CHAPTER 3.3.10.

NEWCASTLE DISEASE (INFECTION WITH NEWCASTLE DISEASE VIRUS)

SUMMARY

Description of the disease: *Newcastle disease (ND) is caused by virulent strains of avian paramyxovirus type 1 (APMV-1), also known as Newcastle disease virus (NDV), of the genus* Orthoavulavirus *belonging to the family* Paramyxoviridae. *At present, there are 21 serotypes of avian paramyxoviruses designated APMV-1 to APMV-21. Each virus belongs to a virus species that are dispersed amongst three genera termed: avian metaavulavirus, avian orthoavulavirus and avian paraavulavirus.*

APMV-1 has been shown to be able to infect over 200 species of birds, but the severity of disease produced varies with both host and strain of virus. Even APMV-1 strains of low virulence may induce severe respiratory and enteric disease when exacerbated by the presence of other organisms or by adverse environmental conditions. The preferred method of diagnosis is validated reverse transcription polymerase chain reaction (RT-PCR) and sequencing, but virus isolation still remains an important laboratory tool.

Detection of the agent: *Suspensions in an antibiotic solution prepared from tracheal or oropharyngeal 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- to11-day-old embryonated chicken eggs. The eggs are incubated at 37°C for 2–7 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 and/or by use of validated specific molecular methods to detect viral genome.*

Any haemagglutinating agents should be tested for specific inhibition with a monospecific antiserum to APMV-1. APMV-1 may show some antigenic cross-relationship with some of the other avian paramyxovirus serotypes, particularly APMV-3, APMV-7 and APMV-12. Real-time RT-PCR on the positive allantoic fluids could also be used as an alternative method for the initial APMV-1 identification.

Virulence of any newly isolated APMV-1 can be evaluated using molecular techniques, i.e. reverse-transcription polymerase chain reaction and sequencing. Alternatively, the intracerebral pathogenicity index (ICPI) can be used to determine virulence. ND is subject to official control in most countries and the virus has a high risk of spread from the laboratory; consequently, appropriate laboratory biosafety and biosecurity must be maintained; a risk assessment should be carried out to determine the level needed. Any APMV-1 with an ICPI $\geq$ 0.7, or with multiple basic amino acids at the C-terminus of the F2 protein and phenylalanine at residue 117 of its F protein, is considered virulent.

Genetic-based tests are most commonly applied to routine diagnosis and offer rapid, sensitive, cost effective alternatives to conventional methods. Real-time RT-PCR targeting a highly conserved gene overcomes wide heterogeneity in the fusion (F) or haemagglutinin-neuraminidase (HN) genes. They can be applied directly to clinical samples once treated to extract viral RNA and can be used to test large sample numbers. Confirmation of detection using highly sensitive and inclusive screening real-time RT-PCR should then be followed by F gene sequencing to determine the virus virulence (the F gene proteolytic cleavage site) and virus genotype. It is important that tests selected have been shown to be able to sensitively detect viruses known to be circulating or an emerging threat to the region in which they are applied.

A screening test by real-time RT-PCR to identify both virulent and avirulent APMV-1 is recommended. For any positive tests, additional testing targeted to the fusion cleavage site can be used to identify viruses with a cleavage site compatible with NDV. Due to variability of the fusion cleavage site, it may require more than one test if using real-time RT-PCR to identify all virulent genotypes circulating in a country or region hence gene sequencing is the preferred approach. For outbreaks that have birds with signs compatible with Newcastle disease virus, which are positive by the real-time RT-PCR screening test, and are negative for the virulent fusion real-time RT-PCR tests, direct sequence of the fusion cleavage site or virus isolation and classical analysis may be required.

Serological tests: *The haemagglutination inhibition (HI) test is used most widely in ND serology, its usefulness in diagnosis depends on the vaccinal immune status of the birds to be tested and on prevailing disease conditions. Enzyme-linked immunosorbent assays also have a place and a number of commercial test kits are available.*

Requirements for vaccines: *Live viruses of low virulence (lentogenic) or of moderate virulence (mesogenic) are used for the vaccination of poultry depending on the disease situation and national requirements. 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 embryonated chickens' 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.

Recombinant Newcastle disease vaccines using viral vectors such as turkey herpesvirus or fowl poxvirus in which the HN gene, F gene or both are expressed have recently been developed and approved. If virulent forms of APMV-1 are used in the production of vaccines or in challenge studies, the facility should meet the requirements for an appropriate biosafety and containment level determined by biorisk analysis. Regulatory oversight may be required in some countries.

A. INTRODUCTION

Newcastle disease (ND) is caused by virulent strains of avian paramyxovirus type 1 (APMV-1) of the genus *Orthoavulavirus* belonging to the subfamily *Avulavirinae*, family *Paramyxoviridae*. The paramyxoviruses isolated from avian species have been classified by serological testing and phylogenetic analysis into 21 serotypes of avian paramyxoviruses designated APMV-1 to APMV-21. Each virus belongs to a virus species that are dispersed amongst three genera: metaavulaulavirus, orthoavulavirus and paraavulavirus (Amarasinghe *et al.,* 2019; ICTV 2019).

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 APMV-1 has been their great variation in pathogenicity for chickens. Strains of APMV-1 have been grouped into five pathotypes on the basis of the clinical signs observed in infected chickens (Alexander & Senne, 2008b). 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. Subclinical: a form that usually consists of a subclinical enteric infection.

Pathotype groupings are rarely clear-cut 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.

APMV-1 may infect humans where the most common sign of infection is conjunctivitis that develops within 24 hours of virulent APMV-1 exposure to the eye (Swayne & King, 2003). Reported infections have been non-life threatening and usually not debilitating for more than a day or two. The most frequently reported and best-substantiated clinical signs in human infections have been eye infections, usually consisting of unilateral or bilateral reddening, excessive lachrymation, oedema of the eyelids, conjunctivitis and sub-conjunctival haemorrhage. Although the effect on the eye may be quite severe, infections are usually transient and the cornea is not affected. There is no evidence of human-to-human spread. There is one report of the isolation of the pigeon variant of APMV-1 (PPMV-1) from immunocompromised patients who died of pneumonia.

