Serial Propagation of Porcine Group C Rotavirus (Pararotavirus) in a Continuous Cell Line and Characterization of the Passaged Virus†

LINDA J. SAIF,* LISA A. TERRETT, KATHY L. MILLER, AND R. F. CROSS

Food Animal Health Research Program, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, Ohio 44691

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The Cowden strain of porcine group C rotavirus (pararotavirus) was adapted to serial passage in a continuous monkey kidney cell line (MA104). Key factors in its successful adaptation included use of virus passaged in primary porcine kidney cells as the initial inoculum, use of roller tubes, and addition of pancreatin to the maintenance medium. A cell culture immunofluorescence test was used to quantitate the virus at each passage level, since a possible cytopathic effect was obscured by the effects of pancreatin. The virus titers dropped after initial passage into MA104 cells but increased thereafter, with peak titers evident after 16 passages (10^12 immunofluorescence U/ml). Immune electron microscopy and genome electropherotyping were used to identify group C rotavirus particles and confirm group C rotavirus double-stranded RNA gel migration patterns, respectively, from infected cell culture supernatants. The electropherotype of the cell culture-propagated group C rotavirus was identical to that of the gut virulent virus from which it was derived. The cell culture-passaged group C rotavirus also retained its infectivity for gnotobiotic pigs. No group A rotavirus was detected in the intestinal contents of the pigs or in cell culture fluids from group C rotavirus-inoculated monolayers with the two former techniques or the cell culture immunofluorescence test. This is the first verified report of serial propagation of a non-group A rotavirus in a continuous cell line.

Group C rotaviruses, also referred to as pararotaviruses, were first detected in swine in 1980 (19) and in humans in 1982 (17). Their occurrence in other species has not been reported. Group C rotaviruses from humans and pigs are antigenically related and have similar double-stranded (ds) RNA genome electropherotypes (6, 20). Group A and C rotaviruses are morphologically identical but antigenically and electropherotypically distinct (15, 19, 20). They are also antigenically and electropherotypically distinct from other non-group A rotaviruses (serogroups B, D, and E) (15, 16, 20).

Porcine group C rotaviruses cause gastroenteritis of a severity similar to that caused by group A rotaviruses in experimentally inoculated gnotobiotic pigs (4). The epidemiologic significance of group C rotaviruses from swine or humans as causes of gastroenteritis have not been well defined, in part because of the absence of simple diagnostic tests like ones used to detect group A rotaviruses. Development of such tests is usually predicated on the ability to produce large quantities of antigen in an in vitro system. Whereas most group A rotaviruses can be serially propagated to high titers in cell culture by using proteolytic enzyme treatment or roller tube cultures, most attempts to propagate group C rotaviruses serially or to propagate other non-group A rotaviruses by similar techniques have failed. Exceptions include the successful serial propagation of two non-group A rotaviruses in primary cell cultures (14, 23). One isolate of avian group D rotavirus was serially propagated in primary chicken embryo liver cells (14), and we recently reported the adaptation of porcine group C rotavirus to serial propagation in primary porcine kidney (PPK) cell cultures (23). However, for many routine serologic assays, propagation of a virus in continuous cell lines is easier and faster. Conditions for adaptation of porcine group C rotavirus to serial passage in a continuous monkey kidney cell line (MA104) are described in this report. Also described are the morphologic, antigenic, ds RNA electrophoretic, and pig infectivity attributes of cell culture-adapted group C rotavirus.

MATERIALS AND METHODS

Viruses. Large-intestinal contents from gnotobiotic pigs infected with the Cowden strain of porcine group C rotavirus were used to inoculate roller tube cultures of PPK cells as described previously (23). Virus from the serial passage 9 in PPK cells was harvested by freeze-thawing of cells, and this suspension was used undiluted as the initial inoculum for the fetal rhesus monkey kidney (MA104) cell line. The viral inoculum for MA104 cells had a titer of 5 × 10^9 fluorescent-focus units (FFU)/ml when titrated in MA104 cells with a cell culture immunofluorescence (CCIF) test (24).

