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Determination of Porcine Rotavirus Serotypes by RT-PCR and RFLP Analysis

Hong-Gi Min, Yong-Hwan Lim and Shien-Young Kang*

Research Institute of Veterinary Medicine/College of Veterinary Medicine, Chungbuk National University, Cheongju 361-763, Korea

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G and P tying of group A porcine rotaviruses (PoRV) from field fecal samples were performed using reverse-transcriptase polymerization chain reaction (RT-PCR) and restriction fragment length polymorphism (RFLP) analysis. After amplifying full length VP7 and partial length VP4 genes, restriction endonucleases were used to digest and analyze the cutting pattern of the gene products. After analysis of digests with restriction endonucleases, seven and six RFLP types were observed for VP7 and VP4, respectively. The G typing analysis of 50 fecal samples revealed that 68% (34/50) were G4, which included G4-like (22/50); 22% (11/50) were G5; 6% (3/50) were G4 and G5 mixed types. The P typing analysis of the same fecal samples revealed that 36% (18/50) were P2B, 52% (26/50) were P9, 1 sample (2%) was a mixture of P2B and P9. Combinations of G and P types, the G4P2B and G4P9 types including G4-like accounted for 26% (13/50) and 32% (16/50), respectively. The G5P2B and G5P9 type also represented 4% (2/50) and 18% (9/50) of the samples. No G3 and G11 or other new P types were identified from the samples tested. Information on the G and P types and G/P combinations in the field fecal samples is useful for developing more effective PoRV vaccines and understanding the epidemiology of PoRV infections in the field.

Key Words: Porcine rotaviruses, G type/P type, RT-PCR, RFLP

INTRODUCTION

Group A rotaviruses are a major cause of viral gastroenteritis in young animals and humans, including young pigs through the world (4,11). The rotavirus genome consists of 11 segments of double-stranded RNA surrounded by triple-shelled capsids. Two outer capsid proteins, VP4 (encoded by gene segment 4) and VP7 (encoded by gene segment 7, 8 or 9 depending on the strain), independently induce neutralizing antibodies (5,9,17). The neutralization specificity related to VP7 is referred to as the G serotype (for glycoprotein), and that associated with VP4 is referred to as the P serotype (for protease-sensitive protein) (4). On the basis of G types (VP7), at least fifteen serotypes of group A rotaviruses have been described in humans and animals (4, 25). At present, six G types (G1, G3, G4, G5, G9 and G11) have been described among porcine rotaviruses with G4 and G5 predominating (1,3,12,15,21). The established Gottfried and OSU strain represent prototypes for G4 and G5, respectively. For the P types (VP4), twenty-two P types have been described in humans and animals (11,23,26) and two P types, P2B and P9, have been mainly reported from pigs (13,16,23). Determination of rotavirus G and P types is important for the development and evaluation of more efficacious rotavirus vaccines. Serotyping has been classically performed by plaque reduction virus neutralization tests using cell culture-adapted strains or directly from fecal

*Corresponding author: Shien-Young Kang. Research Institute of Veterinary Medicine/College of Veterinary Medicine, Chungbuk National University, Cheongju 361-763, Korea.
Phone: 82-43-261-2598, Fax: 82-43-267-3150,
e-mail: sykang@chungbuk.ac.kr

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samples by enzyme-linked immunosorbent assay (ELISA) using serotype-specific monoclonal antibodies (11,14). Recently, new methods, such as nucleic acid hybridization and sequence analyses (5,7,8,10,18~20) have been developed for rotavirus G and P typing. In this study, G and P typing were performed by RT-PCR and subsequent restriction fragment length polymorphism (RFLP) of amplified VP7 (full length) and VP4 gene (partial length including VP8 region; nucleotides 1 to 1096). In addition, the prevalence of G and P types was investigated.

MATERIALS AND METHODS

1. Viruses and cells

Three group A procine rotavirus (PoRV) strains, OSU (G5P2B), Gottfried (G4P9) and YM (G11P9), were used as reference strains for RFLP analysis. The viruses were grown in monkey kidney (MA 104) cells in the presence of pancreatin (50 μg/ml) as described previously (22).

