IMMUNODIFFUSION TEST FOR THE DETECTION OF ROTAVIRUS ANTIGEN IN FAECAL MATERIAL AND GUT HOMOGENATES

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1. Materials and equipment used for the test

1.1. Equipment :

- centrifuge (low speed).
- Ultraturrax (blender)
- magnetic stirrer
- petri-dishes (Ø : 9 cm).

1.2. Reagents :

- anti-bovine rotavirus serum
- faecal homogenates
- standard rotavirus antigen
- Gelose (Difco) Nobel agar
- merthiolate (Eli Lilly)
- PBS pH 7.2
- TNE buffer pH 8.3
- PEG 6000 (Fluka)

2. Preparation of standard rotavirus antigen

- Inoculate a confluent monolayer of PK15, secondary calf kidney or testicle cells with rotavirus.
- Freeze-thaw the cells three times after the appearance of a distinct CPE (2-3 days)
- Titrate the suspension and discard if the titre is less than 4×10^5 $TCID_{50}$/ml
- Clarify by low speed centrifugation (600-1,000 g, 30 min)
- Add 600 g of PEG 6000 to ten litres of the supernatant
- Stir overnight at 4° C
- Collect the precipitate by centrifugation (600-1,000 g, 30 min)
- Resuspend the pellet in 100 ml of PBS and add 1 ml of a 1 % solution of merthiolate
- Store the antigen in one ml vials at -20° C

P. W. de Leeuw et al. (eds.), *Laboratory Diagnosis in Neonatal Calf and Pig Diarrhoea*

3. Preparation of the bovine anti-rotavirus serum

- Inject a 2-3 months old calf subcutaneously with 10 ml of a cellfree rotavirus suspension ($10^6 TCID_{50}$/ml mixed with 10 ml incomplete Freund's adjuvant), divided in two sites in the neck.
- Repeat this injection four times at two weeks intervals
- Test the serum for specificity by indirect immunofluorescence (See NIIF test) two weeks after the last injection. Discard if the titre is less than 3000.

4. Preparation of test samples

- Add two parts of PBS to one part (approximately 5 ml) of faecal material or intestinal tissue and mix with an Ultraturrax (0.5 min)
- Clarify by centrifugation (1,000-1,500 g, 30 min)
- Add one ml of PEG 6000 (60 % in PBS) to nine ml of the supernatant
- Incubate at 4° C overnight
- Collect the precipitate by centrifugation (1,000-1,500 g, 15 min)
- Resuspend the pellet in one ml of PBS

5. Preparation of the Gelose

- Make a 5 % w/v solution of Gelose in distilled water and sterilize at 120° C for 30 min
- Mould the Gelose in pyrex plates to form a 1 cm thick layer
- Cut the Gelose in cubes (1 cm^3)
- Wash the cubes in distilled water twice a day (1 vol. of Gelose in 3 vol. of water) during one week
- Store the cubes at 4° C
- Add 20 g of Gelose cubes to 80 ml of TNE buffer
- Add PEG 6000 to a final concentration of 3 %
- Add 1 ml of a 0.1 % merthiolate solution
- Liquefy the Gelose cubes at 100° C
- Filter the melted Gelose through a selecta filter (m 597 1/2, Ø 320 mm, Schleider & Schull) as hot as possible
- Pour 12 ml of the liquid Gelose in a petri dish (Ø : 9 cm) to form a uniform layer
- Close the petri dishes when the Gelose is gellified and store (upside down) at 4° C

6. Assay proper

- Punch just before use four sets of seven wells (Ø : 5 mm) in each petri dish (fig. 1). The distance between the wells is 5 mm.

- Fill wells nos 1 and 2 with undiluted anti-rotavirus serum
Fill wells nos 4 and 5 with anti-rotavirus serum diluted 1:4
Fill wells nos 3 and 6 with test samples
Fill well no 7 with standard rotavirus antigen
- Incubate at room temperature and read the test after 24-48 h. Samples are scored positive when there is a line of identity with the standard rotavirus antigen

Note :

The sensitivity of the test can be improved by filling the wells twice at six h intervals or by filling the standard antigen and serum wells six h after the sample.

7. Buffer solutions :

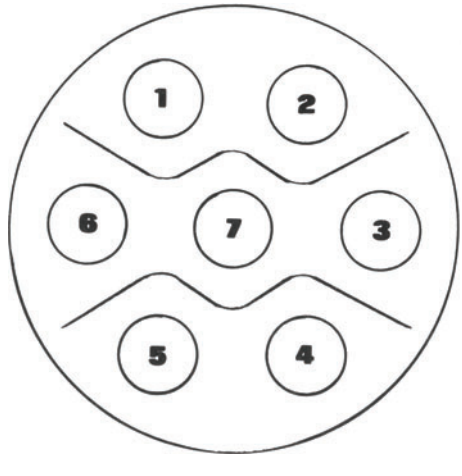
7.1 TNE buffer, pH 8.3 :

Tris	0.106M	1.29 g
Sodium chloride (NaCl)	0,189M	11.09 g
Disodium ethylene diaminotetra-acetic acid (Na_2EDTA)	0.001M	0.39 g
Distilled water to		1000 ml

7.2 Phosphate buffered saline (PBS), pH 7.2 :

Sodium chloride (NaCl)	0.137M	8.0 g
Potassium chloride (KCl)	0.027M	0.2 g
Disodium hydrogen phosphate ($Na_2HPO_4 2H_2O$)	0.809M	1.44 g
Potassium dihydrogen phosphate (KH_2PO_4)	0.015M	0.2 g
Distilled water to		1000 ml

