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* influenzavirus 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 embryonating chicken eggs. The eggs are incubated at 37°C* (*range 35–39°C*) *for 4–7 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 reverse-transcription polymerase chain reaction* (*RT-PCR*).

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.*

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 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 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 biocontainment laboratory. All virulent AI isolates are identified 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 identified as low pathogenicity notifiable avian influenza* (*LPNAI) viruses and non-H5 or non-H7 AI isolates that are not highly pathogenic for chickens are identified 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 *influenzavirus 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 (88). At present, 16 H subtypes (H1–H16) and 9 N subtypes (N1–N9) are recognised. To date, the 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 (3). 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 identified as NAI viruses (89).

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 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 are present. Confirmatory diagnosis of the disease, therefore, depends on the isolation of the causal virus and the demonstration that it fulfils one of the defined criteria described in section B.2. In some specific circumstances this may be achieved by detection of the virus in the infected host, especially using molecular techniques that allow the determination of virus virulence. 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 typing. These definitions evolve as scientific knowledge of the disease increases.

Highly pathogenic notifiable avian influenza (HPNAI) and NAI are subject to official control and the virus has a high risk of spread from the laboratory. Consequently, a risk assessment should be carried out to determine the level of biosecurity needed for laboratory diagnosis and chicken inoculation; characterisation of the virus should be conducted at biocontainment level 3 (at least). The facility should meet the requirements for the appropriate

Containment Group as determined by the risk assessment and as outlined in Chapter 1.1.2 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 embryonating specific pathogen free (SPF) 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 at least five embryonating SPF or SAN chicken eggs of 9–11 days' incubation. The eggs are incubated at 37°C (range 35–39°C) for 4–7 days. Eggs containing dead or dying embryos as they arise, and all eggs remaining at the end of the incubation period, should first be chilled to 4°C and the allantoic fluids should then be 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 haemagglutintin inhibition [HI] and neuraminidase inhibition [NI] tests) as described later (see Section B.3.b). Detection of HA activity, in bacteria-free amnio-allantoic fluids, 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 glycin/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 (8). 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 (37, 52, 72). 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 (87). 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 nine recognised serotypes of avian paramyxoviruses. Most laboratories will have antiserum 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 reverse-transcription polymerase chain reaction (RT-PCR) or real time RT-PCR using nucleoprotein-specific or matrix-specific conserved primers (4, 59). Also, the presence of subtype H5 or H7 influenza virus can be confirmed by using H5- or H7-specific primers (24, 50, 59, 86).

The method recommended for definitive antigenic subtyping of influenza A viruses by the World Health Organization (WHO) Expert Committee (88) involves the use of highly specific antisera, prepared in an animal giving minimum nonspecific reactions (e.g. goat), directed against the H and N subtypes (49). Alternative techniques include antigenic typing using polyclonal antisera raised against a battery of intact influenza viruses, or genotyping using sequence analysis of haemagglutinin and neuramindase 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 3 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, apathy, 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. At the First International Symposium on Avian Influenza held in 1981 (6), it was resolved to abandon the term 'fowl plague' and to define HPAI strains on the basis of their ability to produce not less than 75% mortality within 8 days in at least eight susceptible 4- to 8-week-old chickens inoculated by the intramuscular, intravenous or caudal air sac route. However, this definition proved unsatisfactory when applied to the viruses responsible for the widespread outbreaks in chickens occurring in 1983 in Pennsylvania and the surrounding states of the United States of America (USA). The problem was mainly caused by the presence of a virus of demonstrable low pathogenicity in laboratory tests, but which was shown to be fully pathogenic following a single point mutation. Further consideration of a definition to include such 'potentially pathogenic' viruses was undertaken by several international groups.

The eventual recommendations made were based on the finding that while there have been numerous isolations of strains of H5 and H7 subtypes of LPAI, all the HPAI strains isolated to date have possessed either the H5 or H7 haemagglutinin. Further information concerning the pathogenicity or potential pathogenicity of H5 and H7 subtypes may be obtained by sequencing the genome, as pathogenicity is 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. In 1992, the OIE adopted criteria for classifying an AIV as highly pathogenic based on pathogenicity in chickens, growth in cell culture and the amino acid sequence for the connected peptide. The European Union adopted similar criteria in 1992 (17).

The following criteria, which are a modification of the previous OIE procedure, have been adopted by the OIE for classifying an AIV as HPNAI:

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

   i) any influenza virus that is lethal[1] 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 allantoic fluid

1 When birds are too sick to eat or drink, they should be killed humanely.

or

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 (>2^4 or >$\log_2$ 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 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 death[2].)
- 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 Table 1, which can also be found at: http://www.offlu.net/OFFLU%20Site/Projects/Table%20HPAI%20cleavage%20site%20sequences.pdf).

The OIE has the following classification system to identify viruses for which disease reporting and control measures should be taken (89):

a) All AI isolates that meet the above criteria are identified 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 identified as low pathogenicity notifiable avian influenza (LPNAI).

c) Non-H5 or non-H7 AI isolates that are not virulent for chickens are identified 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. The most commonly used method has been RT-PCR using oligonucleotide primers complementing areas of the gene either side of the cleavage site coding region, followed by cycle sequencing (85). Various stages in the procedure can be facilitated using commercially available kits and automatic sequencers.