The Ohio State University (OSU) isolate of porcine rotavirus was passaged in MA104 cells as described previously (5) and served as the reference strain of a group A rotavirus for comparative tests.

Cell cultures. Approximately 2 × 10^6 MA104 cells were seeded into roller tubes (screw-cap tubes [16 by 125 mm] or Leighton tubes containing cover slips [9 by 35 mm]) and 6- or 24-well plates and grown to confluency in stationary culture at 37°C (6- and 24-well plates were incubated in a 5% CO_2 atmosphere). The growth medium used (supplemented Eagle minimal essential medium [EMEM]) and preparation of the monolayers for viral inoculation were as described previously (23).

Virus propagation. (i) Roller tube cell monolayers. Immediately before viral inoculation, the EMEM was decanted and 0.2 ml of viral suspension was added to duplicate cell monolayers at each passage or enzyme treatment level. Monolayers were incubated at 37°C with rocking every 15 min. At the end of 1 h, 2 ml of serum-free EMEM and various amount of pancreatin (4× NF; GIBCO Laboratories,
Grand Island, N.Y.) were added to the cell monolayers. Some monolayers received no pancreatin, others received 80 or 120 µl of pancreatin diluted 1:10 in phosphate-buffered saline, pH 7.4, and still others received 40 µl of pancreatin diluted 1:200 in phosphate-buffered saline. Control MA104 cell monolayers received 2.2 ml of serum-free EMEM and the various levels of pancreatin described above. Most monolayers were placed in a roller tube apparatus (Conrac Corp., Old Saybrook, Conn.) at 37°C for 2 to 3 days. Monolayers were harvested by being frozen and thawed once. Subsequent passages were conducted in the same manner, with 0.2 ml of the infected-cell suspensions diluted 1:10 to 1:100 in serum-free EMEM as the inoculum for viral passages 7 to 9, 18, and 19. For other viral passages, the inoculum was diluted 1:2 in serum-free EMEM. The cell suspension fluids from each passage were tested for group A and C rotavirus with a CCIF assay (24).

Leighton tube cell monolayers were inoculated with virus in parallel at various passage or enzyme treatment levels to monitor group C rotavirus infectivity. Inoculated cover slip monolayers were removed from the tubes, fixed in acetone, and examined for group C rotavirus-infected cells by using immunofluorescent (IF) staining (23).

The Leighton tube cell monolayer cultures were also used to examine the effect of roller versus stationary cultures on group C rotavirus infectivity and to determine the optimal time postinoculation (p.i.) for harvest of virus-infected cell culture fluids. For these experiments, monolayers received 120 µl of pancreatin diluted 1:10. The infectivity of group C rotavirus in roller versus stationary cell monolayer cultures was compared at viral passages 9 through 11. Duplicate Leighton tube monolayer cultures were inoculated as described previously. One of these was rolled in a roller tube apparatus, while the other remained stationary at 37°C. Cover slips were removed and examined for infected cells at 3 days p.i. The optimal time for harvest of group C rotavirus-infected-cell monolayers was determined with Leighton tube monolayers infected with group C rotavirus from passages 10 and 11. Cover slips were removed at 2, 3, and 4 days p.i., fixed, stained by IF, and examined to determine the approximate numbers of virus-infected cells.

(ii) Twenty-four-well plates. Three-day-old confluent MA104 cell monolayers in 24-well plates were inoculated with 0.1 ml of a 1:2 dilution (prepared in serum-free EMEM) of roller tube viral passage 13 added to duplicate wells. The plates were incubated at 37°C for 1 h in a 5% CO₂ atmosphere with rocking every 15 min. One milliliter of serum-free EMEM and 120 µl of a 1:10 dilution of pancreatin was added to each virus-inoculated or control well. Plates were centrifuged at 1,200 x g for 1 h at room temperature and then incubated at 37°C in a 5% CO₂ atmosphere for 2 to 4 days. Monolayers were harvested by being frozen and thawed once. Four subsequent passages were conducted in the same manner with 0.1 ml of the infected-cell suspensions from the preceding viral passage diluted 1:2 in serum-free medium as the inoculum. The cell suspension fluids from each of the five passages were monitored for group A and C rotavirus with a CCIF assay (24).

