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Avian Reticulo- endotheliosis

Virology and Serology

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1. Introduction

The prototype strain ('T') of reticuloendotheliosis virus (REV) was isolated in 1958 from an outbreak of leukosis among turkeys in the United States of America (Robinson and Twiehaus, 1974). Subsequently, numerous isolates of REV have been obtained, and detailed reviews of the literature on these viruses and REV disease are available (Purchase and Witter, 1975; Witter, 1991). Information relevant to the diagnosis of REV infection in poultry is outlined as follows. The REV group of viruses are C-type oncornaviruses which are morphologically, biochemically and antigenically distinct from avian leukosis/sarcoma viruses. Close antigenic relationships exist between all known strains of REV.

Natural REV disease is known to occur sporadically in turkeys and wild waterfowl and occasionally in other avian species (see 2.1.). It is not usually considered to be an economic problem in chickens and ducks maintained under commercial conditions, but REV contamination of commercial avian vaccines has produced widespread disease in Australia and Japan (Koyama *et al.*, 1976; Jackson *et al.*, 1977).

Pathogenicity and virulence of REV isolates vary greatly with strain and passage history. Both defective and non-defective (less pathogenic) strains of REV have been recognised (Witter, 1991).

Propagation of REV in cell culture may result in a transient and variable cytopathic effect. Additional methods are required to detect the presence of REV reliably. Strains of REV can readily be propagated in duck and chicken embryo fibroblast cell cultures.

Serological responses to REV develop slowly four to eight weeks after infection of young birds.

REV viraemia can occur after infection of embryos and neonatal birds (Ianconescu and Aharonovici, 1978; Bagust *et al.*, 1979). Oral, nasal and cloacal secretions and droppings from viraemic birds can be contagious.

Transmission of infection can occur horizontally, especially in young susceptible poultry housed in close contact. Egg-transmission of REV infection has been proven in both turkeys (McDougall *et al.*, 1978) and chickens (Bagust *et al.*, 1981) and mosquitoes may have a role in the mechanical transmission of REV (Motha *et al.*, 1984).

The maintenance host(s) of natural REV infection is unknown, although wild waterfowl and turkeys are suspected sources (Purchase and Witter, 1975).

In Australia, natural infections of ducks (Grimes and Purchase, 1973), chickens (Bagust and Dennett, 1977; Ratnamohan *et al.*, 1980) and turkeys (Rawlin, 1984) have been demonstrated. Although REV infection of quail and wild waterfowl is suspected, positive isolations have not yet been obtained.

2. Diagnosis

2.1. Disease and Pathology

In turkeys, ducks and quail, affected birds usually appear ill and die suddenly although persistent diarrhoea and leg weakness in turkeys has been observed (McDougall *et al.*, 1978). At autopsy, the liver, kidney, spleen and lymphoid tissues associated with the intestinal tract often appear enlarged and mottled. Diffuse or discrete REV-induced tumors occur most commonly in these organs, although other viscera may also be affected. Histopathological examination (H and E stain) of the neoplasms reveals that the predominant cell type is a large mononuclear lymphoreticular cell with abundant lightly basophilic cytoplasm and an enlarged vesicular nucleus containing prominent nucleoli. Necrosis may be present in the centre of some foci, especially in the liver and spleen. In addition, oedema, anaemia and enlargement of peripheral nerves due to mononuclear cell infiltration have been reported (Witter, 1991).

Young chickens in Australia which were inoculated with a commercial poultry vaccine contaminated with REV (Bagust *et al.*, 1979) developed runting, mild proventriculitis, and occasional nervous signs between two and three weeks of age (Jackson *et al.*, 1977). Additionally, the feathering abnormality of 'nakanuke' (Koyama *et al.*, 1976) was observed. In the field, proventriculitis and runting of chickens do not appear to be caused by REV alone, but in combination with other as yet undefined microbial or environmental influences. Experimental infection of day-old specified pathogen free (SPF) chickens with an Australian strain of REV produced a wide range of clinical, pathological and haematological disorders over 40 weeks (Grimes *et al.*, 1979). Inflammation in the viscera and nervous system, and hypoplasia of the organs of the immune system (bursa, thymus, spleen, caecal tonsil) were observed, as well as development of lymphoreticular-cell tumours of the liver, kidney or spleen in birds aged 22–24 weeks.

