

## CHAPTER 2.3.14.

# NEWCASTLE DISEASE

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### SUMMARY

Newcastle disease (ND) is caused by virulent strains of avian paramyxovirus type 1 (APMV-1) of the genus *Avulavirus* belonging to the family *Paramyxoviridae*. There are ten serotypes of avian paramyxoviruses designated APMV-1 to APMV-10.

ND virus (NDV) 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 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 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 9–11-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 and/or by use of validated specific molecular methods.

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 and APMV-7.

The intracerebral pathogenicity index (ICPI) can be used to determine the virulence of any newly isolated APMV-1. Alternatively, virulence can also be evaluated using molecular techniques, i.e. reverse-transcription polymerase chain reaction and sequencing. 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.

**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.

**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 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.

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 licensed.

If virulent forms of NDV are used in the production of vaccines or in challenge studies, the facility should meet the OIE requirements for Containment Group 4 pathogens, which is generally equivalent to the United States Department of Agriculture's Biosafety Level 3-Agriculture or Enhanced (BSL3-Ag or BSL3-E). Additional 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) serotype of the genus *Avulavirus* belonging to the subfamily *Paramyxovirinae*, family *Paramyxoviridae*. The paramyxoviruses isolated from avian species have been classified by serological testing and phylogenetic analysis into ten subtypes designated APMV-1 to APMV-10 (Miller *et al.*, 2010a); ND virus (NDV) has been designated APMV-1 (Alexander & Senne, 2008b).

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 NDV has been their great variation in pathogenicity for chickens. Strains of NDV have been grouped into five pathotypes on the basis of the clinical signs seen 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. Asymptomatic: a form that usually consists of a subclinical enteric infection.

Pathotype groupings are rarely clear-cut (Alexander & Allan, 1974) 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.

NDV is a human pathogen and the most common sign of infection in humans is conjunctivitis that develops within 24 hours of NDV 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 (Chang, 1981). 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 a pigeon-like APMV-1 from lung tissue, urine and faeces of an immunocompromised patient who died of pneumonia (Goebel *et al.*, 2007).

ND, as defined in Section B.1.f of this chapter, is subject to official control in most countries 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 biosafety and 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 Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities. Within the facility, work should be carried out at biosafety level 2 or above. Countries lacking access to such a specialised national or regional laboratory should send specimens to an OIE Reference Laboratory.

## B. 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), caecal tonsils, spleen, brain, liver and heart tissues. These may be collected separately or as a pool, although brain and intestinal samples are usually processed separately from other samples.

Samples from live birds should include both tracheal or oropharyngeal and cloacal swabs, the latter should be visibly coated with faecal material. Swabbing may harm small, delicate birds, 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), tracheal (or oropharyngeal) 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. Protein-based media, e.g. brain–heart infusion (BHI) or tris-buffered tryptose broth (TBTB), have also been used and may give added stability to the virus, especially during shipping. 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 concentrated stock solution to pH 7.0–7.4 before adding it to the sample. If control of *Chlamydophila* is desired, 0.05–0.1 mg/ml oxytetracycline should be included. 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.

#### b) Virus isolation (the prescribed test for international trade)

The supernatant fluids of faeces or tissue suspensions and swabs, 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. If SPF eggs are not available, at least NDV antibody negative eggs are required. After inoculation, these are incubated at 35–37°C for 4–7 days. To accelerate the final isolation, it is possible to carry out two passages at a 3-day interval, obtaining results comparable to two passages at 4–7-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 4 hours or overnight 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. 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 agglutination may significantly reduce virus titre.

Suspension of homogenated 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. In order 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

PMV-1 (PPMV-1) and of APMV-1, such as the apathogenic Ulster strain, can be isolated in chicken liver or chicken kidney cells but not in embryonated eggs (Kouwenhoven, 1993). 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.

### c) Virus identification

HA activity detected in bacteriologically sterile fluids harvested from inoculated eggs may be due to the presence of any of the ten subtypes of APMV (including NDV) or 16 haemagglutinin subtypes of influenza A viruses, or. Nonsterile fluid could contain bacterial HA. NDV 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 NDV is used.

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) or APMV-7. 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 and APMV-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 becoming increasingly common in diagnostic laboratories. However, the genetic variability of APMV-1 isolates should be considered carefully as potential cause for false negative results of genetic-based laboratory tests. See Sections 1e; 1h and 1i of the present chapter.

### d) Pathogenicity index

The extreme variation in virulence of different NDV 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.f below 'Definition of Newcastle disease'). In the past, such tests as 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 OIE definition (Section B.1.f below) 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. It would not be appropriate to use ICPI for isolates detected in the course of routine surveillance of healthy birds.

The *in vivo* 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 chicken eggs (Alexander & Parsons, 1986.) 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 ( $\geq 10$ ) of young and adult birds with a viral standard dose (e.g.  $10^5 \text{EID}_{50}$ ) administered via natural routes (e.g. oro-nasal route).

