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Genotypic diversity of CSFV field strains: A silent risk reduces vaccination efficacy of CSFV vaccines in Vietnam

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ABSTRACT

Classical swine fever (CSF) is a highly contagious, devastating, and transboundary viral disease that afflicts swine industries worldwide. Immunization with vaccines is one of the most effective strategies for controlling this disease. However, shifts in the antigenicity and pathogenicity of novel evolving viral strains have the potential to evade vaccination. In this study, 352 samples from swines exhibiting fever, hemorrhages, lethargy, and diarrhea in different pig farms located in 9 provinces of Vietnam were collected. CSFV was identified even within farms that had been vaccinated against CSFV. Several farms had swine which had been co-infection with CSFV and other pathogens. Copies of the *E2* gene of 21 samples were isolated, cloned, sequenced, analyzed, and compared with copies of *E2* in four vaccine strains. We identified a total of 42 amino acid substitutions in this glycoprotein, including 11 positions that affect the antigenic properties of E2 and 7 positions that are associated with neutralizing epitopes. The E2 glycoprotein of CSFV strains circulating in Vietnam and vaccine strains differ in their antigenicity. These findings provide deep insights into the molecular characteristics, genetic diversity, pathogenicity, antigenicity, and evolution of CSFV strains in Vietnam. Understanding the pathogenicity, antigenicity, and evolution of circulating CSFV strains will provide avenues for developing new vaccines and efficient approaches to control this disease.

1. Introduction

Classical swine fever virus (CSFV) causes hog cholera, one of the most important viral diseases of both domestic and wild pigs. CSFV can be classified into three major groups which comprise 10 subgroups (Paton et al., 2000b). Vaccination remains the optimal available means of protecting pigs from this disease. Most of the live-attenuated vaccine strains used in Vietnam and other countries have been developed against group 1 strains. However, previous work found that subgroup 2.1 and 2.2 strains lately branched away from the vaccine strains and became dominant in Vietnam (Kamakawa et al., 2006). Modern phylogenetic analyses demonstrate that the viral population had shifted from the classical groups (1 or 3) to group two in some countries in Europe and Asia (Deng et al., 2005; Xing et al., 2019).

E2 glycoprotein is a major antigenic determinant and the most immunodominant protein responsible for the production of neutralizing antibodies. The E2 glycoprotein plays a role in viral attachment and entry into target cells. It has been implicated as one of the determinants of virulence. In addition, this glycoprotein has been used as a target for the development of immuno-diagnostics and immuno-prophylactics against CSFV (Bouma et al., 2000). Thus, changes in this gene may lead to changes in the antigenic profile of the virus and, as a

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consequence, may affect the binding and neutralizing capacity of the neutralizing antibodies produced by vaccinated swine against the CSFV field strain.

There are four antigenic domains in the N-terminal half of E2, and these establish two independent antigenic domains: B/C and A/D (at amino acids positions 1–90 and 91–170, respectively). The E2 glycoprotein contains a series of linear neutralizing epitopes which are responsible for the generation of neutralizing antibodies. Therefore, the E2 glycoprotein has been targeted when designing DNA vaccines against CSFV (Qi et al., 2008). Domains B/C is responsible for specific antigenicity in different CSFV strains; domains D/A is a relatively conserved region (Chang et al., 2012). Antigenic differences among various genotypes of CSFVs can be discerned by reaction patterns with specific monoclonal antibodies (mAbs) to the E2 protein (Chang et al., 2010, 2012; Chen et al., 2010; Luo et al., 2013). E2-specific mAbs confer higher binding and neutralizing efficiency against homologous strains, suggesting that the differences between E2 antigens in different CSFV strains are responsible for the differential neutralization abilities between CSFV strains (Chen et al., 2010; Luo et al., 2013). Single point mutations in the antigen can lead to the inability of mAbs to neutralize E2 (van Rijn, 2007; van Rijn et al., 1994). The difference in antigenicity between the Lapinized Philippines Coronel (LPC) vaccine and the field strains appeared to be a consequence of single amino acids. Amino acid modifications on the E2 protein can affect the antigenicity of E2, thereby affecting the cross-neutralization reaction. Antigen-specific amino acids that contribute to neutralization differences have been found mainly in the B/C domains, suggesting that such regions are involved in antibody escape in the vaccine strain and markedly reduce neutralizing antibody titers among heterologous strains.

This study was conducted to identify and analyze nucleotide and amino acid sequence differences (within the E2 gene) between vaccine strains and field strains in different regions around Vietnam. In particular, we were especially interested in identifying and characterizing those variants which contribute the most to changes in vaccine efficacy. This study provides valuable information for future research efforts aimed at CSFV prevention and vaccine development.

