ASSESSMENT OF THE GENETIC CHANGES OF THE ATTENUATED HANVET1.VN STRAIN COMPARED WITH ORIGINAL VIRULENT 02HY STRAIN OF THE PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

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SUMMARY

The attenuated porcine reproductive and respiratory syndrome virus (PRRSV) strain Hanvet1.vn was developed by Hanvet Pharmaceutical Co., Ltd. by inoculating the virulent strain 02HY on Marc-145 cells for 80 generations and used to produce PRRS vaccine. In this study, we published the results of sequencing, analyzing and comparing the genome of the attenuated PRRSV strain Hanvet1.vn compared with the original pathogenic strain 02HY. The genomes of strains Hanvet1.vn and 02HY have 8 reading frames, coding for 8 non-structural and structural proteins: NSP1a, NSP1b, GP2, GP3, GP4, GP5, MP, NP. After sequencing and translating into proteins, the gene sequence of each open reading frame (ORF) of strain Hanvet1.vn was compared with the sequence of pathogenic strain 02HY to find nucleotide and amino acid changes. The results showed that the Hanvet1.vn pathogenic strain genome (Genbank Accession KU842720) when compared with the pathogenic strain 02HY genome (Submission2490633) had 89 nucleotide mutations that changed 51 amino acids in 7 ORFs and 7 proteins, respectively. Particularly, ORF6 encoding for the M protein is completely unchanged. The size of each reading frame is also exactly the same. It showed that there were no insertion and deletion (Indel) mutations in the ORFs of the attenuated strain after 80 generations of inoculation. There was a change in the genome that made the strain Hanvet1.vn become attenuated, but the gene encoding for the GP5 protein that induces the production of neutralizing antibodies only changed two nucleotides at position 471 (A→G), causing the TCA codon to turn into a TCG codon. This is a silent mutation and both codons code for the amino acid Serine (S). The second mutation at position 587 (A→T) causes Glutamine (Q) to transform into Leucine (L). However, this modification does not belong to the GP5 antigenic epitopes. In conclusion, after 80 passages, despite changes occurred in genes of Hanvet1.vn strain for becoming an attenuated strain, the GP5 protein of the attenuated strain did not change its antigenic amino acids.

Keywords: Hanvet1.vn attenuated strain, 02HY virulent strain, genome sequence, nucleotide and amino acid changes, GP5 antigen epitope.
INTRODUCTION

Porcine Respiratory and Reproductive Syndrome (PRRS) is a contagious disease in pigs of all ages. The disease caused by PRRSV has a complicated course, difficult to control, and with high morbidity rate. The emergence of highly virulent PRRSV strains in China in 2006-2007 caused a series of PRRSV outbreaks in Asia (Tian et al., 2007). The PRRS epidemic has killed millions of pigs since 2006 (Zhou, Yang, 2010). Highly pathogenic strains of PRRSV have also caused outbreaks in other countries in the Asian region. The first case of PRRS appeared in Vietnam in early 2007 (Feng et al., 2008; Metwally et al., 2010) then spread to other Southeast Asian countries such as Laos, Philippines, Cambodia etc. (Jantafong et al., 2010; Ni et al., 2012; Tornimbene et al., 2015).

In Vietnam, from 2007 until now, PRRS has occurred in many provinces and cities throughout the country, causing heavy losses to the livestock industry (Pham Van Son, 2018). There have been many vaccines produced for disease prevention, including the attenuated PRRS vaccine that is being prioritized for development and use in China and Vietnam because of its superior protection compared to other kinds of vaccines. In Vietnam, in order to prevent diseases, many integrated measures have been directed by the Ministry of Agriculture and Rural Development, in which the use of vaccines is the top priority.

