

CHAPTER 3.3.4.

AVIAN INFLUENZA (INCLUDING INFECTION WITH HIGH PATHOGENICITY AVIAN INFLUENZA VIRUSES)

SUMMARY

Influenza A is caused by specified viruses that are members of the family Orthomyxoviridae and placed in the genus Alphainfluenzavirus (Influenzavirus A or influenza A virus). There are seven influenza genera but 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.

Detection 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 nucleoprotein and/or matrix antigens, both of which are common to all influenza A viruses, or by real-time reverse-transcription polymerase chain reaction (real-time RT-PCR) on the allantoic fluids. Isolation in embryos has largely been replaced for initial diagnosis by direct detection in samples, of one or more segments of the influenza A genome using real-time RT-PCR or other validated molecular techniques.

For serological subtyping of 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. Alternatively, the genome of specific H and N subtypes is identified using RNA detection technologies with subtype specific primers and probes (e.g. real-time RT-PCR) or sequencing and phylogenetic analysis.

As the general term 'highly pathogenic avian influenza' and the historical term 'fowl plague' refer to infection with high pathogenicity strains of influenza A virus, it is necessary to assess the pathogenicity of Influenza A virus isolates for domestic poultry. All naturally occurring high pathogenicity avian influenza (HPAI) strains isolated to date have been either of the H5 or H7 subtype, with a subset of H5 or H7 isolates being of low pathogenicity. 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. Regardless of their pathogenicity for chickens, H5 or H7 viruses with a HA0 cleavage site amino acid sequence similar to any of those that have been observed in high pathogenicity viruses are considered to be influenza A viruses with high pathogenicity. H5 and H7 isolates that are not highly pathogenic for chickens and do not have an HA0 cleavage site amino acid sequence similar to any of those that have been observed in highly pathogenic viruses are considered to have low pathogenicity. However in some circumstances it is necessary to verify high or low pathogenicity of a virus isolate using the intravenous inoculation of a minimum of eight susceptible 4- to 8-week-old chickens with infectious virus; strains are considered to be of high pathogenicity 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 highly pathogenic strains of the virus should be conducted in a virus-secure biocontainment laboratory. Low pathogenicity avian influenza (LPAI) in poultry may be accompanied

by a sudden and unexpected increase in virulence (emerging disease) or have proven natural transmission to humans associated with severe consequences. In these disease scenarios there should be formal monitoring in relevant poultry populations by national authorities. The occurrence of H5 and H7 low pathogenicity avian influenza viruses should be monitored as some have the potential to mutate into high pathogenicity avian influenza viruses.

Serological tests: As all influenza A viruses have antigenically similar nucleoprotein and matrix antigens, these are preferred targets of influenza A group serological methods. Enzyme-linked immunosorbent assays (ELISA) are widely used to detect antibodies to these antigens in either host species-dependent (indirect) or species-independent (competitive) test formats. 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.

Requirements for vaccines: The first use of vaccination in an avian influenza eradication programme was against LPAI. The programmes used inactivated oil-emulsion vaccines with the same haemagglutinin and neuraminidase subtypes as the circulating field virus, 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 HPAI and H5/H7 LPAI. During the 1999–2001 outbreak of H7 LPAI in Italy, an inactivated vaccine was used with the same (i.e. homologous) haemagglutinin subtype to the field virus, but with a different (i.e. heterologous) neuraminidase. This allowed the serological 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 H5/H7 LPAI infections, and several countries in Asia, Africa and the Middle East as an aid in controlling HPAI, in China (People's Rep. of) for H7N9, and in Mexico for H7N3 HPAI virus infections. HPAI viruses should not be used as the seed virus for production of vaccine.

If LPAI and HPAI viruses are used in challenge studies, an appropriate level of containment should be used as determined by risk assessment.

A. INTRODUCTION

Influenza in birds is caused by infection with viruses of the family *Orthomyxoviridae* placed in the genus *Alphainfluenzavirus* (*influenzavirus A* or influenza A virus) (International Committee on Taxonomy of Viruses (ICTV), 2019). Influenza A viruses are the only orthomyxoviruses known to naturally affect birds (Swayne & Sims, 2020). 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 nucleoprotein 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 with proposed new subtypes (H17, H18) for influenza A viruses from bats in Guatemala (ICTV 2019; Swayne *et al.*, 2020; Tong *et al.*, 2013). To date, naturally occurring high pathogenicity 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. Low pathogenicity H5 and H7 occur widely in poultry and aquatic wild birds, although intercontinental spread of HPAI has received greater attention in recent years. There is the risk of a H5 or H7 virus of low pathogenicity (H5/H7 low pathogenicity avian influenza [LPAI]) becoming highly pathogenic by mutation. Some avian influenza virus strains have caused sporadic zoonotic infections principally of H5, H7 and H9 subtypes and these three subtypes have been highlighted as potential pandemic risks should additional mutations occur that support sustained human-to-human transmission (Cox *et al.*, 2017).

Throughout this chapter of the *Terrestrial Manual*, the following terms will be used: 1) HPAI as an infection by an avian influenza virus that meets the definition of high pathogenicity, 2) LPAI as an infection with any H1–H16 avian influenza virus that is not of high pathogenicity, and 3) influenza A as an infection with any HPAI or LPAI virus.

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 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 (Swayne *et al.*, 2020). 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 (*Anas platyrhynchos domesticus*) some HPAI viruses do not necessarily produce significant clinical disease. In addition, 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 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.1.1.1. Testing sera from suspect birds using antibody detection methods may supplement diagnosis, but these methods are not suitable for a definitive 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 HAO consistent with HPAI virus) and haemagglutinin subtyping. These definitions evolve as scientific knowledge of the disease increases.

HPAI should be subject to official control by national authorities. In addition LPAI, particularly H5 and H7 subtypes, may be subject to national or state/provincial control. The viruses that cause influenza A have the potential to spread from the laboratory if adequate levels of biosecurity and biosafety are not in place. Avian influenza viruses should be handled with appropriate measures as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*. Biocontainment measures should be determined by risk analysis as described in Chapter 1.1.4. The measures required may vary among the subtypes and pathotypes of influenza A viruses, with higher level containment being indicated for some LPAI and HPAI viruses, and may require additional procedural, equipment and facility enhancements under specific conditions such as high virus concentrations, housing infected animals or conducting procedures with aerosol generating activities. Countries lacking access to such a specialised national or regional laboratory should send specimens to a WOAHP Reference Laboratory.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of avian influenza and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent ^(a)						
Virus isolation	+	+++	+	+++	+	–
Antigen detection	+	+	+	+	+	–
Real-time RT-PCR	++	+++	++	+++	++	–

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of immune response						
AGID	+ (Influenza A)	+ (Influenza A)	++ (Influenza A)	+ (convalescent)	++ (Influenza A)	++ (Influenza A)
HI	+++ (H5 or H7)	++ (H5 or H7)	+++ (H5 or H7)	++ (convalescent)	+++ (H5 or H7)	+++ (H5 or H7)
ELISA	+	+	++	+ (convalescent)	++	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.

