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Serial Propagation of Porcine Group C Rotavirus (Pararotavirus) in Primary Porcine Kidney Cell Cultures†

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A porcine group C rotavirus was adapted to serial propagation in roller tube cultures of primary porcine kidney cells with high concentrations of pancreatin. Infected cells were identified by immunofluorescence staining of cell monolayers. Only group C rotavirus particles were observed in culture supernatants by immune electron microscopy.

Group C rotaviruses, also known as pararotaviruses (PaRV), are morphologically identical to but antigenically distinct from group A rotaviruses (19). PaRV were first detected in swine in 1980 (18) and subsequently verified as a cause of gastroenteritis in swine (5). Rotaviruses with electropherotypes similar to those of PaRV have been identified in humans (2, 7, 8, 15, 16), and several of these have subsequently been verified as serogroup C rotaviruses on the basis of antigenic similarities to the Cowden strain of porcine group C rotavirus (6). Further studies of PaRV have been hindered by the inability to serially propagate these viruses in cell cultures (19). In contrast, most group A rotavirus isolates can be routinely propagated in cell cultures by using proteolytic enzymes or roller tube culture techniques or both (4, 11, 13, 21, 24). Most attempts to serially propagate atypical rotaviruses in cell cultures by similar techniques have failed (19). Only one isolate of avian group D rotavirus has been successfully serially propagated in primary cell cultures of chicken embryo liver cells (14). Conditions for the adaptation of porcine PaRV to serial propagation in primary porcine kidney (PPK) cell cultures are described in this paper.

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PPK cells were prepared and seeded into roller or Leighton tubes (16 by 125 mm) at 2 ml of cell suspension per tube. Cells were grown to confluency (5 to 7 days) in Eagle minimal essential medium (EMEM) supplemented with 0.5% lactalbumin hydrolysate (GIBCO Laboratories, Grand Island, N.Y.), 5% adult bovine serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 25 U of nystatin (Mycostatin; E. R. Squibb & Sons, Princeton, N.J.) per ml. Cells were refed after 2 to 3 days with the same medium, except that the adult bovine serum concentration was decreased to 3%.

Two PaRV samples, both of the Cowden strain (18), were from large intestinal contents collected aseptically from passages 14 and 15 in gnotobiotic piglets. Before use as viral inocula, intestinal contents were diluted 1:25 in serum-free EMEM and clarified by low-speed centrifugation (650 × *g* for 30 min at 4°C). At 3 to 7 h prior to inoculation, the PPK cells were rinsed and refed with serum-free EMEM. Immediately before inoculation, the EMEM was decanted, and 0.2 ml of viral suspension was added to each tube. The tubes were incubated at 37°C for 1 hour, with rocking every 15 min. At the end of 1 h, 2 ml of serum-free EMEM and 120 μl of pancreatin (4X NF; GIBCO; diluted 1:10 in phosphate-buffered saline) were added to each tube. The tubes were placed in a roller tube apparatus (Conrac Corp., Old Saybrook, Conn.) at 37°C for 3 days, removed, and frozen-thawed once. A sample (0.2 ml) of the cell suspension was used for inoculation of each tube for the next virus passage. Subsequent passages were carried out in the same manner, using 0.2 ml of the cell suspension from the prior passage as the inoculum for each tube. The high concentrations of pancreatin which were required for virus propagation caused detachment of the cell monolayers. Therefore, cytopathic effects of the virus could not be determined.

