CHAPTER 2.1.14.

HIGHLY PATHOGENIC AVIAN INFLUENZA

SUMMARY

Highly pathogenic avian influenza (*HPAI*), *also known as fowl plague, is caused by specified influenza A viruses that are members of the family Orthomyxoviridae. There are three influenza types – A, B and C; only influenza A viruses are known to infect birds. Diagnosis is by isolation and characterisation of the virus. This is because infections in birds can give rise to a wide variety of clinical signs that may vary according to the host, strain of virus, the host's immune status, presence of any secondary exacerbating organisms and environmental conditions.*

Identification of the agent: *Suspensions in antibiotic solution of tracheal 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 9–11-day-old embryonated fowl eggs. The eggs are incubated at 35–37°C for 4–7 days. The allantoic fluid of any eggs containing dead or dying embryos as they arise and all eggs at the end of the incubation period are tested for the presence of haemagglutinating activity. The presence of influenza A virus can be confirmed by an immunodiffusion test between concentrated virus and an antiserum to the nucleocapsid or matrix antigens, both of which are common to all influenza A viruses. Isolation in embryos has recently been replaced, under certain circumstances, by reverse-transcription polymerase chain reaction.*

For subtyping the virus, the laboratory must have monospecific antisera prepared against the isolated antigens of each of the 15 haemagglutinin (*H1–H15*) *and 9 neuraminidase* (*N1–N9*) *subtypes of influenza A viruses that can be used in immunodiffusion tests. Alternatively, the newly isolated virus may be examined by haemagglutination and neuraminidase inhibition tests against a battery of polyclonal antisera to a wide range of strains covering all the subtypes.*

As the terms HPAI and 'fowl plague' refer to infection with virulent strains of influenza A virus, it is necessary to assess the virulence of an isolate for domestic poultry. Whereas all virulent strains isolated to date have been either of the H5 or H7 subtype, most H5 or H7 isolates have been of low virulence. The methods used for the determination of strain virulence for birds have evolved over recent years with a greater understanding of the molecular basis of pathogenicity, but still primarily involve the inoculation of a minimum of eight susceptible 4–8-week-old chickens with infectious virus; strains are considered to be highly pathogenic if they cause more than 75% mortality within 10 days. Isolation and characterisation of suspected pathogenic strains of the virus should be conducted in a virus-secure laboratory.

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

Requirements for vaccines and diagnostic biologicals: *In most countries, vaccines specifically designed to contain or prevent HPAI are banned or discouraged by government agencies because they may interfere with stamping-out control policies. During the 1990s the prophylactic use of inactivated oil-emulsion vaccines was employed in Mexico and Pakistan to control widespread outbreaks of HPAI, and a recombinant fowlpox virus vaccine expressing the homologous HA gene was also tested in field trials in Mexico. During the 1999–2001 outbreak in Italy, an inactivated*

vaccine was used with the same haemagglutinin type as the field virus, but with a different neuraminidase. This allowed the differentiation between vaccinated birds and birds infected with the field virus.

If HPAI is used in the production of vaccine or in challenge studies, the facility should meet the OIE requirements for Containment Group 4 pathogens.

A. INTRODUCTION

Highly pathogenic avian influenza (HPAI), which is also known as fowl plague, is caused by infection with influenza A viruses of the family Orthomyxoviridae. Influenza A viruses are the only orthomyxoviruses known to affect birds. Many species of birds have been shown to be susceptible to infection with influenza A viruses; aquatic birds form a major reservoir of these viruses, but the overwhelming majority of isolates have been of low pathogenicity for chickens and turkeys, the main birds of economic importance to be affected. Influenza A viruses have antigenically related nucleocapsid and antigenically related matrix proteins, but are classified into subtypes on the basis of their haemagglutinin (H) and neuraminidase (N) antigens (40). At present, 15 H subtypes (H1–H15) and 9 neuraminidase subtypes (N1–N9) are recognised. To date, the highly virulent influenza A viruses that produce acute clinical disease in chickens and turkeys have been associated only with the H5 and H7 subtypes (with the exception of two H10 subtypes that would also have fulfilled the OIE and European Union [EU] definitions), although the reasons for this are not clear. Many viruses of H5 and H7 subtype isolated from birds have been of low virulence for poultry (1).