2. Field fecal samples of PRV

Fifty fecal samples positive for group A PoRV by electron microscopy were collected from diarrheic piglets. Two fecal samples with reference group A PoRVs (Gottfried and OSU strains) were experimentally infected to gnotobiotic piglets. Before RT-PCR amplification of the VP7 and VP4 genes, all fecal samples were tested again for the presence of PoRV dsRNA by polyacrylamide gel electrophoresis (PAGE). Rotavirus dsRNA was extracted from virus infected cell culture fluids and fecal samples using modifications of previously described procedures (19). Extracted rotavirus dsRNA was resolved in 10% polyacrylamide slab gels using the discontinuous buffer system of Laemmli (12) and electrophoresis was conducted at 12 mA for 14 to 16 h. After electrophoresis, dsRNA bands were visualized by ethidium bromide staining.

3. RT-PCR of VP7 and VP4 genes

RT-PCR was used to produce full length PoRV VP7 and partial length VP4 gene as described previously (19,20). In brief, cDNA was synthesized and amplified in an RT-PCR mixture containing 10 X buffer, dNTP (10 mM, each), 0.2 μg of forward primer (5'-GGCCGGATTTAAAAGCGACAATTT-3' for VP7 and 5'-GGCTATAAAATGGCTTCGCT-3' for VP4), 0.2 μg of reverse primer (5'-GGTCACATCATACAACTCTA-3' for VP7 and 5'-AATGCCTGTGAATCGTCCCA-3' for VP4), 5 U RNasin, 10 U of avian myeloblastosis virus reverse transcriptase and 2.5 U of Taq polymerase. First strand cDNA synthesis was accomplished by incubating the above mixture for 90 min at 42℃. Thirty amplification cycles were conducted, with each cycle consisting of 94℃ for 1 min (denaturation), 48℃ for 1 min (annealing) and 72℃ for 2 min (extension), followed by 7 min extension at 72℃. The full-length PCR products were analyzed on 1% agarose gels using standard procedures.

4. Restriction fragment length polymorphism (RFLP) of amplified VP7 and VP4 genes

Five enzymes, *Eco*RI, *Hind*III, *Vsp*I, *Bam*HI and *Nla*IV for VP7 and three enzymes, *Eco*RI, *Eco*RV and *Hind*III, for VP4 were used for digesting the amplified gene products. These enzymes were chosen because they produced distinct digestion patterns on the basis of published sequence data and empirical investigation. For each digestion, 10 μl of amplified DNA and 10 X buffer and enzyme were mixed and incubated at 37℃ for 2 h and analyzed on 1% agarose gels.

5. Nucleotide sequencing of the VP7 and VP4 genes

To verify the G and P types of selected PoRV field strains, amplified VP7 and VP4 cDNA fragments representing each PoRV RFLP type or showing unusual RFLP patterns were cloned in the pCR II TA cloning vector (Invitrogen) and sequenced using BigDye Terminatior Cycle Sequencing Kit. The sequence data were analyzed using the LAZERGENE program (DNASTAR).

RESULTS

1. RT-PCR

All VP7 and VP4 genes were successfully amplified from the cell culture adapted reference PoRV and 50 field PoRV positive fecal samples by RT-PCR. The amplified genes were 1,062 bp for VP7 and 1,096 bp for VP4 in size, respectively.