(iii) Six-well plates. For the plaque assay, 7-day-old confluent MA104 cell monolayers seeded in six-well plates were used. Tenfold dilutions of the test inoculum (roller tube viral passage 17) through 10⁻⁴ were prepared in serum-free EMEM, and 0.2 ml of each dilution was added to each well after removal of the culture medium. After viral adsorption, 80 µl of a 1:10 dilution of pancreatin was added to each well, followed by 3 ml of the agar overlay medium (serum-free EMEM supplemented with 0.8% Noble agar [Difco Laboratories, Detroit, Mich.] and 0.0007% neutral red) per well. When the agar layer solidified, the plates were inverted, kept at 37°C in a 5% CO₂ atmosphere, and checked daily for appearance of plaques through 10 days p.i.

Detection of group C rotavirus or virus-infected cells. (i) IF staining. Cover slips from infected Leighton tube monolayers were removed and fixed in 100% acetone for 10 min. The cover slips were then incubated with fluorescein isothiocyanate-conjugated hyperimmune globotidiot polyclonal anti-A (OSU) or C porcine rotavirus serum (5) for 1 h at 37°C. Cover slips were examined for percentage of fluorescent cells as described previously (4, 23).

(ii) CCIF. The titers of group C rotavirus in cell culture fluids from roller tube and 24-well plate cultures were quantitated with a CCIF assay (24). The group C rotavirus cell suspension fluids were also tested for group A rotavirus contamination by the CCIF assay (24). Briefly, confluent monolayers of MA104 cells grown in 96-well tissue culture plates were inoculated with the cell culture-adapted group C rotavirus (0.2 ml per well), followed by 40 µl of pancreatin (1:200 in phosphate-buffered saline) per well. Plates were then centrifuged for 1 h at room temperature before being kept in a 5% CO₂ atmosphere at 37°C for approximately 16 h. Infected-cell monolayers were fixed in 80% acetone for 10 min and stained with fluorescein isothiocyanate-conjugated anti-group A (OSU) or C porcine rotavirus serum. Wells were examined for percent fluorescent cells as described previously (4, 23).

(iii) IEM. For immune electron microscopy (IEM), MA104 cell culture supernatants from infected roller tube cultures at passages 1, 10, and 16 through 18 were concentrated 10-fold by high-speed centrifugation (65,000 × g for 60 min at 4°C) before being incubated with anti-group A or C rotavirus serum as described previously (18). Samples were negatively stained and examined for viral particles with a Philips 201 electron microscope (Philips-Norelco, Eindhoven, The Netherlands).

(iv) Polyacrylamide gel electrophoresis of viral ds RNA. Cell culture supernatants harvested from group C rotavirus-infected MA104 cells (passages 13 and 17), as well as the gut virulent Cowden group C rotavirus, and cell culture-propagated OSU group A rotavirus were extracted as described previously to obtain viral ds RNA (26). The ds RNA preparations were electrophoresed in 7.5% polyacrylamide gels and then silver stained as previously described (21, 26) to determine the viral ds RNA electrophoretotypes.

Inoculation of gnotobiotic pigs. Two litters of gnotobiotic pigs of different ages (32 and 4 days old) were used in these studies. Three 32-day-old (pigs 1 to 3) and two, 4-day-old (pigs 4 and 5) gnotobiotic pigs were each inoculated orally with 3 to 5 ml of cell culture fluids from group C rotavirus roller tube passages 13 and 17, respectively (4). Pigs 3 through 5 were swabbed rectally, and clinical signs were noted through 14 days p.i. Pigs 1 and 2 were euthanized at 2 and 3 days p.i., respectively, and segments of the small intestines were removed for histopathologic examination (7) and fluorescent-antibody staining (4). Small-intestinal mucosal smears were stained with fluorescein isothiocyanate-conjugated anti-group A (OSU) or C porcine rotavirus serum as described previously (4, 5). Feces or intestinal contents and rectal swab fluids were examined for rotavirus by IEM (18), and CCIF tests (24), respectively. Group C rotavirus ds RNA was extracted from the intestinal contents of pigs, electrophoresed on polyacrylamide gels, and silver stained as described previously (21, 26).
RESULTS

