豚の下痢便から分離されたパルボウイルスの性状

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Characterization of a Parvovirus Isolated from the Diarrheic Feces of a Pig

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ABSTRACT. A small DNA virus was isolated from the feces of a sow with diarrhea and identified as a parvovirus on the basis of its properties. The virus replicated preferentially in cell cultures of swine origin, including primary porcine thyroid gland and kidney cell cultures in which the cytopathic effect developed. The virus agglutinated erythrocytes of guinea pig, mouse and human group O but not these of chicken. The growth of the virus was inhibited by 5-iodo-2'-deoxyuridine. The virus was resistant to ether and heating at 56°C for 30 min and stable at pH 3.0. The buoyant density of the infectious particles was 1.40 g/m*l* in CsCl density gradient, and the virions were 27 nm in diameter by electron microscopy. The viral protein seemed to be separated into four polypeptides with molecular weights of 81k, 70k, 66k and 62k daltons respectively. Cross serum neutralization test demonstrated that the virus was antigenically different from porcine parvovirus as well as bovine and canine parvoviruses. These findings and the survey on neutralizing antibody distribution indicated indirectly that another parvovirus which could be antigenically distinguished from well-known porcine parvovirus had been widespread among swine in Japan.—KEY WORDS: diarrhea, porcine parvovirus, serotype.

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Isolations of parvoviruses from various mammalian species have been reported. Parvovirus causes enteritis in dogs [2, 19] and feline panleukopenia in cats [5, 10]. Intestinal infections of parvoviruses in calves [1, 8] and rabbits [14] have also been reported. In swine, parvovirus has been established as a causative agent of reproductive disease [9, 16].

Recently, Dea *et al.* [7] detected numerous parvovirus-like particles in the intestinal contents of unweaned piglets with diarrhea by electron microscopy and demonstrated that of the three isolates from these specimens, two were antigenically related to porcine and canine parvoviruses but another differed from porcine parvovirus. Consequently, they have noticed the possibility of a primary etiological role of parvovirus infection in epidemic diarrhea of piglets and that of the presence of other serotypes of porcine parvovirus (PPV) [7].

In this paper, we describe the characteristics of a parvovirus isolated from the feces of a sow and distribution of antibody against this virus among swine in Japan.

MATERIALS AND METHODS

Collection and preparation of specimens: In January 1985, an outbreak of epidemic diarrhea was observed in piglets, breeding pigs and sows on a farm in Osaka Prefecture, Japan. Fecal samples were collected from a sow and two 4-day-old piglets with diarrhea. The suspensions of fecal specimens at 20% were made with Eagle's minimum essential medium (MEM) containing 1,000 units/m*l* penicillin, 1,000 μg/m*l* streptomycin and 500 μg/m*l* kanamycin. The suspensions were centrifuged at 7,000 rpm for 20 min, and resulting supernatant fluids were inoculated into cell cultures for virus isolation.

Cell cultures and media: The cell cultures used in the present study were shown in

Table 1. They were grown in culture bottles in 50 m*l* amount, by the routine method, with Eagle's MEM containing 5% fetal calf serum and antibiotics, 200 units/m*l* penicillin, 200 μg/m*l* streptomycin, 100 μg/m*l* kanamycin and 0.5 μg/m*l* fungisone. Especially, primary porcine thyroid gland (PT) cells were also prepared in culture tubes (11×100 mm) from trypsinized tissues of 2-week-old piglets for virus isolation and titration of virus. The maintenance medium (MM) employed after inoculation of virus material was the same as growth medium, except that fetal calf serum was replaced with 0.1% bovine serum albumin, and for preparation of stock virus and titration of virus, 0.5 μg/m*l* of trypsin type III (Sigma Chemical Co., St. Louis, USA) was added further.

Virus isolation: Each supernant of fecal suspensions was inoculated into PT cells of culture tubes. After adsorption at room temperature for 1 hr, the cell culture to which 1 m*l* of MM was added, was incubated at 37°C and observed daily for cytopathic effect (CPE) microscopically. When no CPE was observed for 7 to 10 days after inoculation, the culture fluid was harvested and inoculated again into the same new cells.

