AUSTRALIAN STANDARD DIAGNOSTIC TECHNIQUES FOR ANIMAL DISEASES

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Avian Influenza

Pathology, Virology and Serology

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1. Introduction

Fowl Plague or virulent avian influenza (AI) was important during the late nineteenth and the early part of this century. Currently clinical outbreaks of avian influenza are more usually associated with less pathogenic strains. Now that the potential for the emergence of virulent strains from non-pathogenic or low pathogenic strains is appreciated, there is general interest in the distribution of all avian influenza viruses in both wild and domestic birds.

The virulence of avian influenza viruses is extremely variable and there are no characteristic disease signs associated with all influenza infections. A wide range of wild and domestic bird species are hosts, especially water fowl, turkeys and domestic ducks with chickens only becoming infected occasionally. Literally thousands of isolations of Influenza A Virus have been made from avian species but the vast majority are not associated with disease (Beard and Easterday, 1973). Both avirulent and virulent strains are found within the same serotype. In the commercial farm species the common signs of infection are increased mortality, drops in egg production and problems associated with the respiratory, enteric and reproductive systems.

The details of the three confirmed cases of avian influenza in Victoria have been recorded by Turner (1976), Barr *et al.* (1986) and Forsyth *et al.* (1993).

2. General Aspects of the Disease

2.1. Clinical Signs

The clinical signs shown by birds infected with avian influenza are extremely variable and are not definitive. The most common clinical signs seen in infected birds include respiratory, enteric, reproductive and occasionally nervous signs. Cyanosis, especially of the comb and wattles, and swollen heads are usually present to some degree in affected flocks. However, the signs which may be present in affected flocks are similar to, and as variable as, those found in flocks affected with Newcastle disease and infection must be confirmed by virus isolation and serological conversion. Of the wild birds which have been infected the only ones to have shown evidence of disease to date were the Common Terns (*Sterna hirundo*) in South Africa in 1961 when many died but little gross pathology was present.

Among the domesticated species significant outbreaks have occurred in commercial turkeys and chickens with general signs varying from decreased egg production and upper respiratory infection to acute, fatal, generalised disease. The outcome of a particular infection depends upon the biological properties of the virus, intercurrent infections, environmental stress, and the age and sex of the affected birds. The resultant interaction of these factors produces the wide variation in morbidity and mortality rates found in different outbreaks.

2.2. Pathology

In general, the main body systems to show signs of infection with AI virus are the respiratory, enteric and reproductive tracts. In particular, the pathological changes associated with virulent influenza virus infection consist of a combination of haemmorhagic, congestive and transudative changes. In the early stages these may be present in the comb and wattles and be followed by necrotic changes in the liver, lungs, spleen and kidneys. Petechial and ecchymotic haemorrhages may be seen in the intestines and internal organs. A greyish-yellow exudate may be present on the air-sacs and peritoneal surfaces and there may be fibrin deposits on the pericardium and liver. Sinusitis of varying intensity has been reported in outbreaks in a wide range of birds species, including game birds.

2.3. Differential Diagnosis

All the infectious diseases which affect the respiratory, enteric and reproductive systems may produce clinical signs and pathology similar to those which have been observed in outbreaks of avian influenza. The likelihood of dual and multiple infections must also be borne in mind.

The most important pathogens to consider under differential diagnosis include the following.

2.3.1. Viruses

Other haemagglutinating agents: Newcastle Disease and other paramyxoviruses. Haemagglutinating adenovirus.

2.3.2. Chlamydia

Especially in turkey and duck flocks.

2.3.3. Mycoplasmosis

Manifested as sinusitis and air-sacculitis.

2.3.4. Bacteria

Pasteurellosis, colibacillosis and salmonellosis.

3. The Virus

Influenza viruses are members of the family *Orthomyxoviridae* and avian influenza viruses all belong to Type A. The virions are 80–120 nm in diameter, have an RNA core, and possess both haemagglutinin (H) and neuraminidase (N) antigens on the surface. Influenza viruses are typed by determining the nucleocapsid and matrix antigens which are both common within a Type. Individual isolates are subtyped by determining the H and N antigens on their surface. Thirteen different haemagglutinins and nine different neuraminidase antigens have been identified. All possible permutations occur between these

two antigens and none are confined to isolates originating from a single host species. Influenza virus isolates are labelled according to the following criteria:

(a) antigenic type;
(b) host of origin;
(c) geographical location;
(d) strain reference number;
(e) year of isolation; and
(f) H and N specificities.

