ELISA FOR THE DETECTION OF BOVINE ROTAVIRUS ANTIGENS IN FAECES

Laboratory manual of the Central Veterinary Institute, Virology Department, 8221 RA Lelystad, The Netherlands.

Reference: Ellens, D.J. and P.W. de Leeuw: Enzyme-linked immunosorbent assay for diagnosis of rotavirus infections in calves. J. clin. Microbiol. 6, 530-532, 1977.

PRINCIPLE OF THE ASSAY

A polystyrene solid phase is coated with antibodies directed against bovine-rotavirus antigens. Upon addition of a faecal extract, rotavirus antigens (if present) will be trapped by specific antibodies covalently linked to the enzyme peroxidase (conjugate). The bound conjugate is visualized through addition of a chromogenic substrate. Specificity of the reaction is checked by blocking the conjugate reaction with a specific anti-rotavirus serum.

MATERIALS AND EQUIPMENT USED

a) equipment

- Polystyrene microtiter plates (Cooke Engeneering, M129-A-E).
- Multichannel pipets + disposable tips.
- Transparent adhesive tape.
- Mechanical vibrators.
- Microtiter plate washer.
- Incubator 37^{o}C.
- Rotator.
- Titertek$^{(R)}$ Multiskan ELISA reader.

b) reagents

- Anti-bovine rotavirus IgG.
- Carbonate-bicarbonate buffer, 0.05 M, pH 9.6.
- Faecal homogenates.
- Standard rotavirus antigen.
- ELISA buffer.
- Conjugate buffer.
- Anti-bovine rotavirus peroxidase conjugate.
- 5-amino-salicylic acid (5-AS) substrate solution.
- Anti-bovine rotavirus blocking serum.

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

PREPARATION OF BOVINE ANTI-BOVINE ROTAVIRUS (Ba-ROTA) SERUM

- Inoculate a new-born colostrum-deprived SPF or a gnotobiotic calf orally with attenuated or virulent bovine rotavirus.
- Thirty days later inject the calf intramuscularly with 2 ml of purified bovine rotavirus ($\geq 2.10^9$ part./ml), emulsified in complete Freund's adjuvant.
- Repeat the intramuscular injection after 20 days; use incomplete instead of complete Freund's adjuvant.
- Bleed the calf 2-3 weeks after the last injection. Store the serum at $-20^{\circ}C$.

SEPARATION OF IgG FROM THE BOVINE ANTI-BOVINE ROTAVIRUS (Ba-ROTA) SERUM

- Add by drops a saturated ammonium sulphate solution to an equal volume (7 ml) of Ba-rota serum of room temperature, which is kept in constant agitation. Let is stand for 8 h at $4^{\circ}C$.
- Collect the precipitate by centrifugation at 1,000 g for 30 min at $4^{\circ}C$.
- Dissolve the pellet in 2 ml of 0.0175 M phosphate buffer, pH 6.3, and dialyse overnight against the same buffer.
- Clarify the solution by centrifugation at 1,000 g for 10 min.
- Apply the sample to a DEAE cellulose column (30 x 1.5 cm) equilibrated against 0.0175 M phosphate buffer, pH 6.3.
- Elute with 0.0175 M phosphate buffer, pH 6.3 and concentrate IgG containing fractions by ammoniumsulphate precipitation.
- Dialyse against 0.15 M NaCl and adjust the IgG concentration to 8 mg/ml.

PREPARATION OF PEROXIDASE CONJUGATED Ba-ROTA IgG ACCORDING TO WILSON AND NAKANE (REF.1)

- Dissolve 8 mg horseradish peroxidase (HRP) (Boehringer, grade I) in 2 ml of distilled water.
- Add 0.4 ml 0.1 M sodium periodate $NaIO_4$ in 0.02 M sodium acetate buffer pH 4.4 and incubate for 20 min at room temperature under constant agitation.
- Dialyse against 1 mM sodium acetate buffer pH 4.4, overnight, $4^{\circ}C$.
- Dialyse 2 ml Ba-rota IgG (8 mg/ml) against 0.01 M carbonate-bicarbonate buffer, pH 9.5.
- Adjust the HRP-$NaIO_4$ solution to pH 9.0 with 0.2 M carbonate-bicarbonate buffer, pH 9.5.
- Add the dialysed Ba-rota IgG solution (2 ml) and incubate for 2 h at room temperature.

- Add 0.2 ml of a freshly prepared solution of sodium borohydride ($NaBH_4$) in distilled water and let it stand for 2 h at room temperature.
- Remove non-conjugated peroxidase by precipitation of the conjugate by adding an equal volume of a saturated ammonium sulphate solution (constant agitation, 4°C, 90 min).
- Wash the precipitate twice with cold (4°C) 50% saturated ammonium sulphate solution, dissolve it in 1 ml demineralized water and dialyse against PBS, pH 7.2.
- Add an equal volume of glycerol and store at -20°C.