Viruses: The newly isolated virus designated H-45 strain was used for the experiment. After the virus was isolated, stock viruses were prepared following 3 to 5 passages in PT cell cultures. The reference parvoviruses used for serological test were the 90HS strain [18], the NADL-2 strain [16] and the 22-1 strain of PPV, the BF15 strain [8] of bovine parvovirus (BPV) and the 29-F strain [3] of canine parvovirus (CPV). PPV, BPV and CPV were grown in ESK, primary bovine kidney, and CRFK cell cultures respectively and each supernatant of the infected cell culture fluids was used for the test. The 22–1 strain was originally isolated from a naturally infected pig and identified in our laboratory (unpublished data).

Titration of virus: Titration of isolated virus was performed in PT cells of culture tubes. Each of serial decimal dilutions of the virus with Eagle's MEM was inoculated into 4 culture tubes. After adsorption at room temperature for 1 hr, MM was added to each tube and then the inoculated cell cultures were incubated at 37°C for 14 days. The infective titer of $TCID_{50}$ was determined both by the development of CPE and the presence of viral hemagglutinin in culture fluid to which guinea pig erythrocytes were added at a concentration of 0.5%.

Hemagglutination test: Two-fold serial dilutions (0.4 m*l*) of the isolate infected cell culture fluid were made with 0.01 M phosphate-buffered saline (PBS; pH 7.2), mixed with 0.4 m*l* of 0.5% suspension of erythrocytes from various species: guinea pig, mouse, pig, cattle, sheep, human group O and chicken. The mixtures in plastic trays were examined after 2 hr of incubation at 4°C. The hemagglutination (HA) titer was expressed as the reciprocal of the highest dilution that showed complete agglutination.

Nucleic acid determination: The type of the nucleic acid of the isolate was indirectly examined by use of 5-iodo-2'-deoxyuridine (IUdR). The virus diluted to contain 10^3 $TCID_{50}$ was inoculated into PT cell culture and after virus adsorption at 37°C for 45 min, the inoculated cell was washed 3 times with PBS. Then the inoculated cell was incubated with MM containing 50 μg/m*l* of IUdR at 37°C for 14 days. The effect of IUdR was determined by infective titer and HA titer of culture fluid harvested daily.

Resistance to ether : Resistance to 20% ether was tested as described [8] by treatment at 4°C for 18 hr.

Acid stability: The virus was diluted 1: 10 in Eagle's MEM adjusted at pH 3.0 with 1 N HCl or at pH 7.2 with 0.1 M Tris (hydroxy-

methyl) aminomethane. After incubation at 22°C for 6 hr, each of virus dilutions was assayed for infectivity.

Heat stability: The virus diluted 1: 10 in distilled water was incubated at 56°C or room temperature for 30 and 60 min and assayed for infectivity.

Size determination: The virus was diluted 1: 10 in 0.15 M NaCl solution containing 0.1% gelatin and filtered through membrane filters of 220, 100 and 50 nm in pore size. These filtrates were assayed for infectivity.

Purification of virus: Supernant culture fluids obtained from the H-45 isolate infected PK cell, after removing cell debris by low speed centrifugation, were centrifuged at 100,000 g for 90 min in a Beckman 45 Ti rotor. The pellets were suspended in CsCl solution based on TN buffer (0.01 M Tris-hydrochloride-0.1 M NaCl; pH 7.2) to a density of 1.35 g/m*l*, and then the suspension was centrifuged at 100,000 g for 40 hr in a Beckman SW 45 rotor. Fractions were collected from the bottoms of tubes and assayed for infectivity and HA activity of guinea pig erythrocytes.

Electron microscopy: The heavy and light HA peak fractions obtained from CsCl density gradients, pooled separately, were negatively stained with 4% uranyl acetate and examined by a JEM-100s (Nippon Denshi Co., Ltd., Tokyo) electron microscope.

