

Virological and Serological Studies of Porcine Respiratory Coronavirus Infection on a Japanese Farm

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(Received 21 January 2008/Accepted 13 May 2008)

ABSTRACT. We detected transmissible gastroenteritis virus (TGEV) antibodies in pig farms in Tochigi prefecture, although the farms had no past record of TGEV vaccination or TGE. Among the farms, Farm A showed a high antibody incidence. We could not confirm if either TGEV or porcine respiratory coronavirus (PRCV) induced the antibodies, since conventional tests failed to discriminate PRCV from TGEV. Therefore, we conducted virological and serological examinations of this farm for 4 years to establish the etiology - TGEV or PRCV. Although no TGEV was detected, PRCVs were isolated from the nasal samples of pigs. Using a commercial ELISA kit, it was found that the antibodies detected in pigs of all the raising stages and sows were raised against PRCV but not TGEV. The phylogenetic analysis of the nucleotide sequences of the isolates showed that they were closely related to each other, and formed a separate cluster apart from the U.S.A. and European strains. In Cesarean-derived, colostrums-deprived piglets inoculated with a PRCV isolate, no clinical signs were seen, and the viruses were mainly isolated from the nasal samples. Moreover, viral genes were detected from the nasal sample of the contact pig. The result suggested that PRCV infection was located in the nasal cavity of pigs, and horizontal transmission easily occurs. From these results, PRCVs with different origins from the exotic PRCVs might be prevalent in pig farms in Japan.

KEY WORDS: PMWS, porcine respiratory coronavirus, PRCV, TGEV.

J. Vet. Med. Sci. 70(9): 929–936, 2008

Porcine respiratory coronavirus (PRCV) was first isolated in Belgium and the United Kingdom in 1986 [19]; subsequently, the virus was detected in other parts of Europe, the U.S.A., Canada, and Korea [4, 8, 10, 28, 31, 32]. Although the antigenic and genetic properties of PRCV are similar to those of transmissible gastroenteritis virus (TGEV), they exhibit different organ tropism. Namely, TGEV grows very well in the epithelial cells of the small intestine of pigs, while the main propagation site for PRCV is the epithelial cells of the nasal cavity, tracheae, lungs, and tonsils [5, 9, 12, 19, 20]. The genes of both viruses showed a high identity; however, the deletion of nucleotides ranging of approximately 600 to 700 bp at the 5' end of the spike (S) gene and the deletion of a part of the open reading frame (ORF) 3 or ORF3–1 gene were found in PRCV [1, 30]. These deletions are considered to be related to the differences in organ tropism between PRCV and TGEV in pigs [1, 15]. Therefore, PRCV is thought to be a variant of TGEV.

Conventional serological techniques can not discriminate PRCV from TGEV because of their common antigenicity [22, 23]. Therefore, the close antigenic similarity between both the viruses may create problems in serological diagnosis in Japan. However, an ELISA system has recently been

developed for differentiation of the antibodies to TGEV and those to PRCV [3]. In Japan, Kamogawa *et al.* [11] reported the first isolation of PRCV from a case of porcine reproductive and respiratory syndrome (PRRS) in 1996. Although they detected serum neutralizing antibodies against PRCV and TGEV in the pigs in the PRRS-affected farms, they did not demonstrate that the antibodies were raised by PRCV but not TGEV. To date, there is no report concerning to seroprevalence of PRCV in pig farms in Japan. Also, pathogenetic or genetic characterization of Japanese PRCV isolates has not been conducted.

By using the micro-indirect fluorescent antibody test (micro-IFAT) [25], we detected antibodies against TGEV in some pig farms in Tochigi prefecture. Surprisingly, in these farms, there had been no incidence of diarrhea due to TGEV infection, and no TGEV vaccination had been carried out. Among these farms, Farm A in particular showed a high incidence of TGEV antibodies with high titers in pigs, although TGEV vaccination or the occurrence of TGE had not been recorded on this farm for the last 20 years. Therefore, we examined the pigs of this farm for 4 years to elucidate whether TGEV alone was responsible for this situation. We determined that PRCV not TGEV was in fact prevalent on this farm and was responsible for the positive results of the TGEV antibodies test. We also report the first detection of antibodies to PRCV, in addition to PRCV isolation and describe the pathogenetic or phylogenetic properties of PRCVs isolated in Japan.

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MATERIALS AND METHODS

Cells: CPK cells derived from porcine kidneys [13] were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and 0.29% tryptose phosphate broth at 37°C in a CO₂ incubator. Maintenance medium contained 2% FCS instead of 10% FCS in the growth medium (GM) described above.

