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Fluorescent Virus Precipitin Test (38994)

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The observation of antibody-aggregated virus particles is the basis of immunoelectron microscopy (IEM). The agents for which IEM has been used include: rubella (1), hepatitis (2), rhinovirus (3), enteroviruses (4), coronavirus (5), and reovirus-like particles (6). The expense encountered in the purchase, maintenance, and use of an electron microscope, however, has made IEM impractical for the majority of diagnostic medical laboratories.

The present study describes a new, rapid serologic test that uses epifluorescence microscopy to facilitate the detection of antibody aggregated virus particles. The test system involves the detection of neonatal calf diarrhea virus (NCDV) in the feces of scouring calves. The test is, however, applicable to other viruses.

Materials and methods. Specimen preparation. Fecal specimens were collected from scouring calves and held at −20°. After storage, the stools were thawed and mixed with equal volumes of distilled water. The mixtures were centrifuged at 5000 rpm (3020*g*) for 10 min using the SS34 rotor in a Sorvall RC-2B centrifuge. The supernatant fluids were decanted and passed through a Pop-Top adaptor (Nucleopore Corp.) holding a 13-mm diameter Millipore membrane filter (Millipore Corp.) with a 0.45-μm pore size.

Fluorescent virus precipitin test (FVPT). A 0.2 ml sample of each filtrate was incubated for 1 hr at 37° with an equal volume of a prefiltered (0.45-μm pore size Millipore) dilution of anti-NCDV conjugate. The fluorescein conjugate, prepared by Norden Laboratories, Lincoln, Nebr., was diluted until optimal conditions were obtained, as determined by using a known positive sample as seen in Table I. After incubation, the tubes were spun at 2000 rpm (700*g*) for 10 min in a Sorvall GLC-1 centrifuge fitted with a HL-4 swinging bucket rotor. The tubes were removed from the centrifuge and gently shaken, and one drop of the contents was placed on a clean glass microscope slide. A coverglass was pressed gently but firmly over the drop before observing with a 100X oil immersion objective and 12.5X wide-angle eyepieces on a Zeiss microscope model GFL. The microscope was equipped with a iv F vertical illuminator and a 2FL reflector housing containing a chromatic reflector LP520, excitor filters KP490 and KP500, and optical barrier filter LP520.

Control tubes consisted of 0.2 ml of distilled water and an equal volume of conjugate.

Brightly fluorescent aggregates of varying sizes appeared in the positive samples. Samples scored as negative displayed only dull background fluorescence devoid of aggregates.

To remove the possibility of bias during the reading of the tests, all preparations were read as unknowns.

Cross-reactions. Goat anti-reovirus and pony anti-*Mycoplasma arthritidis* fluorescent antibody (conjugated with fluorescein isothiocyanate by a technique described elsewhere (7)), and the anti-NCDV conjugate were used in the cross-reaction test. A dilution (0.2 ml) of each of the three conjugates was added, respectively, to three duplicate sets of test tubes. To three of these tubes, each containing a different antiserum, was added 0.2 ml of a reovirus (type 1 Lang strain) suspension containing 2×10^6 infectious particles that had been filtered through a 0.45-μm pore size filter. To the remaining three tubes, was added 0.2 ml of filtered NCDV whose titer was 10^6 infectious particles per milliliter. Titers of the viruses were obtained by the immunofluorescent cell assay (8, 9). The remaining parts of this test followed the FVPT protocol.

Blocking reaction. Unlabeled anti-NCDV serum was added to equal amounts (0.2 ml) of the fecal extracts and incubated for 1 hr at 37°. After incubation, 0.4 ml of diluted

TABLE I. DETERMINATION OF OPTIMAL CONDITIONS FOR CONJUGATE CONCENTRATION, FECAL EXTRACT DILUTION, AND TEMPERATURE.

