

RAPID IDENTIFICATION FOR SEROTYPING OF AFRICAN SWINE FEVER VIRUS BASED ON THE SHORT FRAGMENT OF THE EP402R GENE ENCODING FOR CD2-LIKE PROTEIN

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(Received 28 October, Accepted 31 December 2020)

The first confirmed case of African swine fever (ASF) in Vietnam was officially reported in February 2019. To date, the ASF virus (ASFV) has been detected in all 63 cities/provinces in Vietnam. In order to get a better understanding of the potential role of the EP402R gene in a grouping of ASFV serotypes, thirty ASFV sequences of EP402R genes (accession numbers: MN711757-86) from North Central Coast of Vietnam and 68 well-known references of serotype groups from previous studies were further analyzed. Interestingly, we found that a short fragment of 90 nucleotides was very typical for 8 serological groups of ASFVs. A primer set was designed to amplify the short fragment of 90 nucleotides using the Primer3 program to establish a simplified method for the serotyping of ASFV. Our results indicated that phylogenetic analysis of the short fragment (90 nucleotides) of the EP402R gene is a very specific and useful method for ASFV serotyping when compared to the previous method using a long fragment (816 nucleotides) of this gene and well-known serotype references based on haemadsorption inhibition (HAI) assay.

Keywords: African Swine Fever Virus, Serotyping, EP402R gene, CD2v, Vietnam

INTRODUCTION

Since the first ASF outbreak in China in 2018, the disease has spread to some countries in Asia and the Pacific, including Mongolia, Vietnam, Cambodia, Korea, Laos, Myanmar, Philippines, Timor-Leste, Papua New Guinea and Indonesia. The first case of ASF outbreak in Vietnam was officially reported in February 2019, and to date, the disease has spread in 63/63 provinces/cities of Vietnam. Approximately six million pigs have been culled in infected farms, suggesting the risk of spreading of this virus. Many active measures were conducted by Vietnamese Government to inhibit the rapid spread of ASF virus. In a previous study, we have indicated that ASFVs isolated

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from 30 clinical samples in North Central Coast of Vietnam belonged to genotype II, serotype 8 and contained additional tandem repeat sequences between the I73R and the I329L protein genes [1]. All sequences have been deposited to GenBank with accession numbers as MN711727-56 for p72 genes, MN711757-86 for EP402R genes and MN733925-54 for TRS genes. Similarly, the data obtained from an ASFV isolated in the Red River Delta of Vietnam also indicated that this field strain belongs to genotype II and serotype 8 [2], suggesting that ASFVs circulating in Vietnam are very close to Eastern European and Chinese strains. Additionally, recent reports suggested that the updates of research methods based on current databases of ASFV sequences are very important for ASFV diagnosis, genotyping and serotyping [3,4].

To date, at least eight ASFV serogroups have been reported and some previous studies have suggested that ASF protective immunity appears to be serotype-specific. Additionally, it has been indicated that serotyping of ASFV is very important for vaccine design and development. Currently, two methods have been developed for grouping of ASFV serotypes, including HAI (haemadsorption inhibition) assay and long fragment PCR based on sequencing method developed by Sanna [5]. However, HAI assay has shown a disadvantage in which this method may not apply in the cases of non-haemadsorbing ASFV viruses, suggesting that PCR based on sequencing method is a good candidate for serotyping of ASFV [6,7]. ASFV serotyping method based on a long fragment (816 nucleotides) of EP402R gene has been first described in 2017 [5]. The primer set for this PCR sequencing method was designed based on genotype I sequences and some previous studies have indicated their successes when using this system for ASFV serotyping [5,8,9]. In order to get a better understanding of the potential role of EP402R gene in grouping of ASFV serotypes, thirty sequences of EP402R genes (accession numbers: MN711757-86) were further analyzed. Interestingly, we found that a short fragment of 90 nucleotides was very typical for 8 serological groups of ASFVs. Thus, in this study, we established a simplified method for ASFV serotyping based on the short fragment of 90 nucleotides of EP402R gene coding for CD2-like protein.