- **Intracerebral pathogenicity index**

- i) Fresh infective allantoic fluid 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.

**e) 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 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 APMV-1 viruses that are pathogenic for chickens have the sequence <sup>112</sup>R/K-R-Q/K/R-K/R-R<sup>116</sup> (Choi *et al.*, 2010; Kim *et al.*, 2008a) 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 <sup>112</sup>G/E-K/R-Q-G/E-R<sup>116</sup> and L (leucine) at residue 117. Some of the pigeon variant viruses (PPMV-1) examined have the sequence <sup>112</sup>G-R-Q/K-K-R-F<sup>117</sup>, 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 low ICPI values (Collins *et al.*, 1994). This phenomena has been associated not with the fusion protein (Dortmans *et al.*, 2009), but with the replication complex consisting of the nucleoprotein, phosphoprotein and polymerase (Dortmans *et al.*, 2010).

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 (Miller *et al.*, 2010b). 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.f).

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 NDV 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.

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 (Alexander & Senne, 2008b). Virulent NDV has also been generated experimentally from low virulence virus by passage in chickens (Shengqing *et al.*, 2002).

**f) Definition 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. Thus, the clinical signs are variable and influenced by other factors so that none can be regarded as pathognomonic.

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 ND currently in use in all member states of the European Union is defined in Directive 92/66/EEC of the Commission for European Communities.

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 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.

**h) Phylogenetic studies**

Development of improved techniques for nucleotide sequencing, the availability of sequence data of more APMV-1 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 (Aldous *et al.*, 2003; Cattoli *et al.*, 2010; Czegledi *et al.*, 2006; Kim *et al.*, 2007).

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 (Miller *et al.*, 2010b). Aldous *et al.* (2003) proposed that genotyping of NDV isolates should become part of diagnostic virus characterisation for reference laboratories by producing a 375-nucleotide sequence of the F gene, which includes the F0 cleavage site, routinely for all viruses and comparing the sequences obtained with other recent isolates and 18 viruses representative of the recognised lineages and sub-lineages. Such analysis should allow rapid epidemiological assessment of the origins and spread of the viruses responsible for ND outbreaks.

**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 has been increasing use of molecular techniques to detect NDV in clinical specimens, the advantage being the extremely rapid demonstration of the presence of virus. 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; Nanthakumar *et al.*, 2000). 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 some success. The system used for RNA extraction will also affect the success of RT-PCR on clinical specimens and even with commercial kits care should be taken in selecting the most appropriate or validated for the samples to be analysed.

Usually RT-PCR systems have been used to amplify a specific portion of the genome that will give added value; for example by amplifying part of the F gene that contains the F0 cleavage site so that the product can be used for assessing virulence (Creelan *et al.*, 2002). Perhaps the most serious problem with the use of 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 regimens for handling samples are necessary to prevent this (see Chapter 1.1.5 Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases).

One of the strategies used to avoid post-amplification processing is to employ real-time RT-PCR (rRT-PCR) techniques. The advantages of such assays are that rRT-PCR assays based on the fluorogenic hydrolysis

probes or fluorescent dyes eliminate the post-amplification processing step and that results can be obtained in less than 3 hours. At present, the widest application of an rRT-PCR assay for APMV-1 detection was in the United States of America (USA) during the ND outbreaks of 2002–2003, when the assay described by Wise *et al.* (2004) was employed. The primers and probes in this report were validated on lentogenic, mesogenic and velogenic strains circulating in the USA. At the peak of the outbreak, between 1000 and 1500 samples were tested daily by rRT-PCR. However those protocols do not detect all NDV strains and a more conserved part of the genome should be targeted or a multiple testing approach (i.e. at least two distinct independent laboratory tests for antigen detection) may be needed for detecting the index case.

In fact, one important problem is that APMV-1 isolates have been shown to be genetically distinct. For example, one group of viruses, which were placed in genogroup 6 by Aldous *et al.* (2003) and subsequently Class I by Czegledi *et al.* (2006), are so different from all the other APMV-1 isolates, i.e. Class II viruses (Czegledi *et al.*, 2006) that different primers would be necessary for their detection in RT-PCR tests. Furthermore, it has been recently demonstrated that also within class II APMV-1 viruses, the matrix gene is not truly highly conserved and false negatives occurred in case of outbreak investigations or routine surveillance in poultry using the USDA-validated real-time RT-PCR assay targeting this gene (Cattoli *et al.*, 2009; 2010; Khan *et al.*, 2010). In addition the matrix gene-based real-time PCR that is generally used to identify APMV-1 does not discriminate between lentogenic and mesogenic/velogenic strains, therefore it should be used as screening test for the presence of APMV-1 RNA in the samples and not for detection or confirmation of ND outbreaks. This is particularly true in regions or countries that use live vaccines in poultry routinely. A universal fusion gene-specific rRT-PCR test to detect and pathotype determine virulence would be useful as it would allow for quick pathotyping, however because of the variability of the region encoding for the cleavage site, available tests are of limited use and could fail to detect variants. A promising approach that involves the inclusion of class I viruses within one rRT-PCR was done by Kim *et al.* 2008, combining primers for class I and class II (Kim *et al.*, 2008b). At present, it should be noted that multiplexing RT-PCR or rRT-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; Liu *et al.*, 2010).

