CHAPTER 2.3.4.
AVIAN INFLUENZA

SUMMARY

Avian influenza (AI) is caused by specified viruses that are members of the family Orthomyxoviridae and placed in the genus influenza virus A. There are three influenza genera – A, B and C; only influenza A viruses are known to infect birds. Diagnosis is by isolation of the virus or by detection and characterisation of fragments of its genome. This is because infections in birds can give rise to a wide variety of clinical signs that may vary according to the host, strain of virus, the host's immune status, presence of any secondary exacerbating organisms and environmental conditions.

Identification of the agent: Suspensions in antibiotic solution of oropharyngeal and cloacal swabs (or faeces) taken from live birds, or of faeces and pooled samples of organs from dead birds, are inoculated into the allantoic cavity of 9- to 11-day-old embryonated chicken eggs. The eggs are incubated at 37°C (range 35–39°C) for 2–7 days. The allantoic fluid of any eggs containing dead or dying embryos during the incubation and all eggs at the end of the incubation period are tested for the presence of haemagglutinating activity. The presence of influenza A virus can be confirmed by an immunodiffusion test between concentrated virus and an antiserum to the nucleocapsid and/or matrix antigens, both of which are common to all influenza A viruses. Isolation in embryos has recently been replaced, under certain circumstances, by detection of one or more segments of the influenza A genome using real-time reverse-transcription polymerase chain reaction (rRT-PCR) or other validated molecular techniques.

For subtyping the virus, a reference laboratory should conduct haemagglutination and neuraminidase inhibition tests against a battery of polyclonal or monospecific antisera to each of the 16 haemagglutinin (H1–16) and 9 neuraminidase (N1–9) subtypes of influenza A virus, or identify the genome of specific H and N subtypes using RNA detection technologies with subtype specific primers and probes (e.g. rRT-PCR) or sequencing and phylogenetic analysis.

As the term highly pathogenic avian influenza and the historical term 'fowl plague' refer to infection with virulent strains of influenza A virus, it is necessary to assess the virulence of an isolate for domestic poultry. Any highly pathogenic avian influenza isolate is classified as notifiable avian influenza (NAI) virus. Although all naturally occurring virulent strains isolated to date have been either of the H5 or H7 subtype, most H5 or H7 isolates have been of low virulence. Due to the risk of a low virulent H5 or H7 becoming virulent by mutation in poultry hosts, all H5 and H7 viruses have also been classified as NAI viruses. The methods used for the determination of strain virulence for birds have evolved over recent years with a greater understanding of the molecular basis of pathogenicity, but still primarily involve the intravenous inoculation of a minimum of eight susceptible 4- to 8-week-old chickens with infectious virus; strains are considered to be highly pathogenic if they cause more than 75% mortality within 10 days or inoculation of 10 susceptible 4- to 8-week-old chickens resulting in an intravenous pathogenicity index (IVPI) of greater than 1.2. Characterisation of suspected virulent strains of the virus should be conducted in a virus-secure bioccontainment laboratory. All virulent AI isolates are designated as highly pathogenic notifiable avian influenza (HPNAI) viruses. Regardless of their virulence for chickens, H5 or H7 viruses with a HA0 cleavage site amino acid sequence similar to any of those that have been observed in virulent viruses are considered HPNAI viruses. H5 and H7 isolates that are not pathogenic for chickens and do not have an HA0 cleavage site amino acid sequence similar to any of those that have been observed in HPNAI viruses are designated as low pathogenicity notifiable avian influenza (LPNAI) viruses and non-H5 or non-H7 AI isolates that are not highly pathogenic for chickens are designated as low pathogenicity avian influenza (LPAI) viruses.

Serological tests: As all influenza A viruses have antigenically similar nucleocapsid and matrix antigens, agar gel immunodiffusion tests are used to detect antibodies to these antigens.
Concentrated virus preparations containing either or both type of antigens are used in such tests. Not all species of birds develop demonstrable precipitating antibodies. Haemagglutination inhibition tests have also been employed in routine diagnostic serology, but it is possible that this technique may miss some particular infections because the haemagglutinin is subtype specific. Enzyme-linked immunosorbent assays have been used to detect antibodies to influenza A type-specific antigens in either species-dependent (indirect) or -independent (competitive) test formats.

Requirements for vaccines and diagnostic biologicals: Historically, in most countries, vaccines specifically designed to contain or prevent HPNAI were banned or discouraged by government agencies because they may interfere with stamping-out control policies. The first use of vaccination in an avian influenza eradication programme was against LPAI and LPNAI. The programmes used inactivated oil-emulsion vaccines with the same haemagglutinin and neuraminidase subtypes, and infected flocks were identified by detection of virus or antibodies against the virus in non-vaccinated sentinel birds. During the 1990s the prophylactic use of inactivated oil-emulsion vaccines was employed in Mexico and Pakistan to control widespread outbreaks of NAI, and a recombinant fowl poxvirus vaccine expressing the homologous HA gene was also used in Mexico, El Salvador and Guatemala. During the 1999–2001 outbreak of LPNAI in Italy, an inactivated vaccine was used with the same haemagglutinin type as the field virus, but with a different neuraminidase. This allowed the differentiation of non-infected vaccinated birds from vaccinated birds infected with the field virus and ultimately resulted in eradication of the field virus. Prophylactic use of H5 and H7 vaccines has been practised in parts of Italy, aimed at preventing LPNAI infections, and several countries in Asia, Africa and the Middle East as an aid in controlling HPNAI H5N1 virus infections. HPNAI viruses should not be used as the seed virus for production of vaccine.

If HPNAI is used in challenge studies, the facility should meet the OIE requirements for Containment Group 4 pathogens.

A. INTRODUCTION

Notifiable avian influenza (NAI) is caused by infection with viruses of the family Orthomyxoviridae placed in the genus influenza virus A. Influenza A viruses are the only orthomyxoviruses known to naturally affect birds. Many species of birds have been shown to be susceptible to infection with influenza A viruses; aquatic birds form a major reservoir of these viruses, and the overwhelming majority of isolates have been of low pathogenicity (low virulence) for chickens and turkeys. Influenza A viruses have antigenically related nucleocapsid and matrix proteins, but are classified into subtypes on the basis of their haemagglutinin (H) and neuraminidase (N) antigens (World Health Organization Expert Committee, 1980). At present, 16 H subtypes (H1–H16) and 9 N subtypes (N1–N9) are recognised (Swayne & Halvorson, 2008). To date, naturally occurring highly virulent influenza A viruses that produce acute clinical disease in chickens, turkeys and other birds of economic importance have been associated only with the H5 and H7 subtypes. Most viruses of the H5 and H7 subtype isolated from birds have been of low virulence for poultry. As there is the risk of a H5 or H7 virus of low virulence becoming virulent by mutation, all H5 and H7 viruses have been designated as NAI viruses.

