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DETECTION OF ROTAVIRUS IN FAECAL SPECIMENS WITH A MONOCLONAL ANTIBODY ENZYME-LINKED IMMUNOSORBENT ASSAY: COMPARISON WITH POLYCLONAL ANTIBODY ENZYME IMMUNO-ASSAYS AND A LATEX AGGLUTINATION TEST

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Abstract—Monoclonal antibodies have been produced against the 81/36F strain of rotavirus. One of them, was chosen as diagnostic reagent: it showed high ELISA reactivity with all the bovine, human and porcine rotavirus strains tested and reacted with VP6, structural protein product known to support the common rotavirus antigen.

A sandwich ELISA procedure using the chosen monoclonal as "capture and detecting" antibody was performed to detect rotavirus in faecal samples from experimentally inoculated newborn calves: it always gave a negative response with meconium and a positive response for the stool specimens which rotavirus have been isolated. This assay was compared with Enzygnost and Slidex Rota Kit tests and with a non-commercial sandwich ELISA test using polyclonal antibodies: it showed more sensitivity than the agglutination test and was as sensitive as the other two tests to detect rotavirus in routine diagnostic material. The test evaluated showed no equivocal results.

Key words: Rotavirus, detection, faeces, monoclonal antibodies, diagnostic, ELISA.

DETECTION DE ROTAVIRUS DANS DES MATIERES FECALES A L'AIDE D'UN TEST ELISA UTILISANT UN ANTICORPS MONOCLONAL: COMPARAISON AVEC DES TESTS ELISA UTILISANT DES ANTICORPS POLYCLONAUX ET UN TEST D'AGGLUTINATION AU LATEX

Résumé—Des anticorps monoclonaux ont été produits contre la souche de rotavirus 81/36F. L'un d'entre eux, a été choisi comme réactif de diagnostic: il présentait une réactivité élevée par test ELISA avec toutes les souches bovines, humaines et porcines de rotavirus testées et réagissait avec la VP6, protéine structurale connue pour porter l'antigène de groupe du rotavirus.

Un test ELISA de type "sandwich" utilisant le monoclonal choisi comme anticorps "capteur et

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détecteur" a été réalisé pour détecter du rotavirus dans les matières fécales de veaux nouveau-nés inoculés expérimentalement: il fournissait toujours une réponse négative avec le méconium et une réponse positive avec les fèces à partir desquelles du rotavirus avait été isolé. Ce test a été comparé aux tests Enzygnost et Slidex Rota Kit et à un test ELISA de type "sandwich" non-commercial utilisant des anticorps polyclonaux: il montrait une plus grande sensibilité que le test d'agglutination et était d'une sensibilité égale à celle des deux autres tests pour détecter du rotavirus dans des matières fécales soumises à un diagnostic de routine. Le test évalué ne fournissait aucun résultat équivoque.

Mots-clefs: Rotavirus, détection, fèces, anticorps monoclonaux, diagnostic, ELISA.

INTRODUCTION

Because of the importance of rotavirus as a cause of gastroenteritis in humans and various animal species, numerous diagnostic tests have been developed to detect it in stool specimens [1–13].

Adaptation of these assays has resulted in commercially available diagnostic kits which permit the identification of rotavirus antigen in stool specimens by enzyme linked immunosorbent assays (ELISA) and by latex agglutination assay with polyclonal detecting antibodies. The purpose of this study was to develop a "sandwich" ELISA test based on monoclonal antibodies to detect rotavirus infections and compare it with polyclonal antibody enzyme immuno-assays and a latex agglutination test.

MATERIAL AND METHODS

Viruses and cells

Human rotavirus serotypes Wa (subgroup 2, serotype 1) [14], ST-3 (serotype 4) [15], M (serotype 3) [16] and DS-1 (subgroup 1, serotype 2) [14], porcine rotavirus strain OSU (subgroup 1, serotype 5) [14] and calf rotavirus strain U.K. (serotype 6) [15] were the kind gift of P. Scott (Moredun Research Institute, Edinburgh, Scotland). The porcine rotavirus strain RV277 was donated by M. Pensaert (University of Gent, Belgium). The calf rotavirus strains PQ, NCDV (subgroup 1, serotype 6) [14] and WOODE were respectively supplied by M. Begin (University of Montreal, Quebec), Roy (University of Montreal, Quebec) and G. Woode (Institute for Research on Animal Diseases, Compton, U.K.). The bovine rotavirus strains 81/36F and 81/40F, isolated in Italy [17] were kindly provided by G. Castrucci (University of Perugia, Italy). The bovine rotavirus strains S14 and S77 were isolated in our laboratory [18].

