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Cytopathic Astrovirus Isolated from Porcine Acute Gastroenteritis in an Established Cell Line Derived from Porcine Embryonic Kidney

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A cytopathic astrovirus was isolated from pigs with acute diarrhea in an established cell line that was derived from porcine embryonic kidneys with the aid of trypsin. The virus showed a distinct cytopathic effect characterized by an enlargement of cells and the appearance of fine granules in the cytoplasm. Porcine astrovirus was shown to have an RNA genome, as determined by the effect of 5-iodo-2′-deoxyuridine on its replication, and five polypeptides with molecular masses of 13,000, 30,000, 31,000, 36,000, and 39,000 daltons; and it was shown to be stable to lipid solvents and heating at 50°C for 30 min but somewhat labile to acid (pH 3.0). The buoyant density of the isolate determined in CsCl was 1.35 g/ml. Seroconversion to the virus was evident in the paired serum specimens obtained from pigs with diarrhea that were housed at the farm where the disease occurred. The neutralization test on serum specimens collected randomly from 128 adult pigs of eight herds revealed that 50 of the serum specimens were positive for antibody to porcine astrovirus, although there was considerable variation in the prevalence among herds, ranging from 0 to 83%. Hysterectomy-produced, colostrum-deprived, 4-day-old pigs developed mild diarrhea after oral exposure to porcine astrovirus propagated in the cell culture; and the virus was isolated again from diarrheal stool specimens.

Astrovirus is a small spherical virus with a visible subunit structure which appears white and as a five- or six-pointed star when it is negatively stained. The virus was first detected by Madeley and Cosgrove (11) by electron microscopy (EM) in diarrheal specimens from cases of human infantile gastroenteritis. Subsequently, astroviruses have been demonstrated in diarrheal stool specimens of pigs (1) and other animal species (4, 6, 12, 17, 19, 20), as well as in stool specimens of humans (3, 7, 14).

In most studies, direct EM of fecal specimens has been the sole means available to detect astroviruses in diarrheal stool specimens, because it has been difficult to isolate and propagate them in cell cultures, except only in limited cases. Lee and Kurtz (9) have described the serial passage of human astrovirus in human embryonic kidney cells and the subsequent adaptation to propagation in a continuous line of rhesus monkey kidney ($LLCMK_2$) and primary baboon kidney cells. Woode and Bridger (20) and, more recently, Harbour et al. (4) have successfully cultured bovine and feline astroviruses in bovine and feline embryonic cells, respectively. However, there have been no reports on the isolation of cytopathic astroviruses in cell cultures.

We have previously reported (16) an outbreak of porcine acute diarrhea accompanied by calicivirus, coronavirus, and astrovirus infection. The purpose of this report is to describe the isolation of a cytopathic astrovirus from those diarrheal feces and some of the properties of the cytopathic astrovirus.

MATERIALS AND METHODS

Diarrheal samples. An outbreak of epizootic and acute gastroenteritis occurred among pigs on a small pig farm. All pigs, regardless of age, developed clinical signs characterized by loss of appetite followed by vomiting and watery diarrhea, but death did not occur in pigs of any age. Diarrheal samples were collected from three pigs and submitted to our laboratory for diagnosis. Direct EM of diarrheal feces revealed the presence of coronavirus and calicivirus, but no astrovirus.

Cell culture. An ESK cell culture (18), which is an established cell line derived from porcine embryonic kidneys, was used. The growth medium consisted of Eagle minimal essential medium (EMEM) supplemented with 10% bovine serum, 10% tryptose phosphate broth, and 0.15% sodium bicarbonate. The maintenance medium used was EMEM supplemented with 0.15% sodium bicarbonate and 0.5 μg of crystalline trypsin (type III; Sigma Chemical Co., St. Louis, Mo.) per ml.

Virus isolation. A 10% fecal suspension was made with EMEM, clarified by centrifugation at 2,500 × g for 20 min, and filtered through a membrane filter with a pore size of 450 nm. ESK cells grown in test tubes were washed twice with EMEM, inoculated with 0.2 ml of the fecal suspensions, and incubated at 37°C for 1 h. The inocula were removed, and the cells were washed twice with EMEM. All cultures received 0.5 ml of the maintenance medium and were incubated in a roller drum at 37°C. After 5 days of incubation, culture fluids were harvested and inoculated into fresh ESK cells for the second passage.