Thus the most recent (1992) Australian isolate to be associated with clinical disease in chickens is A/Chicken/Victoria/1/92/H7N3.

4. Laboratory Procedures

4.1. Avian Influenza Virus Isolation

(a) The most suitable samples for virus isolation are cloacal swabs or samples of respiratory tract tissue (trachea, lung, air sac, or sinus exudate). These may be swabs or 10% suspensions of ground tissue in brain/heart infusion supplemented with antibiotics (Beard 1980). Specimens for virus isolation should be collected early in the course of the disease when the virus is present in high concentration. Fresh specimens should be submitted and maintained at 4°C or on dry ice if delays of more than 24 hours are anticipated.

(b) During the preparation of cloacal swab material for egg inoculation a mixture of broad spectrum antibiotics is used to control bacterial and fungal contaminants e.g. penicillin 10 000 units/mL and streptomycin 10 000 µg/mL, gentamycin sulfate 100 µg/mL, and mycostatin at 500 units/mL. Other suitable antibiotics are trimethoprim at 200 µg/mL, and sulfadoxine at 1000 µg/mL. The resultant mixture should be incubated at 37°C for 30 min or at room temperature for an hour prior to inoculation. If antibiotics alone are not sufficient to control contamination the sample should be spun at 500 *g* for 10 min and then filtered through a 0.45 µm membrane filter.

(c) Fertile eggs, which have been incubated for 9–11 days, are inoculated via the allantoic sac with 0.1 mL of suspect material using at least five eggs per inoculum.

(d) The inoculated eggs are incubated at 37°C and candled daily. Allantoic fluid should be harvested aseptically from any eggs which die and tested for haemagglutinating activity (HA). The sample must also be tested for bacterial sterility.

(e) In urgent cases 0.2–0.5 mL of allantoic fluid may be harvested aseptically from surviving eggs two days after inoculation, without harm to the embryos, and tested for HA. The sampled eggs should be resealed and reincubated.

(f) Four days after inoculation the eggs are chilled and the allantoic fluid harvested aseptically and tested for HA (see 4.3.1.).

(g) If HA negative the allantoic fluid harvested from the first passage should be inoculated into a further batch of 5x 9–11-day-old embryonated eggs which are monitored as during the first egg passage.

(h) The HA positive samples should be diluted to contain 8 HA units (for Newcastle Disease Virus, NDV) and four HA units (for AI) per 25 µL and tested in the haemagglutination inhibition (HI) test against known positive Newcastle disease and Avian influenza antisera as outlined in the procedure for the haemagglutination inhibition (HI) assay (see 4.3.2.).

4.2. Electron Microscopy

If an electron microscope is available a pellet should be prepared by centrifugation from the HA positive allantoic fluid, e.g. by spinning for 40 min at 30 000 *g* using an SS34 rotor in a Sorval RC5 high-speed centrifuge. The pellet is resuspended in a small volume of distilled water and a drop added to a drop of phosphotungstic acid (24 WO_3.2 H_3PO_4.48 H_2O) stain on a slide. A grid is then placed over the resultant mixture, removed, drained by capillary action and then examined in the electron microscope (Pease, 1964). Paramyxoviruses (100–250 nm) are larger and more pleomorphic than orthomyxoviruses and the inner core of coiled hollow filaments of RNA can be seen through the virus envelope in the form of herring-bone shaped rods. The surface projections on the envelope of the orthomyxoviruses are slightly larger than those found on the paramyxoviruses, i.e. note the size, morphology, and dimensions of the virions, and the appearance of the helical ribonucleoprotein.

4.3. Serology

Because of the numerous subtypes of avian influenza only centralised reference laboratories have the complete range of H and N antisera which are necessary for subtyping.

The most practical approach to follow in the general diagnostic laboratory is to first test the isolate for NDV and AI using the HI test (see 4.3.2.). As at 1 April 1993 only antiserum to the H7N7 influenza virus is available for distribution from the Australian Animal Health Laboratory (AAHL), PO Bag 24, Geelong, Vic. 3220, Australia. However, isolates of virus can be submitted to AAHL for typing. If this proves to be negative both the isolate and the submitted sera should be tested for avian influenza in a double diffusion agar gel system, incorporating 8% sodium chloride as required for avian sera (Beard, 1980). Positive inactivated antigen and antisera for this purpose are available from AAHL.