PaRV were detected or quantitated by four methods. (i) The percentage of infected cells was determined by using Leighton tube cover slips from control and infected PPK cell cultures supplemented with 40 μl of pancreatin (1:200 in phosphate-buffered saline) and stained with fluorescein isothiocyanate (FITC)-conjugated anti-PaRV serum (immunofluorescence [IF] staining). Anti-PaRV serum was prepared by hyperimmunization of gnotobiotic pigs with the Cowden isolate of PaRV (5). This antiserum was tested against group A and B rotaviruses and by direct IF staining of group A and B rotavirus-infected intestinal mucosal smears. In both tests, the anti-PaRV serum failed to react with group A and B rotaviruses. Cultures were also stained with FITC-conjugated anti-group A rotavirus serum prepared in gnotobiotic pigs (5) as a control. (ii) Virus titers were determined by using a cell culture IF (CCIF) assay (23). Briefly, monolayers of monkey kidney (MA104) cells in 96-well tissue culture plates were inoculated with the viral suspensions followed by 1 drop of pancreatin (1:200 in phosphate-buffered saline) per well. Plates were centrifuged (1,200 × *g* for 1 h) at room temperature and then placed in a 5% CO_2 atmosphere at 37°C for approximately 16 h. Cells were fixed in 80% acetone and incubated with FITC-conjugated anti-PaRV serum or FITC-conjugated anti-group A rotavirus serum. (iii) Immune electron microscopy (17) was also used to confirm the presence of PaRV particles in cell culture supernatants concentrated by ultracentrifugation. (iv) Polyacrylamide gel electrophore-

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TABLE 1. Cell culture infectivity[a] of porcine PaRV at sequential passage levels in PPK cells

Sample[b]	No. of fluorescent cells/well[c] at virus passage:											
	1	2	3	4	5	6	7	8	9	10	11	12
1A	6	100	52	70	200	200	3,000	1,000	1,000	2,000	2,000	NT[d]
1B	17	52	100	60	500	500	2,000	NT	NT	NT	NT	NT
2A	0	5	100	39	49	100	400	500	700	2,000	2,000	2,000
2B	0	48	150	32	100	100	1,700	NT	NT	NT	NT	NT

[a] Determined by a direct CCIF assay in MA104 cell cultures with FITC-conjugated anti-PaRV serum. Samples were diluted 1:10 for the assay.
[b] Duplicate cultures of each of the two Cowden PaRV samples were used.
[c] Number of fluorescing cells per well in a 96-well plate. Counts greater than 99 were estimated as a percentage of fluorescing cells and were then converted to numbers based on 10^4 cells per well.
[d] NT, Not tested.

sis of double-stranded RNA (20, 26) was performed on Gottfried porcine rotavirus (group A) and on both PPK cell culture-adapted PaRV (passage 17) and virulent PaRV obtained from gnotobiotic pig large intestinal contents (the original cell culture PaRV inoculum).

Viral infectivity was detected after passage 1 or 2 in PPK cells by the CCIF assay (Table 1). Infectivity generally increased slowly during the first five or six passages and more dramatically thereafter, with one sample reaching 3.5×10^6 fluorescent focus-forming units per ml by passage 12 (endpoints not shown in Table 1). This titer was based on an endpoint titration of 10-fold dilutions of the viral suspension.

IF seen in monolayers of MA104 cells infected with PaRV from passage 7 in PPK cells is shown in Fig. 1. PaRV-infected PPK cell monolayers on Leighton tube cover slips were observed following IF staining (Fig. 2). Infected cells were visible but often few in number, probably because of the lower concentration of pancreatin needed to prevent cell detachment. The use of a higher concentration of pancreatin to increase infectivity was not possible, owing to cell detachment. At passage 9, 20% of the cell monolayer exhibited IF in infected cells. Shown in Fig. 3 are characteristic double-shelled PaRV particles detected in the cell culture supernatants from passage 10 by immune electron microscopy. No group A rotavirus was observed by CCIF, IF, or immune electron microscopy. Results of polyacrylamide gel electrophoresis of viral double-stranded RNA are shown in Fig. 4. Both cell culture-adapted PaRV and the original PaRV inoculum from intestinal contents exhibited identical electropherotypes, with genome segments distributed among the four size classes in the 4-3-2-2 pattern characteristic of PaRV (19) and no extra bands evident. Polyacrylamide gel electrophoresis of double-stranded RNA from Gottfried rotavirus (group A), shown for comparison, exhibited the 4-2-3-2 migration pattern typical of group A rotaviruses (19).

Group A rotaviruses have been successfully passaged in cell cultures with primary or established cell lines (4, 10, 12, 13, 24). In several reports, some rotaviruses were cultured without enzymes (10, 12, 13), whereas other investigators found that pancreatic enzymes were necessary or greatly enhanced rotavirus infectivity in cell cultures (1, 4, 24, 25). Several studies have also used roller tubes for routine adaptation of human and swine field isolates of group A rotaviruses (11, 21).