Protein analysis of the virus: The polypeptide profiles of purified particles separately obtained from the heavy and light HA peaks in CsCl density gradients were examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a 14 cm 7.5% gel slub with a 1.5 cm 4% stacking gel and Laemmli's discontinuous buffer system [10].

Serological test: Secondary PT cells, the ESK cells, primary bovine kidney cells, and the CRFK cells were used for serum neutralization (SN) tests of the isolate, PPV, BPV, and CPV respectively. Each of two-fold diluted serum was mixed with an equal volume of a virus containing 200 $TCID_{50}$/0.1 m*l* and incubated at 37°C for 1 hr. Each mixture was inoculated into 2 culture tubes. The cultures were examined 14 days later for infection. The antibody titer was expressed as reciprocal of the highest serum dilution that inhibited the production of HA activity in both of the 2 tubes.

Collection of swine sera: During a period of 1980 to 1986, 409 sera were collected from pigs through all ages kept in 14 prefectures between Hokkaido and Kagoshima. Serum samples were assayed for the SN test with the isolated virus.

Antisera: All of antisera were prepared in guinea pigs by two intramuscular injections of inactivated viruses with Freund's complete adjuvant.

RESULTS

Virus isolation and hemagglutinating activity of the isolated virus: An agent which induced a CPE was isolated from the diarrheic feces of a sow following 3 passages in PT cell culture. This isolated virus, designated the H-45 strain, also possessed hemagglutinating (HA) activity. HA activities were demonstrated with guinea pig and mouse erythrocytes at HA titers of 512 and 256 respectively, and with human O erythrocytes at a low titer of 8. The other erythrocytes tested from cattle, pig, sheep and chicken were not agglutinated by the H-45 isolate.

Host cell range: Susceptibility of cell cultures to the isolate were shown in Table 1. The isolate replicated only in cell cultures derived from porcine; primary cell cultures of kidney and thyroid gland and cell lines of ESK and PK-15. In these susceptible cells, infective titers obtained after 14 days' incubation ranged from $10^{2.3}$ to $10^{3.5}$$TCID_{50}$/

Table 1. Susceptibility of cell cultures to the H-45 isolate

Cell culture	Infectivity[a)]	HA titer[b)]	Appearance of CPE[c)]
(Primary cell cultures)			
Porcine thyroid gland	3.3	1024	+
Porcine kideny	2.5	128	+
Bovine kidney	≤0.5	<2	−
Guinea pig kidney	≤0.5	<2	−
(Cell lines)[d)]			
ESK	3.5	4096	±
PK-15	2.3	64	−
Vero	≤0.5	<2	−
HmLu	≤0.5	<2	−
MDCK	≤0.5	<2	−
CRFK	≤0.5	<2	−

a) Determined by production of hemagglutinin using guinea pig erythrocytes. Titers expressed as $\log_{10}TCID_{50}$/0.1 m*l*.
b) Tested with guinea pig erythrocytes.
c) +, present; −, absent; ±, could not be clarified.
d) ESK, Swine embryo kidney; PK-15, Porcine kidney; Vero, African green monkey kidney; HmLu, Hamster lung; MDCK, Canine kidney; CRFK, Feline kidney.

0.1 m*l*, and hemagglutinins were detected in all of these culture fluids. The CPE, which were characterized by forming rounded, refractive cells, were observed both in PT and PK cells. In ESK cells, rounded cells appeared slightly before cells were detached from the surface of the glass bottle. However, whether cytopathic changes in ESK were specific or not could not be determined. No discernible CPE was shown in PK-15 cells.

Inclusion bodies were observed in nucleus of infected PT and PK cells stained with hematoxylin and eosin.