4.3.1. Haemagglutination Assay

Check the titre of the test antigen as follows:

(a) Place 25 µL phosphate buffered saline (PBS) (Dulbecco PBSA) in each V-shaped well of a 96-well microtitre plate. V-shaped plates are recommended as both haemagglutination and red cell buttons are more distinct and clearer to read than in round bottom wells.
(b) Place 25 µL of the antigen (or suspect allantoic fluid) to be tested in the first well of a row.
(c) Place 25 µL PBS (cell control) and 25 µL specific pathogen free (SPF) (negative) serum respectively in the first well of the next two rows.
(d) Serially dilute each row 1 in 2 to the last well (2^{12} dilution).
(e) Add 25 µL PBS to each well.
(f) Add 25 µL of freshly prepared 1% chick red blood cell (RBC) suspension to each well. Chickens from four weeks of age are most easily bled. RBC suspensions should be less than 48 hours old when used. A single donor bird is used and the resulting red cell suspension checked for auto-agglutination.
(g) Shake the plate on a microshaker for 30 s.
(h) Cover and place at +4°C for 30 min (leave longer if control cells have not buttoned).
(i) Record the end-point by tilting the plate and noting the last well in each row in which the blood cells *do not* run (HA titre end-point). This is essential to clearly identify true haemagglutination.
(j) To find the dilution factor required for the test antigen divide the established HA titre of the antigen by the designated number of HA units required for the test (eight for Newcastle disease virus and four for avian influenza). For example, if the HA titre of the antigen is 256 and four HA units are required for the test, then 256/4 = 64 which is the dilution required to provide four HA units of challenge antigen per well.

4.3.2. The Haemagglutination Inhibition Assay

(a) Place 25 µL of PBS in each well of a V-bottomed microtitre plate.
(b) Place 25 µL of each test serum in the first well of separate rows, and 25 µL of the same serum in the last well (serum control) of the same row.
(c) Place 25 µL of known positive serum in the first well of a row (positive control).
(d) Place 25 µL SPF (negative) serum in the first well of a row (negative control).
(e) Place 50 µL of diluted antigen in the first well of a row (retitration of antigen). Remove PBS from this well before adding antigen.
(f) Serially dilute to the second last well of each row.
(g) Add 25 µL of antigen (containing eight HA units for NDV, four HA units for avian influenza) to each well except for those containing the serum controls and retitrated antigen. Add 25 µL PBS to these wells.
(h) Place on a microshaker for 30 s and then incubate at room temperature for 30 min.
(i) Add 25 µL of 1% chicken RBCs to each well and shake on a microshaker for 30 s.
(j) Incubate at +4°C until the serum controls form discrete buttons (about 30 min).
(k) Read the titre of each serum by placing the plate at an angle and recording the maximum dilution at which the RBC buttons run.
(l) The controls are read as follows:
  (i) *Positive*: complete buttoning (to known titre)
  (ii) *Negative*: complete agglutination (no buttoning)
  (iii) *Retitrated antigen*: agglutination in the first three wells for AI, four wells for NDV, with RBC buttons in all other wells.

N.B. If these controls differ by more than one well from the expected value the test must be repeated.

4.3.2.1. Non-specific reactions

These occasionally occur, usually at a titre of less than 1/64. In such cases the reacting serum samples should be treated overnight with Receptor Destroying Enzyme (RDE).

This treatment involves diluting the serum samples 1/5 with RDE (Wellcome Reagents Ltd) and incubating overnight at 37°C. The mixture is then inactivated by heating at 56°C for 30 min. In our experience this treatment reduces most non-specific HI titres to zero. If titres still persist in a few sera after this treatment a further 20 serum samples from the flock concerned should be tested. All the available evidence from the flock, the clinical signs, gross pathology and virological findings must be taken into account before a diagnosis is made.

4.3.3. Preparation of the Chicken Red Blood Cell (RBC) Suspension

(a) Obtain fresh chicken blood by cardiac puncture or via the jugular or brachial vein using a syringe containing some (0.5–1.0 mL in a 10 mL syringe) Alsever's solution attached to a 23 G needle. Remove the needle and quickly transfer the contents to about five times the volume of Alsever's solution (e.g. 2 mL blood to 10 mL Alsevers solution).
(b) Centrifuge the mixture at 500 *g* for five minutes.
c) Remove the supernatant fluid and the layer of white blood cells with a fine pipette.
(d) Resuspend the RBCs in chilled PBS and repeat this procedure twice.
(e) Dilute the red blood cells to 1% in PBS for use in HA or HI tests. The red cell suspension should be stored at 4°C and be less than 48 hours old when used.

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