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Research Article

Genetic Characterization of Open Reading Frame5 (ORF5) of Porcine Reproductive and Respiratory Syndrome Virus in Indonesia Between 2008 and 2014

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Abstract

To investigate the genetic diversity of the Indonesian Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), the complete Open Reading Frame 5 (ORF5) from 11 clinical samples collected between 2008-2014 was sequenced and phylogenetically analyzed. These sequences were compared with the ORF5 of NA referens viruses (VR2332, MLV and JXA1), lelystad virus and 34 ORF5 sequences North America group from Gen Bank. Sequence analysis revealed that the ORF5 have undergone genetic variation. The ORF5 nucleotide and amino acid identities among 11 Indonesia PRRS viruses ranged from 88.5-100 and 87.5-100%, respectively and all belonged to the North American genotype. The ORF5 sequence of nucleotide and amino acids from the North American prototype virus (VR2332) and derived vaccine virus (MLV) were 97.6-100 and 97.0-100% identical to the various ORF5 Indonesia viruses, respectively. In the phylogenetic analysis, of the Indonesian viruses were clusted into two groups. The ORF5 based sequencing analysis can be used to determine the type of PRRS virus and the presence of genetic diversity of PRRS viruses in Indonesia.

Key words: Open reading frame 5, phylogenetic, porcine reproductive, respiratory syndrome, PRRSV Indonesia

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Porcine Reproductive and Respiratory Syndrome (PRRS) has been causing economic losses in the swine industry worldwide (Neumann *et al*., 2005). Recently, out break of porcine high fever diseases in China caused by a Highly Pathogenic PRRSV (HP-PRRSV). The etiology agent of the disease-PRRS virus (PRRSV), which is an envelope, positive-sense, single-stranded RNA virus that belongs to the family Arteriviridae and the order Nidovirales (Cavanagh, 1997). The PRRSV genome is ~15 kbp in length and contains nine Open Reading Frames (ORFs)-ORF 1a, 1b, 2a, 2b, 3, 4, 5, 6 and 7 (Conzelmann *et al*., 1993). The PRRSV is characterized by extensive genetic and antigenic variation laeds to diverse strains in the field (Nelsen *et al*., 1999; Meng, 2000).

The ORF5 encoding the most variable structural protein GP5 (Mardassi *et al*., 1995) and is associated with the neutralizing epitope will be helpful for to understand the genetic relationships among different isolates. The studies on the genetic analysis of the ORF5 have been reported in many countries (Thanawongnuwech *et al*., 2004; Cha *et al*., 2006; Mateu *et al*., 2006). The ORF5 has become the region of choice for monitoring the evolution of PRRSV and of molecular epidemiology research on PRRSV. The virus was first confirmed in Indonesia in 2008 and since then, the North American type PRRSV has spread widely in Indonesia and considerable ORF7 genetic diversity has been identified (Faisal *et al*., 2015). In the present study, to better understand the PRRSV genetic diversity and molecular epidemiology of PRRSV in Indonesia, the ORF5 of PRRSV viruses from 11 clinical samples in Indonesia during the period of 2008-2014 were sequenced and analyzed.

MATERIALS AND METHODS

Sample collection: A total of 21 samples were used in the present study, out of which 18 were originated from previous studies of Faisal *et al.* (2015) and 3 new samples were collected from East Kalimantan (Table 1). The samples were collected from different districts of Indonesia between 2008-2014.

Primer design: Oligonucleotide primers, which amplify the ORF5 of Indonesia PRRSV, were designed based on sequence information available on Gen Bank for strain VR2332 (Accession No. AY150564.1) using primer3 software (http://primer3.ut.ee/). In order to obtain complete ORF5, the primers ORF5F2B5'-CCTGAGACCATGAGGTGGG-3 and ORF5R2 5'-GGCCGCGACTTACCTTTAG-3, were selected in the flanking regions in the ORF4 and ORF6 which generates 778 bp fragment including the whole ORF5 sequence.