2. Materials and methods

2.1. Sample collection

From 2017 to 2019, 352 samples (derived from blood, lymph node, and spleen) from swines exhibiting fever, hemorrhages, lethargy, and diarrhea were submitted to the Viet Han Veterinary Diagnosis Laboratory at Nong Lam University. These samples were derived from various pig farms located in 9 provinces of Vietnam. The provinces from which samples were collected were Ho Chi Minh city, Dong Nai, Ba Ria-Vung Tau, Binh Duong, Lam Dong, Tien Giang, Khanh Hoa, Long An, and Tay Ninh. All available clinical information was also obtained. Samples were processed within 12 h after the reception, and the remains were stored at −20 °C.

2.2. Nucleic acid isolation, reverse transcription/cDNA synthesis, and PCR

We followed the manufacturer's instructions to extract total nucleic acid from samples using the GeneJET Viral DNA/ARN Purification Kit (Thermo Fisher Scientific, USA). Likewise, we followed the manufacturer's protocol to synthesize complementary DNA (cDNA) using the commercial RevertAid Reverse Transcriptase kit (Thermo Fisher Scientific, USA). CSFV was detected by RT-PCR assays using CSF-R (TTYACCACTTCTGTTCTCA) and CSF-F (AGRCCAGACTGGTGGCCNTAYGA) primers (Paton et al., 2000a). The amplicon was verified by its size in 1% agarose gel with Gel-red DNA stain (Merck, Germany).

2.3. Cloning and sequencing of the E2 gene

Among CSFV positive samples, 21 samples were selected for cloning and sequencing. Each sample was selected to represent each farm. The detailed information of the 21 samples was described in Table 1. Amplification of *E2* was performed using a pair of designed primers: CSF-E2F AATTGGATCCCGGTTGTCCTGTAAGGAAG and CSF-E2R TTATGAATTCTTAACCGGCAGCAAGTTGCTC which produced nucleic acids that were 1141 bp in length. The restriction recognition sequences of *Bam*HI and *Eco*RI were included in the primer sequences. The PCR reaction mixture contained 12.5 μl of 2X GoTaq G2 Green Master Mix, 5 μl cDNA, 0.4 μM forward primer, 0.4 μM reverse primer, and 5.5 μl of nuclease-free water to a total reaction volume of 25 μL. Cycling conditions included a 5-min initial denaturation step at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 55 °C, 75 s at 72 °C, and a 7-min elongation step at 72 °C. The E2 gene product was then purified from gel using GeneJET Gel Extraction (Thermo Fisher Scientific, USA) and DNA Cleanup Micro Kit (Thermo Fisher Scientific, USA) per the manufacturer's instructions. The products were then cloned into pGEM-T Easy vector (Promega) and transformed into *E. coli* DH5α (Takara, Japan). The recombinant clones (white colonies on IPTG, X-gal plate) were selected and grown in LB containing 100 μg/ml ampicillin and confirmed using PCR with primers T7 TAATACGACTCACTATAGGG and SP6 ATTTAGGTGACACTATAGAA (Promega), with an expected product size of 1318 bp. Recombinant clones, T7, and SP6 primers were sent to Nam Khoa (Vietnam) Co. Ltd. for sequencing. The *E2* sequences of 21 CSFV strains identified in this study have been deposited in GenBank under the accession numbers MZ869026 - MZ869046.

2.4. Genetic diversity analysis

Sequencing results were processed and analyzed using the Sequencher 5.4.6 software (Genecodes). Nucleotide and amino acid sequences of the samples were analyzed and compared with reference sequences published in Genbank using the BioEdit software (version 7.2.6). Analysis of neutralizing epitopes encoded by the E2 gene of field strains and vaccine strains. The BioEdit software was used to identify amino acid sequence regions that were located on the neutral epitope, relative to the field and vaccine strains.

Table 1
Vaccine status of the 21 CSFV-positive samples.