RT-PCR = reverse-transcription polymerase chain reaction; AGID = agar gel immunodiffusion;

HI = haemagglutination inhibition test; ELISA = enzyme-linked immunosorbent assay.

^(a)A combination of agent identification methods applied on the same clinical sample is recommended.

1. Detection of the agent

Identification of influenza A viruses as the cause of infections and disease in poultry and other birds requires a thorough diagnostic investigation to differentiate from similar diseases caused by other viral agents especially avian paramyxovirus type 1 (APMV-1). Individual influenza A and APMV-1 virus isolates vary greatly in virulence, causing various syndromes evident as subclinical infections, drops in egg production, respiratory disease, and severe and high mortality disease. The latter clinical syndrome can be caused by either HPAI or Newcastle disease viruses. Therefore, it is judicious to have a single sampling procedure and simultaneously conduct specific differentiating diagnostic tests for both influenza A and APMV-1 viruses on field samples to obtain an accurate aetiological diagnosis of a single agent or, on occasion, confirmation of dual infection.

1.1. Samples for virus isolation

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

For investigations of severe disease and high mortality in poultry flocks, it is usual to attempt virus isolation from recently dead birds or moribund birds that have been killed humanely. Samples taken from dead birds should include intestinal contents (faeces) or cloacal swabs and oropharyngeal or tracheal swabs. Samples from trachea, lungs, air sacs, intestine, spleen, caecal tonsils, kidney, brain, liver and heart should also be collected and processed either separately or as a pool. When pooling samples the brain should be collected and processed first (to avoid cross contamination with other tissue types) and kept separate as presence of virus in the brain may be an indicator of HPAI or NDV. Further pools should be made consistent with known virus tropisms between HPAI and LPAI, i.e. grouped at the level of respiratory, systemic and gastrointestinal.

Samples from live birds should include both oropharyngeal or tracheal and cloacal swabs, the latter should be visibly coated with faecal material. To avoid harming them, swabbing of small delicate birds should be done with the use of especially small swabs that are usually commercially available and intended for use in human paediatrics or the collection of fresh faeces may serve as an adequate alternative (caution that some influenza A viruses and type 1 avian paramyxoviruses in birds can have a strong respiratory tropism). Similar swab samples can be pooled from the same anatomical site (i.e. cloacal swabs with cloacal swabs, oropharyngeal swabs with oropharyngeal swabs), and most commonly pooling of 5 or occasionally more, if appropriately validated not to reduce sensitivity of detection, but specific swab types should be used (Spackman *et al.*, 2013). Further the type of swabs used may affect test sensitivity or validity with thin wire or plastic shafted swabs preferred.

The samples should be placed in isotonic phosphate-buffered saline (PBS), pH 7.0–7.4 with antibiotics or a solution containing protein and antibiotics. The antibiotics can be varied according to local conditions, but could be, for example, penicillin (2000 units/ml), streptomycin (2 mg/ml), gentamycin (50 µg/ml) and mycostatin (1000 units/ml) for tissues and oropharyngeal or tracheal swabs, but at five-fold higher concentrations for faeces and cloacal swabs. It is important to re-adjust 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). If control of *Chlamydophila* is desired, 0.05–0.1 mg/ml oxytetracycline should be included. Faeces and finely minced tissues should be prepared as 10–20% (w/v) suspensions in the antibiotic solution. Suspensions should be processed as soon as possible after incubation for 1–2 hours at room temperature. When immediate processing is impractical, samples may be stored at 4°C for up to 4 days. For prolonged storage, diagnostic samples and isolates should be kept at –80°C but for transport on dry ice (≤–50°C) is widely used. Repeated freezing and thawing should be avoided.

1.2. Virus isolation

The preferred method of growing 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, swabs or tissue suspensions obtained through clarification by centrifugation at 1000 *g* for about 10 minutes at a temperature not exceeding 25°C. Clarified preparations can be inoculated using a number of routes including the amniotic sac, chorioallantoic sac or membrane (one of which is recommended for primary isolation) and in all cases allantoic sacs 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. After checking that the embryos have died, the amnio-allantoic fluids should 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 haemagglutination inhibition [HI] and neuraminidase [N] inhibition [NI] tests) or a molecular test to detect influenza A specific nucleic acid signatures (such as real-time reverse transcription polymerase chain reaction [RT-PCR]) as described later (see Section B.1.2.2). 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 orthoavulavirus (formerly avian paramyxovirus). Fluids that give a negative reaction should be passaged into at least one further batch of eggs, and up to three passages.

Routine checks for bacterial contamination should be conducted by streaking samples in Luria Broth agar plates and reading these at 24 and 48 hours of incubation against a light source. BHI agar and blood agar plates may also be used. For larger numbers of sample initial culture could be in tryptose phosphate broth. Contaminated samples can be treated by incubation with increased antibiotic concentrations for 2–4 hours (gentamicin, penicillin G, and amphotericin B solutions at final concentrations to a maximum of 1 mg/ml, 10,000 U/ml, and 20 µg/ml, respectively). Samples heavily contaminated by bacteria that cannot be removed by centrifugation or controlled by antibiotics can be filtrated through 0.45 and 0.2 micron sterile filters. Filtration should be used only when other methods fail because aggregation may significantly reduce virus titre.

1.3. Virus identification

The presence of influenza A virus can be confirmed in AGID tests by demonstrating the presence of the nucleoprotein or matrix antigens, both of which are common to all influenza A viruses (see Section B.2.2). 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 nucleoprotein and matrix polypeptides.

Preparations of nucleoprotein-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 or 1% betapropiolactone.

Use of the AGID test to demonstrate nucleoprotein or matrix antigens is a satisfactory way to indicate the presence of influenza A virus in amnioallantoic fluid, but lacks sensitivity compared to other methods including molecular (see Section 1.2.2) but various experimental and commercial rapid, solid-phase antigen-capture ELISAs (AC-ELISAs) are an effective alternative (Swayne *et al.*, 2020). Most AC-ELISAs have been approved and marketed to detect human influenza A virus in clinical specimens. Some have demonstrated effectiveness for detection of influenza A, but many of these commercial tests have had low sensitivity (Slomka *et al.*, 2012). 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 21 recognised serotypes of avian paramyxoviruses (ICTV, 2019). Most laboratories will have antiserum specific to Newcastle disease virus (avian paramyxovirus type 1, APMV1), 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 3.3.10 *Newcastle disease*).