Depending on the age and type of bird and on environmental factors, the highly pathogenic disease may vary from one of sudden death with little or no overt signs to a more characteristic disease with respiratory signs, excessive lacrimation, sinusitis, oedema of the head, cyanosis of the unfeathered skin and diarrhoea. However, none of these signs can be considered pathognomonic. Diagnosis of the disease, therefore, depends on the isolation of the virus and the demonstration of its virulence for an appropriate host. Testing sera from suspect birds using immunological methods may supplement diagnosis, but these methods are not suitable for a detailed identification. Diagnosis for official control purposes is established on the basis of agreed official criteria for pathogenicity, according to *in-vivo* tests or to molecular determinants (i.e. the presence of multiple basic amino acids at the cleavage site of the haemagglutinin). These definitions evolve as scientific knowledge of the disease increases.

HPAI is subject to official control and the virus has a high risk of spread from the laboratory; consequently, a risk assessment should be carried out to determine the level of biosecurity needed for the diagnosis and characterisation of the virus. The facility should meet the requirements for the appropriate Containment Group as determined by the risk assessment and as outlined in Appendix I.1.6.1 of Chapter I.1.6 of this *Terrestrial Manual.* Countries lacking access to such a specialised national or regional laboratory should send specimens to an OIE Reference Laboratory.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

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

Samples from live birds should include both tracheal and cloacal swabs, although swabs of the latter site are the most likely to yield virus. As small delicate birds may be harmed by swabbing, the collection of fresh faeces may serve as an adequate alternative.

The samples should be placed in isotonic phosphate buffered saline (PBS), pH 7.0–7.4, containing 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 tracheal swabs, but at five-fold higher concentrations for faeces and cloacal swabs. It is important to readjust the pH of the solution to pH 7.0–7.4 following the addition of the antibiotics. Faeces and finely minced tissues should be prepared as 10–20% (w/v) suspensions in the antibiotic solution. Suspensions should be processed as soon as possible after incubation for 1–2 hours at room temperature. When immediate processing is impracticable, samples may be stored at 4°C for up to 4 days. For prolonged storage, diagnostic samples and isolates should be kept at –80°C.

The preferred method of growing avian influenza A viruses is by the inoculation of embryonated specific pathogen free (SPF) fowl eggs, or at least specific antibody negative (SAN) eggs. The supernatant fluids of faeces or tissue suspensions obtained through clarification by centrifugation at 1000 ***g*** are inoculated into the allantoic sac of at least five embryonated SPF fowl eggs of 9–11 days' incubation. The eggs are incubated at 35–37°C for 4–7 days. Eggs containing dead or dying embryos as they arise, and all eggs remaining at the end of the incubation period, should first be chilled to 4°C and the allantoic fluids should then be tested for haemagglutination (HA) activity (see Section B.3.b.). Detection of HA activity indicates a high probability of the presence of an influenza A virus or of an avian paramyxovirus. Fluids that give a negative reaction should be passaged into at least one further batch of eggs.

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

Preparations of nucleocapsid-rich antigen can also be obtained from chorioallantoic membranes for use in the AGID test (5). This method involves removal of the chorioallantoic membranes from infected eggs that have allantoic fluids with HA activity. The membranes are then homogenised or ground to a paste. This is subjected to three freeze-thaw cycles, followed by centrifugation at 1000 ***g*** for 10 minutes. The pellet is discarded and the supernatant is used as an antigen following treatment with 0.1% formalin.

Use of the AGID test to demonstrate nucleocapsid or matrix antigens is a satisfactory way to indicate the presence of avian influenza virus in amnioallantoic fluid, but various enzyme-linked immunosorbent assays (ELISAs) are now also available. There is a sensitive and specific ELISA that demonstrates nucleoprotein of type A influenza virus using a monoclonal antibody against type A influenza nucleoprotein (27, 28, 33). This is available as a commercial kit.

Any HA activity of sterile fluids harvested from the inoculated eggs is most likely to be due to an influenza A virus or to an avian paramyxovirus (a few strains of avian reovirus will do this, or nonsterile fluid could contain HA of bacterial origin). There are currently nine recognised serotypes of avian paramyxoviruses. Most laboratories will have antiserum specific for Newcastle disease virus (avian paramyxovirus type 1), and in view of its widespread occurrence and almost universal use as a live vaccine in poultry, it is best to evaluate its presence by haemagglutination inhibition (HI) tests (see Chapter 2.1.15.).

