

Comparison of Five Diagnostic Methods for the Detection of Rotavirus Antigens in Calf Faeces

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Abstract. Immunelectroosmophoresis, complement fixation, immunofluorescence on inoculated cell cultures, electron microscopy, and an enzyme-linked immunosorbent assay were compared for the detection of rotavirus in faecal samples from calves. Rotavirus particles could be detected in 39 out of 98 faecal samples by electron microscopy. Immunofluorescence, complement fixation, and immunelectroosmophoresis detected rotavirus antigens in 39, 42, and 30 samples, respectively. The enzyme-linked immunosorbent assay demonstrated rotavirus antigens in 49 faecal samples. The assay is not only sensitive but also simple to perform and suitable for large-scale testing.

Introduction

Rotavirus has been implicated as one of the major causative agents in newborn calf diarrhoea [2, 8, 18]. Because many strains of the virus do not grow well in cell culture [18], the diagnosis is based on direct detection in the faeces. Negative contrast electron microscopy has proved to be a reliable method of diagnosis [8]. However, the method is not suitable for the study of large numbers of specimens and its applicability is limited by the lack of general availability and of the expertise needed to perform this technique. A definite need therefore exists for other diagnostic techniques suitable for etiological and epidemiological studies. We have compared a number of previously described techniques with respect to their sensitivity, simplicity, and applicability for large-scale testing. These techniques are immunelectroosmophoresis (IEOP) [5, 9, 15], a complement-fixation test (CFT) [14], an immunofluorescence test (IFT) on inoculated cell cultures [2], and an enzyme-linked immunosorbent assay (ELISA) [3, 13, 19]. In this report we present the results of this study.

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Materials and Methods

Faecal Specimens

Faecal samples were collected from the rectum of calves on three dairy farms on the 2nd and 4th days of life and later when diarrhoea developed. All calves were observed daily for 14 days. Twenty-two samples were obtained from 12 calves that did not develop diarrhoea and 76 samples from 25 calves that had diarrhoea for one or more days. Faecal samples were stored at -20°C. After thawing they were treated ultrasonically in the presence of Genetron 113 (Fluka) as described before [4] and the extract was used as test sample in all assays.

Antiserum Production

Rotavirus was purified from calf faeces as described [7] and emulsified in an equal volume of complete Freund's adjuvant. A volume of 2 ml, containing 2×10^9 rotavirus particles, was injected, divided over four sites, intramuscularly into rabbits. Five weeks later 1 ml was injected intradermally, again divided over four sites. This procedure was repeated twice with weekly intervals. The complement-fixing antibody titer [7] of the hyperimmune rabbit-anti-calf-rotavirus (RArota) serum was 1:1024. Calf-anti-calf-rotavirus (CArota) serum was produced in a colostrum-deprived SPF calf. Three months after vaccination with rotavirus vaccine¹, the calf was injected intramuscularly with 2 ml purified rotavirus, emulsified in complete Freund's adjuvant: 3 weeks later this was followed by intravenous inoculation of 1 ml purified virus. Seven days later the calf was bled for serum.

The complement-fixing antibody titer of the CArota serum was 1:512.

Immunoelectroosmophoresis (IEOP)

The technique employed was similar to that used by Tufvesson and Johnsson [15]. A solution of 0.75% agarose (l'Industrie Biologique Francaise) in barbital tank buffer (0.075 M barbital pH 8.6, including 1.87 mM calcium lactate) was poured on microscope slides. On each slide three troughs (3 x 15 mm) and nine wells (4mm in diameter) were cut. The distance between the trough and the wells was 5 mm. The troughs were closest to the anode and filled with undiluted RArota serum. The wells were filled with undiluted faecal extracts. After electrophoresis for 75 min at 7 V/cm, the slides were washed overnight in tank buffer and then covered with moist filter paper, dried in air at 37°C and stained with 0.5% Coomassie Brilliant Blue in acetic acid-ethanol-water 1:4.5:4.5 for 10 min; excess stain was removed by a threefold rinse in acetic acid-methanol-water 1:4.5:4.5. Samples showing a precipitation line at approximately equal distances from the trough and the well were scored positive.

