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Physicochemical Characterization of Porcine Pararotavirus and Detection of Virus and Viral Antibodies Using Cell Culture Immunofluorescence†

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A cell culture immunofluorescence (CCIF) assay was optimized for detection of porcine pararotavirus (group C rotavirus) in intestinal contents. The greatest viral infectivity was observed when MA104 cells (5 days after subculturing) were rinsed and refed in serum-free medium before inoculation, pancreatin was added to the inocula, and the inocula were centrifuged onto the cells. Gentamicin treatment of pararotavirus samples to reduce bacterial contamination also reduced the viral infectivity of these samples for MA104 cells. An indirect CCIF assay was used to determine the prevalence of pararotavirus and rotavirus antibodies in pig sera. In pigs from four herds, pararotavirus antibodies were detected in 100% (68 of 68) of adults and 59% (24 of 41) of weanling pigs, while 86% (24 of 28) of nursing pigs from 12 herds had pararotavirus antibodies. The physicochemical properties of pararotavirus were examined and compared with those of group A rotaviruses by using the CCIF assay to quantitate in vitro changes in viral infectivity. Pararotavirus was inactivated (≥99% reduction in titer) by heating to 56°C for 30 min, was slightly labile at pH 3 (16 to 34% reduction in titer), and was stable at pH 5 (0 to 17% reduction in titer) and in ether (3 to 19% reduction in titer). One group A rotavirus (Gottfried strain) was stable at 56°C (0% reduction in titer), whereas the OSU strain of group A rotavirus was inactivated at this temperature (99% reduction in titer).

Pararotavirus (PaRV), first detected in diarrheic pigs in 1980 (22), appear morphologically identical to conventional rotaviruses (group A rotaviruses) but are antigenically distinct. Rotaviruslike viruses are also antigenically and genetically distinct from group A rotaviruses and PaRV and have been designated as group B rotaviruses (7). Because of their antigenic and genetic (RNA electropherotype) differences, PaRV have been classified as an additional separate rotavirus group, group C (5, 18).

Until recently, PaRV had been detected only in swine (11, 22). Then, several atypical human rotaviruses with similar characteristics were discovered (12, 13, 16, 19). More recently (9; L. J. Saif, unpublished data), investigators found that several atypical human rotaviruses were antigenically related to porcine group C rotaviruses (PaRV), but two attempts to infect gnotobiotic piglets with one of these viruses were unsuccessful (9). This antigenic cross-reactivity, however, established that PaRV infections do occur in more than one species.

Although the prevalence of group A rotavirus (6, 8, 27) antibodies in swine has been well studied, data on the prevalence of group B rotavirus or PaRV antibodies are limited (8, 17, 27). Detection of PaRV or PaRV antibodies has been hindered by the inability to serially propagate the virus in cell cultures, thus precluding the use of many conventional serological tests.

A cell culture immunofluorescence (CCIF) test was used previously for the detection of many viruses, including group A rotaviruses, which at the time could not be serially propagated in cell cultures (10). This paper describes optimal conditions for a CCIF test for the detection of porcine PaRV. This optimized CCIF test was then used to determine the stability of PaRV to treatment with acid, heat, or ether (29). In addition, infected cell monolayers were used as the antigen source for an indirect immunofluorescence test to assess the prevalence of PaRV antibodies in swine sera from 13 Ohio herds.

MATERIALS AND METHODS

Clinical specimens. Two porcine rotavirus serotypes, OSU (21) and Gottfried (6), obtained from gnotobiotic pig large intestinal contents, were used in this study. Several strains of PaRV from the Ohio Agricultural Research and Development Center or local swine herds were also used. These strains (designated by herd names or initials) were initially collected as large intestinal contents or feces from diarrheic pigs of various ages. The strain designations and ages of the pigs were as follows: Cowden, 27 days old (22); NB, 4 days old; WH, 35 days old; A, 10 days old; and KH, approximately 30 days old. These five PaRV specimens were diluted in serum-free Eagle minimal essential medium (EMEM) (1:5), filtered (0.45-μm-pore filters; Millipore Corp., Bedford, Mass.), and used to experimentally infect gnotobiotic pigs (15). The large intestinal contents and small intestines were collected from these infected pigs and used in subsequent assays. The A strain of PaRV and two additional PaRV strains (S-2, 45 days old; S-3, 27 days old) were from large intestinal contents from pigs naturally infected with PaRV and were used in this form in subsequent assays. Samples were confirmed to contain PaRV by immune electron microscopy (IEM) (21).

