Isolation of Hemagglutinating Encephalomyelitis Virus from Respiratory Tract of Pigs in Japan

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ABSTRACT. Four strains of cytopathic agents with syncytium formation were isolated from the respiratory tract of pigs affected with respiratory diseases in Niigata and Osaka Prefectures in 1984. These viruses agglutinated with erythrocytes from mouse, hamster, rat and chicken. Multiplication of the isolated viruses in cell culture was not inhibited by IUdR. The viruses were inactivated with ether and chloroform, and were also unstable at pH 3.0. The isolated viruses passed readily through a membrane filter of 220 nm pore size, but not through one of 100 nm pore size. Coronavirus-like particles of 120–160 nm in diameter were observed by the electron microscope. In the serum neutralization test, the 4 isolated viruses were immunologically related with the 67N strain of hemagglutinating encephalomyelitis virus (HEV) and were slightly related with the No. 66 strain of bovine coronavirus (BCV). None of them were found to be immunologically correlated with the T0160 strain of transmissible gastroenteritis virus (TGEV). From these results, the 4 isolated viruses were identified to be as the HEV. Conventional and colostrum-deprived piglets were inoculated with isolated HEV by the intranasal or ocular route. They showed mild coughing, sneezing with nasal discharge with some other mild clinical sign. The viruses were successfully recovered from the respiratory tract and lungs during 1 to 9 days after virus exposure. On farms N and T, the field investigations revealed that there was an increase in hemagglutination-inhibiting (HI) antibody in breeding pigs 3 to 4 months old.—**KEY WORDS:** HEV, pig, respiratory disease.

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Hemagglutinating encephalomyelitis virus (HEV) was first isolated by Greig *et al.* [8] from the brain of a suckling piglet affected with encephalomyelitis in Canada, 1962. After that, infections caused by HEV were reported by Cartwright *et al.* [2] in England. In 1972, Mengeling *et al.* [11] isolated HEV from the nasal cavity of an apparently healthy pig in the USA.

The authors isolated 4 strains of HEV from the tracheal and nasal mucosa, and nasal secretions of breeding pigs showing respiratory symptoms in Niigata and Osaka Prefectures in 1984.

This report is about the characterization and transmission of HEV in Japan.

MATERIALS AND METHODS

Specimens for virus isolation: Specimens were collected from 48 autopsied pigs, 143 nasal swabs from pigs showing respiratory signs on farm N in Niigata and farm T in Osaka Prefectures from 1983 to 1984. Ten % (W/V) suspension of a tissue sample was prepared by using Eagle's minimum essential medium (Eagle's MEM) with antibiotics (1,000 UN/ml of penicillin G, 1,000 µg/ml of streptomycin, 500 µg/ml of kanamycin and 5 µg/ml of fungizon) and 5 µg/ml of trypsin type III (Sigma chemical Co., St. Louis, USA). The nasal swabs were also soaked in 1.5 ml of the same medium for the same purpose.

Cell cultures for virus isolation: Primary

porcine thyroid gland (PT) and porcine kidney (PK) cell cultures were prepared by the routine method. For virus isolation or viral assay, 0.1 ml suspension of virus materials were inoculated into a confluent monolayer of PT and PK cell culture tubes. Absorption was allowed to take place at room temperature for 45 min. Thereafter, maintenance medium was added to each culture. Maintenance medium consisted of Eagle's MEM containing antibiotics and trypsin to a final concentration of 1 µg/ml. All tube cultures were set in a rotary drum at 32°C and 37°C.

Viruses: The newly isolated viruses, designated as NPTr32, NNF417, NNa27 and ONS204 strains were employed from the 3rd to the 5th passage levels for PT culture and from the 5th to the 10th passage levels for PK culture. The following viruses were used as references; the 67N strain of HEV, the No. 66 strain of BCV, the TO160 strain of TGEV.

Infective virus assay: Serial tenfold dilutions were made and 0.1 ml of each dilution was inoculated into the cell culture tubes. The cultures were examined daily for cytopathic effects (CPE) until 7 days after the inoculation. Then, a 50% tissue culture infective dose (TCID₅₀) was calculated by Behrens-Kärber method.