There is only one verified report of the successful adaptation of an atypical rotavirus to cell culturing. Avian group D rotavirus (132 virus) was adapted to serial propagation in primary cell cultures by McNulty et al. in 1981 (14). For group D rotavirus adaptation, fecal suspensions were treated with trypsin before inoculation onto primary chicken embryo liver cells, and trypsin was also added to the maintenance medium. Whole cell cultures were frozen at −70°C 24 or 48 h postinoculation and then thawed for the next pas-

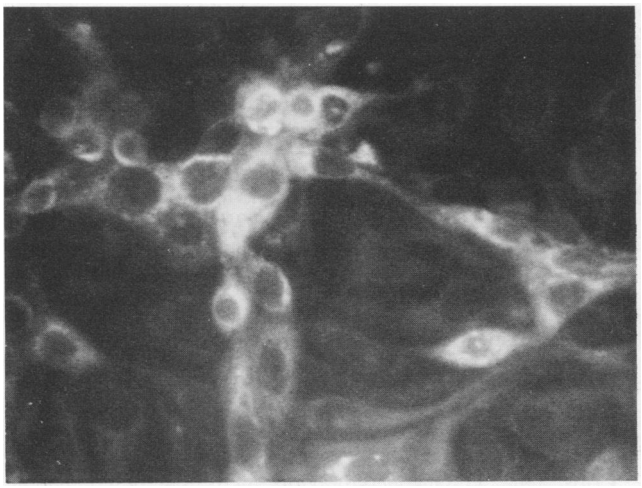

FIG. 1. Characteristic fluorescence observed in MA104 cells infected with tissue culture-adapted PaRV and incubated with FITC-conjugated anti-PaRV serum (CCIF assay). Magnification, ×325.

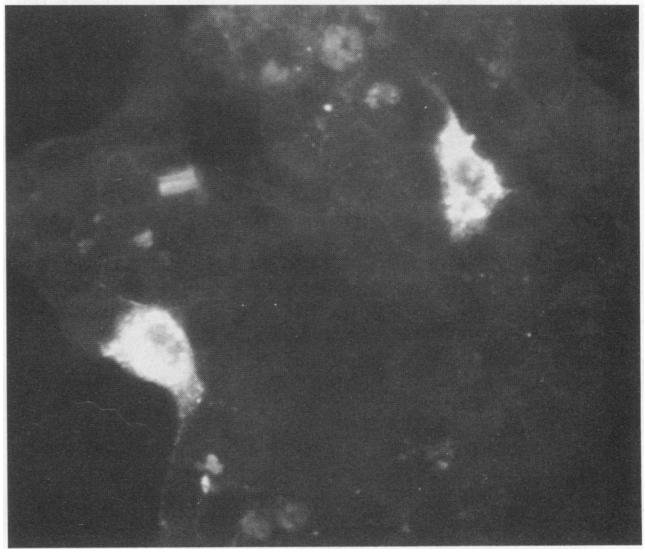

FIG. 2. Characteristic fluorescence observed on Leighton tube slides of PPK cells infected with tissue culture-adapted PaRV and incubated with FITC-conjugated anti-PaRV (IF staining). Magnification, ×325.

sage. In another study, the possible propagation in cell cultures of an atypical porcine rotavirus unrelated to our porcine PaRV was reported (3). However, no further studies were done to verify or characterize the cell culture-propagated virus. For the adaptation of porcine group C rotavirus, primary cells were used, as were high concentrations of pancreatin, and enzyme treatment was used only after viral inoculation. Passage of cell fragments along with supernatant fluids may also have been a key to the successful adaptation, suggesting that perhaps some PaRV particles may remain highly cell associated. Supernatant fluids did contain infectious virus particles, as determined by the CCIF assay, but these fluids were not serially passaged to confirm that serial replication with supernatants alone was possible.

