The Journal of Veterinary Medical Science

FULL PAPER

Virology

Studies on heterologous protection between Japanese type 1 and type 2 porcine reproductive and respiratory syndrome virus isolates

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ABSTRACT. The objective of the present study was to evaluate the cross-protective immunity between type 1 and type 2 porcine reproductive and respiratory syndrome virus (PRRSV) isolates in growing pigs. Japanese type 1 PRRSV, first isolated from a pig with respiratory disorders in a farm in 2009, exhibits unique genetic characteristics. The pathogenicity of a Japanese standard strain of type 2 PRRSV, EDRD1, in pigs immunized by the type 1 PRRSV isolate, Jpn EU 4-37 was determined by evaluating clinical signs, viremia, antibody response, and pathological lesions. Similarly, we evaluated the pathogenicity of Jpn EU 4-37 in pigs immunized by EDRD1 and compared the cross-protective immunity between these isolates. The EDRD1 challenge after Jpn EU 4-37 inoculation reduced viral clearance and shedding in pigs, compared to those treated with the EDRD1 single infection. On the other hand, the pathogenicity of Jpn EU 4-37 after EDRD1 infection did not differ significantly compared to non-immunized pigs treated with Jpn EU 4-37. Therefore, exposure to Jpn EU 4-37 could not induce enough immunity to reduce the viremia against subsequent infection by type 2 PRRSV. However, the immunity induced by Jpn EU 4-37 infection may play a role in reducing viremia caused by type 2 PRRSV. Moreover, the immunity induced by the EDRD1 and other genetically related viruses, which are broadly distributed in Japan, may not contribute to cross-protection against Jpn EU 4-37 as an emerging virus.

KEY WORDS: Immunity, pig, porcine reproductive and respiratory syndrome virus

J. Vet. Med. Sci.
82(7): 935–942, 2020
doi: 10.1292/jvms.20-0122

Received: 3 March 2020
Accepted: 12 May 2020
Advanced Epub: 22 May 2020

Porcine reproductive and respiratory syndrome (PRRS) is characterized by reproductive failure in sows and respiratory symptoms in piglets and growing pigs. Following its emergence in the late 1980s, PRRS has become endemic in most pig-producing countries [10, 29]. The etiologic agent of PRRS is the PRRS virus (PRRSV), an enveloped, single stranded, positive sense RNA virus belonging to the *Arteriviridae* family in the order *Nidovirales* [1]. Two distinct genotypes of PRRSV sharing approximately 60% identity at the nucleotide level have been described: type 1 (European genotype) and type 2 (North American genotype) [20]. Within these two genotypes, however, substantial genetic and antigenic diversity have been demonstrated [32]. Furthermore, the pathogenesis of isolated viruses ranges widely from subclinical to severe manifestations [32]. The broad genetic and antigenic variation in PRRSV and the presence of multiple viral genotypes circulating in swine farms make the control of PRRSV infection extremely difficult. Moreover, the biological and genetic differences between the two genotypes have raised the possibility that they also vary in their virulence and in their ability to cause respiratory disease [20, 21]. Although studies have demonstrated that type 2 PRRSV causes more severe respiratory disease than type 1 [3, 17, 27], these studies compared different genotypes of the virus from different countries.

The first type 2 PRRSV infections in Japan were described in 1994 [19, 26]. Since then, several studies have reported on the molecular characterization of Japanese isolates, all of which have exhibited higher nucleotide identity with type 2 viruses [9, 30, 31]. In 2009, the first type 1 PRRSVs in Japan were isolated from pigs with respiratory disorders in a farrow-to-finish pig farm.

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These isolates exhibited unique genetic characteristics [8]. While the farm had a high number of symptomatic weaning pigs, the pathogenicity of Japanese type 1 PRRSV was difficult to elucidate in the field. Therefore, we investigated the infection of a single isolate, Jpn EU 4-37, in pigs; however, the infected animals showed quite few clinical signs, with light to moderate interstitial pneumonia [7]. These data suggest that Jpn EU 4-37 may be characterized as a strain with low pathogenicity, causing short-term viremia and stimulated by the presence other infections. This combination of characteristics makes it difficult to study this strain in other epidemic farms because most of Japanese pig farms are endemic for type 2 PRRSV. Thus, it is necessary to experimentally demonstrate the complex infection of type 1 and type 2 PRRSV in pigs.