Reverse transcription-polymerase chain reaction: RNA was extracted from nasal secretions, 10% fecal suspensions or oral swabs of pigs by using a commercial RNA extraction kit (TRIzol reagent, Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instruction manual. To detect various viral genes, reverse transcription-polymerase chain reaction (RT-PCR) was performed using a commercial kit (RNA PCR kit ver. 2.1, Takara Bio Inc., Otsu, Japan). cDNA was synthesized using random primers by incubation at 30°C for 10 min and 37°C for 60 min, followed by 99°C for 5 min. Primers for the amplification of the S genes of TGEV and PRCV were 21209 (5'-GGG-TAAGTTGCTCATTAGAAA-3', position in TGEV virulent Pardue strain: 20292-20312) [30] and 2374R (5'-CATAACTAGCACATTGATCA-3', position in TGEV virulent Pardue strain: 21131-21113) which were kindly provided by Dr. S. Yamada (National Institute of Animal Health, Japan). The PCR conditions were as follows: 35 cycles of denaturation at 94°C for 0.5 min, annealing at 55°C for 0.5 min, and extension at 72°C for 1 min. The PCR products were analyzed on a 2% agarose gel stained with ethidium bromide.

Detection of porcine circovirus type 2 (PCV2) genes from serum samples was conducted according to the RT-PCR described by Ellis *et al.* [7], and PRRS virus (PRRSV) genes were also detected from serum samples according to the RT-PCR by Kono *et al.* [14].

Virus isolation: CPK cells were used for PRCV isolation. The percent suspensions of nasal secretion and feces prepared in MEM without serum were centrifuged at 3,000 × g for 15 min at 4°C, and the supernatants were filtered using a 0.45- μ m pore size filter (Millipore, Molsheim, France). The supernatant was inoculated on a monolayer of CPK cells, followed by incubation at 37°C for 60 min. The inoculated CPK cells were maintained in GM at 37°C. If no cytopathic effect (CPE) developed, serial blind passages were performed five times.

Serological tests: A serum neutralization (SN) test was performed in a 96-well microplate containing CPK cells. PRCV isolated in this study was used in the SN test after cloning three times by a limiting dilution method. Two-fold dilutions beginning with a 1:2 dilution of the serum were performed. Fifty μ l (200 median tissue culture infectious dose) of PRCV were incubated with an equal volume of diluted serum at 37°C for 60 min, and each mixture was added to 2 microplate wells containing 0.1 ml of cell suspension (1×10^5 cells). SN antibody titers were defined as the reciprocal of the highest dilution of the serum that completely inhibited the CPE in the 2 wells 7 days after inocula-

tion.

SVANOVIR TGEV/PRCV-Ab enzyme linked immunosorbent assay (TGEV/PRCV-Ab ELISA) kit was used for detection and differentiation of antibodies to TGEV and PRCV in porcine serum samples (Svanova Veterinary Diagnostics, Uppsala, Sweden) [3]. Antibodies against PRRSV and Aujeszky's disease virus (ADV) were measured using a commercial ELISA kit (IDEXX laboratories, Westbrook, ME, U.S.A.). Antibodies against PCV2 were measured using ELISA according to the method of Liu *et al.* [16]. Antibodies against *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) were measured using complement fixation tests according to the method of Mori *et al.* [17]. Antibodies against *Actinobacillus pleuropneumoniae* serotypes 1, 2 and 5 (App-1, 2 and 5) were measured using ELISA. App-1, 2 and 5 antigens were provided by Dr. S. Nagai (Nippon Institute for Biological Science, Tokyo, Japan).

Bacteriological and pathological examinations: Bacteriological examination was conducted by stamping various internal organs collected from growth-retarded pigs on 5% sheep blood agar plates, MacConkey agar plates or chocolate agar plates. The stamped plates were incubated at 37°C for 48 hr under aerophil, 10% CO₂ microaerophilic or anaerobic condition.

Internal organs were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin by standard procedures for histopathological examination. The presence of PCV2, PRRSV, TGEV, *Pasteurella multocida* (*P. multocida*), App-1, 2, 5 and *Pneumocystis carinii* antigens was examined by an immunoperoxidase method using a commercial kit (SAB-PO kit, Nichirei Biosciences, Tokyo, Japan).

Sequencing and phylogenetic analysis: Specific oligonucleotide primers for sequencing were designed based on the sequence of the 3' end of a 1b subunit of polymerase and an S protein of a PRCV 86/137004 strain (GenBank accession no. X60089). Primer sequences used were as follows: PRCV.F (forward primer: GCTCCACGTTGGTTAGTAGC); PRCV.R (reverse primer: CAGTGATCAGATGTGTAAGCT); PRCV-1F (forward primer: CTACTTAGGACCATACTGTG); PRCV-1R (reverse primer: GATCAAAGTCAGCACCTTCA). Sequences of 4 PRCV strains isolated from nasal secretions of pigs in this study were compared with those of eight other PRCV strains available in GenBank (Fig. 1). The purified RT-PCR products were used as templates for sequencing on an ABI 310 automated DNA sequencer using a Big Dye Terminator chemistry kit (Perkins-Elmer/Applied Biosystems, Foster City, CA, U.S.A.). Multiple alignments were conducted by the Clustal X program [24], and the phylogenetic tree was constructed by the neighbor-joining (NJ) method [21].