Dilution fecal extract	Dilution conjugate	Aggregates/field[a]	Aggregate size[b]	Background fluorescence
37°				
1:2	1:10	0.25	Large	Marked
		6	Small	
	1:20	3	Large	Moderate
	1:40	2	Medium	Minimal
	1:80	1	Medium	None
	1:160	0.20	Small	None
1:4	1:10	7	Small	Marked
	1:20	0.25	Large	Moderate
		3	Small	
	1:40	0.33	Medium	Minimal
	1:80	0.25	Large	None
	1:160	0.125	Small	None
1:8	1:10	0.25	Large	Marked
		7	Small	
	1:20	0.33	Medium	Moderate
	1:40	0.25	Medium	Minimal
	1:80	0.33	Small	None
	1:160	0.17	Small	None
20°				
1:2	1:10	7	Small	Marked
	1:20	0.17	Medium	Moderate
	1:40	0.25	Medium	Minimal
	1:80	0.20	Small	None
	1:160	0	—	None
1:4	1:10	7	Small	Marked
	1:20	0.17	Medium	Moderate
	1:40	0.143	Medium	Minimal
	1:80	0.143	Medium	None
	1:160	0	—	None
1:8	1:10	6	Small	Marked
	1:20	1	Small	Moderate
	1:40	0.20	Small	Minimal
	1:80	0.143	Small	None
	1:160	0	—	None
42°				
1:2	1:10	0.25	Large	Marked
		2	Small	
	1:20	1	Medium	Moderate
	1:40	0.5	Medium	Minimal
	1:80	0.33	Small	None
	1:160	0.2	Small	None
1:4	1:10	3	Small	Marked
	1:20	1	Small	Moderate
	1:40	0.5	Small	Minimal
	1:80	0.2	Small	None
	1:160	0.143	Small	None
1:8	1:10	6	Small	Marked
	1:20	1	Small	Moderate
	1:40	0.33	Small	Minimal
	1:80	0.2	Small	None
	1:160	0.143	Small	None

[a] Average number of aggregates from 10 microscopic fields.

[b] Size was scored by determining the largest dimension of an aggregate and placing them in gradations as follows: large = >10 μm, medium = 2–10 μm, small = <2 μm.

anti-NCDV conjugate was added to the mixture. Following an additional 1-hr incubation period, the tubes were centrifuged, slides were prepared, and epifluorescence observations were made as described in the FVPT procedure.

Indirect immunofluorescence. A 0.2-ml sample of the fecal extract was incubated at

TABLE II. AGGREGATION OF VIRUS PARTICLES BY HOMOLOGOUS AND HETEROLOGOUS CONJUGATED ANTIBODY.

Antigen[a]	Conjugate	Final conjugate dilution	Presence of aggregates[b]
Reovirus	Anti-reovirus	1:80	Positive
Reovirus	Anti-NCDV	1:40	Negative
Reovirus	Anti-*M. arthritidis*	1:20	Negative
NCDV	Anti-reovirus	1:80	Negative
NCDV	Anti-NCDC	1:40	Positive
NCDV	Anti-*M. arthritidis*	1:20	Negative

[a] Infectious particles per milliliter: reovirus 10^7; NCDC 10^6.
[b] Determined by FVPT.

37° for 1 hr with 0.2 ml of a 1:40 dilution of adult bovine serum previously shown to be positive for NCDV antibody to at least 1:128 by IEM. After incubation, the mixture was centrifuged 1 hr at 12,000 rpm (17,300*g*) in a Sorvall SS34 rotor. The supernatant fluids were removed carefully using a Pasteur pipette and the resultant pellet was resuspended in 0.2 ml of a 0.15 *M* saline solution. To the resuspended material was added 0.2 ml of various dilutions of fluorescein conjugated rabbit antibovine serum prepared by Microbiological Associates, Bethesda, Md. This mixture was incubated 1 hr at 37°, spun at 2000 rpm (700*g*) for 10 min and observed.

Immunoelectron microscopy. IEM was used in performance comparisons and to confirm the presence of virus particles in the aggregates. Using the method of Vassall and Ray (10), electron microscope grids were dipped into virus–antibody mixtures and observed.

Results. The optimal temperature, concentration of virus, and dilution of labeled antibody for aggregation of NCDV extracted from fecal specimens were determined (Table I). The incubation temperature and fecal extract dilution of choice for all systems were 37° and 1:2 respectively A final conjugate dilution of 1:40 proved superior in these tests. However, because different conjugates vary in quality, there is no single optimal dilution. This parameter must be determined each time a different conjugate is used. Not only were the numbers and sizes of aggregates under these test conditions sufficient to allow rapid and easy positive scoring, but the background fluorescence was low.

In cross-reaction tests, all of the homologous systems were positive, whereas all of the heterologous pairings were negative (Table II).

Centrifugation of NCDV antibody mixtures at 400*g* increased the sensitivity of the FVPT (Table III). After centrifugation, the FVPT results correlated perfectly with those obtained by IEM. Increasing speed or duration of centrifugation did not result in false positive tests.

Specificity of the FVPT was tested by the blocking reaction and indirect immunofluorescence. Unlabeled antibody at low dilution blocked staining of virus aggregates; consequently, no aggregates were observed (Table IV). Indirect immunofluorescence proved to be easily adaptable to this test system (Table V).