Cross-protection conferred by type 2 PRRSV against type 1 PRRSV is an important clinical issue in many Asian countries because of the emergence of type 1 PRRSV. Previous cross-protection studies indicate that the application of a type 2 PRRSV modified live vaccine to control pigs did not induce enough immunity to decrease their viremia against type 1 PRRSV isolated from European countries [4, 16, 28]. In contrast, Ko and Roca have reported that the type 1 PRRSV vaccine showed a certain degree of efficacy against hetero-genotype PRRSV in the early course of infection [14, 25]. Han and Kim have reported that the vaccination of pigs with type 1 is not efficacious in protecting growing pigs from respiratory disease caused by heterologous type 2 PRRSV challenge [12, 23]. Although many researchers have evaluated the cross-protection in the combination of homologous and heterologous viruses, the specific mechanism leading to the immune imbalance between type 1 and 2 PRRSV remains unclear. The extent of cross-protection in type 1 vaccinated pigs infected with virulent type 2 strains seems to be unrelated to the genomic similarity of the strains based on ORF5 sequencing [2, 24]. Unfortunately, the identification of specific genomic determinants of virulence has been elusive and is almost certainly multifaceted; the extent of cross-protection among two genotypes seems to be greatly affected by these combinations. Hence, the aim of this study was to evaluate the antigenic relationship between Japanese type 1 and type 2 PRRSV isolates *in vivo* by assessing their clinical features, viral load in sera and organs, and gross and microscopic lesions in a specific-pathogen-free (SPF) piglet model.

MATERIALS AND METHODS

Animals

Five-week-old crossbred SPF pigs were purchased from a closed SPF herd negative for pathogens causing PRRS, pseudorabies, porcine epidemic diarrhea, transmissible gastroenteritis, atrophic rhinitis, *Mycoplasma* pneumonia, swine dysentery, salmonellosis, toxoplasmosis, and actinobacillosis. The pigs were also negative for antibodies against PRRSV, which was determined before the experiment using a commercially available enzyme-linked immunosorbent assay (ELISA) (HerdChek PRRS ELISA, IDEXX Laboratories Inc., Westbrook, ME, USA). The pigs were kept in a closed animal facility where they were fed a commercial diet. This study was conducted in compliance with the animal experimentation code of the National Institute of Animal Health (NIAH).

Virus

The type 1 PRRSV (Jpn EU 4-37) was originally isolated using porcine alveolar macrophages (PAMs) [7]. The open reading frame (ORF) 5 region of this isolate shares 97.7% nucleotide identity with the equivalent region of the subtype 1 strain, SD-02-10 (GenBank accession number: AY395081). Jpn EU 4-37 was amplified by three passages through PAMs before use. The type 2 PRRSV, EDRD1 (GenBank accession number: AB288356) was isolated in 1994 and is the standard virulent strain in Japan [19]. EDRD1 was propagated in MARC145 cells and stored at −80°C. It was then amplified by one passage through PAMs before use. Culture conditions for both PRRSV strains were previously reported [7, 9].

Experimental design and postmortem examination

The animal experiments were carried out as a type 1 challenge study and a type 2 challenge study, which we designated as "Experiment 1" and "Experiment 2", respectively, as shown in Table 1. In Experiment 1, 14 pigs were randomly allocated into the following three groups: The type2/type1 (n=5) group was inoculated with 1 m*l* of a nasal spray containing $1 \times 10^{5.5}$ 50% tissue culture infectious dose ($TCID_{50}$) /m*l* of EDRD1 using a nasal spray device (TOP Corp., Tokyo, Japan). Five weeks later, the