There were two strains to be attenuated for the use as vaccines including the attenuated PRRSV strain Hanvet1.vn by the Hanvet Pharmaceutical Company Limited and the KTY-PRRS-06 strain by the Vietnam National University of Agriculture (Trinh Dinh Thau et al., 2018), respectively. The attenuated PRRSV strain Hanvet1.vn used in the production of PRRS vaccine by the Hanvet Pharmaceutical Company Limited was created by 80 generations of continuous subculture on Marc-145 cells.

In this paper, we present the results of sequencing, analysis and comparison of changes in the genome between the attenuated virus strain Hanvet1.vn and the original pathogenic strain 02HY in order to evaluate the genetic changes related to the virulence and immunogenicity of the vaccine.

MATERIALS AND METHODS

Materials

The attenuated vaccine strain Hanvet1.vn and the virulent strain 02HY are provided by Hanvet Pharmaceutical Company Limited. cDNA synthesis kit: SuperSciptTM First-StrDNA Synthesis System for RT-PCR (Invitrogen). Cloning Kit: TA cloning Kit (Invitrogen). Gene sequencing kit: BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). High purity chemicals used in molecular biology research are provided by companies such as Sigma, Merck, Invitrogen, include: Taq DNA polymerase, Yeast extract, Tryptone, Agar, Amp (Ampicillin), X-gal, EDTA (Ethylenediaminetetraacetic acid), SDS (Sodium dodecyl sulphate), Chloroform, Trizol, Sodium acetate, Ethanol, NaCl, Tris-HCl, NaOH, TAE (Tris-Acetic acid-Ethylenediaminetetraacetic acid).

Methods

Genetic sequences of North American PRRSV genotypes circulating in Vietnam and the region with numbers FJ393456, FJ393457, FJ393458, FJ393459, FJ394029 and attenuated vaccine strain RespPRRS were used to design primer pairs to amplify the whole genomes of Hanvet1.vn and 02HY viruses. Using GeneDoc 2.7 software to compare and design primer pairs for gene amplification.

Amplification and cloning of 15 gene segments belonging to the attenuated strain Hanvet1.vn and pathogenic strain 02HY were carried out as described previously (Nguyen Thi Nga et al., 2018). The sequencing reaction of clones was performed using the BigDye™ Terminator v3.1 Cycle Sequencing Kit from Applied Biosystems, using two forward and reverse primers M13F and M13R. Automated sequencing on the ABI 3100 system and
sequence analysis using BioEdit and DNA Star software were carried out. Comparison of ORFs and proteins for mutations was performed by the PC/Genet software program. Protein epitopes were analyzed using the Predicting Antigenic Peptides software (http://imed.med.ucm.es/Tools/antigenic.pl).

RESULTS AND DISCUSSION

RESULTS

Amplification and cloning of genes segments of the PRRSV genome

After extraction of total RNA from infected viral fluid of the Marc-145 cells, RNA was converted to cDNA using Invitrogen's SuperScipTMIATMFirst-StrDNA Synthesis kit. cDNA was used as template strand to amplify gene segments of PRRSV attenuated Hanvet1.vn and virulent strain 02HY by PCR with 15 specific primer pairs designed so that the amplified gene fragments have overlapping regions for easily reassembled into a complete genome. To facilitate the storage and sequencing of genes, after purification, the PCR products were ligated to pCR2.1 cloning vector, transformed into E. coli cells with Top F' strain. The recombinant plasmids carrying amplified gene fragments from the PRRSV genome were screened and examined by cutting with the restriction enzyme EcoRI. The gene fragment from the recombinant plasmid was separated from the vector with the same size as the PCR product ligated to the cloning vector (Nguyen Thi Nga et al., 2018).

Sequencing and analyzing the genome of the attenuated strain Hanvet1.vn and the pathogenic strain 02HY

Recombinant plasmids inserted with PCR products were purified and sequenced using an automated gene sequencer (ABI 3100). The resulting data were processed and analyzed using DNAStar and GeneDoc software. After sequencing, assembly and annotation, we obtained the attenuated Hanvet1.vn genome with 8 reading frames, encoding for 8 proteins (GenBank accession number: KU842720). Similarly, strain 02HY also has 8 reading frames, coding for 8 proteins (Submission: 2490633). The length of each reading frame of the attenuated strain Hanvet1.vn is completely unchanged compared to the original pathogenic strain 02HY. The number of nucleotides and amino acids of each reading frame of the attenuated strain Hanvet1.vn and pathogenic strain 02HY are shown in Table 1.