Established cultures of rhesus monkey kidney cells (MA104) were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 50 IU/ml of penicillin, 50 µg/ml of streptomycin and 10% of foetal calf serum.

The myeloma cell line used for fusion [19] was the Sp2/0 cell line. The Sp2/0 cells were grown in DMEM containing 10% horse serum, 5% foetal calf serum, essential and non-essential amino acids, 110 µg/ml sodium pyruvate, 292 µg/ml L-glutamine, 50 µg/ml streptomycin, 50 IU/ml penicillin (complete DMEM).

Production and purification of viruses

All viruses were propagated in MA104 cells in the presence of trypsin as previously described [20]. Rotavirus-infected MA104 cells were frozen and thawed three times. After clarification of the supernatant fluid by centrifugation for 30 min at 10,000 *g*, the virus was pelleted by centrifugation for 3 h at 100,000 *g*. The virus pellet was resuspended in

Tris–NaCl–NaCl₂ (Tris 0.1 M; NaCl 0.15 M; CaCl₂·2H₂O 10 mM–pH 8) buffer and submitted to an isopycnic centrifugation in CsCl (18 h—150,000 *g*). The 1.36 [21] band was collected, pelleted by centrifugation (2 h—300,000 *g*) and suspended in Tris–NaCl–CaCl₂ buffer.

Immunization of mice

Six weeks old female Balb/c mice were injected at days 0 and 21 intraperitoneally and subcutaneously with 25 μ g of purified double-shelled 81/36F rotavirus particles in 250 μ l of NaCl 0.15 M and Freund complete adjuvant (v/v); 6 weeks later, they were given the same preparation in Freund incomplete adjuvant. Four days before fusion, mice were boosted intravenously with 10 μ g of purified virus in 0.5 ml of NaCl 0.15 M.

Production of hybridoma cell lines

Fusion. Four days after the last inoculation, one mouse was sacrificed. The spleen cells were suspended in complete DMEM medium, washed and counted. For the fusion, 5×10^7 myeloma cells and 1×10^8 spleen cells suspended in the medium without serum were mixed in a 15 ml conical centrifuge tube. After centrifugation, the cell deposit was gently mixed for 90 s in 0.5 ml of buffer containing polyethylene glycol 4000 [21]. The tube was progressively filled with medium without serum and centrifuged. Subsequently, the cells were washed and suspended in selective medium (complete DMEM with hypoxanthine, aminopterin and thymidine). They were finally distributed into the wells of tissue culture microplates covered with mouse peritoneal cells as a feeder layer. Two irradiated (500 rads) syngenic mice were injected intravenously with 1×10^8 spleen cells for an adoptive transfer experiment [22]; these mice were sacrificed five days later for the fusion of their spleen cells.

Maintenance and development of the clones. The medium in the microplates was changed 10 days after fusion (complete DMEM medium with hypoxanthine and thymidine).

When the clones had reached a sufficient cell density, the supernatants were removed to test for the presence of anti-rotavirus antibodies, using an indirect ELISA test (see below).

When a positive clone had invaded the whole well, it was transferred to a macroplate well (Tissue culture 24 wells, Gibco, New York, U.S.A.) covered with mouse peritoneal cells. As soon as the cell density was sufficient, three successive clonings were carried out in semi-solid agarose. The final clone was multiplied or stored in liquid nitrogen until needed for production of ascitic fluids.

Screening of hybridomas. The culture medium of the microplate wells containing clones was tested by ELISA using an indirect technique.

The plates (Linbro, Flow Laboratories, U.K.) were coated with rotavirus clarified culture supernatant (25 μ l/well—one night at 4°C). A 25 μ l sample of the supernatant to be tested was allowed to react for one night at 4°C. The presence of rotavirus antibodies was demonstrated by means of a peroxidase-conjugated goat anti-mouse immunoglobulin hyperimmune serum. The test was considered positive when the absorbance measured by a spectrophotometer was at least twice the absorbance of the same clone supernatant tested against a mock-infected clarified culture supernatant of MA104 cells.

Production and purification of ascitic fluids

Production of ascitic fluids. About 1 to 5×10^6 specific antibody-producing hybridoma cells were injected intraperitoneally per mouse, treated with 500 μ l of pristane 10 days before.