EM. Viral particles purified from the supernatants of infected ESK cells and ultrathin sections of infected cells were observed with an electron microscope.

Infected cell culture fluids were harvested when cells exhibited a maximum cytopathic effect (CPE) and were centrifuged at 111,700 × g for 2 h on a sucrose cushion with a density of 1.200 g/ml. The pellet was suspended in 35% (wt/wt) CsCl solution and centrifuged at 111,700 × g for 18 h. The fraction with the highest infectivity was obtained and examined for virus with an electron microscope (JEM-100 CX; Nippon Denshi, Co., Tokyo, Japan), after it was stained with 0.2% phosphotungstic acid in phosphate buffer (pH 7.2).

For the preparation of ultrathin sections, infected ESK

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cells were collected before the maximum CPE appeared, fixed with 2% glutaraldehyde in phosphate buffer, postfixed with 1% osmium tetroxide in phosphate buffer, dehydrated in graded ethanol series, and embedded in an Epon mixture. Ultrathin sections were cut with a glass knife, stained with a lead citrate-uranyl acetate solution, and observed with an electron microscope.

Immune serum. Immune serum was obtained from hysterectomy-produced, colostrum-deprived (HPCD) pigs that were experimentally inoculated with a cloned cytopathic astrovirus.

Immunofluorescence. Confluent monolayers of ESK cells grown in Leighton tubes containing cover slips were inoculated with the isolate, and the cover slips were removed before the maximum CPE appeared. Cells were washed twice with phosphate-buffered saline (pH 7.2), dried, fixed with acetone at room temperature for 10 min, and incubated with the immune serum to the isolate at 37°C for 30 min. Then cells were washed four times with phosphate-buffered saline, stained with fluorescein isothiocyanate-conjugated rabbit anti-porcine immunoglobulin G (IgG) antibody at 37°C for 30 min, washed four times with phosphate-buffered saline, mounted with 50% phosphate-buffered saline–glycerin, and observed with a fluorescence microscope.

Physicochemical examination. The isolate was examined for stability to the treatments with lipid solvents (20% ethyl ether and 10% chloroform), acid (pH 3.0 and 4.0 at 22°C for 3 h), and heat (50°C for 30 min) by ordinary methods by using a porcine enterovirus; a nonenveloped RNA virus; and a pseudorabies virus, which is an enveloped DNA virus, as controls.

The type of nucleic acid of the isolate was determined indirectly by examining the effect of 5-iodo-2′-deoxyuridine (IUdR) on viral replication. The confluent monolayer of ESK cells were inoculated with $10^{3.0}$ 50% tissue culture infective doses ($TCID_{50}$s) of the isolate or the control viruses per ml. After virus adsorption at 37°C for 1 h, cells were washed twice with EMEM and then incubated in either maintenance medium containing $10^{-4.5}$ M IUdR or maintenance medium alone at 37°C. Infected culture fluids were harvested 2 days (pseudorabies virus) and 4 days (the isolate and porcine enterovirus) after inoculation and tested for virus contents.

The composition of polypeptides of the isolate was investigated by an immunoprecipitation method (see below).

Immunoprecipitation. ESK cells were inoculated with the isolate and incubated in a medium containing 20 μCi of L-[^{35}S]methionine per ml and 0.5 μg of trypsin per ml. After 48 h, the supernatant was harvested and centrifuged on a sucrose cushion with a density of 1.200 g/ml at 111,700 × g for 2 h. The pellet was suspended in 0.1 M Tris hydrochloride–0.15 M NaCl solution (pH 7.5). The viral suspension was mixed with the immune serum obtained from an experimentally inoculated HPCD pig, and then the immune complex that formed was precipitated by adding rabbit anti-porcine IgG antibody and immobilized protein A (Pharmacia, Uppsala, Sweden). The virus-antibody-protein A complex was washed five times with 0.1 M Tris hydrochloride–0.15 M NaCl solution and treated with dissociation buffer to remove bound antigen. The supernatant was analyzed by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels (8) with a 4% stacking gel and a 12% resolving gel. The gel was treated for fluorography and autoradiography.

