Enzyme-Linked Immunosorbent Assay for Diagnosis of Rotavirus Infections in Calves

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Received for publication 27 June 1977

An enzyme-linked immunosorbent assay for the diagnosis of rotavirus infection in calves is described. The assay was more efficient for the detection of rotavirus antigens in calf feces than were electron microscopy and immunoelectroosmophoresis.

Infections with serologically related rotaviruses (15) are associated with diarrhea in humans (2, 9) and animals (3, 18). Infections generally are diagnosed by electron microscopy (EM) (2, 3), although several other methods have been described. None of these, including immunofluorescence tests performed on fecal smears (11) or inoculated cell cultures (3), a complement fixation test (14), a fluorescent virus precipitin test (8), or immunoelectroosmophoresis (IEOP) (9, 12, 16), proved substantially more sensitive than EM for the detection of the virus in stools.

In this report, we describe an enzyme-linked immunosorbent assay (ELISA) for the detection of rotavirus antigens in fecal suspensions. The results obtained with this technique are compared with those of EM and IEOP.

The ELISA was first described by Engvall and Perlmann (7). We have used the "double-antibody sandwich" form of ELISA for the detection of viruses (4, 17). Polystyrene cuvettes (LKB, Produkter, AB, Stockholm, Sweden), used as a solid-phase adsorbent, were coated overnight at 37°C with specific antibody. As the source of antibody, the globulin-containing fraction of a hyperimmune calf serum against calf rotavirus, diluted 1:2,500 in 0.05 M bicarbonate buffer (pH 9.6), was used. The antibody-coated cuvettes were washed three times with 0.05% Tween 80 (Merck) in distilled water and were subsequently incubated for 3 h at 37°C with the test samples in duplicate. These samples were 1-ml volumes of fecal extracts prepared as follows: to 1 volume of fecal material was added 4 volumes of 0.15 M phosphate-buffered saline, pH 7.2, containing 0.05% Tween 80 and 2.5 volumes of Genetron 113 (Fluka). The mixtures were homogenized by ultrasonic treatment, and, after low-speed centrifugation, the clear supernatant fluids were used as test samples. After the incubation period, the cuvettes were rinsed three times with 0.05% Tween 80 in distilled

water. The conjugate, horseradish peroxidase (grade I, Boehringer) coupled with calf anti-rotavirus immunoglobulin G by a two-step method using glutaraldehyde as a cross-linking agent (1), was diluted in phosphate-buffered saline-Tween 80 containing 5% fetal calf serum (Flow) and added to the cuvettes. The optimal dilution of the conjugate was 1:2,500, as determined by checkerboard titration. After an incubation period of 1 h at 37°C and a further washing step, 1 ml of the enzyme substrate solution, containing 1 mg of 5-aminosalicylic acid (recrystallized in the presence of Na₂SO₃) and 0.005% hydrogen peroxide adjusted to pH 6.0, was added.

After the reaction had proceeded for at least 1 h at room temperature, the extinction of the reaction product was measured in a colorimeter (Vitatron) at 474 nm. A sample was scored positive if its E_{474} value was equal to or higher than three times that of the negative control (phosphate-buffered saline instead of fecal extract). EM of fecal samples was done as described elsewhere (5). After examining 10 squares of a 400-mesh grid, the sample was scored positive or negative. IEOP for the detection of rotavirus antigen in fecal suspensions was carried out essentially as described by Tufvesson and Johnson (16), using a hyperimmune rabbit serum prepared against purified calf rotavirus. Samples showing a precipitation line at approximately equal distances from the antigen and antibody wells were scored positive.

Fecal specimens were from dairy calves sampled on days 2 and 4 after birth and, later, when diarrhea developed. In the first set, of 98 fecal samples, EM, IEOP, and ELISA detected rotavirus antigens in 39, 30, and 49 samples, respectively. From the 39 samples scored positive for rotavirus by EM, 37 were detected by ELISA and 29 were detected by IEOP (Table 1).