PREPARATION OF THE 5-AS SUBSTRATE SOLUTION (REF.2).

N.B. This procedure is essential to obtain low background values in the test.

- Dissolve 18 g sodium disulphite ($Na_2S_2O_5$, Merck) and 20 g 5-AS (2-hydroxy benzoïc acid, Merck) in 4 litre demineralized water of 80°-90°C.
- Add 2 g of charcoal and stir for 2 min at 80°-90°C.
- Remove the charcoal by filtration and cool-down the filtrate rapidly to 4°C.
- Collect the white precipitate by vacuum filtration through filter paper in a Büchner funnel and wash the precipitate twice with 100 ml demineralized water (4°C).
- Dry the 5-AS powder in the dark at room temperature.
- Dissolve 1 g of the purified 5-AS in one litre substrate buffer, pH 6.8. After the addition of 5-AS the pH of the 5-AS solution should be 5.9 (if not adjust).
- Distribute in 10 to 50 ml volumes and store at -20°C.
- Prior to use an appropriate volume of the 5-AS solution is thawed, minute precipitates if present are dissolved at 37°C, and one tenth of the volume of a freshly prepared hydrogen peroxide solution (0.05%) is added.

COATING THE PLATES

- Dilute Ba-rota IgG in carbonate-bicarbonate buffer 0.05 M pH 9.6 to the optimal[1)] concentration.
- Apply 100 µl of appropriately diluted Ba-rota IgG solution to the wells and cover with tape.
- Incubate for 18 h at 37°C while the plates are rotating at an angle of 45°C.
- Freeze and store at -20°C for up to two months.

PREPARATION OF FAECAL HOMOGENATES AND STANDARD ANTIGEN

Faecal homogenates

- Apply one g of faeces, 4 ml of ELISA buffer and about 7 glass-beads to a 7 ml glass vial (can be frozen and stored at -20°C).
- Before testing, homogenize by shaking for one h at room temperature.

Standard antigen

- Homogenize one volume of diarrhoeic faeces of an orally infected SPF calf in four volumes of PBS.
- Clarify by centrifugation for 30 min at 1,000 g.
- Add an equal volume of Arklone (R) (I.C.I., U.K.) to the supernatant and treat the mixture ultrasonically (3 times, 5 sec, 4°C).
- Separate the phases by centrifugation for 30 min at 1,000 g. (Repeat the Arklone and sonification procedure with the water phase, if it is too viscous).
- Titrate the water phase in the ELISA; determine and express the concentration of rotavirus antigens in ELISA units (EU). One EU is that dilution giving a P/N value of 3.0.

PERFORMANCE OF THE ASSAY

- Thaw the coated microtiter plate and shower three times with demineralized water containing 0.05% Tween 80.
- Fill the wells of rows 1 and 2 with 100 µl volumes of PBS. Apply 100 µl volumes of a two-fold serial dilution of 32 EU standard rotavirus antigen to the wells of rows 3 and 4. Apply, in duplicate, 100 µl volumes of 32 faecal homogenates to the remaining 64 wells. Cover with tape.
- Incubate for 18 h at 4°C.
- Shower six times with demineralized water containing 0.05% Tween 80.
- Apply 100 µl volumes of PBS to the wells of the odd-numbered rows, and 100 µl volumes of appropriately [2] diluted blocking serum to the wells of the even-numbered rows. Cover with tape.
- Incubate for one h at 37°C while the plates are rotating at an angle of 45°.
- Shower three times with demineralized water containing 0.05% Tween 80.
- Fill the wells of the plate with 100 µl volumes of conjugate buffer and an optimal [3] concentration of anti-bovine rotavirus peroxidase conjugate. Cover with tape.
- Incubate for one h at 37°C.
- Shower four times with demineralized water containing 0.05% Tween 80.
- Fill the wells of the plate with 100 µl volumes of the 5-AS substrate

solution, shake for 10 min and cover with tape.
- Read the test between 3 and 24 h.

READING AND INTERPRETATION

With the naked eye

- Read the colour of the well of the standard rotavirus antigen titration that contains one EU rotavirus antigen.
- Score samples positive that have a colour that is equal to, or more intense than, the colour of the "1 EU" well, and that are blocked by the blocking serum.

With the Titertek ^{R} Multiskan ELISA reader at 450 nm

- Add 100 µl volumes of water to all wells of the plate and shake for 10 min.
- Blanking: enter the values of the blanks (row 1 or 2) into the memory of the instrument.
- Measuring: measure the absorbance at 450 nm of all wells.
- Read the absorbance value of the well of the standard rotavirus antigen titration that contains one EU rotavirus antigen.
- Set the matrix range at ten times the absorbance value of the "1 EU" well.
- Start matrix measuring.
- Score samples positive with matrix values $\geq$1 and that are blocked for $\geq$50%.