Serum samples. Serum samples were obtained from six pigs at the acute and convalescent stages of disease that were housed on the farm where epizootic diarrhea occurred, and the serum samples were tested for neutralizing antibody to the isolate. In addition, serum samples were collected randomly from 128 adult pigs of eight herds and tested for the antibody. The history of outbreaks of epizootic gastroenteritis in those herds was unknown.

Neutralization test. Serum samples were diluted to 1:10 with EMEM, heated at 56°C for 30 min, and used in the neutralization test. Serial twofold dilutions of diluted sera were incubated with an equal volume of viral suspension containing 200 $TCID_{50}$s/0.1 ml of the isolate at 37°C for 1 h. Two tubes of ESK cells were inoculated with 0.1 ml of each serum-virus mixture. All cultures received 0.5 ml of the maintenance medium and were incubated in a roller drum at 37°C for 5 days. The neutralizing antibody titer was expressed as the reciprocal of the highest dilution of serum that inhibited a CPE in at least one of two tubes.

Experimental inoculation of HPCD pigs. Three HPCD pigs (age, 4 days) were reared in a vinyl isolator, inoculated orally with 3 ml of a viral suspension of passage 6 with a titer of $10^{5.0}$ $TCID_{50}$s/ml, and observed for clinical manifestations of disease. Fecal and serum samples were collected periodically and tested for virus and neutralizing antibody, respectively.

RESULTS

Isolation of cytopathic astrovirus. ESK cells inoculated with the original materials (fecal suspensions) showed a mild CPE in the presence of trypsin. At the second passage, a distinct CPE appeared in cultures inoculated with two of three fecal samples. A maximum CPE that was characterized by the enlargement of cells and the appearance of fine granules in the cytoplasm was observed 4 to 5 days after inoculation, and cells were eventually detached from the glass surface (Fig. 1). A CPE appeared only when trypsin was added to the maintenance medium and did not occur when the inoculum was mixed with convalescent-phase serum samples obtained from experimentally inoculated HPCD pigs.

The cytopathic agent isolated was successfully titrated by using ESK cells grown in test tubes, and maximum titers of $10^{5.0}$ to $10^{6.0}$ $TCID_{50}$s/ml were obtained constantly after passage 3.

A clone was segregated by terminal dilution on four occasions and examined for morphology, some physicochemical properties, and pathogenicity in pigs. The following experiments were carried out by using viruses from passages 6 to 8.

Morphology. Characteristic astrovirus particles were found in the fraction with a density of 1.35 g/ml that was obtained by CsCl density gradient centrifugation of the viral material that was concentrated from the supernatants of infected ESK cells at passage 7 (Fig. 2). The particles were spherical and were about 30 nm in diameter. A six- or five-pointed starlike structure was evident in many particles, but some empty particles were also observed.

Immunofluorescence and EM. Immunofluorescence of monolayers of ESK cells infected with the isolate at passage 7 is shown in Fig. 3. Fluorescing fine granules were seen to be evenly disseminated in the cytoplasm of the enlarged cells.

On EM of ultrathin sections of infected ESK cells, many virus aggregates that appeared as crystalline arrays were observed in the cytoplasm (Fig. 4).

Physicochemical properties. The isolate was stable to treatments with lipid solvents and heating at 50°C for 30 min, but

