

Isolation, Characterization, and Serial Propagation of a Bovine Group C Rotavirus in a Monkey Kidney Cell Line (MA104)

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A virus (designated the Shintoku strain) which was morphologically indistinguishable from group A rotaviruses was detected in the feces of adult cows with diarrhea in Japan. The virus contained 11 segments of double-stranded RNA and had an electrophoretic migration pattern in polyacrylamide gels similar to that of other group C rotaviruses (4-3-2-2). Feces containing the bovine virus reacted with antiserum to porcine group C rotavirus (Cowden strain) but not group A or B rotaviruses in immunoelectron microscopy. The virus was adapted to serial propagation in roller tube cultures of a rhesus monkey kidney cell line (MA104) by using high concentrations of trypsin. Evidence for viral replication in MA104 cell cultures was demonstrated by immunoelectron microscopy and indirect immunofluorescence by using antiserum to porcine group C rotavirus and by electrophoretic analysis of extracted viral double-stranded RNA. A significant antibody response against the isolate was detected in convalescent-phase sera of cows which excreted the virus: no increased antibody response to bovine group A rotavirus was observed. To our knowledge, this is the first isolation of a group C rotavirus from cattle.

Rotavirus infections are a major cause of viral diarrheas in young animals and children throughout the world (10, 16). Rotaviruses isolated from different species share a common group antigen demonstrable by immunofluorescence (IF), immunoelectron microscopy (IEM), complement fixation, and enzyme-linked immunosorbent assays (34, 36, 37). Recently, atypical rotaviruses, which are morphologically indistinguishable from conventional rotaviruses (group A) but are antigenically distinct, have been found in animals and humans (1, 2, 6–9, 17, 20, 22, 23, 25–27, 29, 35). At present, rotaviruses are divided into seven groups, tentatively designated as groups A to G on the basis of serologic and genome analyses (21, 22, 25, 27, 29, 31). Each group of rotaviruses shares its own common group antigen and shows similar double-stranded RNA (dsRNA) electrophoretic migration patterns, which vary among groups.

Group C rotaviruses have been found in swine and humans (2, 7, 8, 15, 25–27, 35), but there are no reports on the detection of group C rotaviruses in other species, although antibodies to group C rotaviruses have been detected in sera from cattle (5, 27). In addition, attempts to propagate group C rotaviruses in tissue culture have failed, with the exception of a porcine group C rotavirus (28, 33). This report describes the detection and characterization of a group C rotavirus (designated the Shintoku strain) from diarrheic cows and its adaptation to serial propagation in a fetal rhesus monkey kidney cell line (MA104).

Clinical specimens. Feces and sera were obtained from four dairy cows (aged 28 to 41 months) affected with severe diarrhea at a farm in Hokkaido, Japan. The feces were blackish liquid, and diarrhea continued for 5 to 7 days. Fecal samples were collected on day 1 (three cows) and on day 2 (one cow) of illness, diluted 1:10 in 10 mM phosphate-

buffered saline (PBS) (pH 7.4), and clarified by low-speed centrifugation (3000 × *g* for 10 min). These samples were tested for group A rotaviruses by a latex agglutination test (Rotalex; Orion Diagnostica, Espoo, Finland). Serum samples were collected from the affected cows on the same day of fecal sampling and 14 days later.

Viruses. The group A rotavirus, used as a reference virus, was the bovine rotavirus NCDV strain which belongs to serotype 6 (12). This virus was supplied by Y. Murakami, National Institute of Animal Health, Kodaira, Japan, and propagated in MA104 cells as described previously (18). The Cowden strain of porcine group C rotavirus was propagated in MA104 cells or gnotobiotic pigs (2, 28, 33).

Hyperimmune antisera. Hyperimmune antisera to bovine group A rotavirus (NCDV) and porcine group C rotavirus (Cowden) were prepared in gnotobiotic pigs (2, 28). Guinea pig hyperimmune antiserum to the porcine group C rotavirus (Cowden) was supplied by S. Nakata, Sapporo Medical College, Sapporo, Japan (19). These antisera had optimal IEM titers of 400, 300, and 100 against each homologous virus, respectively. Optimal IEM titers were expressed as the reciprocal of the serum dilution which gave maximal numbers of viral aggregates. Hyperimmune antiserum to the bovine group B rotavirus was prepared in a gnotobiotic calf (25, 27, 29). The cell culture-adapted bovine group C rotavirus (Shintoku) at passage 15 was purified with trichlorotrifluoroethane and then by sucrose density gradient ultracentrifugation and was used to inoculate a guinea pig for preparation of hyperimmune antiserum.