Now that the presence of multiple basic amino acids at the HA0 cleavage site is well-established as an accurate indicator of virulence or potential virulence for H5 and H7 influenza viruses, it appears inevitable that determination of the cleavage site by sequencing or other methods will become the method of choice for initial assessment of the virulence of these viruses and incorporated into agreed definitions. This will have the advantage of reducing 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 cultures containing mixed populations 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 definitions for highly pathogenic AI viruses (83) as they killed 7/10 and 8/10 chickens with IVPI values >1.2 when the birds were inoculated intravenously. These viruses did not induce death or signs of disease when inoculated intranasally and did not have multiple basic amino acids at their haemagglutinin cleavage sites. Similarly, other LPAI viruses are nephrotropic and birds that die have high titres of virus in their kidneys indicating a renal pathogenic mechanism (53), 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 (36). Other anomalies are the Chile 2002 (64) and the Canada 2004 (45)

2 When birds are too sick to eat or drink, they should be killed humanely and scored as dead at the next observation.

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 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 heamagglutinin 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; the address of the OFFLU web site is:
http://www.offlu.net/OFFLU%20Site/Projects/Table%20HPAI%20cleavage%20site%20sequences.pdf

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 embryonating chicken eggs (8) 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°C) or 60 minutes at 4°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°C, or for 60 minutes at 4°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.

Chicken sera rarely give nonspecific positive reactions in this test and any pretreatment of the sera is unnecessary. Sera from species other than chickens may sometimes cause agglutination of chicken RBCs. 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.

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 (13).

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 antigen-capture kits that can detect the presence of influenza A viruses in poultry (52, 87). 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.* (15) 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 (87) 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, RT-PCR test methodologies should be validated to the OIE standard (see Chapter 1.1.4 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 (41, 61, 62). The real application of direct RT-PCR tests may be the rapid identification of subsequent outbreaks once the primary infected premises have been detected and the virus characterised. This technique was used with success during the 2003 HPAI outbreaks in The Netherlands. Ring trials conducted recently in the European Union identified H5 and H7 conventional RT-PCR protocols that were sufficiently sensitive for direct amplification from swabs obtained from HPAI-infected poultry (54).

Modifications to the use of RT-PCR have been applied to reduce the time for both identification of virus subtype and sequencing. For example, Spackman *et al*. (59) used a 'real-time' single-step RT-PCR primer/fluorogenic hydrolysis probe system to allow detection of AI viruses and determination of subtype H5 or H7. The authors concluded that 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. The real-time RT-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 (2, 20, 57). 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 (19). 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.

Real-time RT-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, RT-PCR systems can be designed to operate in a 96-well format and combined with high-throughput robotic RNA extraction from specimens (1).

The approach to diagnosis using real-time RT-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 real-time RT-PCR testing for H5 and H7 subtype viruses. For subtype identification, primers used in TaqMan real-time RT-PCRs are targeted at the HA2 region, as this is relatively well conserved within the haemagglutinin genes of the H5 and H7 subtypes (59, 58, 60). It has therefore served as the target region for these subtypes. Spackman *et al.* (59) 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.* (55) described modification of

the H5 oligonucleotide sequences used by Spackman *et al.* (59) 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. This validated Eurasian H5 real-time RT-PCR has proven valuable in the investigation of many H5N1 HPAI clinical specimens submitted to International Reference Laboratories from Europe, Africa and Asia since autumn 2005 (55).

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 real-time RT-PCR protocols (55, 63). In the European Union, National Reference Laboratories have collaborated to define and validate protocols that can be recommended for use within the European Union (54, 55).

Real-time RT-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.* (30) have described a real-time RT-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*. (22) 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 (16, 32). The loop-mediated isothermal amplification (LAMP) system for H5 detection appeared to show high sensitivity and reliable specificity (31).

It seems highly likely that within a very short time molecular-based technology 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 AND DIAGNOSTIC BIOLOGICALS

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 these 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 (34) and is suspected in China (People's Rep. of) and other countries in South-East Asia (46, 56, 70).

Experimental work has shown, for both NAI and LPAI, that vaccination protects 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 (14, 21, 66, 73). However, the virus is still able to infect and replicate in clinically healthy vaccinated SPF birds. 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 transmission (76, 77). 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.

Live conventional influenza vaccines against any subtype are not recommended.

• Conventional vaccines

Conventionally, vaccines that have been used against NAI or LPAI have been prepared from infective allantoic fluid inactivated by beta-propiolactone or formalin and emulsified with mineral oil.

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 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. For example, in the USA, some standardisation of the latter has been carried out by the Center for Veterinary Biologics, which has propagated and stored influenza viruses of several subtypes that can be used as seed viruses for the preparation of inactivated vaccines (7).

