AUSTRALIAN STANDARD DIAGNOSTIC TECHNIQUES FOR ANIMAL DISEASES

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Rotavirus Infection

Virology and Serology

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1. Introduction

The role of rotavirus (reovirus-like agent) as a primary cause of diarrhoea in neonates has been established. The virus causes disease worldwide in newborn calves, pigs, foals, mice, lambs, humans and many other domestic and wild mammals, and in birds such as turkeys.

The incubation period in experimental animals is one to four days. The clinical disease is characterised by depression, followed by severe diarrhoea, vomiting (pigs), dehydration, emaciation and sometimes death. Infection is confined to small intestinal mature epithelial lining cells containing essential digestive enzymes. Destruction of these cells results in altered function and structure of the intestinal villi resulting in diarrhoea. Specific antiviral antibodies normally appear in serum within four to seven days after the appearance of clinical disease, and possibly even sooner in the gut. Rotavirus serotypes depend on variations in two surface proteins, VP4 and VP7. VP4 shows more host specificity, but VP7 (or G, since VP7 is a glycoprotein) serotypes appear to be most important and immunity is G-serotype specific. In Australia (and internationally) pigs are infected by group A rotaviruses of serotypes G3, G4 and G5 (Nagestha and Holmes, 1988; Huang *et al.*, 1989). Bovine serotypes are G6, G8 (rare) and G10 (Snodgrass *et al.*, 1990). Horses, sheep, goats, rabbits, mice, monkeys, cats and dogs, as well as humans, have type G3 rotaviruses, but humans also suffer from types G1, G2, G4, and rarely G8, G9 and G12. Thus particular G serotypes can be found in various hosts, and interspecies transmission is experimentally demonstrable but is probably fairly uncommon in nature. Because rotaviruses have a segmented genome, reassortants can be generated readily both naturally and in the laboratory, and are very useful for more detailed serological studies.

Some of the difficulties associated with the diagnosis and study of rotavirus infection include the following.

(a) Infections are generally acute with transient viral excretion; so diagnosis may be missed unless samples are collected soon after appearance of symptoms.
(b) Infections range over a spectrum from asymptomatic to those resulting in acute diarrhoea and dehydration; due to variations in dose and maternal immunity transferred passively by colostrum and milk.
(c) Presence of maternal antibodies sometimes confuses the interpretation of serological results.
(d) Rotavirus infections are widespread in both mammals and birds; hyperimmune sera for certain serological tests must be produced in gnotobiotic or specific pathogen free animals, or affinity purified.

2. Demonstration of Presence of Rotavirus in Clinically Infected Animals

Demonstration of virus particles in faeces of animals with diarrhoea was historically the most reliable method of diagnosing rotavirus infections. More sensitive and discriminating methods based on detection of viral group antigens or nucleic acids have now largely superseded electron microscopy. Nevertheless electron microscopy remains valuable for research in the diagnosis of enteric viral infections due to group A rotaviruses, the subject of this document, and in addition those caused by rotaviruses of other serogroups (B–F), coronaviruses, adenoviruses, caliciviruses and astroviruses.

The presence of group A rotaviruses in faeces of animals with diarrhoea may be demonstrated by one or all of the following techniques:

(a) enzyme-linked immunosorbent assay (ELISA);
(b) immunofluorescence (IF);
(c) latex agglutination;
(d) electron microscopy (EM); or
(e) polyacrylamide gel electrophoresis (PAGE) of viral RNA.

2.1. Collection and Storage of Specimens

Faecal samples or gut contents should be fresh and collected as close to the onset of diarrhoea as possible. Samples should be chilled during transport to the laboratory. Faeces that are not processed immediately should be stored at 4°C for short periods (weeks) or frozen, preferably below -40°C, for long term storage.

2.2. Preparation of Faecal Material for Examination

Faeces are suspended initially at 1:5–1:20 (v/v), (depending on consistency) in phosphate buffered saline (PBS) and homogenised. The homogenate is then clarified at 800 *g* for 15 min and the supernatant used for ELISA or latex agglutination. A further clarification by centrifugation at 1200 *g* for 30 min is beneficial before pelleting virus for EM or filtering virus prior to infecting cell cultures for IF diagnosis or cell culture adaptation.

