AUSTRALIAN STANDARD DIAGNOSTIC TECHNIQUES FOR ANIMAL DISEASES

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Infectious Bronchitis

Pathology, Virology and Serology

J. Ignjatovic

CSIRO Division of Animal Health, Animal Health Research Laboratory, Parkville, Vic. 3052, Australia.

Contents

Infectious Bronchitis

2

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1. Introduction

Infectious bronchitis (IB) is one of the most common viral infections of chickens in Australia and worldwide (King and Cavanagh, 1991). The virus causes disease in the respiratory and urogenital tracts of the bird. Of these, the renal disease is the form more commonly seen and this has been termed 'uraemia' or IB nephritis.

Infectious bronchitis virus (IBV) may also be the primary factor in serious outbreaks of mixed respiratory tract infections involving Mycoplasma organisms and/or bacteria, particularly *Escherichia coli*. The isolation of IBV from the respiratory tract of the bird should, therefore, be interpreted carefully in view of the complex nature of respiratory diseases in the chicken. A diagnosis of IB should be based on:

(a) the history and clinical features of the disease outbreak;
(b) the pathological findings;
(c) the isolation of IBV.

Serological tests to demonstrate the development of precipitating or neutralising antibodies to the virus can also be used. These are usually restricted, however, to survey, experimental or research projects and not routinely used in the diagnosis of infectious bronchitis.

2. History and Clinical Findings

The chicken is the only natural host for IBV and chickens of all ages are susceptible. The disease spreads rapidly through a flock, with an incubation of 18–48 hours.

Birds with IB show depression, rales, coughing, decreased food consumption and whitish diarrhea (Cumming, 1963). Laying hens may not exhibit these signs, the disease being manifest in these birds by decreased egg production, reduced hatchability and misshapen eggs with thin, watery albumen (Broadfoot and Smith, 1954; Sevoian and Levine, 1957).

3. Pathology

The following account of the pathology is based on the reports of Siller and Cumming (1974), Purcell *et al.* (1976) and the author's experience.

A combination of respiratory and renal lesions in the chicken is highly suggestive of IB and is almost pathognomonic.

3.1. Gross Pathology

At post mortem, affected chickens may show dehydration, congestion and oedema of the tracheal mucosa, pulmonary consolidation, cloudiness of the air sacs, renal enlargement and pallor, distension of the ureters with whitish fluid and excess fluid in the cloaca.

3.2. Histopathology

3.2.1. Trachea

Early lesions include desquamation and cyst formation in the tracheal epithelium, with polymorphonuclear leucocyte reaction and oedematous submucosa. After several days, mitotic figures become prominent and lymphocytes, and to a lesser extent plasma cells, become obvious in the inflammatory reaction. Later, regenerating epithelium undergoes differentiation with the development of cilia and distorted mucus glands.

3.2.2. Lungs

Pulmonary lesions occur mainly near the junction of the primary and secondary bronchi. Epithelial desquamation with purulent exudation occurs, followed by healing.

3.2.3. Air Sacs

The air sacs show oedema and small infrequent lymphocyte accumulations.

3.2.4. Oviducts

There is reduction in the height of the epithelium lining the oviduct, glandular dilation and lymphocytic foci in the lamina propria.

3.2.5. Kidneys

Focal tubular necrosis with desquamation of epithelium and an associated polymorph, lymphocyte and plasma cell reaction occurs. Tubular dilation and stroma oedema are often present. The periodic acid-Schiff (PAS) positive granules are usually found in the cytoplasm of tubular epithelial cells. Infrequently, there is swelling of cells of the visceral layer of Bowman's capsule with a few polymorphs between mesangial cells. Between the eighth and tenth days, mitotic figures are numerous in the regenerating tubular epithelium and there is clearing of much of the epithelial debris and cellular exudate from the lamina of the tubules. Lymphocytes and plasma cells are prominent in the interstitial stroma. Later there is a reduction in PAS positive material in the tubule cells and an increase in interstitial lymph nodules.

In the chronic stage, in the cortex and medulla, there is widespread interstitial nephritis with a predominantly lymphocytic and plasma cell reaction. Scattered throughout the interstitial tissue are plasma cells whose distended cytoplasm stains uniformly deeply with PAS due to the presence of Russell bodies containing immunoglobulins. These have been regarded as pathognomonic for the disease (Siller and Cumming, 1974).

4. Virus Isolation

For diagnosis it is necessary to isolate and identify the IB virus. In most diagnostic laboratories, isolation is undertaken by passaging samples in embryonated eggs or tracheal organ cultures.

