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CHARACTERIZATION OF THE E2 GENE OF CLASSICAL SWINE FEVER VIRUS (CSFV) ISOLATED IN VIETNAM

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ABSTRACT

Classical swine fever is one of the most dangerous infectious diseases, causing significant economic damage to the pig industry globally and in Vietnam. The disease is caused by the *Classical Swine Fever Virus* (CSFV), an RNA virus with a high mutation rate. Classical swine fever remains a complex and persistent threat. In this study, the full E2 gene of CSFV strains collected from Ha Noi (21.028333 N, 105.853333 E), Da Nang (16.079 N, 108.217 E), and Quang Ninh (21.250982 N, 107.193604 E) was analyzed alongside three vaccine strains currently used in Vietnam. The results show that the highly virulent CSFV strains circulating in 2023 belong to genotypes 1.1, 2.1, and 2.2, while the vaccine strains in use belong to genotype 1.1. There are notable differences in amino acids at key antigen epitope sites between the vaccine strains and the circulating CSFV strains in 2023. These findings highlight the need to develop new vaccines that are better suited to the currently circulating virus strains.

Keywords: Classical swine fever virus, E2 gene, Epitope, genotype.

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INTRODUCTION

Classical swine fever is an acute infectious disease in pigs, affecting both domestic pigs and wild boars. CSFV was first observed in the 1830s in Ohio in the United States of America, and since then, it has been reported globally, mainly in countries of America, Asia, and Eastern Europe (Edward et al., 2000). Currently, swine fever has been eradicated in some countries such as Canada, the United States, Australia, and New Zealand. However, it still persists in parts of Asia, Europe, Central and South America, and Africa, causing significant economic damage, especially in poorer countries (Beer et al., 2015). Outbreaks of swine fever spread rapidly and are difficult to control, with an incubation period of 5 to 7 days and a mortality rate of 80% to 100%, leading to severe losses for livestock farmers. Since 2020, African swine fever has spread across the country, leading to mass pig deaths and the destruction of entire herds. This is why outbreaks of classical swine fever have not been as widely publicized. Classical swine fever is classified as a dangerous disease due to its ability to spread quickly through various transmission routes (OIE, 2022). The virulence of the virus strains causing classical swine fever varies. Some strains are highly virulent and cause hyperacute disease (very rapid onset), while others are low-virulence strains that cause chronic disease (prolonged progression). There are also strains with moderate virulence that lead to subacute disease Classical swine fever is a systemic septicemia that damages the vascular endothelium, leading to leukopenia and thrombocytopenia, which in turn cause hemorrhages and thrombosis throughout the body. From 2012 to 2016, CSFV caused serious damage to the pig farming industry in Vietnam, with tens of thousands of cases and mortality rates of up to 90% in some pig herds (Tran et al., 2023). Despite strict vaccination programs, including in Vietnam, the disease continues to occur in various forms due to the emergence of many variants across different genotypes (Van Oirschot, 2003; Ji et al., 2014).

Classical swine fever virus (CSFV) is a single-stranded, positive-sense RNA ((+)ssRNA) virus with an unsegmented genome structure, belonging to the genus *Pestivirus* in the family Flaviviridae (Meyers & Thiel, 1996). The complete genome of CSFV is approximately 12,300 nucleotides long, consisting of two non-coding regions, 5'UTR and 3'UTR, and an open reading frame (ORF) of 11,697 nucleotides, which encodes 3,898 amino acids (Thiel et al., 1991). Among the viral proteins, the antigenic protein E2 (also known as gp55) is a glycoprotein with the highest antigenicity, capable of stimulating the production of neutralizing antibodies (Bouma et al., 2000). CSFV shows a tendency to evolve into multiple genogroups/subgenotypes/ genotypes (Paton et al., 2000), with recombination events occurring between vaccine strains and wild strains, particularly in the amino acid composition of the E2 gene, resulting in significant genomic variation (Ji et al., 2014). To date, CSFV has been classified into three main genotypes, subdivided into 10 subgroups: 1.1, 1.2, 1.3, 1.4, 2.1, 2.2, 2.3, 3.1, 3.2, 3.3, and 3.4. The E2 region of all these genotypes contains the CSFV-specific amino acid sequences 772LFDGSNP778 and 829TAVSPTTLR837, which are recognized by the monoclonal antibody WH303, with the exception of certain strains (e.g., CBR/93 of Thai genotype 3.3) that exhibit an alternative sequence, TAVSSTTLER (Sakoda et al., 1999; Paton et al., 2000). Globally, most live attenuated vaccines have been developed from genotype 1 strains. However, recent studies have reported the increasing prevalence of genotypes 2.1 and 2.2 in various countries, including Vietnam (Deng et al., 2005; Xing et al., 2019; Nguyen et al., 2014). Research on CSFV has largely focused on the sequencing and analysis of genes, particularly the E2 glycoprotein, which is an antigenic determinant responsible for the virus's virulence and the induction of neutralizing antibodies (Bouma et al., 2000).

