AUSTRALIAN STANDARD DIAGNOSTIC TECHNIQUES FOR ANIMAL DISEASES

STANDING COMMITTEE ON AGRICULTURE AND RESOURCE MANAGEMENT

ANIMAL HEALTH COMMITTEE

SUB-COMMITTEE ON ANIMAL HEALTH LABORATORY STANDARDS

Newcastle Disease

Histopathology, Virology and Serology

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1. Introduction

These standard diagnostic procedures for Newcastle disease are for use with avirulent Australian isolates of Newcastle disease virus (NDV) or for NDV serology. They are not intended for use when a virulent exotic strain of NDV is suspected. Follow the procedures designated in Ausvet Plan and the Exotic Diseases Manual and arrange for relevant specimens to be submitted to the Head of Laboratory, Australian Animal Health Laboratory, Geelong, using the agreed protocols and with permission from the relevant State/Territory veterinary authorities (at present your Chief Veterinary Officer and the Chief Veterinary Officer of Victoria).

Newcastle disease is of major importance to the domestic poultry industries in most countries except Australia and New Zealand (Lancaster, 1981; Alexander, 1985). Economic losses occur due to death of birds, decreased egg and meat production, costs of vaccination and the costs to governments of quarantine and other measures to prevent the introduction of the disease into the country or to control the spread within the country. Vaccination is widely practiced to control the diseases and NDV vaccines are the most extensively used of any vaccines (Allan *et al.*, 1978).

The distribution of Newcastle disease has been associated with the spread by the domestic chicken and by the Family *Psittacidae*, with other unspecified methods suspected. Migrating birds may also play a role, although virulent isolates have not been made from such sources (Lancaster, 1981; Alexander *et al.*, 1986; Alexander 1988; Mackenzie *et al.*, 1984, 1985; Rosenberger *et al.*, 1975; Smith and Coakley, 1985).

It has been postulated that three main streams of Newcastle disease infection have occurred (Lancaster, 1981; Alexander, 1988). The first involved the period from 1926 to 1946 (Doyle, 1927; Hanson, 1974), when disease spread to five continents, including Australia (Albiston and Gorrie, 1942; Levine, 1964). During this period, it was believed that the disease moved from continent to continent by live birds or infected frozen poultry carcasses carried by ships.

The second main stream originated in 1962, purportedly in a cockatoo (*Psittacidae*) from Indonesia. Subsequent spread appeared to resemble aspects of the first stream, because in 1969 virulent disease was recognised in imported psittacines in the United States of America. However, there is evidence that velogenic viruses existed in a number of countries prior to 1963 and that a reservoir of infection, perhaps resembling that in Indonesia, existed in countries of Central and South America (Lancaster and Alexander, 1975).

A distinct variation of this second main stream commenced in 1966, when a widely spreading form of velogenic virus was reported in Iran (Hanson *et al.*, 1974) and later in Iraq in 1968. This virus, designated by isolates AG68 (Sokkar and Sawa, 1974) and Essex 70 (Alexander and Allan, 1973), was highly virulent and caused marked respiratory lesions but variable enteric lesions. It was distinct from the viscerotropic velogenic pathotype described in the USA, and spread rapidly across the Middle East and Europe. The velogenic virus form of NDV spread widely between 1966 and 1971.

A third main stream of Newcastle disease commenced around 1981 and is caused by an antigenic variant of NDV associated with pigeons (Russell and Alexander, 1983; Alexander, 1985). This avian paramyxovirus (APMV-1) isolate has been identified with a disease resembling the neurotrophic form of NDV and has spread through Europe and other parts of the world. Isolates have been obtained from diseased racing pigeons in Great Britain, Germany, Belgium, Denmark, Italy, Portugal, Czechoslavakia, Japan, Switzerland, Sweden and United States of America (Alexander, 1985; Pearson *et al.*, 1987). In 1984 an outbreak associated with APMV-1 virus occurred in British chickens. The first isolate obtained from diseased laying hens was shown in monoclonal antibody binding patterns to be identical to the pigeon isolates (Alexander, 1985). Although contact with racing pigeons seemed unlikely, it was known that feral birds in Liverpool docks were infected with the virus and it was subsequently shown that foodstuffs stored at the docks had been fed, untreated, to the affected fowls (Alexander, 1985).