Alternatively, the presence of influenza virus can be confirmed by the use of reverse-transcription polymerase chain reaction (RT-PCR) using nucleoprotein-specific conserved primers (2). Also, the presence of subtype H5 or H7 influenza virus can be confirmed by using H5- or H7-specific primers (13, 26, 39).

The method recommended for definitive antigenic subtyping of influenza A viruses by the World Health Organization (WHO) Expert Committee (40) involves the use of highly specific antisera, prepared in an animal giving minimum nonspecific reactions (e.g. goat), directed against the H and N subtypes (25). An alternative technique is the use of polyclonal antisera raised against a battery of intact influenza viruses. Subtype identification by this technique is beyond the scope of most diagnostic laboratories not specialising in influenza viruses. Assistance is available from the OIE Reference Laboratories (see Table given in Part 3 of this *Terrestrial Manual*).

2. Assessment of pathogenicity

The term highly pathogenic avian influenza implies the involvement of virulent strains of virus. It is used to describe a disease of chickens with clinical signs such as excessive lacrimation, respiratory distress, sinusitis, oedema of the head and face, cyanosis of the unfeathered skin, and diarrhoea. Sudden death may be the only sign. These signs may vary enormously depending on the host, age of the bird, presence of other organisms and environmental conditions. In addition, viruses that normally cause only a mild or no clinical disease may mimic highly pathogenic avian influenza if exacerbating conditions exist.

At the First International Symposium on Avian Influenza held in 1981 (3), it was resolved to abandon the term 'fowl plague' and to define highly pathogenic strains on the basis of their ability to produce not less than 75% mortality within 8 days in at least eight susceptible 4–8-week-old chickens inoculated by the intramuscular, intravenous or caudal air sac route. However, this definition proved unsatisfactory when applied to the viruses responsible for the

widespread outbreaks in chickens occurring in 1983 in Pennsylvania and the surrounding states of the United States of America (USA). The problem was mainly caused by the presence of a virus of demonstrable low pathogenicity in laboratory tests, but which was shown to be fully pathogenic following a single point mutation. Further consideration of a definition to include such 'potentially pathogenic' viruses was undertaken by several international groups.

The eventual recommendations made were based on the finding that while there have been numerous isolations of strains of H5 and H7 subtypes of low pathogenicity, all the highly pathogenic influenza strains isolated to date have possessed either the H5 or H7 haemagglutinin. Further information concerning the pathogenicity or potential pathogenicity of H5 and H7 subtypes may be obtained by sequencing the genome, as pathogenicity is associated with the presence of multiple basic amino acids (arginine or lysine) at the cleavage site of the haemagglutinin. For example, all H7 subtype viruses of low virulence have had the amino acid motif at the HA0 cleavage site of either -PEIPKGR*GLF- or -PENPKGR*GLF-, whereas examples of amino acids motifs for HPAI H7 viruses are: -PEIPKKKKR*GLF-, PETPKRKRKR*GLF-, -PEIPKKREKR*GLF-, -PETPKRRRR*GLF-. Amino acid sequencing of the cleavage sites of H5 and H7 subtype influenza isolates of low virulence for birds should identify viruses that, like the Pennsylvania virus, have the capacity, following simple mutation, to become highly pathogenic for poultry.

The OIE subsequently adopted the following criteria for classifying an avian influenza virus as highly pathogenic:

a) Any influenza virus that is lethal for six, seven or eight of eight 4–8-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.

b) The following additional test is required if the isolate kills from one to five chickens but is not of the H5 or H7 subtype: growth of the virus in cell culture[1] with cytopathic effect or plaque formation in the absence of trypsin. If no growth is observed, the isolate is not considered to be a HPAI isolate.

c) For all H5 and H7 viruses of low pathogenicity and for other influenza viruses, if growth is observed in cell culture without trypsin, the amino acid sequence of the connecting peptide of the haemagglutinin must be determined. If the sequence is similar to that observed for other HPAI isolates, the isolate being tested will be considered to be highly pathogenic.