The Chinese C-strain is an attenuated vaccine virus (classified as genotype 1.1), which originated in China in the 1950s from the

original Shimen strain and was propagated through rabbits (Qiu et al., 2006). Currently, all attenuated swine fever vaccines produced and used worldwide belong to genotype 1. These include the C-strain, propagated through rabbits from the original Shimen strain; the GPE(-) strain (Japanese guinea-pig exaltation-negative strain), transmitted through guinea pigs from the ALD strain; and the Thiverval strain, propagated through cells derived from the original Alfort strain (Van Oirschot, 2003; Qiu et al., 2006; Luo et al., 2014; Ji et al., 2014).

In this study, we present the results of sequencing and analyzing the E2 antigen gene of the classical swine fever virus strain responsible for outbreaks in 2023 in several provinces of Vietnam. E2 is an important gene and supervariable among genotypes of CSFV and commonly used in genetic evolution analysis. Basing on these data, we are comparing them with previously isolated strains in Vietnam and the vaccine strains currently in use.

MATERIALS AND METHODS

Clinical Samples

The specimens were organ samples from pigs suspected of being infected with CSFV, collected from pig farms in Ha Noi (DTLBV), Da Nang (CSF.DN6), and Quang Ninh (CSF.QN7) in 2023 (Table 1). These farms used the vaccine COGLAPEST. The samples were homogenized, stored in ice, and then sent to the Immunology Department. The samples were stored with antibiotics, freeze-thawed three times, and stored at -20 ^{o}C until use. RNA was directly extracted from the samples for further molecular analysis. Additionally, three attenuated C-strain vaccines (BIO-L TCHC contains Hog Choler LPC-PRK strain (DTL-VX1), COGLAPEST contains Thiverval strain (DTL-VX2), and SWIVAC C contains C strain (DTL-VX3)) currently used in Vietnam were included in the molecular investigation and analysis.

RNA Extraction and Reverse Transcription

Viral RNA was extracted from the samples using the QIAamp Viral RNA Mini Kit (Qiagen, Germany), following the manufacturer's instructions. The total RNA was eluted in 30 µL of elution buffer and stored at -80 ^{o}C until use. cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo, USA) with the following components: 4 µL of viral RNA (~100 ng), 1 µL random hexamer primer (100 pmol/µL), 2 µL of dNTP mix (10 mM each), 4 µL of 5X transcriptase buffer, 1 µL RiboLock RNase Inhibitor (20 U/µL), 1 µL RevertAid M-MuLV RT (200 U/µL), and 7 µL of RNase-free water, to a final volume of 20 µL. The Reverse Transcription (RT) reaction was incubated at 25 ^{o}C for 5 minutes, followed by 60 minutes at 42 ^{o}C, and then 5 minutes at 85 ^{o}C to inactivate the enzyme. The resulting cDNA was stored at -20 ^{o}C until further use.

Table 1. List of CSFV strains and genotypes providing the E2 gene for the study

No.	Strains	Place	Vaccine or virulent	Genotype	GenBank Number
1	DTL-VX1	Vaccine, Taiwan	Vaccine	1.1	This study
2	DTL-VX2	Vaccine, Vietnam	Vaccine	1.1	This study
3	DTL-VX3	Vaccine, Japan	Vaccine	1.1	This study
4	DTLBV	Ha Noi, Vietnam	Virulent	2.1	This study
5	CSF.DN6	Da Nang, Vietnam	Virulent	2.2	This study
6	CSF.QN7	Quang Ninh, Vietnam	Virulent	1.1	This study
7	SM1125	Beijing, China	Virulent	1.1	DQ907720
8	ND9-VN	Vietnam	Virulent	2.1	KP702208
9	ND20-VN	Vietnam	Virulent	2.2	MH979232
10	HN2018-VN	Ha Noi, Vietnam	Virulent	2.5	MK782041
11	TB20182-VN	Thai Binh, Vietnam	Virulent	2.5	MK782045