2. Restriction fragment length polymorphism (RFLP) of reference PoRV strains

RFLP patterns of VP7 genes of Gottfried (G4), OSU

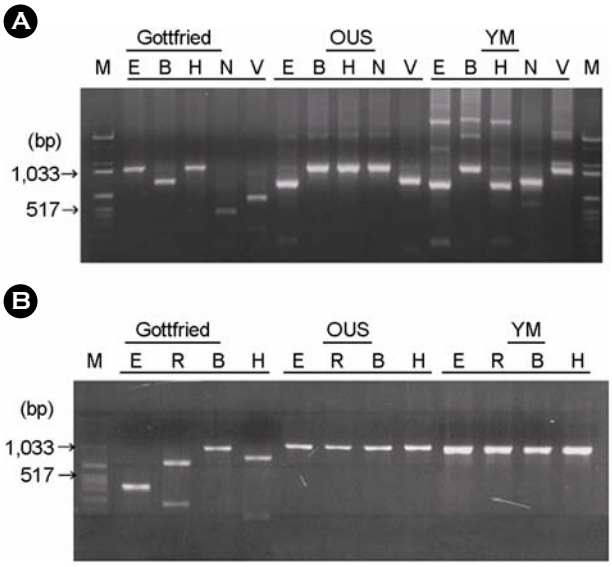

Figure 1. Restriction endonuclease cleavage patterns of reference PoRV VP7 **(A)** and VP4 **(B)** genes.
M: Marker, E: *Eco*RI, B: *Bam*HI, H: *Hind*III, N: *Nla*IV, V: *Vsp*I, R: *Eco*RV

(G5) and YM (G11) rotavirus with five different restriction endonucleases are shown in Fig. 1-A. For VP4, digestion pattern of reference PoRV strains, Gottfried (P2B), OSU (P9) and YM (P9) is shown in Fig. 1-B.

3. RFLP patterns of VP7 and VP4 gene from field PoRV

After digestion of the RT-PCR product of the VP7 genes with *Eco*RI, *Bam*HI, *Hind*III, *Nla*IV and *Vsp*I, PoRV isolates could be classified into four distinct RFLP (I-IV) types (Table 1 and Fig. 2). RFLP type I and IIa represented the typical RFLP pattern of G4 (Gottfried) and G5 (OSU), respectively. RFLP type IIb had same restriction enzyme digestion patterns with those of type IIa except for *Nla*IV digestion. VP7 gene products were cleaved with *Nla*IV in IIb but not in IIa type. RFLP type III isolates had similar restriction enzyme profiles except with *Vsp*I and *Nla*IV. In RFLP type IIIb, the VP7 gene products were digested with only VspI, whereas those were digested with *Nla*IV in RFLP type IIIc. No enzyme digested were found in the VP7 gene products of RFLP type IIIa. The digestion patterns for RFLP IV type differed from those of RFLP type I and IIa. The G types of PoRV isolates belonging to RFLP types III and IV could not be determined using RFLP analysis alone. Therefore, the isolates showing the RFLP types III and IV were

