Isolation of Porcine Respiratory Coronavirus from Pigs Affected with Porcine Reproductive and Respiratory Syndrome

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ABSTRACT. Four cytopathogenic viruses were isolated in CPK cells derived from porcine kidneys from tonsils and lungs of 3 of 15 pigs affected with porcine reproductive and respiratory syndrome virus. Physicochemically and morphologically, the isolates were similar to a coronavirus. The isolates were not distinguished from transmissible gastroenteritis virus (TGEV) by a neutralization test using polyclonal antibodies, but differentiated from TGEV by monoclonal antibodies capable of discriminating between TGEV and porcine respiratory coronavirus (PRCV), indicating that the isolates were PRCV. In a serological survey of 30 serum samples each collected from about 50 days old pigs in the 2 affected farms, 29 (97%) and 15 (50%) sera were positive for neutralizing antibody against the isolate with the titers ranging from 2 to 64, respectively. — KEY WORDS: porcine reproductive and respiratory syndrome, porcine respiratory coronavirus, PRCV.
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Porcine respiratory coronavirus (PRCV) considered as a variant of transmissible gastroenteritis virus (TGEV) with a large deletion in the part of 5' end of the gene encoding spike (S) protein [1, 15, 21, 22] was first isolated in Belgium in 1984 [14]. PRCV and TGEV are antigenically similar to and can not be distinguished from each other by conventional serological tests using polyclonal antibody [3, 19]. PRCV has spread widely in European countries [2, 12, 14, 20] and also been isolated in the United States [21, 23] and Canada [9]. However, there has been no report about PRCV infection in Japanese pigs so far. The purpose of this brief report is to describe the first isolation of PRCV from pigs with respiratory disorders in Japan.

In 1992, a serious respiratory disease characterized by severe abdominal breathing occurred in 1 to 2 month-old pigs in 3 farms, and 23 pigs were submitted to our laboratory for diagnosis [17]. They were diagnosed as being affected with porcine reproductive and respiratory syndrome (PRRS), because PRRS virus (PRRSV) was isolated from their lungs and sera, and pathological examinations on their lungs revealed proliferative and interstitial pneumonia which is the most prominent lesion of PRRS [17].

Then, we made attempts to isolate other viruses from the affected pigs using CPK cells derived from porcine kidneys [6, 10]. The cells grown in test tubes were inoculated with 10 per cent lung and tonsil suspensions obtained from 15 pigs of 2 farms and incubated in a roller drum at 37°C. After 7 days of incubation, culture fluids were harvested and inoculated into fresh CPK cell cultures for the second passage. Of CPK cells inoculated with the tonsil suspensions, three cultures showed cytopathic effect (CPE), while those inoculated with the lung suspensions showed no changes. At the second passage, a distinct CPE appeared

in cell monolayers inoculated with the lung suspension of one pig of which tonsil was positive for CPE agent. CPE produced was characterized by syncytium formation, and the cells were eventually detached from the glass surface. The isolates obtained from tonsils of 3 pigs were designated as CH22, CH32, and CH45, respectively. The isolates were cloned three times by a limiting dilution method in CPK cells and used for further characterization.

One of the isolates, CH32, was tested for stability to the treatments with lipid solvents (20% ethyl ethel and 10% chloroform), acid pH 3.0, and heating at 50°C for 30 min. The nucleic acid of the isolate was determined indirectly by examining the effect of 5-iodo-2'-deoxyuridine (IUdR) on viral replication. All tests were carried out according to the ordinary procedures using a porcine enterovirus, a nonenveloped RNA virus, and a pseudorabies virus, an enveloped DNA virus, as controls. The isolate was unstable to the treatment with lipid solvents, and somewhat labile to the acid and heating. The acid and heating treatments caused reduction of about 1 log $TCID_{50}$ in viral infectivity. IUdR inhibited significantly the replication of pseudorabies virus, but not those of the isolate and porcine enterovirus, implying that the isolate contains RNA genome (data not shown).

For morphological characterization of the isolate CH32, the culture fluid of infected CPK cells was concentrated by centrifugation at 75,000 × g for 2 hr through sucrose cushion with a density of 1.100 g/m*l*. The precipitate was stained with 0.2% phosphotungstic acid solution in phosphate buffer (pH 7.2) and examined for virus with an electron microscope. Figure 1 shows an electron micrograph of the isolate CH32. Characteristic coronavirus-like particles that were pleomorphic and had an envelope with club-shaped projections were found in the material concentrated from culture fluid of the infected cells, suggesting that the isolate CH32 belongs to the family Coronaviridae.