Name	Location	Year	Vaccine status
HVBD1	Binh Duong	2018	NA
HVDN1	Dong Nai	2017	Mycoplasma, PCV2, CSFV
HVDN10	Dong Nai	2018	Mycoplasma, PCV2
HVDN2	Dong Nai	2017	Mycoplasma, PCV2, FMD, CSFV
HVDN3	Dong Nai	2018	NA
HVDN4	Dong Nai	2018	NA
HVDN5	Dong Nai	2018	NA
HVDN6	Dong Nai	2018	NA
HVDN7	Dong Nai	2018	NA
HVDN8	Dong Nai	2018	PRRS, PCV2, CSFV
HVDN9	Quang Ngai	2018	NA
HVHCM1	HCM	2017	NA
HVKH1	Khanh Hoa	2018	NA
HVLA1	Long An	2017	NA
HVLD1	Lam Dong	2017	PRRS, Aujeszky, FMD, PCV2, Mycoplasma
HVTG1	Tien Giang	2017	NA
HVTN1	Tay Ninh	2017	NA
HVVT1	Vung Tau	2017	NA
HVVT2	Vung Tau	2017	NA
HVVT3	Vung Tau	2018	PRRS, CSFV
HVVT4	Vung Tau	2019	NA

CSFV: Classical swine fever virus; PRRS: porcine reproductive and respiratory syndrome virus; PCV2: porcine circovirus type 2; FMD: Foot-and-mouth disease; NA: Not available.

3. Results

3.1. Status of CSFV infection within samples collected from 2017 to 2019

From 2017 to 2019, 352 samples were taken from farms wherein swines exhibited fever, hemorrhages, lethargy, and diarrhea. Among these samples, 91 tested positive for CVFV (Table S1). CSFV was identified in samples from the farms both with and without swine which had been vaccinated against CSFV. These data indicated that the prevalence of CSFV remains high in Vietnam, even within farms that had been vaccinated against CSFV.

3.2. Nucleotide and amino acid sequence comparisons between the E2 gene in CSFV strains and four vaccine strains

The similarities between the nucleotide and amino acid sequences of the *E2* gene copies in the 21 CSFV strains and the four vaccine strains were found to be significantly low, ranging from 80.6 to 83.5% and 87.3–91.1%, respectively. The nucleotide sequence alignments of these strains showed 82.3–83.5% sequence identity with the GPE strain, 80.6–82.2% sequence identity with the C-strain, 81.2–82.5% sequence identity with the LPC strain, and 81.5–82.9% sequence identity with the Thiverval strain. In agreement with these results, an amino acid sequence alignment showed 89.0–91.1% sequence identity with the GPE strain, 87.6–89.8% sequence identity with the C-strain, 87.3–88.7% sequence difference with the LPC strain, and 87.3–88.7% sequence difference with the Thiverval strain (Table 2). Several important and novel variable sites in the E2 glycoprotein between CSFV vaccine strains and the 21 field strains in this study were found and summarized in Table 3 and Table S2.

3.3. Analysis of amino acid positions associated with antigenic variation of E2 between the 21 CSFV strains and four vaccine strains

The amino acid sequence alignment of E2 revealed that the 21 strains shared 87.3–91.1% sequence identity and 42 variable sites with four vaccine strains (Fig. 1). In particular, the data in our study showed that the E2 glycoprotein of CSFV field strains exhibits significant differences relative to the four vaccine strains.

According to previous work (Chang et al., 2010), E 24 and D 40 were responsible for the antigenic specificity of field strains belonging to genotypes 2 and 3, while residues D 16 and K 72 were specific to the vaccine strain belonging to the classical genotype 1. This finding highlights differences between the CSFV strains of this study and vaccine strains at amino acids in four sites (16, 24, 40, and 72). Changed sites in the 21 CSFV strains were as follows: G24E, N40D (compared against four vaccine strains), D16 N (compared against the C- and LPC vaccine strains), and R72K (compared against the C- and LPC vaccine strains), and R72D (compared against the Thiverval vaccine strain) (Table 3).

Furthermore, we found a significant difference between the vaccine and field strains at amino acid positions 20, 24, 90 of the B/C domains, and it highlights the presence of an amino acid change (G24E) in the vaccine strain (subgroup 1.1) relative to subtype 2.1 and 2.2 proteins. Our results may be rationalized by our previous description of the role played by residues at these positions (16, 20, 24, 34, 90) as major determinants of antigenic alternative of E2 between subgroup 2.1 and the C strain (Chen et al., 2010). The G24E substitution causes significantly enhanced binding of E2 protein from the C-strain to anti-QZ-07 serum. Sequence alignment indicated that the glutamic acid residue at this site was invariant among genotype 2. In contrast, the glycine (G) residue was preserved within the vaccine strains, emphasizing the critical function of this site as a significant determinant of antigenic differences in E2. At position 34, 19 CSFV strains belonging to subgroup 2.1c were homologous with 3 vaccine strains, including GPE, the C-strain, and Thiverval (N34); however, those same strains differ from the LPC vaccine strain (T34). The rest of the CSFV strains collected from the provinces of Long An and Tay Ninh belong to subgroup 2.2, and they differed from 3 vaccine strains – namely, the GPE-strain, C-strain, and Thiverval strain (S34 N), as well as LPC strain (S34T). The valine residue at position 90 was invariable among field strains but different from the vaccine strains, wherein serine appeared at position 90.