## 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.

### a) 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 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.

- **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.
- **Haemagglutination inhibition test (an alternative test for international trade)**
    - 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 (Alexander & Allan, 1974), 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).

#### b) Enzyme-linked immunosorbent assay

There are a variety of commercial ELISA kits available 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. 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

#### a) Rationale and intended use of the product

A detailed account of all aspects of NDV vaccines, including their production and use, has been published (Allan *et al.*, 1978) 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 NDV are used in the production of vaccines or in challenge studies, the facility should meet the OIE requirements for Containment Group 4 pathogens as described in Chapter 1.1.2.

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 licensed for use in certain countries.

NDV strains used in conventional commercial live virus vaccines fall into two groups: lentogenic vaccines, such as Hitchner-B<sub>1</sub>, 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.f), 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 NDV 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. The OIE 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 NDV of low virulence for use *in ovo* has been licensed for use in the USA. Some mesogenic strains 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. 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 much larger 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 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. 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) (Allan *et al.*, 1978; Stone *et al.*, 1975). 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 (Allan *et al.*, 1978). 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<sub>1</sub> by conjunctival or spray administration at 1 day of age; live Hitchner-B<sub>1</sub> 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 (Allan *et al.*, 1978). The first protocol is generally applicable to countries where 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<sub>2</sub>, but HI titres as high as 11 log<sub>2</sub> 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 AMPVs (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

### a) Characteristics of the seed

#### i) *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<sub>1</sub> 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 inactivation by high ambient temperatures. Recently, 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.

ii) *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 embryonating chicken eggs as well as by inoculation into healthy chickens that have not been vaccinated against ND.

**b) Method of manufacture**

i) *Procedure*

The vaccine production facility should operate under the appropriate bio-security 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 chapter 1.1.2 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 embryonating fowl 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<sub>50</sub>/0.1–0.2 ml is inoculated into the allantoic cavity of 9- or 10-day-old embryonating 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 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.

ii) *Requirements for substrates and media*

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. In the USA, both live and killed ND vaccines are prepared in SPF eggs.

iii) *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.

iv) *Final product batch tests*

*Sterility/purity*

Tests for sterility and freedom from contamination of biological materials 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.

*Safety*

Some countries also require back passage studies for live NDV vaccine to ensure that the pathogenicity is not increased by cycling through birds (USDA, 2009).

*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 (USDA, 2009), as described in C.4.c above. 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<sub>50</sub> greater than the titer of the virus used in the immunogenicity study described above (USDA, 2009). The minimum titer shall not be less than  $10^{5.5}$  EID<sub>50</sub>.

The infectivity of live virus vaccines is tested by titrating the virus in embryonated fowl eggs to calculate the EID<sub>50</sub>. This involves making tenfold dilutions of virus; 0.1 ml of each dilution is inoculated into five 9 to 10-day-old embryonated fowl 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<sub>50</sub> end-point is calculated using a standard formula such as Spearman–Kärber or Reed Muench (Thayer & Beard, 2008).

c) **Requirements for authorisation**

i) *Safety requirements*

*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.

*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 (USDA, 2009). In the USA the safety test is done with a single dose, administered to chickens 2–6 weeks old (USDA, 2009); 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 (Gould *et al.*, 2001), 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.

*Environmental consideration*

None.

ii) *Efficacy requirements*

*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<sub>50</sub> (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 21–28-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  $10^6$  LD<sub>50</sub> of ND challenge virus, 17–21 days later. Chickens are observed for 21 days. The PD<sub>50</sub> (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<sub>50</sub> is not less than 50 per dose and if the lower confidence limit is not less than 35 PD<sub>50</sub> 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 (USDA, 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.

*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 (> 85%) is sufficiently immunised showing an Ab titre  $\geq$  1:8 (Boven *et al.*, 2008)

iii) *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 (USDA, 2009). 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

#### a) 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 (Boursonnelli *et al.*, 1990; Karaca *et al.*, 1998; Olabode *et al.*, 2010), Pigeonpox virus (Letellier *et al.*, 1991), Herpesvirus of turkeys (Heckert *et al.*, 1996; Morgan *et al.*, 1992; Reddy *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 (Fukanoki *et al.*, 2001; Lee *et al.*, 2008; Mori *et al.*, 1994; Nagy *et al.*, 1991) or plants (Berinstein *et al.*, 2005; Yang *et al.*, 2007) and the use of DNA vaccines, i.e. plasmid DNA encoding relevant immunogenic NDV proteins (Loke *et al.*, 2005; Rajawat *et al.*, 2008). The establishment of a reverse genetics system for NDV (Peeters *et al.*, 1999; Romer-Oberdorfer *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; 2010; Schroer *et al.*, 2009; Steel *et al.*, 2008) 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, and iii) closer immunogenic match with outbreak strains.