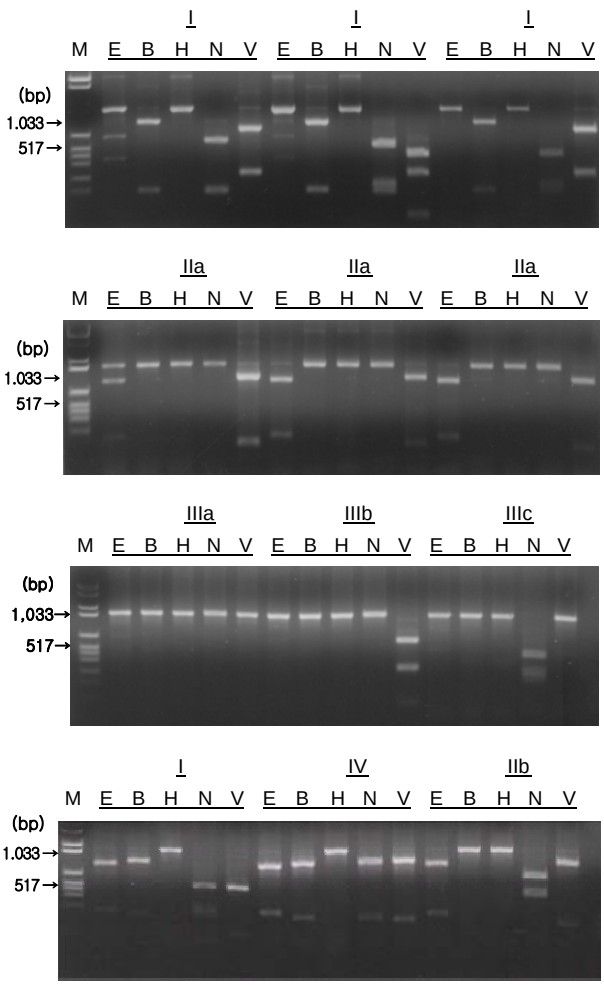

Figure 2. Restriction endonuclease cleavage patterns of PoRV VP7 genes from fecal samples.
M: Marker, E: *Eco*RI, B: *Bam*HI, H: *Hind*III, N: *Nla*IV, V: *Vsp*I I, IIa, IIb, IIIa, IIIb, IIIc and IV represent VP7 RFLP type.

further characterized by nucleotide sequencing of their VP7 genes.

After digestion of VP4 gene products with *Eco*RI, *Eco*RV, *Hind*III, the PoRV field strains could be classified into six different RFLP types (1~6) as shown in Table 2 and Fig. 3. The RFLP types 1 and 2 represented the typical RFLP patterns of P9 (Gottfried) and P2B (OSU), respectively. The RFLP types 3 and 5 differed from type 1 in their *Hind*III and *Eco*RI cleavage patterns, respectively: type 3 did not have *Hind*III cleavage sites and type 5 could not demonstrated *Eco*RI cleavage sites. The RFLP types 4 and 6 differed from type 2 in *Eco*RV and *Hind*III cleavage patterns, respectively. The RFLP types 4 and 6 differed from type 2 in *Eco*RV and *Hind*III cleavage patterns, respectively. The

Table 1. Restriction fragment length polymorphism analysis of the RT-PCR product of the VP7 gene from PoRV field samples

RFLP type	Restriction enzymes					G types
	*Eco*RI	*Bam*HI	*Hind*III	*Nla*IV	*Vsp*I	
I	▪[1]	830/230	▪	500/250/230/80	630/330/100	G4
IIa	800/260[2]	▪	▪	▪	850/210	G5
IIb	800/260	▪	▪	660/400	850/210	G5
IIIa	▪	▪	▪	▪	▪	G4
IIIb	▪	▪	▪	▪	900/160	G4-like
IIIc	▪	▪	▪	860/200	▪	G4-like
IV	760/300	860/200	▪	860/200	850/210	G4-like

[1] Uncleaved (1,062 bp), [2] Size of cleaved RT-PCR product

Table 2. Restriction fragment length polymorphism analysis of the RT-PCR product of the VP4 gene from PoRV field samples

RFLP type	Restriction enzymes				P types
	*Eco*RI	*Eco*RV	*Bam*HI	*Hind*III	
1	600/400	800/200	▪	900/100	P2B
2	▪[1]	▪	▪	▪	P9
3	600/400[2]	800/200	▪	▪	P2B
4	▪	800/200	▪	▪	P9
5	▪	800/200	▪	900/100	P2B
6	▪	▪	▪	900/100	P9

[1] Uncleaved (1,062 bp)
[2] Size of cleaved RT-PCR product

strains of RFLP type 4 had *Eco*RV cleavage sites and the strains of RFLP type 6 had HindIII cleavage sites. One strain belonging to RFLP types 3, 4, 5 and 6 was selected and VP4 partial fragment cloned, sequenced to confirm the P type.

4. Nucleotide sequence analysis

Comparison of the VP7 nucleotide sequence of selected PoRV strains with those of reference PoRV strains are shown in Table 3. The nucleotide sequences of PoRV isolate belonging to RFLP IV type were 94.2% homology with those of Gottfried strain (G4), but 71.1% and 73.5% with those of YM (G9) and OSU (G5) respectively. However, homologies of the nucleotide sequences of PoRV isolates belonging to RFLP IIIb and IIIc type were 84.6% and 83.6%, respectively, with those of Gottfried but were between 71.6% and 72.5% with those of YM and OSU strain.