In addition, residue R156 was found to be constant in genotypes 1 and 3, thereby indicating its importance for antigenic specificity, whereas E213 has a role in determining the antigenic specificity for both genotype groups 1 and 2 (as described previously by Chang et al. (2012)). This finding (shown in Table 3) indicated that the amino acid at position 156 of four vaccine strains was different from that of 19 CSFV strains belonging to subgroup 2.1c (R156K), whereas it was similar to two CSFV group 2.2 strains. At position 213, 18 CSFV strains in subgroup 2.1c and 2 strains in subgroup 2.2 showed differences from the C-strain (E213G) but were identical to the GPE-, LPC, and Thiverval strains. Notably, the HVVT3 CSFV strain (collected from Vung Tau) differed from other field strains, the GPE-, LPC, Thiverval strains (K213E), and the C-strain (K213G). Together, these results demonstrate that the CSFV field strains circulating in Vietnam differ in the antigenicity of their respective E2 glycoproteins.

Table 2
Nucleotide and amino acid sequence identity (%) between the 21 field strains and four vaccine strains.

CSFV strains	D49533/GPE Japan/1995		Z46258/C-strain China/1996		AY526732/LPC AHRI/Taiwan/2005		EU490425/Thiverval France/2008	
	% nu	% aa	% nu	% aa	% nu	% aa	% nu	% aa
HVBD1	82.9	90.6	81.3	89.2	81.4	87.9	82.1	89.2
HVDN1	83.0	90.8	81.4	89.5	81.7	88.7	82.2	89.5
HVDN10	82.8	89.8	81.3	88.4	81.9	88.4	82.2	89.0
HVDN2	82.3	89.0	80.6	87.6	81.2	87.3	81.5	87.6
HVDN3	82.9	89.8	81.2	88.4	81.8	88.4	82.3	89.0
HVDN4	83.0	90.6	81.2	88.7	81.6	88.2	82.2	89.2
HVDN5	83.5	90.6	82.0	88.7	82.2	88.2	82.7	89.2
HVDN6	82.9	90.3	81.3	89.0	81.6	88.2	82.1	89.0
HVDN7	82.7	89.8	81.2	89.0	81.6	88.2	82.1	89.0
HVDN8	82.7	89.8	81.2	89.0	81.6	88.2	82.1	89.0
HVDN9	82.8	90.3	81.3	89.5	81.7	88.7	82.2	89.5
HVHCM1	82.5	89.8	80.9	88.4	81.8	88.4	81.9	89.2
HVKH1	82.9	89.8	81.0	88.4	81.6	88.4	82.3	89.0
HVLA1	83.3	90.8	81.8	89.5	82.0	88.2	82.5	90.0
HVLD1	83.5	90.6	82.0	88.7	82.2	88.2	82.7	89.2
HVTG1	83.5	90.3	82.0	88.4	82.3	87.9	82.9	89.0
HVTN1	83.5	91.1	82.2	89.8	82.5	88.4	82.9	90.3
HVVT1	82.8	90.6	81.4	89.2	81.6	88.4	82.0	89.2
HVVT2	83.0	90.6	81.5	89.2	81.8	88.4	82.2	89.2
HVVT3	82.8	90.0	81.3	89.5	81.7	88.4	82.2	89.2
HVVT4	82.7	90.3	81.4	89.5	81.7	88.7	82.1	89.5

% nu: % nucleodtide identity, % aa: % amino acid identity.

3.4. Amino acid sites associated with changes in virulence

Four selected sites (amino acids 34, 36, 49, and 72) were determined to confer changes in the antigenicity and virulence of CSFV and to contribute to host immunity evasion (Hu et al., 2016; Ji et al., 2014; Perez et al., 2012). As detailed in Table 3, this result indicated that, relative to the four vaccine strains, there were differences at these specific positions of CSFV in our study. At amino acid 36, the glycine (G) residue was invariant among CSFV samples (except for the HVBD1 strain, which harbored a serine residue at that position), and all of the CSFV samples differed from vaccine strains, which harbored aspartic acid at that position. In addition, the amino acid at position 49 of 19 CSFV samples belonging to sub-genotype 2.1c (T), 2 CSFV strains within sub-group 2.2 (I), and four vaccine strains (V) were different. The amino acids changes at positions 19 and 72 are detailed above. All of these differences may potentially contribute to differences in antigenicity.

Table 3
Summary of important variable sites in the E2 glycoprotein between CSFV vaccine strains and the 21 field strains in this study.