Figure 1 shows photomicrographs of the viral aggregates as seen by epifluorescence microscopy accompanied by a corresponding electron micrograph prepared from the same specimen. If viewed at the same magnification, the largest aggregate in Fig. 1a would be slightly smaller than the aggregate shown in Fig. 1c.

Discussion. The results of this study demonstrate that reliable detection of virus–antibody complexes does not require an electron microscope. Any laboratory that has an epifluorescence microscope and a low-speed centrifuge can run valid tests. An epifluorescence microscope is essential because with it, the intensity of exciting light increases as the magnifying power of the objective lens is increased. By contrast, with fluorescence microscopes that use trans-

TABLE III. INFLUENCE OF CENTRIFUGATION ON AGGREGATION OF NCDV IN CALF FECAL EXTRACTS.

Sample	IEM	FVPT			
		Without centrifugation	180 *g*	400 g	700 *g*
1	Negative	Negative	Negative	Negative	Negative
2	Positive	Positive	Positive	Positive	Positive
3	Negative	Negative	Negative	Negative	Negative
4	Negative	Negative	Negative	Negative	Negative
5	Positive	Negative	Negative	Positive	Positive
6	Negative	Negative	Negative	Negative	Negative
7	Negative	Negative	Negative	Negative	Negative
8	Positive	Positive	Positive	Positive	Positive
9	Negative	Negative	Negative	Negative	Negative
10	Negative	Negative	Negative	Negative	Negative
11	Positive	Positive	Positive	Positive	Positive
12	Positive	Negative	Positive	Positive	Positive

mitted light, the intensity of exciting light decreases as the magnification is increased.

The FVPT permits rapid viral aggregate detection that is almost as sensitive as IEM. A positive IEM test requires observation of aggregates of three or more viral particles (11). Size of the viral aggregates is decisive in defining the sensitivity of the FVPT. NCDV measures 70–75 nm. Three of these particles aligned in a linear arrangement would have a dimension that is easily resolved at a magnification of 1250X. In almost all aggregates containing more than eight or nine NCDV particles, the virus–antibody complexes would have two dimensions over 0.2 μm and they would be visible. Aggregates of the smallest viruses would have to be $\sim$10 viruses wide to be visible; this would probably require complexes of several hundred viruses in order to be observed in the FVPT.

Any limitation of the FVPT due to inherent resolution-power problems can be partially offset by the increased volume of sample examined. The FVPT as described examines $\sim$0.05 ml of a sample, whereas the IEM procedure examines only the amount of sample adhering to an electron microscope grid.

Specificity as well as sensitivity is an important consideration in any new serologic procedure. Specificity of the FVPT was demonstrated using the blocking reaction, noting cross-reactions with heterologous reactants, comparing the results with the IEM test, and by an indirect staining procedure.

TABLE IV. SPECIFICITY TESTING BY BLOCKING WITH UNLABELED ANTIBODY.

Final dilution unlabeled antiserum	Final dilution conjugated antiserum	Aggregates present
1:20	1:40	Negative
1:40	1:40	Negative
1:80	1:40	Positive[a]

[a] Only dimly fluorescing aggregates were seen.

TABLE V. SPECIFICITY TESTING BY INDIRECT IMMUNOFLUORESCENCE STAINING.

Final dilution unlabeled antiserum	Final dilution conjugated antiserum	Aggregates present
1:80	1:40	Positive
1:80	1:80	Positive
1:80	1:160	Negative
1:80	1:320	Negative

One problem encountered in any fluorescent antibody procedure is the nonspecificity that can result from an overlabeling of the antibody molecules. The use of such poorly prepared conjugates is not usually possible if virus is to be demonstrated in tissue sections or in tissue cultures. In the FVPT, however, overlabeled globulin preparations should not be a problem since microscopic-