Since the results of physicochemical and morphological examinations suggested that the isolate CH32 was a coronavirus, the isolates CH22, CH32, and CH45 were

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compared serologically with TGEV by a neutralization test. TGEV used was the TO strain [8], and its antibody was prepared in a chicken by multiple injections with the concentrated virus. Based on the assumption that pigs that were housed on the farms where serious respiratory disease occurred had antibody against the isolate, serum collected from one of those pigs was used as a homologous antibody to the isolate. The isolates and TGEV were neutralized to the same extent by both sera, and not distinguished from one another (Table 1). Four coronaviruses, hemagglutinating encephalomyelitis virus, porcine epidemic diarrhea virus, TGEV, and PRCV have been known to infect pigs, and shown to be antigenically distinct from one another, except TGEV and PRCV which are closely related. The results of neutralization tests, therefore, appear to indicate that the isolates are TGEV or PRCV.

Although the common serological tests using polyclonal antibodies can not discriminate between TGEV and PRCV [3, 18], monoclonal antibodies (MAbs) capable of distinguishing both viruses have been reported. Simkins et al. have analyzed epitopes on the S proteins of both viruses with a panel of anti-TGEV MAbs, and mapped 5 epitope subsites designated as A, F, E, V, and D, respectively [18]. The subsites A, F, and E were conserved in both TGEV and PRCV, while the subsite V was specific to the viral strain. The subsite D was mapped on the S protein of only TGEV, but not on that of PRCV. Therefore, the isolates CH22, CH32, and CH45 were further characterized by an indirect immunofluorescent test with MAbs against TGEV and CPK cells infected with either the TGEV TO strain or the isolates. MAbs used were 25C9 and 44C11 which were kindly supplied by Dr. L. J. Saif, Ohio State University, U.S.A. They were generated to the Miller strain of TGEV and had specificity for the subsites A and D of S protein, respectively [18]. As shown in Fig. 2, CPK cells infected with the isolates reacted only with MAb 25C9 but not with MAb 44C11, while both MAbs bound to those inoculated with the TGEV TO strain. These results indicate that the isolates are PRCV.

Then, serum samples collected from 30 pigs each, approximately 50 days old, in 2 farms where the severe respiratory disease occurred were tested for neutralizing antibodies against the isolate CH32 and the TGEV TO strain. The results indicated that 29 (97%) and 15 (50%) sera were positive for neutralizing antibody against the both viruses, respectively. Antibody titers against the CH32 and the TO strain were 14.9 and 8.8 on a geometrical mean in one farm, and 10.1 and 5.8 in the other farm, respectively.

Although PRCV has spread in many European and American countries, PRCV infection among Japanese pigs was obscure. This study indicated the presence of PRCV infection in Japan. The results of serological survey suggest that PRCV infection occurred widely in 2 farms investigated in this study, although the possibility that neutralizing antibodies detected are maternal antibodies can not be excluded. Furthermore, we could not discriminate between PRCV and TGEV antibodies in this study. However, the evidence that the farms had no history of not only the TGE

Table 1. Serological characterization of the isolates by neutralization test with anti-TGEV antibody

Virus		Serum	
		Anti-TGEV	Anti-isolate[a]
Isolate	CH22	256[b]	64
	CH32	256	32
	CH45	512	64
TGEV TO strain		1024	16

TGEV: transmissible gastroenteritis virus.
a) The serum obtained from a pig of the affected herd was used as anti-isolate antibody.
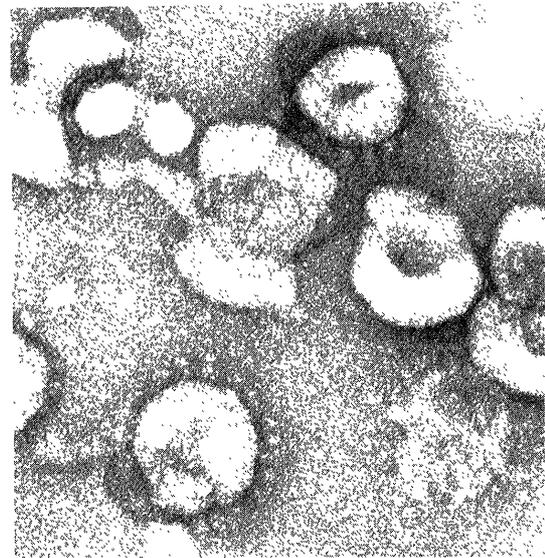b) Neutralizing antibody titers.

Fig. 1. Electron micrograph of the isolate CH32. Characteristic coronavirus particles were found in tissue culture fluids of infected cells. Magnification: × 175,000.

outbreaks but also vaccination against TGE suggests that antibodies detected were induced by PRCV infection. In addition, the results of serological survey in which the antibody titers against the isolate were somewhat higher than those against the TGEV TO strain may support the above conclusion. Since antigenic similarity of both viruses brings about difficulties in serological diagnosis and survey on TGEV and PRCV infections, a method to differentiate between TGEV and PRCV antibodies should be developed.