Only few of the above mentioned biotechnological vaccines have been licensed in certain countries for application in poultry (VectorVax FP-N, Trovac-NDV, Innovax-ND). 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. In addition, 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 or the implementation of the DIVA principle.

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 (Kapczynski & King, 2005). Indeed, it has been shown 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). 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. These results argue for an adaptation of classical vaccine strains to improve the antigenic match between the vaccine and currently circulating virulent NDV strains.

**b) Special requirements for biotechnological vaccines**

Once registered and licensed, biotechnological vaccines have to fulfill the same or similar requirements as classical vaccines as detailed above (Section C: requirements for vaccines and diagnostic biologicals).

**REFERENCES**

- ALDOUS E.W., MYNN J.K., BANKS J. & ALEXANDER D.J. (2003). A molecular epidemiological study of avian paramyxovirus type 1 (Newcastle disease virus) isolates by phylogenetic analysis of a partial nucleotide sequence of the fusion protein gene. *Avian Pathol.*, **32** (3), 239–256.
- ALEXANDER D.J. & ALLAN W.H. (1974). Newcastle disease virus pathotypes. *Avian Pathol.*, **3** (4), 269–278.
- ALEXANDER D.J., MANVELL R.J., LOWINGS J.P., FROST K. M., COLLINS M.S., RUSSELL P.H. & SMITH J.E. (1997). Antigenic diversity and similarities detected in avian paramyxovirus type 1 (Newcastle disease virus) isolates using monoclonal antibodies. *Avian Pathol.*, **26** (2), 399–418.
- ALEXANDER D. J. AND PARSONS G. (1986). Pathogenicity for chickens of avian paramyxovirus type 1 isolates obtained from pigeons in Great Britain during 1983-1985. *Avian Pathology*, **15**, 487-493.
- ALEXANDER D.J., PATTISON M. & MACPHERSON I. (1983). Avian Paramyxovirus of PMV-3 serotype in British turkeys. *Avian Pathol.*, **12**, 469–482.
- ALEXANDER D.J. & SENNE D.A. (2008a). Newcastle Disease, Other Avian Paramyxoviruses, and Pneumovirus Infections. *In: Diseases of Poultry, Twelfth Edition*, Saif Y.M., Fadly A.M., Glisson J.R., McDougald L.R., Nolan L.K. & Swayne D.E., eds. Iowa State University Press, Ames, Iowa, USA, 75–116.
- ALEXANDER D.J. & SENNE D.A. (2008b), Newcastle Disease and Other Avian Paramyxoviruses. *In: A Laboratory Manual for the Isolation, Identification and Characterization of Avian Pathogens*, Dufour-Zavala L. (Editor in Chief) Swayne D.E., Glisson J.R., Jackwood M.W., Pearson J.E., Reed W.M, Woolcock P.R., 4th ed., American Association of Avian Pathologists, Athens, GA, 135–141.
- ALLAN W.H., LANCASTER J.E. & TOTH B. (1978). Newcastle Disease Vaccines. FAO, Rome, Italy.
- BERINSTEIN A., VAZQUEZ-ROVERE C., ASURMENDI S., GOMEZ E., ZANETTI F., ZABAL O., TOZZINI A., CONTE GRAND D., TABOGA O., CALAMANTE G., BARRIOS H., HOPP E & CARRILLO E. (2005). Mucosal and systemic immunization elicited by Newcastle disease virus (NDV) transgenic plants as antigens. *Vaccine*, **23** (48–49), 5583–5589.
- VAN BOVEN M., BOUMA A., FABRI T.H., KATSMA E., HARTOG L. & KOCH G. (2008). Herd immunity to Newcastle disease virus in poultry by vaccination. *Avian Pathol.*, **37** (1), 1–5.
- BOURSNELL M.E., GREEN P.F., SAMSON A.C., CAMPBELL J.I., DEUTER A., PETERS R.W., MILLAR N.S., EMMERSON P.T. & BINNS M.M. (1990). A recombinant fowlpox virus expressing the hemagglutinin-neuraminidase gene of Newcastle disease virus (NDV) protects chickens against challenge by NDV. *Virology*, **178** (1), 297–300.
- BROWN J., RESURRECCION R.S. & DICKSON T.G. (1990). The relationship between the hemagglutination-inhibition test and the enzyme-linked immunosorbent assay for the detection of antibody to Newcastle disease. *Avian Dis.*, **34** (3), 585–587.
- CATTOLI G., DE BATTISTI C., MARCIANO S., ORMELLI S., MONNE I., TERREGINO C. & CAPUA I. (2009). False-negative results of a validated real-time PCR protocol for diagnosis of Newcastle disease due to genetic variability of the matrix gene. *J. Clin. Microbiol.*, **47**, 3791–3792.
- CATTOLI G., FUSARO A., MONNE I., MOLIA S., LE MENACH A., MAREGEYA B., NCHARE A., BANGANA I., MAINA A.G., KOFFI J.N., THIAM H., BEZEID O.E., SALVIATO A., NISI R., TERREGINO C. & CAPUA I. (2010). Emergence of a new genetic lineage of Newcastle disease virus in West and Central Africa – implications for diagnosis and control. *Vet. Microbiol.*, **142**, 168–176.
- CHANG P.W. (1981). Newcastle disease. *In: CRC Handbook Series in Zoonoses. Section B: Viral Zoonoses Volume II*, Beran G.W., ed. CRC Press. Boca Raton, Florida, USA, 261–274.
- CHO S.H., KWON H.J., KIM T.E., KIM J.H., YOO H.S., PARK M.H., PARK Y.H. & KIM S.J. (2008). Characterization of a recombinant Newcastle disease virus vaccine strain. *Clin. Vaccine Immunol.*, **15** (10), 1572–1579.