Viral suspension preparation. Gnotobiotic pigs were orally exposed to rotavirus (OSU or Gottfried strain) or PaRV (Cowden or NB strain) as previously described (15). The pigs were sacrificed shortly after the onset of diarrhea, and their large intestinal contents were collected aseptically.

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Twenty samples (various strains, all from gnotobiotic pigs) containing PaRV were tested for cell culture infectivity. The presence of PaRV in these samples was confirmed by IEM (21). The intestinal contents were serially diluted from 1:25 to $1:10^6$ in serum-free EMEM supplemented with 100 U of penicillin, 100 μg of streptomycin, and 25 U of nystatin (Mycostatin; E. R. Squibb & Sons, Princeton, N.J.) per ml.

One PaRV sample (Cowden isolate), chosen from the others on the basis of its high infectivity for cell cultures [mean CCIF titer = 4.5×10^6 fluorescing-focus units per ml], was used to determine optimal conditions for the CCIF test. A rotavirus sample (OSU strain) with a high CCIF titer was compared with the PaRV sample. Sample treatment variables which were tested included the addition of antibiotics (gentamicin, 100 μg/ml; Schering Corp., Kenilworth, N.J.) with or without incubation at 37°C for 30 min; incubation at 37°C for 30 min without antibiotics; and low-speed centrifugation at 5°C for 30 min.

Cell culturing. Confluent monolayers of rhesus monkey kidney (MA104) cells were prepared in 96-well tissue culture plates (Costar, Cambridge, Mass.) by subculturing 7-day-old monolayers at a 1:4 dilution (approximately 10^6 cells per ml) with EMEM supplemented with 100 U of penicillin per ml, 100 μg of streptomycin per ml, 25 U of nystatin per ml, and 10% fetal bovine serum. Before inoculation, the growth medium was removed, and the monolayers were rinsed once and then refed with serum-free EMEM (27).

CCIF test for antigen detection. This test was adapted from procedures previously used for the detection of rotavirus (3, 10, 28) and PaRV (5). Confluent monolayers of MA104 cells in 96-well plates, prepared as described above, were inoculated with diluted samples (0.2 ml per well). There were two to four wells per specimen for each test, and the test was repeated a minimum of three times. Enzymes were added to the wells as described below. The plates were centrifuged at $1,200 \times g$ for 1 h at room temperature by using microtiter plate centrifuge carriers (Dynatech Laboratories;, Inc., Alexandria, Va.). After centrifugation, the plates were incubated in a 5% CO_2 atmosphere at 37°C for approx 16 h. For detection of PaRV-infected cells, monolayers were fixed in 80% acetone for 10 min and then incubated for 1 h at 37°C with fluorescein isothiocyanate (FITC)-conjugated hyperimmune gnotobiotic pig anti-PaRV serum, prepared as described previously (25). For detection of rotavirus-infected cells, fixed monolayers were incubated for 30 min at 37°C with FITC-conjugated hyperimmune gnotobiotic pig anti-rotavirus serum (25). Glycerin mounting medium was added to the wells, which were then viewed by using an inverted fluorescence microscope with a reflected-light fluorescence attachment and a blue exciter filter (model IMT; Olympus Optical Co., Ltd., Tokyo, Japan). Fluorescing cells were counted, and results were expressed as mean numbers of fluorescing cells per well or fluorescence-forming units per milliliter. Counts greater than 99 fluorescing cells per well were estimated as a percentage of the cell monolayer infected, and then the number of fluorescing cells per well was calculated based on 10^4 cells per well. Cell culture variables tested for effects on viral infectivity were the age (3 to 7 days after subculturing) of cell monolayers at the time of viral inoculation and the length of time (0 to 24 h) between refeeding and inoculation.