Cell cultures. Monolayers of MA104 cells grown for 3 or 4 days in roller tubes were used for virus isolation. The growth medium was Eagle minimum essential medium (EMEM) supplemented with 10% calf serum, 10% tryptose phosphate broth (Difco Laboratories, Detroit, Mich.), 0.15% sodium bicarbonate, and antibiotics (100 U of penicillin per ml and 100 µg of streptomycin per ml).

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Virus isolation and propagation. A fecal suspension obtained from one cow was treated with either 100 or 10 μg of trypsin (type I; Sigma Chemical Co., St. Louis, Mo.) per ml for 30 min at 37°C, and 0.1 ml of each was inoculated onto MA104 cell monolayers which had been grown in roller tubes and washed three times with Earle's balanced salt solution. After adsorption for 60 min at 37°C, the cultures were washed twice with Earle's balanced salt solution and received 0.5 ml of EMEM supplemented with 1 μg of trypsin per ml as maintenance medium. Uninoculated control cells were also treated with 100 or 10 μg of trypsin per ml for 60 min at 37°C and washed, and maintenance medium was added as described above. The cultures were incubated in a roller drum for 3 days at 37°C and then frozen and thawed once for harvest of cell lysates. Subsequent passages were carried out in the same manner by using 0.1 ml of cell suspensions of the previous passage as the inoculum. Roller bottle cell monolayers were inoculated with 10 ml of cell suspensions obtained from the cultures of the respective series from which specimens were treated with 100 or 10 μg of trypsin per ml before inoculation. The cultures were incubated in the same manner as the roller tube passages, and the cell culture fluids (CCF) were harvested and used for electron microscopy (EM), IEM, and dsRNA analyses.

IF. Leighton tube cell monolayers were inoculated with the isolate or bovine group A rotavirus which was pretreated with 10 μg of trypsin per ml, EMEM supplemented with 1 μg of trypsin per ml was added, and the monolayers were incubated in a roller drum at 37°C. For the propagation of the porcine group C rotavirus, pancreatin was substituted for the trypsin (13, 26, 31). After 20 h of incubation, the coverslips from the Leighton tubes were removed, washed with PBS, and fixed in 100% acetone for 10 min. The coverslips were then incubated with serial twofold dilutions (initial dilution, 1:10) of anti-group A or anti-group C rotavirus serum or sera of the cows for 30 min at 37°C. After washes in PBS, coverslips were stained with anti-species globulin conjugated with fluorescein isothiocyanate (ZYMED Laboratories, San Francisco, Calif.). The titer of each serum was expressed as the reciprocal of the highest dilution that produced fluorescent cells.

Titration of the isolate. The titers of the isolate in infected CCF from roller tubes were quantitated by use of a cell culture IF assay (33). Confluent monolayers of MA104 cells grown in 96-well microplates were inoculated with 0.1 ml of the serial 10-fold dilutions of cell suspensions from roller tube cultures that were pretreated with 10 μg of trypsin per ml, 0.1 ml of EMEM supplemented with 1 μg of trypsin per ml was added, and the monolayers were incubated for 20 h at 37°C. Cells were washed with PBS, fixed with 80% acetone, and reacted with guinea pig anti-porcine group C rotavirus serum. After washes in PBS, cells were stained with anti-guinea pig globulin conjugated with fluorescein isothiocyanate. Fluorescent cells were counted, and viral titers were expressed as the total number of fluorescent cell-forming units per milliliter.

EM and IEM. Fecal suspensions and infected CCF were partially purified by ultracentrifugation through a 30% (wt/wt) sucrose cushion, placed on carbon-coated collodion grids, negatively stained with 2% phosphotungstic acid (pH 6.0), and examined for viral particles by use of an electron microscope (model JEM-1200EX; JEOL, Akishima, Tokyo, Japan).