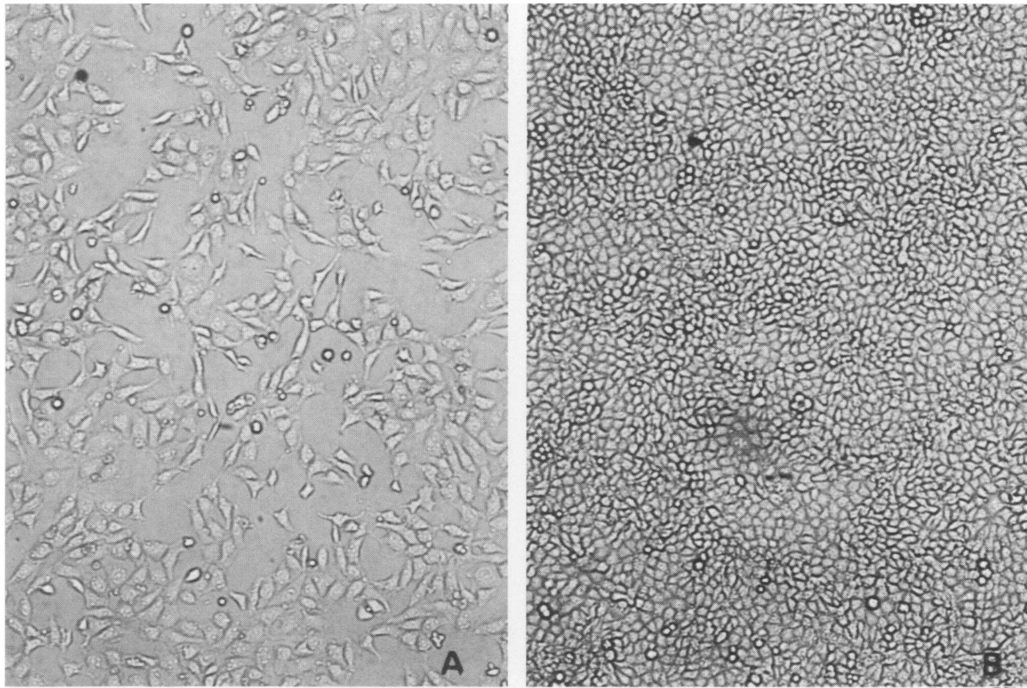

FIG. 1. CPE in ESK cells produced by porcine astrovirus. Infected (A) and noninfected control (B) cells are shown. Enlargement of infected cells with fine granules in the cytoplasm is evident.

was somewhat labile in the acid (Table 1). The pH 4.0 acid treatment for 3 h caused no considerable reduction, but pH 3.0 acid treatment for 3 h caused a reduction of about 2 log $TCID_{50}$s in viral infectivity. IUdR inhibited remarkably the replication of pseudorabies virus, but not that of the isolate, indicating that porcine astrovirus presumably contains an RNA genome.

Polypeptide composition analysis by immnoprecipitation indicated that the isolate contained five polypeptides with molecular masses of 13, 30, 31, 36, and 39 kilodaltons (Fig. 5).

Neutralization test. Seroconversion to the isolates was evident in all convalescent-phase serum samples obtained from six pigs housed on the farm where the disease oc-

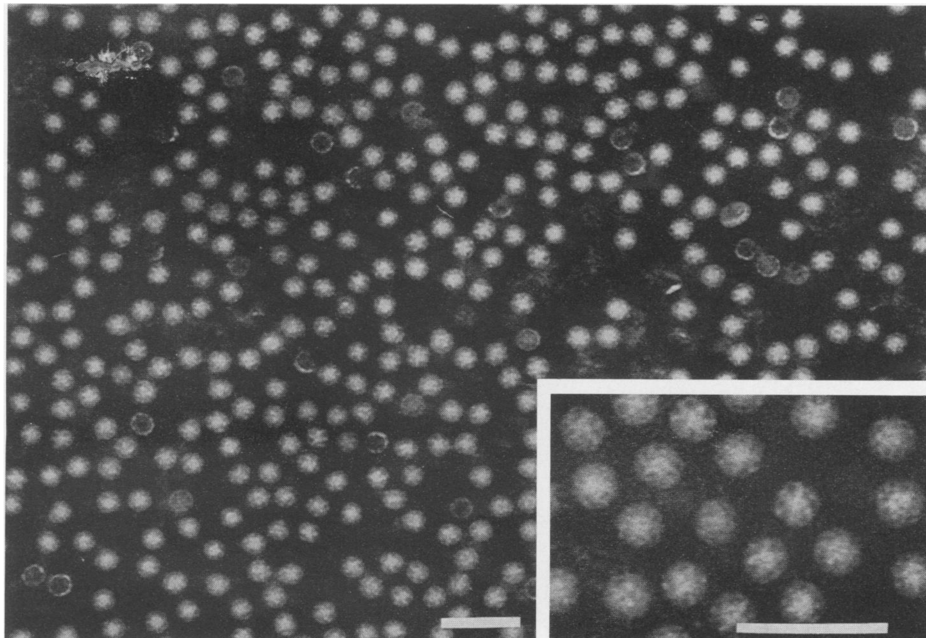

FIG. 2. Electron micrograph of cytopathic porcine astrovirus. The virus was partially purified from the supernatants of infected ESK cells and stained with phosphotungstic acid. The inset is a higher magnification. Bars, 100 nm.