Chemical and physical properties: In some preliminary examinations, it was found out that infective titers measured by incubation with MM containing 0.5 μg/m*l* of trypsin were over 10^2 times higher than that measured with the same medium except trypsin. Therefore, virus titrations following were done using MM containing 0.5 μg/m*l* of trypsin as described before. As shown in Fig. 1, the replication of the isolated virus and the production of hemagglutinin were also inhibited by IUdR, indicating that the

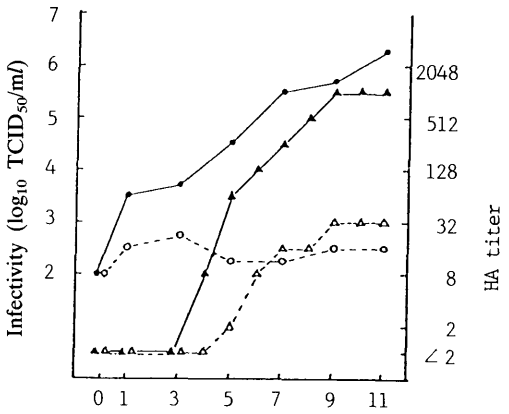

Fig. 1. Effect of IUdR on replication of the isolated virus. Eagle's MEM: ●—● Infective titer, ▲—▲ HA titer. Eagle's MEM containing 50μg/m*l* of IUdR: ○--○ Infective titer, △--△ HA titer.

nucleic acid type of the virus was DNA, indirectly. The other results obtained were summarized in Table 2. The H-45 isolate was resistant to ether and acid treatment. By incubation at 56°C, the virus infectivity was not reduced in 30 min but reduced

Table 2. Physicochemical properties of the H-45 isolate

	Treatment	Infectivity[a]	
		CPE	HA
Ether stability[b]	Untreated	5.3	5.5
	20% ether	5.3	5.5
Acid stability[c]	pH 7.0	4.5	5.0
	pH 3.0	4.8	4.8
Heat stability	untreated	4.5	4.5
	56°C 30 min	4.2	4.2
	60 min	3.5	3.5
Filtration	untreated	4.5	5.3
	220 nm	4.5	5.3
	100 nm	4.3	4.5
	50 nm	4.3	4.5

a) Determined by observation of the CPE (CPE) or by production of hemagglutinin (HA). Titers expressed as $\log_{10}TCID_{50}/0.1$ m*l*.
b) Treated at 4°C for 18 hr.
c) Treated at 22°C for 6 hr.

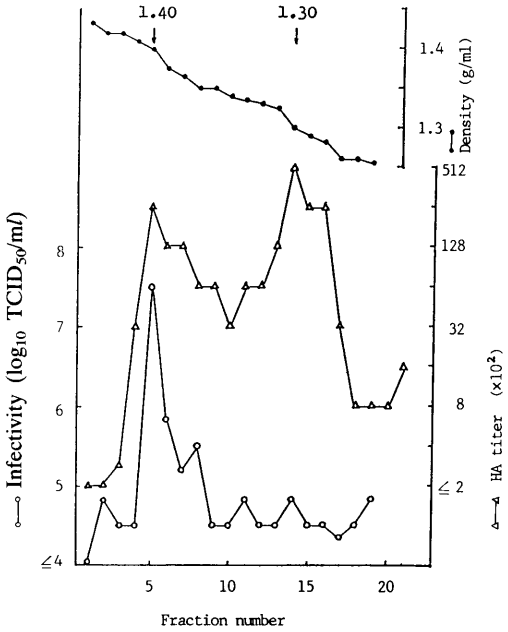

Fig. 2. CsCl density gradient centrifugation of the H-45 isolate.

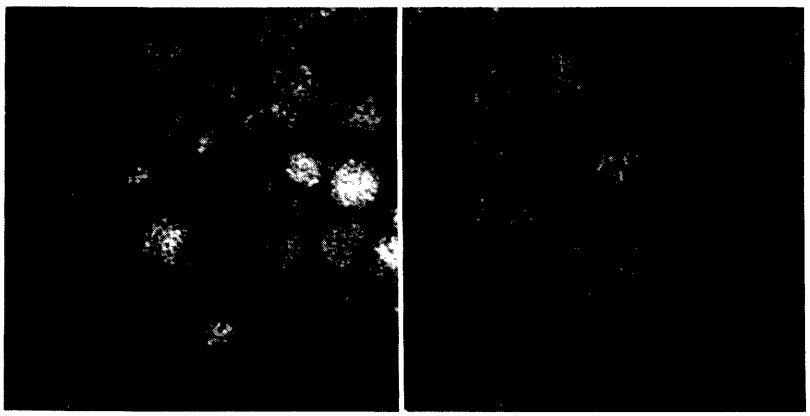

Fig. 3. Negatively stained particles of the H-45 isolate banded at a density of 1.40 g/m*l* (A) and at a density of 1.30 g/m*l* (B) in CsCl gradients. Diameter of full particles is 27 nm.