Amplification of E2 was performed using a pair of primers: CSF-E2F (5'-GCRTTYC TCATYTGTTTGATAA-3') (from 2357 to 2378) and CSF-E2R (5'-GCATGRAACAGC AGYAGTATCC-3') (from 3683 to 3705) (follow position of complete genome of Eystrup strain - NC002657). The pair of primers we designed to amplify a product approximately 1.4 kb in length. The primers were designed based on fifty E2 sequences of CSFV strains available in GenBank. PCR was carried out in 50 µL reaction mixtures containing 25 µL of Taq PCR master mix (Qiagen), 5 µL of cDNA (~50 ng), 2 µL of each primer (10 pmol/µL), 2 µL of DMSO, and 14 µL of nuclease-free water. The PCR amplification was conducted using an MJ PTC-100 thermal cycler (USA) with the following program: an initial denaturation at 94 ^{o}C for 5 minutes, followed by 35 cycles of 94 ^{o}C for 1 minute, 52 ^{o}C for 1 minute, and 72 ^{o}C for 2 minutes, with a final extension at 72 ^{o}C for 10 minutes. The PCR products (10 µL of each) were analyzed on a 1% agarose gel, stained with ethidium bromide, and visualized under UV light (Wealtec, Sparks, NV, USA). Single-band PCR products were purified using the GeneJET PCR Purification Kit. In case where multiple bands were present, the desired DNA bands were excised and purified using the GeneJET Gel Extraction Kit (Thermo).

Nucleotide Sequencing and Computational Analysis

The purified PCR products were cloned into the pCR^{TM}2.1 vector (Thermo Fisher Scientific). Recombinant plasmid DNA was extracted and sequenced using M13F and M13R primers. Sequences were identified using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST). Nucleotide and amino acid sequence alignments of the CSFV strains were analyzed and compared using GENEDOC 2.7 (http://iubio.bio.indiana.edu/soft/molbio/ibmpc/genedoc/readme.html).

RESULTS AND DISCUSSION

Sequence of the E2 gene of three Vietnamese CSFV strains and the C vaccine strains

The PCR amplification of the E2 gene fragment from three virulent CSFV strains and three attenuated vaccine strains produced a product of approximately 1.4 kb in size, using the CSF-E2F and CSF-E2R primer pair. The amplification resulted in a single, high-quality band (Fig. 1). This indicates that the primer pair design and the thermal cycling conditions for the PCR reaction were optimized and suitable for the experiment.

The purified PCR products were cloned into the pCR™ 2.1 vector (Thermo Fisher Scientific). Plasmid DNA was extracted using the GeneJET Plasmid Miniprep Kit (Thermo), and the cloning products were verified using the restriction enzyme *Eco*RI (10U/µL) (Thermo) following the manufacturer's instructions (Fig. 2) before sequencing with the M13F and M13R primer pair.

Based on the sequencing results, the length of the E2 gene for all six CSFV strains was identified as 1,119 nucleotides, coding for 373 amino acids. Phylogenetic analysis indicated that DTLBV belongs to genotype 2.1, CSF.DN6 belongs to genotype 2.2, and CSF.QN7 belongs to genotype 1.1 (data not shown). To date, CSFV strains isolated in Vietnam include genotypes 1.1, 2.1, 2.2, and 2.5. The vaccine strains currently in use belong to genotype 1.1. Therefore, in this study, we focused on analyzing and comparing the uniformity rate as well as the differences in amino acid composition between these genotypes.

The results of comparing nucleotide and amino acid identity percentages of the E2 gene show that, among strains of the same genotype, there is a high similarity rate of over 95% in both nucleotide and amino acid sequences. In contrast, between different genotypes, the similarity rate is much lower, ranging from 87% to 91% for both nucleotide and amino acid sequences. This observed genetic variation between genotypes is

attributed to the high mutation rate and lack of proofreading mechanisms inherent to RNA viral genomes. Specifically, the three vaccine strains exhibit a similarity rate of 93.4% to 94.4% with the field strain of CSFV genotype 1.1. However, they only show a similarity rate of 87% to 89.3% with the field strain of genotype 2.1, 88.3% to 89.2% with the genotype 2.2 strain, and 87.2% to 88.3% with strains of genotype 2.5.