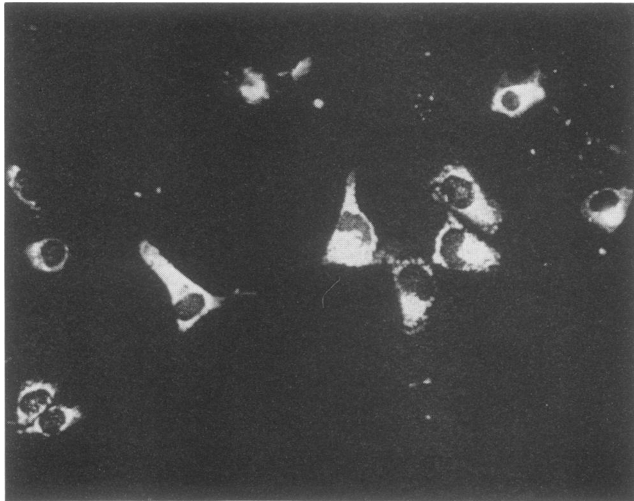

FIG. 3. Immunofluorescence of ESK cells infected with cytopathic porcine astrovirus. Fluorescing fine granules are disseminated in the cytoplasm of enlarged cells.

curred, suggesting that the epizootic gastroenteritis investigated was accompanied by infection with astrovirus.

In a serological survey of serum samples that were collected from 128 pigs of eight herds, 50 pigs (39%) were positive for neutralizing antibody to the isolate (Table 2). However, there was considerable variation in the prevalence among herds, ranging from 0 to 83%.

Experimental inoculation of HPCD pigs. Three HPCD pigs that were exposed orally to the isolate developed mild diarrhea 1 day after inoculation, and it continued for 5 to 6 days. Cytopathic astrovirus was recovered from fecal materials for about 1 week after inoculation (Table 3). All pigs produced neutralizing antibody 14 days after inoculation and survived the infection.

TABLE 1. Some physicochemical properties of cytopathic porcine astrovirus

Treatment	Log $TCID_{50}$/ml		
	Cytopathic astrovirus	Porcine enterovirus	Pseudorabies virus
Ethyl ether (20%)	5.5	6.5	<1.5
Chloroform (10%)	5.1	6.2	<1.5
pH 4.0 for 3 h	5.0	5.9	3.5
pH 3.0 for 3 h	3.8	5.1	2.1
50°C for 30 min	4.6	4.6	<1.5
Not treated	5.7	6.5	7.5
IUdR ($10^{-4.5}$ M)	5.3	6.0	3.5
Control	5.5	6.1	7.4

DISCUSSION

A cytopathic agent was isolated from pigs with acute and epizootic diarrhea in ESK cells with the aid of trypsin. The virus that was concentrated and partially purified from the supernatant of infected ESK cells was morphologically identical to previously reported astroviruses (1, 4, 6, 7, 11, 12, 14, 17, 19, 20). Because there is little few information on the physicochemical properties of astroviruses, the only criteria available for identification of astroviruses are unique and stellate morphology, a diameter of about 28 to 30 nm, and occurrence in stool specimens. The cytopathic virus that we isolated conformed to those criteria, indicating that the virus is porcine astrovirus.