ND, as characterised in Section B.1.6 of this chapter, is subject to official control in most countries and the virus has a high risk of spread from the laboratory. All laboratory manipulations with live virus or potentially infected/contaminated material must be performed at an appropriate biosafety and containment level determined by biorisk analysis, as outlined in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities.* Countries lacking access to appropriate laboratory facilities should send specimens to a WOAH Reference Laboratory for the disease.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent						
Virus isolation	–	+++	+	+++	+	–
Conventional PCR and sequencing	+	++	+	+++	+	–
Real-time RT-PCR	++	+++[1]	++	+++[(a)]	+	–
Detection of immune response						
ELISA	+	+	++	–	++	++
HI	–	–	+	–	++	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay;
HI = haemagglutination inhibition.
[(a)]providing specified inclusive fit for purpose methods are used

1. Detection of the agent

Identification of APMV-1 viruses as the cause of infections and disease in poultry and other birds requires a thorough diagnostic investigation to differentiation from similar diseases caused by other viral agents especially influenza A

viruses. Individual APMV-1 and influenza A viruses isolates vary greatly in virulence, causing various syndromes evident as subclinical infections, drops in egg production, respiratory disease, and severe and high mortality disease. The latter clinical syndrome can be caused by either Newcastle disease (virulent APMV-1) or high pathogenicity avian influenza (HPAI) APMV viruses. It is judicious to have a single sampling procedure and simultaneously run specific differentiating diagnostic tests for both category of agents on field samples to obtain an accurate aetiological diagnosis for a single agent or, on occasion, confirmation of dual infection by APMV-1 and influenza A viruses.

1.1. Samples for virus isolation

Virus isolation is the reference method but is laborious and time intensive, used primarily for diagnosis of a first clinical case in an outbreak and to obtain virus isolates for further laboratory analysis.

For investigations 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 include intestinal contents (faeces) or cloacal swabs and oropharyngeal or tracheal swabs. Samples from lungs, air sacs, intestine, spleen, kidneys, caecal tonsils, brain, liver and heart should also be collected and processed either separately or as a pool. When pooling samples the brain should be collected and processed first (to avoid cross contamination with other tissue types) and kept separate as presence of virus in the brain may be an indicator of NDV or HPAI. Further pools should be made consistent with known virus tropisms between NDV and non-NDV APMV-1 viruses, i.e. grouped at the level of respiratory, systemic and gastrointestinal.

Samples from live birds should include both tracheal or oropharyngeal and cloacal swabs, the latter should be visibly coated with faecal material. To avoid harming them, swabbing of small delicate birds should be done with the use of especially small swabs that are usually commercially available and intended for use in human paediatrics or the collection of fresh faeces may serve as an adequate alternative (caution that some influenza A viruses and type 1 avulaviruses in birds can have a strong respiratory tropism). Similar swab samples can be pooled from the same anatomical site (i.e. cloacal swabs with cloacal swabs, oropharyngeal swabs with oropharyngeal swabs), and most commonly pooling of five or occasionally more, if appropriately validated not to reduce sensitivity of detection, but specific swab types should be used (Spackman *et al.,* 2013). Further the type of swabs used may affect test sensitivity or validity with thin wire or plastic shafted swabs preferred.

The samples should be placed in isotonic phosphate buffered saline (PBS), pH 7.0–7.4 with antibiotics or a solution containing protein and 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 oropharyngeal or tracheal swabs, but at five-fold higher concentrations for faeces and cloacal swabs. It is important to readjust the concentrated stock solution to pH 7.0–7.4 following the addition of the antibiotics. It is recommended that a solution for transport of the swabs should contain protein to stabilise the virus (e.g. brain–heart infusion, up to 5% [v/v] cattle serum, 0.5% [w/v] bovine albumen or similar commercially available transport media). If control of *Chlamydophila* is desired, 0.05–0.1 mg/ml oxytetracycline should be included. Faeces and finely homogenised 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. For prolonged storage, diagnostic samples and isolates should be kept at –80°C but for transport on dry ice (≤–50°C) is widely used. Repeated freezing and thawing should be avoided.

1.2. Virus isolation

The preferred method of growing APMV-1 viruses is by the inoculation of specific pathogen free (SPF) embryonated chicken eggs, or specific antibody negative (SAN) eggs. The supernatant fluids of faeces, swabs 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 three to five embryonated SPF or SAN chickens' eggs of 9–11 days incubation. After inoculation, these are incubated at 35–37°C for 2–7 days. To accelerate the final isolation, it is possible to carry out up to two passages at 1- to 3-day intervals, obtaining results comparable to two passages at 2–7-day intervals (Alexander & Senne, 2008a). 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 for a minimum of 2 hours or

overnight (and checked for embryo death before proceeding) and the allantoic fluids tested for haemagglutination (HA) activity. Fluids that give a negative reaction should be passed into at least one further batch of eggs. Routine checks for contamination should be conducted by streaking samples in Luria Broth agar plates and reading these at 24 and 48 hours of incubation against a light source. BHI agar and blood agar plates may also be used. For larger numbers of sample initial culture could be in tryptose phosphate broth. Contaminated samples can be treated by incubation with increased antibiotic concentrations for 2–4 hours (gentamicin, penicillin g, and amphotericin b solutions at final concentrations to a maximum of 1 mg/ml, 10,000 U/ml, and 20 µg/ml, respectively). Samples heavily contaminated by bacteria that cannot be removed by centrifugation or controlled by antibiotics can be filtrated through 0.45 and 0.2 micron sterile filters. Filtration should be used only when other methods fail because aggregation may significantly reduce virus titre.

Suspensions of homogenised organs, faeces or swabs prepared as for isolation in eggs may also be used for attempted isolation in cell cultures. APMV-1 strains can replicate in a variety of cell cultures of avian and non-avian origin, among which the most widely used are: chicken embryo liver (CEL) cells, chicken embryo kidney (CEK) cells, chicken embryo fibroblasts (CEF), African green monkey kidney (Vero) cells, avian myogenic (QM5) and chicken-embryo-related (CER) cells (Terregino & Capua, 2009). Primary cell cultures of avian origin are the most susceptible. To optimise the chances of viral recovery for isolates of low virulence, trypsin should be added to the culture medium. The concentration of trypsin will vary depending on the type of trypsin and the type of cells used. One example is to add 0.5 µg/ml of porcine trypsin to CEFs. Viral growth is usually accompanied by cytopathic effects typically represented by disruption of the monolayer and formation of syncytia.

The optimal culture system for the virus is to some extent strain-dependent. Some strains of APMV-1 grow poorly in cell culture and replicate to higher titre in embryonated eggs, whereas some strains of Pigeon APMV-1 (PPMV-1) and of APMV-1, such as the avirulent Ulster strain, can be isolated in chicken liver or chicken kidney cells but not in embryonated eggs. If possible, mainly when dealing with samples suspected of being infected with PPMV-1, virus isolation should be attempted using both substrates (embryonated eggs and primary chicken embryo cells). As the viral titre obtained in cell culture is usually very low, additional replication steps in embryonated eggs should be performed prior to characterisation of the isolate by HI or other phenotypic methods. Comparing virus isolation rates for APMV-1 *in ovo* with real-time reverse-transcription polymerase chain reaction (RT-PCR) using positive wild bird surveillance samples, it was demonstrated that there were no significant differences in isolation frequency when using embryonated chickens' eggs, embryonated duck eggs or embryonated turkey eggs. In contrast, largely significant rates of virus isolation were reported with embryonated bird eggs compared with either Madin–Darby canine kidney or Vero cell cultures for APMV-1 real-time RT-PCR positive samples (Moresco *et al.,* 2012). For animal welfare reasons, the number of embryonated eggs should be kept to a minimum, applying the principles of the 3Rs.