CSFV strain	Subgroup	Aa position											
		16	20	24	34	36	40	45	49	72	90	156	213
GPE-/Japan/1995	1.1	N	L	G	N	D	N	K	V	R	S	R	E
S-strain/China/1996	1.1	D	L	G	N	D	N	K	V	K	S	R	G
LPC/AHRI/Taiwan/2005	1.1	D	L	G	T	D	N	K	V	K	S	R	E
Thiverval/France/2008	1.1	N	L	G	N	D	N	K	V	E	S	R	E
HVBD1	2.1c	N	P	E	N	S	D	R	T	R	V	K	E
HVDN1	2.1c	N	P	E	N	G	D	R	T	R	V	K	E
HVDN10	2.1c	N	L	E	N	G	D	R	T	R	V	K	E
HVDN2	2.1c	N	P	E	N	G	D	R	T	R	V	K	E
HVDN3	2.1c	N	L	E	N	G	D	R	T	R	V	K	E
HVDN4	2.1c	N	P	E	N	G	D	R	T	R	V	K	E
HVDN5	2.1c	N	L	E	N	G	D	R	T	R	V	K	E
HVDN6	2.1c	N	P	E	N	G	D	R	T	R	V	K	E
HVDN7	2.1c	N	P	E	N	G	D	R	T	R	V	K	E
HVDN8	2.1c	N	P	E	N	G	D	R	T	R	V	K	E
HVDN9	2.1c	N	P	E	N	G	D	R	T	R	V	K	E
HVHCM1	2.1c	N	L	E	N	G	D	R	T	R	V	K	E
HVKH1	2.1c	N	L	E	N	G	D	R	T	R	V	K	E
HVLA1	2.2	N	L	E	S	G	D	K	I	R	V	R	E
HVLD1	2.1c	N	L	E	N	G	D	R	T	R	V	K	E
HVTG1	2.1	N	L	E	N	G	D	R	T	R	V	K	E
HVTN1	2.2	N	L	E	S	G	D	K	I	R	V	R	E
HVVT1	2.1c	N	P	E	N	G	D	R	T	R	V	K	E
HVVT2	2.1c	N	P	E	N	G	D	R	T	R	V	K	E
HVVT3	2.1c	N	P	E	N	G	D	R	T	R	V	K	K
HVVT4	2.1c	N	P	E	N	G	D	R	T	R	V	K	E

3.5. Amino acid sites associated with reactivity with monoclonal antibodies

Amino acids positions 29, 35, 36, 40, 45, and 49 (which are known to be important for mAb binding to domain C (Chen et al., 2008; Dong et al., 2006)) were variable except amino acids at positions 29 and 35. S35D was found in CSFV samples collected from the province of Binh Duong, and Glu 35 appeared as the alternative amino acid in the remaining 20 CSFV strains. The substitution N40D was present in all field strains. With the exception of two viruses (HVLA1, HVTN1), position 45 in the field strains (which was occupied by an arginine residue) differed from the vaccine strains (which contained lysine at position 45) (Table 3). The remaining positions of interest are detailed above. Changes in amino acid residues in the antigenic domains of the virus may compromise vaccine efficacy.

3.6. Amino acid sites associated with important neutralizing epitopes of E2

Comparisons between neutralizing epitopes located on the B/C and D/A domains of the 21 CSFV viral RNA samples and four vaccine strains enabled us to better evaluate differences in vaccine efficacy against field-related CSFV strains. According to Chang et al., domains B/C contained ^{64}RYLASLHKKALPT^{76} and ^{83}LFDGTNP^{89} neutralizing epitopes, which are known to be critical for maintaining the structural integrity of this epitope's conformation (Chang et al., 2010).

Amino acid sequence alignments identified amino acid substitutions in the epitope regions ^{64}RYLASLHKKALPT^{76} and ^{83}LFDGTNP^{89} of the 21 field strains relative to four vaccine strains, and changes in these two epitopes appear at amino acid positions 72 and 88, respectively. A change from D88T was identified in 18 samples in genotype 2.1c. The D88A substitution was found in an isolate from Tien Giang (subgroup 2.1c), while another strain within subgroup 2.2 (from Long An and Tay Ninh) harbored the substitution D88S (Fig. 1).

In contrast, the sequence ^{140}TAVSPTTLR^{148} on domains D/A is a discontinuous epitope that is highly conserved among the CSFV strains. Except for amino acid position 141 in the GPE vaccine strain, we found that the sequence was unchanged in this region (Fig. 1).