Synthesis of cDNA: The total RNA was isolated from samples using PureLink™ micro to Midi total RNA purification system (invitrogen). A two-step PCR method was employed for the amplification of DNA samples. The cDNA was synthesized using superscript III first strand synthesis (invitrogen) and the RT reaction contained the following ingredients: About 5 μL RNA sample, 1 μL (50 μM) oligo (dT) 20 primer, 1 μL (10 mM) dNTP and 4 μL water. The RNA was denatured at 65°C for 5 min and then cooled on ice. Master mix consisting of 10 μL cDNA synthesis mix was added at 10 μL sample and then incubated in a thermal cycler at 50°C for 50 min, 85°C for 5 min and then stored at -20°C.

The PCRs contained the following ingredients, 12.5 μL of 2×KAPA2G Fast Ready Mix, 1.25 μL (10 μM) of forward primer, 1.25 μL (10 μM) of reverse primer, 5 μL of PCR product and 5 μL of RNAse free water. The thermal profile is as follow, pre-denaturation at 95°C for 2 min and 30 cycles of denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec extension at 72°C for 30 sec and final extension at 72°C for 5 min.

DNA sequencing: The PCR products were sequenced using the Big Dyes TN Kits (Applied Biosystems, USA) following the

Table 1: Sampling information for 11 PRRS viruses Indonesia

Viruses	District	Province	Year	Group
08.267	Dairi	North Sumatera	2008	7
08.313[a]	Tapanuli Utara	North Sumatera	2008	7
08.313[b]	Tapanuli Utara	North Sumatera	2008	7
08.314[a]	Dairi	North Sumatera	2008	7
08.367	Tapanuli Utara	North Sumatera	2008	7
10.420	Deli Serdang	North Sumatera	2010	7
11.407.1	Samarinda	East Kalimantan	2011	1
12.217.1	Kutai Barat	East Kalimantan	2012	1
12.217.5	Kutai Barat	East Kalimantan	2011	1
14.268.2	Deli Serdang	North Sumatera	2014	1
14.268.3	Deli Serdang	North Sumatera	2014	1

PRRS: Porcine reproductive and respiratory syndrome

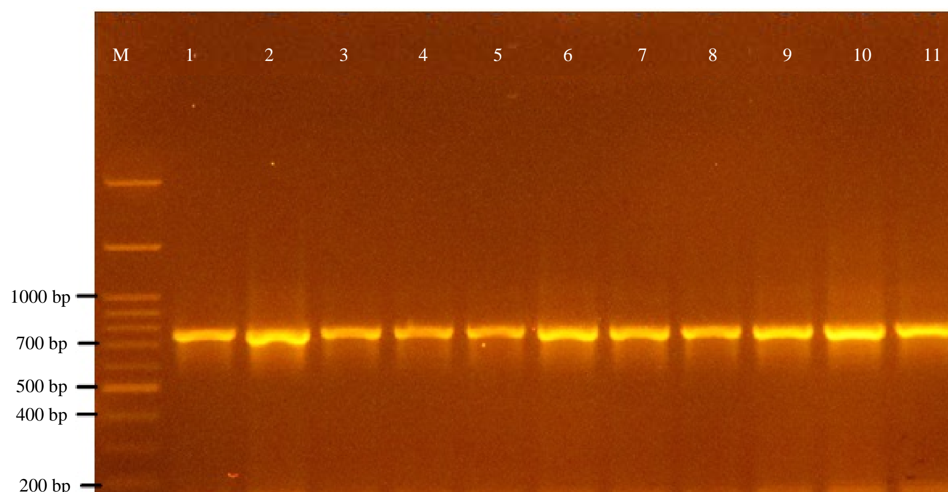

Fig. 1: The results of amplification of Polymerase Chain Reaction (PCR) Open Reading Frame 5 (ORF5) in agarose. The ORF5 was amplified in band 778 bp, using ORF5F2B and ORF5R2 primers. Lane 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 were samples and marker M, (100 bp)

manufactories instructions and analyses on an ABI PRISMA 3700 DNA Analyzer. All PCR products were sequenced in both directions.

