GENETIC CHARACTERIZATION OF E2 GENE OF CLASSICAL SWINE FEVER VIRUS CIRCULATING IN NAM DINH AND HAI DUONG PROVINCES

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ABSTRACT

Classical swine fever (CSF), a highly infectious viral disease of swine, has caused great economic losses to the swine industry worldwide. Previous reports showed that CSF virus (CSFV) circulating in the Mekong delta area of South Vietnam between 2002 and 2003 belonged to group 2. In this study, to identify the predominant CSFV strains currently circulating in North Vietnam, the full-length E2 gene of five CSFV isolates collected from Hai Duong and Nam Dinh provinces of Vietnam in 2014 were sequenced and analyzed. The results of nucleotide (nt) and amino acid (aa) comparison showed that the 5 field CSFV isolates in this study shared 85.5 - 99.7% nt and 92.7 - 99.7% aa identity. Phylogenetic analysis of the complete E2 gene sequences revealed that the CSFV isolates collected from two farms (one in Ninh Giang - Hai Duong and one in My Xa - Nam Dinh) belonged to subgroup 2.1, whereas CSFV isolates collected in the Xuan Truong - Nam Dinh farm clustered within subgroup 2.2.

Keywords: CSFV, E2 gene, Phylogenetic tree.

Đặc điểm di truyền gen E2 của virus dịch tả lợn lưu hành tại tỉnh Nam Định và Hải Dương

TÓM TẮT

Dịch tả lợn là một bệnh truyền nhiễm nguy hiểm trên lợn do virus gây ra, bệnh gây thiệt hại kinh tế nghiêm trọng cho ngành chăn nuôi lợn trên toàn thế giới. Các nghiên cứu trước đây cho thấy virus gây bệnh dịch tả lợn ở đồng bằng sông Cửu Long trong giai đoạn 2002 - 2003 thuộc nhóm 2. Trong nghiên cứu này, để xác định được các chủng virus dịch tả lợn đang lưu hành phổ biến ở miền bắc Việt Nam hiện nay, toàn bộ trình tự gene E2 của 5 chủng virus dịch tả lợn VNUA/HD1, VNUA/ND2, VNUA/ND9, VNUA/ND20 và VNUA/ND21 thu thập được tại tỉnh Nam Định và Hải Dương của Việt Nam năm 2014 đã được giải trình tự và phân tích trình tự gene. Kết quả so sánh trình tự nucleotide (nt) và acid amin (aa) cho thấy 5 chủng virus có tỷ lệ tương đồng với nhau về nt là 85,5 - 99,7% và về aa là 92,7 - 99,7% aa. Kết quả phân tích về mối quan hệ di truyền cho thấy, chủng virus thu thập ở Mỹ Xá - Nam Định và Ninh Giang - Hải Dương thuộc nhóm 2.1, trong khi đó các chủng virus thu thập ở Xuân Trường - Nam Định được xếp vào nhóm 2.2.

Từ khoá: Cây phả hệ, dịch tả lợn, Gen E2.

1. INTRODUCTION

Classical swine fever (CSF) is a highly contagious disease of swine and wild boars, causing significant economical losses in various parts of the world. Classical swine fever virus (CSFV), an enveloped virus containing a single-stranded, positive-sense RNA genome of approximately 12.3 kb, belongs to the genus *Pestivirus* within the family *Flaviviridae* (Meyers *et al.*, 1989). The genome of CSFV consists of 5'- and 3'-untranslated regions flanking a single ORF that encodes four structural (core, E^{rns}, E1, and E2) and eight

non-structural (Npro, p7, and NS2–NS5B) proteins. The E2 protein of CSFV is essential for virus attachment, entry into target cells, and cell tropism (Wang *et al.*, 2004; Liao *et al.*, 2016). The E2 protein also induces neutralizing antibodies and is a determinant of virulence (Risatti *et al.*, 2005; Liao *et al.*, 2016;).