1.3. Virus identification

HA activity detected in bacteriologically sterile fluids harvested from inoculated chicken eggs may be due to the presence of any subtype of APMV (including APMV-1), 16 haemagglutinin subtypes of influenza A viruses from birds, haemagglutinating adenoviruses or bacterial HA. APMV-1 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 APMV-1 is used.

APMV-1 isolated from some wild bird species, e.g. cormorants, does not always demonstrate the ability to haemagglutinate RBCs. Replacement of chicken RBCs with turkey RBCs may be beneficial when testing viral-infected allantoic/amniotic fluid isolated from such species. Even when turkey RBCs are used, the HA activity of the virus remains low making the HA test unreliable for evaluating strains of APMV -1 from some wild bird species (Hines & Miller*,* 2012).

In the HI test, some level of cross-reactivity may be observed among the various avian paramyxovirus serotypes. Cross-reactivity can be observed between APMV-1 and APMV-3 viruses (particularly with the psittacine variant of APMV-3 , commonly isolated from pet or exotic birds), APMV-7 or APMV-12 . The risk of mistyping an isolate can be greatly reduced by using a panel of reference sera or monoclonal antibodies (MAbs) specific for APMV-1, APMV-3, APMV-7 and APMV-12. See also Section B.1.7.

At present, RT-PCR-based techniques for the detection and typing (pathotyping and genotyping) of APMV-1 RNA in allantoic fluid of inoculated fowl eggs is the common standard in diagnostic laboratories. However, the genetic variability of APMV -1 isolates should be considered carefully as potential cause for false negative results when using genetic-based laboratory tests if not shown to have inclusivity. See Sections B.1.5, B.1.8 and B.1.9 of the present chapter.

1.4. Pathogenicity index

The extreme variation in virulence of different APMV-1 isolates and the widespread use of live vaccines means that the identification of an isolate as APMV-1 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.1.6). In the past, tests including the mean death time in eggs, the intravenous pathogenicity test and variations of these tests have been used (Alexander & Senne, 2008b), but by international agreement, a definitive assessment of virus virulence is based on the intracerebral pathogenicity test (ICPI). The current WOAH definition (*Terrestrial Animal Health Code*, Chapter 10.9, *Infection with Newcastle disease virus*) also recognises the advances made in understanding the molecular basis of pathogenicity and allows confirmation of virus virulence, but not lack of virulence, by *in-vitro* tests that determine the amino acid sequence at the F0 protein cleavage site. Because of the severity of the procedure, ICPI should only be used where there is strong justification based on the epidemiological circumstances, for example in the first isolate from an outbreak (index case). It would not be appropriate to use ICPI for isolates detected in the course of routine surveillance of unvaccinated healthy birds. It is recommended to use only when other methods such as gene sequencing and clinical data provide anomalous results.

The *in-vivo* pathogenicity tests on strains isolated from species other than chickens (pigeons or doves for instance) can cause some problems and may not produce accurate readings until passaged in chickens or embryonated chickens' eggs as has frequently been reported for PPMV-1. A more accurate indication of the true pathogenicity of ND viruses for a susceptible species could come from experimental infection of a statistically significant number (≥10) of young and adult birds with a viral standard dose (e.g. $10^5 EID_{50}$) administered via natural routes (e.g. oro-nasal route).

1.4.1. Intracerebral pathogenicity index

i) Fresh infective allantoic fluid, free from influenza A and other extraneous agents, with a HA titre $>2^4$ (>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. (Birds that are alive but unable to eat or drink should be killed humanely and scored as dead at the next observation. 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.

The most virulent viruses will give indices that approach the maximum score of 2.0, whereas lentogenic and asymptomatic enteric strains will give values close to 0.0. Any score of ≥ 0.7 is considered virulent.

1.5. Molecular basis for pathogenicity

During replication, APMV-1 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-translational cleavage is mediated by host-cell proteases. Trypsin is capable of cleaving F0 for all NDV 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 allow the virus to 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, such as epithelial cells of the respiratory and gastrointestinal tracts.

Most APMV-1 viruses that are pathogenic for chickens have the sequence ^{112}R/K-R-Q/K/R-K/R-R^{116} (Choi *et al.*, 2010) 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 ^{112}G/E-K/R-Q-G/E-R^{116} and L (leucine) at residue 117. Some of the pigeon variant viruses (PPMV-1) examined have the sequence ^{112}G-R-Q/K-K-R-F^{117}, but give high ICPI values (Meulemans *et al.*, 2002). 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. However, some PPMV-1 may have virulent cleavage sites with variable ICPI values (Heiden *et al.,* 2014). This phenomenon has been associated not only with the fusion protein, but also with the replication complex consisting of the nucleoprotein, phosphoprotein and polymerase (Dortmans *et al.*, 2011; Heiden *et al.,* 2014).

Several studies have been undertaken 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 nucleotide sequencing with a view to establishing a routine *in-vitro* test for virulence (Miller *et al.*, 2010). Determination of the F0 cleavage sequence may give a clear indication of the virulence of the virus, and this has been incorporated into the definition of ND (see Section B.1.6).

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 APMV-1 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, means that virus isolation and an *in-vivo* assessment of virulence, such as an ICPI, will still be required. Determination of the cleavage site by sequencing or other methods has become the method of choice for initial assessment of the pathogenicity of these viruses and has been incorporated into agreed definitions. This has reduced the number of *in-vivo* tests, although the initial Sanger sequencing result of a F0 cleavage site for APMV-1 should be confirmed by either inoculation of birds or deep sequencing using high throughput sequencing with a minimum of 1000 reads to confirm no virus subpopulations of virulent virus.

Analyses of viruses isolated in Ireland in 1990 and during the outbreaks of ND in Australia since 1998 have given strong evidence that virulent viruses may arise from progenitor viruses of low virulence. Virulent virus has also been generated experimentally from low virulence virus by passage in chickens (Dortmans *et al.,* 2011).