The results in Table 1 showed that there were no deletion and insertion (Indel) mutations in the open reading frames of strain Hanvet1.vn after 80 generations of inoculation.

Table 1. Number of nucleotides and amino acids of each reading frame of the attenuated strain Hanvet1.vn and the virulent parent strain 02HY.

| ORF | Hanvet1.vn 02HY | ORF1a | ORF1b | ORF2 | ORF3 | ORF4 | ORF5 | ORF6 | ORF7 |
|-----|----------------|-------|-------|------|------|------|------|------|------|
| Number of nucleotides in each ORF | 2473 | 1459 | 256 | 254 | 178 | 200 | 174 | 123 |
| Number of amino acids in each protein | 7418 | 4377 | 768 | 765 | 534 | 600 | 522 | 372 |

Genomic changes in the Hanvet1.vn strain compared with pathogenic 02HY strain

The changes in nucleotide sequences and amino acid sequences in each ORF/protein of the attenuated strains of Hanvet1.vn compared to its virulent strain 02HY are shown in Tables 2 and 3, respectively.
The attenuated strain Hanvet1.vn had 89 point mutations in all reading frames that made the changes in 51 amino acids. Among 89 point-mutations there were missense mutations (leading to change of amino acid they encode for) and silent mutations (no change of amino acid) as seen in the attenuated strain Hanvet1.vn.

No nonsense mutations causing stop codons were found. ORF5 encoding for the GP5 protein containing neutralizing antibody-producing epitopes had two-nucleotide changes at position 471 (A→G) and 587 (A→T). The change of TCA to TCG (A→G) is a silent mutation causing no change of amino acid Serine (S). The mutation (A→T) had made a change from Glutamine (Q) to Leucine (L) (Figs 1 and 2).

Table 4 shows epitope amino acids in the epitopicket in the GP5 polypeptide of the attenuated Hanvet1.vn strain. The amino acid change at position 196 (L196Q) occurred in this attenuated strain was outside the GP5 antigen epitope region (Table 4). Thus, it can be concluded, after 80 passages, although the genes of Hanvet1.vn strain have some changes to become attenuated compared to the pathogenic 02HY strain during the attenuation, but the GP5 epitope amino acids responsible for the production of neutralizing antibodies were not affected.

![Figure 1. Comparison of the nucleotide sequence of the ORF5 gene of the attenuated strain Hanvet1.vn with that of the 02HY strain. The attenuated strain Hanvet1.vn appeared with 2 point mutations at positions 471 and 587.](image)
Table 2. Number of nucleotides and amino acids changed in each ORF of the Hanvet1.vn attenuated strain compared with the 02HY pathogenic parent strain.

| Number of nucleotides changes in each ORF | ORF1a | ORF1b | ORF2 | ORF3 | ORF4 | ORF5 | ORF6 | ORF7 | Total |
|------------------------------------------|-------|-------|------|------|------|------|------|------|-------|
|                                           | 45    | 23    | 9    | 4    | 4    | 2    | 0    | 2    | 89    |

| Number of amino acids changes in each protein | NSP1a | NSP1b | GP2 | GP3 | GP4 | GP5 | MP | NP | Total |
|------------------------------------------------|-------|-------|-----|-----|-----|-----|----|----|-------|
|                                                 | 25    | 11    | 6   | 3   | 4   | 1   | 0  | 1  | 51    |

Table 3. Identity of nucleotide and amino acid sequences of Hanvet1.vn attenuated strain compared with 02HY pathogenic strain.