- CHOI K.S., LEE E.K., JEON W.J. & KWON J.H. (2010). Antigenic and immunogenic investigation of the virulence motif of the Newcastle disease virus fusion protein. *J. Vet. Sci.*, **11** (3), 205–211.
- COLLINS M.S., STRONG I. & ALEXANDER D.J. (1994). Evaluation of the molecular basis of pathogenicity of the variant Newcastle disease viruses termed “pigeon PMV-1 viruses”. *Arch. Virol.*, **134** (3–4), 403–411.
- CREELAN J.L., GRAHAM D.A. & MCCULLOUGH S.J. (2002). Detection and differentiation of pathogenicity of avian paramyxovirus serotype 1 from field cases using one-step reverse transcriptase-polymerase chain reaction. *Avian Pathol.*, **31** (5), 493–499.
- CZEGLEDI A., UJVARI D., SOMOGYI E., WEHMANN E., WERNER O. & LOMNICZI B. (2006). Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. *Virus Res.*, **120** (1–2), 36–48.
- DINAPOLI J.M., YANG L., SUGUITAN A., ELANKUMARAN S., DORWARD D.W., MURPHY B.R., SAMAL S.K., COLLINS P.L. & BUKREYEV A. (2007). Immunization of primates with a Newcastle disease virus-vectored vaccine via the respiratory tract induces a high titer of serum neutralizing antibodies against highly pathogenic avian influenza virus. *J. Virol.*, **81** (21), 11560–11568.
- DORTMANS J.C., KOCH G., ROTTIER P.J. & PEETERS B.P. (2009). Virulence of pigeon paramyxovirus type 1 does not always correlate with the cleavability of its fusion protein. *J. Gen. Virol.*, **90** (Pt 11), 2746–2750.
- DORTMANS J.C., ROTTIER P.J., KOCH G. & PEETERS B.P. (2010). The viral replication complex is associated with virulence of Newcastle disease virus (NDV). *J. Virol.*, **84** (19), 10113–10120.
- EUROPEAN COMMISSION (1993). Commission Decision of 8 February 1993 laying down the criteria for vaccines to be used against Newcastle disease in the context of routine vaccination programmes (93/152/EEC): *Official Journal of the European Communities* L 59, 35 (Decision as amended by Decision 2010/633/EC: *Official Journal of the European Union*, L 279, 33).
- FUKANOKI S., IWAKURA T., IWAKI, S., MATSUMOTO K., TAKEDA R., IKEDA K., SHI Z. & MORI H. (2001). Safety and efficacy of water-in-oil-in-water emulsion vaccines containing Newcastle disease virus haemagglutinin-neuraminidase glycoprotein. *Avian Pathol.*, **30** (5), 509–516.
- FULLER C.M., BRODD L., IRVINE R.M., ALEXANDER D.J. & ALDOUS E.W. (2010). Development of an L gene real-time reverse-transcription PCR assay for the detection of avian paramyxovirus type 1 RNA in clinical samples. *Arch. Virol.*, **155**, 817–823.
- GOEBEL S.J., TAYLOR J., BARR B.C., KIEHN T.E., CASTRO-MALASPINA H.R., HEDVAT C.V., RUSH-WILSON K.A., KELLY C.D., DAVIS S.W., SAMSONOFF W.A., HURST K.R., BEHR M.J. & MASTERS P.S. (2007). Isolation of avian paramyxovirus 1 from a patient with a lethal case of pneumonia. *J. Virol.*, **81** (22), 12709–12714.
- GOULD A.R., KATTENBELT J.A., SELLECK P., HANSSON E., LA-PORTA A. & WESTBURY H.A. (2001). Virulent Newcastle disease in Australia: molecular epidemiological analysis of viruses isolated prior to and during the outbreaks of 1998-2000. *Virus Res.*, **77** (1), 51–60.
- HECKERT R.A., RIVA J., COOK S., McMILLEN J. & SCHWARTZ R.D. (1996). Onset of protective immunity in chicks after vaccination with a recombinant herpesvirus of turkeys vaccine expressing Newcastle disease virus fusion and hemagglutinin-neuraminidase antigens. *Avian Dis.*, **40** (4), 770–777.
- HU S., MA H., WU Y., LIU W., WANG X., LIU Y. & LIU X. (2009). A vaccine candidate of attenuated genotype VII Newcastle disease virus generated by reverse genetics. *Vaccine*, **27** (6), 904–910.
- KAPCZYNSKI D.R. & KING D.J. (2005). Protection of chickens against overt clinical disease and determination of viral shedding following vaccination with commercially available Newcastle disease virus vaccines upon challenge with highly virulent virus from the California 2002 exotic Newcastle disease outbreak. *Vaccine*, **23** (26), 3424–3433.
- KARACA K., SHARMA J.M., WINSLOW B.J., JUNKER D.E., REDDY S., COCHRAN M. & McMILLEN J. (1998). Recombinant fowlpox viruses coexpressing chicken type I IFN and Newcastle disease virus HN and F genes: influence of IFN on protective efficacy and humoral responses of chickens following *in ovo* or post-hatch administration of recombinant viruses. *Vaccine*, **16** (16), 1496–1503.



