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Infectious Bursal Disease

Virology and Serology

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1. Introduction

Atrophy of the bursa of Fabricius (BF) is one of the most frequently found lesions in young chickens throughout Australia that are grown under intensive farming conditions. Firth (1974) termed this condition infectious bursal syndrome (IBS) although the nature and type of the lesions found in experimental IBS were characteristic of infectious bursal disease (IBD). Subsequently, one of the agents (designated 002/73) isolated from IBS was demonstrated at the Central Veterinary Laboratory, Weybridge, to be antigenically indistinguishable from infectious bursal disease virus (IBDV) in the gel diffusion precipitin test. It has been shown subsequently to be a type-1 strain of IBDV (Wood *et al.*, 1988). Clinical IBD (Cosgrove, 1962) has not been recognised in Australia although serological studies have shown infection with IBDV is widespread in poultry flocks (Dennett and Bagust, 1980). Field reports have, however, associated a decline in the rate of growth of chickens for three to six days after infection with IBDV, as well as claiming an increased susceptibility of IBDV infected chickens to other poultry diseases. Experimental studies have shown Australian strains of IBDV can affect the serological responses of chickens to Newcastle disease virus (Westbury, 1978).

However, atrophy of the BF has been described in diseases other than IBD (Olson, 1967; Mussman and Twiehaus, 1971) and viruses other than IBDV have been isolated from BF showing atrophy (Bagust and Westbury, 1975). Therefore, it is not possible to conclude that atrophy of the BF is pathognomonic for IBD.

2. Clinical Signs and Pathology

A diagnosis of acute IBD can be based on a consideration of the age and history of the flock, the onset and course of the disease and on an examination of the clinical signs and gross lesions in a number of affected birds. Classic acute IBD is characteristically a disease of rapid onset and recovery with a duration of five to seven days and is most frequently seen in chickens three to five weeks of age. On post mortem examination, affected chickens show dehydration, haemorrhages in skeletal muscles, and the kidney tubules may be so filled with urates that they appear white. The BF is enlarged up to twice the normal size for birds of the age examined, oedematous and yellowish with prominent striations. Petechiation may be present on the peritoneal surface and plicae and necrotic degeneration of the epithelium lining with sloughing may produce a core of caseous material (Cosgrove, 1962; Winterfield and Hitchner, 1964; Hanson, 1967). In these outbreaks 10–20% of chickens in an affected flock can show sudden signs of the disease and mortality may be 1–15% (Cosgrove, 1962).

Enlargement of the BF is only found during the early stages of the disease. Subsequently the BF becomes atrophied and may be only one third to one half its usual size (Cheville, 1967).

The disease does not take this form in Australia. Typically there is no overt disease in an infected flock. Infection with IBDV is first suspected at post-mortem examination because a number of chickens show atrophy of the BF. The BF may be only one half the size it should be for the age of the chicken. Other organs appear normal although what is assumed to be concomitant diseases (airsacculitis, pericarditis, perihepatitis) are occasionally found.

Sometimes bursae showing peribursal oedema, petechiation on the serosal surface and caseous castes are found. Although these signs and lesions may be suggestive of IBD they are not pathognomonic.

Histologically bursae from chickens infected with IBDV show necrosis and depletion of lymphoid cells and of the corticomedullary layer with regression of the bursal follicles. The epithelial lining of the plicae can be hyperplastic and hypertrophic with development of goblet-type cells in an active secretory state. Pseudocystic structures are seen frequently within follicles as is oedema of the interfollicular trabeculae. Also, focal infiltrates of heterophils may be found in the follicles and interfollicular tissues.

These lesions are not, however, diagnostic for IBD, although they are highly suggestive, particularly if the time course of their development is known. IBDV will induce inflammatory and degenerative changes in the BF within 24–72 hours of infection of chickens.

Thus, although the clinical signs and lesions seen in acute IBD are useful in diagnosing the disease, they are not characteristic enough to be used in the diagnosis of mild *or* subclinical IBDV infections. In these circumstances confirmation must be obtained by virus isolation or demonstration of viral antigen and/or specific antibodies.

3. Virus Isolation

This can be undertaken in susceptible chickens, fertile eggs or tissue cultures. The BF is the organ from which isolation is usually attempted.

3.1. Experimental Infection of Chickens

Chickens three to five weeks of age that have no detectable antibody to IBDV should be used. The BF used for isolation attempts should be obtained from birds early after infection as virus is only readily isolated for two to seven days after infection. Tissues are homogenised in cold phosphate buffered saline (PBS) pH 7.2, with 0.5% (w/v) bovine albumin or gelatin and antibiotics (1000 units penicillin, 1000 µg streptomycin/mL) to an appropriate 10% suspension. The homogenate can be stored at -20°C until used.