Purification of ascitic fluids. The ascitic fluids were purified on protein A—Sepharose CL-4B (Pharmacia, Uppsala, Sweden) using the method of Ey *et al.* [23] modified by Seppälä *et al.* [24].

Radio-immuno-precipitation

Preparation of (³⁵S) methionine-labeled rotavirus-infected cell lysates. Confluent monolayers of MA104 cells in 80 cm² bottles were infected with 81/36F virus at a multiplicity of infection of 10 PFU/cell. Virus was allowed to adsorb for 1 h at 37°C; then the inoculum was removed and maintenance medium was added. At 6 h postinfection, the medium was changed to maintenance medium without methionine. After 1 h the medium was again changed to maintenance medium containing 200 µCi of (³⁵S) methionine (Amersham, U.K., 1000 Ci/mM). Incubation was continued for 90 min, after which the cells were washed three times with ice-cold phosphate buffered saline. Cell lysates were prepared by adding 4 ml of lysate buffer containing 0.15 M NaCl, 0.05 M Tris-HCl-pH 7.2, 0.1% SDS, 1% sodium desoxycholate, 1% Triton X-100, 10⁻⁴ M PMSF (1 h—4°C).

The lysates were then centrifuged for 1 h at 100,000 *g* and the supernatants were used as a source of labeled rotavirus proteins for immunoprecipitation. (³⁵S) Methionine-labeled mock-infected cell lysates was also prepared by the same way.

Immunoprecipitation of rotavirus proteins. Ten µl of ascitic fluid were mixed with a 60 µl volume of a 7% suspension of protein A-Sepharose CL-4B pellets (Pharmacia, Uppsala, Sweden); after incubation for 30 min at 4°C, 100 µl of cell lysates was added and incubated one night at 4°C. The protein A-Sepharose was pelleted and washed four times with lysate buffer and once with a Tris-HCl-NaCl buffer.

Adsorbed, labeled viral proteins were recovered by boiling the precipitate in 50 µl Laemmli sample buffer [25] for 2 min. The supernatants were applied to Laemmli discontinuous sodium dodecyl sulfate (SDS)—polyacrylamide (10%) slab gels for analysis. Fluorographic detection of radioactivity was performed following the method described by Chamberlain [26].

Double immunodiffusion

Hyperimmune goat anti-mouse IgA, IgM, IgG, IgG1, IgG2a and IgG2b serums came from Meloy (Virginia, U.S.A.). The anti-mouse IgG3 serum was a Sigma (Deisenhofen, Germany) reagent and the anti-mouse kappa and lambda light chains serums were Nordic Immunology reagents.

Double immunodiffusion reactions were achieved as described by Franssen [27].

Labeling of monoclonal antibodies with biotin

The method described by Goding [28] was applied.

Sandwich ELISA procedure using monoclonal antibodies

The wells of a 96-wells polyvinyl chloride microtiter plate (Linbro, Flow Laboratories, U.K.) were coated with 50 µl of purified monoclonal antibody directed against 81/36F diluted in 0.02 M PBS (pH 7.8), for one night at 4°C. After washing three times with PBS containing 0.02% Tween-80, the wells were postcoated 2 h at 20°C with a solution of 0.5% (wt/v) ovalbumin in PBS to help to prevent non-specific binding. Plates were washed three times and stool suspensions (25 µl/well) were added and incubated for 2 h at 20°C.

Rotavirus clarified culture supernatant and mock-infected clarified culture supernatant were chosen respectively as the positive and the negative controls.

Plates were washed three times, and purified monoclonal antibody labeled with biotin was added at a rate of 25 μ l/well. After 2 h at 20°C, plates were washed three times and 0.025 ml/well of biotinylated streptavidin labeled horseradish peroxidase complex (Amersham, U.K.) (dilution 1/750) was added and the plates were incubated for 2 h at 20°C. The plates were then washed three times and 0.05 ml/well of peroxidase substrate (0.04% O-phenylenediamine + 0.02% urea peroxide in citrate-phosphate buffer 0.1 M, 0.01% Tween-80-pH 4.5) was added.

After 15–30 min at room temperature, the reaction was stopped with 25 μ l of 6 N HCl. The plates were read in a Titertek Multiskan MCC/340 (Flow Laboratories, U.K.) at a wave length of 490 nm. A result was considered positive when the A490 was 3XSD above the negative control.

Sandwich ELISA procedure using polyclonal antibodies

(1) The first ELISA test using polyclonal antibodies is routinely used in the “Centre d’Economie rurale” of Marloie for the detection of rotavirus in field diarrheic bovine faeces samples.