Rotaviral RNA for diagnosis by PAGE or group or serotype determination by hybridisation can simply be extracted directly from the faecal sample.

Quantities are based on the use of Eppendorf tubes (capacity 1.5 mL). Faecal samples (50–100 mg) are weighed into tubes. To each is added 0.5 mL of 0.1 mol/L sodium acetate (CH_3CO_2Na) pH 5 containing 1% sodium dodecyl sulfate (SDS, $C_{12}H_{25}O_4SNa$), and 0.5 mL of 'phenol (C_6H_5OH)– chloroform ($CHCl_3$)' mixture. 'Phenol' is a mixture of 250 g redistilled phenol, 35 g m-cresol ($CH_3C_6H_4OH$) and a solution of

0.25 g 8-hydroxyquinoline in 100 mL water. 'Chloroform' is 24 parts chloroform plus one part isoamylalcohol [$(CH_3)_2CHCH_2CH_2OH$]. The final mixture is three volumes of 'phenol' plus two volumes of 'chloroform', aliquots of which are stored at -20°C until required.

Ensure that tube caps are well sealed and wear gloves since phenol and cresol burn skin. The tubes are Vortex mixed vigorously for one minute, then centrifuged for two minutes at 9980 *g*. The supernatant is removed to another labelled tube — this is the RNA preparation.

2.3. Enzyme-linked Immunosorbent Assay

Many commercial kits for detection of rotaviruses by enzyme immunoassay are available. Both overseas (Flewett *et al.*, 1989) and local experts (Kok and Burrell, 1990) recommend the Dakopatts ELISA rotavirus kit (Bio Scientific). Since the test depends on detection of the common (group A rotavirus) antigen, it is equally applicable to rotavirus diagnosis in animals, birds or people.

Some reagents and solutions can be prepared locally and the most critical components (rotavirus antibody-negative rabbit serum and peroxidase-conjugated rabbit antihuman rotavirus serum) obtained as separate items from Dako. Those wishing to understand more of the rationale should consult Beards *et al.* (1984), but a satisfactory procedure is described below.

(a) Polystyrene flat-bottomed microtitre plates irradiated (for sensitivity, not sterility), such as Nunc Immunoplates (Medos) are coated with rabbit hyperimmune antirotavirus serum (see 3.2.) diluted 1/1000 in 0.1 mol/L sodium carbonate (Na_2CO_3)/sodium bicarbonate ($NaHCO_3$) buffer pH 9.6. Add 50 µL/well and incubate covered plates for 90 min at 37°C or overnight at 4°C. If desired, alternate (control) rows may be coated with the same dilution of Dako X904 rotavirus negative serum.

(b) Wash plates in PBS pH 7.4 containing 0.05% Tween 20 (PBS-Tween) by dipping in a bath of buffer then flicking liquid out of wells, five times, or use an automatic plate washer.

(c) Dilute 10% faecal suspension 1:4 in PBS-Tween plus 2.5% skim milk powder (e.g. carnation instant skim milk powder or equivalent) and 10 mmol/L disodium ethylene diamine tetraacetic acid (EDTA, $C_{10}H_{14}N_2O_8Na_2.2H_20$). Add 50 µL/well in duplicate, incubate for two hours at 37°C. Wash five times as above.

(d) Add 50 µL/well of Dako P219 rabbit antirotavirus peroxidase conjugate, diluted 1/200 in PBS-Tween plus 2.5% skim milk. Incubate for one hour at 37°C. Wash as above.

(e) Just before use, dissolve 0.5 mg/mL ABTS [2,2'-azino-di-(3-ethyl)-benzthiazoline sulfonic acid (Sigma)] in 0.1 mol/L citrate–phosphate buffer pH 4.0 (10 mL needed per plate). Add hydrogen peroxide (H_2O_2) to final 0.003% (10^{-4} dilution of 30%) and mix well. Add 100 µL/well and incubate 30 min at room temperature. Stop reaction with 50 µL/well of sodium fluoride (NaF) solution (1.92 mg/mL in distilled water). Read by eye or in ELISA reader (max. absorbance 405 nm, min. absorbence 492 nm).

