ANNEX III

DIAGNOSTIC PROCEDURES FOR THE CONFIRMATION AND DIFFERENTIAL DIAGNOSIS OF NEWCASTLE DISEASE

The following procedures for the isolation and characterization of Newcastle-disease viruses should be regarded as guidelines and the minima to be applied in the diagnosis of the disease.

The virus responsible for Newcastle disease is the prototype virus of the Paramyxoviridae. At present, there are nine serologically distinguishable groups of Avian Paramyxoviruses, which have been designated PMV-1 to PMV-9. All Newcastle disease viruses are placed in the PMV-1 group. For the purpose of the diagnostic procedures for the confirmation and differential diagnosis of Newcastle disease the following definition shall apply:

‘Newcastle disease’ means an infection of poultry caused by any avian strain of the paramyxovirus 1 with an intracerebral pathogenicity index (ICPI) in day-old chicks greater than 0,7.

CHAPTER 1

Sampling and treatment of samples

1. Samples

Cloacal swabs (or faeces) and tracheal swabs from sick birds; faeces or intestinal contents, brain tissue, trachea, lungs, liver, spleen and other obviously affected organs from recently dead birds.

2. Treatment of samples

The organs and tissues listed in paragraph 1 may be pooled, but separate treatment of faecal material is essential. Swabs should be placed in sufficient antibiotic medium to ensure full immersion. Faeces samples and organs should be homogenized (in an enclosed blender or using a pestle and mortar and sterile sand) in antibiotic medium and made to 10-20 % w/v suspensions in the medium. The suspensions should be left for about two hours at ambient temperature (or longer periods at 4 °C) and then clarified by centrifugation (e.g. 800 to 1 000 g for 10 minutes).

3. Antibiotic medium

Different laboratories have used various formulations of antibiotic medium with success and laboratories referred to in Annex II will be able to offer advice for a particular country. High concentrations of antibiotics are required for faeces samples and a typical mixture is: 10 000 units/ml penicillin, 10 mg/ml streptomycin, 0,25 mg/ml gentamycin and 5 000 units/ml mycostatin in phosphate buffered saline (PBS) These levels can be reduced up to five-fold for tissues and tracheal swabs. For control of Chlamydia organisms, 50 mg/ml oxytetracycline may be added. It is imperative when making the medium that the pH is checked after the addition of the antibiotics and readjusted to pH 7,0-7,4.

CHAPTER 2

Virus isolation

Virus isolation in embryonated fowls' eggs

The clarified supernatent fluid should be inoculated in 0,1-0,2 ml amounts into the allantoic cavity of each of a minimum of four embryonated, fowls' eggs which have been incubated for

8 to 10 days. Ideally, these eggs should be obtained from a specific pathogen-free flock, but when this is impracticable it is acceptable to use eggs obtained from a flock shown to be free of antibodies to Newcastle-disease virus. The inoculated eggs are held at 37 °C and candled daily. Eggs with dead or dying embryos as they arise, and all remaining eggs six days after inoculation should be chilled to 4 °C and the allantoic-amniotic fluids tested for haemagglutination activity. If no haemagglutination is detected the above procedure is repeated using undiluted allantoic/amniotic fluid as inoculum.

When haemagglutination is detected the presence of bacteria should be excluded by culture. If bacteria are present the fluids may be passed through a 450 nm membrane filter, further antibiotics added and inoculated into embryonated eggs as above.

CHAPTER 3

Differential diagnosis

1. Preliminary differentiation

It is intended that all haemagglutinating viruses should be submitted to the national laboratory referred to in Annex II for full identification, characterization and pathogenicity tests. However, it is important that interim control measures for Newcastle disease aimed at limiting the spread of the virus should be implemented as soon as possible and regional laboratories should be able to identify the presence of Newcastle disease virus. The haemagglutinating fluids should, therefore, be used in an haemagglutination inhibition test as described in Chapters 5 and 6. Positive inhibition 1. e. 2^4, or more, with the Newcastle disease virus specific polyclonal antiserum of titre known to be at least 2^9 would serve as preliminary identification enabling the imposition of interim control measures.

2. Confirmatory identification

The national laboratory should undertake full differential diagnosis of any haemagglutinating agent. Confirmation of Newcastle-disease virus would again be by inhibition in haemagglutination inhibition tests with monospecific chicken antisera. Intracerebral pathogenicity index tests as described in Chapter 7 should be carried out on all positive isolates. Pathogenicity indices of greater than 0,7 indicate the presence of virus requiring the full implementation of control measures.