CSFV is classified into three groups (1, 2, and 3), and each group comprises three to four subgroups (1.1 - 1.4, 2.1 - 2.3, and 3.1 - 3.4) *(Lowings et al., 1996; Paton et al., 2000; Postel et al.*, 2013). Previous reports indicated that CSFV strains circulating in China during the 1990s clustered into subgroups 1.1, 2.1, 2.2, and 2.3, however, the majority of CSFV strains belonged to either subgroup 2.1 (49.1%) or 2.2 (36.4%) (Tu *et al.*, 2001; Sun *et al.*, 2013). In Vietnam, although some pigs have been vaccinated against CSF, many pigs still die because commercial vaccines fail to provide sufficient protection (Holland *et al.*, 2003). A previous study showed that CSFV strains isolated in the Mekong delta area of South Vietnam between 2001 and 2003 clustered into one of two subgroups: the majority clustered into subgroup 2.1 and the remainder clustered into 2.2 (Kamakawa *et al.*, 2006). The aim of this study is to understand the molecular characterization and phylogenetic analysis of the complete E2 gene of CSFV isolates collected in 2014 in Hai Duong and Nam Dinh provinces of Vietnam.

2. MATERIALS AND METHODS

2.1. Samples collection

Five CSFV- positive porcine blood plasma samples collected from Nam Dinh and Hai Duong provinces of Vietnam in 2014 were used in this study. Samples are described in table 1 with Hai Duong (N = 1) and Nam Dinh (N = 4).

2.2. RT-PCR and complete E2 gene sequencing

To identify CSFV, total RNA was extracted from whole blood samples using the QiaAmpRNA blood Mini kit (Qiagen, USA) as described by the QIAmp Viral RNA Mini kit protocol (Qiagen, USA). The extracted RNA was then directly subjected to RT-PCR using a one-step RT-PCR kit (Qiagen, USA). The RT-PCR conditions and specific primers used to amplify the complete E2 gene of CSFV were reported previously (Paton *et al.*, 2000). PCR products of the expected size were cloned into the pGEM-T Vector System II™ (Cat. No. A3610; Promega, USA). The cloned genes were sequenced at the Macrogen Institute (Macrogen Co. Ltd) using the T7 and SP6 sequencing primers and an ABI Prism 3730_{XI} DNA Sequencer.

2.3. Phylogenetic analysis

Reference sequences were obtained from GenBank. All sequences were aligned using the CLUSTAL X alignment program (Thompson *et al.*, 1997). The alignment (aln) files were converted to MEGA (meg) files, and a neighbor-joining tree test was run in the MEGA 6.06 program (Tamura *et al.*, 2013) using 1,000 bootstrap replications for each nucleotide sequence.

3. RESULTS AND DISCUSSION

3.1. Phylogenetic analysis

In this study, to determine the distribution profile of CSFV outbreak strains, the full-length E2 genes of 5 CSFV isolates (VNUA/HD1, VNUA/ND2, VNUA/ND9, VNUA/ND20, and VNUA/ND21) collected from Nam Dinh and Hai Duong provinces of Vietnam in 2014 were sequenced and analyzed. Phylogenetic analysis based on the complete E2 gene sequences of CSFV revealed that the CSFV isolates collected from Nam Dinh and Hai Duong provinces in 2014 belonged to two different subgroups, 2.1 and 2.2, of group 2. To be specific, The VNUA/ND9 and VNUA/HD1 strains collected from My Xa-Nam Dinh and Ninh Giang-Hai Duong, respectively, belonged to subgroup 2.1, whereas three strains (VNUA/ND2, VNUA/ND20, and VNUA/ND21) from the Xuan Truong-Nam Dinh belonged to subgroup 2.2 according to phylogenetic analysis (Fig. 1).