The other importance of PRCV is that the virus is suspected as one of causative agents of porcine respiratory diseases. PRCV has a tissue tropism to respiratory tissues and replicates in them to high titers but little in intestinal tissues [4, 5, 13]. On the other hand, TGEV can infect both tissues, but replicates to a greater extent in intestinal than respiratory tissues, and causes severe gastroenteritis [16]. An etiological role of PRCV in the course of the respiratory disease studied in this report was unknown. Initially, PRCV was considered as being nonpathogenic for pigs, because the virus had been isolated from clinically healthy pigs [14]
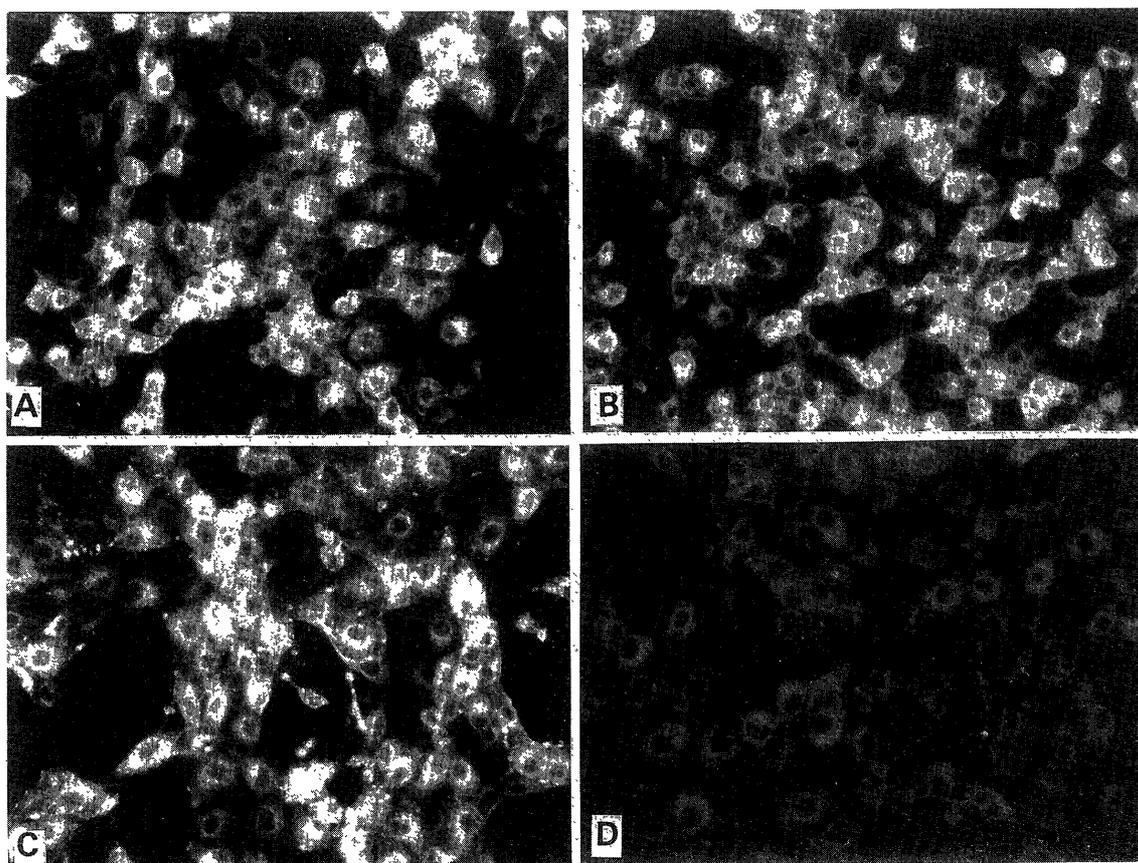
Fig. 2. Immunofluorescence using anti-transmissible gastroenteritis virus (TGEV) monoclonal antibodies (MAbs). CPK cells infected with either TGEV (A and B) or the isolate CH32 (C and D) were stained with either MAb 25C9 (A and C) or 44C11 (B and D). TGEV bound to both MAbs, while the isolate CH32 reacted only with MAb 25C9.

and failed to reproduce the clinical diseases in experimentally inoculated pigs [13]. According to the subsequent experiments, clinical and pathological responses of pigs infected with PRCV varied widely from clinical to subclinical diseases [4, 5, 7, 11, 20]. These appear to imply that the viruses with different pathogenicity are present in the fields, and that the severity of PRCV infection may be affected by some factors such as concomitant infections with other pathogens, multiple environmental factors, and age of infected pigs. The pigs investigated in this study were affected with PRRSV [17]. Halbur *et al.* have also reported the isolation of PRCV from pigs infected with PRRSV [7]. PRRSV infection in young pigs is frequently associated with secondary infection with other pathogens, especially of the respiratory tract [7, 17], and it is suspected that they exacerbate respiratory diseases in infected pigs. Therefore, the etiological role of PRCV in concomitant infection in respiratory tracts with plural pathogens, especially with PRRS virus, should be studied in future.

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