Restriction fragment length polymorphism analysis of open reading frame 5 gene of porcine reproductive and respiratory syndrome virus isolates in Korea

Brief Report

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Summary. The genetic variability of porcine reproductive and respiratory syndrome virus (PRRSV) was studied by restriction fragment length polymorphism (RFLP) of polymerase chain reaction (PCR)-amplified fragments among 50 Korean isolates from open reading frame 5. All Korean PRRSVs were isolated from the field cases after the marketing of an U.S. ATCC VR2332-derived modified live PRRSV vaccine. Combining the restriction enzyme digestion patterns obtained with MluI, HincII, SacII, and HaeIII, we observed 19 distinct RFLP patterns. Seventeen out of 50 PRRSV isolates (34%) exhibited the modified live PRRSV vaccine RFLP pattern. The genomic variations that have been identified in the present study seemed to represent characteristic features of the Korean PRRSV isolates. PCR-based RFLP analysis using several restriction enzymes provides a good genetic estimate for isolate differentiation.

*Porcine reproductive and respiratory syndrome virus (PRRSV) is a member of the genus Arterivirus, family Arteriviridae, order Nidovirales and has a positive single-stranded polyadenylated RNA molecule of approximately 15 kb in length that contains eight open reading frames (ORFs) [3, 18]. The first two open reading frames (ORF 1a and 1b) encode the viral RNA polymerase and associated proteases [8, 18]. ORFs 2, 3, 4 and 5 encode structural proteins, ORF 6 encodes a matrix (M) protein of 18 to 19-kD and ORF 7 encodes a nucleocapsid (N) protein of 14 to 15-kD [14, 18–20].

The porcine reproductive and respiratory syndrome (PRRS) is characterized by reproductive failure of sows and respiratory distress of piglets [10].
Mid-gestation abortions, mummified fetuses, early embryonic death, and infertility have also been reported [16]. The respiratory disease component of PRRS is characterized by pronounced hyperpnea, fever, and interstitial pneumonia in young pigs and mild flu-like signs in nursing, growing, and finishing pigs [10]. PRRSV apparently replicates primarily in interstitial and alveolar macrophages in the lung, and macrophage lineages in several other tissues such as tonsil, Peyer’s patch, liver and adrenal gland [4–6].

Genetic variability exist not only between North American and European isolates but also among North American isolates [11, 15, 20]. However, genetic variations have not been studied in Korean isolates. Restriction fragment length polymorphism (RFLP) analysis is useful in molecular biology for differentiating DNA or cDNA variation. Recently, a PCR-based RFLP analysis method has been developed for differentiation of PRRSV isolates [9, 21]. The objective of the present study was to determine genetic variations among Korean field isolates of PRRSV by PCR-based RFLP.

Materials and methods

**PRRSV isolates**

A total of 50 Korean PRRSV isolates were used for PCR-based RFLP analysis. Forty five field strains were collected from severe dyspneic postweaning pigs with interstitial pneumonia and 5 field strains were collected from aborted fetuses that were submitted to Department of Veterinary Pathology, College of Veterinary Medicine, Seoul National University between 1996 and 1998. Three PRRSV strains were isolated in 1996, 21 were isolated in 1997, and 26 were isolated in 1998. Thirty five were isolated in Kyounggi Province, 10 were isolated in Kyoungsang Province, 3 were isolated in Chungcheung Province, and 2 were isolated in Cholla Province. The original isolation of the Korean isolates was made on both MARC-145 cells and alveolar macrophages as previously described [7, 12]. All PRRSV field strains were isolated from the field cases associated with typical PRRS after the marketing of a modified live virus PRRS vaccine (RespPRRS/Repro vaccine, BI/NOBL Laboratories, Sioux Center, IA).

**Extraction of genomic RNA from cell culture**

PRRSV strains were propagated on MARC-145 cells. The MARC-145 cells were cultured in Eagle’s minimum essential medium, supplemented with 10% fetal bovine serum in 1 humidified 5% CO₂ atmosphere at 37 °C. PRRSV field strains were harvested when 70% to 80% of the cells were affected by cytopathic changes. RNA was extracted from infected cells with the Trizol LS reagent (Gibco BRL, Grand Island, NY) according to the manufacturer’s instructions.

**Reverse transcription**

Twenty-five picomoles of oligo(dT) was added to 1 μg of extracted RNA from either PRRSV-infected MARC-145 cells or lung specimens in 7 μl of diethylpyrocarbonate (DEPC; Sigma Chemical Co., St. Louis, MO)-treated water, denatured at 75 °C for 10 minutes, and then cooled on ice. Reverse transcription reaction was performed in a final volume of 20 μl containing 1 × RT buffer (50 mM Tris-HCl, [pH 8.3], 75 mM KCl, 3 mM MgCl₂), 0.5 mM (each) deoxynucleotide triphosphates (dNTP), 20 U of RNAse inhibitor per μl, and 50 U of murine
leukemia virus reverse transcriptase (Perkin-Elmer). After incubation for 1 h at 42 °C, the mixture was heated at 95 °C for 5 min and chilled on ice.