Enzyme treatments. The effect of proteolytic enzymes on PaRV and rotavirus was tested as described in previous studies (1, 2, 5, 23, 24, 26). Using the CCIF assay, we added either pancreatin diluted from stock (4 X NF; GIBCO Laboratories, Grand Island, N.Y.) at 1:50 to 1:300 in phosphate-buffered saline (PBS) or trypsin (type IX; Sigma Chemical Co., St. Louis, Mo.) diluted from 100 to 5 μg/ml in EMEM, 1 drop per well, immediately after the viral inoculum had been added. Positive control wells contained each inoculum, but PBS was substituted for the enzymes. Two strains of PaRV (Cowden and NB) and two strains of rotavirus (OSU and Gottfried) were assayed with both pancreatin and trypsin.

Physicochemical treatments. The stabilities of PaRV and rotavirus were tested under conditions similar to those described for an infant rat rotaviruslike agent (29). The procedures were modified as follows. Viral suspensions were made from large intestinal contents diluted 1:100 in serum-free EMEM and centrifuged ($1,300 \times g$) to remove large particulate matter. Each sample was further serially diluted in 10-fold dilutions and then treated prior to the assay. The treatments were as follows.

(i) Heat treatment. Samples were incubated in a 56°C water bath for 30 min. They were removed, cooled to room temperature by being placed at 4°C for a few minutes, and then assayed immediately. Untreated controls were stored at 4°C until assayed.

(ii) Ether treatment. Samples were treated with an equal volume of diethyl ether (J. T. Baker Chemical Co., Phillipsburg, N.J.) at room temperature for 1 h and were shaken every 5 min. The ether phase was then aspirated, and the residual was allowed to evaporate from the specimen under a fume hood (for about 15 min) prior to the assay. A mock-treated control was shaken every 5 min at room temperature, but no ether was added.

(iii) Acid treatment. Viral suspensions were treated with 0.1 N HCl to reduce the pH to 3 or 5 and incubated for 30 min at 37°C. The pH was then readjusted to 7 with 0.1 N NaOH. Mock-treated controls received no acid treatment but were readjusted to pH 7 after incubation for 30 min at 37°C.

All samples were assayed in the optimized CCIF test to determine viral infectivity in vitro. They were not tested for in vivo infectivity. Controls at each dilution were included in the test. Except for the heat-treated samples, untreated controls were incubated as for test samples.

Indirect CCIF test for antibody detection. For detection of PaRV antibodies, cell monolayers were inoculated with PaRV, incubated, and then fixed as described for antigen detection. The fixed monolayers were incubated with diluted test sera for 1 h at 37°C, followed by FITC-conjugated rabbit anti-porcine immunoglobulin G (Miles Laboratories, Inc., Elkhart, Ind.) for 1 h at 37°C. Fluorescing cells were detected as described previously (10). For rotavirus antibody detection, monolayers were infected with rotavirus, and the procedure described for PaRV was followed.

Porcine sera. Blood was collected from 137 conventional pigs from 13 Ohio herds in different geographical locations within the state. Sera were from 68 adult (≥8 months of age from 4 herds), 28 nursing (0 to 6 weeks of age from 12 herds), and 41 weanling (1 to 22 days postweaning at 3 to 6 weeks of age from 4 herds) pigs. The sera were separated by centrifugation, heat inactivated (56°C for 30 min), and stored at −20°C until assayed. The sera were diluted 10-fold in PBS (pH 7.4) for indirect CCIF testing.

Each serum sample was tested on a rotavirus-infected monolayer and on a PaRV-infected monolayer. Along with the sera being assayed, three control sera from gnotobiotic pigs, including sera devoid of both PaRV and rotavirus antibodies and hyperimmune sera from PaRV- and rotavirus-inoculated gnotobiotic pigs, were tested. These three con-

trols were included in all PaRV and rotavirus antibody CCIF tests.

Fluorescent-antibody staining. Intestinal mucosal smears were made of the duodenum, jejunum, and ileum of the 10 pigs described above. They were stained with FITC-conjugated hyperimmune gnotobiotic pig anti-PaRV serum as described previously (4, 5, 25).

IEM. Large intestinal contents were collected from pigs and examined by IEM (21) with a Philips 201 electron microscope (Philips-Norelco, Einhoven, The Netherlands).

Comparison of viral detection methods. Samples from 10 pigs (gnotobiotic and naturally infected) were assayed for the presence of PaRV to compare the relative sensitivity and specificity of three detection methods: IEM, fluorescent-antibody staining of mucosal smears, and CCIF.