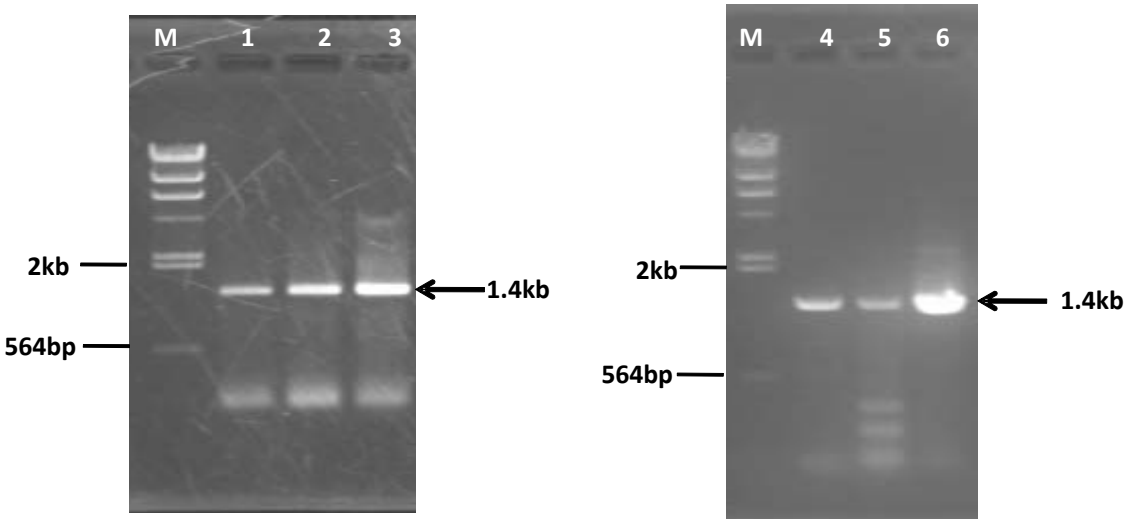

Figure 1. Electrophoresis results of PCR products using the primer pairs CSF-E2F and CSF-E2R for the E2 antigen gene (~1.4 kb) of the classical swine fever virus. M: Lambda DNA/HindIII Marker (Thermo); 1, 2, 3: PCR products in the E2 gene region of three attenuated vaccine virus samples: DTL-VX1, DTL-VX2, DTL-VX3 (1.4 kb); 4, 5, 6: PCR products of the E2 gene region from three virulent CSFV strains: DTLBV, CSF.DN6, and CSF.QN7 (1.4 kb)

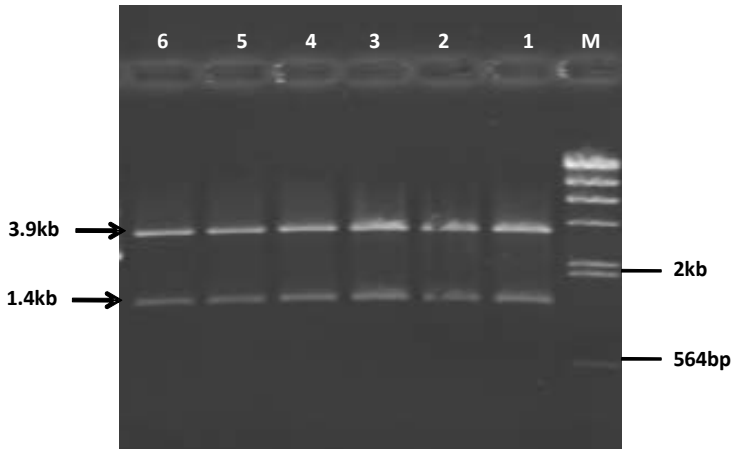

Figure 2. Electrophoresis results of recombinant DNA plasmids from six CSFV strains digested with the *Eco*RI enzyme. 1 µL DNA plasmid (~1µg) each was digested by 10U *EcoRI* enzyme then electrophoresised whole digested product for one well. **M:** Lambda DNA/*Hind*III Marker (Thermo); **1:** DNA plasmid product of the DTLBV strain; **2:** DNA plasmid product of the CSF.DN6 strain; **3:** DNA plasmid product of the CSF.QN7 strain; **4:** DNA plasmid products of the DTL-VX1 strain, 5: DNA plasmid products of the DTL-VX2 strain, 6: DNA plasmid products of the DTL-VX3 strain