Alternatively, the presence of influenza virus can be confirmed by the use of conventional RT-PCR or real-time RT-PCR using nucleoprotein-specific or matrix-specific conserved primers (Nagy *et al.*, 2020; Spackman *et al.*, 2002). Also, the presence of subtype H5 or H7 influenza virus can be confirmed by using H5- or H7-specific primers (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 range of intact influenza viruses and used in HI and NI tests. For laboratories conducting the HI test to H subtype it is strongly recommended that two sera for each H subtype is used but with a heterologous N and should ideally use antisera to contemporary viruses relevant to the region in which the virus is detected. Subtyping can also be accomplished using H and N subtype specific primers in RT-PCR and real-time RT-PCR tests; or using sequence analysis of H and N genes. Subtype identification by these techniques is becoming increasingly common but is beyond the scope of many diagnostic laboratories not specialising in influenza viruses. Assistance is available from the WOAHA Reference Laboratories and Collaborating Centres (see WOAHA website for up-to-date list).

1.4. Assessment of pathogenicity

The term HPAI relates to the assessment of pathogenicity in chickens and implies the involvement of high pathogenicity strains of virus. It is used to describe a disease of fully susceptible chickens with clinical signs that may include one or more of the following: 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/H7 LPAI viruses, all H5/H7 LPAI viruses may potentially become HPAI but predicting which LPAI strains will mutate to HPAI is not possible. 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 influenza A virus gene segments or avian host cellular genome (e.g. 28S rRNA) 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 site¹. Amino acid sequencing of the cleavage sites of H5 and H7 subtype influenza A isolates of low pathogenicity for birds may identify viruses that have the capacity, following simple mutation, to have high pathogenicity for poultry.

The following criteria have been adopted by the WOAHP for determining pathogenicity of an influenza A virus:

- a) One of the two following methods to determine pathogenicity in chickens is used. A high pathogenicity influenza A virus is:
 - i) any influenza A virus that is lethal² 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
or
 - ii) any influenza A virus that has an intravenous pathogenicity index (IVPI) greater than 1.2. The following is the IVPI procedure:
 - Fresh infective allantoic fluid, confirmed free from APMV-1 and other extraneous agents, 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 death³.)
 - 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 molecule (HA0) (i.e. the cleavage site) must be determined. The presence of several basic amino acids, inserts of cellular or viral nucleic acids or loss of specific glycosylation sites in the HA0 cleavage site is the genotypic standard for HPAI strains; therefore, if the isolate being tested has an HA0 cleavage site motif identical to previous HPAI viruses, it should be designated as HPAI irrespective of a low or high pathogenicity determined by pathotyping in chickens (see the table that lists all the reported haemagglutinin proteolytic cleavage sites 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 (see footnote 2). Furthermore any isolate with a new motif must be tested *in vivo* by IVPI. In case of difficulties in the interpretation of the cleavage site motif, WOAHP and/or FAO reference laboratories should be consulted.

The WOAHP classification system to identify influenza A viruses for which disease notification and control measures should be taken is defined in the *Terrestrial Code*.

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

1 http://www.offlu.org/wp-content/uploads/2021/01/Influenza_A_Cleavage_Sites.pdf

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

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

H7 subtypes of avian influenza virus, enabling the amino acids there to be deduced. This can be done by RNA extraction from the sample and direct sequencing of the haemagglutinin proteolytic cleavage site. Various stages in the procedure can be facilitated using commercially available kits and automated sequencers.

Determination of the cleavage site by sequencing or other methods has become the method of choice for initial assessment of the pathogenicity of these viruses and has been incorporated into agreed definitions. This has reduced the number of *in-vivo* tests, although the initial Sanger sequencing result of a HA cleavage site for an H5 or H7 LPAI virus should be confirmed by either inoculation of birds or deep sequencing using high throughput sequencing with a minimum of 1000 reads to to exclude the presence of any HPAI virus.

Although all the truly HPAI viruses isolated to date have been of H5 or H7 subtypes, at least three isolates, all of H10 subtype (H10N1, H10N4 and H10N5), have been reported that would have fulfilled both the WOA and EU *in-vivo* definitions for HPAI viruses (Bonfante *et al.*, 2014; Wood *et al.*, 1996) as they killed 6/10, 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 influenza A 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 is not comparable to multi-organ infection and systemic disease caused by HPAI viruses. An H4N2 virus isolated from quail had a multibasic cleavage site sequence (PEKRRTR/GLF) but with an IVPI value of 0.0 (Wong *et al.*, 2014) suggesting the multibasic cleavage site in viruses other than H5 and H7 alone may not be sufficient for declaration of HPAI virus and the *in-vivo* test should be carried out. Conversely, four viruses (A/chicken/Pennsylvania/1/83 [H5N2] and A/goose/Guangdong/2/96 [H5N1], A/turkey/England/87-92BFC/91 [H5N1] or A/chicken/Texas/298313/04 [H5N2]) have been described that have HA0 cleavage sites containing multiple basic amino acids, but which show low pathogenicity (IVPI <1.2) when inoculated intravenously into 6-week-old chickens (Londt *et al.*, 2007). No single explanation including the presence of a glycosylation site masking the HA0 cleavage site was reported emphasising both intra-haemagglutinin and multigenic influences in rare circumstances upon phenotypic expression of high pathogenicity. The presence of high pathogenicity haemagglutinin cleavage site in H5 and H7 influenza A viruses necessitates declaration of high pathogenicity to facilitate immediate control of the disease, otherwise a delay to complete *in-vivo* testing may result in continued onward transmission and spread between premises with severe consequence for future eradication once confirmed as a HPAI virus.

A table is available on the OFFLU website 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 viruses are characterised; it can be found on the OFFLU site (see footnote 2).

1.5. Antigen capture and molecular techniques

At present, conventional virus isolation and characterisation techniques for the diagnosis of influenza A viruses remain a key method, for initial diagnosis of influenza A infection in a primary disease event and to provide virus for more detailed analyses including *in-vivo* testing and gene sequencing. Further they may be invaluable in confirming or disproving the presence of infectious virus when other test results including conventional and real-time RT-PCR are all weakly positive. 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 are now routinely applied as a first choice for the diagnosis of influenza A infections.

1.5.1. Antigen detection

There are several commercially available AC-ELISA kits that can detect the presence of influenza A viruses in poultry (Swayne *et al.*, 2020). Most of the kits are enzyme immunoassays or are based on immunochromatography (lateral flow devices) 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 influenza A within 15 minutes. The disadvantages are that they may lack sensitivity, they may not have been validated for different

species of birds, H and N 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. Test sensitivities may vary between cloacal and tracheal swabs, whilst the tests can perform less well with samples from waterfowl or wild birds compared with chickens. Improved but moderate sensitivity in so named lateral flow devices was reported when using samples of feather follicles from birds infected with HPAI (Slomka *et al.*, 2012). Because of low sensitivity, antigen detection is mainly used for field screening of high mortality clinical cases for suspected influenza A virus infections followed by confirmation of results using a more sensitive laboratory-based test.

1.5.2. Direct RNA detection

As demonstrated by the current definitions of HPAI, molecular techniques are used preferentially for diagnosis for some time now. Furthermore, there have recently been developments towards their application to the detection and characterisation of influenza A viruses 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 influenza A 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 WOA standard (see Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*) 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.