Table 1. Study design with challenge status

Group		Immunity-conferring virus	Challenge virus
Experiment 1			
	Type2/type1	EDRD1[a)]	Jpn EU 4-37
	Type1		Jpn EU 4-37
	Control		
Experiment 2			
	Type1/type2	Jpn EU 4-37[b)]	EDRD1
	Type2		EDRD1
	Control		

a) EDRD1: Japanese standard strain of type 2 porcine reproductive and respiratory syndrome.
b) Jpn EU 4-37: 1st isolate of type 1 porcine reproductive and respiratory syndrome virus in Japan.

type2/type1 and type1 (n=5) groups were inoculated with 1 m*l* of a nasal spray containing $1 \times 10^{5.5}$ $TCID_{50}$ of Jpn EU 4-37. The remaining 4 pigs served as uninfected controls. In a similar fashion, in Experiment 2, 14 pigs were randomly allocated into three groups: the type1/type2 (n=5) group was inoculated with 1 m*l* of nasal spray containing $1 \times 10^{5.5}$ $TCID_{50}$ of the Jpn EU 4-37. Five weeks later, the type1/type2 and type2 (n=5) groups were inoculated with the EDRD1. The remaining 4 pigs served as uninfected controls. The pigs were monitored for rectal temperature and clinical signs. This study was conducted in compliance with the animal experimentation code of the National Institute of Animal Health (NIAH), National Agriculture and Food Research Organization.

Serology and quantities of PRRSV RNA

Serum was collected from pigs starting from −35 up to 21 dpi of the EDRD1 challenge to analyze antibodies against PRRSV and quantities of PRRSV RNA. Antibodies against PRRSV were determined using the HerdChek PRRS ELISA (IDEX Laboratories Inc.). At necropsy, the lungs, tonsils, tracheobronchial lymph nodes, liver, kidneys and spleen were collected to quantify the amount of PRRSV RNA in these tissues. Kits were used to extract for viral RNA in sera (QIAamp Viral RNA Mini kit; QIAGEN, Venlo, Netherlands) and in tissues (QIAGEN RNeasy Mini kit; QIAGEN). Each RNA extract was used as a template for one-step real-time reverse transcriptase polymerase chain reaction (qRT-PCR) using the TaKaRa One-Step SYBR PrimeScript RT-PCR Kit II (Takara Bio Inc., Kusatsu, Japan). The PCR primers for the detection of type 1 (forward: 5′-GCACCACCTCACCCAAAC-3′, reverse: 5′-CAGTTCCTGCGCCTTGAT) and type 2 (forward: 5′-TCCAGATGCCGTTTGTGCTT-3′, reverse: 5′-GACGCCGGACGACAAATG-3′) PRRSV were designed for TaqMan qRT-PCR [13], which was used to detect a portion of the ORF7 genes (77 and 124 nucleotides) without using a labeled probe. To quantify type 1 PRRSV RNA, real-time RT-PCR was conducted as previously described [8]. The standard curves for the quantitative RT-PCR assay used the equivalent of 1×10^5 $TCID_{50}$/m*l* of Jpn EU 4-37 or EDRD1 RNA extracted from the culture supernatant and serially diluted by 10-fold. To determine the copy number of specific genes in these dilutions, positive control DNA was generated from Jpn EU 4-37 nucleocapsid protein (partial, 86 bp) and EDRD1 nsp2 (partial, 317 bp) genes synthesized by GeneArt® Strings DNA Fragments (Life Technologies Inc., Carlsbad, CA, USA) as described previously [6]. Each serial dilution generated a linear standard curve for each quantitative RT-PCR run. During analysis, the Ct value was considered valid only if it was between the minimum and maximum values obtained using the standard RNA. Fluorescence data were analyzed using PE 7500 Sequence Detection System Software (Version 1.4; Life Technologies, Carlsbad, CA, USA). The standard curves for type 2 PRRSV RNA were constructed in a similar manner, starting from 1×10^3 $TCID_{50}$/m*l* of PRRSV, serially diluted 10-fold.