MATERIALS AND METHODS

Virus isolation

The porcine alveolar macrophages (PAM) cultures used for the isolation of the virus were derived from domestic pigs that were tested negative for ASFV upon diagnosis with conventional PCR as previously described [3,10], and according to the recommendation by the World Organization for Animal Health [10]. Briefly, these cells were obtained and resuspended in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific) supplemented with streptomycin (Sigma-Aldrich), ampicillin (Sigma-Aldrich) and 5% fetal calf serum (FCS, Sigma-Aldrich) to a final concentration of 1×10^6 cells/mL. Six-well culture plate (Thermo Scientific), containing 2 mL of the cell suspension, were inoculated with 50 μ L of a clarified 10 % spleen or lymph

node suspension. After three days of incubation at 37°C, in an atmosphere with 5% CO₂, the first passage virus was harvested by freezing and thawing. For the second passage, 50 µL of this virus harvest was added to 2 mL fresh PAMs, then incubated and harvested as described for the first passage. For the third passage, 50 µL of this virus harvest was added to 2 mL fresh PAMs, then incubated at 37°C, in an atmosphere with 5% CO₂. After one-day incubation, a 20-µl preparation of 1% homologous red blood cells (RBC) in buffered saline was added to each well. The plates were examined for HAD and cytopathic effects over a 4-day period in PAM cells according to the recommendation by World Organization for Animal Health [10].

Conventional PCR method

The DNA of ASFV were extracted from infected pig-organ samples using PureLink Viral RNA/DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) as given by the manufacturer's instructions. Conventional PCR were performed to amplify the partial gene CD2v (CP204L) gene from Vietnam ASFV strains using the specific primer set CD2-2F and 2R, to amplify a 816-bp fragment of EP402R gene coding for the cytoplasmic portion of CD2-like protein as described previously [5,11]. In the second conventional PCR, we designed a set of primers to amplify of short fragment CDS of CD2v gene, named CD2v-F1 (5'-ATTTTCCATCATAATGATGTATTGAT-3', binding site 73,644-73,670) and CD2v-R1 (5'-TGATATTTGGGGGAGTAGCAGG-3', binding site 73,729-73,751), to amplify a 107-bp fragment of EP402R gene. Primer binding sites were based on the comparison of the genome of the ASFV from China (MK333180.1), Vietnam (MN711757-86), Georgia (FR682468), Russia (KM609342) and Belgium (MK543947). PCR was carried out in Agilent PCR System (Agilent, Santa Clara, CA, USA) using Taq polymerase (Thermo Scientific, Waltham, MA USA), according to the manufacturer's instructions. Thermal conditions for performing PCR are as follows: an initial incubation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s; and final incubation 72°C for 7 min.

Genomic characterization

Amplification products were electrophoresed on a 1.5% agarose gel against a 100 bp DNA leader marker (Thermo Scientific) and visualized by UV irradiation and ethidium bromide staining (Sigma-Aldrich, Louis, MO, USA). Amplicons of the correct size were excised from the agarose gel and purified using the QIAQuick gel extraction kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's specifications for sequencing (1st BASE, Selangor, Malaysia). The percentage of identities and similarity scores of the viral gene sequences were calculated using DNASTAR Lasergene (DNASTAR, Inc., Madison, USA). The gene identities and similarities of two ASFV strains from Vietnam were compared with the reference sequences of ASFV strains from the National Center for Biotechnology Information (NCBI).

RESULTS AND DISCUSSION

It has been indicated that CD2v protein of ASFV is required for detecting the HAD phenotype and mediating the HAI serological groups [7]. Additionally, the analysis of CD2v sequence is simple method for ASFV serotyping [6]. The first description of CD2v serogroups of ASFV based on 816 bp long fragment is reported in 2017 by Sanna [5] and this method has been applied successfully for ASFV serotyping [9]. In this study, two ASFV isolates from North Central Coast of Vietnam were used for viral isolation in combination with HAD test and a primer set to amplify the short fragment of CD2v gene was designed using Primer3 software (named as CV2v R1 and CD2v F1). Additionally, PCR primers developed by Sanna [5] with slight modification based on our previous results and GenBank databases for genotypes II were employed to establish a new analytic method for serotyping of ASFVs (Figure 1). Two field ASFV isolates, ASFV/VN/Pig/NA/1299 and ASFV/VN/Pig/TH/1238, were used to examine these primer sets. The HAD positive results and the success to amplify the short and long fragments of EP402R gene of ASFV/VN/Pig/NA/1299 (accession numbers MN711770) and ASFV/VN/Pig/TH/1238 (accession numbers MN711768) isolates by two primer sets were shown in Figure 2A and B.