Sequence analysis of the E2 gene of Vietnamese CSFV strains

The amino acid sequences of the E2 polypeptide from three strains of the attenuated vaccine strain C currently in use in Vietnam, three strains of CSFV collected in this study, and two strains of CSFV previously isolated in Vietnam (ND9 (KP702208), ND20 (MH979232), HN2018 (MK782041), TB20182 (MK782045) were compared and analyzed. This analysis identified the major antigenic epitope sites and other potential amino acid changes (Fig. 3, Table 2).

The analysis results show that the typical amino acid positions for genotype 1 include: 24G, 36D, 88N, 90S, 91T, 174N, 192E, 195V, 212N and 253S. The corresponding positions in genotype 2 are as follows: 24E, 36G, 88T (in G2.1 and G2.5) and 88S (in G2.2), 174K, 192N (in G2.1 and G2.5) and 192D (in G2.2), 212K and 253E.

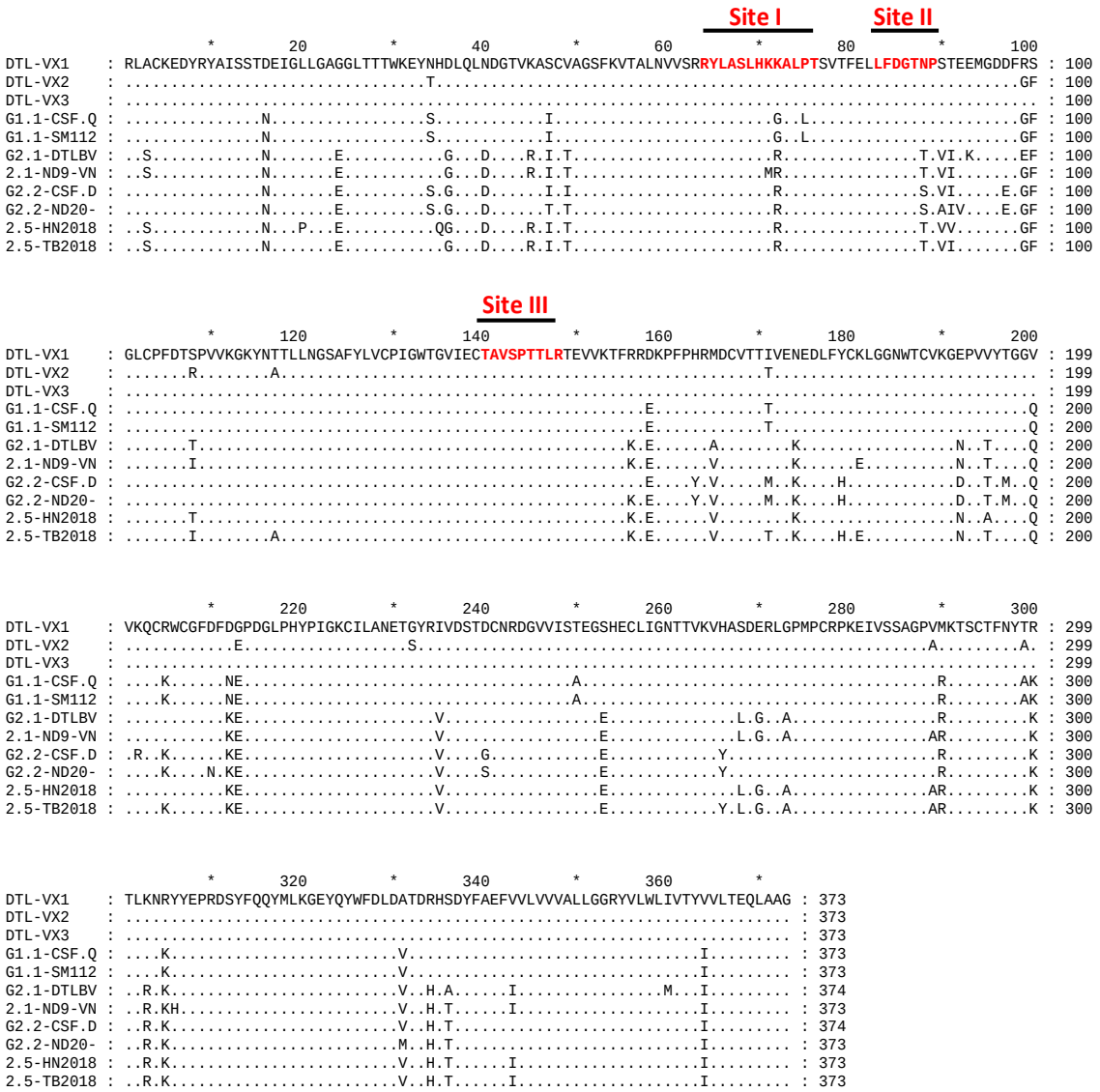

Figure 3. Alignment of the amino acid sequences in the major neutralizing antigenic sites of the E2 protein among Vietnamese field CSFVs compared to vaccine strains

Table 2. The amino acid variations in the E2 protein of Vietnamese CSFV strains

Sites/ Strains	DTL-VX1	DTL-VX2	DTL-VX3	G1.1-CSF.QN7	G1.1-SM1125	G2.1-DTLBV	G2.1-ND9	G2.2-CSF.DN6	G2.2-ND20	G2.5-HY20182	G2.5-TB20182
3	A	A	A	A	A	S	S	A	A	S	S
24	G	G	G	G	G	E	E	E	E	E	E
34	T	N	N	T	T	N	N	T	T	N	N
36	D	D	D	D	D	G	G	G	G	G	G
47	T	I	T	I	I	I	I	I	T	I	I
49	V	V	V	V	V	T	T	V	T	T	T
72	K	E	K	G	G	K	K	K	K	K	K
75	P	P	P	L	L	P	P	P	P	P	P
88	N	N	N	N	N	T	T	S	S	T	T
90	S	S	S	S	S	V	V	V	A	V	V
91	T	T	T	T	T	I	I	I	I	V	I
97	D	D	D	D	D	D	D	E	E	D	D
108	R	S	S	S	S	T	I	S	S	T	I