Pathological examinations

At necropsy, the organs of each pig were visually examined. Then, single sections for microscopic examination were collected from the following tissues: lung lobes, brain, heart, ileum, tonsils, tracheobronchial lymph nodes, superficial inguinal lymph nodes, mandibular lymph nodes, mesenteric lymph nodes, thymus, liver, kidneys, and spleen. Samples were suspended in 10% neutral buffered formalin and then dehydrated, embedded in paraffin wax, sectioned at 4 μm, and stained with hematoxylin and eosin.

Statistical analysis

Data for rectal temperature and viral RNA were analyzed using the unpaired t-test. The microscopic pneumonia scores were analyzed using the Mann–Whitney U-test with the "EZR" package in the R statistical environment (Foundation for Statistical Computing, Vienna, Austria, version 2.13.0). All P-values were two sided and P-values of 0.05 or less were considered statistically significant.

RESULTS

Rectal temperature

The mean rectal temperature ± standard error in all groups of pigs are shown Fig. 1. Among the Jpn EU 4-37 challenged groups, the mean rectal temperatures of type2/type1 and type1 groups did not increase until the end of the experiment. The exception is the type1 group, whose mean rectal temperature was higher than that of the control group at 10 dpi. Following EDRD1 challenge, the mean rectal temperature in type1/type2 and type2 groups increased, starting from 2 dpi, and gradually decreased until 16 dpi. The fever period, during which the mean rectal temperature was significantly higher than that of the control, lasted from 4 to 8 dpi in the type1/type2 group and lasted from 2 to 4 dpi and 8 to 13 dpi in the type2 group. Rectal temperatures of the control group ranged from 39.0°C to 39.5°C throughout the study. Overall, the mean rectal temperatures in the type2/type1 and type1 groups, and the type1/type2 and type2 groups did not differ significantly. None of the animals displayed any clinical signs.

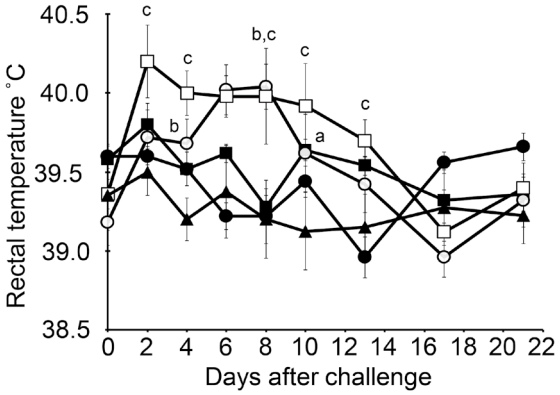

Fig. 1. Changes in the rectal temperature of pigs following challenge with virus. Mean rectal temperatures of pigs in the type1 (■), type2/type1 (●), type2 (□), type1/type2 (○), and non-infectious control (▲) groups. Statistically significant differences (P<0.05) are indicated between a) type2/type1 and control groups, b) type1/type2 and control groups, and c) type2 and control groups. Bars represent standard error of the mean.

Viral load in sera

Levels of greater than 10^3 copies/m*l* of viral RNA were observed in the serum of pigs in type2/type1 and type1 groups from 2 to 21 days after challenge as shown Fig. 2A. Serum viral load in the two inoculated groups sharply increased from 2 to 4 dpi, peaking at 6 dpi with 0.6×10^6 $TCID_{50}$/m*l* in the type2/type1 group and 1.6×10^6 copies/m*l* in the type1 group. Although the viral load in type1 group was significantly higher than those of type2/type1 group at 8 and 10 dpi, the viral loads did not differ significantly during the rest of the experimental period. Following EDRD1 challenge, the serum viral load in the two inoculated groups increased sharply from 2 to 6 dpi, peaking at 8 and 10 dpi with 6.4×10^4 copies/m*l* in the type1/type2 group and 2.1×10^5 copies/m*l* in the type2 group, respectively (Fig. 2B). While the serum viral load in the type2 group remained at approximately 1.9×10^3 copies/m*l* at 22 dpi, the viral load of the type1/type2 group rapidly declined to 76 copies/m*l* at 22 dpi. Notably, the viral loads of the type1/type2 and type2 groups differed significantly by an approximately 25-fold at 22 dpi.