1.6. Description of Newcastle disease

The vast majority of bird species appear to be susceptible to infection with APMV-1 of both high and low virulence for chickens, although the clinical signs seen in infected birds 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. Less acute signs include depression, diarrhoea, oedema of the head and neurological signs including torticollis but with very high levels of mortality. Egg shells may be soft, or birds may stop laying altogether. Moderately virulent (mesogenic) strains typically produce respiratory signs with neurological sequelae and levels of mortality <50%. Some strains, such as those from pigeons, may induce diarrhoea with neurological signs in fowl together with greatly reduced egg production. Low virulence strains produce mild respiratory disease or none at all. Exacerbating circumstances including co-infection with other pathogens, and poor husbandry may cause apparent increases in virulence. Thus, the clinical signs are variable and influenced by other factors so that none can be regarded as pathognomonic. Furthermore, the clinical spectrum of signs cannot easily be distinguished from those of high pathogenicity avian influenza.

Even for susceptible hosts, ND viruses produce a considerable range of clinical signs. Generally, variation consists of clusters around the two extremes in the ICPI test, but, for a variety of reasons, some viruses

may show intermediate virulence. The broad variation in virulence and clinical signs necessitates the careful definition of what constitutes ND for the purposes of trade, control measures and policies.

The definition of Newcastle disease for the purpose of disease notification and control measures is given in the *Terrestrial Animal Health Code.*

1.7. Monoclonal antibodies

Mouse monoclonal antibodies (MAbs) directed against strains of NDV have been used in HI tests to allow rapid identification of NDV without the possible cross-reactions with other APMV serotypes that may occur with polyclonal sera. Many MAbs have been produced that give reactions in HI tests that are specific for particular strains or variant NDV isolates (Alexander *et al.*, 1997).

Panels of MAbs have been used to establish antigenic profiles of NDV isolates based on whether or not they react with the viruses. Typical patterns of reactivity of PPMV-1 strains to MAbs can be used to differentiate these from other APMV-1 rapidly and inexpensively.

1.8. Phylogenetic studies

Development of improved techniques for nucleotide sequencing, the availability of sequence data for an increasing number of APMV-1 viruses in online databases and the demonstration that even relatively short sequence lengths could give meaningful results in phylogenetic analyses, have led to such approaches being widely used. Considerable genetic diversity has been detected, but viruses sharing temporal, geographical, antigenic or epidemiological parameters tend to fall into specific genetic lineages or genotypes and this has proven valuable in assessing both the global epidemiology and local spread of ND (Diel *et al.,* 2012; Dimitrov *et al.,* 2019).

The greater availability and increased speed of production of sequence data using sophisticated, commercially available kits for RT-PCR and automatic sequencing hardware now means that phylogenetic studies are within the capabilities of an increasing number of diagnostic laboratories and can give meaningful results that are contemporaneous rather than retrospective (Miller *et al.*, 2010). To classify new APMV-1 viruses, Diel *et al.* (2012) designed a unified nomenclature and a classification system based on the phylogenetic analyses of complete F gene sequences and on objective criteria to separate APMV-1 into genotypes and sub-genotypes. This work confirmed the existence of two main distinct phylogroups termed class I and II (Miller *et al.,* 2010). Class I largely includes low virulence strains recovered from live bird markets and wild waterfowl worldwide, while class II comprises the vast majority of viruses of high and low virulence isolated from poultry and wild birds. Within classes, a single genotype was revealed for Class I viruses whereas class II NDV isolates have been divided in 18 genotypes. Further revisions to the system of Diel *et al.* have been adopted based on clearly defined metrics for assigning viruses to genotypes and subgenotypes. This system analysed 1956 viruses and defined class II into 22 genotypes and class I into two genotypes (Dimitrov *et al.,* 2019). Through the use of the reference sequence datasets provided, these nomenclatures can be applied to phylogenetic investigations, with any new virus being assigned to a specific class (Class I or II) and to an established or new genotype. However, any virus classification system in use must be reviewed regularly, to ensure that any new or emerging viruses that fall outside the current classification system can be incorporated. Overall this analysis enables rapid epidemiological assessment of the origins and spread of viruses responsible for outbreaks. Furthermore, it enables clear separation of APMV-1 from vaccine viruses that may be detected as part of routine surveillance in poultry and facilitate precise mapping of such viruses including likely vaccine in the case of the latter.

1.9. Molecular techniques in diagnosis

The use of molecular techniques to detect APMV-1 in clinical samples have become the methods of choice in many diagnostic laboratories. In addition, RT-PCR and sequencing is widely used for the determination of the virulence of APMV-1 viruses (see Section B.1.5) or for phylogenetic studies. 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 (Creelan *et al.*, 2002). Tracheal or oropharyngeal swabs are often used as the specimens of choice, because they are easy to process and usually contain little extraneous organic material that can interfere with RNA recovery and amplification by PCR. However, tissue and organ samples and even faeces have been used with success as they may

contain higher viral loads of APMV-1. Strains of APMV-1 can have differential tropisms so it is recommended to collect and test both oropharyngeal and cloacal swabs when using this sample type.

The system used for RNA extraction will also affects the success of the RT-PCR on clinical samples and even with commercial kits care should be taken in selecting the most appropriate or validated system for the processing of samples to be analysed. Increasingly, given the difficulties in shipping biological materials containing infectious substances, commercially available chemically treated cards designed for the shipment of specimens for DNA and RNA analysis have been used (Perozo *et al.,* 2006), particularly in situations where the virus is already known to be endemic in a country.

Due to the large genetic diversity among APMV-1 viruses (Diel *et al.,* 2012; Dimitrov *et al.,* 2019), a laboratory testing algorithm should ideally be developed to take account of the purpose for which the test is being applied. Furthermore, this should consider the relevance of the test for the detection of local contemporaneously circulating strains in particular (but also consider risk for introduction of new viruses) and should be appropriately validated for the purpose for which it is used. This could include testing through inter laboratory ring trials. A molecular testing algorithm for NDV should ideally comprise a screening assay of high sensitivity and inclusivity targeting a conserved gene (i.e. matrix, L protein or polymerase genes) followed by a pathotyping assay. More usually applied to positive samples in screening assays is further examination using methodology directed to the F gene sequence determination enabling classification of virus as virulent (NDV) or avirulent APMV-1.