**Polymerase chain reaction**

The two 20-base oligonucleotides used as primers for the PCR assays were selected from published sequences of the open reading frames 5. The sense and antisense primers were 5′-CCATTCTGTTGGCAATTTGA-3′ and antisense 5′-GGCATATATCATCACTGCGG-3′, respectively [1]. DNA amplification was performed using 25 pmole oligonucleotide primers, 0.2 mM each of dATP, dGTP, dCTP, and dTTP, 10 mM Tris HCl (pH 8.8); 1.25 mM MgCl2; 50 mM KCl; 1.25 unit of Taq polymerase, all in 50 μl of distilled water. The PCR profile used included a denaturing step at 94 °C for 30 sec, followed by annealing of the primers at 53 °C for 30 sec, with an extension step at 72 °C for 30 sec. The thirty cycles of this 3-step procedures were performed in a thermal cycler, followed by a 10 min extension at 72 °C. Specificity of the PCR was evaluated by using non-PRRSV DNA samples from several causative agents of pneumonia. Porcine respiratory coronavirus, Pasteurella multocida and Actinobacillus pleuropneumoniae was used for negative control.

**Restriction fragment length polymorphism analysis**

After amplification, the PCR products were purified with Wizard PCR Preps (Promega Biotech, Madison, WI). The amplified DNA was then digested with the restriction enzyme MluI, HincII, SacII, and HaeIII (Promega) for 3 h at 37 °C in the appropriate buffered solution as previously described except HaeIII [21]. The digested DNA fragments were analyzed by electrophoresis on 2.0% agarose gels. The gels were examined by ultraviolet transillumination and photographed. The size of digested DNA fragments were estimated from migration distances of molecular weight standards.

**Results**

When DNAs prepared from the 50 PRRSV isolates were used as template, the PCR assay yielded a fragment of the expected 716 bp of the same molecular weights as those obtained with the DNA preparation from PRRSV strain VR2332 (Fig. 1). The 716 bp fragments produced by PCR assay were further digested with four restriction enzymes, MluI, HincII, SacII, HaeIII, and analyzed by agarose gel electrophoresis. When the PCR products from the 50 PRRSV isolates were subjected to RFLP analysis, it showed that 2, 6, 3, and 6 distinguishable digestion patterns were generated by MluI, HincII, SacII, HaeIII, respectively (Table 1). To facilitate the reporting and handling of test data, each isolate was given a numerical code for its ORF 5 RFLP pattern with enzymes, MluI, HincII, SacII, HaeIII, in that order. MluI either does not cut (code 1) or does cut (code 2) (Fig. 2A). HincII

![Fig. 1. Agarose gel electrophoresis of PCR-amplified ORF5 of PRRSV. From left to right; M 1-kb DNA ladder; 2 ATCC VR2332-derived modified live PRRSV vaccine strain; 3–6 PRRSV field isolates.](image-url)
Table 1. Restriction fragment length polymorphism code of Korean isolates of PRRSV

| No. of PRRSV isolate | RFLP code | Year isolated | Region (Province) |
|----------------------|-----------|---------------|------------------|
|                      | MluI      | HincII        | SacII            | HaeIII          |
| 1                    | 1         | 1             | 1                | 1               | 1996 | KG^a |
| 1                    | 1         | 1             | 2                | 1               | 1998 | KG   |
| 1                    | 1         | 2             | 2                | 4               | 1997 | KS   |
| 1                    | 1         | 2             | 4                | 4               | 1997 | KG   |
| 2                    | 1         | 2             | 3                | 1               | 1998 | KG, KS |
| 1                    | 1         | 2             | 3                | 2               | 1997 | KG   |
| 1                    | 1         | 3             | 3                | 1               | 1997 | KG   |
| 1                    | 1         | 4             | 1                | 6               | 1997 | KG   |
| 2                    | 1         | 4             | 2                | 4               | 1998 | KG   |
| 3                    | 1         | 4             | 2                | 4               | 1998 | KG   |
| 5                    | 1         | 6             | 3                | 1               | 1998 | KS   |
| 2                    | 2         | 1             | 3                | 1               | 1998 | KG   |
| 17                   | 2         | 3             | 2                | 1               | 1996–98 | KG, KS, CL, CC |
| 5                    | 2         | 3             | 2                | 3               | 1996–97 | KG, CL |
| 2                    | 2         | 3             | 2                | 5               | 1998 | KG   |
| 1                    | 2         | 3             | 3                | 1               | 1997 | KS   |
| 1                    | 2         | 3             | 3                | 3               | 1997 | KG   |
| 2                    | 2         | 5             | 2                | 1               | 1998 | CC   |
| Vaccine strain       | 2         | 3             | 2                | 1               | –     | –    |

^aKG Kyounggi; KS Kyoungsang; CC Chungcheung; CL Cholla

has 6 different cutting patterns, including a no cut pattern, which is designated code 1 (Fig. 2B). SacII has 3 cutting patterns, including a no cut pattern (code 1) (Fig. 2C). HaeIII has 6 different cutting patterns, including a not cut pattern, which is designated code 1 (Fig. 2D). In this manner, all PRRSV strains are given a code based on their ORF 5 cutting patterns. Combining the restriction enzyme digestion patterns obtained with MluI, HincII, SacII, HaeIII, we observed 19 distinct RFLP patterns from the 50 PRRSV isolates tested (Table 1).