slightly in 60 min. The isolate was readily filtered through a membrane filter of 50 nm pore size.

Buoyant density of the virus: By CsCl equilibrium centrifugation, hemagglutinins were banded in two major peaks (Fig. 2). The buoyant density of heavy peak was estimated at 1.40 g/m*l* and that of light peak was at 1.30 g/m*l*, and the former was coincident with the peak of infectivity.

Electron microscopic observation: Electron micrographs of particles obtained separately from the fractions of heavy HA peak and light HA peak are shown in Fig. 3. The fraction of heavy peak contains full particles that have cubic symmetry and a

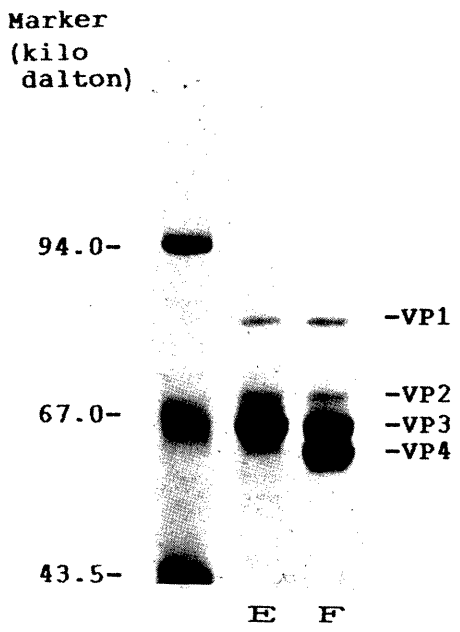

Fig. 4. Protein analysis of purified particles of the H-45 isolate obtained from the fraction with a density of 1.40 g/m*l* (lane F) and the fraction with a density of 1.30 g/m*l* (lane E) in CsCl gradients.

diameter of 27 nm (Fig. 3-A), and on the contrary the fraction of light peak contains mainly empty particles (Fig. 3-B).

Protein analysis of the virus: Fig. 4 shows the staining patterns of purified particles, obtained from the heavy HA peak and the light HA peak, by SDS-PAGE. The heavy particles (lane F) appeared to contain four classified according to month of age. The percentage of serum samples which had antibody titer of 4 or greater varied from 32% to 100%, depending on month of age. Particularly, of 219 sera collected from pigs over 5 months old, 209 (95%) contained antibody.

DISCUSSION

Our virus isolated from the diarrheic feces of a sow could be classified as a member of the parvovirus group, according to its properties such as the nucleic acid type of DNA, the size, acid- and heat-stability, and resistance to ether. Additionally, the HA activity of the isolate was similar to that reported for PPV except the results with chicken erythrocytes [6, 15, 18].

Furthermore, buoyant density and protein composition of the virus were determined to confirm the classification of the virus. Consequently, the buoyant density of purified, infective particles estimated at 1.40 g/m*l* in CsCl density gradient was very similar to that of PPV reported previously [17, 18]. Usually three structural polypeptides can be demonstrated in purified, mature parvovirus [4, 12], including PPV as described by Molitor *et al.* [17], whereas BPV and rabbit parvovirus seem to have four polypeptides [12, 13]. Analysis of the protein composition demonstrated that the particles banding at a density of 1.40 g/m*l* resolved into four polypeptides. This result of the virus were different in number of polypeptide from that of PPV; however, the three of four polypeptides with molecular weights of 81k, 66k and 62k daltons were identical in size to these of PPV termed A, B and C in descending order of size, respectively [17]. On the contrary, the result may suggest that this virus is structurally related rather to BPV, considering the number of polypeptide that the virus contains.