Table 1. Information about the CSFV- positive porcine blood plasma samples used in this study

Sample name	Collection date	Geographic origin (province)	Type of sample
VNUA/HD1	July, 2014	Hai Duong (Ninh Giang)	Whole Blood
VNUA/ND2	June, 2014	Nam Dinh (Xuan Truong)	Whole Blood
VNUA/ND9	June, 2014	Nam Dinh (My Xa)	Whole Blood
VNUA/ND20	June, 2014	Nam Dinh (Xuan Truong)	Whole Blood
VNUA/ND21	June, 2014	Nam Dinh (Xuan Truong)	Whole Blood

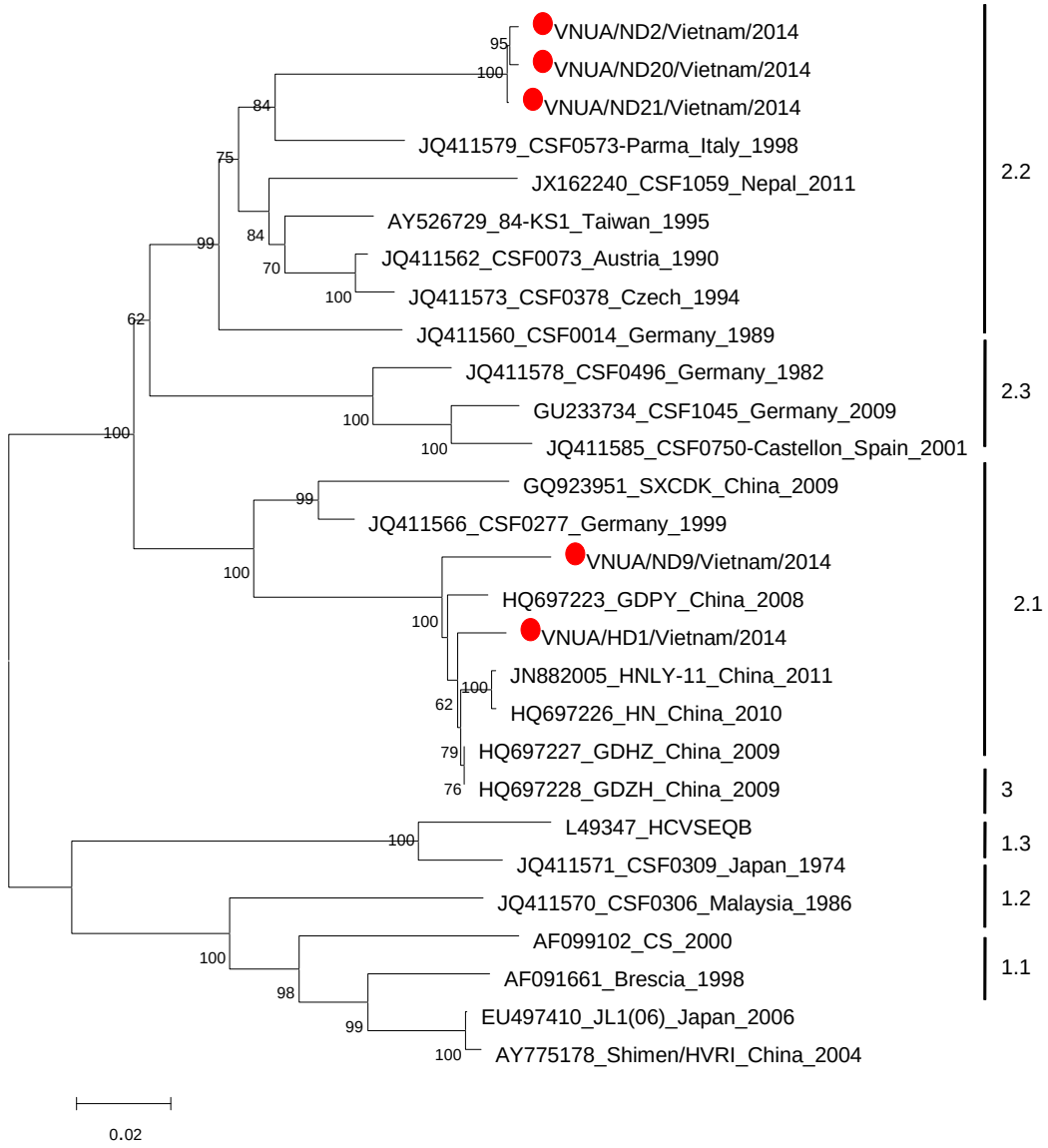

Figure 1. Phylogenetic tree based on the nucleotide sequences corresponding to the full length E2 gene sequences of CSFV