Hemagglutinating ability of the isolated viruses: Twofold dilution of the isolated virus materials were made in 0.4 ml phosphate buffered saline (PBS). Each dilution was mixed with 0.4 ml suspension of 0.5% erythrocytes; human type O, guinea pig, mouse, hamster, rat and chicken. These mixtures were kept at 4°C, 22°C and 37°C for 1 hr and were examined for agglutination of the erythrocytes.

Host range of various cell cultures: Various primary cell cultures were prepared with bovine, canine, feline and chicken kidneys by the routine method. The isolated virus materials were inoculated into each of

the cell cultures which were examined for CPE and hemagglutination (HA) of the culture fluids for 7 days. The infected culture fluids were harvested for 3 and 7 days after inoculation and were subjected to successive passages up to the 5th level.

Nucleic acid type determination: After inoculation with isolated virus materials, the cells were incubated at 37°C, and were washed for 3 times with PBS. Then, the maintenance medium containing 50 µg/ml IUdR was added. The infected cultures with IUdR were examined for virus multiplication.

Stability to chemical reagents: The isolated virus materials were examined for stability to 20% ether, 5% chloroform, 0.1% sodium deoxycolate and 0.5% trypsin by the methods described by Greig and Girard [5, 6].

Stability at pH 3.0 and heat stability with or without 1M MgCl₂: First, 0.5 ml of each of the isolated virus materials were mixed with 4.5 ml veronal buffered solution at pH 3.0. The mixture was incubated at 4°C to estimate infective titers at long-time intervals. For the examination of heat stability the virus materials were mixed with equal volumes of 1M MgCl₂ solution or 0.85% NaCl solution, and was incubated at 22°C and 50°C for 1 hr.

Filtration experiments: Filtrations were performed by using membrane filters 220, 100 and 50 nm in pore size. The resulting filtrates were used to determined infective virus titer assay.

Electron microscopic observation: The infected culture fluids were ultracentrifuged at 100,000 g for 90 min. The resulting sediments were resuspended in small volumes of distilled water. The suspensions of isolated virus materials were stained with 4% uranyl acetate and examined by JEM-100S electron microscope (Nippon Denshi Co., Ltd., Tokyo).

Antisera: Immune sera against all the

isolated viruses were prepared in rabbits by intramuscular injection with Freund's adjuvant. Immunization was carried out in rabbits with 2 weeks' interval for 3 times. Two weeks after the last injection, the animals were bled by the cardiac puncture method. The antiserum against the 67N strain of HEV was kindly supplied by Dr, K. Hirai, of Gifu University.

Serum neutralization test: Inactivated serum (56°C for 30 min) was twofold diluted with PBS. An equal volume of virus suspension containing 200 TCID₅₀/0.1 ml was added to each dilution and incubated at 37°C for 1 hr. Each mixture was divided into 2 culture tubes. After absorption at 37°C for 45 min, 0.75 ml maintenance medium was added to each culture tube, and was incubated at 37°C for 7 days. The antibody titer was expressed, as the reciprocal value of the highest serum dilution.

Experimental infection in pigs and piglets: Four two-month-old pigs and 4 two-day-old colostrum-deprived piglets were inoculated by intranasal and oral routes with 1 to 3 ml suspension of the NPTr32 strain containing 6.5 log₁₀TCID₅₀/ml of virus materials. Each animal was observed for clinical symptoms for 21 days after exposure to the viruses. Nasal, fecal swabs and blood samples were collected from the inoculated animals during the 14 days after exposure. Serum samples were also collected to estimate HI antibody titers.

Survey of HI antibody: From July, 1983 to September, 1984, serum samples were obtained from pigs on farms N and T from where the viruses had been isolated. They consisted of 13 to 40 samples harvested from 1 to 6 months old pigs on each farm. Each serum sample was treated with 25% kaolin at 22°C for 20 min and at 4°C for 18 hr with chicken blood cells for the elimination of non-specific agglutinations. The treated serum was assayed for the HI test using NPTr32 strain virus material as HA antigen.