158	D	D	D	E	E	E	E	E	E	E	E
163	H	H	H	H	H	H	H	Y	Y	H	H
166	N	F	N	N	N	N	N	N	N	N	N
171	T	T	T	T	T	I	I	M	M	I	T
174	N	N	N	N	N	K	K	K	K	K	K
179	Y	Y	Y	Y	Y	Y	Y	H	H	Y	H
192	E	E	E	E	E	N	N	D	D	N	N
195	V	V	V	V	V	T	T	T	T	A	T
197	T	T	T	T	T	T	T	M	M	T	T
200	L	L	L	Q	Q	Q	Q	Q	Q	Q	Q
212	N	N	N	N	N	K	K	K	K	K	K
232	S	G	G	G	G	G	G	G	G	G	G
250	T	T	T	A	A	T	T	T	T	T	T
253	S	S	S	S	S	E	E	E	E	E	E
266	H	H	H	H	H	H	H	Y	Y	H	Y
268	S	S	S	S	S	L	L	S	S	L	L
270	E	E	E	E	E	G	G	E	E	G	G
273	G	G	G	G	G	A	A	G	G	A	A
289	A	V	V	V	V	V	V	A	V	A	A
290	M	R	M	R	R	R	R	R	R	R	R
299	A	A	T	A	A	T	T	T	T	T	T
331	A	V	A	V	V	V	V	V	M	V	V
334	H	R	R	R	R	H	H	H	H	H	H
336	S	S	S	S	S	A	S	S	S	S	S

Note: Amino acids that differ from the vaccine strains are highlighted in red. The amino acids specific to genotype 2.2 are highlighted in purple.

Amino acid sequence comparisons revealed significant deviations between the C strain vaccine strains and virulent strains, with variations observed across genotypes. Specifically, genotype 1 exhibited five distinct amino acid substitutions: 72K/E > G, 75P > L, 158D > E, 200L > Q, and 250T > A. Notably, substitutions at positions 72 (72K > G) and 75 (75P > L) were located within the antigenic epitope Site I. In contrast, genotype 2 displayed a more extensive profile of variations, with thirteen substitutions identified at positions: 24G > E, 36D > G, 49V > T, 88N > T/S, 90S > V/A, 91T > I/V,

158D > E, 174N > K, 192E > N/D, 195V > T/A, 200L > Q, 212N > K, and 253S > E. Furthermore, an amino acid substitution at position 88 (88N > T/S) was observed within the antigenic epitope Site II.