Roller tube cell cultures. Cell suspension fluids from inoculated MA104 cell monolayers treated with 80 to 120 µl of pancreatin (samples 1A and 1B) were positive for group C rotavirus by the CCIF assay (Fig. 1a) at each passage level tested (Table 1). The average virus titers for the first four passages were usually lower than the titer of the initial inoculum but then generally increased thereafter, reaching peak titers (sample 1A) at passage 16. The average virus titers for sample 1A remained at 3 × 10^6 to 5 × 10^6 FFU/ml for the last passages tested (passages 15 to 18). Passage of sample 1B was discontinued after passage 5, since the results were similar to those for sample 1A with 120 µl of pancreatin. Addition of 40 µl of a 1:200 dilution of pancreatin (sample 1C) or no pancreatin (sample 1D) to the MA104 cell monolayers resulted in complete loss of viral infectivity by passage 3 (no fluorescent cells evident in CCIF [Table 1]). No group C rotavirus was detected in supernatant fluids from the un inoculated MA104 cell monolayers.

Cover slips with group C rotavirus-inoculated monolayers of MA104 cells from Leighton tube cultures were also examined at each passage level. An example of the types of fluorescing cells observed at passage 17 is shown in Fig. 1b. Bright cytoplasmic fluorescence and unstained nuclei were evident in infected but not control cell monolayers.

some text and equations would be missing here, but the context seems to be about the propagation of porcine group C rotavirus and the analysis of results from different cultures and passages. The table below shows the cell culture infectivity of porcine group C rotavirus at sequential passage levels in MA104 roller tube cultures.

![Image of the page]

**FIG. 1.** IF observed after incubation of FITC-conjugated anti-group C rotavirus serum on (a) MA104 cells infected with cell culture-passaged (passage 11) group C rotavirus (CCIF assay; magnification, ×330. (b) Leighton tube slides of MA104 cells infected with cell culture-passaged virus (passage 17; magnification, ×530).**

**TABLE 1.** Cell culture infectivity of porcine group C rotavirus at sequential passage levels in MA104 roller tube cultures.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Amt of pancreatin (dilution)*</th>
<th>Avg no. of fluorescent foci/ml at virus passage:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1A</td>
<td>120 (1:10)</td>
<td>5 × 10^3</td>
</tr>
<tr>
<td>1B</td>
<td>80 (1:10)</td>
<td>NT</td>
</tr>
<tr>
<td>1C</td>
<td>40 (1:200)</td>
<td>NT</td>
</tr>
<tr>
<td>1D</td>
<td>None</td>
<td>NT</td>
</tr>
</tbody>
</table>

* The initial inoculum represented replicate cultures of Cowden group C rotavirus, previously passaged nine times in PPK culture, and had a CCIF titer of 5 × 10^4 fluorescent foci per ml.

* Diluted in phosphate-buffered saline. NT, Not tested; -, Passages discontinued.

Leighton tube cultures with cover slips also were used to examine the effect of roller versus stationary culture on the numbers of group C rotavirus-infected cells. Roller tube cultures (serial passages 9 through 11) consistently had about 20-fold greater numbers of infected cells than did stationary cultures at the same passage levels when cover slips were examined after IF staining. Two to three days p.i. was the optimal time for harvest of group C rotavirus-infected MA104 cells to obtain maximal numbers of infected cells as determined by IF staining of Leighton tube cover slip monolayers. After this time, most cells detached from the cover slips, making detection of virus-infected cells difficult. Because of the high levels of pancreatin used in the roller tube cell cultures, a cytopathic effect could not be clearly distinguished in infected-cell monolayers when compared with un inoculated control cell monolayers. No group A rotavirus was detected by the CCIF assay or IF staining of group C rotavirus-inoculated or control monolayers at any passage level.