2.2. Isolation of Reticuloendotheliosis Virus

For diagnostic purposes, priority is given to detecting virus in plasma or serum, kidney and neoplastic tissues.

2.2.1. Collection of Samples

For detection of viraemia, 5–10 mL of whole blood is collected from the wing vein or by cardiac puncture and distributed equally between two bottles. One bottle contains an anticoagulant such as dipotassium ethylenediaminetetraacetate (CEDTA), at a final concentration of 2 mg/mL of blood, or heparin at 50 units/mL of blood. The other is without anticoagulant for serum. REV viraemia may be detected equally well in either the plasma or serum sample, serum

is also required for detection of antibody to REV. Collection of the buffy coat from the plasma sample and inoculation into cell culture may also allow the detection of the presence of other neoplastic avian viruses, e.g. Marek's disease.

The following organs may contain REV: kidney, liver, spleen, thymus, bursa or caecal tonsil, as well as any tumour tissue. Place at least 1 g of specimen in about twice the volume of Transport Medium (see 4.2.7.). Oral, nasal and cloacal swabs may be collected from live birds using sterile cotton wool swabs. Each swab is immediately broken off into a bottle containing Transport Medium.

Vaccine or virus samples may also require examination for contamination by REV. Testing of vaccine should be carried out on material from unopened containers as supplied by the manufacturer.

2.2.2. Transport and Storage

REV is highly thermolabile. Samples may be held and transported on wet ice only if processing and inoculation can be assured within 48 hours of collection. Alternatively, organ samples and swabs should immediately be frozen at -60°C. Plasma and serum samples are processed immediately to remove erythrocytes (see 2.3.) then similarly frozen at -60°C or below.

Samples including virus stocks for testing should only be thawed or vaccines reconstituted immediately prior to inoculation.

2.2.3. Preparation for Inoculation

2.2.3.1. Cell cultures

Eggs used for preparation of cell cultures should be obtained from a flock known to be free of REV infection. Chicken embryo fibroblast (CEF) cultures are prepared from embryos at 9–11 days of incubation by standard methods of trypsinisation. Sterile glass slides or coverslips are inserted in culture vessels (Petri dishes or tubes) prior to seeding with CEF cells in Growth Medium (see 4.2.3.). Cells which are dividing actively are required for the propagation of REV. Hence the seeding rate of the primary CEF cells is adjusted to contain sufficient cells (0.5×10^6 viable cells per mL of growth medium) to just produce a near-confluent monolayer at the time of fixation (see 2.2.5.). The freshly seeded vessels are incubated stationary at 37°C for one hour, to assist cell attachment prior to inoculation.

2.2.3.2. Treatment of specimens

Plasma is removed from the sedimented buffy coat and erythrocytes following centrifugation at 1000 g for 10 min. Serum is collected after extrusion from the clotted blood.

Organ suspensions (10% w/v) are produced by grinding the tissues using a pre-frozen pestle and mortar, and Hanks' Balanced Salt Solution (see 4.2.4.) containing 200 IU/mL of penicillin and 200 µg/mL of streptomycin as diluent.

Resulting suspensions are clarified by centrifugation at 1000 g for 10 min and the supernatant removed for inoculation.

Swabs obtained from the cloaca are thoroughly mixed with the Transport Medium in each bottle, preferably by using a mechanical bench agitator (touch-plate type for 30 s). The fluid is then removed from each bottle, clarified by centrifugation at 1000 g for 10 min and the supernatant taken for inoculation. If fungal contamination is suspected, a sample may be clarified further by using a Swinnex syringe adaptor membrane filter of 0.45 µm average pore diameter. Alternatively, fungistatic antibiotics may be incorporated in the cell culture medium.