The tissue homogenate is centrifuged at 1000 *g* for five minutes, the pellet discarded and the supernatant used for dosing chickens. Chickens can be dosed by ocular instillation, per os or via the cloaca as well as by intramuscular inoculation. The ocular route is the most commonly used method with each chicken being given 25 µL of the test material. Two chickens are killed and examined for lesions in the BF at zero, three, five and seven days after being dosed. A portion of the BF is used for histological examination, the remainder being stored at -20°C until the results of pathology tests are known. The presence of gross and/or histological lesions of IBD in the BF at three, five and/or seven days indicates the presence of IBDV. Demonstration of IBDV antigen in the stored portion of BF provides further evidence that the birds were infected with IBDV (see 4.).

Isolation of the virus in chickens is the simplest technique available for demonstration of IBDV and is recommended for routine isolation purposes. However, care must be exercised in the housing of experimental chickens as IBDV is highly contagious and contamination of facilities with the virus can occur. It is preferable to house birds to be used in IBDV infection studies off the ground at all times and in isolation from other birds.

3.2. Embryonating Chicken Eggs

The fertile eggs must be from a flock free of detectable antibody to IBDV and the chorio-allantoic route of inoculation is the most sensitive route for detecting virus. Fertile eggs 10–12 days of incubation are inoculated with a homogenate of BF (see 3.1.). The eggs are incubated at 37°C and candled daily to determine if deaths occur. Embryos dying in the 48 hours after inoculation are discarded. Embryos dying from two to seven days after inoculation are used for passaging. The chorio-allantoic membrane or whole embryo should be used for subpassage. Chicken embryos alive seven days after inoculation are harvested, homogenised and subpassaged.

Embryonic mortality from infection with IBDV usually occurs between the third and sixth days after inoculation. Embryos show dwarfing, oedema, congestion and haemorrhages in the subcutis and in the region of the kidney, swelling of the liver with greenish discolouration and necrosis, enlargement of spleen and pale foci in the heart muscle.

Up to five passages may be necessary to adapt the virus to produce these lesions. However, none of the lesions can be considered to be specific for IBDV, although in combination they are characteristic of infection with IBDV. The presence of IBDV in the chicken embryo can be confirmed by neutralisation of the effect on chicken embryos by specific antiserum or inoculation of chickens as described above.

Virus isolation in chicken embryos is a useful procedure for routine diagnostic purposes, although the need for passaging the virus means that it is time consuming.

3.3. Tissue Culture

Growth of IBDV has been reported in various cell culture systems (Kosters and Paulsen, 1971; Lukert and Davis, 1974). Growth of IBDV in BF and chicken embryo kidney (CEK) cell cultures was detected by cytopathology and immunofluorescence (Lukert and Davis, 1974). No cytopathic effect (CPE) was seen in either cell type in the first two passages, although fluorescing cells were detected in the infected BF cell culture when stained with specific fluorescent antibody at 48 hours. Adaptation of IBDV to CEK cell cultures appeared to require preliminary growth in BF cell culture. Australian IBDV isolates do not appear to propagate readily and to cause CPE in chicken embryo kidney, liver or fibroblast cell cultures, or in chicken kidney cells (D.P. Dennett, pers. comm. 1975). A strain of Australian IBD virus, GT101, has been adapted to grow in chick embryo fibroblasts (CEFs), while 002/73 and GT101 will both grow in Vero cells without causing CPE. The presence of mixed virus populations in BF submitted for IBDV isolation means that careful identification of agents isolated in cell culture is necessary. Both avian reoviruses and adenoviruses have been readily isolated from the atrophied BF of chickens (Bagust and Westbury, 1975; H.A. Westbury, unpublished data 1976). A standard IBDV monospecific antiserum or monoclonal antibody should be used in tests to differentiate agents isolated in cell cultures.

3.4. Electron Microscopy

McFerran *et al.* (1978) were able to observe IBD viral particles in impression smears of the BF of chickens experimentally infected with virulent IBDV. They were unable to evaluate the use of the procedure in the diagnosis of IBD from field material. Using immunoelectron microscopy they were also able to see IBD viral particles in tissue cultures infected with the virus.