Collection of samples from Farm A: Farm A has a farrow to operation system, where 14 boars and 247 sows are kept. Neonatal pigs (up to 23 days old) were kept in a farrow house, and then the pigs were moved and kept as follows: in a weaner pig house (up to 55 days old), piglet house (up to 90 days old), and feeder pig house (average to 180 days old)

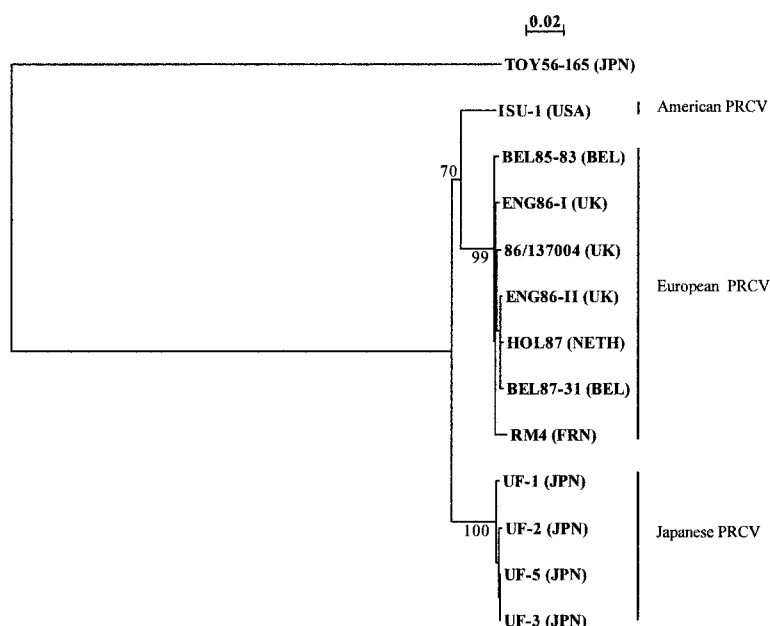


Fig. 1. Phylogenetic relationship among different PRCV strains based on the sequence of the 5' end of the S gene (the first 711 nt) of the virus. Multiple alignments were conducted by the Clustal X program, and the phylogenetic tree was constructed by the NJ method. The tree was rooted to the TOY56-165 strain of TGEV (GenBank accession no. M94103). Sequences of four Japanese PRCV strains, UF-1, UF-2, UF-3, UF-5 and foreign PRCV strains were used for the phylogenetic analysis. The sequences of PRCV strains obtained from GenBank (accession number in brackets) were as follows: Belgium (BEL) BEL85-83 [M94096], BEL87-31 [M94098], France (FRN) RM4 [Z24675], the United Kingdom (UK) ENG86-I [M94100], ENG86-II [M94102], 86/137004 [X60089], Netherlands (NETH) HOL87 [M94097], and USA ISU-1 [DQ811787]. The number on each branch shows the percent occurrence in 1,000 bootstrap replicates. The scale bar represents 2% nucleotide difference.

according to each raising stage.

From December 2000 to December 2003, sampling of sera, nasal secretions, or feces was randomly performed from 4 to 5 healthy pigs per age, including sows, in the pig houses of each raising stage (examination 1) as shown in Table 1.

In examination 2, sampling of sera, nasal secretions, feces and various organs such as liver, spleen, kidney, heart, lung, tonsil, turbinate membranes, cerebrum, intestine, and lymph nodes (tracheobronchial, superficial cervical, popliteal, subiliac, jejunal, and superficial inguinal nodes) was performed from eight growth-retarded piglets from July 2003 to March 2004. In addition, two healthy piglets kept with growth-retarded piglets in the same pen and two healthy piglets kept in a different pen of the same house were examined from September 2003 until March 2004 (Table 1).

Pathogenicity test with Cesarean-derived, colostrums-deprived piglets: Pathogenicity tests were performed in a bio-containment facility. Five heads of 7-day-old Cesarean-derived, colostrum-deprived (CDCD) piglets [6] were divided into 2 groups. Two piglets (nos. 1, 3) were intranasally inoculated with $10^{5.0}$ TCID₅₀ of the PRCV isolate per

head, and each one was kept in a separate pen. One uninoculated piglet (no. 2) was kept in a pen between these pens. Two piglets in another control group (nos. 4, 5) were placed in a different room of the same facility.

We recorded clinical signs and body temperature every day for 14 days post inoculation (d.p.i.). Body weight was measured before inoculation, and at 6 and 14 d.p.i. Nasal secretions, oral swabs, feces and sera were periodically collected from piglets. One piglet (no. 3) was killed at 6 d.p.i., and the remainder were killed at the end of the experiment. Brains, tonsils, tracheae, lungs, thymuses, hearts, livers, spleens, kidneys, stomachs, small and large intestines, and lymph nodes (mandibular, pulmonary and jejunal) were collected from all piglets for virological and pathological examination.