Depending on the species, age and type of bird, specific characteristics of the viral strain involved, and on environmental factors, the highly pathogenic disease, in fully susceptible birds, may vary from one of sudden death with little or no overt clinical signs to a more characteristic disease with variable clinical presentations including respiratory signs, such as ocular and nasal discharges, coughing, snicking and dyspnoea, swelling of the sinuses and/or head, apathy, reduced vocalisation, marked reduction in feed and water intake, cyanosis of the unfeathered skin, wattles and comb, incoordination and nervous signs and diarrhoea. In laying birds, additional clinical features include a marked drop in egg production, usually accompanied by an increase in numbers of poor quality eggs. Typically, high morbidity is accompanied by high and rapidly escalating unexplained mortality. However, none of these signs can be considered pathognomonic. In certain host species such as Pekin ducks some HPAI viruses do not necessarily provoke significant clinical disease. In addition, low pathogenicity avian influenza (LPAI) viruses, which normally cause only a mild or no clinical disease, may in certain circumstances produce a spectrum of clinical signs, the severity of which may approach that of highly pathogenic avian influenza (HPAI), particularly if exacerbating infections and/or adverse environmental conditions are present. Confirmatory diagnosis of the disease, therefore, depends on the isolation or detection of the causal virus and the demonstration that it fulfils one of the defined criteria described in section B.2. Testing sera from suspect birds using antibody detection methods may supplement diagnosis, but these methods are not suitable for a detailed identification. Diagnosis for official control purposes is established on the basis of agreed official criteria for pathogenicity according to in-vivo tests or to molecular determinants (i.e. the presence of a cleavage site of the haemagglutinin precursor protein HA0 consistent with HPNAI virus) and haemagglutinin subtyping. These definitions evolve as scientific knowledge of the disease increases.
NAI are subject to official control. The viruses that cause NAI have the potential to spread from the laboratory if adequate levels of biosecurity and biosafety are not in place. Consequently, a risk assessment should be carried out to determine the level of biosecurity needed for laboratory diagnosis and chicken inoculation; characterisation of the HPAI virus should be conducted at biocontainment level 3 and LPNAI at biocontainment level 2 (at least). The facility should meet the requirements for the appropriate Containment Group as determined by the risk assessment and as outlined in Chapter 1.1.3 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities. 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 (the prescribed test for international trade)**

Samples taken from dead birds should include intestinal contents (faeces) or cloacal swabs and oropharyngeal swabs. Samples from trachea, lungs, air sacs, intestine, spleen, kidney, brain, liver and heart should also be collected and processed either separately or as a pool.

Samples from live birds should include both oropharyngeal and cloacal swabs. 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. Where these are not available, the collection of fresh faeces may serve as an alternative.

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 swabs, but at five-fold higher concentrations for faeces and cloacal swabs. It is important to readjust the pH of the 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). 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. For prolonged storage, diagnostic samples and isolates should be kept at –80°C. Repeated freezing and thawing should be avoided.

The preferred method of growing avian influenza A viruses is by the inoculation of specific pathogen free (SPF) embryonated chicken eggs, or specific antibody negative (SAN) eggs. The supernatant fluids of faeces or tissue suspensions obtained through clarification by centrifugation at 1000 g are inoculated into the allantoic sac of three to five embryonated SPF or SAN chicken eggs of 9–11 days' incubation. The eggs are incubated at 37°C (range 35–39°C) for 2–7 days. Eggs containing dead or dying embryos as they arise, and all eggs remaining at the end of the incubation period, should first be chilled to 4°C for 4 hours or overnight, and the allantoic fluids should then be recovered and tested with a screening test (such as haemagglutination [HA] test), influenza A type-specific test (such as agar gel immunodiffusion test [AGID] or solid-phase antigen-capture enzyme-linked immunosorbent assays [ELISA]) or influenza A subtype-specific test (such as haemagglutinin inhibition [HI] and neuraminidase inhibition [NI] tests) or a molecular test to detect influenza A specific nucleic acid signatures (such as real-time reverse transcriptase polymerase chain reaction [rRT-PCR] test) as described later (see Section B.3.b). Detection of HA activity, in bacteria-free amnio-allantoic fluids verified by microbiological assay, indicates a high probability of the presence of an influenza A virus or of an avian paramyxovirus. Fluids that give a negative reaction should be passaged into at least one further batch of eggs.

The presence of influenza A virus can be confirmed in AGID tests by demonstrating the presence of the nucleocapsid or matrix antigens, both of which are common to all influenza A viruses (see Section B.3.a). The antigens may be prepared by concentrating the virus from infective allantoic fluid or extracting the infected chorioallantoic membranes; these are tested against known positive antisera. Virus may be concentrated from infective allantoic fluid by ultracentrifugation, or by precipitation under acid conditions. The latter method consists of the addition of 1.0 M HCl to infective allantoic fluid until it is approximately pH 4.0. The mixture is placed in an ice bath for 1 hour and then clarified by centrifugation at 1000 g at 4°C. The supernatant fluid is discarded. The virus concentrates are resuspended in glycine/sarcosyl buffer: this consists of 1% (w/v) sodium lauroyl sarcosinate buffered to pH 9.0 with 0.5 M glycine. These concentrates contain both nucleocapsid and matrix polypeptides.

Preparations of nucleocapsid-rich antigen can also be obtained from chorioallantoic membranes for use in the AGID test (Beard, 1970). This method involves removal of the chorioallantoic membranes from infected eggs that have allantoic fluids with HA activity. The membranes are then homogenised or ground to a paste. This is subjected to three freeze–thaw cycles, followed by centrifugation at 1000 g for 10 minutes. The pellet is discarded and the supernatant is used as an antigen following treatment with 0.1% formalin.
Use of the AGID test to demonstrate nucleocapsid or matrix antigens is a satisfactory way to indicate the presence of avian influenza virus (AIV) in amnioallantoic fluid, but various experimental and commercial rapid, solid-phase antigen-capture ELISAs (AC-ELISAs) are an effective alternative (Swayne & Halvorson, 2008). Most AC-ELISAs have been licensed and marketed to detect human influenza A virus in clinical specimens. Some have demonstrated effectiveness for detection of AIV, but many of these commercial tests have had low sensitivity (Woolcock & Cardona, 2005). Those validated for veterinary use are preferred.

Any HA activity of sterile fluids harvested from the inoculated eggs is most likely to be caused by an influenza A virus or an avian paramyxovirus, but a few strains of avian reovirus, as well as nonsterile fluid containing HA of bacterial origin can cause the agglutination of RBCs. There are currently 10 recognised serotypes of avian paramyxoviruses (Miller et al., 2010). Most laboratories will have antisera specific to Newcastle disease virus (avian paramyxovirus type 1), and in view of its widespread occurrence and almost universal use as a live vaccine in poultry, it is best to evaluate its presence by haemagglutination inhibition (HI) tests (see Chapter 2.3.14 Newcastle disease).

Alternatively, the presence of influenza virus can be confirmed by the use of RT-PCR or rRT-PCR using nucleoprotein-specific or matrix-specific conserved primers (Altmuller et al., 1991; Spackman et al., 2002). Also, the presence of subtype H5 or H7 influenza virus can be confirmed by using H5- or H7-specific primers (Monne et al., 2008; Slomka et al., 2007; Spackman et al., 2002).

Antigenic subtyping can be accomplished by monospecific antisera prepared against purified or recombinant H and N subtype-specific proteins, used in HI and NI tests, or polyclonal antisera raised against a battery of intact influenza viruses and used in HI and NI tests. Genotyping can be accomplished using H and N subtype specific primers in RT-PCR and rRT-PCR tests; or 4) using sequence analysis of H and N genes. Subtype identification by these techniques is beyond the scope of most diagnostic laboratories not specialising in influenza viruses. Assistance is available from the OIE Reference Laboratories (see Table given in Part 4 of this Terrestrial Manual).

2. Assessment of pathogenicity

The term HPAI relates to the assessment of virulence in chickens and implies the involvement of virulent strains of virus. It is used to describe a disease of fully susceptible chickens with clinical signs such as ocular and nasal discharges, coughing, snicking and dyspnoea, swelling of the sinuses and/or head, listlessness, reduced vocalisation, marked reduction in feed and water intake, cyanosis of the unfeathered skin, wattles and comb, incoordination, nervous signs and diarrhoea. In laying birds, additional clinical features include a marked drop in egg production usually accompanied by an increase in numbers of poor quality eggs. Typically, high morbidity is accompanied by high and rapidly escalating unexplained mortality. However, none of these signs can be considered pathognomonic and high mortality may occur in their absence. In addition, LPAI viruses that normally cause only mild or no clinical disease, may cause a much more severe disease if exacerbating infections or adverse environmental factors are present and, in certain circumstances, the spectrum of clinical signs may mimic HPAI.