- KHAN T.A., RUE C.A., REHMANI S.F., AHMED A., WASILENKO J.L., MILLER P.J. & AFONSO C.L. (2010). Phylogenetic and biological characterization of Newcastle disease virus isolates from Pakistan. *J. Clin. Microbiol.*, **48** (5), 1892–1894.
- KIM L.M., KING D.J., CURRY P.E., SUAREZ D.L., SWAYNE D.E., STALLKNECHT D.E., SLEMONS R.D., PEDERSEN J.C., SENNE D.A., WINKER K. & AFONSO C.L. (2007). Phylogenetic diversity among low virulence Newcastle disease viruses from waterfowl and shorebirds and comparison of genotype distributions to poultry-origin isolates. *J. Virol.*, **81** (22), 12641–12653.
- KIM L.M., KING D.J., GUZMAN H., TESH R.B., TRAVASSOS DA ROSSA A.P.A., BUENO R., DENNET J.A. & AFONSO C.L. (2008a). Biological and phylogenetic characterization of pigeon paramyxovirus serotype 1 circulating in wild North American pigeons and doves. *J. Clin. Microbiol.*, **46** (10), 3303–3310.
- KIM L.M., SUAREZ D.L. & AFONSO C.L. (2008b). Detection of a broad range of class I and II Newcastle disease viruses using multiplex real-time reverse transcription polymerase chain reaction assay. *J. Vet. Diagn. Invest.*, **20** (4), 414–425.
- KOUWENHOVEN B. (1993). Newcastle Disease *In: Virus Infection of Birds*, McFerran J.B. & McNulty M.S., eds. Elsevier Science Publishers B.V., Amsterdam, Netherlands, 341–4361.
- LEE Y.J., SUNG H.W., CHOI J.G., LEE E.K., YOON H., KIM J.H. & SONG C.S. (2008). Protection of chickens from Newcastle disease with a recombinant baculovirus subunit vaccine expressing the fusion and hemagglutininneuraminidase proteins. *J. Vet. Sci.*, **9** (3), 301–308.
- LENSING H.H. (1974). Newcastle disease – live vaccine testing. *Dev. Biol. Stand.*, **25**, 189–194.
- LETELLIER C., BURNY A. & MEULEMANS G. (1991). Construction of a pigeonpox virus recombinant: expression of the Newcastle disease virus (NDV) fusion glycoprotein and protection of chickens against NDV challenge. *Arch. Virol.*, **118** (1–2), 43–56.
- LIU H., ZHAO Y., ZHENG D., LV Y., ZHANG W., XU T., LI J. & WANG Z. (2011). Multiplex RT-PCR for rapid detection and differentiation of class I and class II Newcastle disease viruses. *J. Virol. Methods*, **171** (1), 149–155. Epub 27 Oct. 2010.
- LOKE C.F., OMAR A.R., RAHA A.R., & YUSOFF K. (2005). Improved protection from velogenic Newcastle disease virus challenge following multiple immunizations with plasmid DNA encoding for F and HN genes. *Vet. Immunol. Immunopathol.*, **106** (3–4), 259–267.
- MAAS R.A., OEI H.L., KEMPER S., KOCH G. & VISSER L. (1998). The use of homologous virus in the haemagglutination-inhibition assay after vaccination with Newcastle disease virus strain La Sota or Clone30 leads to an over estimation of protective serum antibody titres. *Avian Pathol.*, **27** (6), 625–631.
- MEBATSION T., KOOLEN M. J., DE VAAN L. T., DE HAAS N., BRABER M., ROMER-OBERDORFER A., VAN DEN ELZEN, P. & VAN DER MARCEL P. (2002). Newcastle disease virus (NDV) marker vaccine: an immunodominant epitope on the nucleoprotein gene of NDV can be deleted or replaced by a foreign epitope. *J. Virol.*, **76** (20), 10138–10146.
- MEULEMANS G. (1988). Newcastle disease virus F glycoprotein expressed from a recombinant vaccinia virus vector protects chickens against live-virus challenge. *Avian Pathol.*, **17**, 821–827.
- MEULEMANS G., VAN DEN BERG T.P., DECAESSTECKER M. & BOSCHMANS M. (2002). Evolution of pigeon Newcastle disease virus strains. *Avian Pathol.*, **31** (5), 515–519.
- MILLER P.J., AFONSO C.L., SPACKMAN E., SCOTT M.A., PEDERSEN J.C., SENNE D.A., BROWN J.D., FULLER C.M., UHART M.M., KARESH W.B., BROWN I.H., ALEXANDER D.J. & SWAYNE D.E. (2010a). Evidence for a New Avian Paramyxovirus Serotype-10 Detected in Rockhopper Penguins from the Falkland Islands. *J. Virol.*, **84** (21), 11496–11504.
- MILLER P.J., DECANINI E.L. & AFONSO C.L. (2010b). Newcastle disease: Evolution of genotypes and the related diagnostic challenges. *Infect. Genet. Evol.*, **10** (1), 26–35.
- MILLER P.J., KING D.J., AFONSO C.L. & SUAREZ D.L. (2007). Antigenic differences among Newcastle disease virus strains of different genotypes used in vaccine formulation affect viral shedding after a virulent challenge. *Vaccine*, **25** (41), 7238–7246.