Note: The numbers at each branch represent the boostrap values greater than 60% of 1000 replicates. The scale bar indicates the genetic distance

The results of the phylogenetic analysis also showed that Vietnamese CSFV isolates of subgroup 2.1 were closely related to GDPY, GDHZ, HN, and HNLY-11 strains prevalent in China between 2008 and 2011. This finding suggests that the former may have entered Vietnam across the border with southern China. The phylogenetic tree also indicated that subgroup 2.2 strains circulating in Vietnam were separately related to other strains belonging to this subgroup isolated from European countries (Germany, Austria, the Czech Republic, and Italy) as well as CSFV strains isolated in Asia (Taiwan and Nepal) (Fig. 1). This finding involoving the Vietnamese strains within subgroup 2.2 was interesting as it was unclear about the relationship these strains had with other subgroup 2.2 strains around the world, and how they entered into Vietnam, so further genetic analyses of strains from neighboring countries (e.g., Cambodia, Laos, and China) may clarify this.

3.3. Genetic diversity of the E2 gene

The envelope glycoprotein E2 of CSFV is a glycoprotein exposed on the surface of the virion, and is constantly under negative or positive selection forces. Therefore, genetic comparison of the E2 gene sequences of different groups of CSFV may give insight into the diversity of the E2 gene, the potential of immune escape, development of vaccines, and disease control (Chen *et al.*, 2008).

In the present study, comparative nucleotide (nt) and amino acid (aa) sequence analysis of the complete E2 gene of CSFV showed that the Vietnamese strains of subgroup 2.1 (VNUA/HD1 and VNUA/ND9 strain) and subgroup 2.2 (VNUA/ND2, VNUA/ND20 and VNUA/ND21 strain) shared 85.5 - 86.5% nt and 92.7 - 93.5% aa identity (Tables 2 and 3).

When compared within the same subgroup, the VNUA/ND9 strain (from My Xa-Nam Dinh) and the VNUA/VNUA/HD1 strain (from Ninh Giang-Hai Duong) of subgroup 2.1 showed high sequence similarity with complete E2 genes (96.4% at the nucleotide (nt) level and 97.3% at the amino acid (aa) level). Similarity, the three strains (VNUA/ND2, VNUA/ND20 and VNUA/ND21) from the Xuan Truong-Nam Dinh region of subgroup 2.2 also showed very high similarity (99.6 - 99.7% at the nt level and 99.1 - 99.7% at the aa level) with complete E2 gene sequences (Tables 2 and 3).

Table 2. Nucleotide identity among Vietnamese CSFV strains and reference strains

No.	Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	VNUA/HD1/Vietnam/2014																
2	VNUA/ND9/Vietnam/2014	96.4															
3	VNUA/ND2/Vietnam/2014	86.3	85.5														
4	VNUA//ND20/Vietnam/2014	86.3	85.5	99.6													
5	VNUA/ND21/Vietnam/2014	86.5	85.7	99.7	99.7												
6	JN882005 HNLY11 China 2011	98.2	96.6	86.4	86.4	86.5											
7	HQ697227 GDHZ China 2009	98.8	97.3	86.9	86.9	87.1	99.1										
8	HQ697226 HN China 2010	98.2	96.6	86.4	86.4	86.5	99.8	99.1									
9	HQ697223 GDPY China 2008	97.8	96.8	86.6	86.6	86.8	98.2	98.8	98.2								
10	HQ697228 GDZH China 2009	98.8	97.3	86.9	86.9	87.1	99.1	100	99.1	98.8							
11	JQ411579 CSF0573 Italy 1998	87.8	86.9	92.7	92.7	92.8	88.2	88.3	88.2	88.1	88.3						
12	JQ411562 CSF0073 Austria 1990	88.8	87.8	91.7	91.7	91.8	88.7	89.2	88.7	88.6	89.2	94.9					
13	JQ411573 CSF0378 Czech 1994	88.3	87.3	91.5	91.5	91.6	88.2	88.8	88.2	88.3	88.8	94.4	98.9				
14	JQ411560 CSF0014 Germany 1989	88.3	87.1	90.5	90.5	90.6	88	88.5	88	88.2	88.5	93.2	93.9	93.2			
15	JX162240 CSF1059 Nepal 2011	86	85.2	89	89	89	86.2	86.7	86.2	86.3	86.7	91.5	93.4	92.7	90.5		
16	AY526729 84KS1 Taiwan 1995	88.8	87.8	91.6	91.6	91.7	88.5	89.2	88.5	88.8	89.2	94.6	96.6	95.8	93.5	92.8	