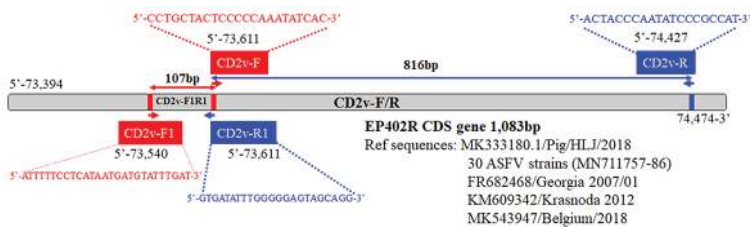


Figure 1. Represent the primer design to amplicon of short fragment (107 nucleotides) and long fragment (816 nucleotides) of EP402R gene coding for CD2-like protein. The ASFV strains Pig/HLJ/2018 in China, Georgia 2007/01 in Georgia, Krasnoda 2012 in Russia and Belgium/2018 in Belgium (Acc. number MK333180.1, FR682468, KM609342 and MK543947, respectively) and 30 ASFV strains isolated in Vietnam (accession numbers MN711757-86), indicated by red color were used as reference genomes.

It is demonstrated that serotyping based on EP402R gene sequences showed to be a useful and convenient tool when compared to hemagglutination inhibition (HAI) assay [5]. Some previous works indicated that the consideration when performing a vaccine design and development should be given to the fact that viruses within a serogroup provide cross-protection from challenge with viruses of the same serogroup. A recent study has found that ASFV isolates, clustered to serogroups 1, 2, or 4, were recognized as genotype I group based on p72 gene sequences. The alignment of short fragments of 90 nucleotide sequences from 30 ASFV sequences in North Central region of Vietnam (accession numbers MN711757-86) and 68 reference sequences of well-known serogroups was shown in Figure 3. Interestingly,

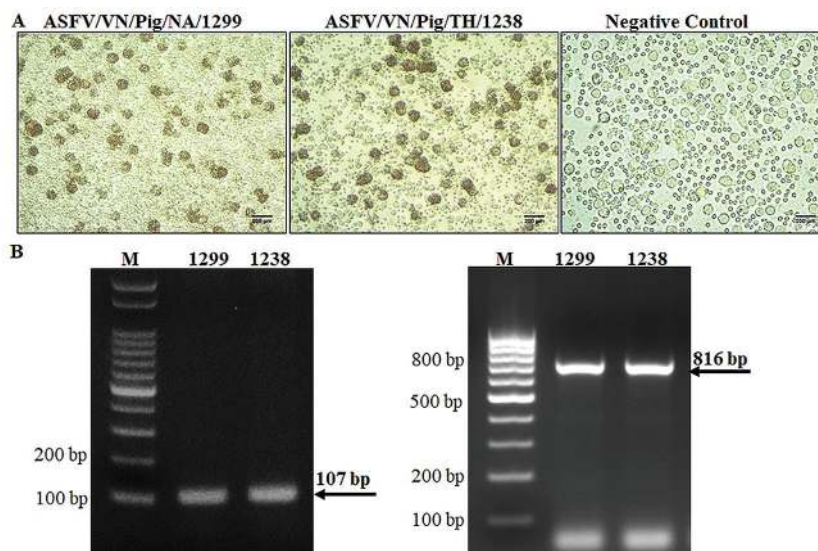


Figure 2. (A) Virus isolation is in combination with the Hemadsorption test in the culture of PAM cells (Original magnification, 100x and scale bar 200µm). (B) The conventional PCR to amplify the short (107 nucleotides) and long (816 nucleotides) fragments of EP402R gene coding for CD2-like protein.