There have been two outbreaks of virulent disease in Australia, both in Victoria, one in 1930 and one in 1932 (Albiston and Gorrie, 1942). From eradication in 1932, until 1966, it was believed that Australia was free of Newcastle disease virus (French, 1964). In 1966 Simmons (1967) isolated a Newcastle disease virus from healthy Queensland chickens, it was subsequently designated the V4 or Queensland strain. This strain was shown to be avirulent, although there have now been many isolates related to V4 taken from Australian poultry and these appear to vary in their biological properties (Westbury, 1981). In 1977 an exotic strain of NDV was isolated from cockatoos illegally imported from Indonesia (Eaves and Grimes, 1978). This isolate, known as the Eaves/Grimes strain has subsequently been shown to be the lentogenic vaccine strain, La Sota (E. Hansson, A.J. Della-Porta, and D.J. Alexander, unpublished data 1991). An additional antigenic group of avirulent NDV isolates have been obtained from wild birds; these are related to the European wild duck isolate MC110 (Mackenzie *et al.*, 1984; Alexander *et al.*, 1986; Della-Porta *et al.*, 1988). These MC110-like isolates are separated from V4-like isolates using panels of monoclonal antibody and in Australia they have only been found in wild birds and not in poultry.

2. Pathogenesis

Newcastle disease virus was first described by Doyle (1927). There are three main classifications of clinical pathotypes: velogenic, or high virulence; mesogenic, or intermediate virulence; and lentogenic, or low virulence, including apathogenic.

The *velogenic viruses* produce an illness of short duration and the mortality in a susceptible flock is between 90 and 100%. The pathotypes include: viscerotrophic velogenic strains or Doyle's form (Doyle, 1927; McDaniel and Orsborn, 1973) and neurotrophic velogenic strains that cause Beach's form (Beach, 1943) or 'avian pneumoencephalitis'.

The *mesogenic viruses* also spread rapidly and produce respiratory distress and coughing. After respiratory symptoms subside, nervous symptoms appear. A feature is the effect on egg production and quality. Mortality may occasionally reach 50% in mature susceptible flocks. The disease caused by mesogenic strains is generally described as Beaudette's form (Beaudette and Black, 1946).

The *lentogenic viruses* are generally associated with respiratory symptoms and a sudden drop in egg production. The disease caused by lentogenic strains is generally described as Hitchner's form (Hitchner and Johnson, 1948). Asymptomatic infections have been reported in a number of countries, including Ireland (Ulster 2C strain) and Australia (V4 strain), (Simmons, 1967; McFerran *et al.*, 1968; McFerran and Nelson, 1971). Asymptomatic Newcastle disease virus may often be diagnosed by chance or by routine serological surveillance.

A wide range of pathogenic lesions can be seen in birds infected with NDV. There is no gross lesion which is pathognomonic for Newcastle disease. Lesions can resemble those seen in avian influenza, infectious laryngotracheitis and infectious bronchitis. Sudden onset of disease within the flock and post mortem lesions can be highly suggestive of virulent NDV. Common lesions for velogenic viscerotrophic NDV are tracheal lesions with peritracheal lesions near the thoracic inlet; haemeorrhage or necrosis, the proventriculus and necrosis, ulceration, or both, of the lymphoid aggregates in the intestine such as Peyer's patches or caecal tonsils. Care needs to be taken to diagnose Newcastle disease on lesions alone.

3. Serological Tests

There are a number of serological tests used for monitoring the immune status of chickens. These usually detect the presence of specific antibodies that react with the surface antigens on the virus. The most commonly used are the haemagglutination-inhibition test and the enzyme-linked-immunosorbent assay (ELISA). Less widely used are a range of virus neutralisation tests (Lancaster, 1981; Beard and Hansson, 1984; Ishida *et al.*, 1985).

3.1. Haemagglutination-Inhibition Test

The haemagglutination-inhibition (HI) test is very widely used for NDV serology and in identification of NDV isolates. It involves the inhibition of agglutination of chick red blood cells by 8–10 units of NDV haemagglutinin (HA) in the presence of antibody. The antiserum is usually diluted to 1:8 or higher to avoid non-specific agglutinins sometimes found in the sera and a microtitre assay employed (Allan and Gough, 1974*a*, 1974*b*). In Australia, low HI titres are seen in birds infected with endemic avirulent NDV isolates, with titres of 1:4–1:8 being common. Overseas, titres of 1:8 or greater are usually taken as positive. A detailed description of the method is in 6.1.