| Nucleotide identity in the two strains (%) | ORF1a | ORF1b | ORF2 | ORF3 | ORF4 | ORF5 | ORF6 | ORF7 |
|------------------------------------------|-------|-------|------|------|------|------|------|------|
|                                           | 99.39 | 99.47 | 98.83| 99.48| 99.26| 99.67| 100.00| 99.46|

| Amino acid identity in the two strains | NSP1a | NSP1b | GP2 | GP3 | GP4 | GP5 | MP | NP |
|---------------------------------------|-------|-------|-----|-----|-----|-----|----|----|
|                                       | 98.99 | 99.25 | 97.66| 98.82| 97.75| 99.50| 100.00| 99.19|

Figure 2. Comparison of the amino acid sequence of the GP5 protein of the attenuated strain Hanvet1.vn with that of the 02HY strain. The silent mutation at nucleotide position 471 does not change the amino acid Serine. The missense mutation at position 587 causes Glutamine (Q) to transform into Leucine (L), however this amino acid is outside the antigenic epitope region (Table 4).

Table 4. GP5 protein epitopes of the attenuated strain Hanvet1.vn

| Number | Starting position | Epitope sequence | Ending position |
|--------|-------------------|------------------|-----------------|
| 1      | 4                 | KCLTACCCSRLLFLCIVPFYLAVLVN | 30 |
| 2      | 37                | SHIQIYNLTLCE     | 49             |
| 3      | 62                | WAVETFVFIPVVLTHIVSYGALTTSHTFLDTVLGATVSTAGYYHGRYVLSSSAYVCALAALICFVIRLAK | 129 |
| 4      | 133               | SWRYSCTR         | 140            |
| 5      | 154               | RWRSPFVIV        | 161            |
| 6      | 165               | GKVVEVEGHLIDLKRVLVDG | 183   |
| 7      | 185               | AATPLTRVSA       | 194            |
DISCUSSION

In addition to imported vaccines, domestically researched and manufactured vaccines have also played an important role in the prevention of PRRSV in Vietnam. The Hanvet Pharmaceutical Co., Ltd. has succeeded in creating an attenuated strain Hanvet1.vn by 80 generations of inoculation on Marc-145 cells and using this strain to produce vaccine against porcine reproductive and respiratory syndrome virus.

Sequencing and analyzing the genome and finding genetic changes of the attenuated strain Hanvet1.vn compared with the original pathogenic strain 02HY, in this study, have provided important information for the monitoring of genetic changes following to decide safety and protective immunogenicity of vaccines during production. The results of comparing nucleotide and amino acid sequences of all 8 reading frames of the attenuated strain Hanvet1.vn with the original pathogenic strain 02HY showed that there were 89 nucleotide mutations that changed 51 amino acid mutations scattered in seven open reading frames and seven proteins, respectively. However, ORF6 encoding the M protein is completely unchanged. In the open reading frames, only silent mutations and missense mutations appeared, no nonsense mutations generating stop codons. The size of each reading frame is also exactly the same. It showed that there were no insertion and deletion (Indel) mutations in the open reading frames (ORFs) of the attenuated strain after 80 generations of inoculation.

There was a change in the genome that made the Hanvet1.vn strain become attenuated, but the gene encoding the GP5 protein changed only two nucleotides at position 471 (A->G) causing the TCA codon to turn into TCG. This is a silent mutation and both codons code for the amino acid Serine (S). The mutation at position 587 (A->T) causes Glutamine (Q) to turn into Leucine (L). However, this modification does not belong to the GP5 antigenic epitopes. Thus, it can be said that, after 80 passages in Marc-145 cell line, although the genes of strain Hanvet1.vn have changed to become an attenuated strain, the gene encoding GP5 protein has not change the immunogenicity. GP5 is an essential protein playing an important role in stimulating the immune response to generate PRRSV-neutralizing antibodies (Popescu et al., 2017). Therefore, the conservation of the antigenicity of the GP5 protein of the attenuated strain Hanvet1.vn compared with the original pathogenic strain 02HY is decisive for the effectiveness of the vaccine.