4. Demonstration of Infectious Bursal Disease Virus Antigens

Precipitating antigen can be demonstrated in the BF of IBDV infected chickens by the gel diffusion precipitin test (GDPT) or the enzyme-linked immunosorbent assay (ELISA). Both techniques are appropriate for testing the BF of experimentally infected chickens (e.g. virus isolation in chickens) but can also be used for testing field material. However, the bursae used should be from chickens in the early *or* acute stages of infection (three to seven days) as IBDV antigen can only rarely be demonstrated in the BF of

chickens that have been infected for seven days or longer. The bursae used for antigen testing are processed as in 3.1. It is then tested against a monospecific IBDV serum and a positive control IBDV precipitating antigen in a GDPT (see 3.1.) or the ELISA (see 3.4.). In the GDPT the presence of IBDV antigen in the test preparation is indicated by the development of one or more precipitin lines between the positive serum and test antigen. Line(s) of identity with precipitin line(s) between the serum and control positive antigen must be demonstrated. In the ELISA the quantity of IBDV antigen is indicated by the intensity of the enzyme catalysed colour reaction and the specificity is dependent on the reactivity of either the monospecific antiserum or preferably a monoclonal antibody.

5. Serology

5.1. Gel Diffusion Precipitin Test

The GDPT used is essentially that developed by Faragher (1971). The gel is 1.25% Agar No.1 (Oxoid) in 8% (1.37 mol/L) sodium chloride and 0.5% (0.5 mol/L) phenol (C_6H_5OH) at pH 7.0 and is pipetted onto 7.5 x 2.5 cm microscope slides. Patterns of seven wells, one central and six peripheral in hexagon pattern are cut in the agar and plugs removed. Wells are 5 mm in diameter with 2 mm between the circumferences of each well.

The antigen is prepared from the BF of specific pathogen free (SPF) chickens that have been experimentally infected with IBDV strain 002/73. The virus used to infect chickens should be free of extraneous agents. The bursae are weighed and equivalent volumes of trichlorotrifluoroethane ($C_2Cl_3F_3$,Arklone P, ICI) and distilled water are added. The mixture is homogenised in a tissue blender, centrifuged at 2000 *g* for 30 min, the supernatant harvested and stored at -70°C.

IBDV specific chicken antiserum is used as the control positive serum in the test. A control negative serum should also be used. The control positive serum is obtained from SPF chickens at 10–14 days after experimental infection with purified IBDV strain 002/73.

The reference antigen is dispensed into the central well, control positive serum into wells 2, 4 and 6, while negative control serum is placed in wells 1, 3 and 5. To ensure that wells are filled to accurate levels dispensing of reagents should be done on a well lit, flat surface. The wells should be filled until the surface of the reagent is slightly convex and then taken down to the level of the agar.

The control positive serum and reference antigen are then diluted in two-fold steps and tested against one another, in various combinations to determine the optimum dilution of each for clarity of precipitin lines.

Table 1. Interpretation of the gel diffusion precipitin test

+++	Denotes test line equivalent to reference line.
++	Denotes test line weaker than reference line, but producing a continuous line across the face of the test well.
+	Denotes a distinct turn, or hook, toward a test well on the end of the reference line.

For routine testing, test sera replaces the negative control serum in well 1, 3 and 5. The reaction of test serum and reference antigen can be roughly quantitated by comparison with the precipitin line formed with the control positive serum (Table 1).

Non-specific reactions occur as lines of precipitation which either cross, or fail to establish a line of identity with the reference line. These are not commonly encountered.

Precipitating antibody to IBDV has been demonstrated to persist up to 138 weeks after experimental infection (Faragher, 1971).

A technique for quantitation of antibody to IBDV using the GDPT has been described by Cullen and Wyeth (1975). In this procedure wells are cut in agar in a offset linear pattern, wells being 6 mm in diameter with 3 mm interspace in agar 3 mm thick. One row of wells is filled with antigen. The opposite row is filled with dilutions of serum in PBS. Control sera are also included. The endpoint is the last dilution of serum at which a precipitin line is formed with antigen. Repeat titrations of sera have shown the endpoint determination to be reproducible.

5.2. Neutralisation Test (In Ovo)

Neutralisation test in embryonating chicken eggs have been used to study the serological responses of chickens naturally and experimentally infected with IBDV (Winterfield, 1969). The test proved valuable in assessing the immune status of chickens.

5.3. Neutralisation Test (In Vitro)

The virus neutralisation assay is most conveniently performed in flat-bottomed 96-well microtitre trays (Fahey *et al.*, 1985*a*).

Chicken antiserum is heat-inactivated at 56°C for 30 min and initially diluted in serum-free medium 199 containing 1.5% of a 5.6% (0.66 mol/L) solution of sodium bicarbonate ($NaHCO_3$). After this initial dilution the antiserum is serially diluted in 25 µL aliquots of medium 199 containing 2% heat-inactivated foetal calf serum, 10% tryptose phosphate broth, 1.5% of 5.6% stock solution of sodium bicarbonate, 0.5% of 0.3 mol/L Hepes solution, penicillin (100 µg/mL), streptomycin (100 µg/mL) and fungizone (2.5 µg/mL). An equal volume of IBDV-GT101, diluted in the complete medium to give 100–1000 50% tissue culture infective doses ($TCID_{50}$) per 25 µL, is added to each well. The trays are gently shaken to mix the reagents and incubated at 37°C for one hour.