The wells of a 96-wells polystyrene microtiter plate (Immuno-Plate I, Gibco, New York, U.S.A.) were coated with 1 μ g of protein A-Sepharose purified hyperimmune rabbit anti-81/36F rotavirus serum diluted in PBS (one night, 4°C). After washing with PBS, the wells were saturated 30 min with a solution of 2% (wt/v) bovine serum albumin in PBS. The plate was dried, sealed with plastic foil, enclosing a dessicant and kept at 4°C until use. Hundred μ l of each stool sample were placed in each well of the microtiter plate. Faeces samples were distributed undiluted if their consistency allowed it; otherwise they were diluted in PBS. The plate was incubated 1 h at room temperature and washed with PBS.

The detecting antibody added (100 μ l/well) was the hyperimmune rabbit antiserum described above but conjugated with peroxidase and diluted 1:100 in PBS-2% BSA. After 1 h, the wells were rinsed out with PBS and substrate (0.04% O-phenylenediamine + 0.05% H₂O₂ in citrate 35 mM-phosphate 66 mM buffer-pH 5) was added (100 μ l/well). The reactions were stopped with the addition of 50 μ l of 6 N HCl per well.

(2) The second ELISA test using polyclonal antibodies is a commercial test (Enzygnost, Behring Diagnostics, Brussels, Belgium) and was performed as described by the producer.

Latex agglutination test

The Slidex Rota Kit (Bio-Merieux, Brussels, Belgium) was carried out as described by the producer.

Faecal specimens

Calf faeces were obtained from two separate sources.

Experimental animal. Four colostrum-deprived newborn calves were used in the experiments reported here. They were isolated immediately after birth in individual containers in thoroughly decontaminated stable and inoculated orally with 81/36F rotavirus strain.

The experimental procedure has been reported in detail in a separate paper [20]. Stool samples were collected before and twice a day for at least 10 days after inoculation. Virus

isolation from stool samples have been performed following the method of Babiuk *et al.* [29].

Routine diagnostic material. Bovine faeces samples submitted to the veterinary Centre of Marloie, for routine diagnosis were examined. Thirty were tested by all tests described before, 21 other samples by the ELISA tests and 50 other samples by the non-commercial polyclonal and monoclonal ELISA tests.

Treatment of faecal samples. Approximately 0.2 g of faecal sample was diluted in 2 ml of a solution of 0.5% (wt/v) ovalbumin in PBS. After stirring, the suspension was centrifuged for 30 min at 2000 g.

RESULTS

Production and characterization of monoclonal antibodies directed against group specific antigen of rotaviruses

Fusion frequencies of 3.34×10^{-5} and 2.50×10^{-5} and specific fusion frequencies of 2×10^{-7} and 10×10^{-7} were obtained respectively for the immunized mouse and for the fusion coming from adoptive transfer experiment.

Hybridoma clones BR27, 42 and 71 were chosen as candidate diagnostic reagents. The cultures showed characteristics of *in vitro* and *in vivo* rapid growth, stability during storage in liquid N₂ and stable antibody production. Monoclonal antibodies BR27, 42 and 71 showed high ELISA reactivity (OD \geq 1.5) not only with 81/36F virus but also with all other viruses of bovine, human or porcine origins tested.

Monoclonal antibodies BR27, 42 and 71 are IgG2a kappa. They are stable to concentration, purification and storage at -20°C . The antibodies bind satisfactorily to polyvinyl microtiter wells. Radioimmunoprecipitation was used to demonstrate that they react with VP6, the 42,000-dalton product of the sixth rotaviral gene.

Choice of the best pair of monoclonal antibodies for the sandwich ELISA

Figure 1 illustrates optical density versus dilution curves given by three biotinylated monoclonal antibodies when used to detect rotavirus of clarified culture supernatant.

Figure 2 illustrates the inhibition curves of the fixation of the biotinylated monoclonal antibody BR71 on coated rotavirus clarified culture supernatant by dilutions of unconjugated monoclonal antibodies. The fixation of biotinylated BR71 were not inhibited by monoclonal antibodies BR27 and BR42. Similar results were obtained when BR27 or BR42 were used as biotinylated monoclonal antibodies and BR71 as unconjugated antibody. On the other hand, the fixation of biotinylated BR71 (2 ng/well) on rotavirus were strongly inhibited by concentrations of unconjugated BR71 greater than 30 ng/well.