In addition to the attenuated strain generated by the Hanvet Pharmaceutical Company Limited, the another attenuated strain named KTY-PRRS-06 has also been recently succeeded by the Vietnam National University of Agriculture using the serial passages for 90 generations on Marc-145 cell line. The attenuated strain causes cytopathic effect (CPE) 12 hours after infection and completely damage the cells after 48 hours. The attenuated strain KTY-PRRS-06 is not pathogenic when administered to pigs and induces a specific antibody response against PRRSV. The authors compared the ORF5 sequence of the attenuated strain KTY-PRRS-06 with the pathogenic strain and found 13 different positions in the nucleotide sequence and 8 different positions in the amino acid sequence (Trinh Dinh Thau et al., 2018). Thus, the attenuated strain KTY-PRRS-06 after 90 generations of inoculation on the Marc-145 cells appeared more point mutations than the attenuated strain Hanvet1.vn. In the work by Trinh Dinh Thau et al. (2018), the authors did not analyze each mutation in the ORF5 gene and did not clarify whether these mutations affect the ability to produce PRRSV-neutralizing antibodies belonging to the epitopes.

Allende et al., 2000 sequenced and analyzed the variation in the genome of the attenuated RespPRRS strain used in PRRS vaccine production and compared with the virulent parent strain 16244B. The attenuated strain RespPRRS was created by subculture of 25 generations on MA-104 cells at 35-37°C, then 20 generations in the same cell line but at 31°C. The
results showed that there were no Indel mutations in the genome and discovered 212 point mutations in the whole genome. Thus, through 55 generations of inoculation on MA-104 cells with 25 generations at 35-37°C and 20 generations at 31°C, the authors created an attenuated strain with a higher number of point mutations compared with our study (212 mutations compared with 89 mutations in our study).

The authors also identified the changes of 9 amino acids located on 7 proteins Nsp1β, Nsp2, Nsp10, ORF2, ORF3, ORF5, ORF6 and suggested that the modifications of these amino acids may provide the molecular bases for the observed attenuated phenotype.

CONCLUSION

In this study, the genome of the attenuated PRRSV strain Hanvet1.vn was sequenced, analyzed and compared with the genome of the parental pathogenic strain 02HY to find out the genetic variation of the attenuated strain Hanvet1.vn after 80 passages on Marc-145 cells. The results showed that the genomes of strains Hanvet1.vn and 02HY have 8 reading frames, coding for 8 non-structural and structural proteins: NSP1a, NSP1b, GP2, GP3, GP4, GP5, MP, NP. After sequencing and translating into proteins, the gene sequence of each open reading frame (ORF) of strain Hanvet1.vn was compared with the sequence of pathogenic strain 02HY to find nucleotide and amino acid changes. The results showed that, after 80 generations of inoculation, in the genome of strain Hanvet1.vn, there were 89 nucleotide mutations that changed 51 amino acids in seven open reading frames and seven proteins respectively. Remarkably, ORF6 encoding for the M protein is completely unchanged.

The size of each reading frame is also exactly the same. It showed that there were no insertion and deletion (Indel) mutations in the open reading frames (ORFs) of the attenuated strain after 80 generations of inoculation. There was a change in the genome that made the strain Hanvet1.vn become attenuated, but the gene encoding the GP5 protein that induces the production of the neutralizing antibodies only changed two nucleotides at position 471 (A->G) is a silent mutation causing no change in amino acid Serine (S). The mutation at position 587 (A->T) changed Glutamine (Q) to Leucine (L), but this Leucine amino acid change is outside of the GP5 antigenic epitopes. It is concluded, after 80 passages, the Hanvet1.vn strain have changed at some sites compared to the pathogenic strain to become an attenuated strain, but the gene encoding GP5 protein responsible for production of the neutralizing antibodies has not been altered.

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