The main challenge with the use of conventional RT-PCR in diagnosis is the necessity for post-amplification processing because of the high potential for contamination of the laboratory and cross contamination of samples. Extreme precautions and strict regiments for handling samples are therefore necessary to prevent this (see Chapter 2.1.2 *Biotechnology advances in the diagnosis of infectious diseases*). However, one of the strategies used to circumvent this issue is to apply real-time RT-PCR techniques. The advantages are that real-time RT-PCR assays are based on the fluorogenic hydrolysis probes or fluorescent dyes which eliminates the post-amplification processing step and can provide results in a few hours. These types of assay have been successfully used during ND outbreaks when the laboratory may be required to test large numbers of samples. It may be that during an outbreak a bespoke assay with a perfect match and high sensitivity to the circulating strain may be applied. In principle, when confirming a new event, it is appropriate to use a combination of tests (i.e. at least two distinct independent laboratory tests for antigen or nucleic acid detection) and is certainly recommended for the confirmation of an index case.

This could be a sensitive real-time RT-PCR screening assay using a highly conserved gene followed by gene sequencing to determine virulence.

It is possible that bespoke assays that amplify a specific portion of the genome might provide added value, for example by amplifying part of the F gene that contains the F0 cleavage site so the product can additionally be used for simultaneous pathotyping (Fuller *et al.,* 2009; Steyer *et al.,* 2010) or sequence analysis. However due to the genetic diversity described in Section B.1.8 *Phylogenetic studies* universal F gene specific RT-PCR tests to detect and pathotype virulence remains a challenge. Furthermore, due to the variability in the F0 region encoding the cleavage site, currently available tests are of limited use and may fail to detect variants.

Most of the viruses affecting poultry reside in APMV-1 class II, but within this class there is extensive genetic diversity and even highly conserved genes such as the matrix or L protein contain some heterogeneity that might lead to false negatives during investigations. Furthermore, given the extensive use of live vaccine strains, such assays will not reliably discriminate between lentogenic, mesogenic and velogenic viruses. A detection system based on the L gene has been extensively validated with a wide range of genotypes (Sutton *et al.,* 2019) and provides a sensitive and reliable assay for the detection of both class I 1 and class II strains. Furthermore, it has been proven to have high utility during inter-laboratory trials producing reliable and reproducible results. Other assays have been developed to include both class I and class II viruses (Kim *at al.,* 2008). There is a wide range of other assays that have been reported and used, but they generally lack consistency on a broad spectrum of analyses including sensitivity, so should be carefully selected according to the local environment and strains circulating.

Some assays use more than one gene for detection and pathotyping APMV-1, for example both the F-gene and the M-gene in a single-tube reaction. In addition, duplex real-time assays have been developed

that can simultaneously detect and differentiate NDV and avian influenza virus (AIV) (Nguyen *et al.,* 2013). However, more recent developments include the application of analyser-based multiplex PCR technologies, for the simultaneous detection and differentiation of an even wider range of avian pathogens (Xie *et al.*, 2014). Rapid high-throughput methods have been also developed, which combine one-tube multiplex RT-PCR with bead-based hybridisation and detection technology, to simultaneously identify a number of avian respiratory viruses in a single or mixed infection (Laamiri *et al.,* 2016). At present, it should be noted that multiplexing RT-PCR or real-time RT-PCR assays aiming at broadening the range of virus detection frequently result in reduced sensitivity of the test compared with single target assays (Fuller *et al.*, 2010).

1.10. Gene sequencing

Currently real-time RT-PCR is the preferred method of virus surveillance because the test provides rapid sensitive diagnostics for NDV and is available in high throughputs. However, greater use of sequencing technologies, particularly as unit costs reduce with improvement in technology, offer powerful opportunities to simultaneously detect and sequence from clinical samples in a laboratory or field setting, for example applying nanopore technology (Butt *et al.,* 2018).

Sanger sequencing methodology has been widely used for decades and enables the rapid determination of typically a single (F) target gene in 24–36 hours to define virus virulence and still has widespread utility. However, as genomic data can be rapidly determined using high throughput sequencing technology, it enables a broader analysis using a range of bioinformatics tools. For example, with the advent of greater access to sequencing methodology either through specialised laboratories or commercial providers it is now possible to determine the genomic sequences of NDV from birds to provide a level of characterisation important in rapid pathogen identification and outbreak intervention. Conventionally nucleotide sequences have been used in outbreak epidemiology to infer virus origin and precise relationships between different viruses associated within the same event (by phylogeny) to support outbreak management. Virus gene sequences of F or HN neuraminidase can rapidly be compared with known sequences in gene databases and used to reveal closest match thereby identifying the virus. This often avoids the need to culture the virus for rapid identification although reliability and quality of data reduces with increasing cycle threshold values in samples from real-time RT-PCR testing. Occasionally such analyses have been applied at the whole genome level and can offer greater analytical specificity to the analyses when dealing with groups of very closely related ND viruses.

2. Serological tests

NDV may be employed as an antigen in a wide range of serological tests, enabling neutralisation or enzyme-linked immunosorbent assays (ELISA) and HI to be used for assessing antibody levels in birds. At present, the HI test is most widely used for detecting antibodies to APMV-1 in birds while the use of commercial ELISA kits to assess post-vaccination antibody levels is common. In general, virus neutralisation or HI titres and ELISA-derived titres correlate at the flock level rather than at the level of individual birds. Serological assays are also used in diagnostic laboratories to assess antibody response following vaccination, but have limited value in surveillance and diagnosis of ND because of the almost universal use of vaccines in domestic poultry. Furthermore, reference antigens produced with historic strains may reduce the sensitivity of HI assay when used for the detection of antibodies against ND viruses currently circulating. For this reason it is important to investigate the antigenic relationships between the antigen used in the laboratory and current circulating viruses.

2.1. Haemagglutination and haemagglutination inhibition tests

Chicken sera rarely give nonspecific positive reactions in the HI 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 (U-bottomed can be used but care in reading is required as the clarity is less defined) 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 (0.01 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 NDV). 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.

2.1.1. 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 or inactivated 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.

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.

2.1.2. 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 (positive serum, virus/antigen and PBS controls) 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 (>2^2 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 (2^4 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 (2^3 or $\log_2 3$) or more. Back titration of antigen should be included in all tests to verify the number of HAU used.

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, 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 NDV (Alexander *et al.*, 1983).

2.2. Enzyme-linked immunosorbent assay

There is a variety of commercial ELISA kits available including those formally registered with WOAH[1] and these are based on several different strategies for the detection of NDV 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 and their performance verified within the laboratory. The HI test and ELISA may measure antibodies to different antigens; depending on the system used ELISAs may detect antibodies to more than one antigen while the HI test is probably restricted to those directed against the HN protein. However, comparative studies have demonstrated that the ELISAs are reproducible and have high sensitivity and specificity; they have been found to correlate well with the HI test (Brown *et al.*, 1990). Conventional ELISAs have the disadvantage that it is necessary to validate the test for each species of bird for which they are used. Competitive ELISAs may not recognise all strains of APMV-1 if they use MAb known for their specificity for single epitopes.