Vaccines and virus samples must first be freed from viruses which produce a cytopathic effect in CEF cell cultures. The following method from the Therapeutic Goods Administration Laboratories (Viral Products Branch, PO Box 100, Woden ACT 2606, Australia) is suitable for use with commercial Marek's disease (herpesvirus of turkeys, HVT) (Calnek *et al.*, 1970) vaccine. The contents of a vial of reconstituted HVT vaccine is incubated at room temperature for 30 min with an equal volume of a dilution of a monospecific antiserum known to possess a titre of antibody sufficient to neutralise the infectivity of the HVT. The mixture is then inoculated into a small flask or other vessel of about 75 cm² surface area containing a sub-confluent monolayer of CEF cell cultures. After two days incubation at 40°C, supernatant fluid is harvested and clarified to remove any floating cells which may contain virus. To a 1 mL volume of clarified fluid an equal volume of the diluted HVT antiserum is added and the mixture incubated for 30 min. The procedure for inoculation of cell cultures (see 2.2.4.) is then followed.

2.2.4. Inoculation of Cell Cultures

A volume of 0.1 mL is inoculated into the growth medium of at least two replicate CEF cultures for each sample. Uninoculated CEF cultures are also maintained to provide negative controls. All cultures are incubated at 37°C or 40°C for about three days. The supernatant fluid from each group of cultures is then passaged into fresh CEF cultures using the above procedure. At least 1 mL of fluid from each sample is retained when passaging, and stored at -60°C should further examination be required. A total of three serial passages for each sample is performed as small amounts of REV infectivity may not be detectable in early passages.

2.2.5. Preparation for Fluorescent-Antibody Test

Immediately after passaging is completed, coverslips are removed from the culture vessels and washed in phosphate buffered saline pH 7.2 (PBS) (see 4.2.5.). Coverslips are fixed in acetone at 4°C for 10 min, air-dried and stored in an air

tight container at -20°C until required for testing. Control coverslips are similarly harvested from uninoculated CEF cultures.

2.2.6. Identification of Reticuloendotheliosis Virus

The indirect fluorescent antibody (IFA) test is used for staining coverslips. Preliminary screening for REV infection may be performed using coverslips harvested from the second passage of a sample. Controls should include a CEF coverslip known to show focal infection with REV (see 4.1.1.) and one which is negative for REV infection. For ease of handling, up to six coverslips may be held on a glass slide by spots of nail polish, with the cell culture side facing upwards. The surfaces of all the coverslips are divided into two equal areas using nail polish or a water proof marking pencil. If glass slides are used, mark out and subdivide an equivalent area on each slide for staining.

Dilutions of reference chicken antisera (negative or positive for REV antibody, see 4.1.2.) are placed in a separate area on each coverslip or slide. These are incubated at 37°C in a humidified chamber for 30 min, then washed at least three times with PBS before draining. Each coverslip is then flooded with a standardised appropriate dilution of commercial fluorescein-conjugated rabbit antichickens IgG and incubated in a humidified chamber for 30 min at 37°C. Preparations are then washed as described previously, air-dried and mounted with the culture facing downwards on a clean glass slide. The mounting medium consists of nine parts of analytical reagent (AR) grade glycerol and one part of veronal buffer pH 8.6 (see 4.2.6.). Coverslips are examined for specific fluorescence by ultra-violet (UV) microscopy using transmitted light and dark field illumination. Rapid and accurate scanning of each stained area may be performed at a total magnification of about 100.

2.2.7. Interpretation of Results

2.2.7.1. Control coverslips

2.2.7.1.1. Uninfected. Positive and negative sera should not show fluorescence.

2.2.7.1.2. Infected.

- (a) Negative sera — should not show fluorescence.
- (b) Positive sera — focal areas of cells showing bright cytoplasmic fluorescence characteristic of REV (Purchase and Witter, 1975; Bagust *et al.*, 1981). Apple-green foci are clearly visible against a darker background of cells which are stained light-green but do not fluoresce.