it is clear that a short fragment of EP402R genes is very typical for 8 serotype groups of ASFVs. Phylogenetic analyses of nucleotide sequences of EP402R gene encoding for CD2-like protein was further constructed using the neighbor-joining method with a bootstrap value of 1,000 in the MEGA7 program [12]. The results in Figure 4A and 4B indicated an agreement exists between short fragment analysis (Figure 4B) and the previous method based on 816 nucleotide long sequences (Figure 4A) in which the two methods shares 22 nucleotides in the downstream sequences of 90 nucleotides short fragment. Additionally, ASFV serotyping based on 90 nucleotide short fragments is clearer and more specific when compared to long fragment analysis as seen in Figure 4. In previous studies, a good correlation for ASFV serotyping between phylogenetic analysis of EP402R gene coding for CD2-like protein and HAI serogrouping were observed and the information of ASFV serotypes is very necessary for vaccine design and development [6]. HAI assay is the golden method for ASFV serotyping. However, this method required a live virus, reference serum and may not apply in the cases of non-haemadsorbing ASF viruses. Additionally, ASFV antibodies may appear late or at low titre during infection, affecting directly to this assay [13-16]. Thus PCR based on sequencing method of EP402R gene encoding for CD2-like protein is good candidate and simple method for serotyping of ASFV [6]. Our results suggested that analysis of short fragments of EP402R gene is good tool for serotyping of ASFV strains circulating in Vietnam.

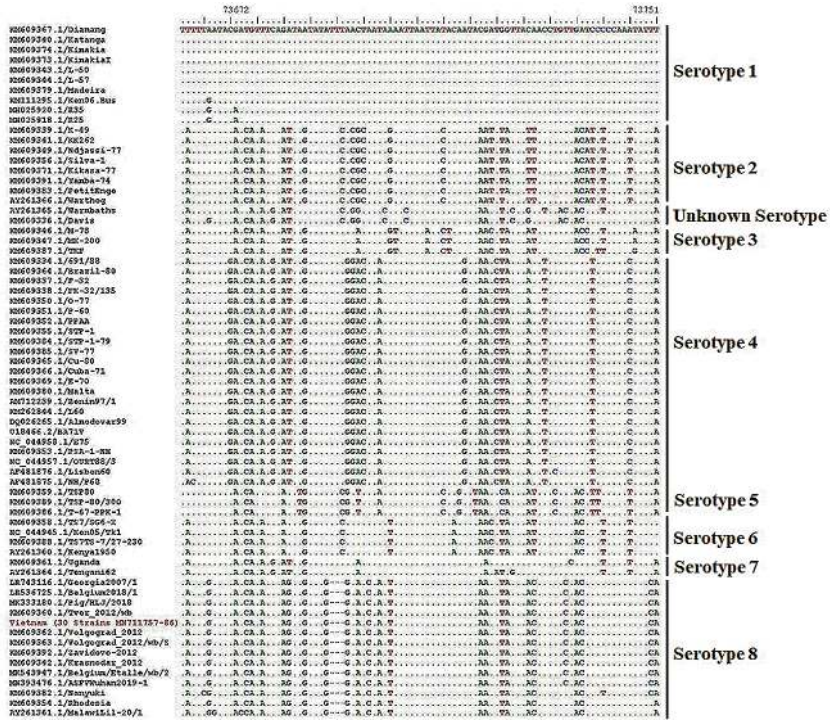


Figure 3. Alignment of the 90 nucleotide sequences of the EP402R genes in African swine fever virus isolated from the North Central Coast of Vietnam and some well-known references of ASFV strains.