A second set, of 367 fecal samples, was examined by IEOP and ELISA. With the ELISA, 110 samples were scored positive, of which 54

were scored positive by IEOP (Table 2). In 5 ELISA-negative samples, a faint precipitation line could be observed in the IEOP. Thus, of a total of 465 specimens, the IEOP detected rotavirus antigens in only 53% of the samples that were positive in the ELISA. Although 2 samples scored positive with EM and 5 scored positive with IEOP were not confirmed by the ELISA, the results indicate that the ELISA is a more efficient technique than EM and IEOP for the detection of rotavirus antigens in fecal extracts.

The detection limit of the ELISA with calf antibody was investigated, using serial dilutions

TABLE 1. Detection of rotavirus antigen with EM, ELISA, and IEOP in 98 faecal samples from calves

No. of samples	Rotavirus antigen detected ^a		
	EM	ELISA ^b	IEOP
2	+	—	—
8	+	+	—
29	+	+	+
0	+	—	+
11	—	+	—
1	—	+	+
0	—	—	+
47	—	—	—

^a +, Detected; —, not detected.

^b ELISA was performed in cuvettes coated with antibody obtained from an SPF calf hyperimmunized with homologous rotavirus.

of fecal extract and a purified rotavirus preparation. Rotavirus was detected in the fecal extract diluted 1:5,000, corresponding to about 10^6 rotavirus particles per ml as determined by semi-quantitative EM (13) performed on the undiluted specimen (Fig. 1a). With IEOP, viral antigens were detected at a dilution of 1:32 with the rabbit hyperimmune serum and at a dilution of 1:4 with the calf hyperimmune serum. In a gradient-purified viral preparation (5), rotaviruses could be detected with the ELISA at a concentration of 10^7 particles per ml (Fig. 1b), as determined with quantitation EM on the undiluted material. These results suggest that fewer rotavirus particles may be detectable by ELISA in crude material than in purified material. However, the composition of the material may influ-

TABLE 2. Detection of rotavirus antigen with ELISA and IEOP in 367 faecal samples from calves

No. of samples	Rotavirus antigen detected ^a	
	ELISA ^b	IEOP
54	+	+
56	+	—
5	—	+
252	—	—

^a +, detected; —, not detected.

^b ELISA was performed in cuvettes coated with antibody obtained from an SPF calf hyperimmunized with homologous rotavirus.

FIG. 1. (a) ELISA extinction values for a serial dilution of a fecal extract. E_{474} was measured after the enzyme reaction had proceeded for 1 h (·····) and 18 h (-----); the E_{474} values of the negative control measured 0.02 and 0.09, respectively. (b) ELISA extinction values for a purified rotavirus preparation diluted in phosphate-buffered saline-Tween 80. E_{474} was measured after the enzyme reaction had proceeded for 1 h (·····) and 18 h (-----); the E_{474} values of the negative control measured 0.04 and 0.12, respectively.

ence the reliability of quantitation EM. Further work is in progress to determine more precisely the amount of viral antigen present at the detection limit of the ELISA.

The use of the ELISA as described here is not restricted to the diagnosis of calf rotavirus infections, but it can also be used for the diagnosis of rotavirus infections in humans (6). In our hands, the ELISA proved to be a reproducible, rapid, and quantitative method for the detection of rotavirus antigens. The detection limit of this technique is about 1 ng of viral protein per ml, and its sensitivity for the detection of rotavirus antigens in fecal homogenates is about 100-fold higher than that of IEOP. The test can also be carried out in round-bottomed polystyrene microtiter plates (Cooke), which can be read by the naked eye. The microtiter procedure is particularly suited for the large-scale testing of field samples that may be required for etiological and epidemiological investigations.

We thank J. A. M. van Balken, A. P. Timmer, and P. de Kreek for technical assistance.