RESULTS

Viral suspension preparation. Maximum PaRV infectivity for MA104 cells was observed with viral suspensions clarified by low-speed centrifugation alone. Treatment of PaRV or Gottfried rotavirus samples with gentamicin reduced their CCIF titers by approximately 2- to 11-fold, whereas OSU rotavirus CCIF titers were reduced only 1.2- to 4-fold (results not shown). Incubation of samples in the absence of gentamicin had little effect on viral infectivity.

CCIF test for antigen detection. Maximum numbers of PaRV-infected fluorescing cells were detected when MA104 cell monolayers were rinsed and refed with serum-free EMEM 4 days after subculturing and inoculated with viral suspensions 12 to 24 h later. PaRV-infected fluorescing cells, detected by using a direct staining method in the CCIF test, are shown in Fig. 1. Bright, cytoplasmic fluorescence and unstained nuclei of infected cells were evident.

Enzyme treatments. The effects of enzyme treatment on the infectivity of two PaRV and two rotavirus isolates are shown in Table 1. Both trypsin and pancreatin enhanced the infectivity of PaRV and OSU rotavirus as compared with the untreated controls. Gottfried rotavirus infectivity was enhanced by pancreatin but reduced by trypsin at the enzyme concentrations indicated in Table 1. Pancreatin generally produced a greater increase in infectivity for PaRV and rotavirus and was therefore chosen for regular use in the CCIF test. The enzyme concentrations used for this comparison were optimal for each enzyme (pancreatin, 1:200 dilution; trypsin, 5 μg/ml; results not shown), as determined by previous CCIF testing. Optimal enzyme concentrations were based only on the infectivity of Cowden PaRV and OSU rotavirus.

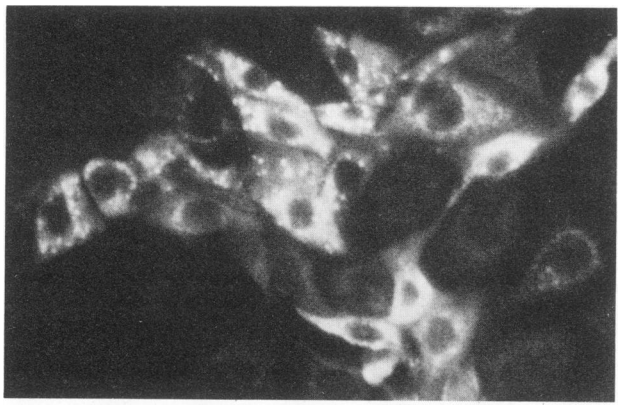

FIG. 1. Typical fluorescence observed in MA104 cells infected with PaRV (Cowden isolate) and incubated with FITC-conjugated PaRV antiserum. Magnification, ×325.

TABLE 1. Cell culture infectivity of PaRV and rotavirus samples following enzyme treatment[a]

Strain	Virus dilution	Mean no. of fluorescent cells/well[b] after indicated treatment		
		PBS (pH 7.4)	Pancreatin	Trypsin
PaRV				
Cowden	10^{-2}	29	500	300
	10^{-3}	3	18	23
	10^{-4}	0	8	8
	10^{-5}	0	7	0
NB	10^{-2}	800	1,300	1,000
	10^{-3}	300	500	300
	10^{-4}	10	60	62
	10^{-5}	0	9	11
Rotavirus				
OSU	10^{-4}	500	3,000	2,500
	10^{-5}	54	700	700
	10^{-6}	5	100	100
	10^{-7}	0	15	15
	10^{-8}	0	2	1
Gottfried	10^{-2}	36	500	2
	10^{-3}	100	69	7
	10^{-4}	35	54	8
	10^{-5}	1	5	1

[a] Based on a direct CCIF assay with PBS as a control, pancreatin diluted 1:200 in PBS, or trypsin diluted to 5 μg/ml in EMEM added 1 drop per well to 96-well plates.

[b] Mean numbers of fluorescing cells in 12 wells. Counts greater than 99 were estimated as a percentage of fluorescing cells and then converted to numbers based on 10^4 cells per well.

