The use of chickens for the testing of vaccines involves the inoculation of ten or more birds of stated age that originate from an SPF flock. Ten doses of live vaccine are administered supraconjunctivally to each bird and the birds are then observed for 21 days. No chicken should show serious clinical signs and none should die from causes attributable to the vaccine (19).

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For inactivated vaccines, a double dose is administered by the recommended route to ten 3-week-old birds, and these are observed for 2 weeks for the absence of clinical signs of disease or local lesions.

c) Potency

Various methods for the testing of ND virus vaccines for potency have been proposed. The importance of using a suitable challenge strain for assessment has been stressed (11). A suitable strain is the Herts 33 strain. For live vaccines, the method recommended (19) involves the vaccination of 20 SPF or other fully susceptible birds at the minimum recommended age by the suggested route using the minimum recommended dose. After 14-21 days, each vaccinated bird and ten control birds are challenged intramuscularly with 105 LD50 (50% lethal dose) of ND challenge virus. The vaccine passes the test if at the end of 10 days, 90% of the vaccinated chickens survive with no signs of disease, but all controls die within 6 days.

For inactivated vaccines, 21-28-day-old SPF or susceptible chickens are used. Three groups of 20 birds are injected intramuscularly with volumes of vaccine equivalent to 1/25, 1/50 and 1/100 of a dose. A group of ten chickens is kept as controls. All the birds are challenged by intramuscular injection of 106 LD50 of ND challenge virus, 17-21 days later. Chickens are observed for 21 days. The PD50 (50% protective dose) is calculated by standard statistical methods. The test is only valid if challenged control birds all die within 6 days. The vaccine complies with the test if the PD50 is not less than 50 per dose and if the lower confidence limit is not less than 35 PD50 per dose. Some control authorities accept a test at 1/50 only, for animal welfare reasons.

It is not necessary to repeat the potency test on each batch if it has been shown that a representative batch of the final product from the master seed has passed the test.

d) Duration of immunity

The level of immunity reached with any single dose or regimen of ND vaccination will vary enormously with both vaccine and host species. The level of immunity required in a given host (i.e. to protect against death, disease, meat or egg production losses) is extremely complex and difficult to evaluate.

Generally some assessment of the longevity of serum antibodies should be made and vaccine regimens adopted to maintain these above an acceptable level (11).

e) Stability

When stored under the recommended conditions the final vaccine product should maintain its potency for at least 1 year. Accelerated stability tests such as reduction of infectivity following incubation at 37°C for 7 days (26) may be used as a guide to the storage capabilities of a batch of live vaccine. Oil emulsion vaccines should also be subjected to accelerated ageing by storing at 37°C, for a minimum of 1 month, without separation of the aqueous and oil phases. Live virus vaccines must be used immediately after reconstitution. Inactivated vaccines must not be frozen.

f) Preservatives

For live vaccines preservatives must not be included in the freeze-dried product, but antimicrobial preservatives may be incorporated in the diluent used to reconstitute the vaccine.

g) Precautions (hazards)

Live ND virus vaccines may represent a hazard to humans. ND viruses, both virulent and of low virulence for chickens have been reported to have infected humans, usually causing acute conjunctivitis following direct introduction to the eye. Infections are usually transient and the cornea is not involved.

Mineral oil emulsion vaccines represent a serious hazard to the vaccinator. Accidental injection of humans should be treated promptly by incision and washing of the site, as for a 'grease-gun' injury.

5. Tests on the final product

a) Safety

See Section C.4.b. above

b) Potency

See Section C.4.c. above.

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