Recent developments in typing Newcastle-disease viruses, particularly monoclonal antibody techniques, has enabled grouping of the strains and isolates. In particular, some monoclonal antibodies are available which are specific for the vaccinal strains used on the territory of the Community and can be employed in simple haemagglutination inhibition tests.

Since live vaccine strains may often be isolated from sampled poultry the advantage of their rapid identification at the national laboratory referred to in Annex II is obvious. Such monoclonal antibodies should be obtained by the Community reference laboratory as referred to in Article 14 and supplied to the national laboratories to enable confirmation of the isolation of vaccinal viruses.

The national laboratories should submit all haemagglutinating agents to the Community reference laboratory.

3. Further typing and characterization of isolates

The Community reference laboratory should receive all haemagglutinating viruses from the national laboratories for further antigenic and genetic studies to enable a greater understanding

of the epizootiology of the disease(s) within the Community in keeping with the functions and duties of the reference laboratory.

CHAPTER 4

Rapid tests for detection of Newcastle-disease virus and antibodies

Rapid tests for the detection of Newcastle disease virus in vaccinated birds and the detection of antibodies in unvaccinated birds are outlined below:

1. Detection of Newcastle disease virus

Several rapid test that directly detect Newcastle-disease antigens have been employed in the diagnosis of infections in vaccinated birds. Those most commonly used to date are fluorescent antibody tests on longitudinal sections of the trachea and peroxidase antibody tests on the brain. There seems no reason to doubt that other direct antigen detection tests could be applied to Newcastle-disease virus infections.

The drawback to such tests is that it is impracticable to examine all the potential sites of replication of Newcastle-disease virus in the vaccinated birds. So that, for example, absence of evidence of virus in the trachea does not preclude virus replication in the gut. No direct detection method is recommended for routine use in the diagnosis of Newcastle disease, although in specific circumstances such tests may have a useful role.

2. Detection of antibodies in unvaccinated birds

The majority of laboratories involved in Newcastle disease diagnosis are familiar with the haemagglutination inhibition test and the recommendation described below relate to this test for the measurement of antibodies to the virus. However, enzyme-linked immunosorbent assays (Elisa) may be successfully used to detect antibodies to the virus. It is suggested that if there is a wish to employ an Elisa test at regional laboratory level the test should be monitored by the national laboratory referred to in Annex II.

(a) Samples

Blood samples should be taken from all birds if the flock size is less than 20 and from 20 birds from larger flocks (this will give a 99 % probability of detecting at least one positive serum if 25 % or more of the flock is positive, regardless of flock size). The blood should be allowed to clot and serum removed for testing.

(b) Examination for antibodies

Individual serum samples should be tested for their ability to inhibit Newcastle disease virus haemagglutinating antigen in standard haemagglutination inhibition tests as defined in Chapter 6.

There is some debate as to whether 4 or 8 haemagglutinin units should be used for the HI test. It would appear that either is valid and the choice should be left to the discretion of the national laboratories. However, the antigen used will affect the level at which a serum is considered positive: for 4 HAU a positive serum is any showing a titre of 2^4 or greater and for 8 HAU a positive serum is any showing a titre of 2^3 or greater.

CHAPTER 5

Haemagglutination (HA) test

Reagents

1. Isotonic saline buffered with phosphate (PBS) (0,05 M) to pH 7,0-7,4.

2. Red blood cells (RBC) taken and pooled from a minimum of three specific pathogen free chickens (if not available blood may be taken from birds regularly monitored and shown to be free of NDV antibodies) into an equal volume of Alsever's solution. Cells should be washed three times in PBS before use. For the test a 1 % suspension (packed cell v/v) in PBS is recommended.

3. NDV strain Ulster 2C is recommended for use as standard antigen.

Procedure

(a) Dispense 0,025 ml PBS into each well of a plastic microtitre plate (V-bottomed wells should be used).

(b) Place 0,025 ml of virus suspension (i.e. allantoic fluid) in the first well.

(c) Use a microtitration diluter to make two-fold dilutions (1:2 to 1:4096) of virus across the plate.

(d) Dispense a further 0,025 ml of PBS to each well.