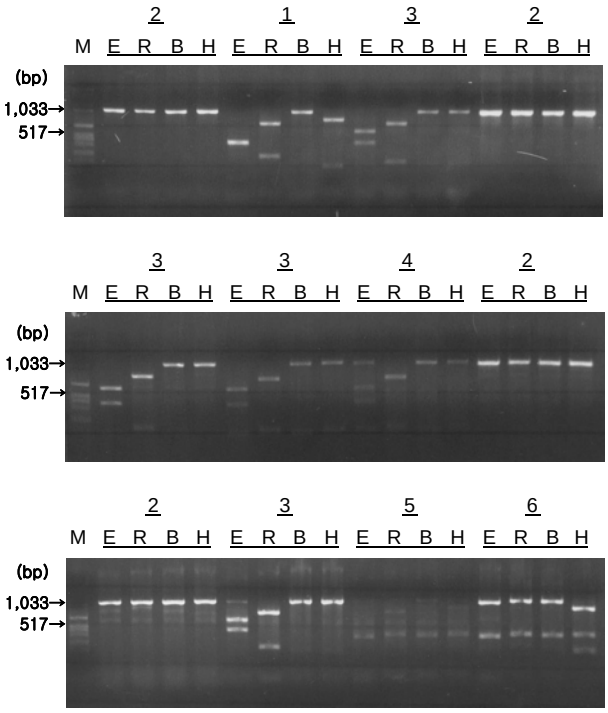

Figure 3. Restriction endonuclease cleavage patterns of PoRV VP4 genes from fecal samples.
M: Marker, E: *Eco*RI, R: *Eco*RV, *Bam*HI, H: *Hind*III 1, 2, 3, 4, 5, and 6 represent VP4 RFLP type.

5. Prevalence of G, P types and G/P combinations of PoRV strains

The results of the G and P type analysis of 50 PoRV field samples using RT-PCR/RFLP and sequence analysis are presented in Table 4. The G type from 50 fecal samples revealed that 24% (12/50) were G4; 22% (11/50) were G5; 44% (22/50) were G4-like; 6% (3/50) were G4 and G5

Table 3. Comparison of the VP7 nucleotide sequences of the three PoRV field isolates with those of reference PoRV

Isolate	Similarity (%)					
	A	B	C	Gottfried	OSU	YM
A (IIIb)	▪	82.6	84.3	84.6	71.7	71.6
B (IIIc)		▪	82.0	83.6	72.5	71.9
C (IV)			▪	94.2	73.5	71.1
Gottfried				▪	76.0	74.9
OSU					▪	72.9
YM						▪

▪ 100% homology

Table 4. G and P type combinations of PoRV field samples analyzed by RT-PCR and RFLP

G/P types	No. of samples (%)				
	P2B	P9	P2B+P9	Untypable	Total (%)
G4	4	7	0	1	12 (24)
G4-like	9	9	0	4	22 (44)
G5	2	9	0	0	11 (22)
G4+G5	2	0	1	0	3 (6)
Untypable	1	1	0	0	2 (4)
Total (%)	18 (36)	26 (52)	1 (2)	5 (10)	50 (100)

mixtures. The P type from 50 fecal samples revealed that 36% (18/50) were P2B, 52% (26/50) were P9, and 2% (1/50) were mixtures of P2B and P9. All possible combinations of G and P types existed in the field fecal samples (Table 4).

The G4P9 including G4-like was predomonant (32%) followed by G4P2B types including G4-like (26%), G5P9 (18%) and G5P2B (4%). The G and P type mixed infections, G4+G5/P2B and G4+G5/P2B+P9, were also observed.

DISCUSSION

It is very important to determine the prevalence and distribution of rotavirus serotypes in order to develop effective vaccine strategies. Several methods such as plaque reduction neutralization test, enzyme-linked immunosorbent assay (ELISA) employing serotype-specific monoclonal antibodies, nucleic acid hybridization and RT-PCR, have been developed for determination of rotavirus serotypes (7,8,10,14,18~20). The unreliability of serological methods for the G and P typing are well recognized problems. This may be originated from an accumulation of points mutations which leads to amino acid changes in antigenic determinants recognized by serotype-specific monoclonal antibodies (MAbs). Also, limited availability of serotype-specific MAbs is another big obstacles for G and P typing. This has resulted in the gradual replacement of serotyping by molecular typing methods. In this study, G and P typing were performed by RT-PCR and restriction fragment length polymorphism (RFLP) of amplified VP7 and VP4 genes and the prevalence of G and P types was investigated.