2.2.7.2. Test coverslips

The square stained with antibody-positive serum is examined first.

- (a) If there is no fluorescence, the specimen is negative for REV infection.

- (b) If there is fluorescence for REV, examine the area of this coverslip that was stained with the antibody-negative serum. If fluorescence is absent in the latter field, the specimen is positive for REV infection.
- (c) If REV fluorescence is suspected but difficult to discriminate from the background staining, then the test result is equivocal. This material requires to be repassaged from the stored duplicate at serial dilutions from undiluted to 10⁻³. The criteria of (b) must be satisfied for a positive result. Overstaining of background may result from inadequate washing of coverslips or using inappropriate dilutions of reagent sera.

2.2.8. Chicken Inoculation Test

Although somewhat superseded by isolation of REV in cell culture, inoculation of day-old chickens can be a useful adjunct in diagnosis of REV infection. It can be readily carried out as part of a transmission trial for a suspected infectious aetiology.

2.2.8.1. Procedure

A group of about 20 SPF chickens are each injected by either the intraperitoneal route or subcutaneously with 0.2 mL of inoculum. A control group of SPF chickens is inoculated with saline and maintained separately. Both groups are inspected daily for signs of illness, ruffling and development of feathering defects ('nakanuke'). Abnormalities in feathering are most pronounced two to four weeks after inoculation. Histopathological examination of organs of some of the chickens at this stage may aid diagnosis. All birds are bled at four and eight weeks of age from the wing vein to provide at least 0.5 mL of clean serum at each bleeding. These are frozen at -20°C to test for the presence of antibody to REV (see 2.3.).

2.2.9. Enzyme-linked immunosorbent assay

An antigen detection enzyme-linked immunosorbent assay (ELISA) can be used to assay for the presence of REV in a variety of samples including field samples. ELISA is less sensitive than culture assay; however, samples obtained from turkey and chicken flocks infected with REV are usually positive in an antigen detection ELISA (Ignjatovic *et al.*, 1987; Witter and Salter, 1989). Samples such as serum, vaginal/cloacal swab and egg albumen are all suitable for detection of infection; however some serum samples from non-infected birds might give 'false positive' reactions in ELISA. Egg albumen contains the highest amount of REV antigen and is the sample of choice, particularly for flock screening and identification of shedding and transmitting hens. The reagents for ELISA are available from the CSIRO Division of Animal Health, Parkville, Vic. 3052, Australia.

Rabbit anti-p30 IgG is diluted in 0.05 mol/L carbonate-bicarbonate buffer pH 9.6 to 5 µg/mL and 0.2 mL added to the wells of polystyrene microplates (Disposable Products Pty Ltd, 810 Princes Highway, Springvale, Vic. 3171, Australia). The plates are incubated for four hours at 37°C, or overnight at 4°C, and stored at 4°C until required. Test plates are washed three times with washing buffer (PBS containing 0.1% Tween 20) and 0.2 mL of test samples added to the wells in duplicate. After one hour at room temperature the samples are removed and plates washed three times with washing buffer. Rabbit anti-p30 IgG-horseradish peroxidase (HRP) conjugate is diluted to 1:1600 (in washing buffer supplemented with 0.5% bovine serum albumin) and 0.2 mL added to each well. The plates are incubated for one hour, washed three times and 0.2 mL of substrate (5-amino-2 hydroxybenzoic acid at a concentration of 1 mg/mL in phosphate buffer, pH 5.95) added to each well. Prior to use, 0.2 mL of freshly prepared 0.35% hydrogen peroxide (H₂O₂) is added to each 10 mL of substrate solution. After one hour the absorbance is measured at 450 nm (A₄₅₀) using an automatic microplate reader (Multiscan, ICN Biomedicals Australasia, 31 Seven Hills Rd, North Seven Hills, NSW 2147, Australia). Samples are considered positive if the mean of A₄₅₀ exceed 0.15 (signal to noise ratio greater than 2:1)