Although direct EM demonstrated calicivirus and corona-

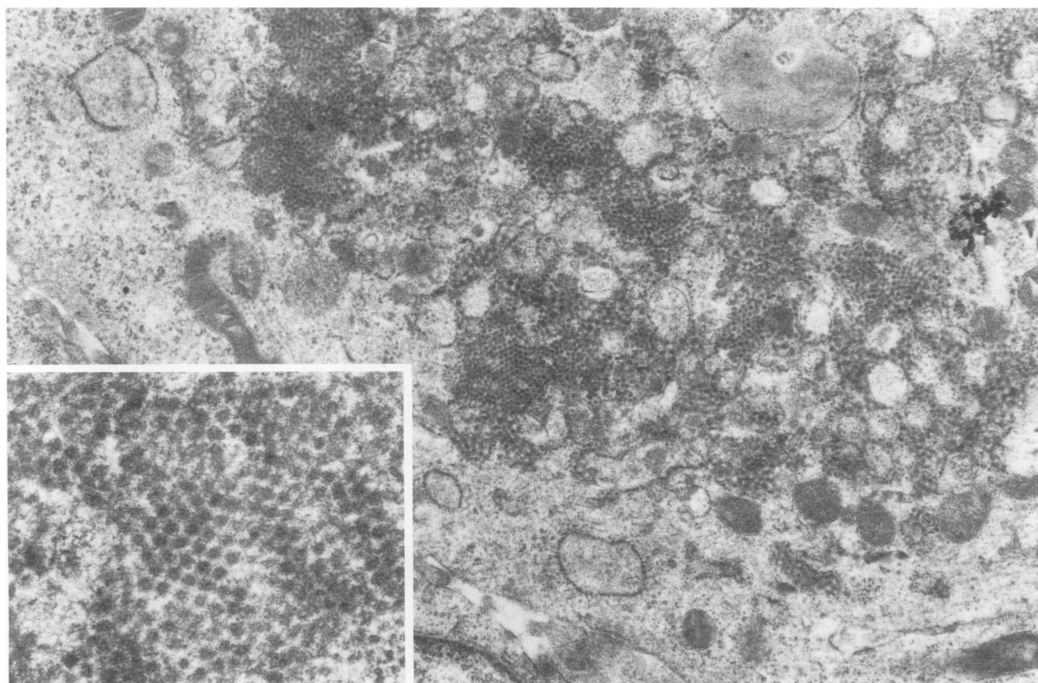

FIG. 4. Electron micrograph of an ultrathin section of ESK cells infected with cytopathic porcine astrovirus (magnification, ×22,000). Many viral aggregates (arrows) are present in the cytoplasm. The inset is a higher magnification (×66,000) of a viral aggregate.

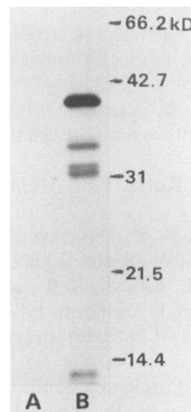

FIG. 5. Autoradiogram of the viral polypeptides of porcine astrovirus. The virus was labeled with L-[^{35}S]methionine and was immunoprecipitated with a convalescent-phase serum sample obtained from an HPCD pig (lane B). Lane A, Immunoprecipitate of the supernatant of noninfected ESK cells. Numbers at the right of the column indicate the positions of molecular mass markers (in kilodaltons [kD]).

virus but no astrovirus in the original fecal samples, only astrovirus was isolated in ESK cell cultures. This was probably due to the facts that a small amount of astrovirus was present in feces and that coronavirus and calicivirus are difficult to propagate in cell cultures (1, 13, 15).

Astroviruses have been also difficult to cultivate in cell cultures. Lee and Kurtz (9) passaged human astrovirus in primary human embryonic kidney cells and subsequently adapted the virus to $LLCMK_2$ and primary baboon kidney cells without evidence of diminishing infectivity. In addition, adaptation of bovine (20) and feline (4) astroviruses to bovine and feline embryonic cells has been reported, respectively. However, there have been no reports on the isolation of a cytopathic astrovirus.

It seems to be possible to conclude that the CPE induced in ESK cells was caused by porcine astrovirus based on the following evidence. (i) Virus particles characteristic of astroviruses were found only in the supernatants of infected ESK cells but not in those of noninfected control cells, (ii) no CPE was observed in noninfected control cells that were strictly comparable to inoculated cells, (iii) specific fluorescence was demonstrated in infected cells by immunofluorescence by using immune serum to the isolate, (iv) many aggregates of small viral particles were found in the cytoplasm of infected cells, and (v) no CPE appeared when the inoculum was mixed with the immune serum.

TABLE 2. Neutralizing antibody to cytopathic astrovirus in porcine sera

Herd	No. of serum samples tested	No. (%) of positive serum samples	Antibody titer[a]
A	43	10 (23)	10–160
B	34	21 (62)	10–160
C	8	5 (63)	20–80
D	4	2 (50)	10–40
E	15	6 (40)	10–40
F	4	0 (0)	
G	14	1 (7)	80
H	6	5 (83)	20–160
Total	128	50 (39)	10–160

[a] Range of antibody titers of positive serum samples.