RESULTS

Virus isolation: Specimens were inoculated into PT and PK cell cultures for virus isolation. The cell cultures were subjected to 5 successive passages. They were examined daily, and CPE was recognized 2 to 4 days after each passage. It was observed by the formation of syncytia within 18 hr after inoculation. Four CPE agents (NPTr32, NNF417, NNa27 and ONS204) were isolated from tracheal, nasal mucosa, and nasal secretion of affected pigs showing respiratory and debilitating symptoms on two farms, in Niigata and Osaka Prefectures.

HA test: Erythrocytes from mouse, hamster, rat and chicken showed positive HA with titers ranging from 32 to 128. HA was negative with human type O, pig and guinea pig erythrocytes.

Host cell range: The four viruses showing CPE multiplied well in PT and PK cells, having infective titers ranging from 6.0 to 7.25 log₁₀TCID₅₀/ml. They failed, however, to propagate in bovine, dog, cat, and chicken kidney cell cultures.

Physicochemical properties: All the results obtained from NPTr32 strain is summarized in Table 1. Multiplication of the isolated virus was not inhibited in the presence of IUdR in PK cells.

Therefore, it indicated that the isolated virus nucleic acid type was RNA. The isolated virus was inactivated completely with 20% ether, 5% chloroform and 0.1% sodium deoxycolate, but not with 0.5% trypsin. The infective titers of the isolated virus that was treated for long time (72 hr) in pH 3.0 solution (3.5 log₁₀TCID₅₀/ml) was lower than those treated with pH 7.0 solution (5.0 log₁₀TCID₅₀/ml) with the same time. The infective titer of the isolated virus that was examined with 1M MgCl₂ solution at 50°C for 1 hr (3.5 log₁₀TCID₅₀/ml) was lower than the titer that was examined with

Table 1. Physicochemical properties of the NPTr32 isolate

	Treatment	Infectivity (log ₁₀ TCID ₅₀ /ml)
Nucleic acid type	IUdR	5.5
	Control	5.75
Chemical reagent sensitivity	Ethyl ether	<1.5
	Chloroform	<1.5
	Sodium deoxycholate	<1.5
	Trypsin	5.25
	Control	5.5
Acid stability	pH 3.0	3.5
	pH 7.0	5.0
Heat stability	1M MgCl ₂ 22°C	5.0
	50°C	3.5
Heat stability	0.85% NaCl 22°C	5.0
	50°C	2.5
Filtrability	Unfiltered	5.75
	220 nm	5.5
	100 nm	<1.5
	50 nm	<1.5

Fig. 1. Electron micrograph of negatively stained viral particle of the isolate NPTr32 strain. Bar=100 nm.

the same solution at 22°C for 1 hr (5.0 log₁₀TCID₅₀/ml).

At the same time, the infective titer of the isolated virus that was examined with 0.85% NaCl solution at 50°C for 1 hr (2.5 log₁₀TCID₅₀/ml) was lower than the titer that was examined at 22°C for 1 hr (5.0 log₁₀TCID₅₀/ml).

The isolated virus passed readily through a 220 nm pore size filter, but not through 100 and 50 nm pore size filters.

Electron microscopic observation: Many virus-like particles were observed in the preparation. They were 120 to 160 nm in diameter showing the characteristics of coronavirus particles. They have a dense, featureless core 90 to 130 nm in diameter surrounded by projections that looks like a corona (Fig. 1, 2).

Cross neutralization test: The isolated viruses were completely neutralized by immune serum against 67N strain of HEV. There was a slight antigenic relationship between each isolated virus and strain No.

Table 2. Cross neutralization test among the isolates, 67N (HEV), TO160 (TGEV), No. 66 (BCV) and Shiga (IBV) stains

Virus	Antiserum							
	NPTr32	NNF417	NNa27	ONS204	67N (HEV)	TO160 (TGEV)	No. 66 (BCV)	Shiga (IBV)
NPTr32	1024	512	512	256	64	<2	4	<2
NNF417	512	512	256	256	64	<2	2	<2
NNa27	512	256	512	256	64	<2	2	<2
ONS204	512	256	256	512	64	<2	2	<2
TO160	<2	<2	<2	<2	<2	2048	<2	<2
No. 66	4	2	2	2	<2	<2	8192	<2

Fig. 2. Electron micrograph of negatively stained viral particle of the isolate NPTr32 strain. Bar=100 nm.