4.1. Samples for Virus Isolation

The virus can be isolated from many tissues of IBV-infected birds. The preferred sources of samples are trachea, lung or kidney. IBV from layers and breeders can also be isolated from caecal tonsils, cloacal swabs and reproductive organs (Cook, 1984). The virus persists in these tissues for up to 28 days after infection or vaccination, although isolation is more successful early in the infection.

4.1.1. Tracheal Mucous

For isolation of IBV from the trachea, tracheal mucus can be used as a source of virus. The whole trachea is excised and placed on flat, sterile surface (such as a tissue culture dish), cut longitudinally with scissors and the tracheal mucus scraped with a scalpel. The tracheal mucus is transferred to 0.5 mL of sterile phosphate buffered saline (PBS) containing antibiotics (10 000 units of penicillin, 100 µg streptomycin and 100 µg of gentamicin per mL) and the solution is vortexed vigorously for 30 s.

4.1.2. Tissue Homogenates

Tissues are homogenised with a glass tissue grinder or with a mortar, pestle and abrasive in cold nutrient broth or PBS containing antibiotics in a volume to give a 10% suspension. The homogenate is then centrifuged at 2000 g for 10 min, preferably in a refrigerated centrifuge, the supernate removed and allowed to stand at 4°C for 30–45 min. The supernate can then be inoculated immediately into embryonated eggs, or stored at -20°C, or lower, if some time is to elapse before inoculation.

Alternative methods of preparation of the tissues for virus isolation have been demonstrated to be adequate, and may suit some laboratories. For kidney preparations, the tissue is cut into small pieces and these are forced through the nozzle of a 5 mL sterile syringe into a sterile centrifuge tube, filling it to about the 3 mL mark. An equal volume of nutrient broth or PBS containing antibiotic is then added, the suspension thoroughly and vigorously shaken, and the tube allowed to stand at room temperature for 10 min. The suspension is then used as described in the above paragraph.

For a tracheal specimen, the tissue is cut into pieces 1–2 mm wide and placed in a sterile centrifuge tube. About 3 mL of diluent is dispensed into the tube, which is then shaken vigorously for three minutes. The tube is allowed to stand at room temperature for 10 min and then used as before.

4.2. Isolation of Infectious Bronchitis Virus by Propagation in Embryonated Eggs

Some tissue suspension, usually 0.2 mL, is inoculated into the allantoic sac of six 9–10-day-old embryonated eggs. The eggs are incubated in a humidified incubator at 37°C. The eggs are candled daily and embryos dying within 24 hours of incubation are discarded. The allantoic fluid is harvested from three eggs 48 hours after inoculation and stored at -20°C (this represents the first 'blind' passage). The remaining eggs are incubated for a further 120 hours (total of seven days). At day seven, the inoculated embryos are chilled to 4°C, opened and examined for lesions typical of IBV. Lesions are not found in chicken embryos after 48 hours of incubation. The harvested fluid from the first 'blind' passage is diluted two-fold in PBS and inoculated into embryonated eggs in the same way as before. Fluid harvested from these eggs after 48 hours of incubation represents the second 'blind' passage.

At least three serial 'blind' passages of 48 hours are made, although six to seven passages may be necessary for the adaptation of some variant field IBV isolates, in order to induce lesions typical of IBV in the chicken embryo. At the final passage, at day seven, the eggs are opened and the embryos examined for lesions. Uninoculated control embryos of the same age as those being examined should be used for comparison.

IBV is considered to have been isolated if embryos show some or all of the following lesions:

(a) tight curling of the embryo, with the feet compressed over the head;
(b) dwarfing — the embryo may be one-third the size of control specimens;
(c) thickening of the amnionic membrane;
(d) abnormal feather development or 'clubbed' down;
(e) urate deposits in the mesonephros.

Curling is considered to be the most characteristic lesion, although some isolates of IBV fail to induce this lesion and only cause dwarfing.

If the lesions described above are found on the first passage of the virus in embryonated eggs, then it is possible that the virus is egg adapted and therefore could be a vaccine strain. However, Fabricant (1949) found that 46% of field isolates induced these lesions on first passage in chicken embryos, a further 33% on the second, 20% on the third and 1% on the fourth. Therefore, such lesions on the first passage cannot be strictly interpreted as indicative of the virus being a vaccine strain.