Table 3. Amino acid identity among Vietnamese CSFV strains and reference strains

No.	Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	**VNUA/HD1/Vietnam/2014**																
2	**VNUA/ND9/Vietnam/2014**	97.3															
3	**VNUA/ND2/Vietnam/2014**	93.2	93														
4	**VNUA//ND20/Vietnam/2014**	93	92.7	99.1													
5	**VNUA/ND21/Vietnam/2014**	93.5	93.2	99.7	99.4												
6	JN882005 HNLY11 China 2011	97.8	97.8	93.2	93	93.5											
7	HQ697227 GDHZ China 2009	98.6	98.6	93.8	93.5	94.1	99.1										
8	HQ697226 HN China 2010	97.8	97.8	93.2	93	93.5	99.4	99.1									
9	HQ697223 GDPY China 2008	98.3	98.3	93.5	93.2	93.8	98.9	99.7	98.9								
10	HQ697228 GDZH China 2009	98.6	98.6	93.8	93.5	94.1	99.1	100	99.1	99.7							
11	JQ411579 CSF0573 Italy 1998	93.5	93.2	95.4	95.1	95.7	93.8	94.1	93.8	93.8	94.1						
12	JQ411562 CSF0073 Austria 1990	94.6	93.8	96.2	95.9	96.5	94.1	94.6	94.1	94.3	94.6	96.7					
13	JQ411573 CSF0378 Czech 1994	94.3	93.5	95.9	95.7	96.2	93.8	94.3	93.8	94.1	94.3	96.5	99.1				
14	JQ411560 CSF0014 Germany 1989	93.5	92.7	95.1	94.9	95.4	93	93.5	93	93.2	93.5	95.7	96.7	96.5			
15	JX162240 CSF1059 Nepal 2011	92.7	92.2	93.8	93.5	94.1	92.7	93	92.7	92.7	93	95.4	96.2	95.4	94.9		
16	AY526729 84KS1 Taiwan 1995	93.8	93	95.7	95.4	95.9	93.2	93.8	93.2	93.5	93.8	96.5	97.8	97.5	96.5	95.4	

When compared to other referenced CSF strains, the VNUA/HD1 strain in subgroup 2.1 showed high E2 gene sequence similarity with five Chinese isolates (GDPY, GDHZ, GDZH, HNLY-11, and HN) within subgroup 2.1 (97.8 - 98.8% at nt and 97.8 - 98.6% at aa levels), whereas the Vietnamese strains in subgroup 2.2 showed much lower similarity (nt/aa): VNUA/ND2 (86.4 - 86.9%/93.2 - 93.8%), VNUA/ND20 (86.4 - 86.9%/93.0 - 93.5%), and VNUA/ND21 (86.5 - 87.1%/93.5 - 94.1%). However, the complete E2 nucleotide sequences of strains VNUA/ND2, VNUA/ND20, and VNUA/ND21 of subgroup 2.2 were more similar to those of strains of subgroup 2.2 isolated from European countries (CSF0378, CSF0573, CSF0073, and CSF0014), sharing 90.5 - 92.8% at nt and 94.9 - 96.5% at aa (Tables 2 and 3).