2.3. Serological Diagnosis of Reticuloendotheliosis Virus Infection

2.3.1. Indirect Fluorescent Antibody Test
CEF coverslips or slides, which are known to be infected with REV (see 4.1.1.), are stained with the sera under test for antibody to REV. Test sera are diluted 1:20 in PBS and inactivated at 56°C for 30 min. If large numbers of sera are to be examined, pools may be prepared from up to five separate birds and the final dilution of each serum adjusted to 1:20 with diluent. Pre-centrifugation of the diluted sera at 1000 g for five minutes is sometimes necessary to remove floccules which can obscure staining reactions in the IFA test. The REV-positive coverslips or slides preferably containing focal REV fluorescence (see 4.1.1.) are each marked into areas of about 1 cm² and separate squares are used to test each serum or pooled serum sample. The procedure for the IFA test, including the system of controls for reagents previously detailed (see 2.2.6.), is then followed.

2.3.1.1. Interpretation of results

2.3.1.1.1. Controls. As in 2.2.6.

2.3.1.1.2. Test sera.

- (a) If there is no fluorescence, the serum sample is negative for REV antibody.
- (b) If there is moderate to brilliant focal fluorescence for REV, contrasting with the dark background of the uninfected cells of the CEF monolayer, the serum sample is positive

for REV antibody. However, if characteristic REV cytoplasmic fluorescence is produced by a serum with a monolayer containing a generalised (non-focal) REV infection, the specificity of the serum reaction must be confirmed by the absence of fluorescence when restaining using CEF monolayers known to be free of REV infection.

- (c) If a weak fluorescent reaction occurs at a serum dilution of 1:20, the test result is equivocal. If a similar result occurs on retesting, this serum sample is conservatively scored as negative (*N.B.* below).
- (d) If non-specific (background) fluorescence occurs to levels which would mask the presence of specific REV fluorescence, then the test serum requires to be absorbed with a preparation of uninfected SPF CEF cell cultures until the non-specific activity is removed.

N.B. Individual chickens which develop a persistent REV viraemia may fail to produce detectable antibody to REV. At least 25 sera from each group of birds housed together should be tested to determine if REV is present. Positive IFA reactions to REV infection by field chickens usually occur within a serum dilution range of 1/20–1/320 and can persist for several months after infection (Bagust and Grimes, 1979).

2.3.2. Agar Gel Precipitin Test

The Agar Gel Precipitin (AGP) test can be used for detection of either antibody to REV or REV antigen in serum and plasma samples (Ianculescu and Aharonovici, 1978). Although generally less sensitive for antibody detection than the IFA test, AGP is a relatively simple and useful test for screening flocks for REV infection. The AGP test, but not the IFA system, can also detect a non-infectious (immune complex formation) phase of REV viraemia which may occur as systemic humoral antibody develops following REV infection of chickens (Bagust and Grimes, 1979).

The AGP test for REV antibody or antigen is performed using reagents (REV AGP agar, antigen and reference sera) prepared as described in 4.2. Sera are tested undiluted after inactivation at 56°C for 30 min. Wells are punched in the agar gel using a template. A hexagonal arrangement of a set of wells of diameter 3 mm equidistant from a central well, with a centre-to-centre distance of 7 mm, has proved useful for diagnostic screening purposes.

Sera to be tested are placed in the peripheral wells with a positive antibody reference serum interspersed at regular intervals among the test sera. For detection of antibody, REV AGP antigen is then placed in the central well of each set. For detection of antigen in the sera under test, a reference serum known to be capable of precipitin formation with REV antigen is placed in the central well of each set. Reagent controls for

tests carried out on any one day should include appropriate reactions between REV positive and negative cell culture antigens and the reference antisera.