Confluent primary CEF cultures are prepared in Petri dishes and the cells suspended by trypsinisation. The cells are washed once in complete medium and resuspended to 75 x 10^4 viable cells/mL of the complete medium 199.

Following the incubation of antiserum with virus, 50 µL of CEF suspension is added to each well. The trays are again shaken gently and incubated for three to four days at 37°C in a humidified atmosphere of 5% carbon dioxide in air. The culture medium is then removed from the wells of the microtitre trays and replaced with 50 µL of 1% crystal violet in 90% ethanol (C_2H_5OH). After one minute the trays are washed gently with water several times and allowed to dry. As the crystal violet stains living cells they appear dark purple, while areas where the cells have died or sloughed off because of the replication of the IBDV appear much lighter. The reciprocal of the serum dilution which neutralises the virus totally, so as to inhibit all CPE, is taken as the titre of the antiserum.

Control neutralisation assays are performed with both antibody positive and antibody negative serum. Also the virus stock is assayed in quadruplicate from 10^{-2} to 10^{-9}, to ensure that the stated amount of virus was added to each well. In addition to these controls the highest concentration of each antiserum being titrated (the initial dilution) is incubated with the CEF cells in the absence of virus to detect any toxic effects of the antiserum on the cells.

5.4. Enzyme-linked Immunosorbent Assay

The ELISA can be used to detect both IBDV antigen in infected tissues and also chicken antibody to IBDV. The assay is conveniently performed at room temperature in flat-bottomed 96-well polystyrene microtitre trays (Fahey *et al.*, 1985*a*). The wells of the trays are coated with 100 µL of the IgG fraction of hyperimmune rabbit antiserum to IBDV diluted in 0.05 mol/L carbonate–bicarbonate buffer pH 9.6. The IgG fraction of the rabbit antiserum can be obtained by Rivanol precipitation (York *et al.*, 1983) or column chromatography using blue dextran (Pharmacia) or protein A (Pharmacia). The trays are sealed with cellotape and stored at room temperature overnight. The trays are then washed three times with PBS containing 0.05% Tween 20 (PBST); the wash buffer remaining in each well for at least one minute. The IBDV antigen (see 3.1.) is diluted in filtered wash buffer containing 5% instant skim milk powder (diluting buffer) and 100 µL added to each well. The trays are then incubated for one hour before being washed three times with the wash buffer. The antiserum is serially diluted in the diluting buffer and 100 µL added to the coated wells. The trays are again incubated for one hour and washed three times with the wash buffer. Sheep antichicken Ig conjugated to horseradish peroxidase (KPL) is diluted to 1:1000 in diluting buffer and 100 µL added to each well. The trays are incubated for a further hour and washed three times with PBST and finally with distilled water. Recrystallised (100 µL) 5-aminosalicyclic acid (Merck) containing 0.2 mL of 0.1 mol/L hydrogen peroxide (H_2O_2) is added to each well (York *et al.*, 1983) and the trays placed on a shaker for 30 min. The optical density (OD) in each well is read at 450 nm using a plate reader (Titertek Multiscan).

The ELISA must be optimised by titrating each batch of the coating antibody, the IBDV antigen and the enzyme-linked antichicken Ig reagents to obtain maximal OD readings of 1.10–1.20, while keeping background readings to a minimum.

The end point for the ELISA is taken as 0.15 OD units above the background binding (York *et al.*, 1983) and the initial dilution of negative sera or sera with low concentrations of antibody is usually 1:200. Controls should include wells without the antigen, but with the coating antibody, chicken serum and conjugate, and wells in which only the chicken serum is omitted and replaced by diluting buffer. The plate reader should be 'blanked' on a row of wells which have remained untreated, except for the addition of substrate.

In practice the ELISA for antibody is as sensitive as the *in vitro* virus neutralisation assay, and both are up to 100-fold more sensitive than the GDPT.

The ELISA can be readily adapted to detect the concentration of IBDV antigen in tissue extracts. To achieve this the unknown antigen preparation was titrated by serial dilution and then a predetermined concentration of chicken antiserum was added which gave a maximum OD of 1.0 following the addition of conjugate and substrate (Fahey *et al.*, 1985*a*). A similar end point was used as when titrating antibody levels.

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