The historical term ‘fowl plague’ has been abandoned in favour of the more accurate term HPAI. Because all naturally occurring HPAI viruses to date have been H5 and H7 subtypes and genomic studies have determined HPAI viruses arise by mutation of H5 and H7 LPAI viruses, all H5 and H7 LPAI have been recognised as potentially pathogenic. Pathogenicity shifts have been associated with changes to the proteolytic cleavage site of the haemagglutinin including: 1) substitutions of non-basic with basic amino acids (arginine or lysine); 2) insertions of multiple basic amino acids from codons duplicated from the haemagglutinin cleavage site; 3) short inserts of basic and non-basic amino acids from unknown source; 4) recombination with inserts from other gene segments that lengthen the proteolytic cleavage site; and 5) loss of the shielding glycosylation site at residue 13 in combination with multiple basic amino acids at the cleavage site. Amino acid sequencing of the cleavage sites of H5 and H7 subtype influenza isolates of low virulence for birds should identify viruses that have the capacity, following simple mutation, to become highly pathogenic for poultry.

The following criteria have been adopted by the OIE for classifying an AIV as HPAI:

a) One of the two following methods to determine pathogenicity in chickens is used. A HPAI virus is:

i) any influenza virus that is lethal1 for six, seven or eight of eight 4- to 8-week-old susceptible chickens within 10 days following intravenous inoculation with 0.2 ml of a 1/10 dilution of a bacteria-free, infective amnioallantoic fluid

or

1 When birds are too sick to eat or drink, they should be killed humanely.
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ii) any virus that has an intravenous pathogenicity index (IVPI) greater than 1.2. The following is the IVPI procedure:

- Fresh infective allantoic fluid with a HA titre >1/16 (>24 or >log2 4 when expressed as the reciprocal) is diluted 1/10 in sterile isotonic saline.
- 0.1 ml of the diluted virus is injected intravenously into each of ten 4- to 8-week-old SAN susceptible chickens; if possible, SPF chickens should be used.
- Birds are examined at 24-hour intervals for 10 days. At each observation, each bird is scored 0 if normal, 1 if sick, 2 if severely sick, 3 if dead. (The judgement of sick and severely sick birds is a subjective clinical assessment. Normally, ‘sick’ birds would show one of the following signs and ‘severely sick’ more than one of the following signs: respiratory involvement, depression, diarrhoea, cyanosis of the exposed skin or wattles, oedema of the face and/or head, nervous signs. Dead individuals must be scored as 3 at each of the remaining daily observations after death2.)
- The IVPI is the mean score per bird per observation over the 10-day period. An index of 3.00 means that all birds died within 24 hours, and an index of 0.00 means that no bird showed any clinical sign during the 10-day observation period.

b) For all H5 and H7 viruses of low pathogenicity in chickens, the amino acid sequence of the connecting peptide of the haemagglutinin must be determined. If the sequence is similar to that observed for other highly pathogenic AI isolates, the isolate being tested will be considered to be highly pathogenic (see the table that lists all the reported haemagglutinin proteolytic cleavage site of HA0 protein for H5 and H7 LPAI and HPAI viruses based on deduced amino acid sequence, which can be found on the OFFLU site at: http://www.offlu.net/fileadmin/home/en/resource-centre/pdf/Influenza_A_Cleavage_Sites.pdf).

The OIE has the following classification system to identify viruses for which disease reporting and control measures should be taken:

a) All AI isolates that meet the above criteria are designated as HPNAI.

b) H5 and H7 isolates that are not virulent for chickens and do not have an HA0 cleavage site amino acid sequence similar to any of those that have been observed in HPNAI viruses are designated as low pathogenicity notifiable avian influenza (LPNAI).

c) Non-H5 or non-H7 AI isolates that are not virulent for chickens are designated as LPAI.

A variety of strategies and techniques have been used successfully to sequence the nucleotides at that portion of the HA gene coding for the cleavage site region of the haemagglutinin of H5 and H7 subtypes of avian influenza, enabling the amino acids there to be deduced. This can be done by extraction the specimen and direct sequencing of the hemagglutinin proteolytic cleavage site, or first, cloning the hemagglutinin and then sequencing the cDNA. Various stages in the procedure can be facilitated using commercially available kits and automatic sequencers.

Determination of the cleavage site by sequencing or other methods has become the method of choice for initial assessment of the virulence of these viruses and has been incorporated into agreed definitions. This has reduced the number of in-vivo tests, although at present the inoculation of birds is still required to confirm a negative result as the possibility of virus populations containing mixtures of viruses of high and low virulence cannot be ruled out.

Although all the truly HPAI viruses isolated to date have been of H5 or H7 subtypes, at least two isolates, both of H10 subtype (H10N4 and H10N5), have been reported that would have fulfilled both the OIE and EU in vivo definitions for highly pathogenic AI viruses (Wood et al., 1996) as they killed 7/10 and 8/10 chickens with IVPI values >1.2 when the birds were inoculated intravenously. However, these viruses did not induce death or signs of disease when inoculated intranasally and did not have a haemagglutinin cleavage site sequence compatible with HPAI virus. Similarly, other intravenously inoculated LPAI viruses are nephrotropic and birds that die have high titres of virus in their kidneys indicating a renal pathogenic mechanism (Slemons & Swayne, 1990), but such laboratory-induced pathobiology does not equal to the multi-organ infection and systemic disease caused by HPNAI viruses. Conversely, four viruses have been described that have HA0 cleavage sites containing multiple basic amino acids, but which show low virulence (IVPI <1.2) when inoculated intravenously into 6-week-old chickens (Londt et al., 2007). Other anomalies are the Chile 2002 (Suarez et al., 2004) and the Canada 2004 (Pasick et al., 2005) H7N3 HPAI viruses, which show distinct and unusual cleavage site amino acid sequences of PEKPKTCSPLSRCRETR*GLF and PENPKQAYRKRMTR*GLF, respectively. These viruses appear to have arisen as a result of a recombination between the HA, nucleoprotein and matrix genes, respectively, resulting in

2 When birds are too sick to eat or drink, they should be killed humanely and scored as dead at the next observation.
an insertion at the HA0 cleavage site of 11 amino acids for the Chile virus and 7 amino acids for the Canadian virus. Both are extremely virulent when inoculated into 6-week-old chickens intravenously.

A table is available on the OFFLU web site that lists all the reported haemagglutinin proteolytic cleavage site of HA0 protein for H5 and H7 LPAI and HPAI viruses based on deduced amino acid sequence. This table will be updated as new virus are characterised; it can be found on the OFFLU site at:


### 3. Serological tests

#### a) Agar gel immunodiffusion (an alternative test for international trade)

All influenza A viruses have antigenically similar nucleocapsid and antigenically similar matrix antigens. Owing to this fact AGID tests are able to detect the presence or absence of antibodies to any influenza A virus. Concentrated virus preparations, as described above, contain both matrix and nucleocapsid antigens; the matrix antigen diffuses more rapidly than the nucleocapsid antigen. AGID tests have been widely and routinely used to detect specific antibodies in chicken and turkey flocks as an indication of infection, but AGID tests are less reliable at detecting antibodies following infection with influenza A viruses in other avian species. These have generally employed nucleocapsid-enriched preparations made from the chorioallantoic membranes of embryonated chicken eggs (Beard, 1970) that have been infected at 10 days of age, homogenised, freeze–thawed three times, and centrifuged at 1000 g. The supernatant fluids are inactivated by the addition of 0.1% formalin or 1% betapropiolactone, recentrifuged and used as antigen. Not all avian species may produce precipitating antibodies following infection with influenza viruses.