RT-PCR/RFLP has been described for the subgroup and serotype characterization of rotaviruses and differentiation of wild and vaccine strains in other viruses (2,6,24). Using five restriction enzymes (*Eco*RI, *Bam*HI, *Hind*III, *Nla*IV and *Vsp*I), VP7 gene of PRV isolates could be classified into four RFLP (I-IV) types. In brief, RFLP type I and IIa represented the typical RFLP pattern of Gottfried (G4) and OSU (G5), respectively. Therefore, there were no problems to determine the G type belonging to these RFLP types. However, several PoRV isolates showed different RFLP patterns (IIb, IIIa, IIIb, IIIc and IV) from those of reference strains. In case of RFLP type IIb, one of the fecal samples was derived from gnotobiotic pig experimently infected with OSU strain. But, RFLP type IIb showed different cleavage patterns with those of OSU strain (RFLP type IIa): there were cleavage sites for *Eco*RI, *Nla*IV and *Vsp*I in RFLP type IIb compared to cleavage sites for *Eco*RI and *Vsp*I in RFLP type IIa. In case of RFLP type IIIa, one of the fecal samples was derived from gnotobiotic pig experimently infected with Gottfried strain. But, there were no cleavage sites for *Eco*RI, *Bam*HI, *Hind*III, *Nla*IV and *Vsp*I compared to cleavage sites for *Bam*HI, *Nla*IV and *Vsp*I in RFLP type I. It could be assumed that there were some point mutations in VP7 gene during replication in pigs and existing restriction enzyme sites disappeared or new sites generated. In RFLP type IIIb and IIIc, the VP7 gene product was digested with only *Vsp*I and *Nla*IV, respectively. The G types of PoRV isolates belonging to RFLP types III and IV could not be determined using RFLP analysis alone. Therefore, the isolates showing the RFLP types IIIb, IIIc and IV were further characterized by nucleotide sequencing of their VP7 genes. The homologies of PoRV isolate (A and B) belonging to type IIIb and IIIc with those of Gottfried strain were

84.6% and 83.6% in nucleotide sequences and 91.3% and 89.7% in amino acid sequences, respectively. In case of type IV (C), the nucleotide sequence homologies with Gottfried strain were 94.2%. For these reasons, PoRV isolates of RFLP type IIIb, IIIc and IV were tentatively classified as a G4-like type. Even though there were several cleavage patterns for G and P types by RT-PCR/RFLP, this methods have some advantage over traditional tests. First, it may overcome the problem of antigenic variants within serotypes which fail to react with MAbs used in serotyping assays. Second, it can be used for serotyping of rotavirus isolates in specimens in which degradation of the outer virion capsid has occurred. Finally, it can provide more accurate information on the diversity of the VP4 and VP7 genes of the rotavirus strains circulating in the pig populations.

There is limited data on the prevalence of P types in PoRV field samples. One reason for the difficulty in P typing is that the VP4 protein is much less abundant in the outer capsid than VP7, which means that assays based on VP4 antigen are harder to apply for routine P typing (11). Therefore, assays based on genomic P typing seem to be more consistent and easier to apply at this time.

In the present study, the combination of G and P types were determined. Although the G4P2B (Gottfried strain) and G5P9 (OSU strain) types were dominant in the field samples, but all possible combinations existed in the PoRV field samples. This could be caused by naturally occurring mixed infections and reassortment in the host. We demonstrated in the present study that RT-PCR/RFLP procedure is a reliable method for monitoring of the antigenic diversity of rotaviruses in swine. Prevalence of G and P types and G/P combinations in the field samples should be useful for understanding the epidemiology of PoRV and designing vaccination strategies to control PoRV infection.

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