- MORGAN R.W., GELB J., SCHREURS C.S., LUTTICKEN D., ROSENBERGER J.K. & SONDERMEIJER P.J. (1992). Protection of chickens from Newcastle and Marek's diseases with a recombinant herpesvirus of turkeys vaccine expressing the Newcastle disease virus fusion protein. *Avian Dis.*, **36** (4), 858–870.
- MORI H., TAWARA H., NAKAZAWA H., SUMIDA M., MATSUBARA F., AOYAMA S., IRANTI Y., HAYASHI Y. & KAMOGAWA K. (1994). Expression of the Newcastle disease virus (NDV) fusion glycoprotein and vaccination against NDV challenge with a recombinant baculovirus. *Avian Dis.*, **38** (4), 772–777.
- NAGY E., HUBER P., KRELL P.J. & DERBYSHIRE J.B. (1991). Synthesis of Newcastle disease virus (NDV)-like envelopes in insect cells infected with a recombinant baculovirus expressing the haemagglutinin-neuraminidase of NDV. *J. Gen. Virol.*, **72** (Pt 3), 753–756.
- NAKAYA T., CROS J., PARK M.S., NAKAYA Y., ZHENG H., SAGRERA A., VILLAR E., GARCIA-SASTRE A. & PALESE P. (2001). Recombinant Newcastle disease virus as a vaccine vector. *J. Virol.*, **75** (23), 11868–11873.
- NAYAK B., KUMAR S., DINAPOLI J.M., PALDURAI A., PEREZ D.R., COLLINS P.L. & SAMAL S.K. (2010). Contributions of the avian influenza virus HA, NA, and M2 surface proteins to the induction of neutralizing antibodies and protective immunity. *J. Virol.*, **84** (5), 2408–2420.
- NANTHAKUMAR T., KATARIA R.S., TIWARI A.K., BUTCHAIH G. & KATARIA J.M. (2000). Pathotyping of Newcastle disease viruses by RT-PCR and restriction enzyme analysis. *Vet. Res. Commun.*, **24**, 275–286.
- OLABODE A.O., NDAKO J.A., ECHEONWU G.O., NWANKITI O.O. & CHUKWUEDO A.A. (2010). Use of cracked maize as a carrier for NDV4 vaccine in experimental vaccination of chickens. *Virol. J.*, **7**, 67.
- PEETERS B.P., DE LEEUW O.S., KOCH G. & GIELKENS A.L. (1999). Rescue of Newcastle disease virus from cloned cDNA: evidence that cleavability of the fusion protein is a major determinant for virulence. *J. Virol.*, **73** (6), 5001–5009.
- PEETERS B.P., DE LEEUW O.S., VERSTEGEN I., KOCH G. & GIELKENS A.L. (2001). Generation of a recombinant chimeric Newcastle disease virus vaccine that allows serological differentiation between vaccinated and infected animals. *Vaccine*, **19** (13–14), 1616–1627.
- PEROZO F., VILLEGAS P., ESTEVEZ C., ALVARADO I.R., PURVIS L.B. & SAUME E. (2008). Avian adeno-associated virus-based expression of Newcastle disease virus hemagglutinin-neuraminidase protein for poultry vaccination. *Avian Dis.*, **52** (2), 253–259.
- RAJAWAT Y.S., SUNDARESAN N.R., RAVINDRA P.V., KANTARAJA C., RATTA B., SUDHAGAR M., RAI A., SAXENA V.K., PALIA S.K. & TIWARI A.K. (2008). Immune responses induced by DNA vaccines encoding Newcastle virus haemagglutinin and/or fusion proteins in maternal antibody-positive commercial broiler chicken. *Br. Poult. Sci.*, **49** (2), 111–117.
- REDDY S. K., SHARMA J. M., AHMAD J., REDDY D.N., McMILLEN J.K., COOK S.M., WILD M.A & SCHWARTZ R.D. (1996). Protective efficacy of a recombinant herpesvirus of turkeys as an in ovo vaccine against Newcastle and Marek's diseases in specific-pathogen-free chickens. *Vaccine*, **14** (6), 469–477.
- ROMER-OBERDORFER A., MUNDT E., MEBATSION T., BUCHHOLZ U.J. & METTENLEITER T.C. (1999). Generation of recombinant lentogenic Newcastle disease virus from cDNA. *J. Gen. Virol.*, **80** (Pt 11), 2987–2995.
- SAKAGUCHI M., NAKAMURA H., SONODA K., OKAMURA H., YOKOGAWA K., MATSUI K. & HIRA K. (1998). Protection of chickens with or without maternal antibodies against both Marek's and Newcastle diseases by one-time vaccination with recombinant vaccine of Marek's disease virus type 1. *Vaccine*, **16** (5), 472–479.
- SCHROER D., VEITS J., GRUND C., DAUBER M., KEIL G., GRANZOW H., METTENLEITER T.C. & ROMER-OBERDORFER A. (2009). Vaccination with Newcastle disease virus vectored vaccine protects chickens against highly pathogenic H7 avian influenza virus. *Avian Dis.*, **53** (2), 190–197.
- SHENGQING Y., KISHIDA N., ITO H., KIDA H., OTSUKI K., KAWAOKA Y. & ITO T. (2002). Generation of Velogenic Newcastle Disease Viruses from a Nonpathogenic Waterfowl Isolate by Passaging in Chickens. *Virology*, **301** (2), 206–211.
- STEEL J., BURMAKINA S.V., THOMAS C., SPACKMAN E., GARCIA-SASTRE A., SWAYNE D.E. & PALESE P. (2008). A combination *in-ovo* vaccine for avian influenza virus and Newcastle disease virus. *Vaccine*, **26** (4), 522–531.