Furthermore, Fig. 1 shows the differences between the neutralizing epitope in the vaccine strains and field strains. The substitution of Arg by Lys was found at position aa 156 of the CSFV strain belonging to subgroup 2.1c. M165 and D174 in the 21 field strains were substituted by V65 and K174, respectively. Except for HVDN5, CSFVs collected from Khanh Hoa, Binh Duong, Dong Nai, and Ba Ria Vung Tau harbored the substitution T171I, while the T171 in CSFVs obtained from Long An and Tay Ninh was changed to M171.

4. Discussion

In general, RNA viruses exhibit significantly greater mutation rates than DNA viruses. The substitution rates range between 1.03×10^{-4} substitutions per site per year. This difference is attributed to the error-prone nature of viral RNA polymerases (Kwon et al., 2015), and the resultant increased variation in RNA viruses gives rise to greater genetic distance, thereby making it more likely to diminish the efficacy of the vaccines against CSFV. E2 is the major immunogenic among the CSFV proteins. It induces neutralizing antibodies and protection against lethal infection (Hulst et al., 1993). To better understand the factors that affect vaccine efficacy, and to broaden our knowledge of CSFV evolution and epidemiology in Vietnam, we analyzed the nucleotide and amino acid sequences of E2 from 21 field strains and then compared these sequences to those in four vaccine strains.

We found that CSFV infection was highly prevalent in swine farms throughout the provinces of Vietnam and CSFV infection occurs in farms with or without swine that had been vaccinated against CSFV. Although CSFV infection has generally been well controlled by vaccination efforts, recent reports have indicated that the immunity induced by live attenuated vaccines may not always protect hosts in clinical applications (Coronado et al., 2019). CSF outbreaks in a large number of CSFV-vaccinated farms throughout China have been reported previously (Luo et al., 2017). In Vietnam, many herds contracted the disease, and fatal epizootics were found in farms vaccinated against CSFV (Izzati et al., 2021; Kamakawa et al., 2006). Several reasons have been given to explain how CSFV may escape CSF vaccination-induced immunity. On prominent theory postulates that novel CSVF subtypes (with new genetic variations and antigenic alterations) may be responsible for vaccine failure (Kamakawa et al., 2006; Luo et al., 2017). Recent studies reported that viral populations have switched from the classical subtype