Data analysis: The specific primers ORF5F2B and ORF5R2 were designed to amplify gene target, which flanks from the partial of ORF4-ORF6 as long as 778 bp. This flanking region including the whole ORF5 sequence. The whole sequences have the same nucleotides length in size of 600 nt, which encoded 200 amino acids for each virus and analyzed using MEGA 6.0 version software (Tamura *et al*., 2013). The ORF5 sequences of nucleotide and amino acids Indonesian PRRS virus were aligned along with referens sequences (VR2332, MLV and JXA1) from Gen Bank using clustal W located in the MEGA program. Genetic variations analysis were seen based on the differences in ORF5 sequences. Phylogram was generated using the neighbor joining by the computer program MEGA6 based on the formula of Kimura 2 parameter (Kimura, 1980). The robustness of the phylogenetic analysis was determined by bootstrap analysis with 1000 replications (Felsenstein, 1985).

RESULTS

PCR amplification of the target cDNA: In this study, the investigation was carried out from 21 clinical samples but for the other 9 samples due to the small quantity of initial tissue. Eleven samples were investigated on the ORF5 for PCR and sequencing (Table 1). The ORF5 from the 11 PRRS viruses were amplified with two steps PCR. Eleven samples were detected positive used primers ORF5 F2B and ORF5R2 with the amplicon size of 778 bp (Fig. 1).

Sequence analysis of ORF5: The complete ORF5 of 11 PRRSV from clinical samples were sequenced and blast searched in Gen Bank. Genetic analysis revealed that the ORF 5 of the all 11 sequences had 600 bp, which encoded 200 amino acids as those most other PRRS virus indicating there were no deletion or insertion in the viruses compared with referens virus type 2 (VR2332).

Variety of amino acids based on ORF5 sequences: In Indonesian PRRS viruses, the amino acid mutations found in the signal sequence at position 3 (E3G), 9 (G9C), 16 (S16F), 24 (C24Y) and 25 (F25L), when compared to VR2332 and this area was previously reported as variable region. The mutations were also observed in ectodomain area at positions 34 (D34N), 35 (S35N), 39 (L39I) and 58 (N58Q). In the transmembrane area mutations were observed in position 66, (S66T), 92 (A92G), 94 (V94A), 101 (F101Y), 102 (V102Y), 121 (T1 21I) and 127 (F127L). Some mutations were present in endodomain area; 137 (S137A), 151 (R151G), 161 (V161I), 164 (G164R), 185 (A185V), 189 (L189I) and 200 (L200P). These mutations might play an important role in grouping the viruses.

Phylogenetic analysis: To decipher genetic relatedness of Indonesian PRRS viruses, a phylogenetic tree was contructed using 11 sequences of current study and 34 sequences of