Furthermore, these evaluations enable the appropriate setting of test thresholds for interpretation between positive and negative samples. The increased sensitivity of real-time RT-PCR leads to the detection of viral RNA in samples in the absence of infectious virus and care should be taken when interpreting outputs with small detection limits that may not be indicative of active infection. This problem can be overcome, through the testing of multiple samples from the same cohort of infected birds, especially relevant when testing samples from domestic poultry for disease investigation.

In settings with more limited facilities, RT-PCR techniques on clinical samples can, with the correctly defined primers, result in rapid detection and subtype identification (at least of H5, H7 and H9 subtypes, and more recently developed assays are also available for other subtypes), including a cDNA product that can be used for nucleotide sequencing. However, these approaches have now been largely replaced by the preferred molecular detection tests for influenza A virus by real-time RT-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 real-time RT-PCR primer/fluorogenic hydrolysis probe system to allow detection of influenza A 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 real-time RT-PCR was shown to have sensitivity and specificity equivalent to virus isolation in numerous settings but updates to primer/probe design can be beneficial over time to accommodate genetic evolution in gene regions targeted by assays (Laconi *et al.*, 2020). These tests provide high sensitivity and specificity similar to those of virus isolation when used on 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, improvements in RNA extraction methods have been developed to eliminate most PCR inhibitors from test samples.

Real-time RT-PCR, usually based around the hydrolysis probe 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 influenza A 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, real-time

RT-PCR systems can be designed to operate in a 96-well format and combined with high-throughput robotic RNA extraction from specimens (Aguero *et al.*, 2007).

The approach to diagnosis using real-time RT-PCR adopted in most laboratories has been based on initial generic detection of influenza A virus in clinical samples, primarily by initially targeting the matrix (M) gene, which is highly conserved for all influenza A viruses, followed by specific real-time RT-PCR testing for H5 and H7 subtype viruses. Numerous assays have been reported for highly sensitive detection of M (or NP) gene fulfilling the criteria for a suitable screening test. For subtype identification, primers used in real-time RT-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.* (2007) described modification of the H5 oligonucleotide sequences used by Spackman *et al.* (2002) to enable the detection of the Eurasian 'Goose/Guangdong lineage' (Gs/GD) H5N1 subtype and other Eurasian H5 subtypes that have been isolated within the past 15 years in both poultry and wild birds. As the group of 'Gs/GD' viruses diversified and spread across several continents it has become important that diagnostics in all settings have proven fit for purpose detection of this H5 lineage of viruses divided into multiple clades (World Health Organization/World Organisation for Animal Health/Food and Agriculture Organization & H5N1 Evolution Working Group, 2014). Newer rapid methods have been developed that enable simultaneous detection and subtyping speeding the time to achieve rapid identification of an influenza A virus using arrays (Hoffmann *et al.*, 2016) or microchip (Kwon *et al.*, 2019) technologies. The validated Eurasian real-time RT-PCR have proven valuable in the investigation of many H5Nx HPAI clinical samples and other subtypes submitted to International Reference Laboratories from Europe, Africa, Asia and North America since 2005 (Liu *et al.*, 2018; 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 in a diverse range of avian species, and in viruses from broad geographic areas and time periods. In addition, real-time RT-PCR methods are now widely used for the rapid and accurate determination of the neuraminidase subtype (James *et al.*, 2018)

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. In the European Union, National Reference Laboratories have collaborated to define and validate protocols that can be recommended for use within Europe (Hoffmann *et al.*, 2016; Nagy *et al.*, 2020; Slomka *et al.* 2007). Importantly this should include routine analysis of detected viruses (coordinated through WOAHP Reference Laboratories) in standard assays to ensure reliable specific detection of contemporary viruses affecting poultry and other populations. In addition, given the high variability in the influenza A genome it is imperative that assays used in routine diagnosis and surveillance have ongoing demonstration of their fitness for detection of contemporary viruses validated for use in the region where they are applied. There should be an appropriate match for local strains taking account of significant regional and intercontinental variability amongst particular endemic viruses. Laconi *et al.* (2020) in reviewing five validated well used real-time RT-PCR methods concluded that continuous monitoring of assay performance using both *in silico* and *in-vitro* methodology was important as the emergence of new strains containing mutations within primer and probe binding areas might significantly affect the positive outcome of a test. Increasingly with improvements in assay design and using novel biochemical approaches screening assays relevant to all influenza A viruses from all hosts (animal and human) have been developed (Nagy *et al.*, 2020) with high relevance to an avian-'other' host interface.

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.* (2007) have described a real-time RT-PCR test specific to the Eurasian 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. In situations where large numbers of positive samples/cases are detected during disease events, specific targeted real-time RT-PCR assays have been developed for the simultaneous sensitive detection and pathotyping of viruses. This can prove to be very useful, particularly when applying to early warning systems such as

surveillance of wild bird populations for local presence of HPAI (Graaf *et al.*, 2017; Naguib *et al.*, 2017).

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. The loop-mediated isothermal amplification (LAMP) system for H5 and H7 detection appears to show high sensitivity and reliable specificity (Ahn *et al.*, 2019; Bao *et al.*, 2014), but may have limited application because of susceptibility to viral mutations affecting the target regions, reducing virus detection (Postel *et al.*, 2010).

Increasing innovation and technological improvements have made it possible that molecular based and improved antigen detection technologies have developed sufficiently to permit rapid flock side tests for the detection of presence of influenza A virus specific subtypes and pathogenicity markers (Inui *et al.*, 2019). Furthermore, innovations in test design have enabled for example the development of point of care chip based ultrafast PCR approaches (Kwon *et al.*, 2018) with increasing application anticipated in the future.

1.5.3. Gene sequencing

Currently real-time RT-PCR is the preferred method of virus surveillance because the test provides rapid sensitive diagnostics for influenza H5, H7 and H9 and is available in high throughputs. However, greater use of sequencing technologies particularly as unit costs reduce with improvement in technology, offer powerful opportunities to simultaneously detect and sequence from clinical samples in a laboratory or field setting, for example applying nanopore technology (King *et al.*, 2020).

Increasingly gene sequencing is being applied not only to detailed characterisation of viruses for use in molecular epidemiology but also in virus subtyping and defining markers for host range including zoonotic risk. Sanger sequencing methodology has been widely used for decades and enables the rapid determination of typically a single (H) target gene in 24-36 hours to define virus pathogenicity (see Section B.1.1.1) and still has widespread utility. However, as genomic data can be rapidly determined using next generation sequencing technology it enables a broader analysis using a range of bioinformatics tools (Zhang *et al.*, 2017). For example, with the advent of greater access to sequencing methodology either through specialised laboratories or commercial providers it is now possible to determine the genomic sequences of influenza A viruses from birds to provide a level of characterisation important in rapid pathogen identification and outbreak intervention. Conventionally nucleotide sequences have been used in outbreak epidemiology to infer virus origin and precise relationships between different viruses associated within the same event (by phylogeny) to support outbreak management. Virus gene sequences of haemagglutinin and neuraminidase can rapidly be compared to known sequences of all subtypes in gene databases and used to reveal closest match thereby identifying the virus subtype and phylogenetic relationships. This often avoids the need to culture the virus for rapid identification although reliability and quality of data reduces with increasing cycle threshold values in samples from real-time RT-PCR testing.