Within genotype 2, subgenotype 2.2 strains share conserved residues with genotype 1 at positions 3A, 34T, 108S, 268S, 270E, and 273G, while differing from subgenotypes 2.1 and 2.5. Notably, subgenotype 2.2 also displays unique conserved residues at positions 88S, 171M, 197M, and 331M, which are absent in all other genotypes.

To date, CSFV strains causing disease in Vietnam include genotypes 1.1, 2.1, 2.2, and 2.5 (Kamakawa et al., 2006; Tran et al., 2009; Izzati et al., 2021; Nguyen et al., 2014). In particular, CSFV genotype 2.5 strains were reported by Izzati and colleagues when isolating pathogenic CSFV strains in some northern provinces in 2018 based on the E2 gene nucleotide sequence phylogeny (Izzati et al., 2021). However, this study did not identify specific amino acid differences between genotypes 2.1 and 2.5 (Fig. 3, Table 2).

Previous studies have demonstrated that mutations at four amino acid positions 34, 36, 49, and 72 have altered the antigenicity and virulence of CSFV, helping the virus evade host immunity (Ji et al., 2014; Hu et al., 2016). Amino acid positions 29, 35, 36, 40, 45, and 49 play an important role in binding monoclonal antibodies to domain C (Chen et al., 2010). The results of this study show that the field strains in Vietnam differ from the currently used vaccine strain C at the following corresponding positions: 72K > G for genotype 1; 36D > G and 49T > V for genotype 2. There are no differences at amino acid positions 29, 34, 35, 40, and 45.

The E2 polypeptide contains three critical antigenic epitope regions: 64RYLASLHKKALPT76 (Site I), 83LFDGTNP89 (Site II), and 140TAVSPTTLR148 (Site III). Sequence comparison of field and vaccine virus strains in Vietnam demonstrated that Site III (140TAVSPTTLR148) is conserved across all field isolates. However, significant amino acid substitutions were observed at antigenic Site I. Specifically, lysine (K) and proline (P) residues in vaccine strains were replaced by glycine (G) and leucine (L) residues in genotype 1 field isolates, resulting in the sequence 64RYLASLHKGALPT76. Furthermore, at antigenic Site II, the asparagine (N) residue in vaccine strains was substituted by threonine (T) in genotypes 2.1 and 2.5, and by serine (S) in genotype 2.2.

Given that many circulating strains belong to genotype 2, while commercial vaccines utilize genotype 1 strains, this discrepancy may contribute to the reduced protection observed with current vaccines. Consequently, antibodies elicited by the vaccine may fail to neutralize field viruses effectively. These epitope site variations likely alter the virus's ability to infect host cells, thereby impacting its pathogenicity.

Amino acid differences at the two antigenic epitope positions (Site I and Site II) are likely to diminish the level of immune protection conferred by vaccine strains against pathogenic strains of genotypes 2.1, 2.2, and 2.5 circulating in our country. Although CSFV genotype 2.5 circulated and caused numerous outbreaks in Northern Vietnam in 2018, our research at that time did not isolate any viruses of that genotype. The development of effective vaccines against the circulating genotypes remains a critical issue in Vietnam, requiring both practical solutions and thorough laboratory research.

CONCLUSION

This study analyzes the characteristics of the E2 gene and has identified three strains of classical swine fever virus circulating in Vietnam, including genotypes 1.1, 2.1, and 2.2. The three vaccine strains currently in use belong to genotype 1.1. There is a conserved antigenic epitope (Site III) between the vaccine strains and field strains; however, however, mutations that alter amino acid residues exist in the remaining two epitopes (Site I and Site II). Additionally, there are

amino acid differences at significant positions in the E2 glycoprotein between genotype 1 and genotype 2. The results of the study highlight the need to monitor the molecular epidemiology of CSFV in Vietnam, as well as to evaluate the protective efficacy of currently used vaccines against virus strains of genotype 2. Developing a vaccine for CSFV genotype 2 is essential to enhance the effectiveness of CSFV disease prevention in our country.

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