3.2. Enzyme-linked Immunosorbent Assay

The enzyme-linked immunosorbent assays (ELISAs) have been developed for assays of antibodies to NDV (Miers *et al.*, 1983; Nishikawa *et al.*, 1983; Piela et al., 1984; Wilson *et al.*, 1984; Della-Porta and Spencer, 1989). These ELISAs have no significant advantage over the HI test in specificity but do enable tests to be automated and the computer collection and analyses of data. A standard ELISA for NDV serology (see 6.2.) and is based on the method of Della-Porta and Spencer (1989). This ELISA has been extensively field evaluated and has been found to relate to HI titres and not to be affected by non-specific red blood cell agglutinins. Testing is carried out at a dilution of 1:200 of serum, thus enabling the use of very small serum volumes. NDV-ELISA kits are available from Head of Laboratory, CSIRO Australian Animal Health Laboratory, PO Bag 24, Geelong, Vic. 3220, Australia [Tel. (052) 275 000; Fax (052) 275 555] for a small fee. Kits will test up to 4000 sera and include antigen, positive and negative control sera and sheep antichicken IgG-horseradish peroxidase labelled conjugate (conjugate prepared by Dr Terry Spencer, Regional Veterinary Laboratory, DARA, Benalla, Vic. 3672, Australia. Tel. (057) 622 933; Fax (057) 623 953).

3.3. Future Serological Tests

Tests with an increased specificity compared to the HI and NDV chicken antibody ELISA are at present under investigation. These involve the use of monoclonal antibodies in blocking/competition ELISAs. Such ELISAs might have application in differentiating V4-infected or vaccinated birds from other infected birds, as in an exotic NDV outbreak.

4. Virus Isolation and Identification

4.1. Isolation in Chick Embryos

Separate sterile cotton wool swabs are used to obtain trachael and cloacal specimens from tests birds. The swabs used should not contain alginate. The swabs are immediately immersed individually in 3–5 mL of brain heart infusion broth (Oxoid) containing 10 000 international units (IU) of crystalline penicillin and 1 mg streptomycin per mL.

The suspension is then refrigerated at 4°C for 30–40 min and 0.1 mL inoculated into the allantoic sac of at least four 9–10-day-old embryonated chicken eggs. Eggs from a specific pathogen free (SPF) flock should be used if possible or, if not available, eggs should be sourced from a Newcastle disease virus antibody negative flock. The inoculated eggs are incubated at 37–38°C for five days, the eggs being candled each day.

The allantoic fluid from embryos dying during this period, except those dying within 24 hours of inoculation, is harvested. The fluid is tested for haemagglutinating (HA) activity with chicken erythocytes. Allantoic fluid not causing haemagglutination is diluted two-fold in broth and reinoculated into chicken embryos.

Chicken embryos alive five days after inoculation are chilled at 4°C for at least two to three hours, and the allantoic fluid harvested. The fluids from the eggs inoculated with the one swab are pooled and tested for HA activity. A second passage is undertaken for fluid that shows no HA activity. Swabs not showing HA activity after two passages in embryonating eggs are considered negative.

4.2. Identification Procedures

Agents showing HA activity are tested in a HI test with specific NDV antibody to confirm the identity of the agent. (*N.B.* Other agents that might produce HA activity include avian influenza or avian paramyxoviruses). A constant virus-varying serum test in microtitre HA trays is used. The HA titre of the agent to be used in the test is determined. A dilution containing 8–10 HA units of the agent is prepared. This is then used in the HI test using a standard reference positive NDV antiserum. The end point is the highest dilution of serum in which HA is completely inhibited. It is advisable to include a positive control of 8–10 HA units of NDV antigen and titrate the positive antiserum with both the unidentified (test) agent and the reference antigen. The procedures for the HA and HI tests are included in 6.1.

4.3. Pathotyping Tests

A range of pathotyping tests are available to characterise the virulence of any NDV isolates. It should be noted that where pathogenic (exotic) isolates of NDV are suspected, the pathogenicity tests should be carried out at the Australian Animal Health Laboratory, Geelong. The relevant sections of Ausvet Plan and Exotic Diseases Manual should be consulted and the Chief Veterinary Officer of the State or Territory must be informed. For completeness the pathotyping procedures are briefly summarised below.