In the EU, a similar definition was adopted in Directive 92/40/EEC (11), although in this case the intravenous pathogenicity index (IVPI) test was used as a method of assessing virulence. For the purposes of confirming disease and implementing the control measures in the Directive, the following definition applies:

'an infection of poultry caused by an influenza A virus that has an intravenous pathogenicity index in 6-week-old chickens >1.2 or any infection with influenza A viruses of H5 or H7 subtype for which nucleotide sequencing has demonstrated the presence of multiple basic amino acids at the cleavage site of the haemagglutinin.'

The IVPI test is carried out as follows.

i) Fresh infective allantoic fluid with a HA titre >1/16 (>2^4 or >$\log_2$ 4 when expressed as the reciprocal) is diluted 1/10 in sterile isotonic saline.

ii) 0.1 ml of the diluted virus is injected intravenously into each of ten 6-week-old SPF chickens.

iii) Birds are examined at 24-hour intervals for 10 days. At each observation, each bird is scored 0 if normal, 1 if sick, 2 if severely sick, 3 if dead. (The judgement of sick and severely sick birds is a subjective clinical assessment. Normally, 'sick' birds would show one of the following signs and 'severely sick' more than one of the following signs: respiratory involvement, depression, diarrhoea, cyanosis of the exposed skin or wattles, oedema of the face and/or head, nervous signs. Dead individuals must be scored as 3 at each of the remaining daily observations after death.)

iv) The intravenous pathogenicity index (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.

A variety of strategies and techniques have been used successfully to sequence the nucleotides at that portion of the HA gene coding for the cleavage site region of the haemagglutinin of H5 and H7 subtypes of avian influenza, enabling the amino acids there to be deduced. The most commonly used method has been RT-PCR using oligonucleotide primers complementing areas of the gene either side of the cleavage site coding region, followed

1 For example primary cells such as chick embryo cells or cell lines such as MDCK cells, although most cell cultures support the growth of HPAI influenza viruses or those of low pathogenicity in the presence of trypsin.

by cycle sequencing (38). Various stages in the procedure can be facilitated using commercially available kits and automatic sequencers.

Now that the presence of multiple basic amino acids at the HA0 cleavage site is well-established as an accurate indicator of virulence or potential virulence for H5 and H7 influenza viruses, it appears inevitable that determination of the cleavage site by sequencing or other methods will become the method of choice for initial assessment of the virulence of these viruses and incorporated into agreed definitions. This will have the advantage of reducing the number of *in-vivo* tests, although at present the inoculation of birds may still be required to confirm a negative result as the possibility of virus cultures containing mixed populations of viruses of high and low virulence cannot be ruled out.

Although all the truly HPAI viruses isolated to date have been of H5 or H7 subtypes, at least two isolates, both of H10 subtype (H10 N4 and H10 N5), have been reported that would have fulfilled both the OIE and EU definitions for HPAI viruses (36) as they killed 7/10 and 8/10 chickens with IVPI values >1.2 when the birds were inoculated intravenously. However, they produced no deaths or disease signs when inoculated intranasally and these viruses did not have multiple basic amino acids at their haemagglutinin cleavage sites.

3. Serological tests

a) Agar gel immunodiffusion

All influenza A viruses have antigenically similar nucleocapsid and antigenically similar matrix antigens. This fact enables the presence or absence of antibodies to any influenza A virus to be detected by AGID tests. Concentrated virus preparations, as described above, contain both matrix and nucleocapsid antigens; the matrix antigen diffuses more rapidly than the nucleocapsid antigen. AGID tests have been widely and routinely used to detect specific antibodies in chicken and turkey flocks as an indication of infection. These have generally employed nucleocapsid-enriched preparations made from the chorioallantoic membranes of embryonated fowl eggs (5) that have been infected at 10 days of age, homogenised, freeze–thawed three times, and centrifuged at 1000 ***g***. The supernatant fluids are inactivated by the addition of 0.1% formalin or 1% betapropiolactone, recentrifuged and used as antigen. Not all avian species may produce precipitating antibodies following infection with influenza viruses.