Complement Fixation Test (CFT)

The CFT was carried out in microtiter plates according to standard laboratory techniques. Faecal extracts were serially diluted in twofold steps in veronal buffer pH 7.2. Two dilution series per sample were made. To one series inactivated RArota serum was added, which was anticomplementary up to the 1:64 dilution, in a dilution of

¹ Scourvax-reo, kindly supplied by Dr. N. Zygraich, R.I.T., Rixensart, Belgium

1:128. The other series received only veronal buffer. Antigen and serum were incubated for 20 min at room temperature and 60 min at 4°C before complement was added. This mixture was incubated overnight at 4°C before the haemolytic system was added. End points were taken as the dilution leading to 50% haemolysis. Samples were scored positive when the end point in the presence of serum was reached at at least a fourfold higher antigen dilution than the end point in the presence of buffer.

Immunofluorescence Test (IFT)

Secondary bovine embryonic kidney (BEK₂) monolayers were grown on coverslips in Leighton tubes in Hanks' BSS containing 10% foetal calf serum (FCS), 0.5% LAH, and appropriate antibiotics. Monolayers were inoculated in duplicate with 0.3-ml volumes of faecal extracts diluted 1:5 in Hanks' BSS. After 1-hour incubation at 37°C the cells were washed and maintenance medium (Eagle) with 2% FCS was added. After inoculation with the faecal extracts the tubes were kept in a roller tube drum. After 20 h at 37°C the cultures were washed with PBS, fixed in acetone, and stained with a 1:35 dilution of FITC conjugated RArota serum. This was followed by washing in PBS before mounting in buffered glycerol and examination with an UVmicroscope for the presence of fluorescent cells.

Electron Microscopy (EM)

For negative contrast EM the faecal extracts were applied to carbon-coated grids and stained with a solution of 2% PTA adjusted to pH 6.2 with KOH. Ten squares of a 400-mesh grid were examined using a Jeol 100C electron microscope at a magnification of 50,000 for the presence of rotavirus particles.

Enzyme-Linked Immunosorbent Assay (ELISA)

Coating: The globulin fraction of the CArota serum was prepared by precipitation of serum with 50% saturated ammonium sulfate. The precipitate was washed twice, dissolved in distilled water, and dialysed against PBS overnight at 4°C. Wells of a polystyrene microtiter plate (Cooke) were coated with 250 µl of the globulin fraction diluted 1:2500 in 0.05 M carbonate buffer pH 9.6. After an incubation period of 18 h at 37°C the plates were frozen and stored at -20°C. Prior to use the plates were thawed and rinsed three times with deionized water containing 0.05% Tween 80.

Conjugate: Conjugation of peroxidase with the globulin fraction of the CArota serum was carried out essentially as described by Avrameas and Ternynck [1]. Ten mg of horseradish peroxidase (grade I, Boehringer) were dissolved in 0.2 ml 0.1 M phosphate buffer pH 6.8 containing 1.25% glutaraldehyde (Merck). After standing overnight at room temperature the unreacted glutaraldehyde was removed by exclusion chromatography in Sephadex G25 (Pharmacia Ltd.). The brown-coloured fractions were pooled (approximately 1 ml) and mixed with 0.5 ml of the globulin fraction of the CArota serum, and then 0.15 ml 1 M carbonate buffer pH 9.6 was added. After the coupling reaction had proceeded for 18 h at 4°C the unreacted glutaraldehyde was blocked by addition of 0.15 ml 0.2 M lysine. The conjugate was precipitated in 50% saturated ammonium sulfate, washed, dissolved in 0.2 ml PBS, and dialysed overnight against PBS. The conjugate was stored in 20-µl quantities at -20°C and prior to use diluted in PBS containing 0.5 M NaCl, 0.05% Tween 80, and 5% FCS. The optimal dilution of the conjugate was determined by checkerboard titration to be 1:2500.