High concentrations of pancreatin seem to be necessary for the optimal cell culture infectivity of PaRV. The high concentration of pancreatin caused detachment of the cells from roller tubes, but this did not prevent virus replication. Lower concentrations of pancreatin were shown to allow some viral infectivity, but the infectivity was much lower than that in cultures with higher enzyme concentrations. It is not known whether this enzyme affects the virus, the cell, or both in allowing viral infectivity. Studies of group A rotaviruses have demonstrated that pretreatment of the viruses with trypsin acts upon the viruses to cleave major outer capsid polypeptides. This, in turn, allows penetration of the viruses into the cytoplasm for replication (9, 22). Pancreatin treatment may affect PaRV replication in a similar manner.

To our knowledge, this is the first verified report of the adaptation of an atypical mammalian rotavirus to serial propagation in cell cultures. The PaRV titers have continued to increase throughout most of the 12 passages studied to date. Attempts to adapt porcine PaRV to an MA104 cell line are now in progress. Methods used in this study may be helpful in adapting other atypical mammalian rotaviruses, including human PaRV, to serial propagation in cell cultures.

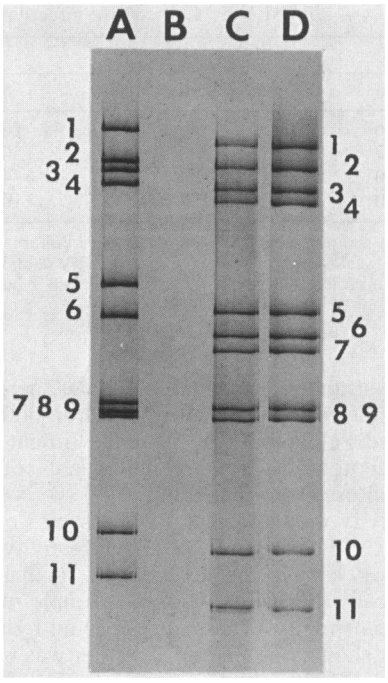

FIG. 4. Genome electropherotypes of a group A rotavirus and PaRV. Lanes: A, Gottfried group A rotavirus; B, blank; C, PPK cell culture-propagated PaRV (passage 17); D, gut virulent PaRV (Cowden isolate) used to originally infect PPK cell cultures. The numbers on the right designate segments of the porcine PaRV genome, and those on the left represent segments of the porcine group A rotavirus genome. Migration was from top to bottom.

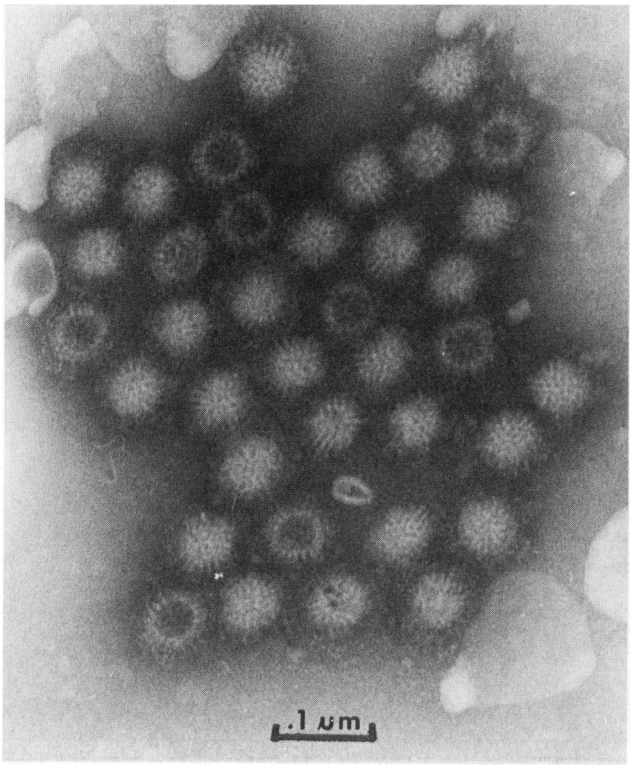

FIG. 3. Typical aggregate of double-shelled PaRV particles obtained from cell culture supernatants incubated with anti-PaRV (immune electron microscopy).

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