

LateX Agglutination Test for Detecting Feline Panleukopenia Virus, Canine Parvovirus, and Parvoviruses of Fur Animals

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A latex agglutination (LA) test for the detection of parvoviruses of fur animals, cats, and dogs was developed, and its sensitivity and specificity were compared with those of hemagglutination (HA) and the enzyme-linked immunosorbent assay (ELISA). Tissue culture isolation was used to confirm the specificity results. Fecal samples from various sources were tested, including specimens from raccoon dogs and mink which were experimentally infected with parvoviruses by oral exposure. LA compared favorably with the other tests. The ELISA was the most sensitive. When it was considered as a reference test, the corresponding sensitivities for HA and LA were 96 and 91%, respectively. The specificities were 93% for the ELISA, 95% for the HA test, and 92% for the LA test. LA seems to be a suitable technique for screening animals in the field and in laboratories in which sophisticated techniques are not available.

For several years enteritis caused by feline panleukopenia virus (FPV) and mink enteritis virus (MEV) has been known to occur in cats and mink. Recently, a viral diarrhea of dogs which is associated with canine parvovirus (CPV) type 2 has been recognized. Antigenically the virus is related to FPV and MEV but differs from them in biological characteristics. When the first isolates of CPV were reported in Finland it was speculated that the virus may cause disease outbreaks in fur animals as well. In June 1980, pathological examinations revealed a condition identical to canine parvoviral enteritis among raccoon dogs (*Nyctereutes procyonoides*) on fur farms in eastern Finland (13). The parvovirus was first isolated from blue foxes (*Alopex lagopus*) in 1983 (16). This isolate was found to be more closely related to FPV and MEV than to CPV. All the virus strains mentioned possess cross-reacting antigens.

Parvoviral enteritis seems to have become a problem on fur farms. Owing to the highly contagious nature of the infection, rapid diagnosis is essential. Several techniques for the detection of parvoviruses in feces have been reported. These include the hemagglutination (HA) test with pig erythrocytes (4), isolation in cell cultures combined with immunofluorescence staining (1), electron microscopy (7), and the enzyme-linked immunosorbent assay (ELISA) (10). The method most widely used is HA combined with HA inhibition with known parvopositive serum. It is inexpensive and easy to perform. The major disadvantage is that a continuous source of pig erythrocytes must be available. The test is mainly performed in diagnostic laboratories. The other tests require specialized equipment, and some of them, such as isolation in cell cultures, are time-consuming.

Simplification of methods, as has been achieved with latex agglutination (LA), avoids the need for laboratory equipment and reduces time and expense. The test can be used in the field, and the results are available within minutes. It has already proved to be useful for detecting rotaviruses in feces (6).

In the work described in the present report the LA test for parvoviruses was developed, and LA, HA, and the ELISA were compared. Specificity results were also confirmed by

isolation in cell cultures; this has proved to be one of the most reliable methods for the detection of small numbers of infectious virus in stool samples and vaccines (11, 14).

MATERIALS AND METHODS

Samples. Most of the stool samples were submitted to the National Veterinary Institute for laboratory diagnosis of suspected viral enteritis during the period 1980 through 1983. Some of the samples were from test animals artificially infected with raccoon dog parvovirus or MEV.

A total of 674 specimens were tested, of which 256 were from blue foxes, 165 were from mink, 144 were from raccoon dogs, 65 were from ferrets, and 41 were from domestic dogs. Fecal samples from three cats were studied and reported with the mink samples.

Preparation of fecal suspensions. Several buffers, including Tris hydrochloride (pH 8.2) and barbital buffers (pH 6.12 and 7.00), were used in preparing stool specimens. The Tris buffer has been recommended for the ELISA (5), and good results were achieved in the HA test when barbital buffer was used. For the LA test, however, conventional phosphate-buffered saline (PBS) was the most suitable and was therefore used throughout the study for all suspensions.