The E2 protein is the most immunogenic CSFV protein, containing four antigenic domains (A - D) within the N-terminal half. The domain A itself includes three subdomains (Weiland *et al.*, 1990; Van Rijn *et al.*, 1993; Van Rijn *et al.*, 1994; Wonnemann *et al.*, 2001). Previous reports identified a series of linear neutralizing epitopes located in the B, C, and A1 domains (Lin *et al.*, 2000; Dong and Chen, 2006; Dong *et al.*, 2006; Zhang *et al.*, 2006).

In the present study, an epitope (aa ^{141}-AVSPTTLRTE-^{150}) recognized by a CSFV-specific murine mAb was found to be conserved among all the Vietnamese strains. Another epitope (aa ^{306}-YYEP-^{309}) recognized by an pestivirus-reactive murine mAb was conserved within VNUA/HD1, VNUA/ND2, VNUA/ND20, and VNUA/ND21, but the epitope in VNUA/ND9 was slightly different (aa ^{306}-HYEP-^{309}). Analyses using overlapping peptides revealed that aa sequence ^{4}-CKEDYRY-^{10} was conserved in all Vietnamese strains; however, epitopes aa 103 - 125 and aa 155 - 176 were less well-conserved. The transmembrane domain (aa 342 - 366) located within the C-terminal half of the E2 protein expressed by the VNUA/ND2, VNUA/ND20, and VNUA/ND21 strains was well-conserved, although a single aa substitution in the E2 transmembrane domain of VNUA/HD1 and VNUA/ND9 (aa ^{343}V↔^{343}I) was identified.

Three conserved regions (CRs) at aa 109 - 155, 306 - 330, and 337 - 373 (Garry and Dash, 2003) within the E2 protein of the Vietnamese strains were hydrophobic. One variable region (VR1) was located within the antigenic domains B and C of the N-terminal region (aa 3 - 59), whereas another (VR2) was located in the

central part of E2 (at aa 156 - 212 within antigenic domain A) (Chen *et al.*, 2008). A previous study reported that the variable amino acids in the putative VR1 and VR2 of subgroups 2.1 and 2.2 were thought to be located at aa positions 8, 29, 35, 36, 40, 45, 49, 156, 158, 165, 171, and 174 (Chen *et al.*, 2008). Among those aa positions, the variable amino acids at positions 8, 156, 158, 165, 171, and 174 were important because they were located within neutralizing epitopes (Dong and Chen, 2006; Dong *et al.*, 2006), whereas those at positions 29, 35, 36, 40, 45, and 49 were important for mAb binding (Van Rijn *et al.*, 1994). In this study, two aa substitutions were found at positions 45 (R↔K) and 171 (I↔M) when we compared the Vietnamese CSF virus subgroups 2.1 and 2.2 (Fig. 2).

The analysis results of the deduced aa sequence variation of the complete E2 gene showed that there were 19 aa substitutions at positions ^{3}S↔^{3}A, ^{34}N↔^{34}S, ^{45}R↔^{45}K, ^{47}I↔^{47}T, ^{88}T↔^{88}S, ^{90}V↔^{90}A, ^{92}E↔^{92}V, ^{97}D↔^{97}E, ^{108}I↔^{108}S, ^{163}H↔^{163}Y, ^{171}I↔^{171}M, ^{179}Y↔^{179}H, ^{192}N↔^{192}D, ^{197}T↔^{197}M, ^{205}R↔^{205}K, ^{210}D↔^{210}N, ^{240}D↔^{240}S, ^{273}A↔^{273}G, and ^{243}I↔^{243}V when we compared the Vietnamese CSFV strains subgroups 2.1 and 2.2 (Fig. 2). This finding demonstrated that the present Vietnamese CSFV isolates subgroups 2.1 and 2.2 were completely different and not related to each other. Interestingly, all five CSFV strains used in this study were collected from Hai Duong and Nam Dinh provinces, which are northern provinces of Vietnam. This means that the CSFV strains were circulating in northern Vietnam and classified into two distinct subgroups, 2.1 and 2.2.