Physicochemical treatments. The stabilities of PaRV and rotavirus to heat, acid, and ether treatments are summarized in Table 2. Incubating samples in a 56°C water bath decreased the cell culture infectivity of both PaRV strains (Cowden and NB) and one rotavirus strain (OSU) by ≥99%. Gottfried rotavirus was unaffected by heat treatment. The infectivities of samples treated with ether were reduced by 3 to 19% for both PaRV strains and 0 to 8% for both rotavirus strains. The addition of acid to samples reduced the titers by 16 to 34% for PaRV and 17 to 27% for rotavirus at pH 3 and

TABLE 2. Percent reduction in CCIF titers of PaRV and rotaviruses following various physicochemical treatments[a]

Strain	% Reduction in CCIF titer[b] after indicated physicochemical treatment			
	Heat	pH 3	pH 5	Ether
PaRV				
Cowden	≧99	16	17	3
NB	≧99	34	0	19
Rotavirus				
OSU	≧99	27	0	0
Gottfried	0	17	11	8

[a] Heat, 56°C for 30 min; pH adjusted to pH 3 or 5 for 30 min at 37°C and then readjusted to pH 7; ether, equal volume of ether added for 1 h and then aspirated.

[b] As compared with mock-treated control titers determined by a CCIF assay.

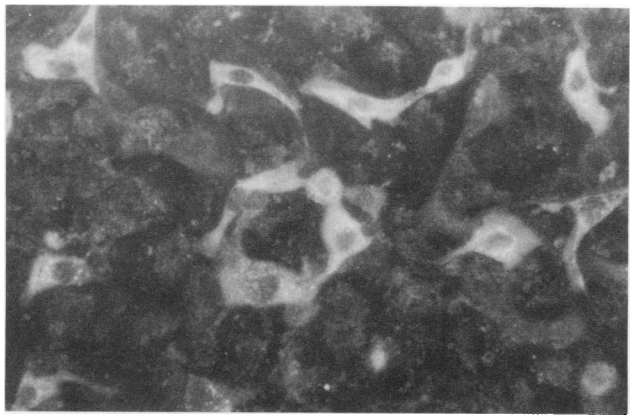

FIG. 2. Typical fluorescence observed in MA104 cells infected with PaRV (Cowden isolate) and incubated with PaRV antiserum followed by FITC-conjugated anti-porcine immunoglobulin G. Magnification, ×160.

by 0 to 17% for PaRV and 0 to 11% for rotavirus at pH 5.

Indirect CCIF test for antibody detection. Typical immunofluorescence observed after incubation of PaRV-infected MA104 cells with conventional swine sera containing PaRV antibodies followed by FITC-conjugated anti-porcine immunoglobulin G is shown in Fig. 2. Cells exhibited a distinct uniform or granular cytoplasmic fluorescence, depending on the serum sample tested. The nuclei of the cells were dark and unstained. Further dilution of the sera (1:100) dramatically decreased the brightness of the fluorescent cells, making it difficult to detect them. A total of 100% (68 of 68) of adult swine, 86% (24 of 28) of nursing pigs, and 59% (24 of 41) of weanling pigs had PaRV antibodies. A total of 100% of pigs of all age groups tested had rotavirus antibodies.

Comparison of viral detection methods. The sensitivities of the various tests for detection of PaRV are shown in Table 3. All 10 of the selected pigs were positive for PaRV by IEM. Nine (90%) were positive by fluorescent-antibody staining of intestinal mucosal smears from the same pigs, whereas five (50%) were positive by the CCIF test.

DISCUSSION

In addition to antigenic differences between group A rotaviruses and PaRV, these two viruses possess distinctive biological differences in their in vitro propagation. While group A rotaviruses have been adapted to serial propagation in cell cultures, PaRV have not. A CCIF test routinely used for the detection of group A rotaviruses was optimized to detect PaRV. Conditions which enhanced PaRV infectivity for MA104 cells included using 5-day-old cell monolayers, a viral inoculum without pretreatment with gentamicin, the addition of pancreatin to the inoculum, and centrifugation of the inoculum onto the cells. Since group C rotaviruses antigenically related to porcine PaRV (Cowden isolate) have also been detected in stools from human infants (9; L. J. Saif, unpublished data), these same techniques, may facilitate the development of a similar CCIF assay for human PaRV. Alternatively, porcine PaRV could serve as the antigen source for the detection of antibodies to human PaRV.