STONE H.D., BONEY W.A. JR, CORIA M.F. & GILLETTE K.G. (1975). Viscerotropic velogenic Newcastle disease in turkeys: vaccination against loss of egg production. *Avian Dis.*, **19** (1), 47–51

SWAYNE D.E. & KING D.J. (2003). Avian influenza and Newcastle disease. *J. Am. Vet. Med. Assoc.*, **222** (11), 1534–1540.

TERREGINO C. & CAPUA I. (2009). Clinical traits and pathology of Newcastle disease infection and guidelines for farm visit and differential diagnosis. *In: Avian Influenza and Newcastle Disease*, Capua I., ed. AD Springer Milan, Milan, Italy.

THAYER S.G. & BEARD C.W. (2008). Serologic Procedures. *In: A Laboratory Manual for the Identification and Characterization of Avian Pathogens*, Fifth Edition, Dufour-Zavala L., ed. American Association of Avian Pathologists, USA, pp. 222–229.

UNITED STATES DEPARTMENT OF AGRICULTURE (USDA) (2009). United States Department of Agriculture, Code of Federal Regulations, Title 9, Parts 1–199. Washington, DC, USA.

WAMBURA P.N. (2011). Formulation of novel nano-encapsulated Newcastle disease vaccine tablets for vaccination of village chickens. *Trop. Anim Health Prod.*, **43** (1), 165–169.

WISE M.G., SUAREZ D.L., SEAL B.S., PEDERSEN J.C., SENNE D.A., KING D.J., KAPCZYNSKI D. & SPACKMAN E. (2004). Development of a real-time reverse-transcription PCR for detection of Newcastle disease virus RNA in clinical samples. *J. Clin. Microbiol.*, **42**, 329–338.

YANG Z.Q., LIU Q.Q., PAN Z.M., YU H.X. & JIAO X.A. (2007). Expression of the fusion glycoprotein of Newcastle disease virus in transgenic rice and its immunogenicity in mice. *Vaccine*, **25** (4), 591–598.

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**NB:** There are OIE Reference Laboratories for Newcastle disease  
(see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list:  
<http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/> ).  
Please contact the OIE Reference Laboratories for any further information on  
diagnostic tests, reagents and vaccines for Newcastle disease