66 of BCV. The isolated viruses were not neutralized with the TO160 strain of TGEV or the Shiga strain of IBV (Table 2).

Experimental infection in pigs and piglets: The colostrum-deprived piglets inoculated with the viruses showed clinical sign from 2 to 9 days after the inoculation. The sign were slight coughing, sneezing with nasal discharges, diarrhea, fever up to 40°C, inappetence and listlessness (Fig. 3). The conventional 2-month-old pigs showed only

respiratory sign such as slight coughing, sneezing with nasal discharges, 2 to 7 days after exposure to the viruses (Fig. 4). Both experimentally infected pigs and piglets showed no vomiting and central nervous sign. Virus was successfully recovered from the respiratory tract and lungs of the infected pigs and piglets during 1 to 9 days after the infection. HI antibody of pigs and piglets were detected 7 days after exposure to the viruses.

Fig. 3. Experimental infection of colostrum-deprived piglets with the NPTr32 strain. RS: Respiratory sign, DR: Diarrhea, IA: Inappetence and listlessness, VR: Virus recovery, HI: Hemagglutination inhibition titer, i/n: intranasal route, i/o: oral route.

Survey on HI antibody: The results of survey on HI antibody from farms N and T are shown in Table 3. The positive ratio of HI antibody ranging from 9.1% to 65.4% in pigs 1 to 2 months old on farm N and from 0% to 36.7% in pigs on farm T. There were significant differences in positive ratio between the pigs 2 and 3 months old on farm N. The positive ratio was higher than 50% in all the pigs after 3 months excluding group 4 pigs 4 months old.

DISCUSSION

The authors isolated 4 strains of HEV from tracheal, nasal mucosa, and nasal discharges of pigs suffering from respiratory diseases and debilitation. The isolated viruses propagated well in PT and PK cells. The tissue culture fluids showed hemagglutinating activity on chicken erythrocytes. CPE appeared more clearly in PT cells than PK cells. Syncytia was formed in cell cultures about 18 hr after virus inoculation. The CPE was generally complete within

Table 3. Survey of HI antibody on farms from where virus was isolated

Farm	Group No. of pigs	Age of pigs in months					
		1	2	3	4	5	6
N	1	50.0*($\frac{8}{16}$)**	65.4($\frac{17}{26}$)	81.5($\frac{22}{27}$)	100 ($\frac{23}{23}$)	95.7($\frac{22}{23}$)	100 ($\frac{23}{23}$)
	2	12.0 ($\frac{3}{25}$)	25.0($\frac{7}{28}$)	77.3($\frac{17}{22}$)	77.3($\frac{17}{22}$)	70.0($\frac{14}{20}$)	88.9($\frac{16}{18}$)
	3	9.1 ($\frac{2}{22}$)	20.0($\frac{3}{15}$)	53.8($\frac{7}{13}$)	66.7($\frac{12}{18}$)	73.7($\frac{14}{19}$)	77.8($\frac{14}{18}$)
	4	30.8 ($\frac{8}{26}$)	9.1($\frac{2}{22}$)	50.0($\frac{12}{24}$)	43.5($\frac{10}{23}$)	95.8($\frac{23}{24}$)	80.0($\frac{16}{20}$)
T	5		0 ($\frac{0}{40}$)	86.2($\frac{25}{29}$)	84.0($\frac{21}{25}$)	92.3($\frac{24}{26}$)	
	6		36.7($\frac{11}{30}$)	80.0($\frac{24}{30}$)	93.3($\frac{28}{30}$)	96.4($\frac{27}{28}$)	

* Positive rate (%)

** No. of positive serum/No. of serum examined.

Fig. 4. Experimental infection of conventional pigs with the NPTr32 strain. RS: Respiratory sign, DR: Diarrhea, IA: Inappetence and listlessness, VR: Virus recovery, HI: Hemagglutination inhibition titer, i/n: intranasal route, i/o: oral route.