Antibody to PRRSV

Antibodies against both types of PRRSV were observed in type2/type1 and type1/type2 groups from 7 days after immunization of the pigs, as shown in Fig. 3. From this point, the sample-to-positive (S/P) ratio rose gradually except for steeper increases observed at 10 and 4 dpi after challenges by Jpn EU 4-37 and EDRD1, respectively. In type1 and type2 groups, the positive detection of PRRSV (S/P>0.4) was first observed at 10 and 12 dpi, respectively; thereafter, the S/P ratio of viral antibody gradually rose. No antibodies against PRRSV were observed in control group.

Viral RNA in tissues

Type 1 PRRSV RNA was detected in all the tested organs of the type2/type1 and type1 groups (data not shown). Type 1 PRRSV RNA was present at levels of more than 2×10^6 copies/g in the lungs and tonsils collected from both groups. In all organs, no significant differences were noted in the levels of type 1 PRRSV RNA in the type2/type1 and type1 groups (Fig. 4). Type 2 PRRSV RNA was also detected from the all organs tested in the type1/type2 and type2 groups (data not shown). The quantity of type 2 PRRSV RNA in the tonsils in the type1/type2 group was significantly lower than in that in the type2 group, as shown in Fig. 5B. However, no other significant differences were noted between type1/type2 and type2 groups in their levels of type 2 PRRSV RNA in other organs.

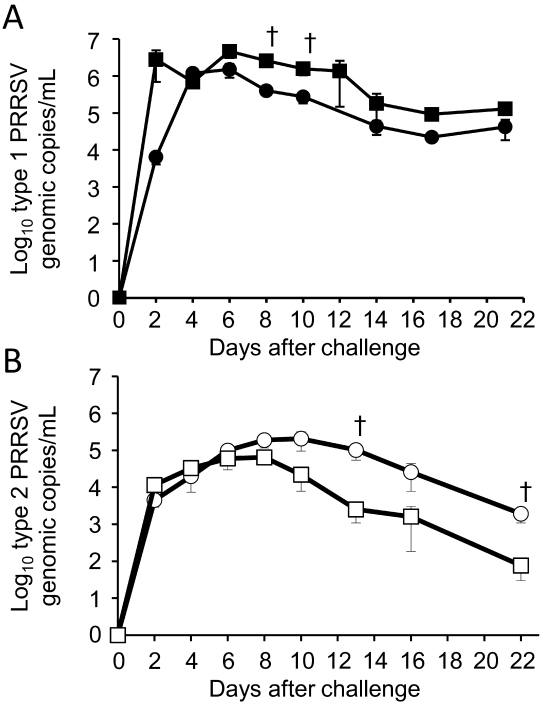

Fig. 2. Mean values of the quantity of type 1 (A) and type 2 (B) porcine reproductive and respiratory syndrome virus (PRRSV) RNA in the serum of pigs from type1 (■), type2/type1 (●), type2 (□), and type1/type2 (○) groups. Viral RNA (mean ± SE) content was measured by one-step RT-PCR. † indicates significant difference between groups (P<0.05). Error bars represent standard errors.

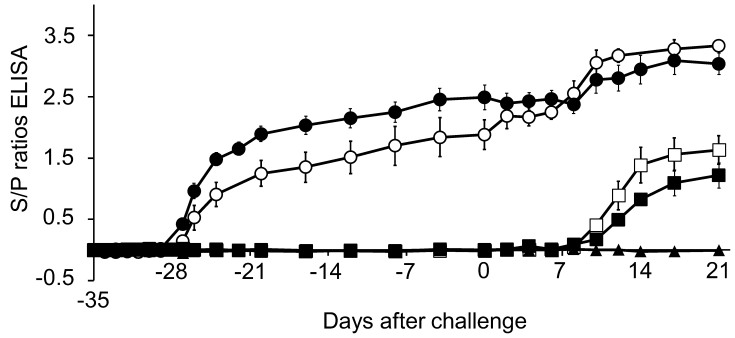

Fig. 3. Mean S/P ratio of ELISA in the serum from type1 (■), type2/type1 (●), type2 (□), type1/type2 (○), and non-infectious control (▲) groups. Error bars represent standard errors.