The samples, which had been stored at -70°C , were suspended in PBS (sample/PBS ratio, 1:9 [vol/vol]) by shaking or rotating at room temperature for periods varying from 5 min to 2 h and clarified by centrifugation in an International rotor 840 at 1,500 rpm for 10 min or by filtration through a coarse paper (Whatman no. 1) or nitrocellulose filter (pore size, 0.45 μm ; Schleicher & Schuell, Inc.). The supernatant fluids were used without further purification for LA and the ELISA.

For HA testing, 1 part of chloroform was added to 9 parts of supernatant. After vigorous mixing for 1 h at 21°C , the samples were cooled and centrifuged at 1,500 rpm for 10 min at 4°C as described above. The HA activity was measured in clarified supernatants.

Production of antiserum. A specific immune serum for the ELISA and LA was raised against raccoon dog parvovirus isolated from fecal samples. For antiserum production, chloroform-treated suspensions of stool samples from a field

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case were made as described above. The virus was pelleted by centrifugation through a 20% sucrose cushion in a Beckman SW25 rotor for 3 h at 23,000 rpm at 4°C. The pellet was suspended in TE buffer (0.01 M Tris hydrochloride [pH 7.4], 0.1 M NaCl, 0.001 M EDTA) and further purified by the method of Lei (8). Sucrose gradients were made by pipetting 4.5 ml of 17.5% sucrose solution (wt/wt) in 0.04 M Na₂HPO₄ with 0.2 M NaCl (pH 7.6) into the tubes of a Beckman SW50.1 rotor. The tubes were frozen at -20°C and thawed at 4°C overnight; 0.5 ml of the virus suspension was laid on the top of the gradient and run for 40 min at 45,000 rpm at 4°C. Fractions containing the HA activity were pooled and subsequently purified by banding in an isopycnic CsCl gradient; 2 ml of 55% CsCl (wt/wt) in TE buffer (pH 7.4) was placed in tubes as described above and frozen at -70°C. The tubes were thawed at 4°C overnight, and 3 ml of the virus pool was added to the CsCl solution, which was run for 4 h at 45,000 rpm at 4°C. Peak fractions were detected as described above and desalted by being dialyzed against PBS.

Rabbits were inoculated intramuscularly three times 2 weeks apart with purified parvovirus (30 µg of protein in 0.5 ml of PBS) combined in equal parts with Freund incomplete adjuvant. Blood was collected 10 days after the final booster inoculation.

ELISA. Rabbit anti-parvovirus immunoglobulin G was purified by ammonium sulfate precipitation and by ion-exchange chromatography. Horseradish peroxidase and immunoglobulin G fractions were conjugated by the method of Wilson and Nakane (17).

The double-antibody ELISA was done by the procedure of Grauballe et al. (5). Specific anti-CPV (raccoon dog parvovirus) antibodies (100 µl) in bicarbonate buffer (pH 9.6) or an equal amount of preimmunized rabbit immunoglobulin G, as a control, was added to wells in such a manner that each sample could be tested twice with a double control. The plates were incubated for 1 h at room temperature and washed with PBS-Tween 20. Fecal suspensions (50 µl) were added as 1:50 dilutions, along with the same antibodies (50 µl) as earlier but conjugated to horseradish peroxidase. During the incubation period of 2 h at room temperature (or overnight at 4°C) the antibodies on the wells reacted with any parvoviral antigens present in the samples; at the same time the antigens were detected by the conjugate. After washing, the reaction was visualized by adding ortho-phenylenediamine hydrochloride and H₂O₂ in a citrate-phosphate buffer (pH 5). When the color reaction in the test wells was at least twice as strong as that in the control wells, the samples were considered to be positive. In cases when control and test wells were both colored, the results were considered inconclusive.