RESULTS

Isolation of PRCV and detection of antibodies against PRCV: We attempted to detect PRCV genes by RT-PCR from nasal secretions and fecal specimens of pigs listed in Table 1. In examination 1, the PRCV genes were detected

Table 1. Collection of materials from pigs

Examination	Date of sampling	No. and description of pigs	age in days	Number of samples			
				Nasal	Fecal	Serum	Tissue ^{c)}
1	Dec. 2000	4 healthy sows	Adult	4	2	4	— ^{d)}
		16 healthy pigs ^{a)}	28, 50, 85, 150	16	8	16	—
	Dec. 2001	4 healthy sows	Adult	4	2	4	—
		30 healthy pigs	20, 50, 85, 120, 150, 180	30	10	30	—
	Jun. 2002	4 healthy sows	Adult	4	2	4	—
		20 healthy pigs	28, 50, 85, 120, 150	20	14	20	—
	Jun. 2003	5 healthy sows	Adult	5	5	5	—
		25 healthy pigs	28, 50, 85, 120, 150	25	25	25	—
	Dec. 2003	5 healthy sows	Adult	5	5	5	—
		25 healthy pigs	28, 60, 85, 120, 150	25	25	25	—
2	Jul. 2003	Growth-retarded piglet no.1	85	1	1	1	15
	Aug. 2003	Growth-retarded piglet no.2	70	1	1	1	15
	Sep. 2003	Growth-retarded piglet no.3	80	1	1	1	15
		4 healthy piglets ^{b)}	80	4	4	4	—
	Oct. 2003	Growth-retarded piglet no.4	70	1	1	1	15
		4 healthy piglets	70	4	4	4	—
	Nov. 2003	Growth-retarded piglets nos. 5, 6	70	2	2	2	30
		4 healthy piglets	70	4	4	4	—
	Jan. 2004	Growth-retarded piglet no.7	60	1	1	1	15
		4 healthy piglets	60	4	4	4	—
Mar. 2004	Growth-retarded piglet no.8	75	1	1	1	15	
	4 healthy piglets	75	4	4	4	—	

a) Neonatal pigs, weaner pigs, piglets, and feeder pigs.

b) Each two heads were kept with growth-retarded pig(s) in the same and different pens, respectively.

c) Cerebrum, nasal mucosa, tonsils, lung, heart, liver, spleen, kidney and intestines, and lymph nodes (tracheobronchial, popliteal, superficial cervical, subiliac, superficial inguinal and jejunal).

d) Samples were not collected.

from nasal secretions of two weaner pigs of 50-day-old and four piglets of 60-day-old (Table 2). In examination 2, PRCV genes were detected from each of the four growth-retarded and healthy piglets. Two healthy piglets were kept with the retarded ones (nos. 5 and 6) in the same pen, and the other two were in a different pen of the same house.

Five strains of PRCV (designated UF-1, -2, -3, -4 and -5) were isolated from 5 out of the 10 nasal samples that showed positive results in the RT-PCR (Table 2). No PRCV gene was detected from the fecal samples and organs of any of the pigs examined. The TGEV gene was not detected in any nasal swabs, fecal or organ samples.

SN antibodies against the PRCV strain UF-1 were detected from most of the pigs examined during the period from December 2000 to March 2004 (Table 3). In examination 1, SN titers ranged from 1:16 to 1:2,048 in 22 healthy sows and from <1:2 to 1:2,048 in 116 healthy pigs. In examination 2, SN antibodies were detected from five out of the eight growth-retarded piglets and 19 out of the 20 healthy piglets. The titers in both retarded and healthy piglets ranged from <1:2 to 1:128, respectively.

Because the SN test can not differentiate the antibodies to TGEV from those to PRCV, it is possible that this SN test detected the antibodies to TGEV. Therefore, serum samples

were examined by TGEV/PRCV-ab ELISA for distinguishing the antibodies to TGEV from those to PRCV. Antibodies to PRCV were detected in 101 out of the 112 serum samples tested by the TGEV/PRCV-Ab ELISA kit; however, no antibodies to TGEV were detected (Table 3).

Involvement of PRCV or other pathogens in growth-retarded piglets: In examination 2, antibodies to PRCV, PCV2 or PRRSV were detected from both the growth-retarded and healthy piglets as shown in Table 4. There was no significant difference in the incidence of antibody against each virus and its viral gene between the growth-retarded and healthy piglet groups.

Antibodies against App serotype 5 were detected in four out of the eight growth-retarded piglets in addition to all the healthy ones; however, no App was isolated from the lungs of the retarded piglets, and no antigens were detected by the immunoperoxidase method. No antibodies against App-1,2, *M. hyopneumoniae* or ADV were detected. In bacteriological examination, *P. multocida* was isolated from one growth-retarded piglet, and *Streptococcus* spp. was found in three growth-retarded piglets.