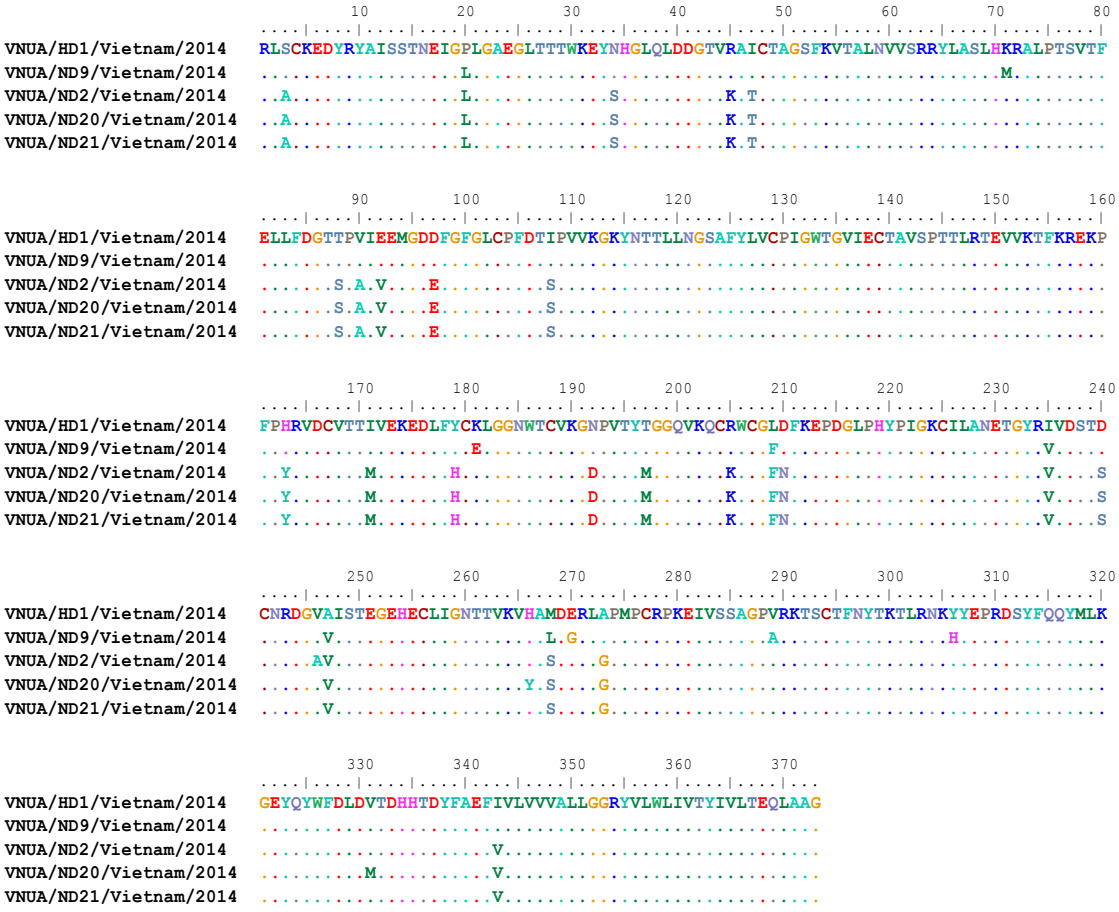

Figure 2. Alignment of deduced amino acid sequence encoded by the E2 gene among Vietnamese CSFV strains

4. CONCLUSION

The full-length E2 genes of five CSFV strains, VNUA/HD1, VNUA/ND2, VNUA/ND9, VNUA/ND20 and VNUA/ND21, collected from Hai Duong and Nam Dinh provinces of Vietnam in 2014, have been sequenced and analyzed. The results of the phylogenetic analysis based on the complete E2 gene sequences revealed that the CSFV strains were circulating in northern Vietnam, and belonged to two different subgroups, 2.1 and 2.2. The nucleotide (nt) and deduced amino acid (aa) comparison of the E2 genes showed that the 5 field CSFV isolates in the present study shared 85.5 - 99.7% nt and 92.7 - 99.7% aa identity.

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