4.3.1. Mean Death Time (MDT)

The mean death time of the minimum lethal dose (MDT/MLD) (Hanson and Brandly, 1955) is based on the highest dilution (usually 10^{-7}) in which all of the embryos have died or possess HA at 128 hours. It involves the inoculation of 10 nine-day-old embryos with 10-fold dilutions of virus from 10^{-1} to 10^{-10} and use of the formula:

MDT = (No. dead at X hour)(X hours)+ (No. dead at Y hour)(Y hours)+etc./Total No. dead

The chick embryos are usually divided into two sets of five embryos per dilution, inoculated eight hours apart. The embryos are monitored at least twice daily to obtain the number of deaths (after the first 24 hours). The MDT is the least reliable of the bird pathogenicity tests and the results can be subject to considerable variation.

4.3.2. Intracerebral Pathogenicity Index (ICPI) for One-day-old Chickens

Eight chickens at least 24 hours old, but not more than 40 hours, are inoculated intracerebrally with 0.1 mL of undiluted inoculum. The chicks are then housed together and observed daily until death or for eight days. The survival index, based on time of death, is calculated by scoring each chick daily for eight days (0, normal; 1, diseased; 2, dead). The resulting sum is divided by the number of observations. High intracerebral pathogenicity indices (ICPIs) (1–2) usually indicate velogenic or mesogenic NDV isolates, whereas a low ICPI (<0.5) would indicate a lentogenic or apathogenic isolate.

4.3.3. Intravenous Pathogenicity Index (IVPI) for Six-week-old Chickens

Birds that have not been exposed to NDV are inoculated intravenously with 0.1 mL undiluted inoculum as described by Alexander and Parsons (1984). The birds are then observed daily until death or for eight days. The intravenous pathogenicity index (IVPI) is calculated by scoring each chicken daily for eight days (0, normal; 1, diseased; 2, paralysed; 3, dead). The resulting sum is divided by the number of observations. High IVPIs (1.5–3) are indicative of velogenic isolates of NDV.

4.3.4. Other Pathogenicity Tests

There are a number of additional tests sometimes applied to give an indication of the pathogenicity of NDV isolates. These include evaluation of pathogenicity in six- to eight-week-old chickens by conjunctiva and cloacal inoculation

and ability to plaque in chick embryo fibroblast cells with or without DEAE-dextran and Mg^{2+} ions (Barahona and Hanson, 1968). Additional tests include rate of elution from red blood cells, thermostability of HA and ability to agglutinate red blood cells from horses. All these tests give data of variable usefulness for pathotyping and for differentiating NDV isolates.

4.4. Other Tests

4.4.1. Monoclonal Antibody Typing

Alexander (1990) has used panels of monoclonal antibodies to place NDV isolates into 12 groups; these roughly correlate to virulence but are not necessarily specific for pathotype. They are extremely useful for epidemiological studies. Della-Porta *et al.* (1988) have developed a panel of monoclonal antibodies against the Australian avirulent isolate Queensland/V4. This panel is also useful for separating viruses into groups, especially the Queensland/V4-like and MC110-like isolates from wild birds (Alexander *et al.*, 1986; Della-Porta *et al.*, 1988).

Russell and Alexander (1983) employ an immunoperoxidase technique on fixed infected cells and read the test using light microscopy. This involves infecting the cells, incubation, fixation, and subsequent detection with the monoclonal antibody, immunoperoxidase staining and observation. The technique described by Della-Porta *et al.* (1988) involves the use of antibody precoated ELISA plates to capture virus from allantoic fluids and subsequent screening with the monoclonal antibodies and detection in an ELISA. This test can be carried out in less than one day.

4.4.2. Antigen Distribution in Chorioallantoic Membrane

Studies on the distribution of NDV strains in chick embryos showed that the tissue distribution was related to the pathogenicity of the isolate (Nagai *et al.*, 1979). A diagnostic test based on the localisation of NDV antigens in the chorioallantoic membrane (CAM) of infected chick embryos has been developed (A.J. Della-Porta, G. Russell, J.G. Young, H. Hamid, Darminto, P. Daniels, unpublished data 1991). The CAM is fixed in 10% neutral buffered formalin, paraffin embedded and thin sections prepared (Lillie and Fullmer, 1976). These are fixed and stained with a pool of NDV group-reactive monoclonal antibodies. The bound monoclonal antibodies are detected using immunofluorescence, immunoperoxidase or immunogold with silver counter-staining techniques. The antigens for avirulent and lentogenic isolates of NDV are restricted to the endodermal layer of cells, whereas mesogenic and velogenic isolates have their antigens distributed through all cell layers of the CAM.