Six- and twenty-four-well plates. The titer of group C rotavirus dropped from the initial titer of 5 × 10^5 FFU/ml (roller tube passage 13) through each of five successive passage levels in the stationary 24-well plate cultures of inoculated MA104 cells. By passage 5, no group C rotavirus was detected in the inoculated cell suspension fluids (titer,
<10 FFU/ml and passages were discontinued. No plaques were detected during a 10-day period for any dilution of the inoculum (initial parrotavirus titer, 5 × 10<sup>6</sup> FFU/ml) on monolayers grown in six-well plates.

**Inoculation of gnotobiotic pigs.** The clinical signs, lesions, and viral detection in gnotobiotic pigs inoculated with MA104 cell culture-propagated (roller tube passage 13 or 17) group C rotavirus are summarized in Table 2. Both older (32-day-old) and younger (4-day-old) gnotobiotic pigs showed clinical signs or evidence of group C rotavirus infection as detected by IEM, CCIF tests, or IF staining of intestinal smears. The onset of clinical signs was earlier and the duration was slightly longer in the younger than in the older gnotobiotic pigs. However, diarrhea in the pigs given the cell culture virus was mild (semiliquid). IF was observed only in the cytoplasm of enterocytes as illustrated in the IF-stained mucosal smear from the duodenum of pig 2 (Fig. 2). All intestinal smears and rectal swab fluids of group C rotavirus-inoculated pigs were negative for IF when stained for group A rotavirus antigens.

**IEM.** Aggregates of typical double-capsid (about 70 nm in diameter) or single-capsid (about 55 nm in diameter) group C rotaviruses were detected after incubation of cell culture supernatants with hyperimmune anti-porcine group C rotavirus serum (Fig. 3A). The group C rotaviruses were not aggregated by hyperimmune anti-porcine group A rotavirus serum (Fig. 3B). All passage levels examined contained typical group C rotaviruses, and the total numbers of viral particles observed for each passage correlated well with the CCIF titers, with the greatest numbers evident at passage 16.

The presence of aggregates of typical group C rotaviruses in feces or intestinal contents from each gnotobiotic pig inoculated with a cell culture-propagated virus was also confirmed by IEM with specific antiserum, as was the absence of group C rotavirus aggregation in the corresponding samples reacted with anti-group A rotavirus serum.

**Electrophoresis of viral ds RNA.** The electrophoretic migration patterns (electropherotypes) of the MA104 cell culture-passaged porcine group C rotavirus at the passage levels examined were identical to those of the gut virulent virus (Cowden) from which they were derived (Fig. 4). The genome segments were distributed among the four size classes in the 4-3-2-2 pattern characteristic of group C rotaviruses, and no extra bands were evident. Moreover,
passage of the cell culture-adapted virus in gnotobiotic pigs did not alter its electropherotype (Fig. 4). The electrophoretic migration pattern of the cell culture-passaged and gut virulent porcine group C rotavirus was clearly distinguishable from that of the cell culture-passaged OSU porcine group A rotavirus (Fig. 4), which displayed a 4-2-3-2 genome banding pattern, typical of group A rotaviruses.

DISCUSSION

Many strains of group A rotaviruses from humans and other animals have been successfully adapted to serial propagation in primary cells or continuous cell lines (3, 9-13, 22, 25, 27). The key factor in the successful routine adaptation of many rotavirus strains to serial propagation in vitro has been the use of pancreatic enzymes (1, 3, 25, 27) or roller tube cultures (10, 22). We have found similar factors to be important in the adaptation of porcine group C rotavirus to serial propagation in PPK cells as described previously (23) or MA104 cells as described in this report. Whereas some isolates of group A rotaviruses were first propagated in primary cell cultures before adaptation to continuous cell lines (6, 13, 25), other isolates were passaged directly into MA104 cell cultures (5, 10, 22) or other continuous cell lines (3, 9). Attempts to passage a porcine group C rotavirus directly in MA104 cell cultures by using similar techniques were unsuccessful (4, 23).