The results of serological test demonstrated that the isolate was antigenically different from PPV as well as BPV and CPV. While, the limited ability to replicate preferentially in cell cultures of porcine origin and the presence of antibody among pigs suggest that the isolate can be classified as a parvovirus of porcine origin.

Overall, the results represented here prove the existence of another parvovirus which could be antigenically distinguished from well-known PPV, and indirectly indicate that this virus had been widely preva-

Table 3. Cross serum neutralization test with the isolate and reference parvoviruses

Test virus	SN titer[a] of antiserum to					
	H-45	90HS	NADL-2	22–1	BF15	29-F
H-45 (isolated virus)	512	<4	<4	<4	<4	<4
90HS (PPV)	4	512	64	512	<4	<4
NADL-2 (PPV)	<4	1024	128	1024	—[b]	—
22–1 (PPV)	4	1024	128	1024	—	—
BF15 (BPV)	<4	<4	—	—	1024	<4
29-F (CPV)	<4	<4	—	—	<4	4096

a) Titer were expressed as reciprocal of the highest dilution of serum that neutralized a virus of 100 $TCID_{50}$.
b) Not tested.

lent throughout the swine population of Japan.

To our knowledge, no evidence of any antigenic difference between strains of PPV has been reported. There may be a few reason why this virus has not been previously isolated. PK cell cultures, which are mainly used for virus isolation, are less sensitive than PT cell cultures, and it takes a long term of cultivation, at least for more than 9 days, to detect the CPE on early passage even in PT cells. PT cell cultures have also an advantage to be easily maintained better for a long term. Adding a certain concentration of trypsin to medium is likely to shorten the cultivation term before appearance of the CPE.

Further studies are needed to elucidate a role of the virus infection in porcine diarrhea. Detailed results of our further attempts to confirm its pathogenicity, including already obtained evidences of the possible intestinal infection of this virus in gnotobiotic piglets, will be described elsewhere.

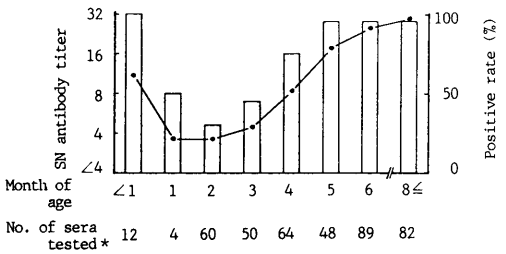

Fig. 5. Distribution of serum neutralizing (SN) antibody for the isolate among swine of different ages. ●—●: Geometric mean of SN antibody titer. 凵 : Positive rate (A titer of 4≤ was adopted as positive). * Sera tested were collected from 14 prefectures in Japan during a period of 1980 to 86.

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要　約

豚の下痢便から分離されたパルボウイルスの性状：安原寿雄・松井　修・平原　正・扇谷年昭・田中雅之・児玉和夫・中井正久・佐々木文存（微生物化学研究所）——1985年，2月，某大規模養豚場に発生した下痢症例において，繁殖母豚の下痢便より豚甲状腺初代培養細胞に円形化細胞変性を示す因子が分離された．分離ウイルスはIUdR存在下でその増殖が阻害され，エーテル，熱，酸の各処理に抵抗性を示した．感染培養液中には電顕により27nmの粒子が認められ，塩化セシウム密度勾配による浮上密度は1.40g/m*l*であった．SDS-PAGEにより，ウイルス蛋白は分子量81k，70k，66k及び62kダルトンの4種類のバンドに分かれた．また，赤血球凝集性はモルモット，マウス，ヒトO型の各赤血球で認められた．一方，既知の豚パルボウイルス（PPV）との交差中和試験では，分離ウイルスの抗原性は血清学的にPPVとは明らかな差異が認められたが，以上の成績から，分離ウイルスはパルボウイルスに属すると考えられた．抗体調査から，この分離ウイルスはわが国の豚の間に広く存在することが示唆された．