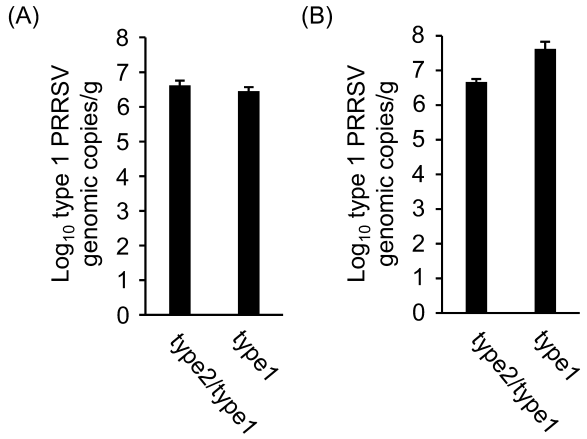

Fig. 4. Quantitative detection of type 1 porcine reproductive and respiratory syndrome virus (PRRSV) RNAs in lungs (A) and tonsils (B) collected from type2/type1 and type1 groups at 21 dpi of Jpn EU 4-37. Viral RNA (mean ± SE) content was measured by one-step RT-PCR. There were no significant differences between both organs of the 2 groups.

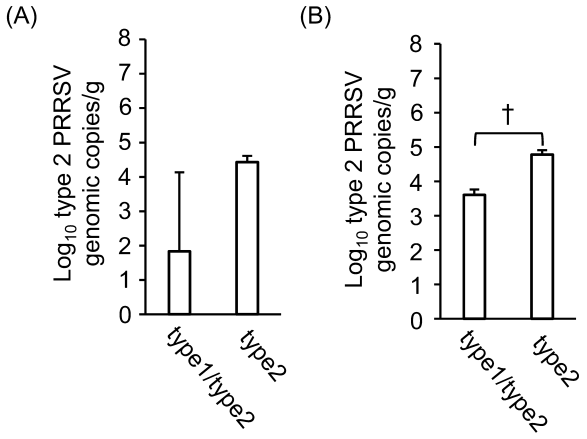

Fig. 5. Quantitative detection of type 2 porcine reproductive and respiratory syndrome virus (PRRSV) RNAs in lungs (A) and tonsils (B) collected from type1/type2 and type2 groups at 21 dpi of EDRD1. Viral RNA (mean ± SE) content was measured by one-step RT-PCR. (A) No significant difference in the levels of RNA of EDRD1 in the lungs of both groups was detected. However, viral RNAs of the type1/type2 group tended to be lower than that of the type2 group. (B) A significant difference was observed when comparing the tonsils of both groups (†, $P<0.05$).

Gross and histological lesions

At necropsy, the lungs of the pigs in all groups had not collapsed, although the lungs in type 1- and type 2-PRRSV-infected groups showed swollen consolidation with a variable amount of tan and red mottling. Lymphadenopathy was also observed in all the infected groups. The typical microscopic lesions found in the lungs of infected groups are summarized in Fig. 6. Pneumonic lesions are characterized by multifocal, mild to moderate interstitial pneumonia with septal thickening characterized by type II pneumocyte hypertrophy and hyperplasia. There were no obvious gross lesions although slight septal inflammatory reactions were observed in the type1/type2 group (6B). However, type2/type1 (Fig. 6A), type1 (Fig. 6C), type2 (Fig. 6D) groups exhibited mild interstitial alveolar thickening with perivascular lymphohistiocytic infiltration. Moreover, the lymph nodes of both type1 and 2 groups displayed germinal center hyperplasia and hypertrophy (data not shown). No gross or microscopic lesions were observed in controls.