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

A detailed account of all aspects of NDV vaccines, including their production and use, has been published (Brown *et al.,* 2019) and should be referred to for details of the procedures outlined here. 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. If virulent forms of APMV-1 (NDV) are used in the production of vaccines or in challenge studies, the facility should meet the requirements for an appropriate biosafety and containment level determined by biorisk analysis as described in chapter 1.1.4.

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 NDV are expressed. Several of these recombinant viruses have been approved for use in certain countries.

NDV strains used in conventional commercial live virus vaccines fall into two groups: lentogenic vaccines, such as Hitchner-B_1, LaSota, V4, NDW, I2 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.1.6), but as these vaccines are used primarily in countries where ND is endemic this may not necessarily preclude their use. In the USA, the 9CFR 121.3b.818 states that NDV strains with ICPI values equal to or greater than 0.7 are virulent and reportable, leaving APMV-1 isolates of low virulence to be used as vaccines. The European Union stated in their Commission Decision 93/152/EEC (European Commission, 1993) that for routine ND vaccination programs the viruses used as live NDV vaccines are to be tested under specific conditions and have an ICPI of less than 0.4 or 0.5, depending on the dose of vaccine given. WOAH Biological Standards Commission similarly recommended in 2000 that in principle vaccines should have an ICPI <0.7. However, 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.

Live virus vaccines may be administered to birds by incorporation in the drinking water, delivered as a coarse spray (aerosol), or by intranasal or conjunctival instillation. A live vaccine formulated from a APMV-1 of low virulence for use *in ovo* has been approved for use in the USA. Some mesogenic strains

1 https://www.woah.org/en/scientific-expertise/registration-of-diagnostic-kits/background-information/

are given by wing-web intradermal inoculation. Vaccines have been constructed to give optimum results through application by specific routes.

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 (BPL). This is incorporated into an emulsion with mineral oil or vegetable 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 greater amount of antigen is required for immunisation than for live virus vaccination.

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 2–4 weeks later. Vaccination of fully susceptible 1-day-old birds, even with live vaccines of the lowest virulence, may result in respiratory disease, especially if common pathogenic bacteria are present in significant numbers.

Re-vaccination of layers should be performed 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. Layers that have high serological titres for NDV are protected against drop in egg production and poor egg quality (shell-less, soft shelled eggs, off-coloured eggs) (Brown *et al.,* 2019). The level of homology between the vaccine strain and the field virus can influence the degree of protection against reduced egg production (Cho *et al.,* 2008).

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 (Brown *et al.,* 2019). Two examples of vaccination programmes that may be used in different disease circumstances are listed here. For the first example, when the disease is mild and sporadic, it is suggested that the following order of vaccination be adopted: live Hitchner-B_1 by conjunctival or spray administration at 1 day of age; live Hitchner-B_1 or LaSota at 18–21 days of age in the drinking water; live LaSota 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 LaSota 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 (Brown *et al.,* 2019). The first protocol is generally applicable to countries were virulent NDV is not endemic and is intended to minimise productivity losses by using a milder vaccine during the initial vaccination. Considering possible constraints of ND vaccination, particularly applying to live vaccines, proper immunisation should be validated by serological testing of vaccinated flocks. Regardless of which test system would be applied, i.e. ELISA or HI, humoral immune response should be demonstrated at the flock level.

When HI is used to evaluate the immune response after vaccination, it should be taken into account that HI titres are greatly influenced by the quality of vaccine, the route and method of administration, environmental and individual factors, but also depend on the species (e.g. generally the HI response of some species, such as turkey and pigeon, is lower than that of chicken). It is also recommended to inactivate nonspecific haemagglutinating agents often present in the serum of some species such as game birds (pheasant, partridge, etc.), quails, ostriches and guinea fowl, by heat treatment in a water bath at 56°C for 30 minutes.

Single vaccinations with live lentogenic virus may produce a response in susceptible birds of about 4–6 $\log_2$, but HI titres as high as 11 $\log_2$ or more may be obtained following a vaccination programme involving oil-emulsion vaccines. The actual titres obtained and their relationship to the type of protection and duration of immunity for a given flock and programme are difficult to predict. Variation in HI titres

may occur for nonspecific factors, for instance due to the antigenic correlations, infection with other APMVs (e.g. APMV-3) may result in significant increased titres to NDV. The HI titre is also influenced by the characteristics of antigen used. For instance, the use of the homologous La Sota antigen in the HI assay after vaccination with this virus resulted in significantly higher titres than when heterologous Ulster virus was used (Maas *et al.,* 1998). Furthermore, reference antigens produced with historic strains may reduce the sensitivity of HI assay when used for the detection of antibodies against ND viruses currently circulating. For this reason, it is important to investigate the antigenic relationships between the antigen used in the laboratory and current circulating viruses, and between vaccine strains and reference HA antigens, to avoid misjudgements in estimating serum antibody titres.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

The first principle to consider when selecting a strain for a live NDV 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. 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 LaSota strain will cause considerably greater problems in young susceptible birds than the Hitchner-B_1 strain, the Ulster based vaccines, or specific LaSota clones, although in general the regular LaSota vaccine induces a stronger immune response. There is detectable variation in the antigenicity of different circulating strains, which may indicate a need to tailor vaccines more carefully to relate antigenically to any prevalent field virus (Miller *et al.*, 2007).

Live vaccines using either of two avirulent Australian NDV strains selected for their heat stability, V4 or I-2, have been used with animal feed acting as carriers to combat the specific problems associated with village chicken rearing in developing countries with variable success. The intention is that this vaccine could be coated on food easily fed to roaming chickens while being slightly more resistant to inactivated by high ambient temperatures. Vaccines with both viral strains have been formulated that produce sufficient HI antibody titres (Olabode *et al.*, 2010) and in some instances prevent mortality after virulent challenge (Wambura, 2011).

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. Some lentogenic strains grow to very high titres in eggs.

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

The master seed should be checked after preparation for sterility, safety, potency and extraneous agents. The master seed should be free of bacterial (including Salmonella), fungal, and mycoplasma contamination, and should be free of extraneous viruses. In addition to laboratory tests for the detection of avian lymphoid leukosis, cytopathic and hemadsorbing agents, chicken anaemia virus and reticuloendotheliosis virus, the master seed used in live vaccines should be evaluated for pathogens by inoculation into embryonated chickens' eggs as well as by inoculation into healthy chickens that have not been vaccinated against ND.