Fig. 1



1,2 - undiluted anti-rotavirus serum
4,5 - anti-rotavirus serum, diluted 1:4
3,6 - test samples
7 - standard rotavirus antigen

NEUTRALISATION OF THE INDIRECT IMMUNOFLUORESCENCE TEST (NIIF) FOR THE DETECTION OF ROTA, CORONA AND BVD VIRUS IN BOVINE FAECAL MATERIAL

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REFERENCE

La neutralisation de l'immunofluorescence indirecte (NIFI) : une technique spécifique et quantitative pour la mise en évidence d'antigènes viraux. G.Wellemans, E.Van Opdenbosch, D.De Kegel, Ann.Méd.Vét.,123, 185-194, 1979.

1. Principe of the test

The test is based on the decrease of the antibody titre of a particular serum which will occur when it is previously incubated with homologous antigen. The test can be used to detect rotavirus, bovine coronavirus or bovine viral diarrhea virus (BVD) in faecal extracts or tissue homogenates. The sera are selected on the basis of their titre in an indirect immunofluorescence (IIF) test.

Cells containing the reference antigen are fixed on a slide. This antigen forms a complex with its corresponding antibody. This complex is evidenced by staining with rabbit anti bovine IgG conjugate. If reference Ab is previously brought in contact with the suspected antigen, a complex will be formed. Therefore, reference antibody is no longer available to react with the infected cells fixed to the slide. Thus there is an inverse relationship between the amount of antigen in the sample and the indirect immunofluorescence titre of the reference serum.

2. Equipment and reagents used in the test

- low speed centrifuge
- Ultraturrax (blender)
- magnetic stirrer
- coated glass slides with ten wells (Glasdekoratie BV, Vigor, Nuland, The Netherlands)
- UV-microscope : indecent leight

Reagents :

- hyperimmune antiserum to bovine rotavirus, bovine coronavirus, and BVD virus.
- standard bovine rotavirus, bovine coronavirus and BVD virus antigen suspensions.
- Rabbit anti-bovine IgG conjugated with fluoresceine isothiocyanate (FITC), free of antibody against the viruses to be detected.

3. Preparation of glass slides with infected cells

- inoculate confluent monolayers of secondary bovine embryo kidney cells with a virus suspension.
- trypsinize the cell cultures when approximately 30 % of the cells is expected to contain antigen detectable by fluorescence (rotavirus one day, bovine coronavirus four days, BVD virus two days).
- wash the cells three times in PBS and resuspend the cells of one Roux flask in 40 ml of PBS.
- put one drop of the cell suspension in each well of the coated glass plates.
- dry the cells at 37° C and fix them in acetone at -20° C during ten min.
- store the slides at -40° C.

4. Selection of reference sera

- carry out an IIF-test (using two-fold dilution steps) with inactivated bovine sera prepared against one viral agent (the serum does not have to be mono-specific).
- discard if the titer is less than 1:810.
- store selected sera in small volumes at -40° C.

5. Preparation of control antigens

- rotavirus and BVD virus : see manual ID-test section A.
- bovine coronavirus : prepare a concentrate of calf rectum found positive for bovine coronavirus by direct immunofluorescence as described in the ID-manual under C.

6. Preparation of test samples

- See ID manual under section C.

7. NIIF test proper

- prepare in each row of a microtiter plate two-fold dilutions of a previously tested hyperimmune serum in PBS (the two highest dilutions should give a negative IIF result).
- add to one row an equal volume of control antigen and to another row an

volume of PBS. The remaining rows are used for the test samples added in equal volumes.

- cover the plate with tape and incubate at 37° C for 12 h.
- put one drop of each of the mixtures in a well of the glass slides containing fixed infected cells and incubate in a humidified atmosphere at 37° C during one h.
- dip the slides in distilled water and wash in PBS at room temperature for one h by magnetic stirring.
- dip again in distilled water, dry the slides, and stain with one drop of an appropriate dilution of a FITC-conjugated anti-bovine IgG preparation.
- incubate the slides in a humidified atmosphere at 37° C for 0.5 h., dip in distilled water, repeat washing in PBS as above, dip in distilled water, and dry.
- add one drop of buffered glycerine pH 9.6 to each well and cover the slides with a cover slip.
- read the results using a UV-microscope.

A negative test is when there is no difference between the titre of the reference serum mixed with the test sample and with PBS. A positive test is when there is four-fold or more reduction of the titre of the reference serum incubated with the test sample as compared to the PBS titre. When a higher titre of the reference serum is found in the presence of the test sample, this means that the test sample contains antibodies directed against the viral antigens present in the fixed cells.

Expression of the antigen titre of a test sample :

$$\frac{\text{reciprocal of the highest positive serum dilution in the presence of PBS}}{\text{idem in the presence of the test sample}}$$

8. Buffers and materials

Carbonate-bicarbonate buffer pH 9.6 :

- $NaHCO_3$ 21 g 0.250 M
- Na_2CO_3 26.5 g 0.250 M
- dissolve in one litre of distilled water.

Buffered glycerine, pH 9.6 :

- glycerine 9 ml
- carbonate-bicarbonate buffer pH 9.6 1 ml

PEG 6000 : Fluka AG, Buchs, Switserland.