Figure 3 shows the relative sensitivities of the sandwich ELISA test using BR71 as "capture" antibody and one of the three biotinylated monoclonal antibodies as "detecting" antibody.

The greatest sensitivity was obtained with the pair of monoclonal antibodies BR71–BR42. The pair BR71–BR27 was more sensitive than the pair BR71–BR71 only for the antigen dilutions less than 8.

Detection of rotavirus in faecal samples

Experimental animal. Faeces samples known to be positive by virus isolation were all found to be positive in the ELISA using monoclonal antibodies. Pre-inoculation samples

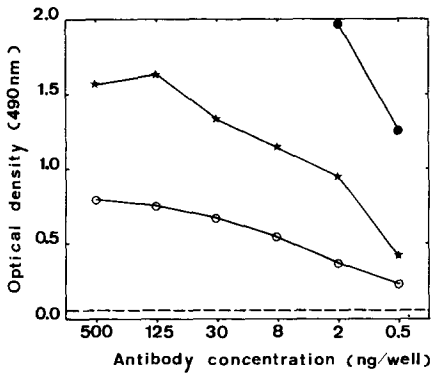


Fig. 1. Comparison of the efficiency of three monoclonal antibodies as "detecting antibody". Symbols ● BR71; ★ BR42; ○ BR27. The plates were coated with rotavirus clarified culture supernatant. This antigen was detected by dilutions of biotinylated monoclonal antibodies.

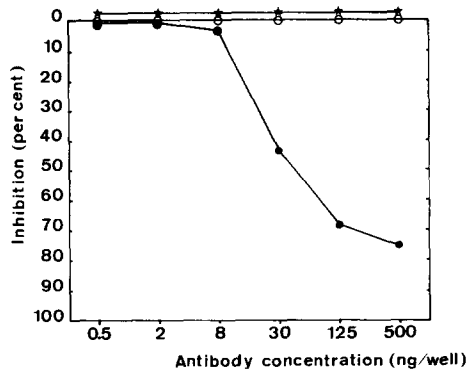


Fig. 2. Inhibition of the fixation of the biotinylated monoclonal antibody BR71 on rotavirus by dilutions of unconjugated monoclonal antibodies. Symbols: ● BR71; ★ BR42; ○ BR27. The plates were coated with rotavirus clarified culture supernatant. The antigen reacted first with different dilutions of unconjugated monoclonal antibodies and then with biotinylated monoclonal antibody BR71 (2 ng/well).

were used as virus-negative controls; all gave negative results both by ELISA and virus isolation.

Faecal samples from one calf infected with bovine rotavirus were ELISA and virus isolation positive on days 2 (a.m.) to 3 (p.m.) after infection (Fig. 4). On the other days, virus isolation were negative; however optical densities were positive by ELISA on days 1 (p.m.) and 4 (a.m.) after inoculation.

Routine diagnostic material: comparison of the monoclonal antibody ELISA with the polyclonal antibody ELISAs and the latex agglutination test. All results are presented in Table 1. Agreement on the detection of bovine rotaviruses was reacted by ELISA with monoclonal antibodies and ELISA with polyclonal antibodies, Enzygnost and Slidex Rota

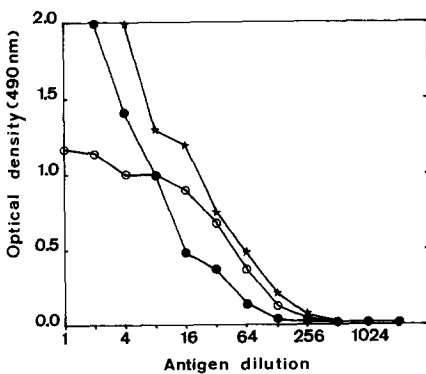


Fig. 3. Comparison of the sensitivities of the sandwich ELISA-test using three monoclonal antibodies as "detecting antibody". Symbols: ● BR71; ★ BR42; ○ BR27. The plates were coated with monoclonal antibody BR71 (50 ng/well). The antigen, rotavirus clarified culture supernatant, was detected by biotinylated monoclonal antibodies (10 ng/well).