Increasingly such analyses are now being applied at the whole genome level to reveal virus genotypes and provide greater analytical specificity to the analyses. Such approaches are especially valuable to track since virus evolution which can be more precisely mapped including change through genetic reassortment, a key mechanism associated with virus diversity and fitness for birds. This approach is especially valuable for early or first incursions in a new event as it enables greater precision in determining virus origin and the mechanisms leading to the emergence of virus. This has become increasingly important in characterising the rapid evolution and wide diversity of Gs/GD lineage viruses associated with transcontinental spread. Translation of nucleotide sequences of all genomic segments into amino acid sequences enables data mining for other virus characteristics or traits such as tropism, host range markers including zoonotic and predicted antiviral drug susceptibility which are invaluable for informing outbreak management.

2. Serological tests

2.1. Enzyme-linked immunoassay (ELISA)

Commercial ELISA kits that detect antibodies against the nucleoprotein are available. Kits with an indirect and competitive/blocking format have been developed and validated, and are now being used to detect influenza A virus-specific antibodies. Several avian influenza competitive ELISA (AIV C-ELISA) or blocking ELISA (AIV B-ELISA) have been developed and validated as a more sensitive alternative to the AGID test for the detection of influenza A group reactive antibodies in sera from chickens and other bird species (SCAHL, 2009). This AIV ELISA platform, as either a “competitive” or “blocking” format, detects antibodies to influenza A viruses by allowing these antibodies to compete for antigen binding sites with a monoclonal antibody against an epitope on the nucleoprotein that is conserved in all influenza A viruses.

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 WOAHP Register for kits certified by the WOAHP⁴. ELISA kits are of moderate cost and are amenable to high throughput screening for influenza A virus infections and have strong utility for application to large-scale serosurveillance programmes and compare favourably to HI (Arnold *et al.*, 2018). However, all positive results must be followed by HI test for subtyping to H5 and H7. Some subtype-specific ELISA kits are available, e.g. for antibodies to H5, H7, H9 and some N subtypes i.e. N1 but generally are of lower sensitivity than influenza A ELISA.

2.2. Agar gel immunodiffusion

All influenza A viruses have antigenically similar nucleoprotein 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 nucleoprotein antigens; the matrix antigen diffuses more rapidly than the nucleoprotein 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 nucleoprotein-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, for example ducks. The AGID is a low-cost serological screening test of reduced sensitivity for detection of generic influenza A infections, but must be followed by HI tests for subtyping influenza A positives as to H5 and H7.

Tests are usually carried out using gels of 1% (w/v) agarose or purified type II agar and 8% (w/v) NaCl in 0.01 M phosphate buffer, pH 7.2, poured to a thickness of 2–3 mm in Petri dishes or on microscope slides, and incubated in a humidified chamber. Using a template and cutter, wells of approximately 5 mm in diameter are cut into the agar at a distance of about 3 mm from each other. A pattern of wells must place each suspect serum adjacent to a known positive serum and antigen. Each well should have reagent added to fill the well, corresponding to the top of the meniscus with the top of the gel, but do not over fill. Approximately 25–30 μ l of each reagent should be required per well, but this depends on thickness of the gel, with thicker gels requiring an additional volume of reagent.

Wells should be examined for precipitin lines at 24 hours, and weak positive samples or samples for which specific lines have not formed should be incubated longer and examined again at 48 hours. The time to formation of visible precipitin line is 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

4 <https://www.woah.org/en/what-we-offer/veterinary-products/#ui-id-5/>

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.

Whilst the AGID is relatively inexpensive and suitable for resource limited settings it is being increasingly replaced by other platforms such as ELISA for flock level serological investigations including pre export/import screening of birds for historical exposure to influenza A.

2.3. 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. U- bottomed plates can be used but care in reading is required as the clarity is less defined. The reagents required for these tests are isotonic PBS (0.01 M), pH 7.0–7.4, 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.

2.3.1. 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.

2.3.2. Haemagglutination inhibition test

- 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.

The HI test is primarily used to determine if antibodies indicating influenza A virus infections are subtyped as H5 and H7 or other H subtypes (H1-4, H6, H8-16). 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 tested serum contains antibodies against the same N subtype as the H antigen used in the HI test. The steric inhibition reaction can result in RBC buttoning in the bottom of the plate or streaming at the same rate as the control. If using whole virus antigen in HI test for subtyping, it is important to ensure that two antigens for each haemagglutinin subtype are used with heterologous neuraminidase i.e. H5N1 and H5N6 to eliminate the possibility of interference in the assay with anti N antibodies that can lead to false typing results. Alternatively the H antigen used 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 influenza A.

2.4. Neuraminidase inhibition test

The neuraminidase-inhibition test has been used to identify the influenza A 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 a WOAH Reference Laboratory. The DIVA (differentiating infected from vaccinated animals) strategy used previously 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. REQUIREMENTS FOR VACCINES

1. Background

Vaccination alone is not the solution to the control of HPAI if eradication is the desired result. Without the application of monitoring systems, strict biosecurity and depopulation in the face of infection, HPAI will become endemic in vaccinated poultry populations. Long-term circulation of the virus in a vaccinated population may result in both antigenic and genetic changes as has occurred with H5Nx (Gs/GD lineage), H7N3, H7N9 and H9N2 influenza A viruses in Mexico, and various Middle Eastern and Asian countries (Swayne & Sims, 2020). Currently used vaccines and the use of vaccination have been reviewed (FAO, 2016; Swayne & Sims, 2020). The haemagglutinin is the primary influenza A viral protein that elicits a protective immune response used in officially approved poultry vaccines and such immunity is haemagglutinin subtype specific.

To date, the majority of influenza A vaccines used in poultry have been inactivated whole virus vaccines prepared from infective allantoic fluid of embryonated chicken eggs, inactivated by beta-propiolactone or formalin and emulsified with mineral oil adjuvants. Because of the potential for reassortment leading to increased virulence, live conventional influenza vaccines against any subtype are not recommended. However, biotechnology holds great potential to generate live avian influenza virus vaccines with altered gene segments which reduce the risk of reassortment, limit replication and abrogate negative aspects of live influenza A virus vaccines (Song *et al.*, 2007). The existence of a large number of haemagglutinin subtypes (i.e. H1–16), together with the known variation of different strains within a subtype, pose serious problems when selecting strains to produce inactivated influenza A

vaccines. 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 biologically characterised, fully approved seed strain viruses possessing the same haemagglutinin subtype as the field virus and capable of yielding high concentrations of antigen. Historically, inactivated vaccines for LPAI and HPAI control have used LPAI viruses with a matching haemagglutinin subtype from outbreaks as seed strains, but with development of resistance in the field associated with prolonged vaccine use, the majority of inactivated vaccine seed strains are now reverse genetic derived virus with antigenically close matching haemagglutinin, and sometimes neuraminidase, to circulating field viruses. Use of HPAI viruses as inactivated vaccine seed strains is strongly discouraged because of biosafety concerns.