N.B. The rows containing rotavirus-negative rabbit serum are to detect false positives. If none or very few are being encountered, this precaution can be omitted. For a positive control, use a known positive faecal sample or cell culture virus (see 3.1.). For a conjugate control, omit antigen, use antigen diluent only.

2.4. Immunofluorescence Technique

2.4.1. Preparation of Material

2.4.1.1. Cell culture (infectivity assay)

For rotavirus cultivation the monkey kidney continuous cell line MA104 is recommended, but other lines such as CV-1, MDBK, BSC-1, or primary porcine or bovine kidney cells can also be used. Rotaviruses in filtered (0.22 µm membrane) faecal extracts are activated by trypsin (Sigma) at final concentration of 10 µg/mL. Incubate 20 min at 37°C, then add foetal bovine serum to final 2%. Aspirate growth medium off cells, rinse with maintenance medium (e.g. Eagles minimum essential medium with no serum). For cell monolayers on coverslips in tubes, inoculate 0.2 mL, or 0.05 mL per well of microtitre plates. Adsorb for one hour at 37°C, remove inocula, replace maintenance medium, incubate 16–24 hours at 37°C in a carbon dioxide (CO_2) incubator. For assay, use four-fold dilutions of virus in maintenance medium.

2.4.1.2. Faecal smears

Faecal material or gut content from an infected animal is thinly spread on a microscope slide and air dried.

2.4.1.3. Intestinal section

Segments for immunofluorescent staining are filled with an embedding medium for frozen tissue specimens and frozen with liquid nitrogen. Pieces of intestine are cut from the frozen segments and mounted on microtome chucks. Sections about 5 µm thick are cut on a freezing microtome, mounted on glass slides and air dried.

2.4.2. Staining

For coverslips, faecal smears and tissue sections (on glass) the following procedure should be carried out: wash off medium with PBS if necessary; fix in cold acetone (CH_3COCH_3)(2°C) for 10 min, then air dry. Wash twice with PBS pH 7.2, five minutes each. Flood with hyperimmune rabbit antirotavirus serum diluted (usually 1/500–1/2000) in PBS. Incubate in a humid box

for 30 min at 37°C. Wash three times with PBS. Flood with fluorescent sheep antirabbit immunoglobulin (e.g. code RF, Silenus Laboratories) at optimal dilution (about 1/200 in PBS) and incubate in humid box 30 min at 37°C. Wash three times for five minutes each with PBS. Mount in glycerol/PBS 9:1, pH 8 and examine under an ultraviolet (UV) microscope with appropriate filters.

Microtitre plates must be fixed with 80% acetone, as they lose their transparency if exposed to 100%. *N.B. Gently* remove medium, rinse once with PBS, and aspirate excess. Add one drop of PBS per well, then four drops of acetone. Alternatively, predilute the acetone to 80%.

2.4.3. Interpretation and Comments

For adaptation to cell culture, most animal rotaviruses require a few passages with trypsin activation of inocula (see 2.4.1.1.), and serum-free maintenance medium containing 1 µg/mL of trypsin. However, even those difficult to adapt will infect cells following primary inoculation. Trypsin activation is essential, and serum is avoided because mammalian sera contain trypsin inhibitors. Infected cells show discrete, intracytoplasmic inclusions by IF.

A faecal smear is considered positive when it contains numerous brightly fluorescing cells. Smears containing an occasional cell with low-intensity fluorescence against a background of non-fluorescing cells are considered negative.

Excessive background fluorescence can result from using conjugate that is not sufficiently diluted, or from smears that are too thick. Non-specific immunofluorescent debris is often orange rather than the expected yellow–green. Non-specific fluorescence can be detected by preparing two faecal smears from the same specimen, staining one with the proper antiserum and the other with antiserum to an unrelated virus.

For this application, it does not matter if the anti-immunoglobulin conjugate also contains antirotavirus antibodies: sheep sera usually do.

2.5. Examination by Electron Microscopy

A portion of the faecal extract supernatant is pelleted by ultracentrifugation (120 000 *g* for 90 min) and the pellet resuspended in a few drops of distilled water. The sample is mounted on a carbon coated EM grid and negatively stained with either 2% phosphotungstic acid ($H_3PO_4.12WO_3$) pH 5.0 or 10% saturated ammonium molybdate [$(NH_4)_2MoO_4$]. If excessive debris is present, ultracentrifuge the sample through a cushion of 20% sucrose. This can be done in a Beckman airfuge. It is then important to wash specimen on the grid to remove sucrose, before negative staining. Their regular size, icosahedral symmetry and characteristic appearance make rotaviruses relatively easy to identify by EM even in presence of much debris.