Histological examination revealed various lesions in the organs of the growth-retarded piglets, such as lymphocyte depletion, reticular cell proliferation, multinucleated giant

Table 2. Detection of PRCV genes by RT-PCR and isolation of PRCV from nasal samples

Examination	Date of sampling	Description of pigs	Age in days	Detection of PRCV genes with RT-PCR	Isolation (strain)
1	Jul. 2002	Healthy weaner pig	50	+	-
	Dec. 2003	Healthy weaner pig	50	+	+(UF-1)
		Healthy piglet	60	+	+(UF-2)
		Healthy piglet	60	+	-
		Healthy piglet	60	+	-
		Healthy piglet	60	+	-
2	Nov. 2003	Growth-retarded piglet no.5	70	+	+(UF-3)
		Growth-retarded piglet no.6	70	+	+(UF-4)
		Healthy piglet ^{a)}	70	+	NT ^{c)}
		Healthy piglet ^{a)}	70	+	NT
		Healthy piglet ^{b)}	70	+	NT
	Healthy piglet ^{b)}	70	+	NT	
	Jan. 2003	Growth-retarded piglet no.7	60	+	-
Mar. 2004	Growth-retarded piglet no.8	75	+	+(UF-5)	

a) A piglet was kept in the same pen as the growth-retarded piglets nos. 5 and 6.
 b) A piglet was kept in the different pen as the growth-retarded piglets nos. 5 and 6.
 c) Not tested.

Table 3. Detection of antibodies against the strain UF-1 of PRCV

Examination	Date of sampling	Description of pigs	Head	Serum neutralizing antibody to the UF-1 strain											ELISA		
				<2	2	4	8	16	32	64	128	256	512	1024	2048	PRCV	TGEV
1	From Dec. 2000 to	Healthy sows	2					2 ^{b)}	3	2	6	3	2	2	2	12/14 ^{c)}	0/14
	Dec. 2003	Healthy pigs ^{a)}	116	11	7	11	14	20	19	10	13	5	4	1	1	66/70	0/70
2	From Jul. 2003 to	Growth-retarded piglets	8	3	1	1		1		1						5/8	0/8
	Mar. 2004	Healthy piglets	20	1	1	4	2	6	2	3	1					18/20	0/20

a) Neonatal pigs, weaner pigs, piglets, and feeder pigs.
 b) Number of pigs.
 c) Number of positive/a total numbers examined.

Table 4. Detection of antibodies to PRCV, PCV2 and PRRSV and viral genes from growth-retarded and healthy piglets kept in the same or different pens

Description of pigs	Head	% detection rate of antibodies to			% detection of viral genes		
		PRCV	PCV2	PRRSV	PRCV	PCV2	PRRSV
Growth-retarded	8	62.5	87.5	87.5	50	100	62.5
Healthy	20	95	90	95	20	100	55

cell infiltration, and basophilic cytoplasmic inclusion bodies in lymphoid tissues as well as interstitial pneumonitis, bronchopneumonia, and rhinitis. PCV2 antigens were detected in all but one of the growth-retarded piglets (pig no. 4), and PRRSV antigens were also detected in the lungs of five growth-retarded piglets (pig nos. 2, 3, 5 to 7) by the immunoperoxidase method (data not shown).

From these results, most of growth-retarded piglets seemed to suffer from postweaning multisystemic wasting syndrome (PMWS) associated with PCV2 and/or PRRSV infection (pig nos. 1-3 and 5-7).

Phylogenetic analysis of PRCV isolates: The sequences of the 5' end of the S gene (the first 711 nt) of our PRCV isolates (strains UF-1, -2, -3, -5) were compared with those of the eight PRCV strains isolated in U.S.A. and European countries (the United Kingdom, Belgium, France and the Netherlands). The phylogenetic trees indicated that all PRCV strains were clustered into one clade, suggesting that PRCV strains share a common ancestor (Fig. 1). However, the Japanese PRCV strains were most closely related to each other, and formed a separate cluster apart from other PRCV strains. The Japanese strain (UF-1) showed a higher

Table 5. Detection of viral gene, antibody and isolation of PRCV in Cesarean-derived, colostrums-derived piglets infected with the UF-1 strain of PRCV

Pig no.	Samples	Method	Days post-inoculation					
			0	1	3	5	7	14
1 (inoculated)	Nasal	RT-PCR	- ^{a)}	+	+	+	+	-
		Isolation	-	2.25 ^{b)}	5.0	4.0	2.5	-
	Oral	RT-PCR	-	+	+	+	+	-
		Isolation	-	-	-	-	-	-
	Fecal	RT-PCR	-	-	-	-	-	-
		Isolation	-	-	-	-	-	-
	Serum	Neutralization test	<2 ^{c)}	NT ^{d)}	NT	NT	NT	8
	2 (contact)	Nasal	RT-PCR	-	-	-	+	+
Isolation			-	-	-	-	-	-
Oral		RT-PCR	-	-	-	-	-	-
		Isolation	-	-	-	-	-	-
Fecal		RT-PCR	-	-	-	-	-	-
		Isolation	-	-	-	-	-	-
Serum		Neutralization test	<2	NT	NT	NT	NT	<2
3 (inoculated) ^{e)}		Nasal	RT-PCR	-	+	+	+	
	Isolation		-	1.5	2.75	2.75		
	Oral	RT-PCR	-	+	+	+		
		Isolation	-	-	0.75	-		
	Fecal	RT-PCR	-	-	-	-		
		Isolation	-	-	-	-		
	Serum	Neutralization test	<2	NT	NT	<2		

a) +: positive; -: negative.