Tests are usually carried out using gels of 1% (w/v) agarose or purified agar and 8% (w/v) NaCl in 0.1 M phosphate buffer, pH 7.2, poured to a thickness of 2–3 mm in Petri dishes or on microscope slides. Using a template and cutter, wells of approximately 5 mm in diameter are cut into the agar. A pattern of wells must place each suspect serum adjacent to a known positive serum and antigen. This will make a continuous line of identity between the known positive, the suspect serum and the nucleocapsid antigen. Approximately 50 µl of each reagent should be added to each well.

Precipitin lines can be detected after approximately 24–48 hours, but this may be dependent on the concentrations of the antibody and the antigen. The precipitin lines are best observed against a dark background that is illuminated from behind. A specific, positive result is recorded when the precipitin line between the known positive control wells is continuous with the line between the antigen and the test well. Crossed lines are interpreted to be caused by the test serum lacking identity with the antibodies in the positive control well.

#### b) Haemagglutination and haemagglutination inhibition tests

Variations in the procedures for HA and HI tests are practised in different laboratories. The following recommended examples apply to 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 red blood cells (RBCs) taken from a minimum of three SPF or SAN chickens and pooled into an equal volume of Alsever’s solution. 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) Dispense 0.025 ml of PBS into each well of a plastic V-bottomed microtitre plate.

  ii) Place 0.025 ml of virus suspension (i.e. infective allantoic fluid) 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, 1/4, 1/5, 1/6, etc.

  iii) Make twofold dilutions of 0.025 ml volumes of the virus suspension across the plate.

  iv) Dispense a further 0.025 ml of PBS to each well.

  v) Dispense 0.025 ml of 1% (v/v) chicken RBCs to each well.

  vi) Mix by tapping the plate gently and then allow the RBCs 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, by which time control RBCs should have formed 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) Dispense 0.025 ml of PBS into each well of a plastic V-bottomed microtitre plate.
  
  ii) Place 0.025 ml of serum into the first well of the plate.
  
  iii) Make twofold dilutions of 0.025 ml volumes of the serum across the plate.
  
  iv) Add 4 HAU of virus/antigen in 0.025 ml to each well and leave for a minimum of 30 minutes at room temperature (i.e. about $20^\circ$C) or 60 minutes at $4^\circ$C.
  
  v) Add 0.025 ml of 1% (v/v) chicken RBCs to each well and mix gently, allow the RBCs to settle for about 40 minutes at room temperature, i.e. about $20^\circ$C, or for 60 minutes at $4^\circ$C if ambient temperatures are high, by which time control RBCs should have formed a distinct button.
  
  vi) The HI titre is the highest dilution of serum causing complete inhibition of 4 HAU of antigen. The agglutination is assessed by tilting the plates. Only those wells in which the RBCs stream at the same rate as the control wells (containing 0.025 ml RBCs and 0.05 ml PBS only) should be considered to show inhibition.
  
  vii) The validity of results should be assessed against a negative control serum, which should not give a titre $>1/4$ ($>2^2$ 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.

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. The meaning of a minimum positive titre should not be misinterpreted; it does not imply, for example, that immunised birds with that titre will be protected against challenge or that birds with lower titres will be susceptible to challenge. Appropriate virus/antigen control, positive control serum and RBC control well should be included with each batch of HI tests.

Chicken sera rarely give nonspecific positive agglutination reactions in this test and any pretreatment of the sera is unnecessary. Sera from species other than chickens may sometimes cause agglutination of chicken RBCs resulting in nonspecific agglutination. Therefore, each serum should first be tested for this idiosyncrasy and, if present, it should be inhibited 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, mixing 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. Alternatively, RBCs of the avian species under investigation could be used. Nonspecific inhibition of agglutination can be caused by steric inhibition when the H antigen and serum in the HI test have the same N subtype. The steric inhibition reaction can result in RBC buttoning in the bottom of the plate or streaming at the same rate as the control. To prevent steric nonspecific inhibition the H antigen used to test unknown serum must be of a different N subtype than the unknown sera, or the H antigen use can be recombinant or purified H protein that lacks N protein. The HI test is based on antigenic binding between the H antigen and antisera and thus other factors may cause nonspecific binding of the H antigen and sera leading to a nonspecific inhibition reaction. At this time there are no documented cross reactions or nonspecific inhibition reactions between the different haemagglutinin subtypes of avian influenza.

The neuraminidase-inhibition test has been used to identify the AI neuraminidase type of isolates as well as to characterise the antibody in infected birds. The procedure requires specialised expertise and reagents; consequently this testing is usually done in an OIE Reference Laboratory. The DIVA (differentiating infected from vaccinated animals) strategy used in Italy also relies on a serological test to detect specific anti-N antibodies; the test procedure has been described (Capua et al., 2003).

c) **Enzyme-linked immunoassay (ELISA) (an alternative test for international trade)**

Commercial ELISA kits that detect antibodies against the nucleocapsid protein are available. Kits with an indirect and competitive format have been developed and are now being used to detect AIV-specific antibodies. The kits should be validated for the specific species of interest and for the specific purpose(s) for which they are to be used. Several different test and antigen preparation methods are used. Such tests have usually been evaluated and validated by the manufacturer, and it is therefore important that the instructions specified for their use be followed carefully. Please see the OIE Register for kits certified by the OIE (http://www.oie.int/vcda/eng/en_vcda_registre.htm).
4. Antigen capture and molecular techniques

At present, the conventional virus isolation and characterisation techniques for the diagnosis of AI remain the methods of choice, for at least the initial diagnosis of AI infections. However, conventional methods tend to be costly, labour intensive and slow. There have been enormous developments and improvements in molecular and other diagnostic techniques, many of which have been applied to the diagnosis of AI infections.

a) Antigen detection

There are several commercially available AC-ELISA kits that can detect the presence of influenza A viruses in poultry (Swayne & Halvorson, 2008; Woolcock & Cardona, 2005). Most of the kits are enzyme immunoassays and use a monoclonal antibody against the nucleoprotein; they should be able to detect any influenza A virus. The main advantage of these tests is that they can demonstrate the presence of AI within 15 minutes. The disadvantages are that they may lack sensitivity, they may not have been validated for different species of birds, subtype identification is not achieved and the kits are expensive. The tests should only be interpreted on a flock basis and not as an individual bird test. Oropharyngeal or tracheal samples from clinically affected or dead birds provide the best sensitivity. Nevertheless, the lack of sensitivity is a major drawback to the use of available antigen detection tests. Chua et al. (Chua et al., 2007) evaluated five detection tests and showed overall sensitivities from 36.3% to 51.4%; these authors pointed out that in terms of sensitivity using cloacal and tracheal swabs, the tests performed less well with samples from waterfowl or wild birds than they did with samples from chickens. Woolcock & Cardonna (Woolcock & Cardona, 2005) examined five commercial tests licensed for human clinical use and found a wide variation in the ability to detect AIV in poultry specimens with minimal detection limits of $10^{4.7}$ EID$_{50}$ (50% egg-infective dose) of virus per ml with the best test, and a minimum of $10^{5.7}$ EID$_{50}$ per ml for the remaining tests.

b) Direct RNA detection

As demonstrated by the current definitions of HPNAI, molecular techniques have been used in the diagnosis of AI for some time now. Furthermore, there have recently been developments towards their application to the detection and characterisation of AI virus directly from clinical specimens of infected birds. It is imperative that when using highly sensitive molecular detection methods that allow rapid direct detection of viral RNA for confirmatory laboratory diagnosis of avian influenza infections, stringent protocols are in place to prevent the risk of cross-contamination between clinical samples. In addition, RNA detection test methodologies should be validated to the OIE standard (see Chapter 1.1.5 Principles and methods of validation of diagnostic assays for infectious diseases) using clinical material to demonstrate the tests as being ‘fit for purpose’ for application in a field diagnostic setting, which may include the use of internal test standards. The control reactions enable greater confidence in the integrity of the molecular reactions, clinical samples and results.