Since the 1970s in the USA, inactivated influenza A vaccines have been used primarily in turkeys against LPAI viruses under emergency vaccination programs, but since the 2000s, most vaccines have been against H1 and H3 swine influenza A viruses used under a routine preventative vaccination program in breeder turkeys (Swayne *et al.*, 2020). Since the early 1990s, vaccination against H9N2 LPAI virus has been used extensively in Asia and the Middle East using billions of inactivated vaccine doses (Swayne & Sims, 2020). Vaccination against HPAI was first used in Mexico during the H5N2 outbreaks of 1994–1995 (Villarreal, 2007), and in Pakistan (Naeem, 1998) during the H7N3 outbreaks of 1995. Beginning with H5N1 goose/Guangdong (Gs/GD) lineage HPAI outbreaks in Hong Kong in 2002 (Sims, 2003), a vaccination policy was adopted using H5N2 LPAI vaccine seed strains and subsequently replaced with H5Nx reverse genetic vaccine seed strains, as the field virus spread throughout and outside of China. Between 2002 and 2010, 113 billion doses of vaccine was used to control HPAI with 95% being inactivated and 5% recombinant vaccines, and a similar usage rate continues (Swayne *et al.*, 2011; Swayne & Sims, 2020). As the H5Nx Gs/GD lineage HPAI spread across the globe, additional countries have implemented emergency and/or preventative vaccination programs for HPAI control. Similarly, preventive vaccination against H5N1 HPAI has been permitted for outdoor poultry and zoo birds in several European Union countries in the 2000s.

Live recombinant virus-vectored vaccines with H5 influenza A virus haemagglutinin gene inserts have been approved and used in a few countries since 1997, mostly in chickens, and include recombinant fowl poxvirus (rFPV), recombinant Newcastle disease virus (rNDV) and recombinant herpesvirus turkey vaccines (rHVT). Since 2015, non-replicating, haemagglutinin based H5 RNA particle, H5 expressed baculovirus and H5 DNA vaccines have been approved for poultry but have had limited use (Swayne & Sims, 2020).

1.1. Rationale and intended use of the product

Experimental work has shown, for HPAI and LPAI, that potent and properly administered vaccines increase resistance to, or prevent infection, protect against clinical signs and mortality, prevent drops in egg production, reduce virus shedding from respiratory and intestinal tracts, protect from diverse field viruses within the same haemagglutinin subtype, protect from low and high challenge exposure, and reduce excretion and thus prevent contact transmission of challenge virus (Capua *et al.*, 2004; Swayne & Sims, 2020). Although, in experimental vaccination studies, a challenge virus is still able to infect and replicate in clinically healthy vaccinated SPF birds when exposed to high doses, the quantities shed may be insufficient for onward transmission of the virus (Van der Goot *et al.*, 2005). 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. Most national HPAI and LPAI 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 regulatory approval of influenza A 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 3.3.10).

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 HPAI virus is to be used in challenge studies, the facility used for such studies should meet the competent veterinary authority within the country minimum requirements for Containment Group 3 pathogens as outlined in chapter 1.1.4.

2.1. Characteristics of the seed

2.1.1. 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 vaccine. For HPAI, reverse genetic produced vaccine seed strains based on haemagglutinin gene of the HPAI virus are preferred, but should have the cleavage site sequence altered to that of a H5/H7 LPAI virus.

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).

2.1.2. 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.

2.2. Method of manufacture

2.2.1. 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 lyophilised 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 EID₅₀ 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 and surfactants. The exact formulations are generally commercial secrets.

2.2.2. Requirements for substrates and media

The inactivated influenza A 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.

2.2.3. 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.

2.2.4. 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.

i) Sterility and purity

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) 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.

iii) Batch potency

Potency of influenza A 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 HPAI virus (e.g. chapter 3.3.10) may also be used for vaccines prepared to give protection against LPAI subtypes. For inactivated vaccines against HPAI or LPAI virus, potency tests may rely on the measurement of immune response or challenge and assessment of morbidity, mortality (HPAI only) and quantitative reduction in challenge virus replication in respiratory (oropharyngeal or tracheal) and intestinal (cloaca) tracts. Assessment of haemagglutinin antigen content could allow for *in-vitro* extrapolation to potency for subsequent vaccine batches.

iv) Preservatives

A preservative may be used for vaccine in multidose containers.

2.3. Requirements for regulatory approval

2.3.1. Safety requirements

i) Target and non-target animal safety

Most inactivated influenza A vaccines are approved 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 influenza A 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.

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

Only inactivated influenza A 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 pathogenic field viruses.

iii) Environmental consideration

None

2.3.2. Efficacy requirements

i) For animal production

For regulatory purposes, influenza A vaccines should pass an efficacy challenge test using a statistically relevant number of SPF or SAN chickens per group. The challenge should occur at a minimum of three weeks post-vaccination, using a challenge HPAI virus dose that

causes 90% or greater mortality in the sham population. A standardised challenge dose of 10^6 mean chicken embryo infectious doses is most widely used. Protection from mortality in the vaccine group should be a minimum of 80%. For LPAI, 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. Other metrics of protection can be used to determine efficacy such as prevention of drops in egg production.

In establishing minimum antigen requirements, 50 PD₅₀ or 3 µg of haemagglutinin per dose have been recommended (Swayne & Sims, 2020). 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 antigenically close related vaccine and challenge viruses.

- ii) For control and eradication

Efficacy should be the same as for animal production.

2.3.3. 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

3.1. Vaccines available and their advantages

Recombinant live vaccines for influenza A viruses have been produced by inserting the gene coding for the influenza A virus haemagglutinin into a non-influenza live virus vector and using this recombinant virus to immunise poultry against influenza A (Swayne & Sims, 2020). Recombinant live vector vaccines have several advantages over inactivated influenza A vaccines: 1) they induce mucosal, humoral and cellular immunity; 2) they can be mass administered *in ovo* or to 1-day-old birds in the biosecure hatchery to induce early protection; and 3) they enable easy serological differentiation of infected from non-infected vaccinated birds because they do not induce the production of antibodies against the nucleoprotein or matrix antigens that are common to all influenza A viruses; i.e. differentiation of infected from vaccinated (DIVA) animals. 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, recombinant live vaccines have limitations in that they may have reduced replication and thus induce no or only partial protective immunity in birds that have had field exposure to or vaccine induced immunity against the vector virus or the H gene insert (Bertran *et al.*, 2018; Swayne & Sims, 2020). 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; i.e. most severe inhibition in decreasing order for Newcastle disease virus, fowl poxvirus and HVT vectors. In addition, because the vectors are live viruses that may have a restricted host range, the use of such vaccines must be restricted to species in which efficacy has been demonstrated.