For IEM, 10 μl of the partially purified isolate from feces or from infected CCF was incubated for 2 h at 37°C with an

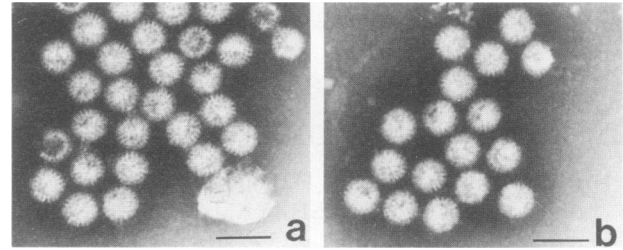


FIG. 1. IEM of bovine group C rotavirus incubated with guinea pig anti-porcine group C rotavirus (Cowden) serum diluted 1:100. Bar, 100 nm. (a) Aggregate of rotavirus particles observed in the feces of a cow with diarrhea; (b) aggregate of rotavirus particles observed in CCF from MA104 cells infected with bovine group C rotavirus (passage 15).

equal volume of hyperimmune antiserum to group A, B, or C rotavirus (diluted 1:100) and examined for viral aggregation.

Electrophoresis of viral dsRNA. Viral dsRNA was extracted from the partially purified isolate from feces and CCF and from partially purified porcine group C rotavirus (Cowden) and purified group A rotavirus (NCDV) with phenol-chloroform and then precipitated with ethanol. Electrophoresis of the extracted dsRNA was conducted in 7.5 or 10% polyacrylamide slab gels for 18 h at 4°C as described previously (14, 24). Gels were then stained with a silver stain kit (Bio-Rad Laboratories, Richmond, Calif.).

Fecal examination. Particles morphologically indistinguishable from rotavirus were found in fecal samples from all four cows by EM. Other viruslike particles were not observed. Most of the particles were double shelled and approximately 70 nm in diameter (Fig. 1). The particles were agglutinated by both anti-porcine group C porcine rotavirus sera (gnotobiotic pig and guinea pig) diluted 1:100 (Fig. 1a) but not with anti-bovine group A or B rotavirus serum diluted 1:100. All fecal samples were negative by a latex agglutination test for group A rotaviruses. Analysis of viral dsRNA by gel electrophoresis showed the same electrophoretic migration pattern, with 11 genome segments evident for all fecal samples. The migration pattern of the fecal virus was similar to that of group C rotaviruses isolated previously from pigs and humans, which is described as a 4-3-2-2 pattern (Fig. 2, lanes B, E, and F), but differed from the pattern typical for group A rotaviruses, described as a 4-2-3-2 pattern (Fig. 2, lanes A and C).

Virus isolation in cell cultures. The isolation and serial propagation of the virus in MA104 cells was confirmed by IF, EM, IEM, and electrophoresis of viral dsRNA. Virus infectivity was detected by a cell culture IF test in cell suspensions from the culture series in which inocula were pretreated with 100 μg of trypsin per ml. Fluorescent cells were observed when cultures were stained with either anti-porcine group C rotavirus serum or anti-bovine group C rotavirus serum. Virus titers were low in cell suspensions at passage 1 (5×10^2 fluorescent cell-forming units per ml) but increased gradually after additional passages, reaching titers of 8×10^4 fluorescent cell-forming units per ml at passage 15. Shown in Fig. 3 is specific IF in Leighton tube cell monolayers infected with bovine group C rotavirus cell suspensions at passage 13 and stained by indirect IF with gnotobiotic pig anti-porcine group C rotavirus serum diluted 1:640. In contrast, no fluorescent cells were observed in monolayers inoculated with cell suspensions from the culture series in which inocula were pretreated with 10 μg of trypsin per ml

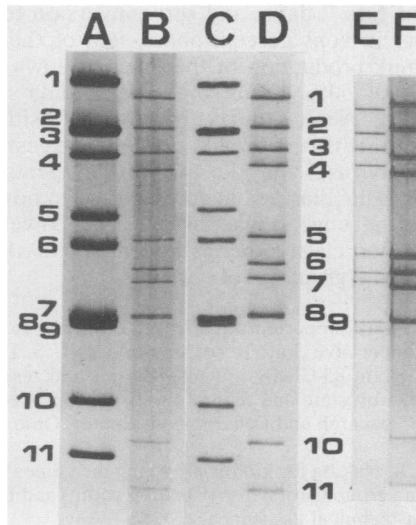


FIG. 2. Comparison of the electrophoretic migration patterns of rotaviral dsRNA. Samples were analyzed in 7.5% (lanes E and F) or 10% (lanes A to D) polyacrylamide gels, and gels were stained with silver nitrate. Lanes: A and C, bovine group A rotavirus (NCDV); B, dsRNA extracted from the feces of a diarrheic cow; D and F, dsRNA extracted from CCF of MA104 cells infected with bovine group C rotavirus (passages 15 and 30, respectively); E, porcine group C rotavirus (Cowden). Lanes A and B, lanes C and D, and lanes E and F were run on separate gels. The numbers indicate segments of the genome, and migration is from top to bottom.

or from the noninfected culture series reacted with anti-group A or anti-group C rotavirus serum.