48 hr after inoculation, but usually after this time there remains a residual population of apparently normal cells. These findings corresponded to that reported by Greig *et al.* [5] and Greig and Girard [6].

In 1972, Mengelling *et al.* [11] isolated 67N strain of HEV from the nasal cavity of an apparently healthy pig in the United States.

Girard *et al.* [4] and Mengeling *et al.* [11] observed that HEV agglutinated erythrocytes from chicken, rat, mouse, hamster and turkey. The isolated viruses agglutinated erythrocytes from rat and hamster, but were not tested for agglutination of erythrocytes from turkey.

HEV was unstable to lipid solvents, acid and heat [6, 7]. The infectivity of the isolated virus decreased gradually in pH 3.0 solution during a long term incubation.

When observed by the electron microscope, the isolated viruses appeared to be coronaviruses. Each particle contained a core 90 to 130 nm in diameter surrounded by regularly spaced club-like projections about 20 nm in mean length. These results corresponded to those of previous studies [8, 13].

The serum neutralization tests revealed that the isolated viruses were identical with HEV.

Colostrum-deprived piglets, as well as conventional pigs, showed respiratory sign such as slight coughing, sneezing with nasal discharges when inoculated with these isolated viruses. By the experimental infection, colostrum-deprived piglets showed fever up to 40°C, diarrhea, inappetence and listlessness, though conventional pigs did not show such sign. These results indicate that conventional pigs were resistant to virus infections under general conditions. But colostrum-deprived piglets manifested general sign, showing sensitivity to the isolated viruses.

Greig *et al.* [5] first succeeded in isolating

HEV from a brain of a piglet affected with encephalomyelitis in 1962. Since then, many investigators [1, 2, 3, 10] reported that HEV was a causative agent of encephalomyelitis, and also as a vomiting-wasting disease. Mengeling and Cutlip [12] observed some clinical sign, including hyperesthesia, vomiting and respiratory sign of pneumonitis, in specific pathogen-free piglets exposed to the 67N strain of HEV. The authors' virus strains were isolated from a pig showing respiratory sign. It is very interesting to note that these sign were manifested by pigs that were exposed to these strains.

In Japan, the isolation of HEV has not been reported, but a serological survey on this virus has been conducted by Hirai *et al.* [9] to show that approximately 50% of the tested sera were positive for HI antibodies against HEV. On 2 farms N and T where the virus was isolated by the authors, the positive ratio of antibodies was less than 30% among pigs that were examined at 1 to 2 months old in groups 2 to 5, and increased rapidly to more than 70% in pigs that were examined at 3 to 4 months old, excluding groups 3 and 4. Consequently, it was speculated that pigs might be naturally infected in the field some time between 2 to 3 months of age when there is a decrease in maternal antibodies.

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要 約

豚気道からの血球凝集性脳脊髄炎ウイルスの分離：平原 正・安原壽雄・児玉和夫・中井正久・佐々木文存（微生物化学研究所）——1984年，新潟県と大阪府下の2養豚場で，発咳や鼻漏を呈する約3か月齢豚の気道から融合性の細胞変性を示す agent 4 株が分離され，マウス，ラット，ハムスターおよびニワトリの赤血球を凝集した。分離株は IUdR により増殖を阻害されず，エーテル，クロロホルムおよび酸（PH 3.0）に感受性で，220 nm のフィルターを通過したが，100 nm のフィルターは通過しなかった。電子顕微鏡観察により，直径約120～160 nm のコロナウイルス様粒子を認めた。抗血清による交差中和試験では，豚伝染性胃腸炎ウイルスとは反応せず，牛コロナウイルスとは弱い反応を示し，血球凝集性脳脊髄炎ウイルス（HEV）と強い交差を示した。以上の成績から，分離株は HEV と同定された。2 か月齢の仔豚と初乳未摂取 2 日齢仔豚に分離ウイルスを経鼻および経口接種したところ，軽い発咳やくしゃみ，鼻漏などを呈し，気道および肺よりウイルスが回収され，HI 抗体は 7 日目に最初に検出された。抗体分布調査では，約 3～4 か月齢で陽性率が上昇する傾向がみられた。