4.3. Isolation of Infectious Bronchitis Virus in Tracheal Organ Cultures

Isolation of IBV can also be attempted in tracheal organ cultures (tracheal rings) made from specified-pathogen-free chickens (Cook, 1984). This method of isolation is used when adaptation of IBV in embryonated eggs is not desired. Tracheal organ cultures are also used for cloning of IBV in which case an isolate is passaged five to six times, at limited dilution, in tracheal rings (Cook, 1984).

5. Identification of Infectious Bronchitis Virus

Until recently identification of IBV was most often performed on egg passaged materials. Because of the need for adaptation of field isolates to embryonated eggs the isolation of IBV by this method has been time consuming and cumbersome. Identification of IBV can, however, be attempted directly on the samples collected from chickens suspected to be suffering from IB using the agar gel precipitation (AGP) test or the more recently introduced enzyme-linked immunosorbent assay (ELISA).

5.1. Agar Gel Precipitation Test

The AGP test is regarded as a relatively insensitive method for the detection of IBV. However, it can be used, particularly in the early stages of infection, if appropriate precipitating antisera are available (Lohr, 1981). For the test, tracheal mucus is collected by scraping the trachea, resuspended in 0.2 ml of PBS and used as antigen in the AGP test. Four of the six peripheral wells are filled with different reference antisera known to contain IBV-precipitating antibodies. One well is filled with known negative serum and one with PBS. Even if precipitating lines form only with two of the four reference antisera (due to limited cross-reaction of IBV isolates) the sample is considered to contain IBV.

5.2. Enzyme-linked Immunosorbent Assay

Samples of tracheal mucus or tissue homogenate can also be used in a sandwich ELISA. For an ELISA, 96-well microtitre plates are coated with a monoclonal antibody that recognises all Australian IBV strains. Tracheal mucus or tissue homogenate is added to duplicate wells. Different IBV-specific polyclonal antibody is added, followed by an enzyme conjugate. After addition of substrate, color develops if IBV was present in the sample. The test is specific for IBV and furthermore enables the discrimination of subtype B vaccine viruses from other field isolates of IBV. This ELISA can also be used for egg passaged material. Reagents and full test details can be made available from the CSIRO Division of Animal Health, Parkville, Vic. 3052, Australia, Tel. (03) 342 9700; Fax (03) 347 4042.

5.3. Curling and Dwarfing of the Embryos

Identification of IBV is most commonly achieved by propagating samples suspected to contain IBV in embryonated eggs for a minimum of three 'blind passages' and examining inoculated embryos for lesions characteristic of IBV (see 4.2.).

5.4. Fluorescent Antibody Technique

The use of specific fluorescence staining of allantoic cells of IBV inoculated eggs is described by Clarke *et al.* (1972). They found the technique to be faster, more economical and more sensitive than methods depending on the ability of IBV to cause dwarfing of chicken embryos.

5.5. Identification of Virus by Electron Microscopy

The most distinctive morphological feature of IBV in negatively stained preparations is the possession of a corona of pear shaped projections attached to the virus envelope (Berry *et al.*, 1964). These can be used in the characterisation of the virus. However, morphological variation among IBV strains has been described (Harkness and Bracewell, 1974). They observed pleomorphism in virion shape, the presence or absence of a corona as well as variation in the shape of the projection. Considerable experience is, therefore, needed in interpreting the significance of particles observed in this way.

6. Serological Identification of Infectious Bronchitis Virus

There is a considerable antigenic variation among IB viruses and at least seven serotypes have been identified in the USA and in Europe. In Australia at least nine subtypes (designated A–I) of IBV are recognised (Wadey and Faragher, 1981) and reference sera for identification of these subtypes can be made available from the CSIRO Diviion of Animal Health, Parkville, Vic. 3052. Three Australian IB vaccines viruses, Steggles, Webster's strain Vic S and strain Inghams, are all of subtype B, whereas Webster strain A3 IB vaccine virus is of subtype C.

Serotyping of an IBV isolate is most commonly performed by the virus neutralisation test in embryonated eggs although virus neutralisation in tracheal organ cultures and chicken embryo kidney cells have been used extensively (Cook *et al.*,1984; Wadey and Faragher, 1981). Far more cross-reactions between various IBV serotypes occur when virus neutralisation is performed in eggs and chicken kidney cells, whereas neutralisation in tracheal organ cultures gives rise to less cross-reactions between serotypes.