Rotavirus particles have a diameter of 65–75 nm. In faecal samples, they may have either a double or single shell. It has been estimated that about 10^6 virus particles per gram of faeces must be present before they can be reliably detected by EM.

2.6. Animal Inoculation

The inoculation of susceptible animals with material suspected of containing rotavirus is a relatively sensitive method. However, it is not practical for routine diagnosis as it requires the use of animals, maintained under germ-free conditions. The ubiquity of rotaviruses and their persistence in previously contaminated environments make experiments on unprotected animals uninterpretable.

2.7. Diagnosis by Polyacrylamide Gel Electrophoresis of Rotaviral RNA

Originally developed as a research tool, this technique is slightly less sensitive and more labour-intensive than ELISA, but is often cheaper and gives much more epidemiological information. It is the most practical method for distinguishing different strains of rotavirus. It is also the best method for routine checking of the identity of standard cultivable rotaviruses and absence of cross contamination. It can also be used to determine whether outbreaks or sporadic infections in particular areas are due to single or multiple strains. Rotaviral genomic RNA is double stranded and thus resistant to degradation. Sequence variations result in minor variations of mobility of the 11 genome segments, resulting in an almost infinite variety of recognisable patterns or 'electropherotypes' following electrophoresis and silver staining (Herring *et al.*, 1982).

2.7.1. RNA Extraction

RNA is prepared from faeces as described in 2.2.

2.7.2. Electrophoresis

For electrophoresis, the discontinuous buffer system is based on that of Laemmli (Herring *et al.*, 1982) but SDS is omitted since it would interfere with silver staining. Stock solutions of acrylamide (C_3H_5NO)[30% plus 0.8% methylene bisacrylamide ($C_7H_{10}N_2O_2$) in distilled water], 'Lower Tris' (four times) buffer (1.5 mol/L Tris HCl, pH 8.8), 'Upper Tris' (four times) (0.5 mol/L Tris HCl pH 6.8), Tris–glycine reservoir buffer (four times) (12 g Tris base + 57.6 g glycine/L) and sample buffer [Upper Tris (four times) 7.5 mL, glycerol 2.5 mL, bromophenol blue 10 mg] are prepared in advance and stored at 4°C.

N.B. Unpolymerised acrylamide is toxic: breathing powder or skin contact with solutions should be avoided — wear gloves. Ammonium persulfate [$(NH_4)_2S_2O_8$] (20 mg/mL) is unstable and must be made up fresh, immediately before use.

For a Bio Rad or similar electrophoresis apparatus, slabs 14 x 16 cm long and 0.75 mm

thick, the lower gel (final 10% acrylamide) requires 6.65 mL of acrylamide stock, 5 mL of four times Lower Tris, and 8.05 mL water. After mixing and degassing, 5 µL TEMED (N,N,N',N'-tetramethylethylenediamine, $C_6H_{16}N_2$) and 0.3 mL ammonium persulfate are added and the gel is poured or pipetted into the mould. The lower gel is overlaid with water-saturated n-butanol to produce a flat interface. When the acrylamide has set, the butanol is removed and rinsed off with Lower Tris buffer.

The upper (stacking) 3% gel is made from 0.5 mL acrylamide stock, 1.25 mL Upper Tris (four times) and 3.1 mL water, then 5 µL TEMED and 150 µL ammonium persulfate. The stacking gel is pipetted into the mould with the well-forming comb in place. A 15-well comb is ideal (tooth width 5.5 mm) — wells hold 50 µL.

For routine tests, 20 µL of RNA sample mixed with 10 µL of sample buffer are loaded in each well. For an 0.75 mm thick gel, an overnight (16 hour) run at room temperature at constant current 8–10 mA per gel is convenient and satisfactory. The voltage starts at about 60 V and increases to about 180 V by the end of the run.