4.4.3. Molecular Biology Based Tests

The structure of the external glycoproteins, the haemagglutinin-neuraminidase (HN) and the fusion (F) proteins has been demonstrated to be related to the virulence of NDV isolates (Klenk *et al.*, 1977; Nagai and Yoshida, 1984; Rott and Klenk, 1988).

The F protein is synthesised as a precursor protein, Fo, that must be cleaved into two subunits, F_1 and F_2, linked by a disulfide bond, to be active. The sequence of amino acids at the cleavage site contains pairs of basic amino acids (Arg-Arg or Arg-Lys) for the velogenic and mesogenic isolates of NDV. The lentogenic and avirulent isolates only contain single basic amino acids (Arg) at the cleavage site (McGinnes and Morrison, 1986; Espion *et al.*, 1987; Sato *et al.*, 1987*a*; Toyoda *et al.*, 1987; Gorman *et al.*, 1988). This difference in amino acid composition at the cleavage site(s) affects the ability of endoproteases to cleave the Fo protein; the proteases able to cleave at two basic amino acids being much more common and widely distributed in cell types. The trypsin-like protease that cleaves at a single arginine is much more limited in its cellular distribution. Viruses in which the Fo is not cleaved are not infectious, and the ability of avirulent or low virulence isolates is limited to those tissues in which the relevant protease is present, hence their restricted invasiveness.

The HN protein is not usually synthesised as a precursor protein, HNo, in virulent NDV isolates (Jorgensen *et al.*, 1987; Sato *et al.*, 1987*b*; Wemers *et al.*, 1987; McGinnes *et al.*, 1987; Gorman *et al.*, 1988; Gotoh *et al.*, 1988; Millar *et al.*, 1988). In avirulent strains the stop-codon has been mutated so that a read through of the gene continues for an additional 45 amino acids past the termination of the HN protein found in virulent isolates. Subsequent cleavage of this precursor HNo, to about the size of HN, is required for infectivity. However, this cleavage is restricted because it is a single basic amino acid (Arg), similar to that found for Fo cleavage region in avirulent and low virulent isolates of NDV.

Antipeptide antisera have been produced to a variety of short synthetic peptides of NDV F- and HN- proteins. These antipeptide sera (or other future antisera) will form the basis of tests to distinguish between viruses with and without the HNo extension and with different Fo cleavage sequences (Gorman *et al*, 1990).

Tests based on the RNA nucleotide sequences for the genes covering these areas are also being developed. The polymerase chain reaction (PCR) is being used to amplify the gene sequence and analyse for the Fo cleavage site and for the HNo extension (E. Hansson, J. Bashiruddin, A.R. Gould and A.J. Della-Porta, unpublished data 1992). This test is available at the Australian Animal Health Laboratory to supplement other pathotyping tests.

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6. Appendixes

6.1. Appendix 1 — Haemagglutination and Haemagglutination-Inhibition Tests

6.1.1. Introduction

Haemagglutination (HA) is used to detect the presence of agents, including NDV, that agglutinate chicken red blood cells (RBCs) and haemagglutination-inhibition (HI) tests are used to determine the identity of an agent or for detection and titration of specific antibodies. A macrotest employing standard WHO perspex plates, or disposable WHO plates, has generally been replaced with a microtitre test that employs round bottom 96-well microtitre plates (Allen and Gough, 1974*a*, 1974*b*).

6.1.2. Preparation of Chicken Red Blood Cells

Blood is collected from mature chickens that are free of HI antibody to NDV. The blood is dispensed into Alsever's solution immediately following collection, in order to stop clotting of the RBCs. The RBCs are prepared by three cycles of washing in saline and centrifugation. The final concentration of RBCs is adjusted to 0.5% in saline. This preparation is stored at 4°C and can be used for up to 72 hours after preparation.

6.1.3. Haemagglutination Test

(a) Dispense 50 µL of normal saline (or Dulbecco's complete phosphate buffered saline, PBS) into each well of a round-bottommed microtitre plate using a multi-channel pipettor.

(b) For one sample, titrate in duplicate across the plate (columns 1–12) by placing 50 µL of the allantoic fluid into the first wells of the first two rows of the plate and dilute across to the eleventh well using a multichannel pipettor. Remove 50 µL from the 11th well (leaving 50 µL).
N.B. The twelfth well is left as a RBC control, and contains 50 µL of saline.

(c) Add 50 µL of the 0.5% RBC suspension to each well. Mix by lightly tapping the plate and incubate at 4°C for at least 40 min. The RBC control well should show a clear button at that time.
N.B. Examine the plate periodically for evidence of HA activity, as subsequent elution may occur — especially if the plate warms to room temperature.