FOOTNOTES

1) The optimal concentration of Ba-rota IgG for coating should be determined by checkerboard titration:
- Coat a microtiter plate by applying two-fold serial dilutions of Ba-rota IgG in 0.05 M carbonate-bicarbonate buffer pH 9.6 to the wells of rows A-H.
- Shower three times with 0.05% Tween 80.
- Apply two-fold serial dilutions of 32 EU standard rotavirus antigen to the wells of rows 3-12. Fill the wells of rows 1 and 2 with 100 µl volumes of PBS.
- Proceed the ELISA in the normal way.
- The optimal Ba-rota IgG dilution for coating is that dilution which will give positive/negative (P/N) values $\geq$3 with the highest dilution of antigen.

2) Appropriately diluted blocking serum: minimal amount of serum blocking for 90% 32 EU of standard rotavirus antigen.

3) The optimal concentration of Ba-rota-peroxidase conjugate should be determined by checkerboard titration:

- Thaw a coated microtiter plate and shower three times with demineralized water containing 0.05% Tween 80.
- Apply 100 µl volumes of two-fold serial dilutions of 32 EU standard rotavirus antigen to the wells of rows 3-12 and fill the wells of rows 1 and 2 with 100 µl volumes of PBS. Cover with tape.
- Incubate for 18 h at 4°C.
- Shower three times with demineralized water containing 0.05% Tween 80.
- Apply 100 µl volumes of two-fold serial dilutions of the Ba-rota-peroxidase conjugate to the wells of rows A-H and cover the plate with tape.
- Proceed the ELISA in the normal way.
- The optimal Ba-rota-peroxidase conjugate dilution is that dilution which gives P/N values $\geq$3 with the highest dilution of antigen.

BUFFER SOLUTIONS

Phosphate-buffered saline (PBS), pH 7.2

Sodium chloride (NaCl)	8.0 g (136.7 mM)
Potassium chloride (KCl)	0.2 g (2.7 mM)
Disodium hydrogen phosphate ($Na_2HPO_4 . 2H_2O$)	1.44 g (8.1 mM)
Potassium dihydrogen phosphate (KH_2PO_4)	0.2 g (1.5 mM)
Demineralized water to	1000 ml

Stock solutions for phosphate buffers

A. 0.1 M solution of potassium dihydrogen phosphate, 13.61 g KH_2PO_4 in 1000 ml.

B. 0.1 M solution of disodium hydrogen phosphate, 17.80 g $Na_2HPO_4 . 2H_2O$ in 1000 ml.

- 0.1 M phosphate buffer, pH 6.8. Adjust the pH of solution A to pH 6.8 with solution B (approximately 510 ml of A and 490 ml of B).
- 0.0175 M phosphate buffer, pH 6.3. Adjust the pH of solution A to pH 6.3 with solution B (about 773 ml of A and 227 ml of B) and dilute 1: 4.71 (4710 ml distilled water to 1000 ml buffer).

Acetate buffer 0.02 M, pH 4.4

A. 0.02 M solution of acetic acid,

1.18 ml acetic acid (96%, d = 1.06 g/ml) in 1000 ml

B. 0.02 M solution of sodium acetate,

1.64 g $C_2H_3O_2Na$ in 1000 ml.

Adjust the pH of solution A to pH 4.4 with solution B (about 305 ml A and 195 ml B).

HAEMADSORPTION - ELUTION - HAEMAGGLUTINATION ASSAY (HEHA) FOR THE DETECTION OF BOVINE CORONAVIRUS ANTIGENS IN FAECES

Laboratory manual of the Central Veterinary Institute, Virology Department, 39 Houtribweg, 8221 RA Lelystad, The Netherlands.

Reference: J.A.M. van Balken, P.W. de Leeuw, D.J. Ellens and P.J. Straver: Detection of coronavirus in calf faeces with a haemadsorption - elution - haemagglutination assay (HEHA). Vet. Microbiol. 3, 205-211, 1978/1979.

PRINCIPLE OF THE ASSAY

The Haemadsorption - Elution - Haemagglutination Assay is based on the observation that the haemagglutinating activity of bovine coronavirus present in faecal samples is temperature-dependent. The method consists of adsorption of the virus onto mouse erythrocytes at 4°C, removal of unadsorbed material and elution of adsorbed viral material at 37°C. The eluate is then used in a haemagglutination test. Specificity of the reaction is checked by a blocking assay.