A rFPV-H5 vaccine, with H gene insert for A/turkey/Ireland/1378/1983 (H5N8), was developed in the early 1980s and authorised beginning in 1998 for use against H5N2 LPAI of Mexico (Swayne & Sims, 2020). This vaccine has principally been used in Mexico with expansion into several other countries within Central America and Vietnam with over 9 billion doses used between 1998 and 2016. This rFPV-H5 has had the H gene insert updated to A/chicken/Mexico/P-14/2016 (H5N2) (Bertran *et al.*, 2020). An rFPV-H7 with haemagglutinin insert from A/chicken/Guanajuato/07437-15/2015 (H7N3) has been developed and approved with deployment to Mexico in 2018 against H7N3 HPAI, and a rFPV-H5 with H and N gene inserts from A/goose/Guangdong/1996 (H5N1, clade 0) was used in China against the H5N1 HPAI during 2005 (Chen & Bu, 2009; Criado *et al.*, 2019; Swayne & Sims, 2020). rFPV can be effective when given to 1-day-old chicks with varying levels of maternal immunity (Arriola *et al.*, 1999). However, when very high levels of inhibitory immunity is anticipated because of previous infection or vaccination, the efficacy of the recombinant live vaccine in such day-old chicks should be confirmed and may require a prime-boost application of recombinant vaccine followed at a minimum 10 days later by inactivated influenza A vaccine boost to give optimal immunity (Richard-Mazet *et al.*, 2014; Swayne & Sims, 2020).

Newcastle disease virus can also be used as a vector for expressing influenza haemagglutinin genes. A recombinant Newcastle disease vaccine virus (rNDV) expressing a H5 HA gene (rNDV-H5) was shown to protect SPF chickens against challenge with both virulent Newcastle disease virus and a HPAI H5N2 virus (Veits *et al.*, 2006). A similar recombinant virus based on Newcastle disease virus vaccine strain La Sota and expressing H gene of A/goose/Guangdong/1996 (clade 0)(H5N1) 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 rNDV-H5 (clade 0) vaccine has been used widely with subsequent updating of HA insert twice with clade 2.3.4 and 2.3.2 clade haemagglutinin inserts (Swayne & Sims, 2020). An rNDV-H5 with H gene insert from A/chicken/Mexico/435/2005 (H5N2) has been developed, approved and deployed in Mexico against H5N2 LPAI (Swayne & Sims, 2020). An rNDV-H5 vaccine with H gene insert from A/chicken/Iowa/04-20/2015 (H5N2) (Gs/GD lineage, clade 2.3.4.4) insert was effective in protecting chickens against challenge with homologous H5N2 HPAI virus in chickens lacking immunity to the Newcastle disease virus vector or the H gene insert, but rNDV-H5 vaccine was ineffective as a primary or booster vaccine in poultry with maternal immunity or well-immunised against Newcastle disease or the H5 haemagglutinin protein (Bertran *et al.*, 2018). rNDV-H5 vaccines are effective as a primary vaccine if used in Newcastle disease or H5 antibody negative chickens, or as a priming vaccine followed by a boost with an inactivated influenza A vaccine in Newcastle disease or H5 antibody positive chickens. The major advantage of rNDV-H5 is the ability for low cost mass application by spray in the hatchery or field (Swayne & Sims, 2020).

Since 2010, a rHVT-H5 with haemagglutinin insert of A/swan/Hungary/4999/2006 (Gs/GD lineage, clade 2.2) has been approved and used in Egypt and Bangladesh against H5Nx Gs/GD lineage HPAI and in Mexico against H5N2 LPAI (Rauw *et al.*, 2011; Swayne & Sims, 2020). This rHVT-H5 vaccine has produced broad protection across diverse H5 HPAI viruses (Rauw *et al.*, 2011). Furthermore, maternally derived antibodies to rHVT vector or H5 haemagglutinin protein have had minimal negative impact on the effectiveness of the vaccine in broiler chickens after a single vaccination at 1 day of age (Bertran *et al.*, 2018). The rHVT-H5 is limited to application only *in ovo* or at 1 day of age to chickens in the hatchery, as application later on the farm is not feasible because of the ubiquitous infection by Marek's disease viruses or use of Marek's disease vaccines.

Because of the induction of broader immunity across mucosal, humoral and cellular areas, recombinant live vectored vaccines have had a longer use life in the field before appearance of field viruses that are resistant to the vaccine strains as compared to inactivated whole virus vaccines which produce primarily a strong humoral immunity. A recombinant duck enteritis virus in domestic ducks has been developed and shown efficacy but is pending regulatory approval and deployment in China (People's Rep. of) (Liu *et al.*, 2011).

Non-replicating haemagglutinin-based RNA particle and DNA vaccines with H gene from A/Gyrfalcon/Washington/40188-6/2014 (H5N8) (Gs/GD lineage, clade 2.3.4.4) have been approved for poultry use in the USA (Swayne & Sims, 2020). The H5 RNA particle vaccine is part of the USA emergency vaccine bank, along with rHVT-H5 and an inactivated H5N2 vaccines. The H5 RNA particle vaccine has been demonstrated to be an effective booster vaccine to replace rg inactivated H5Nx vaccine (Bertran *et al.*, 2017). A baculovirus with H gene insert from A/duck/China/E319-2/2003 (Gs/GD lineage, clade 2.3.3) has been approved for poultry use in Bangladesh, Egypt and Mexico (Swayne & Sims, 2020). Since this category of vaccine only contain the specific influenza A haemagglutinin protein, they are easily amenable to serological DIVA testing using assays designed for identifying antibodies to the nucleoprotein/matrix protein. However, field reports of protection with vectored and conventional influenza A vaccines suggest that protection by single dose of the vectored vaccines for long lived poultry is not feasible, with long-term field protection requiring a booster with inactivated influenza A vaccine or non-replicating, haemagglutinin-based vaccine (Swayne & Sims, 2020).

In addition to these approved vaccines, various experimental haemagglutinin-based H5 and H7 influenza A vaccines have been described using *in-vivo* or *in-vitro* expression systems including recombinant adenoviruses, salmonella, vaccinia, avian leucosis virus, various eukaryotic systems (plants or cell cultures) and infectious laryngotracheitis virus (Swayne & Sims, 2020).

3.2. Special requirements for biotechnological vaccines, if any

Live recombinant vectored vaccines with influenza A haemagglutinin 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 HPAI and H5/H7 LPAI 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 of infected flocks. 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 field 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 (Swayne & Kapczynski, 2008).