(d) The plates are read for HA activity. The endpoint is the highest dilution at which complete, or near complete, haemagglutination occurs. Partial agglutination is scored as negative.

(e) Where the identity of the haemagglutinating isolate is required, 8–10 units of this and a reference NDV isolate should be used to titrate a specific positive NDV reference antiserum in the HI test.

6.1.4. Haemagglutination-Inhibition Test

(a) Dilute the antigen to 8–10 HA units.

(b) To the wells in column 1 of a round bottom microtitre plate, add 25 µL of saline (or PBS) and then add 25 µL of the test serum, in duplicate, to wells 1A and 1B, etc. down the plate. Column 1 is for the serum control.

(c) To the wells in columns 2–12 add 50 µL of saline. Columns 2–11 are for the titration of the serum in the HI test and column 12 is for the RBC control.

(d) Add 50 µL of each serum to duplicate wells in column 2 on the plate (e.g. 2A and 2B etc.) and dilute them using a 50 µL multi-channel pipettor. Discard 50 µL from the wells in column 11.

(e) Add 50 µL of antigen to each well in columns 2–11.

(f) After mixing by lightly tapping, incubate the plate at room temperature for one hour. This allows any antibodies present to bind to the NDV–HA antigen.

(g) Add 50 µL of 0.5% RBC suspension to each well. Mix as above and incubate the plate at 4°C until the cells in the RBS control have clearly settled into a button (about 40 min) [see 6.1.3.(c.)].

(h) The plates are read for HI activity as follows.
(i) *Column 1.* The serum control. Should have RBC buttons, as for RBC control. This indicates absence of non-specific agglutinins in the serum. If the serum agglutinates the RBCs then the serum might be reacting non-specifically.
(ii) *Column 12.* The RBC control. Should have RBC buttons indicating that the RBCs are working properly.
(iii) Columns 2–11. The titration of the serum. If specific antibodies are present in the serum, HA will be inhibited. The endpoint is the highest dilution which completely inhibits HA. If the serum is positive, the wells will appear like the RBC control and change to HA as the serum is diluted. Dilutions are from 1:2, in two-fold steps, to 1:1024.

(i) Antigen Control. The antigen used in this test must be titrated, at the same time this test is carried out, to determine the HA titre (HA test, see 6.1.3.). The HA titre should be between 1:8 and 1:10 (8–10 HA units).

(j) Non-specific serum agglutinins can cause non-specific activity, often up to 1:8. Care should be exercised in interpreting low HI antibody titres. Titres of 1:8 or greater are taken as positive. Titres between 1:2 and 1:4 may be positive and are often present in chickens infected with avirulent Australian NDV isolates.

6.2. Appendix 2 — Newcastle Disease Virus Chicken Antibody Enzyme-linked Immunosorbent Assay

6.2.1. Introduction

This ELISA will measure antibodies in chicken serum against Newcastle disease virus (NDV). The antigen is prepared from the V4 vaccine strain of NDV grown in specific pathogen free (SPF) eggs, harvested and purified by differential and sucrose density gradient centrifugation. Inactivation is performed using β-propiolactone. This test will not differentiate between titres due to infection or exposure to V4 or field strains of NDV nor between vaccination or post-infection antibody titres.

6.2.2. Summary of Method

(a) Dilute V4 NDV antigen at 0.5 µg/mL in sodium carbonate buffer pH 9.5.

(b) Coat U-bottom plate at 50 µL per well.

(c) Incubate at room temperature overnight.

(d) Wash plate with ELISA wash buffer or PBS/Tween.

(e) Washed plates may be stored at 4°C for up to one month.

(f) Add 50 µL per well of serum diluted 1:200 in PBS/Tween.

(g) Use positive and negative control sera on every plate.

(h) Incubate, with shaking, for 30 min at room temperature.

(i) Wash plate with ELISA wash buffer or PBS/Tween.
(j) Add 50 µL per well of conjugate diluted to working strength in PBS/Tween.
(k) Incubate, with shaking, for 30 min at room temperature.
(l) Wash plate with ELISA wash buffer or PBS/Tween.
(m) Add 100 µL per well of ABTS/hydrogen peroxide (H_2O_2) in citrate buffer. ABTS is 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate).
(n) Incubate for one hour at room temperature.
(o) Read at 414 nm wavelength (405 nm is used if 414 nm is not available).