Substrate: The enzyme substrate used was hydrogen peroxide plus 5-aminosalicylic acid (5-AS, Merck). 5-AS was purified by adding equal amounts of 5-AS and $\text{Na}_2\text{S}_2\text{O}_5$ to distilled water of 80°C until saturation. After filtration through charcoal and cooling to 4°C the white precipitate was collected, dried, and stored in the dark.

Before use 100 mg of the purified 5-AS were dissolved in 90 ml hot distilled water. After cooling, the solution was brought to pH 6.0 with 1 M NaOH and 10 ml 0.05% hydrogen peroxide was added.

Assay proper: The ELISA was essentially carried out as described for plant viruses [16] and hepatitis B surface antigen [17]. Volumes of 200 µl of undiluted faecal extracts were added to the wells in duplicate. Each plate contained a twofold serial dilution of a standard positive faecal extract and a PBS-control. After an incubation period of 3 h at 37°C the plates were washed three times, and 200 µl of the conjugate was added. A further incubation period of 1 h at 37°C was followed by a threefold rinse and addition of the substrate. The plates were read by visual inspection 1 h and 18 hours after the substrate was added, using the serially diluted standard positive sample and the blank as reference.

Blocking assay: All samples scored positive in the direct assay were examined with the blocking assay to demonstrate the specificity of the ELISA. The blocking tests were performed with serum obtained from a gnotobiotic calf hyperimmunized with purified rotavirus (kindly donated by Dr. J.C. Bridger). The serum was used 1:100 diluted in PBS, containing 0.5 M NaCl and 0.05% Tween 80 (PBSST). The coated plates were incubated with the test samples as described above, however three wells were used per sample. To the first well 200 µl PBSST, to the second well 200 µl of a rotavirus negative serum 1:100 diluted in PBSST, and to the third well 200 µl of the gnotobiotic calf-anti-rotavirus serum 1:100 diluted in PBSST were added. After an incubation period of 1 h at 37°C and an additional washing procedure, the assay was carried out as described above and examined for rotavirus-specific blocking.

Results

From 98 faecal samples 30 were scored positive for rotavirus antigens by IEOP. In 39 faecal samples virus was detected by IFT and EM, whereas the CFT demonstrated the presence of the agent in 42 samples. The ELISA detected viral antigens in 49 samples (Fig. 1). No rotavirus antigens were detected by the techniques in 40 samples. The 30

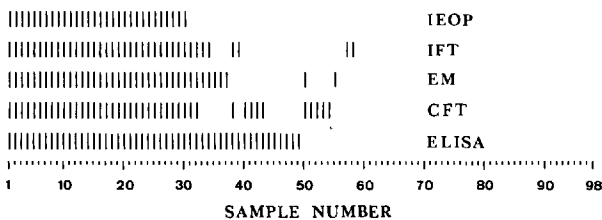


Fig. 1. Detection of rotavirus or rotavirus antigens in 98 faecal samples from calves with IEOP, IFT, EM, CFT, and ELISA

samples scored positive for rotavirus by IEOP were confirmed by all other techniques used. Two out of 39 samples scoring positive by IFT, were not confirmed by any of the other techniques. These two samples, derived from calves that did not develop diarrhoea during the observation period, led to only a few fluorescent cells in BEK₂ cell cultures. In one sample a few rotavirus particles were detected by EM. All other techniques failed to demonstrate the presence of the virus in this sample. In four cases the CFT result was not confirmed by one of the other techniques. The ELISA detected rotavirus antigens in 49 faecal samples, all confirmed by the results of the blocking assay. Eleven other samples were scored weakly positive in the direct assay, but could not be confirmed in the blocking assay. These samples were considered to be negative. Of the 49 samples scored positive by the ELISA, 43 were confirmed by at least one of the other techniques (Fig. 1).

ELISA, CFT, EM, IFT, and IEOP demonstrated the presence of rotavirus or rotavirus antigens in faecal samples of 24, 22, 21, 21, and 18 calves that developed diarrhoea, respectively. Rotavirus antigens were detected by ELISA in the faeces of two calves that remained healthy, and in the faeces of two other calves by IFT.