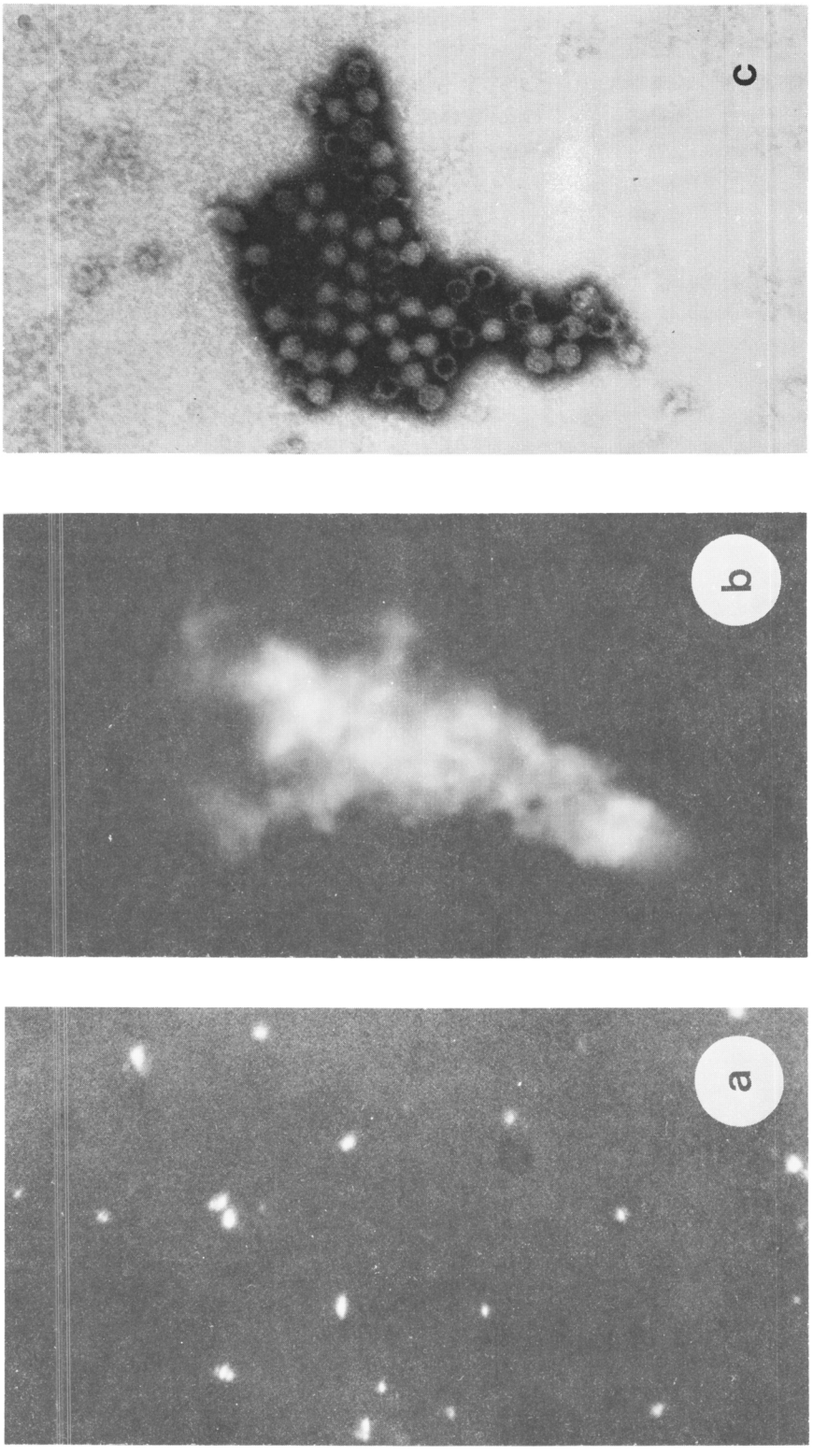

FIG. 1 Photomicrographs of virus aggregates. (a) FVPT small aggregates, × 7000. (b) FVPT Large aggregate, × 7000. (c) IEM aggregate, × 60,700.

ally visible debris is removed by centrifugation and filtration. The virus-antibody aggregates could be seen easily even when conjugates were used at concentrations so high that the suspending fluids in the test fluoresced.

The FVPT is versatile and can be adapted easily to the indirect method of fluorescent antibody staining. The adaptation adds to the practicability of the test by eliminating the problem of maintaining a large stock of conjugates.

Summary. A fluorescent virus precipitin test (FVPT) for the serologic identification of small particulate antigens such as viruses has been described. The test has several advantageous characteristics: (a) It is probably as sensitive as any serologic test (i.e., aggregates with dimensions of 0.2 μm are detectable; therefore, complexes containing as few as three large viruses would give a positive test). (b) Cultivation of the virus is not required. (c) Since an indirect test can be used, only a single fluorescent conjugate is needed to permit the detection of a number of viruses. (d) The indirect test can be used to detect antiviral antibody. (e) The FVPT is rapid and reliable. (f) Its simplicity should enhance its general acceptance and application.

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