RT-PCR techniques on clinical specimens can, with the correctly defined primers, result in rapid detection and subtype identification (at least of H5 and H7), including a cDNA product that can be used for nucleotide sequencing (Starick et al., 2000; Suarez, 2007). This technique was used with success during the 2003 HPAI outbreaks in The Netherlands.

However, the preferred molecular detection tests for AI virus is the rRT-PCR, a modification to the RT-PCR that reduces the time for both identification of virus subtype and sequencing. For example, Spackman et al. (2002) used a single-step RT-PCR primer/fluorogenic hydrolysis probe system to allow detection of AI viruses and determination of subtype H5 or H7. The test performed well relative to virus isolation and offered a cheaper and much more rapid alternative, with diagnosis on clinical samples in less than 3 hours. In additional studies, the rRT-PCR was shown to have sensitivity and specificity equivalent to virus isolation based on field validation in the live poultry market control programme of New York and New Jersey during the winter of 2002, and the H7N2 LPAI outbreak and eradication programme in Virginia during 2002 (Elvinger et al., 2007; Spackman et al., 2003). The test provides high sensitivity and specificity similar to virus isolation from tracheal and oropharyngeal swabs of chickens and turkeys, but may lack sensitivity for detection of influenza A virus in faecal swabs, faeces and tissues in some bird species, because of the presence of PCR inhibitors resulting in false-negative results (Das et al., 2006). Incorporation of a positive internal control into the test will verify a proper test run. In addition, improved RNA extraction methods have been developed to eliminate most PCR inhibitors from test samples.

rRT-PCR, usually based around the hydrolysis probe or ‘TaqMan’ method for generation of the target-specific fluorescence signal, has become the method of choice in many laboratories for at least partial diagnosis directly from clinical specimens. The method offers rapid results, with sensitivity and specificity comparable to virus isolation. These are ideal qualities for AI outbreak management, where the period of time in which an unequivocal diagnosis can be obtained is crucial for decision making by the relevant
Veterinary Authority. In addition, rRT-PCR systems can be designed to operate in a 96-well format and combined with high-throughput robotic RNA extraction from specimens (Agüero et al., 2007).

The approach to diagnosis using rRT-PCR adopted in most laboratories has been based on initial generic detection of AIV in clinical specimens, primarily by initially targeting the matrix (M) gene, which is highly conserved for all type A influenza viruses, followed by specific rRT-PCR testing for H5 and H7 subtype viruses. For subtype identification, primers used in TaqMan rRT-PCR are targeted at the HA2 region, as this is relatively well conserved within the haemagglutinin genes of the H5 and H7 subtypes (Spackman et al., 2008; Spackman & Suarez, 2008). It has therefore served as the target region for these subtypes. Spackman et al. (2002) demonstrated specific detection of these subtypes, but cautioned that their H5 and H7 primer/probe sequences had been designed for the detection of North American H5 and H7 isolates and might not be suitable for all H5 and H7 isolates. This proved to be the case. Slomka et al. (Slomka et al., 2008) described modification of the H5 oligonucleotide sequences used by Spackman et al. (2002) to enable the detection of the Asian lineage HPAI H5N1 AI virus and other Eurasian H5 AI viruses that have been isolated within the past decade in both poultry and wild birds. Validated rRT-PCR protocols for the simultaneous detection and typing of H5, H7 and H9 RNA have been developed (Monne et al., 2008). These validated Eurasian rRT-PCR have proven valuable in the investigation of many H5N1 HPAI clinical specimens and other subtypes submitted to International Reference Laboratories from Europe, Africa and Asia since autumn 2005 (Monne et al., 2008; Slomka et al., 2007). Each set of primers and probes needs to be validated against a diverse set of viruses to make the test applicable to in diverse avian species, and in viruses from broad geographic areas and time periods.

One of the problems with rapidly emerging new tests is that methods and protocols may be developed and reported without the test being properly validated. This has been addressed for some of the rRT-PCR protocols (Slomka et al., 2007b; Suarez et al., 2007). In the European Union, National Reference Laboratories have collaborated to define and validate protocols that can be recommended for use within the European Union (Monne et al., 2008; Slomka et al. 2007b).

rRT-PCR protocols have been described that amplify regions across the cleavage site of the HA0 gene. This may result in useful tests for specific viruses. For example, Hoffman et al. (2007) have described an rRT-PCR test specific to the Asian HPAI H5N1 Qinghai-like clade 2.2 viruses that represents a rapid means of determining the pathotype for this subgroup of H5N1 HPAI viruses without sequencing. Fereidouni et al. (2008) have developed a restriction fragment polymorphism-based assay that enables the pathotyping of NAI of subtype H5 independent of sequencing or animal experiments after RT-PCR and restriction enzyme digest of the amplificate.

Modifications to the straightforward RT-PCR method of detection of viral RNA have been designed to reduce the effect of inhibitory substances in the sample taken, the possibility of contaminating nucleic acids and the time taken to produce a result. For example, nucleic acid sequence-based amplification (NASBA) with electrochemiluminescent detection (NASBA/ECL) is a continuous isothermal reaction in which specialised thermocycling equipment is not required. NASBA assays have been developed for the detection of AIV subtypes H7 and H5 in clinical samples within 6 hours (Ko et al., 2004). The loop-mediated isothermal amplification (LAMP) system for H5 detection appeared to show high sensitivity and reliable specificity (Imai et al., 2006), but may have limited application because of susceptibility to viral mutations affecting the target regions, reducing virus detection (Postel et al., 2010).

It seems highly likely that within a very short time molecular-based and improved antigen-based technologies will have developed sufficiently to allow rapid ‘flock-side’ tests for the detection of the presence of AIV specific subtypes and virulence markers. The extent to which such tests are employed will depend very much on the agreement on and adoption of definitions of what constitutes statutory infections for control and trade purposes.

C. REQUIREMENTS FOR VACCINES

1. Background

It is important that vaccination alone is not considered the solution to the control of NAI or LPAI subtypes if eradication is the desired result. Without the application of monitoring systems, strict biosecurity and depopulation in the face of infection, there is the possibility that NAI viruses could become endemic in vaccinated poultry populations. Long-term circulation of the virus in a vaccinated population may result in both antigenic and genetic changes in the virus and this has been reported to have occurred in Mexico, China (People's Rep. of), Egypt, Indonesia and other countries (Grund et al., 2011; Lee et al., 2004; Smith et al., 2006; Swayne & Kapczynski, 2008b). Currently used vaccines and the use of vaccination have been reviewed (Capua & Alexander, 2008; Swayne, 2003, 2004; Swayne & Kapczynski, 2008a, 2008b).
In this chapter, conventional vaccines are limited to inactivated avian influenza virus vaccines. These vaccines have been used against NAI or LPAI, having been prepared from infective allantoic fluid inactivated by beta-propiolactone or formalin and emulsified with mineral oil. Live conventional influenza vaccines against any subtype are not recommended.