DISCUSSION

The results of this study demonstrate that pigs in the acute phase are partially protected against heterologous type 2 PRRSV challenge by the Japanese type 1 PRRSV isolate; however, immunity conferred by the type 2 PRRSV infection did not decrease the persistence of the virus in the blood of the infected pigs. Park and his colleague have reported a similar phenomenon in their concurrent vaccination study using type 1 and 2 PRRSV vaccines [23]. They suggested that the difference of the induction level of IFN-ɤ-SC on viremic reduction between two genotypes may result in the different viremic levels [23]. The results of cross-protection study should be interpreted carefully because it might not be a consistent feature of the antigenic relationship between type 1 and type 2 PRRSV worldwide. Our study is important for understanding what happens when the farm which is contaminated by one of the genotypes is threatened by another genotype of PRRSV.

In this study, intranasal inoculation quickly resulted in viremia and dissemination of PRRSV to several tissues in both groups challenged with type 1 and 2 PRRSV. Viremia contributed to the dissemination of the virus throughout the respiratory and lymphoid tissues. Viral loads in blood and pathological lesions have been used to assess cross-protective immunity in PRRSV [25]. Here, we analyzed the actual quantity of viral nucleic acid measured by real-time PCR. Immunization with Jpn EU 4-37 reduced the level of viremia after challenge with EDRD1, but EDRD1 was not able to reduce the level of viremia after the Jpn EU 4-37 challenge despite EDRD1 showing higher pathogenicity compared with that by Jpn EU 4-37. PRRSV-caused viremia plays a central role in the development of respiratory disease [5], the level of viremia in sera is related to the viral load in tissues. In the present study, immunization of pigs with Jpn EU 4-37 was able to reduce the amount of EDRD1 RNA in the tonsils as shown Fig. 5B, and the severity of EDRD1-induced lung lesions as shown Fig. 6B. On the other hand, EDRD1-induced immunity was unable to reduce the severity of lung lesions caused by Jpn EU 4-37 and the amount of Jpn EU 4-37 RNA (Fig. 4).

ELISA results show that the type 1 isolate induced lower S/P values than type 2 PRRSV. It is assumed that virulent isolates, due to their high rate of *in vivo* growth, trigger a faster and more intense humoral response than attenuated isolates or vaccine strains