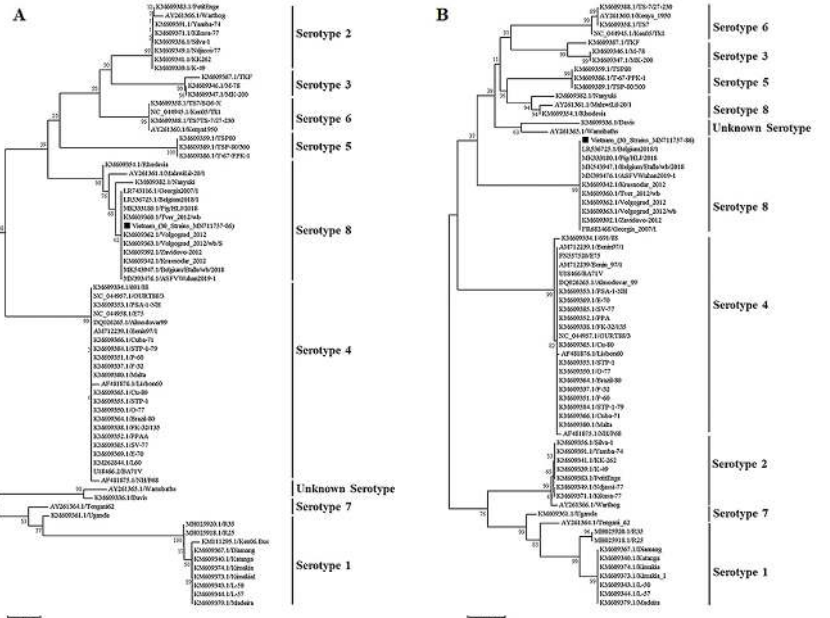


Figure 4. Phylogenetic analysis of the 90 nucleotide sequences (**A**) and 816 nucleotide sequences (**B**) of EP402R gene encoding for CD2-like protein. The sequences of the EP402R gene coding for CD2v-like protein of representative ASF virus were downloaded from the NCBI database. The neighbor-joining method was used to construct phylogenetic trees using MEGA 7 software. Numbers along branches indicate bootstrap values >80% (1,000 replicates). The black square indicates the ASF virus isolate from this study. Scale bars indicate nucleotide substitutions per site.

CONCLUSION

To our knowledge, the information of serogroups of ASFV is very important for vaccine development, as well as the strategy of vaccine use in the future. Taken together, our data suggested that phylogenetic analysis of 90 nucleotide sequences of EP402R gene amplified by CD2v R1/F1 primers is a useful and specific tool for serotyping of circulating ASFV strains when compared to the previous method using a long fragment of 816 nucleotides and well-known serotyping references based on HAI assays.

Acknowledgments

This work was supported by the Ministry of Science and Technology to Hoang Vu Dang (Project code: ĐTĐL.CN-75/19). The authors would like to thank Dr. Andrew G. Yersin of Kemin Industries, Inc. Scott Avenue Des Moines, IA 50317, USA, for English edition.

Ethics approval and consent to participate

The study was conducted in compliance with the institutional rules for the care and use of laboratory animals and using the protocol approved by the Ministry of Agriculture and Rural Development of Vietnam (TCVN 8402:2010).

Availability of data and materials

All sequences have been deposited to GenBank under accession numbers MN711757-86 for CD2v genes.

Authors' contributions

TTTTH, TAD, and DVH carried out the conceptualization, molecular genetic studies, participated in the sequence alignment, methodology, software, validation, investigation, and drafted the manuscript. LDV, HVT, CTN, NTH and NTC participated in the design of the study, performed the data curation and helped to draft the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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BRZA SEROTIPIZACIJA VIRUSA AFRIČKE KUGE SVINJA ZASNOVANA NA ANALIZI KRATKIH FRAGMENTA EP402R GENA KOJI KODIRA CD2-LIKE PROTEIN

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Prvi potvrđen slučaj afričke kuge svinja (ASF) u Vijetnamu bio je zvanično prijavljen u februaru 2019. godine. Do danas, ASF virus (ASFV) je detektovan u 63 provincije Vijetnama. Radi boljeg razumevanja potencijalne uloge EP402R gena u grupisanju ASFV serotipova, analizirano je trideset ASFV sekvenci EP402R gena (pristupni brojevi: MN711757-86) poreklom iz Centralne severne obale Vijetnama i 68 referentnih uzoraka serotip grupa koji su dobro poznati iz prethodnih studija. Interesantan je bio nalaz da je kratak fragment od 90 nukleotida bio tipičan za 8 seroloških grupa ASF virusa. Dizajniran je set prajmera sa ciljem amplifikacije kratkih fragmenata (90 nukleotida), korišćenjem Primer3 programa, a za uspostavljanje jednostavne metode serotipiziranja ASFV. Rezultati ukazuju da je filogenetska analiza kratkih fragmenata (90 nukleotida) EP402R gena je veoma specifična i predstavlja korisnu metodu serotipizacije ASFV u poređenju sa prethodno opisanim metodama koje su koristile duge fragmente (816 nukleotida) ovog gena kao i dobro poznate referentne serotipove dobijene metodom inhibicije hemadsorpcije.