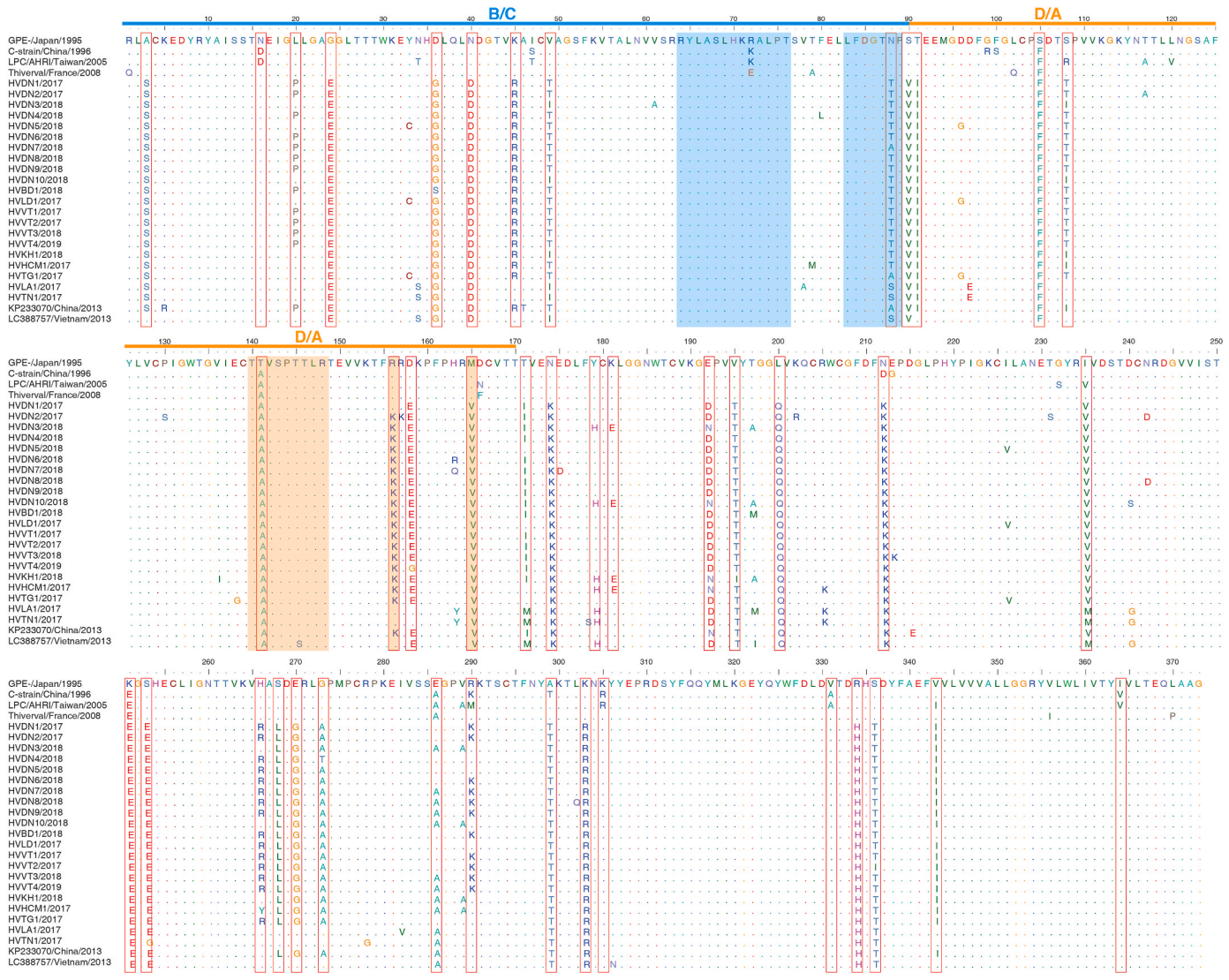

Fig. 1. Mutations in the E2 protein of CSFV of four vaccine strains compared with the 21 field strains. Amino acid sequence alignments of E2 from the 21 field strains and four reference vaccine strains. Dots indicate the amino acid position in the GPE vaccine strain; letters indicate amino acid changes. The blue and orange lines indicate the position of domains B/C and D/A, respectively. The red box demarcates the position of the important linear epitope. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

1 or 3 to subtype 2 in most Asian and European countries (Deng et al., 2005; Xing et al., 2019). Notably, circulating CSFV branched away from the vaccine strains and became more dominant (Chen et al., 2008). A change in the nucleotide sequence can change the amino acid sequence in such a way that leads altered antigenicity of E2. Recombinant E2 proteins are effective against homologous CSFV strains (Bouma et al., 2000; Hulst et al., 1993). We found that all 21 CSFV strains in this study clustered into subgroups 2.2 or 2.1c (Nguyen et al., 2021), whereas all current vaccines in Vietnam, were developed against strains in subtype 1 (Kamakawa et al., 2006). Together, these findings support the notion that circulating CSFV strains may carry genetic variations and antigenic alterations that may indeed reduce vaccine efficacy.

Naturally, any mutation that changes the position and the composition of amino acids may lead to changes in the properties and functionality of a given variant-harboring protein. Changes in several sites (17, 34, 36, 49, and 72) have previously been implicated as contributing to changes in virulence and promoting immune evasion (Hu et al., 2016; Ji et al., 2014; Perez et al., 2012). Amino acids at 29, 35, 36, 40, 45, and 49 sites are essential for recognition by mAb (Chen et al., 2008; Dong et al., 2006). In addition, single mutations at amino acid positions 21, 35, 36, 40, 45, 49, 134, 144, 145, and 148 can ablate the neutralizing ability of mAbs or swine antisera (van Rijn et al., 1994). Our findings identify several amino acid sites (34, 36, 40, 45, 49, and 72) that differ from those of the four vaccine strains.

This suggests these same mutations might diminish the ability of existing antibodies to neutralize CSFV field strains, making current vaccines less effective in Vietnam.

Domains D/A are relatively conserved, while domains B/C are responsible for antigenic specificity among different CSFV strains (Chang et al., 2010). Several important antigenic regions that are associated with both conformational and linear epitopes have been identified in E2. The residues 1–84/111 and 77–111/177 of the B/C/D/A domains mediate the ability of mAbs to recognize conformation-dependent epitopes (van Rijn et al., 1993, 1994). Residues E24 and D40 were constant in all CSFV field strains, whereas residues G24 and N40 were present in current vaccine strains. D16 N, R72K, and R72D were found in CSFV field strains compared to vaccine strains. Residues E24 and D40 have a function in the antigenic specificity of field strains, whereas residues D16 and K72 play a role in the antigenic specificity of the vaccine strain (Chang et al., 2010). E2 variations of

field strains affect the reaction patterns and cross-neutralization ability of anti-CSFV sera and mAbs against themselves (Chen et al., 2010). These findings provide molecular evidence that the vaccines fail to protect swine against circulating CSFV.