To use serological DIVA schemes, vaccination systems that enable the detection of field exposure in vaccinated populations should be used. Several systems have been used. First, use of a vaccine containing a virus of the same haemagglutinin 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 was used in Italy following the re-emergence of a H7N1 LPAI virus in 2000, and used an H7N3 inactivated vaccine with the detection of N3 antibodies indicating a vaccinated flock, N1 antibodies indicating infection, and both N1 and N3 antibodies indicating an infected, vaccinated flock (Capua *et al.*, 2003). 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 as is present in many low and middle income countries with H5Nx (Gs/GD lineage), H9N2 and other NA subtypes in live poultry markets (Swayne & Sims, 2020). A second serological DIVA option is the use of vaccines that contain only HA, e.g. replicating or non-replicating recombinant vaccines, which allows validated, classical AGID and nucleoprotein (NP)- or matrix protein-based ELISAs to be used to detect antibodies indicative of infection in vaccinated birds. Finally, for inactivated vaccines, a test that detects antibodies to the nonstructural viral or M2e proteins have been described (Avellaneda *et al.*, 2010; Lambrecht *et al.*, 2007). These systems are 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 LPAI inactivated vaccine seed strains and recombinant fowl poxviruses with H5 gene inserts have shown broad cross protection in chickens against challenge by diverse H5 HPAI viruses from Eurasia and North America (Swayne & Kapczynski, 2008). In 1995, Mexico implemented influenza A vaccine use for poultry as one tool in the HPAI control strategy, with eradication of HPAI strain by June 1995, but as H5N2 LPAI viruses continued to circulate, H5N2 vaccination was maintained (Villarreal, 2007). Within a few years, multiple lineages of antigenically variant H5N2 LPAI field viruses emerged that escaped from immunity induced by the original 1994 inactivated vaccine seed strain (Lee *et al.*, 2004). Similarly, emergent H5Nx HPAI Gs/GD lineage field viruses have arisen in China (the People's Rep. of), Indonesia, Egypt and various other Asian and Middle Eastern countries since 2005 that escaped from immunity induced by classical H5 inactivated LPAI vaccine seed strains and even rg generated H5 vaccine seed strains used in commercial vaccines (Grund *et al.*, 2011; Liu *et al.*, 2020; Swayne & Sims, 2020). Similarly, H9N2 LPAI field viruses resistant to inactivated vaccine seed strains have arisen in multiple countries in Asian and Middle East after prolonged usage of a single inactivated vaccine seed strain. It is not clear whether the emergence of these antigenic variants is related to use of vaccines or improper use of vaccines, but the emergence of resistance necessitated the change in vaccine seed strains to antigenically match the circulating field strains (Cattoli *et al.*, 2011; Lee *et al.*, 2016). China as the largest user of avian influenza vaccines has updated its inactivated H5Nx (Gs/GD lineage) and H7N9 seed strains eight times and once, respectively, with the life span of a seed strain ranging from 3 to 7 years (Liu *et al.*, 2020; Swayne & Sims, 2020). Mexico has updated its H5N2 inactivated seed strains twice and its rFPV-H5 once over a 20-year period of H5 vaccine use (Swayne & Sims, 2020). Initially H9N2 inactivated vaccine usage in South Korea, was associated with decreased field virus diversity, as vaccinal immunity completely inhibited antigenically closely related field virus replication (Lee *et al.*, 2016). However, over time, field

virus diversity increases as antigenic variants arise in the field and expand their populations. The live recombinant vectored vaccines have been updated less frequently, suggesting a broader immunity, requiring less frequent insert updates as compared inactivated vaccine seed strains.

All influenza A vaccination programmes should have an epidemiologically relevant surveillance programme that includes all relevant geographical regions and production sectors. The resulting isolates, along with viruses obtained from outbreaks, should be assessed for genetic and antigenic variation as part of an ongoing program for assessing vaccine effectiveness in the field. Initially, the viruses should be sequenced and analysed for critical amino acid changes within the five major antigenic epitopes of the HA. A representative subset of antigenic variants should be tested for cross-reactivity in a HI test using a panel of standard antisera produced against diverse influenza A viruses from the same HA subtype and the data analysed for quantitative changes by antigenic cartography (Fouchier & Smith, 2010). Based on this cartographic data, a few of the predominant circulating influenza A viruses and selected antigenic variants should be used in challenge efficacy studies (Swayne *et al.*, 2015). Vaccines that are not protective should be discontinued and replaced with vaccines containing updated inactivated vaccine seed strains or HA inserts within other vaccine platforms. Based on the timeline for emergence of antigenic variants for H5N1 viruses in China (People's Rep. of), vaccines should be assessed at a minimum every 2–3 years for efficacy against predominant circulating field viruses of the country or region. Alternatively, vaccine seed strains should be updated when a vaccine-escape mutant accounts for more than 30% of the relevant AIV subtype (Liu *et al.*, 2020). 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 approved for use in control programmes. Proper administration of high quality, potent vaccines is critical in inducing protective immunity in poultry populations.

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NB: There are WOA Reference Laboratories for avian influenza (please consult the WOA Web site: <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).

Please contact the WOA Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for avian influenza

NB: FIRST ADOPTED IN 1989 AS AVIAN INFLUENZA (FOWL PLAGUE). MOST RECENT UPDATES ADOPTED IN 2021.

APPENDIX 3.3.4.1.

BIOSAFETY GUIDELINES FOR HANDLING HIGH PATHOGENICITY AVIAN INFLUENZA VIRUSES IN VETERINARY DIAGNOSTIC LABORATORIES

INTRODUCTION

The spread of high pathogenicity H5Nx avian influenza throughout Asia, Africa and Europe has led to an increase in the number of laboratories performing diagnostics for this pathogen. High pathogenicity avian influenza (HPAI) viruses, 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 approximately 60% mortality reported in humans infected with H5N1 HPAI virus. In recognition of the need for guidance on how to handle these viruses safely, the WOAAH has established the following biocontainment guidelines for handling specimens that may contain HPAI virus. They are based on Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*, the World Health Organization⁵, and Centers for Disease Control and Prevention⁶.

BIOCONTAINMENT LEVELS

Samples for diagnostic testing for HPAI virus using the following techniques do not require high-level containment but should be carried out at an appropriate biosafety and containment level determined by risk analysis (see chapter 1.1.4.):

- Conventional and real-time reverse transcriptase polymerase chain reaction (RT-PCR)
- Antigen-capture assays
- Serology

Virus isolation and identification procedures for handling specimens that may contain high-titred replication-competent HPAI virus should as a minimum, 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.

5 WHO laboratory biosafety guidelines for handling specimens suspected of containing avian influenza A virus, 12 January 2005.

6 Biosafety in Microbiological and Biomedical Laboratories, 5th edition. HHS Publication No. (CDC) 21-1112. <https://www.cdc.gov/labs/pdf/CDC-BiosafetyMicrobiologicalBiomedicalLaboratories-2009-P.PDF> 1 December 2009.

- 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 appropriate containment including:

- Inoculated chickens should be held in animal isolation cabinets or other primary containment devices, or non-isolation cages/floor pens in specially designed containment rooms
- Animal isolation cabinets should be in a separate facility that is equipped to handle the appropriate biocontainment for HPAI.
- The room should be under negative pressure to the outside and the animal isolation cabinets should be under negative pressure to the room.
- Animal isolation cabinets 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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