Treatment with antibiotics is a standard procedure for inhibiting bacterial growth in fecal samples. When gentamicin was used in samples, viral infectivity for MA104 cells was generally reduced. Therefore, the PaRV and rotavirus samples used in this study were not treated with gentamicin. Because microbial contamination of clinical specimens may interfere with viral infection of cells and subsequent fluorescence of positive samples, these samples may need to be filter sterilized or treated with other antibiotics prior to their use as inocula for CCIF tests. The use of gentamicin for inhibiting PaRV infectivity requires further study in vitro and in vivo.

In general, treatment of PaRV or rotavirus with proteolytic enzymes increased the infectivity of the viruses in the CCIF test; pancreatin was generally more effective than trypsin. In a previous study (5), it was noted that the sensitivity of the CCIF test for PaRV detection was increased 5- to 10-fold by the addition of pancreatin to the medium. As previously reported (23, 24, 26), the infectivity of group A rotaviruses for MA104 cells was also enhanced by the use of pancreatin in this study. The infectivity of the Gottfried strain of group A rotavirus was enhanced by pancreatin, whereas trypsin reduced the infectivity. Since optimal enzyme concentrations were not determined for Gottfried rotavirus, it is possible that other concentrations of trypsin could enhance its infectivity.

PaRV and rotavirus were relatively stable to ether and pH 5; viruses of both groups were slightly labile at pH 3. These results agree with those of previous stability studies of rotavirus (14, 20, 21, 30). Cowden and NB PaRV and OSU rotavirus were inactivated by incubation at 56°C, but Gottfried rotavirus was unaffected. The reasons for this latter finding are unknown. Both group C (PaRV) and group A rotaviruses were more stable than a group B rotavirus (29), which was inactivated at pH 3 as well as by heat.

PaRV and rotavirus antibody prevalence differed in swine serum samples. Rotavirus antibodies were present in all pig sera tested. PaRV antibodies were less prevalent in sera from the same pigs. The results of this study agree with those of a previous study (8) in which the prevalence of rotavirus and PaRV antibodies in swine in England was determined, except for the lower prevalence of PaRV antibodies in adult pigs in that study. Studies of the prevalence of rotaviruslike virus (group B rotavirus) antibodies indicated that only 23% of sera from three Ohio herds were positive (27), whereas 86% of swine sera in England were positive (8).

The following are possible explanations for the lower incidence of PaRV antibodies than of rotavirus antibodies in swine. First, PaRV infections may be less severe, of shorter duration, or less frequent, resulting in lower serum antibody titers that are less readily detected by the indirect CCIF test.

TABLE 3. Comparison of viral detection methods for various PaRV isolates

PaRV isolates	Specimen source[a]	Result[b] by:		
		CCIF	IEM	FA[c]
Cowden	G	+	+	+
Cowden	G	+	+	+
NB	G	+	+	+
WH	G	+	+	+
WH	G	−	+	+
KH	G	−	+	+
A	G	−	+	−
A	NI	+	+	+
S-2	NI	−	+	+
S-3	NI	−	+	+

[a] G, Experimentally infected gnotobiotic pig; NI, naturally infected pig.
[b] +, Positive; −, negative.
[c] FA, Mucosal smear fluorescent-antibody test.

Second, PaRV may be less widespread than rotaviruses, although 100% of herds (13 herds) tested in this study were seropositive for both viruses. Finally, the indirect CCIF assay may be more sensitive for detecting rotavirus than PaRV antibodies. These same reasons may explain the lower prevalence of rotaviruslike virus antibodies as well (8, 27).

Pigs positive for PaRV by IEM were used to compare the relative sensitivities of direct fluorescent-antibody staining of mucosal smears and the CCIF test for detection of PaRV. While fluorescent-antibody staining was positive for 9 of 10 pigs, the disadvantages of this diagnostic method is that it can be used only if the animals are sacrificed. Fecal smears cannot be easily evaluated by this method to monitor the onset or duration of an experimental or natural infection. The CCIF test was positive only for 50% of the samples tested, but all of the samples negative by CCIF had low numbers of viral particles [≤10 virus particles per grid square (300-mesh grid) by IEM]. Additional refinements in the CCIF test may improve its sensitivity further for routine detection of PaRV from clinical specimens.

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