LITERATURE CITED

1. Avrameas, S., and T. Ternynck. 1971. Peroxidase labeled antibody and F_{ab} conjugates with enhanced intracellular penetration. *Immunochemistry* 8:1175-1179.
2. Bishop, R. F., G. P. Davidson, J. H. Holmes, and B. J. Ruck. 1974. Detection of a new virus by electron microscopy of faecal extracts from children with acute gastroenteritis. *Lancet* ii:149-150.
3. Bridger, J. C., and G. N. Woode. 1975. Neonatal calf diarrhoea: identification of a reovirus like (rotavirus) agent in faeces by immunofluorescence and immune electron microscopy. *Br. Vet. J.* 131:528-535.
4. Clark, M. F., and A. N. Adams. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. Gen. Virol.* 34:475-483.
5. de Leeuw, P. W., A. P. K. M. I. van Nieuwstadt, J. A. M. van Balken, and D. J. Ellens. 1977. Rotavirus infections in calves. II. Experimental infections with a Dutch isolate. *Neth. J. Vet. Sci.* 102:515-524.
6. Ellens, D. J., and P. W. de Leeuw. 1977. Detection of infantile gastroenteritis virus (rotavirus) by ELISA. *Lancet* i:1363.
7. Engvall, E., and P. Perlmann. 1972. Enzyme-linked immunosorbent assay (ELISA). III. Quantitation of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen-coated tubes. *J. Immunol.* 109:129-135.
8. Foster, L. G., M. W. Peterson, and R. S. Spendlove. 1975. Fluorescent virus precipitin test. *Proc. Soc. Exp. Biol. Med.* 150:155-160.
9. Grauballe, P. C., J. Genner, A. Meyling, and A. Hornsleth. 1977. Rapid diagnosis of rotavirus infections: comparison of electron microscopy and immunoelectrophoresis for the detection of rotavirus in human infantile gastroenteritis. *J. Gen. Virol.* 35:203-218.
10. Kapikian, A. Z., H. W. Kim, R. G. Wyatt, W. L. Cline, J. A. Arrobio, C. D. Brandt, W. J. Rodriguez, D. A. Sack, R. M. Chanock, and R. H. Parrott. 1976. Human reovirus-like agent as the major pathogen associated with "winter" gastroenteritis in hospitalized infants and young children. *N. Engl. J. Med.* 294:965-972.
11. Mebus, C. A., N. R. Underdahl, M. B. Rhodes, and M. J. Twiehaus. 1969. Calf diarrhea (scours): reproduced with a virus from a field outbreak. *Nebr. Agric. Exp. Stn. Res. Bull.* 233.
12. Middleton, P. J., M. Petric, C. M. Hewitt, M. T. Szymanski, and J. S. Tam. 1976. Counter-immunoelectrophoresis for the detection of infantile gastroenteritis virus (orbi-group) antigen and antibody. *J. Clin. Pathol.* 29:191-197.
13. Monroe, J. H., and P. M. Brandt. 1970. Rapid semi-quantitative method for screening large numbers of virus samples by negative staining electron microscopy. *Appl. Microbiol.* 20:259-262.
14. Spence, L., M. Fauvel, S. Bouchard, L. Babiuk, and J. R. Saunders. 1975. Test for reovirus-like agent. *Lancet* ii:322.
15. Thouless, M. E., A. S. Bryden, T. H. Flewett, G. N. Woode, J. C. Bridger, D. R. Snodgrass, and J. A. Herring. 1977. Serological relationships between rotaviruses from different species as studied by complement fixation and neutralization. *Arch. Virol.* 53:287-294.
16. Tufvesson, B., and T. Johnsson. 1976. Immunoelectrophoresis for detection of reo-like virus: methodology and comparison with electron microscopy. *Acta Pathol. Microbiol. Scand. Sect. B* 84:225-228.
17. Voller, A., A. Bartlett, D. E. Bidwell, M. F. Clark, and A. N. Adams. 1976. The detection of viruses by enzyme-linked immunosorbent assay (ELISA). *J. Gen. Virol.* 33:165-176.
18. Woode, G. N., and J. C. Bridger. 1975. Viral enteritis of calves. *Vet. Rec.* 96:85-88.