The gel plates are incubated in a humidified container at 37°C for 24 hours and examined using a darkground or indirect illumination. Incubating is then continued to 48 hours when a final reading is performed.

2.3.2.1. Interpretation of results

2.3.2.1.1. Control reagents. Precipitin reactions between the various reagent preparations must be appropriate or the test is not valid. One clear precipitating line usually forms between REV antigen and antibody, although a second more diffuse region of precipitation may occur with strongly reacting sera.

2.3.2.1.2. Test sera

- In the absence of precipitin reactions, the test serum is negative.
- When a positive reaction occurs, the sample should be retested and confirmed for specificity by reacting with the appropriate REV reagent preparations.

N.B. Occasional spurious precipitin reactions can occur between fowl sera of different blood group (iso-antigen) types. These reactions are considerably more intense than those obtained with the REV AGP system.

2.3.3. Serum Neutralisation Test

The Serum Neutralisation (SN) test can be used with those strains of REV which are known to produce a cytopathogenic effect, or plaques, in cell culture. The sensitivity of the SN test for detection of antibody to REV is similar to that of the more widely used IFA test. However, if required, the SN test can also detect the minor antigenic differences that have been reported to occur among isolates of REV (Purchase and Witter, 1975).

In Australia and other countries a SN test has been developed which utilises a cytopathogenic strain of REV propagated in duck embryonic fibroblast (DEF) cell cultures (Sinkovic and Choi, 1978). In outline, sera to be screened for SN antibody are diluted 1:20 and inactivated at 56°C for 30 min. A stock of REV of known titre of infectivity is diluted to contain between 10^3 and 10^4 cell culture infective doses per mL. Equal volumes of diluted serum and virus are mixed and incubated at 37°C for 30 min. The infectivity of each virus-serum mixture is then assessed by inoculating 25 μ L onto microculture plates of DEF cell cultures. After incubation at 37°C for two to three days, the test is read by examining the cultures with an inverted microscope. REV infection produces microplaques of focal granulated and rounded refractile cells, and an increased number of detached cells.

2.3.3.1. Interpretation of results

- If there is no inhibition of REV cytopathic effect, the serum is negative for REV antibody.
- If marked inhibition of REV cytopathic effect occurs at a dilution of 1:20 or higher, the serum is positive for REV antibody.
- If only slight inhibition of REV cytopathic effect occurs at a serum dilution of 1:20, the test result is equivocal. If a similar result is obtained on a repeat test, the sample is negative for antibody to REV.

Alternative methods of assay of neutralising antibody using the IFA test and macroplaque systems have been used in countries other than Australia (Purchase and Witter, 1975; Witter, 1980) but do not appear to offer additional advantages.

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4. Appendixes

4.1. Appendix 1 — Methods for Preparation of Reticuloendotheliosis Virus Serological Reagents

4.1.1. Antigens

4.1.1.1. Agar gel precipitin test antigen

- (a) Prepare primary CEF cultures and seed 6×10^6 cells in Growth Medium (see 4.2.3.) into 90 mm plastic Petri dishes. Incubate the dishes at 37°C for one to two hours in a 5% (v/v) carbon dioxide in air atmosphere.
- (b) Inoculate REV into supernatant fluid of most of the cultures and continue the incubation for two to three days. Uninoculated cultures are handled and processed similarly to infected cultures, to prepare a negative cell culture control antigen.
- (c) When monolayers are confluent, harvest the cells with trypsin-versene. Reseed cells in fresh Growth Medium into three secondary Petri dishes for each primary Petri dish. Add two glass coverslips (22 x 11 mm) to the cells in each of an uninfected and infected new Petri dish. Incubate these secondary CEF cultures for three to four days.
- (d) Remove the coverslips, stain and examine by IFA procedure to assess the extent of REV infection of monolayer.
- (e) Harvest the supernatant fluids for concentration if 90% or more of the cells in the

infected monolayers show REV fluorescence. If the extent of REV infection is insufficient, repeat steps (c) and (d), then harvest supernatant fluids.