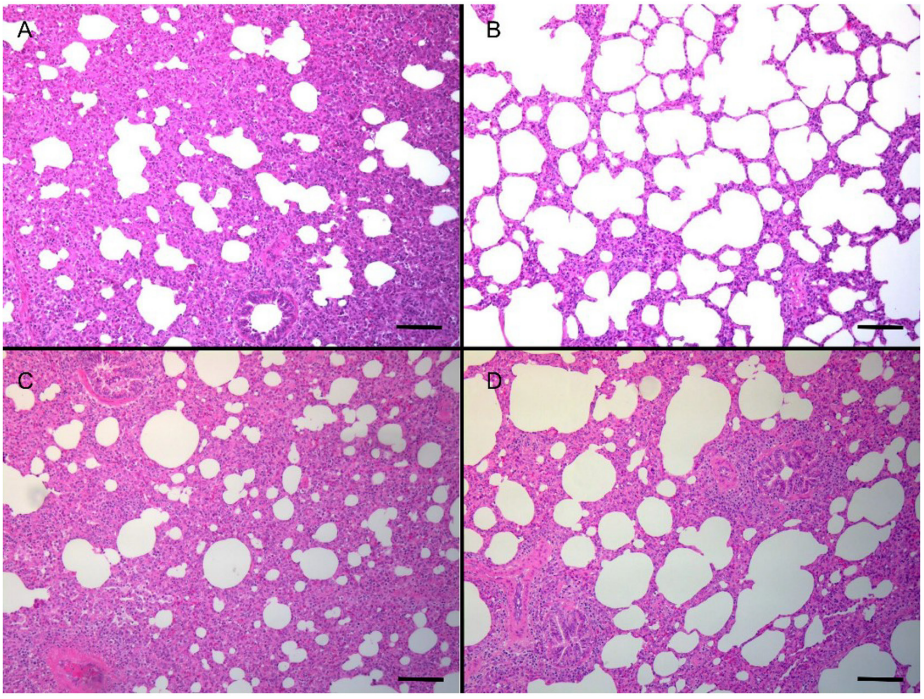

Fig. 6. Histopathological findings in the lungs collected from type2/type1 (A), type1/type2 (B), type1 (C), and type2 (D) groups. Pathologically, no obvious gross lesions were detected although slight septal inflammatory reactions were observed in the type1/type2 group (B). Discoloration in the cranial and middle lobes and mild to moderate interstitial pneumonia were observed in the type1/type2 (A), type1 (C), and type2 (D) groups.

[18]. We confirmed that EDRD1 induces a more intense humoral response than Jpn EU 4-37 in both the single infection model (type1 group) and the heterologous infection model (type1/type2 group). Interestingly, despite the strong antibody response induced by EDRD1 in pigs, the immunity raised by EDRD1 infection did not reduce the persistence of the virus in the infected animals. These results contrast with those of previous studies showing that type 2 PRRSV barely reduces the viral load in blood and clinical signs in pigs after heterologous type 1 PRRSV challenge [16, 28]. These contrasting results may be due to differences between the antigenic variation of the challenging strain and the immunization strain. Different PRRSV isolates already show a remarkable antigenic variation [15].

Although the mechanism of host immunity against PRRSV has not been fully determined, a degree of cross-protection was observed among different viral isolates [18, 22]. However, it has been difficult to predict the strength of protective immunity against PRRSV infections among heterogeneous viral strains, based on the analysis of contributing factors such as the production of neutralizing antibodies [11] and genetic characteristics [18]. We previously reported the high mortality rate for all stages of pig growth at a Japanese farm experiencing a type 1 PRRSV outbreak [8]. During the outbreak, however, the farm already had an epidemic of type 2 PRRSV; PRRSV-specific antibodies had been already detected in approximately 50% of the pigs before the invasion of the type 1 PRRSV. This discrepancy can be partly explained by the difference in the extent of antigenic relationship between types 1 and 2 PRRSV based on this study. Following the invasion and spread of type 1 PRRSV in the farm, pigs were quickly and broadly infected by the virus because immunity against type 2 PRRSV confers no cross-protection. Because of the high seroconversion rate (94.3%) in the epidemic season, no pigs with type 2 PRRSV viremia were detected [8]. This suggests that viral replication is inhibited due to the cross-protective immunity induced by type 1 PRRSV infection, leading to reduced type 2 PRRSV load in the circulating blood, thus reducing the amount of virus excreted from the body.

Partial cross-protection against type 2 PRRSV challenge by type 1 PRRSV immunity provides swine practitioners and producers with clinically meaningful information because type 2 PRRSV infection is prevalent in Japan [9]. However, these results should be interpreted carefully because this study was conducted with only one type 1 and type 2 isolate combination. Nevertheless, the information that the prevalent type 2 PRRSV did not reduce the persistence of type 1 PRRSV in the infected animals is likely to be more important for swine practitioners and producers. Future work is needed to determine the level of cross-protection that a type 2-based PRRSV vaccine can confer against various type 1 PRRSV field strains; this is important because no type 1 PRRSV-based vaccine is commercially available in Japan. One of the limitations of this study is the fact that we conducted only a single replicate of the experiments. Therefore, conclusions indicated from this study may not have enough statistical power. Further studies are necessary to evaluate if the results found in this study can be reproducible or not.

CONFLICT OF INTEREST. We declare no commercial or associated interests that would represent a conflict of interest in connection with the work submitted.

ACKNOWLEDGMENTS. We thank Ms. Nachiko Hattori (NIAH) for her great technical assistance. The authors thank the staff of the NIAH for animal handling and technical assistance. This research was supported by a Grant-in-Aid from the regulatory Research Project of the Ministry of Agriculture, Forestry, and Fisheries.

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