(e) Add 0,025 ml of 1 % red blood cells to each well.

(f) Mix by tapping gently and place at 4 °C.

(g) Plates are read 30 — 40 minutes later when controls are settled. Reading is done by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. Wells with no HA should flow at the same rate as the control cells with no virus.

(h) The HA titre is the highest dilution that causes agglutination of the RBCs. That dilution may be regarded as containing one HA unit (HAU). A more accurate method for determining the HA titre is to do HA tests on virus from a close range of initial dilutions i.e. 1:3, 1:4, 1:5, 1:6 etc. This is recommended for the accurate preparation of antigen for haemagglutination inhibition tests (see Chapter 6).

CHAPTER 6

Haemagglutination inhibition (HI) test

Reagents *(see Chapter 5)*

(a) Phosphate buffered saline (PBS)

(b) Virus-containing allantoic fluid diluted with PBS to contain 4 or 8 HAU per 0,025 ml.

(c) 1 % chicken RBCs.

(d) Negative control chicken serum.

(e) Positive control serum.

Procedure

(a) Dispense 0,025 ml PBS into all wells of a plastic microtitre plate (with V-bottomed wells).

(b) Place 0,025 ml of serum into first well of plate.

(c) Use microtitration diluter to make two-fold dilutions of serum across plate.

(d) Add 0,025 ml of diluted allantoic fluid containing 4 or 8 HAU.

(e) Mix by tapping and place plate at 4 °C for a minimum of 60 minutes or room temperature for a minimum of 30 minutes.

(f) Add 0,025 ml 1 % RBCs to all wells.

(g) Mix by gentle tapping and place at 4 °C.

(h) Plates are read after 30-40 minutes when control RBCs are settled. This is done by tilting and observing the presence or absence of tear-shaped streaming at the same rate as control wells containing RBCs (0,025 ml) and PBCs (0,05 ml) only.

(i) The HI titre is the highest dilution of antiserum causing complete inhibition of 4 or 8 units of virus (an HA titration to confirm the presence of the required HAU should be included in each test).

(j) The validity of the results is dependent on obtaining a titre of less than 2^3 for 4 HAU or 2^2 for 8 HAU with the negative control serum and a titre of within one dilution of the known titre of the positive control serum.

CHAPTER 7

Intracerebral pathogenicity index test

1. Infective freshly harvested allantoic fluid (HA titre must be greater than 2^4 is diluted 1:10 in sterile isotonic saline (anti-bodies must not be used).

2. 0,05 ml of the diluted virus is injected intracerebrally into each of 10 one-day old chicks (i.e. 24 hours; 40 hours after hatching). The chicks should be hatched from eggs obtained from a specific pathogen-free flock.

3. The birds are examined at intervals of 24 hours for eight days.

4. At each observation each bird is scored: 0 = normal; 1 = sick; 2 = dead.

5. The index is calculated as shown in the following example:

Clinical signs	**Day after inoculation(number of birds)**									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**Total**	**Score**
normal	10	4	0	0	0	0	0	0	14 × 0	= 0
sick	0	6	10	4	0	0	0	0	20 × 1	= 20
dead	0	0	0	6	10	10	10	10	46 × 2	= 92
									Total = 112	

Index is a mean score per bird per observation = 112/80 = 1,4

CHAPTER 8

Assessment of plaque-forming ability

1. It is usually best to use a dilution range of virus to ensure that an optimum number of plaques are present on the plate. Ten-fold dilutions up to 10^{-7} in PBS should be sufficient.

2. Confluent monolayers of chick embryo cells or a suitable cell line (Madin-Darby bovine kidney for example) are prepared in 5 cm diameter Petri dishes.

3. 0,2 ml of each virus dilution is added to each of two Petri dishes and the virus allowed to absorb for 30 minutes.

4. After washing three times with PBS the infected cells are overlaid with the relevant medium containing 1 % w/v agar and either 0,01 mg/ml trypsin or no trypsin. It is important that no serum is added to the overlay medium.

5. After 72 hours, incubation at 37 °C the plaques should be of sufficient size. They are best seen by removing the agar overlay and staining the cell monolayer with crystal violet (0,5 % w/v) in 25 % v/v ethanol.

6. All viruses should give clear plaques when incubated in the presence of trypsin in the overlay. When trypsin is absent from the overlay only viruses virulent for chickens will produce plaques.