Because both infected and noninfected (control) cell monolayers were damaged considerably by the high trypsin concentration contained in the inocula pretreated with 100 μ g of trypsin per ml, cytopathic effects were not observable through passage 12. After passage 13, however, it was possible to distinguish cytopathic effects in infected cultures as compared with noninfected controls. Infected cells de-

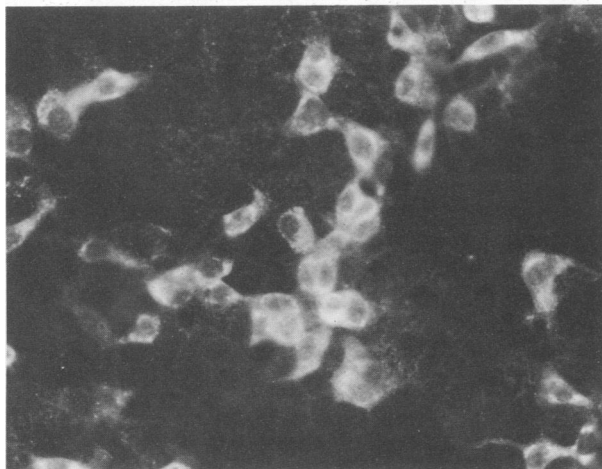


FIG. 3. MA104 cells infected with the bovine group C rotavirus (passage 13) and reacted with gnotobiotic pig anti-porcine group C rotavirus (Cowden) serum diluted 1:640 and then stained with fluorescein isothiocyanate-conjugated rabbit anti-porcine IgG diluted 1:20 (indirect IF). Magnification, $\times 223$.

TABLE 1. Antigenic relationships between group A and group C rotaviruses as determined by indirect IF

Antiserum	Indirect IF titer ^a against rotavirus		
	Bovine group C (Shintoku)	Porcine group C (Cowden)	Bovine group A (NCDV)
Gnotobiotic pig anti-bovine group A rotavirus (NCDV)	160	160	81,920
Gnotobiotic pig anti-porcine group C rotavirus (Cowden)	10,240	10,240	80
Guinea pig anti-bovine group C rotavirus (Shintoku)	10,240	10,240	160
Gnotobiotic pig preimmune serum	<10	<10	<10

^a Determined as the reciprocal of the highest antiserum dilution which resulted in fluorescing cells.

tached from the surface of culture tubes within 3 days after inoculation, while control cells remained attached, although they appeared damaged by the trypsin. No cytopathic effect was observed in the culture series in which inocula were pretreated with 10 μ g of trypsin per ml.

Passages 7 and 15 of infected CCF were examined by EM, IEM, and electrophoresis of viral dsRNA. Rotavirus particles were detected by EM in infected CCF of the culture series in which inocula were pretreated with 100 μ g of trypsin per ml. Other viral particles were not observed. The particles were similar in morphology to the particles in the original feces, which were mostly double shelled and approximately 70 nm in diameter. The particles were agglutinated with both anti-group C porcine rotavirus sera (gnotobiotic pig and guinea pig) but not with gnotobiotic pig anti-group A rotavirus serum diluted 1:100 (Fig. 1b). No viral particles were observed in CCF of the culture series in which viral inocula were pretreated with 10 μ g of trypsin per ml or in noninfected control cultures. The electrophoretic migration pattern of dsRNA extracted from infected CCF of the culture series in which inocula were pretreated with 100 μ g of trypsin per ml was identical to that from the original feces (Fig. 2, lanes B and D).

Antigenic reactivity. MA104 cell monolayers infected with the bovine group C rotavirus at passage 20, the porcine group C rotavirus (Cowden), and the bovine group A rotavirus (NCDV) were used for serological assays by indirect IF (Table 1). Antisera to the bovine and porcine group C rotaviruses reacted with the heterologous virus at the same titers as they did to the homologous virus. These sera also reacted with the group A rotavirus, but the reactions against the group A rotavirus were 64- to 128-fold lower than those against the group C rotaviruses. Antiserum to the group A rotavirus reacted with all of the viruses but had 512-fold-lower IF titers when tested against the group C rotaviruses than when tested against the homologous virus.