- (f) Supernatant fluids are placed in Visking dialysis tubing. Evaporate in front of a fan until their volume is reduced to about one-tenth of the original.
- (g) Re-tie tubing to concentrate medium. Dialyse against PBS to remove the deep red colour of the phenol red indicator.
- (h) Antigens may be stored at 4°C for three months or -20°C for longer. These should be dispensed in small aliquots for storing at -20°C to avoid repeated thawing and freezing.

4.1.1.2. IFA test antigen

- (a) Place sterile glass coverslips or slides in plastic Petri dishes of 90 mm diameter.
- (b) Each Petri dish is seeded with 6×10^6 primary CEF in 12 mL of Growth Medium (see 4.2.3.). Stationary incubation at 37°C for one day in a 5% (v/v) carbon dioxide in air atmosphere. About 75% coverage of surface area of Petri dish should occur during this period.
- (c) Control uninfected dishes are inoculated with 1 mL of Growth Medium. Inoculate 1 mL containing 10^4 focus-forming units of a known strain of REV into the Growth Medium of the remainder of the Petri dish cultures. Disperse the inoculum by gentle agitation of the dishes and continue incubation at 37°C for one day.
N.B. If wishing to produce discrete foci of REV fluorescence [d(ii)], the required dilution of the stock virus preparation is made in Growth Medium prior to inoculation.
- (d) Remove the supernatant fluid from the cell cultures and replace as follows:
 - (i) For generalised REV fluorescence, add 12 mL of fresh Growth Medium per dish and incubate at 37°C for a further two days.
 - (ii) For focal REV fluorescence, prepare and add 12 mL of Agarose Overlay (see 4.2.2.) per dish and incubate similarly.
- (e) Fix both control and infected CEF coverslips or slides as noted in 2.2.6.
- (f) Stain and examine for REV fluorescence by the IFA test procedures detailed in 2.2.6.

4.1.2. Antiserum (negative, positive for REV antibody)

- (a) A minimum of 9-10 chickens are obtained from a source flock known to be free of REV infection. Wings are tagged for identification of individual birds.
- (b) Each chicken is bled from the wing vein at three to six weeks of age and just prior to inoculation with REV. Serum from this bleeding should be negative in IFA, AGP or SN tests for REV antibody.

- (c) Inoculate each chicken intraperitoneally or intramuscularly (I/P or I/M) with $>10^3$ fluorescent focus forming units of a known strain of REV in a volume of 0.5 mL. The suspending medium for inoculation should be as free of serum as possible.
- (d) Hold inoculated chickens in isolation for up to six weeks as required. The strongest serological responses usually occur between three and five weeks post inoculation. Clinical signs of infection are unusual in chickens infected at this age.
- (e) Bleed all chickens from the wing vein at two weeks and again at three weeks after inoculation. Carry out serological screening for early development of antibody to REV by each chicken using serological systems of choice (see 2.3.).
- (f) Bleed and test at subsequent intervals of one week. When the antibody response is satisfactory, i.e. IFA or SN titres of ≥ 1 in 320 and intense precipitin formation in the AGP test, individual chickens are exsanguinated by cardiac puncture.
- (g) Process the serum, dispense into small aliquots and store undiluted at -20°C or colder. Thaw an aliquot as required and dilute with PBS to reach the working range. Diluted sera may be stored at 4°C for up to three months.

4.2. Appendix 2 — Laboratory Solutions and Media

4.2.1. Agar for AGP Test

- Ionagar No. 2 (Oxoid) 10.0 g
 - (or other purified agar) 80.0 g
 - Sodium chloride, NaCl 80.0 g
 - Potassium dihydrogen phosphate, KH_2PO_4 , 0.066 mol/L 29.6 mL
 - Disodium hydrogen phosphate, Na_2HPO_4 , 0.66 mol/L 70.4 mL
 - Sodium azide, NaN_3 0.1 g
 - Add distilled water to 1 L
- Mix while boiling, dispense into 50 mL volumes and autoclave at 10 psi (70 kPa) for 30 min. When cool, store at room temperature.