6.1. Virus Neutralisation in Embryonated Eggs

An undiluted reference IB serum and a normal serum are mixed with an equal volume of the virus preparation and incubated at 37°C for 60 min. Volumes of 0.1 mL of each mixture are inoculated into the allantoic sac of eight embryos 9–10 days old and incubated at 37°C for seven days. Deaths within 24 hours of inoculation are considered to be non-specific. The embryos are examined at the completion of incubation for lesions characteristic of IBV (see 4.2.). The virus preparation should be completely neutralised by the reference antiserum.

6.2. Haemagglutination Inhibition Test

The haemagglutination inhibition (HI) test has been used for typing IBV strains, particularly in the United States of America and in Europe (Alexander *et al.*, 1976; Lashgari and Newman, 1982) where it was found that after treatment with phospholipase C, most IBV isolates will haemagglutinate chicken red blood cells. The HI test has very limited value, however, for serotyping in Australia, since only two of nine the IBV subtypes were found to haemagglutinate chicken red blood cells (Faragher, 1987). The primary response to IBV gives rise to antibodies that are strain specific in the HI test, whereas the secondary response give rise to antibodies that are broadly cross-reactive in the HI test. Therefore, the HI is not suitable to detect exposure to a particular serotype of IBV in field sera.

7. References

Alexander, D.J., Bracewell, C.D., and Gough, R.E. (1976). Preliminary evaluation of the hemagglutination and hemagglutination inhibition tests for avian infectious bronchitis virus. *Avian Pathology* **5**, 125–34.

Berry, D.M., Cruikshank, J.G., Chu, H.P., and Wells, R.J.H. (1964). The structure of infectious bronchitis virus. *Virology* **23**, 403–7.

Broadfoot, D.I., and Smith W.M. Jr. (1954). Effect of infectious bronchitis in laying hens on egg production, percent unsettable egg and hatchability. *Poultry Science* **35**, 653–4.

Clarke, J.K., McFerran, J.B., and Gay, F.W. (1972). Use of allantoic cells for the detection of avian infectious bronchitis virus. *Archives fur die gesamte Virusforschung* **36**, 62–70.

Cook, J.K.A. (1984). The classification of new serotypes of infectious bronchitis virus isolated from poultry flocks in Britain between 1981 and 1983. *Avian Pathology* **13**, 733–41.

Cook, J.K.A., Darbyshire, J.H., and Peters, R.W. (1976). The use of chicken tracheal organ cultures for the isolation and assay of avian infectious bronchitis virus. *Archives of Virology* **50**, 109–18.

Cumming, R.B. (1963). Infectious avian nephrosis (uraemia) in Australia. *Australian Veterinary Journal* **36**, 145–7.

Faragher, J.T. (1987). A haemagglutination inhibition test for avian infectious bronchitis virus antibody. *Australian Veterinary Journal* **64**, 250–2.

Fabricant, J. (1949). Studies on the diagnosis of Newcastle disease and infectious bronchitis of fowls. II. The diagnosis of infectious bronchitis by virus isolation in chick embryos. *Cornell Veterinarian* **39**, 414–31.

Harkness, J.W., and Bracewell, C.D. (1974). Morphological variation among avian infectious bronchitis virus strains. *Research in Veterinary Science* **16**, 128–31.

King, D.J., and Cavanagh, D. (1991). 'Infectious Bronchitis.' *In* 'Diseases of Poultry'. 9th Edn. pp. 471–84. (The Iowa State University Press: Ames, Iowa, USA.)

Lashgari, M.S., and Newman, J.A. (1982). Preparing hemagglutinating antigen from isolates of infectious bronchitis virus. *Avian Diseases* **26**, 508–19.

Lohr, J.E. (1981). Diagnosis of infectious bronchitis (IB) by examination of tracheal mucus for IB–precipitating antigens. *Avian Diseases* **25**, 1058–64.

Purcell, D.A., Thain, V.L., and Surman, P.G. (1976). The histopathology of infectious bronchitis in fowls infected with nephrotropic "T" strain of virus. *Australian Veterinary Journal* **52**, 85–91.

Sevoian, M., and Levine, P.P. (1957). Effects of infectious bronchitis on the reproductive tracts, egg production, and egg quality of laying chickens. *Avian Diseases* **1**, 136–64.

Siller, W.G., and Cumming, R.B. (1974). The hisotopathology of an interstitial nephritis in the fowl produced experimentally with infectious bronchitis virus. *Journal of Pathology* **114**, 163–73.

Wadey, C.N., and Faragher, J.T. (1981). Australian infectious bronchitis viruses: identification of nine subtypes by a neutralisation test. *Research in Veterinary Science* **30**, 70–4.