Antibody responses in affected cows. Sera of all cows against the isolate were seronegative in the acute phase (antibody titers, <10) but were seropositive in the convalescent phase, with antibody titers ranging from 1,280 to 2,560 by indirect IF. Against group A rotavirus, sera of all cows were seropositive (antibody titers ranged from 160 to 640 by indirect IF), but less than fourfold increases between acute- and convalescent-phase sera were observed.

Group C rotaviruses which share a common group antigen have been reported in swine and humans. Their genomes consist of 11 segments of dsRNA which show similar migration profiles in polyacrylamide gel electrophoresis, identified as a 4-3-2-2 migration pattern (2, 7, 21, 25, 27). In this study, viral particles detected in diarrheic cows were agglutinated by anti-porcine group C rotavirus serum, and dsRNA extracted from those particles revealed 4-3-2-2 electrophoretic migration patterns characteristic of group C rotaviruses. These data indicate that the virus detected from cases of bovine diarrhea in the present study belongs to group C rotavirus. Thus, to our knowledge, this is the first report of the detection and isolation of a group C rotavirus in cattle. We propose that the virus found in this study be designated the Shintoku strain of bovine group C rotavirus.

Although most group A rotaviruses are cultivated readily in MA104 cell cultures, many attempts to propagate non-group A rotaviruses in cell culture have failed, except for one porcine group C rotavirus (Cowden) (28, 33). This virus was propagated in primary porcine kidney cells and subsequently adapted to MA104 cells by using pancreatin and roller tubes for virus cultivation (28, 33). We tried to directly adapt the bovine group C rotavirus to serial propagation in MA104 cells with the aid of high concentrations of trypsin and roller tube cultures. The results of examination of infected cells and CCF by IF, IEM, and analysis of viral dsRNA indicate that the bovine group C rotavirus was successfully adapted to serial propagation in MA104 cells. The bovine group C rotavirus propagated in MA104 cells only when inocula were treated with 100 μ g of trypsin per ml, not when they were treated with 10 μ g of trypsin per ml, although the latter concentration was successfully used to propagate group A rotaviruses (11, 18, 30). This suggested that treatment of inocula with high concentrations of proteolytic enzymes and rolling of cultures are important factors for propagation of non-group A rotaviruses in cell cultures, as previously described for porcine group C rotavirus by Saif et al. (28). Why considerably higher concentrations of proteolytic enzymes are required for propagation of non-group A rotaviruses, as compared with most group A rotaviruses, is unknown. Further studies on the interaction between non-group A rotaviruses and proteolytic enzymes are needed to elucidate mechanisms to enhance the infectivity of these viruses.

There was a weak cross-reaction between group A and C rotaviruses detected by IF (Table 1). This result suggests that group A and group C rotaviruses may share a minor common antigen or that nonspecific background reactivities were present. Support for the former possibility comes from studies by Bremont et al. (3) who reported a higher-than-expected homology level (55 and 42% for nucleotides and amino acids, respectively) between the VP6 gene of the porcine group C rotavirus (Cowden) and the corresponding gene of group A rotavirus. This finding lends support to the possible existence of a minor common antigen between group A and group C rotaviruses recognized by the polyclonal antisera in this study. However, because previous investigators reported that there was no serological cross-reactivity between group A and group C rotaviruses (2, 4, 13, 19, 21, 26, 31), further studies are needed to examine the cross-reactivity with additional antisera, including use of monoclonal antibodies and other immunological methods to test for antigenic relatedness.

It has been reported that porcine group C rotavirus is as virulent for pigs as group A rotavirus (2). In this study, all of the cows in which the virus was detected showed severe

diarrhea for 5 to 7 days, and seroconversion to the isolate was evident in convalescent-phase sera of these cows. In addition, milk production of the infected cows declined by an average of 50% during the illness (data not shown). Although no screening for bacterial and parasitic pathogens was done during this outbreak, our data suggest that bovine group C rotaviruses may be one of the agents capable of causing epizootic diarrhea in cows similar to bovine coronavirus (32). Challenge studies in cows and calves are necessary to further clarify the pathogenicity of the Shintoku strain of bovine group C rotavirus.

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