b) Virus titer (Log_{10} TCID₅₀/0.05 ml).

c) Neutralizing antibody titer.

d) Not tested.

e) This piglet was euthanized 6 d.p.i.

genomic sequence identity with the European strains, ranging from 94.1 to 94.8%, than with the U.S.A. strain (93.8%) (data not shown).

Pathogenicity of the PRCV isolate in CDCD piglets: In two piglets (nos. 1 and 3) inoculated intranasally with the UF-1 strain of PRCV, viral genes were detected from the nasal and oral samples at 1, 3, 5 and 7 d.p.i. (pig no.1) and at 1, 3 and 5 d.p.i. (pig no. 3), respectively (Table 5). Conversely, the viral genes were only detectable from the nasal samples of the uninoculated contact piglets (pig no. 2) at 5 and 7 d.p.i. Although no viruses were isolated from the contact piglets, viruses were isolated from the nasal samples of the inoculated piglets up to 7 d.p.i. Two piglets of the uninoculated control group placed in the pen of a different room remained free from PRCV infection during the experimental period.

DISCUSSION

Conventional serological techniques can not discriminate PRCV from TGEV because of their common antigenicity [2, 22, 26]. Therefore, the close antigenic similarity between both the viruses may create problems in serological diagnosis. Our preliminary study showed that antibodies to

TGEV were detected in pigs from some pig farms in Tochigi prefecture using micro-IFAT [25], although these farms did not have a past record of TGEV vaccination or occurrence of TGE. However, at that time, we could not confirm if these antibodies were induced by either TGEV or by PRCV infection. Therefore, we selected one farm (Farm A), which in particular showed a high positive rate of TGEV antibodies, and continued the virological and serological examination in the pigs for 4 years in order to establish the etiology - TGEV or PRCV.

Although TGE was expected based on the TGEV antibodies test, no TGEV genes were detected from pigs and the occurrence of disease was also not observed on Farm A. In contrast, PRCV genes or PRCVs were isolated from nasal samples of healthy and growth-retarded piglets (Table 2). Most of these PRCVs and their genes were obtained from piglets during the period of November 2003 to March 2004, indicating that the infection of PRCV occurred among the piglet group during this period. Prior to this study, no PRCV isolation had been reported since the first description reported by Kamogawa *et al.* [11] in Japan in 1996. Our PRCV isolation with serological and genetic findings may indicate that PRCV is widely prevalent among pig farms in Japan. In the present study, phylogenetic analysis indicated

that the Japanese PRCV strains formed a separate cluster apart from the U.S.A. strain and the European strains (Fig. 1).

The high incidence of SN antibodies against the UF-1 strain of PRCV isolated from the weaner pig was observed throughout the 4-year examination period (Table 3). Conventional serological assays such as SN tests have not differentiated between PRCV and TGEV [3, 18, 23]. We confirmed this fact based on the absence of a significant difference between the SN antibody titers against both viruses (data not shown). To our knowledge, there have been no reports on the detection of antibodies against PRCV in Japan. In this study, we clearly demonstrated the presence of antibodies against PRCV using ELISA for detection and differentiation of antibodies against TGEV and PRCV in porcine serum samples. The long-term observation indicated that PRCV is prevalent on this farm, since antibodies to PRCV were detectable in most pigs examined in all the raising stages regardless of season. In the present study, we were able to isolate PRCVs on a pig farm since the first isolation of PRCVs in 1996 in Japan [11]. It is likely that PRCV is widely present throughout the country.

Since PRCVs or viral genes were detectable from pigs at the end of the weaning stage and at the beginning of the piglet stage (Table 2), it was strongly suggested that pigs became infected with PRCV around these stages. Therefore, it may be important to isolate and raise young susceptible pigs under strict hygienic conditions.

Most growth-retarded piglets examined were diagnosed with PMWS or with interstitial pneumonitis due to PRRSV infection based on histological and immunohistochemical examination. Although PRCVs and its viral genes were detected from the nasal samples of the growth-retarded piglets, they were also derived from the healthy ones and not from the pathological lesions observed in the growth-retarded piglets. Hence, it seemed that PRCV was not directly associated with the cases of growth retardation.