For Use: Boil again thoroughly to liquify agar and dispense onto clean glass slides.

4.2.2. Agarose Overlay for Cell Cultures

4.2.2.1. Part A: Agarose 1% (w/v) gel

- Agarose (Miles Laboratories) 10.0 g
 - Distilled water 1 L
- Mix while boiling, dispense into 50 mL volumes and autoclave at 10 psi (70 kPa) for 30 min. When cool, store at room temperature.

4.2.2.2. Part B: Overlay medium (2x)

- Medium 199 - 10 x (CSL) 140 mL
- Tryptose phosphate broth (Difco) 5% (w/v) 175 mL
- Calf serum (inactivated) 87.5 mL

- Sodium bicarbonate, NaHCO_3 , 5.6% (w/v) 17.5 mL
- Add sterile distilled water to 1 L

Dispense in 50 mL volumes and store at 4°C .
For Use: Boil 50 mL of Agarose Gel (Part A) until completely liquid, then place the container in a water bath at 46°C for 20 min. Warm 50 mL of 2x overlay medium (Part B) to 46°C for 20 min. Mix equal volumes of Parts A and B and allow this single-strength mixture to stand at 46°C for 10 min. Gently pipette the Overlay onto the cell cultures.

4.2.3. Growth Medium for Cell Cultures

- Medium 199 (CSL) 90 mL
- Tryptose phosphate broth (Difco) 5% (w/v) 5 mL
- Calf serum (inactivated, 56°C for 30 min) 5 mL

4.2.4. Hank's Balanced Salt Solution

- Sodium chloride, NaCl 8.00 g
 - Potassium chloride, KCl 0.40 g
 - Calcium chloride, CaCl_2 0.14 g
 - Disodium hydrogen phosphate, Na_2HPO_4 0.06 g
 - Potassium dihydrogen phosphate, KH_2PO_4 0.06 g
 - Magnesium sulfate, MgSO_4 0.2 g
 - Glucose 1.0 g
 - Phenol red 0.02 g
 - Add distilled water to 1 L
- Sterilise by filtration and dispense in volumes of 100 mL. Store at room temperature.

4.2.5. Phosphate Buffered Saline pH 7.2

4.2.5.1. Method 1

- (a) Solution A
- NaCl 8.50 g
- Na_2HPO_4 9.47 g
- Add distilled water to 1 L

(b) Solution B

- NaCl 8.50 g
- KH_2PO_4 9.07 g
- Add distilled water to 1 L

For Use: Mix 720 mL of (a) and 280 mL of (b).

Check the pH and adjust as necessary. If required, sterilise by autoclaving at 15 psi (105 kPa) for 30 min.

4.2.5.2. Method 2

Use commercially available PBS (Dulbecco 'A') tablets (*Oxoid Code BR14a*).

Dissolve 10 tablets in 1 L of distilled water, and add 5 mL of calcium/magnesium stock solution (CaCl_2 , 0.1 g; MgCl_2 , 0.1 g; distilled water to 5 mL).

4.2.6. Transport Medium (SPGA-EDTA, as Calnek *et al.*, 1970)

Sucrose, $C_{12}H_{22}O_{11}$	7.46 g
KH_2PO_4	0.05 g
K_2HPO_4	0.13 g
Monosodium glutamate	0.09 kg
Bovine albumen (Fraction V)	1.0 g
EDTA	0.2 g

Sterilise by filtration. Dispense and store at room temperature.

4.2.6. Veronal Buffered Glycerol (pH 8.6)

Glycerol (analytical reagent grade)	9 parts
Sodium barbitone, $C_8H_{11}N_2NaO_3$,	
0.05 mol/L	
(1.03 g to 100 mL distilled water)	1 part

Adjust pH to 8.6 with 0.1 mol/L hydrochloric acid (HCl), stirring constantly.