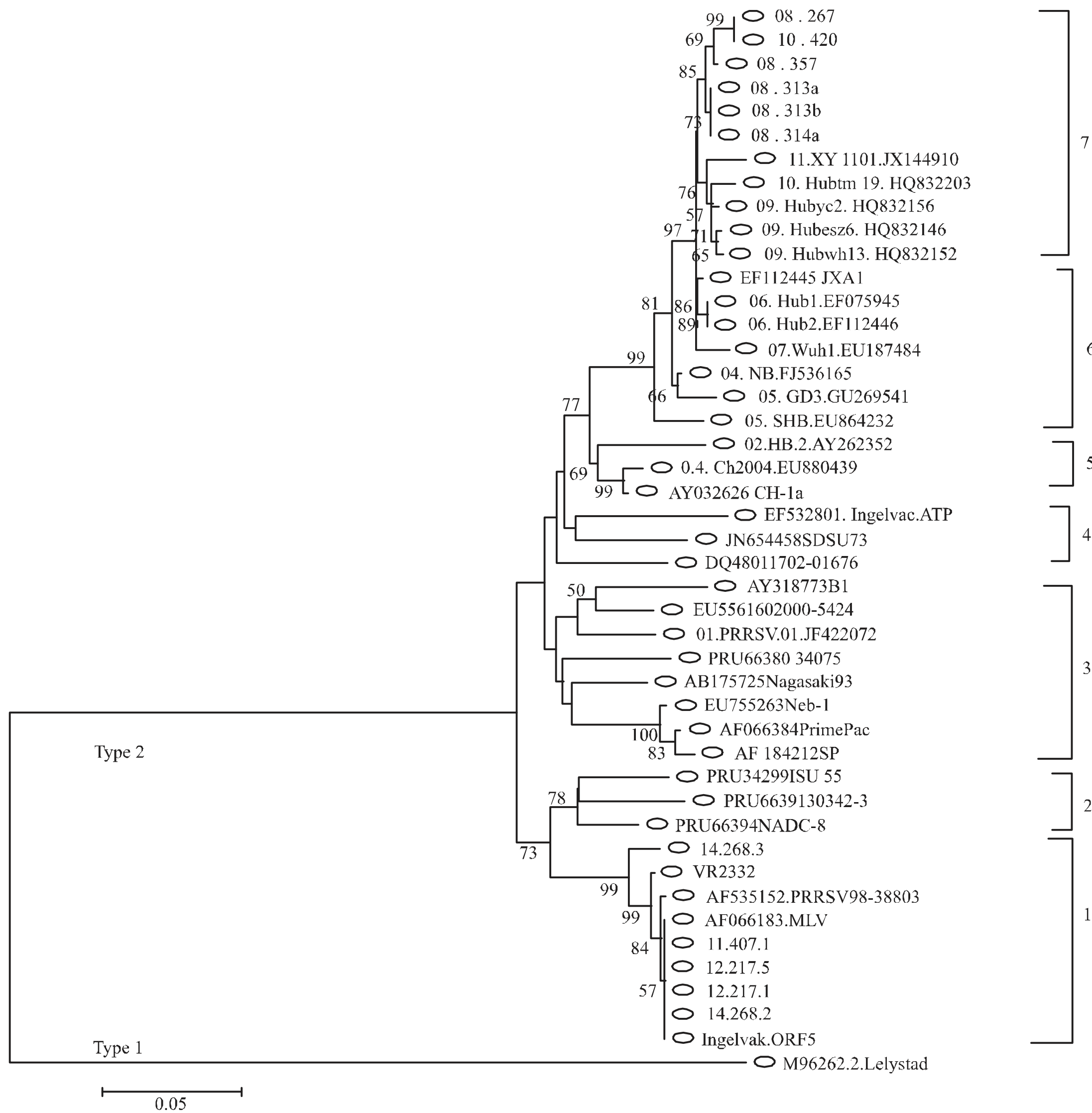

Fig. 2: Phylogenetic analysis of Indonesia PRRSV viruses of the North American genotype based on the nucleotide sequence of complete ORF5. The tree was constructed using the neighbor joining method with the Kimura 2 parameter model. The number at the branch node indicates the percentage occurrence in 1000 boot strap replications. The bar scale indicates the genetic distance among ORF5 sequences

reference NA viruses. The nucleotide and amino acids divergence of the Indonesian PRRS viruses ranged between 88.5-100 and 87.5-100%, respectively. The Indonesian PRRS viruses were divided into two genotype, genotype 1 and 7 (Fig. 2). There were five sequences clustered with genotype 1 (08.407.1, 12.217, 12.217.5, 14.268.2 and 14.268.3). These viruses have a closeness with viruses VR232 and MLV and shared nucleotide and amino acid sequence identities of 97.6-100 and 97.0-100%, respectively. Remaining six sequences cluster with group 7 and shared the nucleotide and amino acids levels of 88.8-99.1 and 87.0-99.0%, respectively with J×A1 virus.

DISCUSSION

The sequencing analyis indicates that the origin of all the 11 PRRS virus from Indonesian between 2008-2014 is the North American genotype. The whole ORF5 of the Indonesian PRRSV sequences have the same nucleotide length of 600 nucleotides, which encoded 200 amino acids with no deletion or insertion of nucleotides, but silent or missense mutations are detected compared to references PRRS virus (VR2332, MLV and JXA1).

The phylogenetic analysis shows that all the 11 Indonesian PRRS virus are divided into two genotype (Fig. 2).