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

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

b) Haemagglutination and haemagglutination inhibition tests

Variations in the procedures for HA and HI tests are practised in different laboratories. The following recommended examples apply in the use of V-bottomed microwell plastic plates in which the final volume for both types of test is 0.075 ml. The reagents required for these tests are isotonic PBS (0.1 M), pH 7.0–7.2, and red blood cells (RBCs) taken from a minimum of three SPF chickens and pooled in an equal volume of Alsever's solution. (If SPF chickens are not available, blood may be taken from birds that are regularly monitored and shown to be free from antibodies to avian influenza). Cells should be washed three times in PBS before use as a 1% (packed cell v/v) suspension. Positive and negative control antigens and antisera should be run with each test, as appropriate.

- **Haemagglutination test**

i) Dispense 0.025 ml of PBS into each well of a plastic V-bottomed microtitre plate.

ii) Place 0.025 ml of virus suspension (i.e. infective allantoic fluid) in the first well. For accurate determination of the HA content, this should be done from a close range of an initial series of dilutions, i.e. 1/3, 1/4, 1/5, 1/6, etc.

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

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

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

vi) Mix by tapping the plate gently and then allow the RBCs to settle for about 40 minutes at room temperature, i.e. about 20°C, or for 60 minutes at 4°C if ambient temperatures are high, by which time control RBCs should be settled to a distinct button.

vii) HA is determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. The titration should be read to the highest dilution giving complete HA (no streaming); this represents 1 HA unit (HAU) and can be calculated accurately from the initial range of dilutions.

- **Haemagglutination inhibition test**

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 after gentle mixing, 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 be settled to a distinct button.

vi) The HI titre is the highest dilution of serum causing complete inhibition of 4 HAU of antigen. The agglutination is assessed by tilting the plates. Only those wells in which the RBCs stream at the same rate as the control wells (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 >$\log_2$ when expressed as the reciprocal), and a positive control serum for which the titre should be within one dilution of the known titre.

HI titres may be regarded as being positive if there is inhibition at a serum dilution of 1/16 (2^4 or $\log_2$ 4 when expressed as the reciprocal) or more against 4 HAU of antigen. Some laboratories prefer to use 8 HAU in HI tests. While this is permissible, it affects the interpretation of results so that a positive titre is 1/8 (2^3 or $\log_2$ 3) or more.

Chicken sera rarely give nonspecific positive reactions in this test and any pretreatment of the sera is unnecessary. Sera from species other than chickens may sometimes cause agglutination of chicken RBCs, so this property should first be determined and then removed by adsorption of the serum with chicken RBCs. This is done by adding 0.025 ml of packed chicken RBCs to each 0.5 ml of antisera, shaking gently and leaving for at least 30 minutes; the RBCs are then pelleted by centrifugation at 800 ***g*** for 2–5 minutes and the adsorbed sera are decanted. Alternatively, RBCs of the avian species under investigation could be used.

The neuraminidase-inhibition test has been used to identify the AI neuraminidase type of isolates and to characterise the antibody in infected birds. The procedure requires specialised expertise and reagents; consequently this testing is usually done in an OIE Reference Laboratory. The DIVA (differentiating infected from vaccinated animals) strategy also relies on using a serological test to detect specific anti-N antibodies; the test procedure has been described (9).

Commercial ELISA kits that detect antibody against the nucleocapsid protein are available. 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.

4. Developing techniques for the diagnosis of avian influenza

At present the conventional isolation and virus characterisation techniques for the diagnosis of AI remain the method of choice, for at least the initial diagnosis of AI infections. However, conventional methods tend to be costly, labour intensive and slow. The past 10 years or so has seen enormous developments and improvements in molecular and other diagnostic techniques, many of these have been applied to the diagnosis of AI infections.

a) Antigen detection

The commercially available Directigen® Flu A kit (Becton Dickinson Microbiology Systems), which is an antigen-capture enzyme immunoassay system, has been used for detecting the presence of influenza A viruses in poultry (28), particularly in the USA. The kit uses a monoclonal antibody against the nucleoprotein

and should therefore be able to detect any influenza A virus. Although it was developed to detect virus in mammalian infections it has been successfully applied to detecting viruses in poultry and other birds, although there may be some variation in the sensitivity for different specimens. The main advantage of the test is that it can demonstrate the presence of AI within 15 minutes. The disadvantages are that it may lack sensitivity, it has not been validated for different species of birds, subtype identification is not achieved and the kits are expensive.

b) Direct RNA detection

Although, as demonstrated by the current definitions of HPAI, molecular techniques have been used in the diagnosis of AI for some time, recently there have been developments in their application for detection and characterisation of AI virus directly from clinical specimens from infected birds.