6.2.3. Detailed Method

6.2.3.1. Coating

Volumes (50 µL) of V4 NDV ELISA antigen, diluted to 0.5 µg/mL in coating buffer (see 6.2.4.), are added to each well of polystyrene 'U'-well microtitre plates. The plate is incubated at room temperature overnight. Enhanced binding plates should not be used. Suggested types include Disposable Products #23154, NUNC #2-62170, Cooke M24A or NUNC #4-75434 (Polysorp®). About 5 mL of coating antigen solution is required per plate.

6.2.3.2. Washing

Wash the plates at least three times with PBS/Tween (see 6.2.4.). The plates are either used immediately or stored, after sealing, for up to month at 4°C.

6.2.3.3. Serum samples

Dilute the serum samples 1:200 for testing in PBS/Tween. Suggested volumes for diluting purposes are 10 µL of serum in 2 mL of PBS/Tween. If care is taken one micropipette tip can be used for all samples. Ensure that the tip is rinsed several times after discharging into the PBS/Tween diluent. Duplicate 50 µL volumes are added to the microtitre plate, i.e. sample 1 to A1/B1, sample 2 to A2/B2, sample 10 to A10/B10, sample 11 to CI/DI up to 40. Again one tip can be used for all samples providing that the tip is rinsed, this time in the new diluted sample before, addition to the plate. Incubate the plate for 30 min at room temperature shaking on a microtitre plate shaker. Samples may be prediluted and stored at 4°C for up to one day before assay.

6.2.3.4. Controls

Negative and positive 1:10 stock dilutions are prepared by diluting each ampoule of 0.1 mL freeze dried serum in 1 mL of PBS/Tween. The negative standard is prepared by diluting the 1:10 stock a further 1:20 in PBS/Tween to give a final dilution of 1:200. This is added to wells G11/G12. The positive standard serum is diluted at the recommended dilution; at present 1:400 by making a 1:40 dilution in PBS/Tween. This is then added to wells A11/A12 and doubly diluted down the plate to row F to generate dilutions of 1:800, 1:1600, 1:3200, 1:6400 and 1:12 800.

Both control standards can be stored frozen in aliquots at a dilution of 1:10. Wells H11/H12 are left blank and reserved for conjugate controls. Note that the suggested dilutions of the control sera may vary from batch to batch. Ensure that the control samples are added to the plate as soon as possible after the test samples. This minimises any standardisation problems due to incubation time differences.

6.2.3.5. Washing

Wash the plates at least three times with PBS/Tween.

6.2.3.6. Conjugate

Conjugate (50 µL) diluted in PBS/Tween are added to all wells of the microtitre plate(s). The optimum dilution of the conjugate is supplied by the manufacturer or should be determined by titration of the conjugate. The plates are incubated, with shaking for 30 min at room temperature. About 5 mL of conjugate is required per plate.

6.2.3.7. Washing

Wash the plates at least three times with PBS/Tween.

6.2.3.8. Substrate

ABTS/hydrogen peroxide substrate (100 µL) are added to each well of the microtitre plate. Incubate at room temperature for one hour. About 10 mL is required per plate. (*N.B.* Other substrates, such as TMB or OPD may be used. ABTS is recommended for its stability and because its reaction is self limiting and there is no need to add a stop solution).

6.2.3.9. Optical density

The optical density (OD) of the plate is read at 414 nm or 405 nm if a 414 nm filter is not available as the green colour produced has a broad absorption peak. Full colour development takes about 1.5 hours although measurements can be taken at any time up to 24 hours as long at the plates are sealed with cellotape or similar to stop evaporation of the liquid. Results are calculated and expressed as ELISA units (see 6.2.5.).

6.2.4. Reagents

6.2.4.1. Warning

All reagents should be formulated using only *glass distilled* water, other types of distilled water may inactivate the conjugate producing low or no colour reaction. We suggest that all reagents be prepared as concentrated stock solutions which are conveniently diluted prior to use.

6.2.4.2. Coating buffer

This consists of a 50 mmol/L mixture of sodium carbonate (Na_2CO_3)/sodium hydrogen carbonate ($NaHCO_3$) at a pH of about 9.5. This reagent is prepared as a 10 times concentrate as below:

Na_2CO_3, 170 mmol/L	18.0 g
$NaHCO_3$, 330 mmol/L	27.7 g

Make to one litre with glass distilled water.