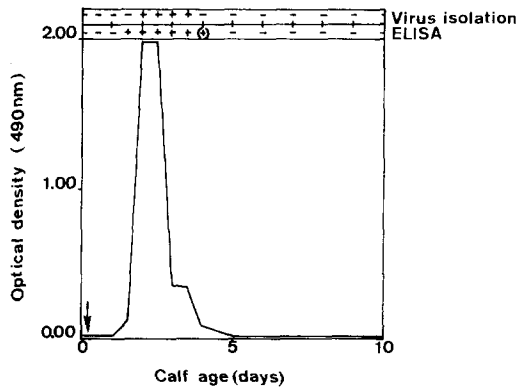


Fig. 4. Results of using an ELISA and virus isolation for detection of bovine rotavirus in the faeces of a SPF calf. Arrow—oral inoculation with bovine rotavirus; + rotavirus positive; - no rotavirus detected.

Table 1. Results obtained by ELISA with monoclonal antibodies, non-commercial available ELISA with polyclonal antibodies, Enzygnost and Slidex Rota Kit

Number of samples	ELISA (monoclonal antibodies)	ELISA (polyclonal antibodies)	Enzygnost	Slidex Rota Kit
7	+	+	+	+
9	-	-	-	-
1	+	+	NT	-
2	+	+	NT	+
1	-	-	NT	-
3	-	-	*	NT
3	-	-	+	NT
6	+	+	+	NT
7	-	-	-	NT
2	+	-	NT	NT
2	-	+	NT	NT
6	+	+	NT	NT
91	-	-	NT	NT
1	-	-	+	**
1	+	+	+	-
1	-	+	-	-
1	+	+	*	-
2	+	-	+	-
1	-	-	+	-
1	-	+	-	-
1	-	+	-	**
1	-	-	*	-

NT—not tested.

*Equivocal results (once positive, once negative).

**Equivocal results (agglutination with the control reagent).

Kit in 94%, 78% and 77% respectively. The Slidex Rota Kit gave 7% of equivocal results: agglutination occurred with both latex particles sensitized with specific antiserum and negative control latex. The Enzygnost showed 11% of equivocal results: in the first well of the microtiter plaque, the result must be considered as positive and in the repetitive one as negative, if one refers to the manufacturer's recommendations.

Samples which produced discrepant results (17%) between ELISA with monoclonal antibodies and Slidex Rota Kit were all positive by ELISA and negative by the other test. Faecal specimens which provided different answers by ELISA with monoclonal antibodies and Enzygnost were all negative by ELISA with monoclonal antibodies and positive by Enzygnost.

As far as the two non-commercial available ELISAs were concerned, half samples giving contradictory results were positive by ELISA with monoclonal antibodies and negative by ELISA with polyclonal antibodies; the other half showed the opposite.

DISCUSSION

We have presented a new diagnostic test for rotavirus in stool specimens. The key diagnostic reagent in this test is monoclonal antibody BR71. This monoclonal antibody showed high reactivity with rotavirus of human (Wa, ST-3, M, DS-1), porcine (OSU, RV277) and bovine (PQ, NCDV, UK, WOODS, 81/36F, 81/40F, S14, S77) origin. By radioimmunoprecipitation, BR71 reacted with VP6, the inner capsid 42,000—molecular weight structural protein product of the sixth rotaviral gene [30]. Subgroup specificity and the common rotaviral antigen are known to be two different antigenic regions of VP6 [31].

The high reactivity of BR71 with both subgroup 1 (DS-1, OSU, NCDV) and subgroup 2 (Wa) rotavirus was indicative of its group specificity and apparent lack of subgroup prejudice. Therefore, BR71 monoclonal antibody would be used to detect rotavirus in bovine faeces as well as in human, porcine and other animal species faecal samples.

BR71 monoclonal antibody was chosen to be used as "capture and detecting" antibody. Greatest sensitivities could be obtained with another pair of monoclonal antibodies (BR71-BR42) but the differences observed were not statistically significant. The use of only one reagent in a sandwich ELISA test would simplify the standardization procedures and would significantly reduce the cost price of the test.

Detection of rotavirus in faecal samples from experimentally inoculated newborn calves showed that the sandwich ELISA test with the chosen monoclonal antibody always gave a negative response with meconium and pre-inoculation faecal samples and a positive response for the stool specimens from which rotavirus have been isolated.

We also compared our assay with the well established and widely used Enzygnost and Slidex Rota Kit tests and with a non-commercial sandwich ELISA test using polyclonal antibodies. The sandwich ELISA test using monoclonal antibodies showed more sensitivity than the agglutination test and was as sensitive as the ELISA tests with polyclonal antibodies to detect rotavirus in routine diagnostic material. The test evaluated showed no equivocal results as Enzygnost and Slidex Rota Kit did.

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