Discussion

Electron-microscopic examination showed that faecal samples from calves (Ellens, unpublished) and pigs [12] may contain rotavirus-antibody complexes. These samples often failed to cause fluorescence in inoculated BEK₂ cell cultures. Ultrasonic treatment in the presence of Genetron thoroughly homogenized faecal samples and increased the percentage of samples causing fluorescence. It also reduced the anticomplementary activity of the test samples and resulted in a 'cleaner' preparation for EM examination. The sensitivity of the different tests was not influenced or was enhanced by the use of treated instead of untreated specimens (Ellens, unpublished).

Faecal samples of SPF calves raised in isolation sometimes gave rise in the IEOP to precipitation lines close to the antibody trough. Precipitation lines at approximately equal distances from the antibody trough and the antigen well were never observed using these samples. Thus, only these lines were considered specific for the presence of rotavirus antigens. Use of CArota serum instead of RArota serum did not improve the sensitivity of the IEOP [4]. The IEOP is easy to perform and can be used for the examination of large numbers of samples. However the results are not rapid available and small amounts of antigen may not be detected. This same conclusion was reached by others working with human rotavirus [9,14]. No further attempts were made to improve the sensitivity of this test. It is conceivable, however, that the use of a rabbit serum prepared against immunoprecipitates [5] may improve the results.

Tubes containing inoculated cell cultures for diagnosis by immunofluorescence were always rolled. Compared to stationary cultures, more samples were scored positive and the number of cells showing fluorescence after staining was greatly enhanced. As mentioned before, the way test samples were treated also influenced the results obtained. No difference was found when staining was performed 1 or 3 days after inoculation. The IFT appears to be useful as a diagnostic method, although laborious when large numbers of samples have to be examined.

Detection of rotavirus in faeces by complement-fixation has been hampered by the anticomplementary activity present in faecal samples from calves and in human stools

[9, 15]. This problem can be overcome by comparison of the results in the presence of buffer and serum and by treatment of the samples with Genetron. The sensitivity of the CFT for the detection of rotavirus in faeces compares favourably with that of EM. However, it is difficult to rule out the possibility of occasional false positive results.

Of the techniques compared in this study, ELISA appears to be the most promising. Its sensitivity compares favourably with that of the other techniques, and its specificity can easily be checked by the blocking assay. Serial dilution experiments, using the standard positive faecal extract, showed that rotavirus antigens could be detected by ELISA in a dilution of 1:5000, compared to 1:32 in the IEOP and 1:128 in CFT [4]. The blocking assay requires an additional incubation step of 1 h at 37°C in comparison with the direct ELISA. During this period the antigen-antibody complexes in the cups are exposed to PBS containing 0.5 M NaCl and 0.05% Tween 80. This may explain the fact that by the use of this procedure rotavirus antigens in the standard positive faecal extract could only be detected up to the 1:2500 dilution, i.e., a twofold loss in sensitivity. The same effect was also observed when other samples were used. Thus weakly positive samples in the direct assay may be negative in the blocking assay: the 11 faecal samples found weakly positive in the direct assay but scored negative in the blocking assay, in fact remain doubtful.

In addition to its sensitivity the ELISA has several other advantages. It can be used to examine a large number of samples and the results are available the same day. All reagents used are stable. Expensive equipment is not required and reading can be done by the naked eye.

Two other methods described for diagnosis of rotavirus infections were not included in this study, a solid phase-radioimmunoassay (RIA) [6,10] and a fluorescent virus precipitin test [11]. Both tests were reported to be as sensitive as EM. For large-scale testing the fluorescent precipitin test seems rather laborious. The RIA appears suitable for studying large numbers of faecal specimens in a short period, but the need for unstable radioactive reagents and an expensive counting device render this technique impractical in many situations.

The results of this study indicate that, until some sensitive tissue culture method is available, ELISA is the most useful technique available for diagnosis of rotavirus infections in calves.

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