Dilute one volume of the 10 times stock with nine volumes of glass distilled water prior to use. Store the concentrate in a tightly capped container to stop the entry of carbon dioxide. An alternative coating buffer is PBS pH 7.2 prepared (see 4.3.). *Do not* add Tween 20 to the coating solution as no antigen will bind.

6.2.4.3. PBS/Tween

This consists of 140 mmol/L NaCl, 10 mmol/L sodium phosphate and 0.05% Tween 20. EDTA (1 mmol/L final) can be added if heavy metal contamination is of concern. This reagent is conveniently prepared as a 10 or 20 times concentrate without Tween 20 (PBS) or with Tween 20 (PBS/Tween) as below (Table 1).

Dilute one volume of the 10 times stock with nine volumes of glass distilled water or one volume of the 20 times stock with 19 volumes of glass distilled water as required. This 10 times stock solution will be pH 6.8–6.9 and will increase to pH 7.2 when working strength solution is prepared. It can be stored at 4°C for at least one year (watch for precipitation of buffer salts), or at room temperature for several months. Working strength solution should be monitored carefully for bacterial or fungal growth and discarded if any growth is noticed.

6.2.4.5. Conjugate diluent buffer

Affinity purified sheep antichicken IgG-horseradish peroxidase conjugate is diluted to the recommended dilution in PBS/Tween prepared as above. Although the optimal dilution of conjugate is usually supplied any new batch of reagents should be titrated using the positive and negative control sera.

Table 1. Composition of serum diluent buffer

	Concentration (mmol/L)	*10 times (g)*	*20 times (g)*
NaCl	1400	81.8	1636.6
$NaH_2PO_4.2H_2O$[1]	30	4.4	8.8
Na_2HPO_4—anhydrous[1]	70	10.2	20.4
Na_2EDTA	10	3.7	7.4
Tween 20[2]	0.05%	5.0	10.0

Make to 1 L with glass distilled water.

[1] The weight of sodium phosphate used per litre is dependent on the hydration of the salt, i.e. anhydrous, H_2O, $2H_2O$, etc. Check the salt you are using and compensate for any differences in the hydration.

[2] If Tween 20 is not included in the 10 times or 20 times stock it should be added at the rate of 0.5 g/L of working strength reagent.

6.2.4.6. Substrate solution

The recommended chromogen is ABTS. Final concentrations are 1.0 mmol/L ABTS and 2.5 mmol/L hydrogen peroxide in 50 mmol/L sodium citrate buffer pH 4.2. Stock solutions of the necessary components are prepared as below:

(a) ABTS (50 mmol/L) 27.5 mg/mL in water. This solution can be stored at 4°C for several months. The addition of a single granule of zinc metal or a small amount of zinc dust (laboratory reagent grade) to 10 mL of the ABTS stock will gradually remove the slight background green colour of the substrate without adversely affecting the activity.

(b) Hydrogen peroxide (125 mmol/L) (30% v/v) 12.1 µL/mL in water. This solution is stable for several days at room temperature. Alternatively urea peroxide adduct tablets may be used at the rate of one tablet (1.0 g) to 10 mL of glass distilled water. Use 15 µL per 10 mL of substrate solution prepared as below.

(c) Citrate buffer stock
Trisodium citrate.$2H_2O$, m.w. 294 (0.23 mol/L) 67.6 g
(0.27 mol/L)
Citric acid.H_2O, m.w. 210 (0.23 mol/L) 56.7 g
Make to 1 L with glass distilled water.
N.B. The weight of the citrate salts used per litre is dependent on the hydration of the salt, i.e. anhydrous, H_2O, $2H_2O$, etc. Check the salt you are using and compensate for any differences in the hydration.

(d) The final working strength of the substrate solution is prepared by mixing:
One volume ABTS stock (1 mmol/L final);
One volume hydrogen peroxide stock (2.5 mmol/L final);
Five volumes of citrate buffer stock (50 mmol/L final); and
Forty-three volumes of distilled water.

The working strength solution is stable for at least several days if kept in a sealed container.

6.2.5. Assessment of Results

The positive control series have been assigned ELISA unit values ranging from 512 for the 1:400 dilution to 16 for the 1:12 800 dilution and 0 for the 1:200 negative control serum. A computer program or hand produced graph is then used to compare the ODs of the test samples with the ODs of the standards and thus determine the relative ELISA units or titres. ELISA unit values <30 are usually associated with negative sera. The negative cut-off can be determined by running a large number of negative chicken sera in the ELISA.