Table 2: Amino acid variation in the important sites of ORF5

	Derivatives vaccine			China-like, PRRSV												
Virus name	13	137	151	34	39	66	92	102	121	127	161	164	189	199	PNE (37-45)	DCE (27-30)
VR2332	R	A	R	D	L	S	A	V	T	F	I	R	I	P	SHLQLIYNL	VLAN
MLV	Q	-	G	-	-	-	-	V	-	L	-	-	-	-	SHLQLIYNL	VLAN
Ch-1a (classic China)	-	S	-	N	F	T	G	Y	I	L	V	G	L	L	SHFQLIYNL	VLVN
JXA1 (HP-PRRS China)	-	S	-	N	I	T	G	Y	I	-	V	G	L	L	SHIQLIYNL	VLVN
08.267.Dairi	-	S	-	N	I	T	G	Y	I	L	V	G	L	L	SHIQLIYNL	VLAN
08.Taput.313A	-	S	-	N	I	T	G	Y	I	L	V	G	L	L	SHIQLIYNL	VLAN
08.Taput.313B	-	S	-	N	I	T	G	Y	I	L	V	G	L	L	SHIQLIYNL	VLAN
08.Dairi.314A	-	S	-	N	I	T	G	Y	I	L	V	G	L	L	SHIQLIYNL	VLAN
08.Taput.357	-	S	-	N	I	T	G	Y	I	L	V	G	L	L	SHIQLIYNL	VLAN
10.Deli S.420	-	S	-	N	I	T	G	Y	I	L	V	G	L	L	SHIQLIYNL	VLAN
11.Kal.407.1	Q	-	G	-	-	-	-	-	-	-	-	-	-	-	SHLQLIYNL	VLAN
12.Kal.217.1	Q	-	G	-	-	-	-	-	-	-	-	-	-	-	SHLQLIYNL	VLAN
12.Kal.217.5	Q	-	G	-	-	-	-	-	-	-	-	-	-	-	SHLQLIYNL	VLAN
14.Deli S.268.2	Q	-	G	-	-	-	-	-	-	-	-	-	-	-	SHLQLIYNL	VLAN
14.Deli S.268.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	SHLQLIYNL	VLVN

Abbreviation amino acids base on one letter symbols, PNE: Primary neutralization epitope, DCE: Decoy epitope, ORFS: Open reading frame 5 and PRRSV: Porcine reproductive and respiratory syndrome virus

The first genotype is the viruses that genetically close related to PRSSV virus from China (JXAI) with the viruses shared 98.5-99.1% in the level of nucleotide and 98.5-99.1% level amino acids. The second genotype comprises of viruses that clustered in the same lineage with the VR2332 virus. The viruses within this group shared 98.0-99% nucleotide similarities in the ORF5 and 98.0-99.0% amino acid similarities compared to VR 2332 virus and into the first genotyoe. Both genotypes viruses from Indonesia entered PRRS virus type 2, this indicates that type 2 PRRS virus had spread widely in Indonesia. When the ORF5 sequences of Indonesian viruses are compared to Modified Live Vaccine (MLV) virus, the study viruses show 88.5-100 and 87.5-100% similarities in the nucleotide and amino acid sequences, respectively. High phylogenetic relatedness with 100% similarity in both nucleotide and amino acid sequences to the MLV virus are found among the four viruses (10.407.1, 12.217.1, 12.217.5 and 14.268.2) belonging to the first group in this study indicating that these viruses originated from the MLV-like vaccine which has been used in North Sumatera, since 2008.

In term of genetic relationships, the PRRS viruses from Indonesia seems to have close relatedness to those from China virus, which this can be seen from the patterns of amino acid mutations between these viruses (Table 2). To investigate the amino acid differences among the virus groups, the GP5 amino acid sequences of our 11 representative PRRS viruses are aligned together with North American type viruses (VR2332, MLV and JXA1). The functional domains of GP5 such as the signal sequence, ectodomain, transmembrane regions and endodomain were divided according to previous reports (Han *et al*., 2006). Important motifs in GP5 including decoy epitope (DCE) and Primary Neutralizing Epitope (PNE) as described previously (Ostrowski *et al*., 2002; Plagemann *et al*., 2002; Plagemann, 2004).

It was suggested that the PNE was located at AA 37-45, a segment similar to that of the NA isolate (Ostrowski *et al*., 2002; Plagemann *et al*., 2002; Plagemann, 2004). In of VR2332 virus, the DCE and PNE of the NLVN American isolate were identified as (A/V)27 and other North American isolate were identified as (A/V)27LVN and S37H(F/L)QLIYN, respectively (Ostrowski *et al*., 2002; Plagemann *et al*., 2002; Plagemann, 2004). The NA sequence was more variable at the DCE and at the sequences between DCE and PNE. Substitutions are found at amino acid positions 29 and 30 and common substitutions are are A29V and N30D. Decoy epitope of Indonesian PRRS virus has been undergone mutation at amino acid 39 (L39I) when compared with the VR2332 virus. Amino acids sequences of 13Q and 151G are found in some Indonesian PRRS virus (Table 2).