According to previously published results, a series of C-strain-based recombinant E2 mutants (with single N-terminal amino acid alterations between subtype 2.1 and the C strain) were used to identify specific residues that are essential for recognition by mAb-2B6 derived from the B/C domains of the C-strain (Tong et al., 2015). In this study, the N88S substitution at the C-terminus of the B/C domains was found in two CSFV strains in subgroup 2.2. This substitution induces a ~30% reduction in binding to mAb-2B6, relative to wild-type rE2-BC. Each of the three residues within the C-strain epitope ^{85}DGXNP^{89} has an equal role in binding to mAb-2B6 (Tong et al., 2015). Amino acid sequence alignments of E2 (positions 1–111) from many CSFV isolates have demonstrated that the mAb-2B6 epitope is conserved in group 1 but not in group 2 strains. The residue N88 is strongly conserved in genotype 1, but it varies in those of genotype 2, thereby revealing its potential to constitute a principal determinant of differences in antigenicity. These results may be rationalized in light of previous reports that residue 88 of the E2 epitope is recognized by the mAb HQ06 and significantly affects its specificity (Peng et al., 2008). Although not involved in neutralization, the mAb-2B6 epitope ^{85}DGXNP^{89} may play a role in maintaining the conformational structure of the B/C domains that influence binding to polyclonal sera (Chang et al., 2010).

Various reactivity patterns with mAbs have provided clues regarding the antigenic variation in E2 between different CSFV genotypes (Chang et al., 2010; Luo et al., 2013). E2-specific mAbs have higher binding and neutralizing efficiency against homologous strains. It suggests that variation in the E2 antigens in CSFV strains is responsible for the differential neutralization abilities between CSFV isolates (Chen et al., 2010; Luo et al., 2013). Single point alterations that may induce complete loss of mAbs or pig anti-CSFV serum binding against CSFV were identified at N36, D40, and K45 in domains B/C (van Rijn et al., 1994). Furthermore, the difference in antigenicity between the LPC vaccine and the field strains appeared in the amino acids at D16, E24, D40, and K72 on the antigenic units B/C, as well as at R156 in domain A and E214 at the C-terminal region of genotypes 1, 2, and 3 (Chang et al., 2010). The amino acid substitutions D16 N, L20P, G24E, N34S, or S90A between the antigenic B/C units of vaccine and field strains significantly enhanced heterologous binding to pig anti-CSFV serum (Chen et al., 2010). These substitutions were also found in CSFV circulating in Vietnam. Amino acid substitutions on the E2 glycoprotein may also change antigenicity and cross-neutralization. Specific amino acids of the antigen that contribute to the differential neutralizing abilities have been located mainly in the B/C domains, thereby implicating those positions as major determinants of antigenic variation associated with antibody escape in the vaccine strains and markedly reduce neutralizing antibody titers among heterologous strains.

5. Conclusion

These results demonstrate that coinfection of CSFV with other pathogens is common in swine farms. CSFV vaccine strains and circulating strains in Vietnam differ in the antigenicity of their respective E2 glycoproteins. A total of 42 amino acid positions were found to differ between the 21 RNA viral samples in our study and four vaccine strains. In particular, 11 amino acid positions were found to affect the antigenic properties of the E2 protein (16, 20, 24, 36, 40, 45, 49, 72, 90, 156, 213) and 7 amino acid sites were found to be associated with neutralizing epitopes (72, 88, 141, 156, 165, 171, 174). The antigenic alterations in the E2 glycoprotein of the CSFV strains in Vietnam (relative to the four vaccine strains) may provide a compelling explanation for the limitations of current vaccination efforts. As a result, it will be crucial for novel vaccine development to take these factors into account.

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Availability of data and material

The *E2* sequences of 21 CSFV strains identified in this study have been deposited in GenBank under the accession numbers MZ869026 - MZ869046.

CRediT authorship contribution statement

Ngoc Hai Nguyen: Data curation, Writing – review & editing, Formal analysis, designed the study, analyzed the data, wrote the manuscript. **Binh Nguyen Thi Phuong:** Data curation, Formal analysis. **Trung Quan Nguyen:** performed experiments. **Duy Do Tien:** Writing – review & editing, designed the study, wrote the manuscript. **My Duyen Nguyen Thi:** Data curation, Writing – review & editing, Formal analysis, analyzed the data, wrote the manuscript. **Minh Nam Nguyen:** Data curation, Writing – review & editing, Formal analysis, designed the study, analyzed the data, wrote the manuscript.

Declaration of competing interest

All authors have read the journal's policy on disclosures of potential conflicts of interest, and we declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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