LA. Latex particles (Latex 0.81; Difco Laboratories) were coated with specific rabbit anti-parvovirus antibodies by the methods of Severin (15) and Haikala et al. (6). Immunoglobulins were precipitated from immune and nonimmune rabbit sera with saturated ammonium sulfate solution. The precipitates were dissolved and dialyzed in 0.9% NaCl solution. The ammonium sulfate-free immunoglobulins were diluted 1:20 with 0.054 M glycine-saline buffer (pH 8.2). Equal volumes of immunoglobulin dilutions and latex suspension (10%, wt/vol) were mixed and incubated at 37°C in a water bath for 2 h. Latex particles were then washed by centrifugation at 2,500 rpm for 15 min. The supernatants were discarded and replaced by fresh 0.27 M glycine-saline buffer (pH 8.2). The washing procedure was repeated twice. The remaining latex protein-binding sites were blocked by suspending the beads in 0.1% bovine serum albumin in the same

buffer at 0.27 M (pH 8.2). The final latex concentration in test and control reagents was 0.5%.

The LA test was performed by mixing 1 drop (30 µl) of fecal suspension with 1 drop of latex reagent coated with anti-CPV antibodies. Latex particles coated with immunoglobulin from nonimmunized rabbits served as a control. A positive and a negative control antigen were included in the test. The reactions were examined visually after 3 min of gentle rocking. Any agglutination found was counted as positive, provided that the control suspension remained negative.

HA testing. HA tests were performed in microtiter plates by the method of Carmichael et al. (4). MEV and FPV require slightly acidic conditions for agglutination; a barbital buffer (pH 6.12) was therefore used throughout the study (12). Agglutination titers of 64 or more were considered specific for parvovirus.

Isolation of parvoviruses in cell cultures. Isolation in cell cultures was used to confirm the specificity in cases in which any of the three methods described above gave an inconclusive result or when there were discrepancies among the tests.

A continuous cell line of fetal feline lung was used to determine the presence of FPV, MEV, and blue fox parvovirus, and the canine cell line A-72 (2) was used for CPV and raccoon dog parvovirus. Monolayers were trypsinized, split (1/3 or 1/4), and subcultured in 25-cm² plastic bottles (Becton Dickinson Labware). Minimal essential medium supplemented with fetal calf serum (10%) and antibiotics was used as the growth medium. Since the replication of parvoviruses requires actively multiplying host cells, the cultures (5 ml) were infected with 0.2 ml of fecal suspension at the time of seeding. These cultures were incubated at 37°C for 5 days and harvested by freezing and thawing three times; they were then used to infect the second cell culture passage. The cells (1 ml) and the inocula (0.1 ml) were grown on flying cover slips (1.5 cm²) in culture tubes at 37°C for 48 h. The cover slips were removed and fixed with acetone for 10 min, and the demonstration of viral replication was performed by standard indirect fluorescent-antibody tests. Rabbit anti-parvovirus antibodies (mentioned above) at a dilution of 1 to 20 and conjugated anti-rabbit globulins (Dako Corp.) at a dilution of 1 to 10 were used as reagents. Virus-positive cells showed strong nuclear fluorescence.

RESULTS

Comparison of tests. (i) **Sensitivity.** Table 1 shows the sensitivity of the ELISA, HA, and LA. The ELISA was apparently the most sensitive test, detecting 67 (100%) positive samples.

The HA test failed to reveal virus in three raccoon dog fecal samples obtained in the field; the ELISA gave a strong

TABLE 1. Sensitivity of the isolation methods

Source of samples	No. of samples	No. of positive results by ^a :		
		ELISA	HA	LA
Dogs	41	8	8	8
Raccoon dogs	144	42	39	36
Mink and three cats	168	16	16	16
Blue foxes	256	1	1	1
Ferrets	65	0	0	0

^a Isolation with the ELISA was considered a reference test with which the others were compared. The percent positive results were as follows: ELISA, 100; HA, 95.5; LA, 91.