The SHLQLINK motif sequences and L39I mutation are detected in the PNE of Indonesian PRRS viruses when they are compared to VR2332 (Table 2). These suggest that the Indonesian PRRS viruses may have been undergone high immunological pressure originating from vaccination or natural immunity derived from persistent infection or multi-host of different strains of pigs. Analysis of the decoy epitope shows that all Indonesian PRRS viruses prepared by amino acid VLAN (27V) and without the mutation, when compared with VR2332 virus (Table 2). Decoy epitope responsible to reduce and to deliberate the production of neutralizing antibodies against the virus so that the virus is able to evade the host immune system (Ostrowski *et al*., 2002).

Alanine at position 137 in the derivatives vaccine strains and serine at position 137 in the field strains

Table 3: Number and distribution of the potential N-glycosylation sites in the GP5

	Positions of the glycosylation sites						
Sample	30	33	34	35	44	51	Number
08.267.Dairi	+	+	-	+	+	+	5
08.Taput.313A	+	+	-	+	+	+	5
08.Taput.313B	+	+	-	+	+	+	5
08.Dairi.314A	+	+	-	+	+	+	5
08.Taput.357	+	+	-	+	+	+	5
10.Deli S.420	+	+	-	+	+	+	5
11.Kal.407.1	+	+	-	-	+	+	4
12.Kal.217.1	+	+	-	-	+	+	4
12.Kal.217.5	+	+	-	-	+	+	4
14.Deli S.268.2	+	+	-	-	+	+	4
14.Deli S.268.3	+	+	-	-	+	+	4

+: Exiting potential N-glycosylation sites, -: Not exiting

(Wesley *et al*., 1999) are detected in all viruses of group 1 from Indonesia viruses. Previous studies have shown that amino acid mutations arginine to glutamine (R13Q) and arginine to glycine (R51G) appear to be involved in PRRS virus attenuation (Madsen *et al*., 1998; Yang *et al*., 1998; Allende *et al*., 2000). Both the amino acid mutations indicate the live virus can be re-isolated from a derivative vaccine. If this position compared with the VR 2332 virus, mutations of R13Q and R151G are detected in four viruses (10.407.1, 12.217.1, 12.217.5 and 14.268.2) suggesting that all four viruses are originated from vaccine derivatives. The same positions in Ingelvac PRRSV® MLV and MLV virus vaccine are occupied by 13Q and 151G.

The potential glycosylation sites in Indonesia viruses have been analyzed (Table 3). N-glycosylation sites on the glycoprotein 5 are found at positions 30, 32, 33, 34, 44 and 51 (Ansari *et al*., 2006; Wissink *et al*., 2004). Various glycosylation sites are found i n the Indonesian PRRS viruses; genotype 1 have four glycosylation sites at positions 30, 33, 44 and 51, while genotype 7 have five glycosylation sites at positions 30, 33, 35, 44 and 51 (Table 3). The diversity of glycosylation site may have critical point on virus neutralization that can inhibit and degrade the host immune response against PRRS virus (Johnson *et al*., 2003; Wei *et al*., 2003). Mutations in the area of the N-glycosylation sites affect protein structure, accessibility of antibodies and immunogenicity of proteins of the PRRS virus. In addition, N-glycosylation plays an important role in infection, replication and immune response to virus (Ansari *et al*., 2006).

CONCLUSION

This study provides information that since identified in 2008 PRRS virus isolated from Indonesia has shown genetic diversities on nucleotide and amino acids sequences. Genetic diversity of the ORF5 is important to role in the classification of the PRRS group. The Indonesian PRRS virus is divided into two groups: First and the seven genotype. Based on the analysis of amino acid sequences of ORF5 in the Indonesia PRRS viruses obtained three amino acids as derivates or attenuated from the virus vaccine is 13G, 151G and 137A.

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