The existence of a large number of virus subtypes, together with the known variation of different strains within a subtype, pose serious problems when selecting strains to produce inactivated avian influenza vaccines, especially for LPAI. In addition, some isolates do not grow to a sufficiently high titre to produce adequately potent vaccines without costly pre-concentration. While some vaccination strategies use autogenous vaccines, i.e. vaccines prepared from isolates specifically involved in an epizootic, others rely on vaccines prepared from viruses possessing the same haemagglutinin subtype and capable of yielding high concentrations of antigen.

Since the 1970s in the USA, inactivated AI vaccines have been used primarily in turkeys against LPAI and LPNAI viruses. These viruses may cause severe clinical signs, especially in exacerbating circumstances. Significant quantities of this vaccine have been used (Swayne & Halvorson 2008). In recent years in the USA, most of the inactivated Al vaccine has been used in breeder turkeys to protect against H1 and H3 swine influenza viruses. Vaccination against H9N2 LPAI virus has been used extensively in Asia and the Middle East (Swayne & Kapczynski, 2008a). Vaccination against HPNAI of H5N2 subtype was used in Mexico following outbreaks in 1994–1995 (Villareal, 2007), and against H7N3 subtype in Pakistan (Naeem, 1998) following outbreaks in 1995. In Mexico, the HPNAI virus appears to have been eradicated, but LPNAI virus of H5N2 has continued to circulate, while in Pakistan, HPAl viruses genetically close to the original HPAI virus were still being isolated in 2004. Following the outbreaks of HPNAI caused by H5N1 virus in Hong Kong in 2002 (Sims, 2003), a vaccination policy was adopted using H5N2 vaccine. Beginning in 2004, the widespread outbreaks of HPAI H5N1 in several countries of South-East Asia and Africa resulted in emergency and prophylactic vaccination being applied in China (the People’s Rep. of), Indonesia, Vietnam and Egypt. Inactivated H7N7 AI vaccine was used in Korea (Dem. Rep. of) during 2005 to control a HPAI outbreak. Similarly, preventive vaccination against H5N1 HPAI has been permitted for outdoor poultry and zoo birds in several European Union countries in recent years. Italy has extensively used the tool of serological (heterologous neuraminidase) DIVA with vaccination to control recurrent epidemics of LPNAI caused by viruses of the H7 subtype. A bivalent H5/H7 prophylactic vaccination programme was also developed as a result of an evolving epidemiological situation (Capua & Marangon, 2008).

Live recombinant virus vectored vaccines with avian influenza virus haemagglutinin gene inserts have been licensed and used in a few countries since 1997, mostly in chickens, and include recombinant fowl poxvirus, recombinant Newcastle disease virus and recombinant herpesvirus turkey vaccines.

a) Rationale and intended use of the product

Experimental work has shown, for both NAI and LPAI, that properly administered AI vaccines protect against clinical signs and mortality, reduces virus shedding and increases resistance to infection, protects from diverse field viruses within the same haemagglutinin subtype, protects from low and high challenge exposure, and reduces excretion and thus contact transmission of challenge virus (Capua et al., 2004; Swayne, 2003; Swayne & Suarez, 2000). However, the virus is still able to infect and replicate in clinically healthy vaccinated SPF birds when given in high challenge doses. Most of the work evaluating vaccines has been done in chickens and turkeys and some care must be taken in extrapolating the results obtained to other species. For example, in an experimental system using HPAI H7N7 as a challenge virus it was shown for chickens and ringed teal ducks, Callonetta leucophrys, that single vaccination sufficiently reduced excretion and increased the infective dose required and the transmission between birds was dramatically reduced. However, for golden pheasants, Chrysolophus pictus, even though a single vaccination provided clinical protection, there was no effect on the excretion of challenge virus and no influence on reducing virus transmission (Van der Goot et al., 2007). In some countries, vaccines designed to contain or prevent NAI are specifically banned or discouraged by government agencies because it has been considered that they may interfere with stamping-out control policies. However, most AI control regulations reserve the right to use vaccines in emergencies.

2. Outline of production and minimum requirements for conventional vaccines

The information below is based primarily on the experiences in the USA and the guidance and policy for licensing avian influenza vaccines in that country (United States Department of Agriculture, 1995 [updated 2006]). The basic principles for producing vaccines, particularly inactivated vaccines, are common to several viruses e.g. Newcastle disease (chapter 2.3.14).

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 Principles of veterinary vaccine production. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

The vaccine production facility should operate under the appropriate biosecurity procedures and practices. If HPNAI virus is to be used in challenge studies, the facility used for such studies should meet the requirements for Containment Group 4 pathogens as outlined in chapter 1.1.3.
a) Characteristics of the seed

i) Biological characteristics

For any subtype, only well characterised influenza A virus of proven low pathogenicity, preferably obtained from an international or national repository, should be used to establish a master seed for inactivated vaccines. HPAI viruses should not be used as seed virus for AI vaccine.

A master seed is established from which a working seed is obtained. The master seed and working seed are produced in SPF or SAN embryonated eggs. 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).

ii) Quality criteria (sterility, purity, freedom from extraneous agents)

The established master seed should be controlled/examined for sterility, safety, potency and absence of specified extraneous agents.

b) Method of manufacture

i) Procedure

For vaccine production, a working seed, from which batches of vaccine are produced, is first established in SPF or SAN embryonated eggs 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 below –60°C as lyophilized virus does not always multiply to high titre on subsequent first passage.

The routine procedure is to dilute the working seed in sterile isotonic buffer (e.g. PBS, pH 7.2), so that about $10^3$–$10^4$ EID$_{50}$ in 0.1 ml are inoculated into each allantoic cavity of 9- to 11-day-old embryonated SPF or SAN chicken 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 collected by suction. 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.

In the manufacture of inactivated vaccines, the harvested allantoic fluid is treated with either formaldehyde (a typical final concentration is 1/1000, i.e. 0.1% formalin) or beta-propiolactone (BPL) (a typical final concentration is 1/1000–1/4000, i.e. 0.1–0.025% of 99% pure BPL). The time required must be sufficient to ensure freedom from live virus. Most inactivated vaccines are formulated with non-concentrated inactivated allantoic fluid (active ingredient). However, active ingredients may be concentrated for easier storage of antigen. The active ingredient is usually emulsified with mineral or vegetable oil. The exact formulations are generally commercial secrets.

ii) Requirements for substrates and media

The inactivated influenza vaccines prepared from conventional virus are produced in 9- to 11-day-old embryonated SPF or SAN chicken eggs. The method of production is basically the same as for propagating the virus aseptically; all procedures are performed under sterile conditions.

iii) In-process controls

For inactivated vaccines, completion of the inactivation process should be tested in embryonated eggs, taking at least 10 aliquots of 0.2 ml from each batch and passaging each aliquot at least twice through SPF or SAN embryos. Viral infectivity must not remain.

iv) Final product batch tests

Most countries have published specifications for the control of production and testing of vaccines, which include the definition of the obligatory tests on vaccines during and after manufacture.

Sterility and purity

Tests for sterility and freedom from contamination of biological materials may be found in chapter 1.1.7.

Safety

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

Batch potency

Potency of avian influenza vaccine is generally evaluated by testing the ability of the vaccine to induce a significant HI titre in SPF or SAN birds. Conventional potency testing involving the use of three diluted doses and challenge with virulent virus (e.g. chapter 2.3.14) may also be used for vaccines
prepared to give protection against HPNAI or LPNAI subtypes. For inactivated vaccines to other subtypes, where virulent viruses are not available, potency tests may rely on the measurement of immune response or challenge and assessment of morbidity and quantitative reduction in challenge virus replication in respiratory (oropharyngeal or tracheal) and intestinal (cloaca) tracts. Assessment of haemagglutinin antigen content (Wood et al., 1985) could allow for in-vitro extrapolation to potency for subsequent vaccine batches.