TABLE 2. Specificity of the isolation methods

Source of samples	No. of samples	No. of correct results by ^a :		
		ELISA	HA	LA
Dogs	41	38	38	40
Raccoon dogs	144	141	137	129
Mink and three cats	168	150	160	160
Blue foxes	256	232	241	229
Ferrets	65	63	65	63

^a When the tests used gave different or inconclusive results the samples concerned were examined with isolation tests in cell cultures. The figures represent confirmed correct answers of each method. The percent specificities were as follows: ELISA, 92.6; HA, 95.1; LA, 92.1.

positive reaction for these samples. HA found 64 of 67 positive samples (96%).

The LA test was slightly less sensitive; in addition to the above three false-negative reactions, it failed to detect virus in three samples which contained low concentrations of parvovirus with low HA titers: 64, 256, and 256. The ELISA reactions were weak in these cases, too. The samples were obtained from experimentally infected raccoon dogs and represented the first signs of infection in these animals. Of the 67 positive samples, 61 (91%) were also rated positive by the LA test.

No differences in reaction were found between parvovirus strains from different animal species when tested by the ELISA, HA, and LA.

(ii) **Specificity.** The HA test was the most specific and gave the correct result in 95% of samples; the ELISA and LA gave the correct result in 93 and 92% of samples, respectively (Table 2).

Some of the false-positives showed considerably high HA titers of up to 1,024 (6 samples had titers of 64; 11 had titers of 128; 4 had titers of 256; 10 had titers of 512; and 2 had titers of 1,024). In the ELISA, the false-positives could be detected by using proper controls. Accordingly, false-positive reactions in the LA test were found by using as controls latex covered with a preimmunization serum.

DISCUSSION

The LA test used was similar to the procedure described by Haikala et al. (6) for rotavirus.

The test seems to be sufficiently specific and sensitive to detect parvovirus infections; it compared favorably with LA and the ELISA, which are the assays most commonly used at present. The LA method had some advantages over the ELISA and HA when used for parvovirus diagnosis. The simplicity and rapidity of the test permit its use in circumstances under which special instrumentation is not available.

A centrifuge is the only equipment needed for diagnosis in the laboratory, and in the field it can be omitted by using coarse filters combined with syringes or small plastic bottles in which stool suspensions can be prepared by shaking.

All the parvovirus strains from fur animals, cats, and dogs were equally reactive when tested by LA, HA, and the ELISA. This is because parvovirus strains share common antigenic determinants and because most of them have the ability to agglutinate pig erythrocytes, although the optimum pH for the reaction varies.

The comparison of sensitivities revealed that the ELISA was the most reproductive test. It detected six more positive samples than did the LA test. The discrepancies found in the first three cases are probably due to the presence of coproantibodies. The ELISA apparently competes with

these antibodies and reacts with immunoaggregates. In the LA and HA tests, only free viruses are reactive. In artificially infected dogs, coproantibodies appear in the intestinal contents very soon (a day or two) after the appearance of the first clinical signs (3, 9). The appropriate samples for parvovirus isolation by LA and HA must be collected before the disease has progressed so far that the virus is neutralized in the intestine.

The other three positive cases not detected by the LA test were found to contain very low parvovirus titers. On fur farms and kennels, such failure can be compensated for by collecting samples from several animals at different stages of the disease. One of them has usually reached the peak of virus shedding at the time of collection.

The specificity of all three tests was good. The overall correlation with isolation in cell cultures for the HA was 95%; the corresponding figures for the ELISA and LA were close to that: 93 and 92%, respectively. The false-positive findings seemed to be related to the condition of the samples rather than to the animal species studied. Suspensions made from hemorrhagic feces which originated from obduction material were especially problematic. They showed high nonspecific HA titers and gave inconclusive results by binding to both pre- and postimmunization immunoglobulin G on ELISA wells and LA particles. Some of the false-positives found with the LA test may also be because weak agglutinations were also taken into account.

The LA test described in the present study provides a rapid and sensitive method for detecting parvoviruses in feces. The results are reliable if the samples are collected at the early stage of the disease. In suspected cases it is advisable to examine several samples per farm.

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