RT-PCR techniques on clinical specimens could, with the correctly defined primers, result in rapid detection and subtype (at least of H5 and H7) identification, plus a cDNA product that could be used for nucleotide sequencing (22, 30, 31). Results obtained by Koch (19) indicated that care should be taken in clinical specimens used as while tracheal samples from infected birds showed high sensitivity and specificity relative to virus isolation, RT-PCR tests on faecal samples lacked sensitivity. The real application of direct RT-PCR tests may be on rapidly identifying subsequent outbreaks once the primary infected premises has been detected and the virus characterised. This technique was used with success during the 2003 HPAI outbreaks in The Netherlands.

Modifications on the use of RT-PCR have been applied to reduce the time for both identification of virus subtype and sequencing. For example Spackman *et al.* (29) used a 'real time' single-step RT-PCR primer/ fluorogenic hydrolysis probe system to allow detection of AI viruses and determination of subtype H5 or H7. The authors concluded that the test performed well relative to virus isolation and offered a cheaper and much more rapid alternative.

Modifications to the straightforward RT-PCR method of detection of viral RNA have been designed to reduce the effect of inhibitory substances in the sample taken, the possibility of contaminating nucleic acids and the time taken to produce a result. For example, nucleic acid sequence-based amplification (NASBA) with electro-chemiluminescent detection (NASBA/ECL) is a continuous isothermal reaction in which specialised thermocycling equipment is not required. NASBA assays have been developed for the detection of AI virus subtypes H7 and H5 in clinical samples within 6 hours (10, 18).

It seems highly likely that within a very short time molecular based technology will have developed sufficiently to allow rapid 'flock-side' tests for the detection of the presence of AI virus, specific subtype and virulence markers. The extent to which such tests are employed in the diagnosis of AI will depend very much on agreement on and adoption of definitions of statutory infections for control and trade purposes.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

In some countries, vaccines designed to contain or prevent HPAI are specifically banned or discouraged by government agencies because they may interfere with stamping-out control policies. However, most HPAI control regulations reserve the right to use vaccines in emergencies, and this has been done in Mexico and Pakistan.

Since the 1970s in the USA there has been widespread use of inactivated vaccines produced under special licence on a commercial basis (16, 21, 24). These vaccines have been used primarily in turkeys against viruses that are not highly pathogenic but which may cause serious problems, especially in exacerbating circumstances. Significant quantities of vaccine have been used (15, 21). Inactivated vaccine was prepared from the virus of low virulence of H7N3 subtype responsible for a series of outbreaks in turkeys in Utah in 1995 and used, with other measures, to bring the outbreaks under control (17). Following the stamping out of the HPAI H7N1 outbreaks in Italy, low virulence virus of the same subtype re-emerged and vaccination under strict control measures was allowed.

The existence of a large number of virus subtypes, together with the known variation of different strains within a subtype, pose serious problems when selecting strains to produce influenza vaccines. In addition, some isolates do not grow to a sufficiently high titre to produce adequately potent vaccines without costly prior concentration. The vaccines produced have either been autogenous, i.e. prepared from isolates specifically involved in an epizootic, or have been prepared from viruses possessing the same haemagglutinin subtype that yield high concentrations of antigen. In the USA, some standardisation of the latter has been carried out in that the National Veterinary Services Laboratories have propagated and hold influenza viruses of each subtype for use as seed virus in the preparation of inactivated vaccines (4). These vaccines and those for use in Italy (9, 12) against

viruses of low pathogenicity, and against HPAI in Mexico (14) and Pakistan (23), have been prepared from infective allantoic fluid inactivated by beta-propiolactone or formalin and emulsified with mineral oil.

During the 1999–2001 outbreak in Italy, the HPAI H7N1 subtype was eradicated by stamping-out, but a low pathogenic AI virus re-emerged. In order to supplement direct control measures, a DIVA strategy was developed based on the used of an inactivated oil emulsion vaccine containing the same H subtype as the field virus, but a different N, in this case H7N3. Vaccinated and naturally infected birds were differentiated using a serological test to detect specific anti-N antibodies (7, 8).