Successful adaptation of a porcine group C rotavirus to serial propagation in MA104 cell cultures as described in this report was contingent upon a number of factors. These included (i) prior passage of virus (nine passages) in PPK cells, (ii) continued use of high levels of pancreatin in the maintenance medium, (iii) use of roller tube cell monolayers, and (iv) use of a sensitive assay for detection of infectious virus (CCIF) or virus-infected cells (IF). Use of whole-cell lysate suspensions as inocula at each passage also may have contributed to viral infectivity, if these viruses tend to remain highly cell associated.

We were unsuccessful in adapting group C rotavirus to serial propagation in stationary cultures of MA104 or PPK cells. This failure probably also accounts for our inability to adapt a group C rotavirus to plaque formation in stationary cultures of either MA104 or PPK cell cultures (L. J. Saif and K. L. Miller, unpublished data). Reasons for the failure to passage this virus serially in stationary cultures are unknown. Although the group C rotavirus adapted to MA104 roller tube cell cultures currently has a titer of $8 \times 10^5$ FFU/ml after the last passage (18), group A rotaviruses adapted to MA104 cell cultures frequently have titers of $10^6$ FFU/ml or greater at the same passage level. Additional work is needed to try to increase the virus titers further and identify the factor(s) in pancreatin, and its optimal concentration, which enhances group C rotavirus infectivity. Whether the mechanism of this enzyme enhancement resembles that seen for group A rotaviruses; i.e., proteolytic cleavage of the outer capsid polypeptides (8) is unknown. Further studies are also necessary to adapt group C rotaviruses to grow and form plaques in stationary cultures and to clone the virus to assure homogeneity for future serologic tests.

This is the first verified report of adaptation of a mammalian atypical rotavirus to serial propagation in a continuous cell line. In two earlier reports (14, 23), primary cell cultures and proteolytic enzymes were required to adapt a porcine group C or avian group D rotavirus to serial propagation. In another study, the possible cell culture propagation of a porcine atypical rotavirus, antigenically unrelated to porcine group C rotavirus, was reported (2), but no further studies were done to verify or characterize the cell culture-propagated virus. In the present study, the identity of the MA104 cell culture-propagated group C rotavirus was confirmed by using IF, IEM, and ds RNA electropherotyping. The electropherotype of the MA104 cell culture-passaged group C rotavirus was identical to that of the gut virulent group C rotavirus and resembles the previously published electropherotypes described for group C rotaviruses from swine or humans (4, 6, 15, 17, 20). No extra segments were evident which might otherwise suggest mixed infections. Likewise, no other viruses were detected in the concentrated cell culture supernatant fluids by electron microscopy. No group A rotaviruses were detected at any passage level with these same procedures.

The cell culture-passaged group C rotavirus retained its infectivity for gnotobiotic pigs after 13 or 17 passages in MA104 cells. In contrast to previous studies with gnotobiotic pigs given virulent group C rotavirus (4), only mild diarrhea was observed in the five pigs given the cell culture-passaged virus. However, the lesions observed and the patterns of IF staining in the intestines were similar between pigs given a cell culture-passaged virus and those given a virulent virus (4). No group A rotaviruses or other viruses were detected in the feces or intestinal contents of the inoculated pigs with any of the virus detection methods described.

Successful adaptation of porcine group C rotavirus to propagation in a continuous cell line has many important implications. For instance, similar techniques may be useful for adaptation of human group C rotaviruses or other strains of group C porcine rotaviruses to in vitro propagation, thereby facilitating development of methods for routine
detection of group C rotaviruses or antibodies in either swine or humans. Alternatively, the in vitro propagated swine group C rotavirus might be used to create reassortants with the fastidious human group C rotaviruses, thereby rescuing these viruses, as was done earlier for noncultivatable human group A rotaviruses (11).

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LITERATURE CITED