2.7.3. Silver Staining

Gloves must be used when handling gels or fingerprints will stain. The gel is removed from the glass 'sandwich' and placed in a glass or plastic tray on a slow rocker; the stacking gel is cut off and discarded. First the gel is washed for 30 min in 200 mL of 10% ethanol, 0.5% (0.9 mol/L) acetic acid (CH_3CO_2H) in distilled water, then the washing solution is drained off and replaced with 0.011 mol/L silver nitrate ($AgNO_3$)(0.37 g in 200 mL distilled or deionised water). After rocking for 30 min, the gel is drained, rinsed briefly twice with distilled water and developing solution is added. This contains 7.5 g sodium hydroxide in 250 mL distilled water, plus 1.9 mL of 38% formaldehyde solution (HCHO) or 0.75 g paraformaldehyde [$CH_2O)_n$]. Degassing of the silver and hydroxide solutions before use, and final addition of 22 mg of sodium borohydride ($NaBH_4$) to the developer sometimes improves staining. The gel is rocked in developer until RNA bands appear brown or black, with a yellow background (about 5–10 min). To stop, developer is drained off and replaced with 5% acetic acid (0.9 mol/L). Gels are examined and photographed on a light box, no filters needed. If desired, they can be stored in 0.07 mol/L sodium carbonate (Na_2CO_3).

RNA patterns vary slightly depending on electrophoretic conditions, especially temperature, so if minor differences or identity of strains is the issue, they must be compared in the same gel. Despite the minor variations, all group A rotaviruses have a recognisable RNA pattern of four, two, three (closely spaced) and two RNA segments counted from the top of the gel. Rotaviruses of serological groups B–F are generally recognisable by deviations from the usual group A pattern and RNA electrophoresis is the best method for detecting them. Serological reagents for detection of non-group A rotaviruses by, e.g. ELISA and immunofluorescence, are not yet available in Australia. Although group A rotaviruses are the major pathogens in most species, rotaviruses of other groups are significant in sheep, and well documented in calves and piglets.

3. Demonstration of Antirotavirus Antibodies by Serological Tests

Sera submitted for serology are heat inactivated at 56°C for 30 min and stored at -20°C. Known negative and positive sera preferably derived from gnotobiotic or specific pathogen free animals must be included with each test.

Antigen is prepared either from clarified and concentrated faecal homogenate containing virus or from culture fluids infected with a tissue culture-adapted rotavirus strain. Cultivable and well-characterised rotavirus strains such as simian SA11 (serotype G3) and bovine UK strains (serotype G6) are obtainable from the author's laboratory.

3.1. Antigen Preparation

Cultures of MA 104 or CV-1 cells are infected with SA11 or UK bovine rotavirus (see 2.4.1.) and harvested after about two days when cytopathic effects are extensive. Remaining cells are removed from the glass (or plastic) by shaking with sterile glass beads. Batches (200 mL) are extracted by homogenising twice for 30 s with 0.25 volume of fluorocarbon (Arklone or Genetron 113, ICI Australia). Centrifuge 1600 *g* for 10 min at 4°C to separate phases, then reextract the fluorocarbon (lower) phase with an equal volume of PBS. Pool supernatants from the two extractions, and centrifuge for one hour at 90 000 *g* to pellet virus and fragments. Resuspend pellets in 1/50 of the original volume of PBS, aliquot and store at -70°C. Avoid repeated freezing and thawing: aliquots in use can be kept at 4°C for a few weeks. Control antigen is prepared in parallel from uninfected cultures.

3.2. Production of Hyperimmune Sera

An emulsion of equal volumes of antigen and adjuvant (Montanide ISA50 from Tall-Bennett) is inoculated subcutaneously or intramuscularly into multiple sites on rabbits or guinea pigs, or intraperitoneally into mice. Five weeks later, the rabbits or guinea pigs are injected intradermally with 0.25 mL antigen in each of four sites, repeating twice at weekly intervals. For mice use 0.05 mL of antigen intravenously at the same intervals. Ten days after the last injection, the animals are bled and the hyperimmune sera separated. Montanide ISA50 is a less irritating substitute for Freund's adjuvant.

Guinea pigs and inbred mice are generally free of preexisting rotavirus antibodies. Rabbits

may have antibodies to group A rotavirus, serotype G3. This is unimportant if the serum is intended for group-specific rotavirus detection.