1. PREPARATION OF FAECAL EXTRACTS (AND STANDARD ANTIGEN)

- Homogenize one volume of faeces in four volumes of PBS (pH 7.2).
- Clarify by low speed centrifugation (10 min, 1,500 g).
- Use the supernatant fluid in the test.

2. COLLECTION OF WHOLE MOUSE BLOOD

- Anaesthetize adult female mice with chloroform.
- Collect whole mouse blood by heart puncture.
- Mix the blood with an equal volume of an Alsever dextrose solution.
- Centrifuge at low speed (10 min, 200 g).
- Wash the red blood cells several times with PBS until the supernatant fluid is clear.

3. ASSAY PROPER

3.1. Haemadsorption

- Mix a 40% suspension of mouse erythrocytes in PBS pH 7.2 with an equal volume (0.5 ml) of a faecal extract.
- Incubate for 1 h at 4°C.
- Centrifuge at low speed (10 min, 200 g).
- Wash the erythrocytes twice with 4.5 ml of PBS of 4°C.

3.2. Elution

- Add 1 ml of warm PBS (37°C) and mix gently.
- Incubate for 1 h at 37°C.
- Centrifuge at low speed and use the supernatant (eluate) in the haemagglutination assay.

3.3 Haemagglutination assay

- Prepare two-fold serial dilutions of the supernatant in a microtiter plate, using PBS with 0.2% bovine serum albumin as the diluent (PBS-BSA).
- Add an equal volume (25 µl) of a 1% mouse erythrocyte suspension in PBS-BSA and mix on a mechanical shaker for 5 min.
- Incubate for 90 min at 4°C.
- Read the test with the naked eye.
- Express the HEHA-titres as the reciprocal of the highest antigen dilution showing complete haemagglutination.

4. HAEMAGGLUTINATION INHIBITION (CONFIRMATION) TEST

4.1. Pre-treatment of positive and negative reference sera.

- Inactivate the sera at 56°C for 30 min.
- Mix the sera with equal volumes of 25% Kaolin in PBS and incubate with occasional stirring at room temperature for 45 min.
- Clarify by low speed centrifugation (20 min, 1,000 g).
- Mix the treated serum with an equal volume of a 10% mouse erythrocyte suspension in PBS.
- Incubate for 60 min at 4°C, stir every 10 min.
- Clarify by low speed centrifugation (10 min, 200 g).
- Use the supernatant in the confirmation test.
- Store the treated reference sera in small volumes at -20°C.

4.2. Test proper

- Dilute the eluate of the test samples to contain eight haemagglutinating units in 25 µl.
- Mix with equal volumes of 1:8 in PBS-BSA diluted positive and negative (pre-treated) reference sera.
- Incubate for 45 min at room temperature.
- Add 25 µl of a 1% mouse erythrocyte suspension in PBS-BSA.
- Incubate for 90 min at 4°C and read the test.

The haemagglutinating activity of a test sample is considered coronavirus specific if complete blocking occurs in the presence of the positive serum, but not in the presence of the negative serum.

5. INGREDIENTS

(Unless stated otherwise, analytical grade chemicals from Merck).

5.1. PBS pH 7.2:

8.0 g NaCl		0.14 M
0.2 g KCl		2.7 mM
1.44 g $Na_2HPO_4 . 2H_2O$		8.1 mM
0.2 g KH_2PO_4		1.5 mM
H_2O	ad	1000 ml

5.2. Alsever-dextrose solution pH 6.1:

20.5 g $C_6H_{12}O_6$	0.11 M	(Brocasef, Deventer, The Netherlands)
8.0 g $C_6H_5Na_3O_7 . 2H_2O$	27.2 mM	
4.2 g NaCl	71.9 mM	
6.01 g $C_6H_8O_7 . H_2O$	28.6 mM	
H_2O ad	1000 ml	

Sterilize for 15 min at 110°C.

5.3. Kaolin

- Prepare a 25% suspension of Kaolin (Brocasef, Deventer, The Netherlands) in 5N hydrochloric acid, shake and centrifuge (10 min, 1,000 g).
- Wash three times with PBS, each time followed by centrifugation.
- Sterilize a 25% suspension in PBS for 1 h at 121°C and store it at 4°C.

5.4. Reference sera

A reference antiserum against bovine coronavirus was prepared in a 19-day-old SPF calf deprived of colostrum. The calf was inoculated orally with a British isolate and subsequently hyperimmunized with the same strain multiplied in bovine trachea organ cultures. The virus was pelleted from the culture medium at 250,000 g for 30 min, resuspended in PBS, pH 7.2, and emulsified in an equal volume of incomplete Freund's adjuvant. Two ml of this emulsion were injected intramuscularly at 19 and again at 23 weeks of age. Blood was withdrawn 10 days after the last injection.

Pre-serum obtained from the same calf was used as the reference negative serum.