To date, there have been no reports on the pathogenicity of PRCV isolates against pigs in Japan. We evaluated the pathogenicity of the UF-1 strain isolated in this study using CDCD piglets (Table 5). Since the UF-1 strain was mainly recovered from the nasal samples of the inoculated piglets, but not from the fecal samples and organ samples, it was suggested that the employed PRCV strain caused infection limited to nasal cavities of the infected piglets. In addition, the UF-1 strain failed to cause any clinical diseases and lesions in the infected piglets, which suggested that the PRCV strain was nonpathogenic. Van Reeth *et al.* [27] failed to induce clinical diseases in the pigs inoculated with PRCV 91V44 strain. In contrast, Vannier [29] succeeded in inducing clinical signs such as difficulty in breathing, fever, and poor growth in pigs experimentally infected with PRCV isolated in France. These results may suggest differences in pathogenicity among the PRCV strains used.

Van Reeth *et al.* [27] and Saif and Sestak [20] reported that respiratory signs due to PRCV infection were enhanced by concomitant infection with other pathogens such as

PRRSV and/or ADV. In Japan, Kamogawa *et al.* [11] isolated PRCVs from pigs affected with PRRS. In this study, we also could isolate PRCVs from pigs infected with PRRSV and PCV2. Even if PRCV is nonpathogenic or has low pathogenicity, it may cause adverse situations in pigs in combination with some other factors such as concomitant infections with other pathogens and/or multiple environmental factors.

REFERENCES

1. Ballesteros, M. L., Sánchez, C. M. and Enjuanes, L. 1997. Two amino acid changes at the N-terminus of transmissible gastroenteritis coronavirus spike protein result in the loss of enteric tropism. *Virology* **227**: 378–388.
2. Callebaut, P., Correa, I., Pensaert, M., Jiménez, G. and Enjuanes, L. 1988. Antigenic differentiation between transmissible gastroenteritis virus of swine and a related porcine respiratory coronavirus. *J. Gen. Virol.* **69**: 1725–1730.
3. Carman, S., Josephson, G., McEwen, B., Maxie, G., Antochi, M., Eernisse, K., Nayar, G., Halbur, P., Erickson, G. and Nilsson, E. 2002. Field validation of a commercial blocking ELISA to differentiate antibody to transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus and to identify TGEV-infected swine herds. *J. Vet. Diagn. Invest.* **14**: 97–105.
4. Chae, C., Kim, O., Min, K., Choi, C., Kim, J. and Cho, W. 2000. Seroprevalence of porcine respiratory coronavirus in selected Korean pigs. *Prev. Vet. Med.* **46**: 293–296.
5. Costantini, V., Lewis, P., Alsop, J., Templeton, C. and Saif, L. J. 2004. Respiratory and fecal shedding of porcine respiratory coronavirus (PRCV) in sentinel weaned pigs and sequence of the partial S-gene of the PRCV isolates. *Arch. Virol.* **149**: 957–974.
6. Debouck, P. and Pensaert, M. 1980. Experimental infection of pigs with a new porcine enteric coronavirus, CV 777. *Am. J. Vet. Res.* **41**: 219–223.
7. Ellis, J. A., Bratanich, A., Clark, E. G., Allan, G., Meehan, B., Haines, D. M., Harding, J., West, K. H., Krakowka, S., Konoby, C., Hassard, L., Martin, K. and McNeilly, F. 2000. Coinfection by porcine circoviruses and porcine parvovirus in pigs with naturally acquired postweaning multisystemic wasting syndrome. *J. Vet. Diagn. Invest.* **12**: 21–27.
8. Groschup, M. H., Brun, A. and Haas, B. 1993. Serological studies on the potential synergism of porcine reproductive and respiratory syndrome virus and influenza-, corona- and paramyxoviruses in the induction of respiratory symptoms in swine. *J. Vet. Med. B* **40**: 681–689.
9. Halbur, P. G., Paul, P. S., Vaughn, E. M. and Andrews, J. J. 1993. Experimental reproduction of pneumonia in gnotobiotic pig with porcine respiratory coronavirus isolated AR310. *J. Vet. Diagn. Invest.* **5**: 184–188.
10. Jabrane, A., Girard, C. and Elazhary, Y. 1994. Pathogenicity of porcine respiratory coronavirus isolated in Québec. *Can. Vet. J.* **35**: 86–92.
11. Kamogawa, O., Tomita, Y., Kaneko, M., Yamada, S., Kubo, M. and Shimizu, M. 1996. Isolation of porcine respiratory coronavirus from pigs affected with porcine reproductive and respiratory syndrome. *J. Vet. Med. Sci.* **58**: 385–388.
12. Kim, L., Hayes, J., Lewis, P., Parwani, A. V., Chang, K. O. and Saif, L. J. 2000. Molecular characterization and pathogenesis of transmissible gastroenteritis coronavirus (TGEV) and porcine respiratory coronavirus (PRCV) field isolates co-circulat-