Preservatives
A preservative may be used for vaccine in multidose containers.

c) Requirements for authorisation
i) Safety requirements

Target and non-target animal safety
Most inactivated avian influenza vaccines are licensed for use in chickens and turkeys. Field trials in the target species should be conducted to determine tolerance and safety of the vaccine at full dose. Recently the use of inactivated avian influenza vaccines has been expanded to ducks, geese, other poultry and zoo birds. Any extra-label use of the vaccines should be done cautiously and under the supervision of a veterinarian experienced in disease control through vaccination in the test species. Care must be taken to avoid self-injection with oil emulsion vaccines.

Reversion-to-virulence for attenuated/live vaccines
Only inactivated avian influenza virus vaccines are recommended. Live conventional influenza vaccines against any subtype are not recommended because of the risk for reassortment of gene segments of vaccine virus with field virus, potentially creating more virulent field viruses.

Environmental consideration
None

ii) Efficacy requirements

For animal production
For licensing purposes, avian influenza vaccines should pass an efficacy challenge test using a minimum of 24 SPF chickens per group. The challenge should occur at a minimum of three weeks post-vaccination, using a challenge HPNAI virus dose that causes 90% or greater mortality in the sham population. A standardised challenge dose of $10^5$ mean chicken embryo infectious doses is most widely use. Protection from mortality in the vaccine group should be a minimum of 80%. For LPNAI, mortality is not a feature of challenge models, therefore a statistically significant reduction in virus shedding titre and/or the number of birds shedding virus from oropharynx or cloaca should be observed between sham and test vaccine groups.

In establishing minimum antigen requirements, 50 PD$_{50}$ or 3 µg of haemagglutinin per dose have been suggested (Swayne & Kapczynski, 2008a). Minimum HI serological titres in field birds should be 1:32 to protect from mortality or greater than 1:128 to provide reduction in challenge virus replication and shedding.

For control and eradication
Efficacy should be the same as for animal production.

iii) Stability
When stored under the recommended conditions, the final vaccine product should maintain its potency for at least 1 year. Inactivated vaccines must not be frozen.

3. Vaccines based on biotechnology

a) Vaccines available and their advantages
Recombinant vaccines for AI viruses have been produced by inserting the gene coding for the influenza virus haemagglutinin into a live virus vector and using this recombinant virus to immunise poultry against AI (Swayne, 2004). Recombinant live vector vaccines have several advantages: 1) they are live vaccines able to induce mucosal, humoral and cellular immunity; 2) they can be administered to young birds and induce an early protection, e.g. the fowl poxvirus can be administered at 1 day of age, is compatible with the Marek’s disease vaccine, and provides significant protection 1 week later; 3) they enable differentiation between infected and vaccinated birds, as, for example, they do not induce the production of antibodies against the
nucleoprotein or matrix antigens that are common to all AI viruses. Therefore, only field-infected birds will exhibit antibodies in the AGID test or ELISAs directed towards the detection of influenza group A (nucleoprotein and/or matrix) antibodies. However, these vaccines have limitations in that they will replicate poorly and induce only partial protective immunity in birds that have had field exposure to or vaccination with the vector virus, i.e. fowl poxvirus or infectious laryngotracheitis viruses for currently available recombinant vaccines (Swaye & Kapczynski, 2008a, b). If used in day-old or young birds, the effect of maternal antibodies to the vector virus on vaccine efficacy may vary with the vector type. In the case of fowl poxvirus recombinant vaccine, it has been reported that effective immunisation was achieved when given to 1-day-old chicks with varying levels of maternal immunity (Arriola et al., 1999). However, when very high levels of maternal antibodies are anticipated because of previous infection or vaccination, the efficacy of the fowlpox vector vaccine in such day-old chicks should be confirmed and may require a prime-boost application of recombinant vaccine followed in 2–3 weeks by inactivated AI vaccine boost. In addition, because the vectors are live viruses that may have a restricted host range (for example infectious laryngotracheitis virus does not replicate in turkeys), the use of these vaccines must be restricted to species in which efficacy has been demonstrated.

The use of recombinant vaccines is restricted to countries in which they are licensed and legally available. The recombinant fowlpox-AI-H5 vaccine has been licensed in El Salvador, Guatemala, Mexico, China (the People’s Rep. of) and the USA (Swaye & Kapczynski, 2008a). Recombinant fowlpox virus vaccines containing H5 HA have been prepared and evaluated in field trials, but the only field experience with this vaccine has been in Mexico, El Salvador, Guatemala and China (the People’s Rep. of) where it has been used in the vaccination campaign against the H5N2 LPNAI and H5N1 HPNAI viruses.

Newcastle disease virus can also be used as a vector for expressing influenza HA haemagglutinin genes. A recombinant Newcastle disease vaccine virus expressing a H5 HA gene was shown to protect chickens against challenge with both virulent Newcastle disease virus and a HPNAI H5N2 virus (Veits et al., 2006). A similar recombinant virus based on Newcastle disease virus vaccine strain La Sota and expressing the Asian lineage H5 HA gene was produced in China (the People’s Rep. of) (Ge et al., 2007) and reported to be efficacious in protection studies with either virus. This latter virus has been licensed in China (the People’s Rep. of) and used widely. Recombinant Newcastle disease virus (rNDV) vaccines are effective in poultry lacking immunity to the Newcastle disease virus vector, but rNDV vaccines are largely ineffective as single dose primary vaccine in poultry with maternal immunity or well-immunised against Newcastle disease. rNDV vaccines are effective if used as a priming vaccine followed by a boost with an inactivated AI vaccine. In addition to these licensed vaccines, various experimental haemagglutinin-based AI vaccines have been described using in vivo or in vitro expression systems including recombinant adenoviruses, salmonella, baculovirus, vaccinia, avian leucosis virus, alphavirus and infectious laryngotracheitis virus (Swaye & Kapczynski, 2008a). DNA encoding H5 haemagglutinin has been evaluated as a potential vaccine in poultry.

b) Special requirements for biotechnological vaccines, if any

Live recombinant vectored vaccines with avian influenza gene inserts should have an environmental impact assessment completed to determine the risk of the vaccine to be virulent in non-target avian species and will not increase in virulence in the target avian species.

4. Surveillance methods for detecting infection in vaccinated flocks and vaccinated birds

A strategy that allows differentiation of infected from vaccinated animals (DIVA), has been put forward as a possible solution to the eventual eradication of NAI without involving mass culling of birds and the resulting economic damage, especially in developing countries (FAO, 2004). This strategy has the benefits of vaccination (less virus in the environment), but the ability to identify infected flocks would still allow the implementation of additional control measures, including stamping out. DIVA strategies use one of two broad detection schemes within the vaccinated population: 1) detection of influenza A virus (‘virus DIVA’), or 2) detection of antibodies against influenza A virus infection (‘serological DIVA’). At the flock level, a simple method consists of regularly monitoring sentinel birds left unvaccinated in each vaccinated flock, but this approach does have some management problems, particularly with regards to identifying the sentinels in large flocks. As an alternative or adjunct system, testing for field exposure may be performed on the vaccinated birds either by detection of field virus or antibodies against the virus. To detect the field virus, oropharyngeal or cloacal swabs from baseline daily mortality or sick birds can be tested, individually or as pools, by molecular methods, such as rRT-PCR or AC-ELISA of the vaccinated populations (Swaye & Kapczynski, 2008a).