3.3. Enzyme-linked Immunosorbent Assay

Serum IgG or IgA are indicators of past infection by rotavirus, and IgA coproantibody is a more transient (one month) indication. Polyvinyl microtitre plates are slightly preferable to polystyrene plates for binding rotavirus antigen, prepared as described above (see 3.1.). For more details see McLean *et al.* (1980). Antigen is diluted optimally (about 1/100, determined by checkerboard titration) in 0.1 mol/L carbonate buffer pH 9.6 [see 2.3.(a)] and plates are coated overnight at 4°C. After washing [see 2.3. (b)], add dilutions of serum or faecal extract in PBS-Tween plus 2.5% skim milk (for serum) or 5% (for faeces). Incubate sera for one hour at 37°C, faecal extracts overnight at 4°C. The main cause of insensitivity in detection of faecal IgA is removal of antigen from the plate by proteolytic enzymes, but the higher concentration of skim milk and low temperature incubation counteracts this. After washing as before, antibody is measured by enzyme-conjugated anti-immunoglobulin G or A (if available) binding [see 2.3. (d–e)]. In this case the anti-immunoglobulin sera must be specific for the animal species and the class of immunoglobulin. They must also be free of antirotavirus antibody, so the anti-immunoglobulin sera must be either affinity purified, or prepared in rotavirus antibody-negative animals such as guinea pigs, but not rabbits, goats, swine or sheep.

3.4. Neutralisation Tests

To measure levels of glycoprotein (G) specific antibodies in sera, cell culture stocks of prototype G-serotype strains are required. Mixtures of serum dilutions and sufficient trypsin activated virus to produce 1–200 fluorescent cells per microtitre well (see 2.4.1.1.) are incubated for one hour at 37°C and then assayed (see 2.4.1.–2.4.3.). The titre is defined as the reciprocal of the dilution of serum causing a 50% reduction in the number of infected (fluorescent) cells. Cross reactions occur (by definition) at titres 1/10–1/20 of those of homologous neutralisation reactions, but more commonly at 1/1000 or less.

Neutralisation tests are gaining importance for monitoring performance of rotavirus vaccines.

4. Concluding Remarks

Rotavirus diagnostic methods are evolving rapidly. Latex agglutination for antigen detection (agglutination of antibody-coated polystyrene particles) was not discussed because the commercially available tests, although very rapid, are expensive and less sensitive than ELISA or PAGE. Direct serotyping of rotaviruses by ELISA is possible but expensive, and requires VP7-specific (neutralising) monoclonal antibodies which are not generally available. Nucleotide sequences of the epitope regions of genome segments encoding VP7 are known, so RNA prepared for diagnosis can also be transferred to nylon membranes and hybridised with serotype-specific oligonucleotide probes to determine G-serotypes (Sethabutr *et al.* 1990). By the time these procedures are next revised, non-radioactively labelled probes for all serotypes will probably be available, as well as group-specific reagents for rotaviruses of groups B, C, etc. I suspect that these will soon replace serological reagents since they are so much easier to produce and standardise.

5. References

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6. Appendix

6.1. Appendix 1 — Suppliers

Dako. Bio Scientific Pty Ltd, PO Box 78, Gymea, NSW 2227. Tel. (02) 521 2177; Fax (02) 542 3037.

Medos Company. PO Box 137, Burwood, Vic. 3125. Tel. (03) 808 9077; Fax (03) 808 0926. Nunc Immunoplate No. 439454.

Sigma Aldrich. Unit 2, 10 Anella Avenue, Castle Hill, NSW 2154. Tel. (008) 800 097; Fax (008) 800 096. No.T0134 Porcine trypsin type ix.

Silenus Laboratories. 5 Guest Street, Hawthorn, Vic. 3122. Tel. (03) 819 5000; Fax (008) 818 6977. Code RF.

ICI Australia. 1 Nicholson Street, Melbourne, Vic. 3000. Tel. (03) 665 7111; Fax (03) 288 4940. Arklone.

Tall-Bennett. 9 Moncroft House, 93–95 Rose Street, Essendon, Vic. 3040. Tel. (03) 337 0444; Fax (03) 331 0252. Montanide ISA50.