Since the 1970s in the USA, there has been some use of inactivated vaccines produced under special licence on a commercial basis (28, 39, 47). These vaccines have been used primarily in turkeys against viruses that are not highly pathogenic, but that may cause severe clinical signs, especially in exacerbating circumstances. Significant quantities of this vaccine have been used (29, 39). In recent years in the USA, most of the special license inactivated vaccine has been used in breeder turkeys to protect against H1 and H3 swine influenza viruses (65). Conventional vaccination against the prevailing strain of LPAI has also been used in Italy for a number of years (18). Vaccination against H9N2 infections has been used in Pakistan (43), Iran (79), China (People's Rep. of) (35), as well as several countries in the Middle East.

Inactivated vaccine was prepared from the LPNAI virus of H7N3 subtype responsible for a series of outbreaks in turkeys in Utah in 1995. In combination with additional measures, it was used to bring the outbreaks under control (29). Similarly, in Connecticut in 2003, vaccination of recovered hens and replacement pullets with a H7N2 or H7N3 vaccine was implemented following an outbreak of LPNAI caused by a H7N2 virus (68).

Vaccination against HPNAI of H5N2 subtype was used in Mexico following outbreaks in 1994–1995 (24, 25, 34), and against H7N3 subtype in Pakistan (42) 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, HPAI 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 (51), a vaccination policy was adopted using H5N2 vaccine. In 2004 the widespread outbreaks of HPAI H5N1 in several countries of South-East Asia resulted in emergency and prophylactic vaccination being applied in the China (People's Rep. of), Indonesia and Vietnam. Inactivated H7N7 AI vaccine was used in Korea (Dem. Rep. of) during 2005 to control a HPAI outbreak. Prophylactic vaccination has also been used in limited areas in Italy to aid in the control of H5 and H7 LPNAI viruses. Similarly, preventive vaccination has been permitted for outdoor poultry and zoo birds in several European Union countries in recent years.

- **Recombinant vaccines**

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 (67). 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 (38, 69). 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 (5). 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. 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 (66, 90). Recombinant fowlpox virus vaccines containing H5 HA have been prepared and evaluated in field trials (9, 26, 48, 71), 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 LPAI and H5N1 HPAI viruses. Between 1995 and 2006, Mexico used more than 1.788 billion doses of inactivated H5N2 vaccine in their H5N2 control programme (80, 81). In addition, Mexico, Guatemala and

El Salvador have used over 1.6 billion doses of the recombinant fowlpox-AI-H5 vaccine for control of H5N2 LPNAI from 1997 to 2005 and China (the People's Rep. of) used 606 million doses in 2005 (90).

Newcastle disease virus can also be used as a vector for expressing influenza HA genes (44). A recombinant Newcastle disease vaccine virus (clone 30) containing and expressing a H5 HA gene was shown to protect chickens against challenge with either virulent Newcastle disease virus or a HPAI H5N2 virus (78). 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) (27) 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 as one of the four H5 vaccines allowed under the compulsory vaccination policy currently in place; this policy resulted in the vaccination of 8.2 billion birds between January and September 2006 (40). As with other recombinant vaccines, it seems doubtful that this vaccine will be appropriate for use in older birds that are well-immunised against Newcastle disease, and it is not clear to what extent the presence of maternal immunity to either the vector or the AI HA in young chicks will affect efficacy. In addition to these licensed vaccines, various experimental approaches have been described including recombinant adenoviruses, salmonella, etc. A baculovirus-expression system has been used to produce recombinant H5 and H7 antigens for incorporation into vaccines (82) and DNA encoding H5 haemagglutinin has been evaluated as a potential vaccine in poultry (33).

• Detection of 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 (23). 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 ('genetic 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 real-time RT-PCR or AC-ELISA of the vaccinated populations.

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 (11, 12). The same strategy was used to control LPNAI caused by H7N3 in Italy in 2002–2003 (10), 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 (74).This system is yet to be validated in the field.

• Production of 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 (75). 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 1.1.8 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.

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 1.1.2.

1. Seed management

a) Characteristics of the seed

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.

b) Method of culture

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

c) Validation as a vaccine

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

2. Method of manufacture

For vaccine production, a working seed, from which batches of vaccine are produced, is first established in SPF or SAN embryonating 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 lyophilised virus does not always multiply to high titre on subsequent first passage.

The inactivated influenza vaccines prepared from conventional virus are produced in embryonating chicken eggs. The method of production is basically the same as for propagating the virus aseptically; all procedures are performed under sterile conditions.

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 embryonating 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) or beta-propiolactone (a typical final concentration is 1/1000–1/4000). 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.

3. In-process control

For inactivated vaccines, completion of the inactivation process should be tested in embryonating 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.

4. Batch control

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.

a) Sterility

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

b) 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.

c) 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 (84) could allow for *in vitro* extrapolation to potency for subsequent vaccine batches.

d) 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.

e) Preservatives

A preservative may be used for vaccine in multidose containers.

f) Precautions (hazards)

Care must be taken to avoid self-injection with oil emulsion vaccines.

5. Tests on the final product

a) Safety

See Section C.4.b. above

b) Potency

See Section C.4.c. above.

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NB: There are OIE Reference Laboratories for Avian influenza (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

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 the OIE *Terrestrial Manual* (1) and the World Health Organization (2).

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.

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