The information below is based primarily on the experiences in the USA and the guidance and policy for licensing avian influenza vaccines in that country (34). The basic principles for producing vaccines, particularly inactivated vaccines, are common to several viruses e.g. Newcastle disease (Chapter 2.1.15.).

Guidelines for the production of veterinary vaccines are given in Chapter I.1.7. Principles of veterinary vaccine production. The guidelines given here and in Chapter I.1.7 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 used for vaccine production or for vaccine–challenge studies, that part of the facility where this work is done should meet the requirements for Containment Group 4 pathogens as outlined in Appendix I.1.6.1 of Chapter I.1.6 of this *Terrestrial Manual.*

Live conventional influenza vaccines against any subtype are not recommended.

1. Seed management

a) Characteristics of the seed

For any subtype only well characterised influenza A virus of proven low pathogenicity, preferably obtained from an international or national repository, should be used to establish a master seed for inactivated vaccines.

b) Method of culture

A master seed is established, and from this a working seed. If the strain has been cloned, the establishment of a master culture may only involve producing a large volume of infective allantoic fluid (minimum 100 ml), which can be stored as lyophilised aliquots (0.5 ml).

c) Validation as a vaccine

The master seed should be checked after preparation for sterility, safety, potency and absence of specified extraneous agents.

2. Method of manufacture

For vaccine production, a working seed, from which batches of vaccine are produced, is first established by expansion of an aliquot of master seed to a sufficient volume to allow vaccine production for 12–18 months. It is best to store the working seed in liquid form at below –60°C as lyophilised virus does not always multiply to high titre on subsequent first passage.

The inactivated influenza vaccines prepared from conventional virus are produced in embryonated fowl eggs. The method of production is basically that of propagating the virus aseptically; all procedures are performed under sterile conditions.

It is usual to dilute the working seed in sterile PBS, pH 7.2, so that about 10^3–10^4 EID_{50} (50% egg-infective dose) per 0.1 ml are inoculated into the allantoic cavity of 9- or 10-day-old embryonated SPF fowl eggs. These are then incubated at 37°C. Eggs containing embryos that die within 24 hours should be discarded. The incubation time will depend on the virus strain being used and will be predetermined to ensure maximum yield with the minimum number of embryo deaths.

The infected eggs should be chilled at 4°C before being harvested. The tops of the eggs are removed and the allantoic fluids aspirated after depression of the embryo. The inclusion of any yolk material and albumin should be avoided. All fluids should be stored immediately at 4°C and tested for bacterial contamination before large pools are made for inactivation.

In the manufacture of inactivated vaccines, the harvested allantoic fluid is treated with either formaldehyde (a typical final concentration is 1/1000) or beta-propiolactone (a typical final concentration is 1/2000–1/4000). The time required must be sufficient to ensure freedom from live virus. Most inactivated vaccines are not concentrated; the inactivated allantoic fluid is usually emulsified with mineral or vegetable oil. The exact formulations are generally commercial secrets.

3. In-process control

For inactivated vaccines, the efficacy of the process of inactivation should be tested in embryonated eggs, taking 25 aliquots of 0.2 ml from each batch and passaging each aliquot three times through SPF embryos.

4. Batch control

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

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter I.1.5.

b) Safety

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

c) Potency

Conventional potency testing involving the use of three diluted doses and challenge with virulent virus (e.g. Chapter 2.1.15.) may be used for vaccines prepared to give protection against HPAI or H5 and H7 subtypes generally. For inactivated vaccines to other subtypes where virulent viruses are not available, potency tests may rely on the measurement of immune response or challenge and assessment of morbidity. Assessment of haemagglutinin antigen content (37) could allow *in-vitro* extrapolation to potency for subsequent vaccine batches.

d) Stability

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

e) Preservatives

A preservative may be used for vaccine in multidose containers.

f) Precautions (hazards)

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

5. Tests on the final product

a) Safety

See Section C.4.b. above

b) Potency

See Section C.4.c. above.

6. Novel vaccines

Recombinant fowl pox virus vaccines containing H5 HA have been prepared, evaluated (6, 32) and used in field trials in Mexico (15).

A baculovirus-expression system has been used to produce recombinant H5 and H7 antigens for incorporation into vaccines (35).

DNA encoding H5 haemagglutinin has been evaluated as a potential vaccine in poultry (20).

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