- ing in a swine herd. *Arch. Virol.* **145**: 1133–1147.
13. Komanawa, H., Fukusho, A. and Shimizu, Y. 1987. Micro-method for neutralization test of transmissible gastroenteritis virus using porcine kidney cell line, CPK cells. *Jpn. J. Vet. Sci.* **49**: 141–144.
 14. Kono, Y., Kanno, T., Shimizu, M., Yamada, S., Ohashi, S., Nakamine, M. and Shirai, J. 1996. Nested PCR for detection and typing of porcine reproductive and respiratory syndrome (PRRS) virus in pigs. *J. Vet. Med. Sci.* **58**: 941–946.
 15. Laude, H., Van Reeth, K. and Pensaert, M. 1993. Porcine respiratory coronavirus: molecular features and virus-host interactions. *Vet. Res.* **24**: 125–150.
 16. Liu, C., Ihara, T., Nunoya, T. and Ueda, S. 2004. Development of an ELISA based on the baculovirus-expressed capsid protein of porcine circovirus type 2 as antigen. *J. Vet. Med. Sci.* **66**: 237–242.
 17. Mori, Y., Yoshida, Y., Kuniyasu, C. and Hashimoto, K. 1983. Improvement of complement fixation test antigen for the diagnosis of *Mycoplasma hyopneumoniae* infection. *Natl. Inst. Anim. Health. Q (Jpn)*. **23**: 111–116.
 18. Paton, D. J., Brown, I. H. and Vaz, E. K. 1991. An ELISA for the detection of serum antibodies to both transmissible gastroenteritis virus and porcine respiratory coronavirus. *Br. Vet. J.* **147**: 370–372.
 19. Pensaert, M., Callebaut, P. and Vergote, J. 1986. Isolation of a porcine respiratory, non-enteric coronavirus related to transmissible gastroenteritis. *Vet. Q.* **8**: 257–261.
 20. Saif, L. J. and Sestak, K. 2006. Transmissible gastroenteritis and porcine respiratory coronavirus. pp. 489–516. *In: Diseases of Swine*, 9th ed. (Straw, B. E., Zimmerman, J. J., D'Allaire, S. and Taylor, D. J. eds.), Blackwell Publishing, Ames.
 21. Saitou, N. and Nei, M. 1987. The neighbor-joining methods: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**: 406–425.
 22. Simkins, R. A., Weilnau, P. A., Bias, J. and Saif, L. J. 1992. Antigenic variation among transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus strains detected with monoclonal antibodies to the S protein of TGEV. *Am. J. Vet. Res.* **53**: 1253–1258.
 23. Simkins, R.A., Weilnau, P. A., Van Cott, J., Brim, T. A. and Saif, L. J. 1993. Competition ELISA, using monoclonal antibodies to the transmissible gastroenteritis virus (TGEV) S protein, for serologic differentiation of pigs infected with TGEV or porcine respiratory coronavirus. *Am. J. Vet. Res.* **54**: 254–259.
 24. Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. 1997. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**: 4876–4882.
 25. Usami, Y., Fukai, K., Yamaguchi, O. and Imai, K. 2001. Micro-indirect immunofluorescent antibody test for the detection of antibody to transmissible gastroenteritis virus. *Aust. Vet. J.* **79**: 640–641.
 26. van Nieuwstadt, A. P. and Boonstra, J. 1992. Comparison of the antibody response to transmissible gastroenteritis virus and porcine respiratory coronavirus, using monoclonal antibodies to antigenic sites A and X of the S glycoprotein. *Am. J. Vet. Res.* **53**: 184–190.
 27. Van Reeth, K., Nauwynck, H. and Pensaert, M. 1996. Dual infections of feeder pigs with porcine reproductive and respiratory syndrome virus followed by porcine respiratory coronavirus or swine influenza virus: a clinical and virological study. *Vet. Microbiol.* **48**: 325–335.
 28. Van Reeth, K. and Pensaert, M. 1994. Prevalence of infections with enzootic respiratory and enteric viruses in feeder pigs entering fattening herds. *Vet. Rec.* **135**: 594–597.
 29. Vannier, P. 1990. Disorders induced by the experimental infection of pigs with the porcine respiratory coronavirus (P. R. C. V.). *J. Vet. Med. B* **37**: 177–180.
 30. Vaughn, E. M., Halbur, P. G. and Paul, P. S. 1995. Sequence comparison of porcine respiratory coronavirus isolates reveals heterogeneity in the S, 3, and 3–1 genes. *J. Virol.* **69**: 3176–3184.
 31. Wesley, R. D., Woods, R. D., Hill, H. T. and Biber, J. D. 1990. Evidence for a porcine respiratory coronavirus, antigenically similar to transmissible gastroenteritis virus, in the United States. *J. Vet. Diagn. Invest.* **2**: 312–317.
 32. Wesley, R. D., Woods, R. D., Mckean, J. D., Senn, M. K. and Elazhary, Y. 1997. Prevalence of coronavirus antibodies in Iowa swine. *Can. J. Vet. Res.* **61**: 305–308.