2.2. Method of manufacture

2.2.1. Procedure

The vaccine production facility should operate under the appropriate bio-security procedures and practices. If ND, as defined in Section B.1.6 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 biosafety and biosecurity as outlined in chapter 1.1.4 of this *Terrestrial Manual.*

A master seed is established, and from this a working seed. If the strain has been cloned through a 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). Seed viruses of unknown pedigree should be passed through SPF eggs and cloned before producing the master seed. Some passage through SPF chickens may also be desirable (Allan *et al.*, 1978).

For vaccine production, a working seed, from which batches of vaccine are produced, is first established by expansion 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 (Allan *et al.*, 1978).

Most ND vaccines are produced in embryonated chickens' eggs, and live virus vaccines should be produced in SPF eggs. The method of production is large-scale aseptic propagation of the virus with all procedures performed under sterile conditions. It is usual to dilute the working seed in sterile PBS, pH 7.2, so that roughly 10^3–10^6 EID_{50}/0.1–0.2 ml is inoculated into the allantoic cavity of 9- or 10-day-old embryonated SPF chickens' 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 BPL (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 or BayolF, plus one volume of emulsifying agent, such as Arlacel A, Montanide 80 or Montanide 888. 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. If the viscosity is too high viscosity and the vaccine is difficult to inject; too low viscosity and the vaccine is unstable.

2.2.2. Requirements for substrates and media

Most live virus vaccines are grown in the allantoic cavity of embryonated chickens' eggs but some, notably some mesogenic strains, have been adapted to a variety of tissue culture systems. In the USA, both live and killed ND vaccines are prepared in SPF eggs.

2.2.3. In-process controls

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. In the USA, passage is not required unless the results are inconclusive.

2.2.4. Final product batch tests

i) Sterility/purity

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9. In the USA, several purity tests are conducted on each serial of a live vaccine. Most of these may be omitted for killed products if the inactivating agent renders the test results meaningless.

ii) Safety

Some countries also require back passage studies for live NDV vaccine to ensure that the pathogenicity is not increased by cycling through birds (Code of Federal Regulations [CFR], 2019).

iii) Batch potency

Each batch of live vaccine virus should be tested for viability and potency. 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 passing each three times through SPF embryos (Allan *et al.*, 1978).

Most countries have published specifications for the control of production and testing of NDV vaccines, which include the definition of the obligatory tests on vaccines during and after manufacture. In Europe, the European Pharmacopoeia states that 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 potency test.

In the USA, each serial batch of inactivated ND vaccine is tested for potency by vaccination-challenge (CFR, 2019). At least ten vaccinates and ten control birds, 2–6 weeks of age, must be used. At least 90% of the control birds must show typical signs of Newcastle disease or die, and at least 90% of the vaccinates must remain normal during the 14 day post-challenge period. In the USA, each serial batch and each subserial of live ND vaccine must have a virus titer that is at least $10^{0.7}$ EID_{50} greater than the titer of the virus used in the immunogenicity study described above (CFR, 2019). The minimum titre shall not be less than $10^{5.5}$ EID_{50}.

The infectivity of live virus vaccines is tested by titrating the virus in embryonated chickens' eggs to calculate the EID_{50}. This involves making tenfold dilutions of virus; 0.1 ml of each dilution is inoculated into five 9 to 10-day-old embryonated chickens' eggs. After 5–7 days of 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 EID_{50} end-point is calculated using a standard formula such as Spearman–Kärber or Reed Muench (Thayer & Beard, 2008).

2.3. Requirements for regulatory approval

2.3.1. Safety requirements

i) Target and non-target animal safety

Live NDV 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 washing of the site with removal of the material, including incision of tissues, as for a 'grease-gun' injury.

ii) Reversion-to-virulence for attenuated/live vaccines

The 9CFR 113.329.768 states that in the USA the use of chickens for the testing of NDV vaccines involves the inoculation of twenty-five SPF birds, five days of age or younger. 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. An alternative is to use the prechallenge part of the potency test, described below, as a safety test and if unfavourable reactions that are attributable to the product occur, the test is declared inconclusive and the safety test is repeated. If not repeated satisfactorily, the batch is declared unsatisfactory (CFR, 2019). In the USA the safety test is done with a single dose, administered to chickens 2–6 weeks old (CFR, 2019); the prechallenge part of the potency test can serve as the safety test.

In view of the finding that virulent NDV can emerge by mutation from virus of low virulence, the introduction of wholly new strains of ND in live vaccines should be considered carefully and the vaccines subjected to evaluation before use. Recombinant strains that are used in live vaccines in the USA are subject to additional safety requirements. The genetic stability of the virus should be demonstrated at the highest passage level to be used in production. The phenotypic effect of any genetic modification(s) should be thoroughly assessed to ensure that the genetic modifications have not resulted in any unexpected effects *in vivo*. Studies should be performed in chickens to evaluate possible alterations in tissue tropism, as well as to evaluate whether the vaccine virus is shed. Recombinant strains that are shed into the environment must be evaluated for safety in non-target avian species as well as in mammalian species, and the ability to persist in the environment under field conditions should be addressed.

iii) Environmental consideration

None.

2.3.2. Efficacy requirements

i) For animal production

Various methods for the testing of NDV vaccines for potency have been proposed. The importance of using a suitable challenge strain for assessment has been stressed (Allan *et al.*, 1978). Challenge strains used in Europe and the USA are Herts 33 or GB Texas, respectively. For live vaccines, the method recommended involves the vaccination of 10 or more SPF or other fully susceptible birds, some countries specify 20 birds, at the minimum recommended age by the suggested route using the minimum recommended dose. After 14–28 days, each vaccinated bird and ten control birds are challenged intramuscularly with at least 10^4 EID (50% egg infectious dose) or 10^5 LD_{50} (50% lethal dose) of ND challenge virus. Challenged birds are observed for 14 days; at least 90% of the control birds must develop clinical signs and die within 6 days of Newcastle disease. If at least 90–95% of the vaccinates do not remain free of clinical signs, the master seed is unsatisfactory.

For inactivated vaccines, in Europe 21- to 28-day-old SPF or susceptible chickens are used. Three groups of 20 birds each 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 10^6 LD_{50} of ND challenge virus, 17–21 days later. Chickens are observed for 21 days. The PD_{50} (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 PD_{50} is not less than 50 per dose and if the lower confidence limit is not less than 35 PD_{50} 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.

The recommended efficacy test for inactivated vaccines in the USA is a vaccination–challenge study (CFR, 2009). At least ten SPF chickens, 2–6 weeks old, are vaccinated with the minimum recommended dose. The 9CFR 113.205.727 states that after 14 days post-vaccination, the vaccinates and at least ten unvaccinated controls are challenged with the GB Texas strain of Newcastle disease virus and the vaccinates are observed for 14 days. At least 90% of the control birds must develop clinical signs of Newcastle disease during the observation period. If at least 90% of the vaccinates do not remain free of clinical signs, the master seed is unsatisfactory.

ii) For control and eradication

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 (Allan *et al.*, 1978). Most commercial vaccines have been designed to control clinical signs however they do not prevent viral replication and are not suitable for eradication.