RFLP code 2-3-2-1 was the most common cutting pattern for PRRSV isolates. Field viruses with RFLP code for 2-3-2-1 were isolated from postweaning pigs only throughout the 4 Provinces from 1996 to 1998. Among the 17 isolates known to carry RFLP code for 2-3-2-1, 1 was isolated in 1996, 7 were isolated in 1997, and 9 were isolated in 1998. Among the 5 isolates known to carry RFLP code for 2-3-2-3, 1 was isolated in 1996 and 4 were isolated in 1997. Among the 5 isolates from aborted fetus, 4 had RFLP code for 1-6-3-1 and 1 had RFLP code for 1-2-3-1. The RFLP code for 1-2-3-1 were also observed in PRRSV strain isolated from postweaning pigs.
Fig. 2. General types of RFLP patterns for PRRSV field isolates. PCR products (716 bp containing ORF5 were digested with restriction enzymes *Mlu*I (A), *Hinc*II (B), *Sac*II (C), and *Hae*III (D).

**Discussion**

In the present study, we are unable to distinguish field Korean isolates and the U.S. ATCC VR2332-derived modified live PRRSV strain, by comparing the RFLP patterns of a PCR-amplified genomic fragment of the ORF 5 gene. However, the genomic variations that have been identified in the present study seemed to represent characteristic features of the Korean PRRSV isolates. RFLP analysis can be used to differentiate PRRSV isolates and results can be used to aid in epidemiologic investigations of outbreaks of PRRS in swine farms. With this method, PRRSV isolates are assigned a 4-digit RFLP code; the first digit is the *Mlu*I RFLP pattern, the second is the *Hinc*II pattern, the third is the *Sac*II pattern, and the last digit is the *Hae*III pattern. Thus, 17 out of 50 PRRSV isolates (33%) were same RFLP code for the vaccine virus which is 2-3-2-1. These data suggest the possibility that wild type PRRS viruses have 2-3-2-1 pattern or that, instead, the PRRSV isolates were actually the vaccine strain. Thus, to distinguish a PRRSV vaccine strain from other PRRSV field isolates in Korea, it may be necessary a series of alternative restriction enzyme cuts to obtain other RFLP patterns. Alternatively, study such as nucleotide sequence would be required to differentiate field wild type PRRS viruses from PRRS vaccine virus.

It would be possible that the field isolates that prevailed in Korea before the introduction of modified live vaccine, had a very similar genetic structure to the vaccine and thus they coincidentally exhibit the same RFLP as the modified live PRRSV strain in use. Alternatively, one possibility that is tempting to be considered from the results reported herein is that the vaccine that started to be used on Korea since mid-1996 may have reverted to a more virulent form...
and that many isolates that are supposedly of field origin (i.e. those isolated from non-vaccinated herds which we used in this study) may have originated from the vaccine in the field, as has already been reported to occur in United States and Denmark [13, 17]. Notably, whereas the vaccine strain did not cause detectable pathologic changes other than some lymph node enlargement, field strains were associated with macroscopic and microscopic pulmonary lesions [17]. All field strains which were similar to vaccine strain in RFLP pattern in this study, were isolated from pigs with severe interstitial pneumonia. Analysis of the data reported here and elsewhere [2, 17] suggest that, under certain conditions, virus in an vaccine can persist, mutate, and eventually, as a result of mutation, cause a clinically relevant response in pigs.

Differences at restriction sites can provide a meaningful genetic marker for diagnostic and epidemiological purposes. RFLPs produced by one enzyme in the selected DNA fragment represent one nucleotide difference, therefore, if mutation occurs at that nucleotide, the vaccine virus can no longer be identified by the RFLP patterns of the enzyme. Seven PRRSV isolates have the same RFLP code for three digits, 2-3-2 but have different RFLP code for last digit from PRRSV vaccine virus. Therefore, the profile of PCR-RFLP patterns of several enzyme is meaningful for critical discrimination of the vaccine virus.

The diversity of RFLP pattern accounts for the sequence variation of the ORF5 from different field PRRSV isolates. Sequence analysis of ORF5 from North American and European isolates showed a high level of variation [11, 15]. Results of the present study demonstrate that ORF5 is also diverse in Korean isolates. The genetic diversity of ORF5 gene together with the observation that antigenicity of E protein encoded by ORF5 is diverse may have important information for development of a vaccine. The E protein encoded by ORF5 will not be a suitable candidate for the design of a subunit vaccine. Nowadays, as shown here, PCR-based RFLP methods are simple but powerful tools that allow the genetic diversity of PRRSV isolates. This may also contribute to a better understanding of the epidemiology of this pathogen.

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