To use serological DIVA schemes, vaccination systems that enable the detection of field exposure in vaccinated populations should be used. Several systems have been developed in recent years. These include the use of a vaccine containing a virus of the same haemagglutinin (H) subtype but a different neuraminidase (N) from the field virus. Antibodies to the N of the field virus act as natural markers of infection. This system has been used in Italy following the re-emergence of a LPNAI H7N1 virus in 2000. In order to supplement direct control measures, a ‘DIVA’ strategy was implemented using a vaccine containing H7N3 to combat a H7N1 field infection. Vaccinated and field exposed birds were differentiated using a serological test to detect specific anti-N antibodies (Capua et al., 2006).
al., 2003). The same strategy was used to control LPNAI caused by H7N3 in Italy in 2002–2003 (Capua & Alexander, 2004), in this case with a H7N1 vaccine. In both cases, vaccination combined with stamping out, using the described DIVA strategy resulted in eradication of the field virus. Problems with this system would arise if a field virus emerges that has a different N antigen to the existing field virus or if subtypes with different N antigens are already circulating in the field.

Alternatively the use of vaccines that contain only HA, e.g. recombinant vaccines, allows classical AGID and nucleocapsid protein (NP)- or matrix-based ELISAs to be used to detect infection in vaccinated birds. For inactivated vaccines, a test that detects antibodies to the nonstructural virus protein has been described (Tumpey et al., 2005). This system is yet to be validated in the field.

5. Continued evaluation and updating of vaccine seed strains to protect against emergent variant field virus strains

Historically, H5 LPNAI inactivated vaccine seed strains and recombinant fowl poxviruses with H5 gene inserts have shown broad cross protection in chickens against challenge by diverse H5 HPNAI viruses from Eurasia and North American (Swayne & Kapczynski, 2008a). However, avian influenza vaccines have had limited use in the field until 1995 when the H5N2 HPAI outbreak occurred in Mexico and vaccine use was implemented as part of the control programme (Villareal 2007). The HPAI strains were eradicated by June 1995, but as H5N2 LPNAI viruses have continued to circulate, vaccination was maintained as one of the control tools for these H5N2 LPNAI strains. Within a few years, multiple lineages of antigenically variant H5N2 LPNAI field viruses emerged that escaped from immunity induced by the original 1994 vaccine seed strain used in the conventional inactivated vaccine (Lee et al., 2004). Similarly, emergent H5N1 HPNAI field viruses have arisen in China (The People’s Rep. of), Indonesia and Egypt since 2005 that escaped from immunity induced by classical H5 inactivated vaccine seed strains used in commercial vaccines (Chen & Bu, 2009; Grund et al., 2011; Swayne & Kapczynski, 2008b). It is not clear whether the emergence of these antigenic variants is related to use of vaccines or improper use of vaccines.

All avian influenza vaccination programmes should have an epidemiologically relevant surveillance programme to check for emerging variants and representative isolates of AI viruses obtained should be assessed for genetic and antigenic variation. Screening can be done by HI testing using genetic variant field virus isolates and vaccine seed strains as antigen, and isolates suspected of being antigenic variants should then be analysed by methods to quantify antigenic changes, e.g. antigenic cartography (Fouchier & Smith, 2010). H5 and H7 LPNAI vaccine seed strains used in inactivated vaccines and recombinant vaccine viruses with AI haemagglutinin gene inserts should be re-evaluated and seed strains that are not protective should be discontinued: a) whenever there is evidence of emergence of antigenic variants or vaccine failure (clinical disease in vaccinated flocks with a solid immune response to the vaccine antigen); or b) every 2–3 years for efficacy against circulating field viruses, and the use of seed strains that are not protective should be discontinued. The vaccine seed strain evaluation should include field viruses from all relevant geographical regions and production sectors, and sequence analyses of such viruses to identify genetic variants that can be further evaluated for antigenic change that may reduce the efficacy of the vaccine(s) in use. Strains representative of the major circulating antigenic lineage(s) plus selected antigenic variants should be used in challenge trials against current licensed vaccine seed strains, as well as potential future seed strains. Based on this scientific information, the competent veterinary authority within the country should establish, in consultation with leading veterinary vaccine scientists and international organisations, naturally isolated or reverse genetics LPAI vaccine seed strains for conventional inactivated vaccines, and H5 and H7 haemagglutinin gene insert cassettes for recombinant vaccines. In some situations, more than one seed strain may be necessary to cover all production sectors within a country. Only high quality and potent vaccines should be licensed and used in AI control programmes. Proper administration of high quality, potent vaccines is critical in inducing protective immunity in poultry populations.

REFERENCES


Chapter 2.3.4. — Avian influenza


Chapter 2.3.4. — Avian influenza


Chapter 2.3.4. – Avian influenza


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NB: There are OIE Reference Laboratories for Avian influenza
(see Table in Part 4 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 avian influenza.
APPENDIX 2.3.4.1.

BIOSAFETY GUIDELINES FOR HANDLING HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUSES IN VETERINARY DIAGNOSTIC LABORATORIES

INTRODUCTION

The spread of highly pathogenic H5N1 avian influenza throughout Asia, Africa and Europe has led to an increase in the number of laboratories performing diagnostics for this pathogen. Highly pathogenic avian influenza viruses (HPAIV), in general, are a serious threat to birds and mortality is often 100% in susceptible chickens. In addition, the agents can also pose a serious zoonotic threat, with over 50% mortality reported in humans infected with HPAIV H5N1. In recognition of the need for guidance on how to handle HPAI viruses safely, the OIE has established the following biocontainment level guidelines for handling specimens that may contain HPAI virus. They are based on biosafety guidelines published in this OIE Terrestrial Manual (2012) and the World Health Organization (2005).

BIOCONTAINMENT LEVELS

Samples for diagnostic testing for HPAI using the following techniques can be processed using the OIE containment level for group 2 pathogens:

- Polymerase chain reaction (PCR)
- Antigen-capture assays
- Serology

Virus isolation and identification procedures for handling specimens that may contain high-titred replication-competent HPAIV should be performed at the OIE containment level for group 3 or group 4 pathogens, which would include the following:

- Personnel protective equipment should be worn, including solid-front laboratory coats, gloves, safety glasses and respirators with greater than or equal to 95% efficiency.
- Specimens from potentially infected birds or animals should only be processed in type II or type III biological safety cabinets (BSC).
- Necropsies of birds should be performed in a Type II BSC while wearing respiratory protection, such as a N95 respirator, or in a Type III biological safety cabinet, or other primary containment devices with 95% efficient air filtration.
- Centrifugation should be performed in sealed centrifuge cups.
- Centrifugation rotors should be opened and unloaded in a BSC.
- Work surfaces and equipment should be decontaminated after specimen processing.
- Contaminated materials should be decontaminated by autoclaving or disinfection before disposal or should be incinerated.

If chickens or other birds or mammals are inoculated with HPAI viruses, inoculation should be done in a containment level for group 4 pathogens and should include:

- Inoculated chickens should be held in isolation cages or other primary containment devices, or non-isolation cages/floor pens in specially designed rooms such as biosafety level 3 agriculture (BSL-3Ag) as designed by the US Department of Agriculture.
- Cages should be in a separate facility that is equipped to handle containment level for group 3 pathogens.
The room should be under negative pressure to the outside and the cages should be under negative pressure to the room.

- Cages should have HEPA-filtered inlet and exhaust air.
- Biosafety cabinet or other primary containment devices should be available in the animal facility to perform post-mortem examinations and to collect specimens.

REFERENCES


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