TABLE 3. Responses of HPCD pigs orally inoculated with cytopathic astrovirus

Pig no.	Log $TCID_{50}$ of virus per ml in feces on the following days after inoculation:						Neutralizing antibody titer on the following days after virus inoculation:				
	0	1	3	5	7	10	0	7	14	21	28
1	Neg[a]	2.0	4.5	4.8	4.6	Neg	<10	<10	20	40	80
2	Neg	3.1	4.7	5.3	2.1	Neg	<10	<10	10	20	40
3	Neg	2.8	5.6	4.2	3.6	2.3	<10	<10	10	80	80

[a] Neg, Negative for virus isolation.

In previous reports (4, 9, 20), as well as in the present study, astroviruses were cultivated in cell cultures only when serum-free maintenance medium containing trypsin was used. These results suggest that propagation of astroviruses in cell cultures is trypsin dependent.

There have been several reports dealing with the serologic properties of astroviruses. Human astroviruses are divided into five serotypes (J. B. Kurtz and T. W. Lee, Letter, Lancet **ii**:1405, 1984) and have been shown to be unrelated to bovine, ovine (10), or feline (4) astroviruses. Porcine and bovine astroviruses are also serologically different from each other (1). However, the characterization and classification of astroviruses have not been established because of the difficulty of propagating them in cell cultures. Herring et al. (5) have purified ovine astrovirus from epithelial cells of the small intestinal villi of an infected gnotobiotic lamb and have shown that the virus has two polypeptides with similar molecular masses (about 33 kilodaltons) and a 34S single-stranded RNA genome with a poly(A) tail. From these results, it has been proposed that astroviruses should be placed in a separate group of the family *Picornaviridae*. In this study, it was suggested that porcine astrovirus contains an RNA genome, since its replication was not inhibited by IUdR and it was shown to have five polypeptides with molecular masses of 13 to 39 kilodaltons. There is no reasonable explanation for the discrepancies in the polypeptide compositions of ovine and porcine astroviruses determined by Herring et al. (5) and in the present study, respectively. The other physicochemical properties of porcine astrovirus determined in the present study seem to be similar to those of enteroviruses. Further characterization of astroviruses is expected to establish their classification. In this regard, the cytopathic porcine astrovirus isolated in this study appears to be a useful tool for characterizing the physicochemical properties of astroviruses.

Although astroviruses are frequently associated with nonbacterial gastroenteritis in human and animals, their etiological significance is still undefined. Human astrovirus is considered as an occasional cause of gastroenteritis in both children and adults (2). Gnotobiotic calves inoculated with bovine astrovirus alone suffered from no clinical disease (20), while those inoculated with astrovirus combined with either rotavirus or Breda virus developed severe diarrhea (21). Ovine (17) and feline (4) astroviruses have been shown to be pathogenic in gnotobiotic lambs and specific-pathogen-free kittens, respectively, although the diarrhea that was experimentally produced in kittens was milder than that which occurs during the naturally occurring disease (4). Bridger (1) has reported that gnotobiotic piglets inoculated with porcine astrovirus developed anorexia and diarrhea and failed to gain weight, which was followed by death. This may not represent the pathogenicity of the virus, however, because a fecal filtrate used for the experimental inoculation

contained not only astrovirus but also calicivirus, atypical rotavirus, and enterovirus (1).

The pigs investigated in this study had severe diarrhea, and seroconversion to cytopathic astrovirus occurred in their convalescent-phase serum samples. In addition, calicivirus and coronavirus were demonstrated in the fecal specimens by direct EM (16). It seems to be possible, therefore, that the severe diarrhea observed in pigs with the naturally occurring disease was probably due to mixed infection with those viruses.

HPCD pigs orally exposed to the cloned porcine astrovirus developed mild diarrhea and shed virus in feces, but all survived the infection. This may indicate that porcine astrovirus is pathogenic in piglets. However, its pathogenicity seems to be lower than those of porcine transmissible gastroenteriris coronavirus, epidemic diarrhea coronavirus, and rotavirus, since piglets infected with those viruses often die.

The results of the serologic survey of porcine sera suggest that the spread of porcine astrovirus is common among pigs, although there likely is considerable variation in its prevalence among herds.

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