Transmission of the ND virus in an area might be interrupted only if a very high percentage of the resident susceptible population (> 80%) is sufficiently immunised showing an Ab titre ≥ 1:8 (Brown *et al.,* 2019).

2.3.3. Stability

When stored under the recommended conditions the final vaccine product should maintain its potency for at least the designated shelf life of the product. Accelerated stability tests such as reduction of infectivity following incubation at 37°C for 7 days (Lensing, 1974) 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. The USA requires real-time stability to be demonstrated on at least three sequential serials of NDV vaccine (CFR, 2019). Each serial should be evaluated at multiple intervals until the expiration date has been reached in order to develop a degradation profile for the product.

Live virus vaccines must be used immediately after reconstitution. Inactivated vaccines must not be frozen. In most countries, preservatives must not be included in the freeze-dried live product, but antimicrobial preservatives may be incorporated in the diluent used to reconstitute the vaccine. An alternative used in the USA is to allow the use of certain preservatives, but they must be indicated on the labelling.

3. Vaccines based on biotechnology

3.1. Vaccines available and their advantages

The advent of recombinant DNA technology has resulted in the development of novel NDV vaccines. One class consists of vector vaccines, which consist of a suitable carrier virus that expresses one or more immunogenic NDV proteins (usually F and/or HN), thereby inducing an immune response against both NDV and the vector virus itself. Examples of such vector vaccines are recombinants based on Vaccinia virus (Meulemans, 1988), Fowlpox virus (Boursnell *et al.*, 1990; Olabode *et al.*, 2010), Pigeonpox virus (Letellier *et al.*, 1991), Herpesvirus of turkeys (Heckert *et al.*, 1996), Marek's disease virus (Sakaguchi *et al.*, 1998) and avian adeno-associated virus (Perozo *et al.*, 2008).

Other approaches include the development of subunit vaccines based on the large scale expression of NDV proteins (usually F and/or HN) using baculovirus vectors (Lee *et al.*, 2008); or plants (Berinstein *et al.*, 2005) and the use of DNA vaccines, i.e. plasmid DNA encoding relevant immunogenic NDV proteins (Loke *et al.*, 2005). The establishment of a reverse genetics system for NDV (Peeters *et al.*, 1999) has made it possible to genetically modify the NDV genome and to develop NDV strains with new properties. These include the implementation of serological differentiation (DIVA vaccines (Mebatsion *et al.*, 2002; Peeters *et al.*, 2001) and the incorporation and expression of foreign genes, thereby making NDV itself a vaccine vector for application in poultry (Nakaya *et al.*, 2001) and other species, including primates (Dinapoli *et al.*, 2007).

The desired profile for NDV vaccines include: 1) prevention of transmission; 2) differentiation of infected from vaccinated animals (DIVA); 3) induction of protection with a single dose; 4) maternal antibody override; 5) mass vaccination; 6) cross-protection against variant strains, 7) Increased safety and minimal side effects. Some of the above-mentioned recombinant vaccines reach or surpass the efficiency of conventional vaccines in terms of antibody induction or protection against a virulent challenge strain, and thus they show great promise for future use. Moreover, they offer a number of advantages compared

to conventional NDV live vaccines, such as i) improved safety for vaccinated birds due to the absence of residual virulence, ii) implementation of the DIVA principle, iii) closer immunogenic match with outbreak strains, iv) possibility of an optimally controlled *in ovo* mass vaccination, v) long lasting immunity provided by a single vaccine shot (Palya *et al*., 2014).

Only few of the above-mentioned biotechnological vaccines have been approved in certain countries for application in poultry. A problem for some of the vaccines mentioned here may be that existing immunity against the vector might interfere with generic application of such vaccines in the field. As most vector vaccines are based on viruses that are themselves potential avian pathogens, it is difficult to guarantee complete safety under field circumstances. These problems can be overcome with the apathogenic turkey herpesvirus based recombinant vaccine (Palya *et al.,* 2012). The fact that most of these vaccines are genetically modified organisms (GMO) means that they have to go through a rigorous and tedious testing and registration process. Furthermore, the production of biotechnological vaccines is likely more expensive than that of classical NDV vaccines. As currently used classical vaccines are cheap and adequate, at least for the protection of poultry against clinical signs and death, a real incentive for veterinary pharmaceutical companies to develop new vaccines is lacking. It is likely that poultry farmers would be willing to pay a higher price for a vaccine only if it offers significant advantages over conventional vaccines. It is unlikely that this situation will soon change unless national or international authorities modify the requirements for ND vaccines such as a minimum requirement for the reduction of shedding of challenge virus, interruption of virus transmission or the implementation of the DIVA principle. It must also be considered that the application of new, safe and efficacious vectored vaccines *in ovo* or at 1 day of age, when used alone in countries with a high risk of ND, will not provide the necessary protection. In these epidemiological scenarios early protection must be reinforced by the administration at the hatchery of a live attenuated vaccine followed by a booster vaccination at approximately 2 weeks of age as a protective immunity induced by vector-based vaccines is only achieved some weeks after the administration.

Recurrent outbreaks of ND in the face of vaccination has raised the question whether currently used ND vaccines are still adequate, not only for the protection against clinical disease, but also for the inhibition of virus transmission (Mayers *et al.,* 2017). Some studies have indicated that the extent of homology between vaccine and challenge strain is important in reducing the shedding of virulent virus (Hu *et al.,* 2009; Miller *et al.,* 2007) but this may not necessarily correlate with disease or transmission. It has been demonstrated that exchanging the F and HN genes of a vaccine strain with the corresponding genes of an outbreak strain resulted in a vaccine that was much better able to reduce virus shedding of the outbreak strain than the unmodified vaccine. However, vaccine failure may be attributable to many factors including importantly, poor vaccination practices. Dortmans *et al.* (2014) demonstrated that susceptibility of vaccinated poultry to NDV infection was not a result of vaccine mismatch, when they challenged field vaccinated chickens with contemporary NDV of different genotypes. Further work is required to fully define and understand factors that will lead to reduced vaccine efficacy in a field environment.

3.2. Special requirements for biotechnological vaccines

Once regulatory approval has been gained, biotechnological vaccines have to fulfil the same or similar requirements as classical vaccines as detailed above (Section C: Requirements for vaccines and diagnostic biologicals).

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NB: There are WOAH Reference Laboratories for Newcastle disease (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for Newcastle disease

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2021.