Genetic analysis of African swine fever virus based on major genes encoding p72, p54 and p30

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ABSTRACT

African swine fever (ASF) is reported as a highly contagious hemorrhage lethal disease of domestic and wild swine. The causative agent of ASF is a large enveloped DNA virus with a complex structure. There are twenty-four ASFV genotypes described to date. However, in Vietnam, only genotypes II had been previously described. The genetic characterization of ASFV enhances the understanding of ASF epidemiology in terms of the extent, severity, source, and potential genetic variations among ASFV strains circulating in Southern Vietnam. Twenty ASFV strains were collected from pigs with ASFV-infected clinical signs from 10 provinces during 2019 - 2020. Partial B646L (p72) gene, complete E183L (p54), and CP204L (p30) genes were amplified, purified, and sequenced. Phylogenetic analysis confirmed the circulation of genotype II by both the partial B646L (p72) and full-length E183 (p54) gene sequencing. Analysis of the p72, p54, and p30 regions did not indicate any change in the nucleotide and amino acid sequences among these strains. The results of this study revealed that these ASFVs shared high homology to ASFV isolates detected in Northern Vietnam and China.

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1. Introduction

African swine fever (ASF) is reported as a highly contagious hemorrhage lethal disease by the World Organization for Animal Health (OIE, 2019). First described in Kenya in 1921, it happened on the African continent until 1957, when it spread from Angola to Lisbon. From then on, African swine fever virus (ASFV) has been repeatedly identified in many countries of Europe, Central America, and South America. The introduction of ASFV into Georgia from Southeast Africa in 2007 was described by Rowlands (2008). After that, ASFV crossed into the Eastern European Union (EU) territory in 2014 and affected Lithuania, Poland, Latvia, and Estonia in 2014, the Czech Republic and Romania in 2017, and Hungary and Bulgaria in 2018 (Gallardo et al., 2017; OIE, 2018). In 2018, the disease reached China having the largest pig production (Ge et al., 2018), followed by Vietnam (2019) (Le et al., 2019), and other Asian countries, which caused the devastation of pig industry, leading to substantial economic loss due to the lack of an effective vaccine (FAO, 2020).

African swine fever is caused by the ASFV that is the only member of the family *ASFarviridae* (Dixon et al., 2020). ASFV was classified into 24 genotypes (Quembo et al., 2018). The different strains lead to variable pathogenesis ranging

from highly lethal to asymptomatic. The highly virulent genotype II is confirmed as a circulating strain in China, and Vietnam which showed high mortality (91-100%), making ASF the most severe restriction on domestic pig production, food security, and livelihood and socioeconomic effects (Ge et al., 2018; Gallardo et al., 2019).

In Vietnam, the previously characterized ASFV have been reported only genotype II based on sequencing of the C-terminal region of the B646L gene (Le et al., 2019; Tran et al., 2020; Mai et al., 2021). Further differentiation between close strains and identifying virus subtypes of the 24 genotypes was achieved by nucleotide sequencing of the whole E183L gene regions encoding p54 proteins and the CP204L gene encoding the phosphoprotein p30. Previous studies have shown that using the three encoding regions of the ASFV DNA including p72, p54, and p30, to characterize ASFV is much sufficient despite the presence of many other markers (Nix et al., 2006; Gallardo et al., 2009).

This study was set out to characterize African swine fever virus genotypes from 20 farms in 10 provinces, which occurred ASF outbreaks in 2019 and 2020. The results are expected to contribute sequence data for molecular epidemiology studies and provide further understanding of ASF disease patterns in Southern Vietnam.

2. Materials and Methods

2.1. Study design and sample collection

This study was conducted in 10 Southern provinces of Vietnam (Figure 1). Samples were collected from clinical pigs in ASF outbreaks reported in the period of 2019 - 2020. Sets of lymph nodes, spleens, kidney, lung, and whole blood from 20 pigs were collected for diagnosis according to the OIE-recommendation, then the whole blood used for DNA extraction submitted to sequencing. PCR amplification of ASFV detection was performed using primers PPA-1/PPA-2 described in previous studies (Table 1). Sample source, temporal and spatial distribution of specimens were summarized in Table 2.

Table 1. Primers used for PCR detection and PCR genomic characterization

Gene name	GPrimers	Sequences (5'-3')	Size of products	References
B646L	PPA-1	AGTTATGGGAAACCCGACCC	257bp	Aguero et al., 2004
	PPA-2	CCCTGAATCGGAGCATCT		
B646L	P72U	GGCACAAGTTCGGACATGT	478bp	Bastos et al., 2003
	P72D	GTACTGTAACGCAGCACAG		
E183L	PPA722	CGAAGTGCATGTAATAAACGTC	676bp	Gallardo et al., 2008
	PPA89	TGTAATTTCATTGCGCCACAAC		
CP204L	P30F	ATGAAAATGGAGGTCATCTTCAAAAC	521bp	Rowlands et al., 2008
	P30R	AAGTTTAATGACCATGAGTCTTACC		

2.2. DNA extraction and PCR assay

The genomic DNA of ASFV was extracted following the manufacturer's instructions of the commercial kit Promega^{TM} Wizard^{TM} SV

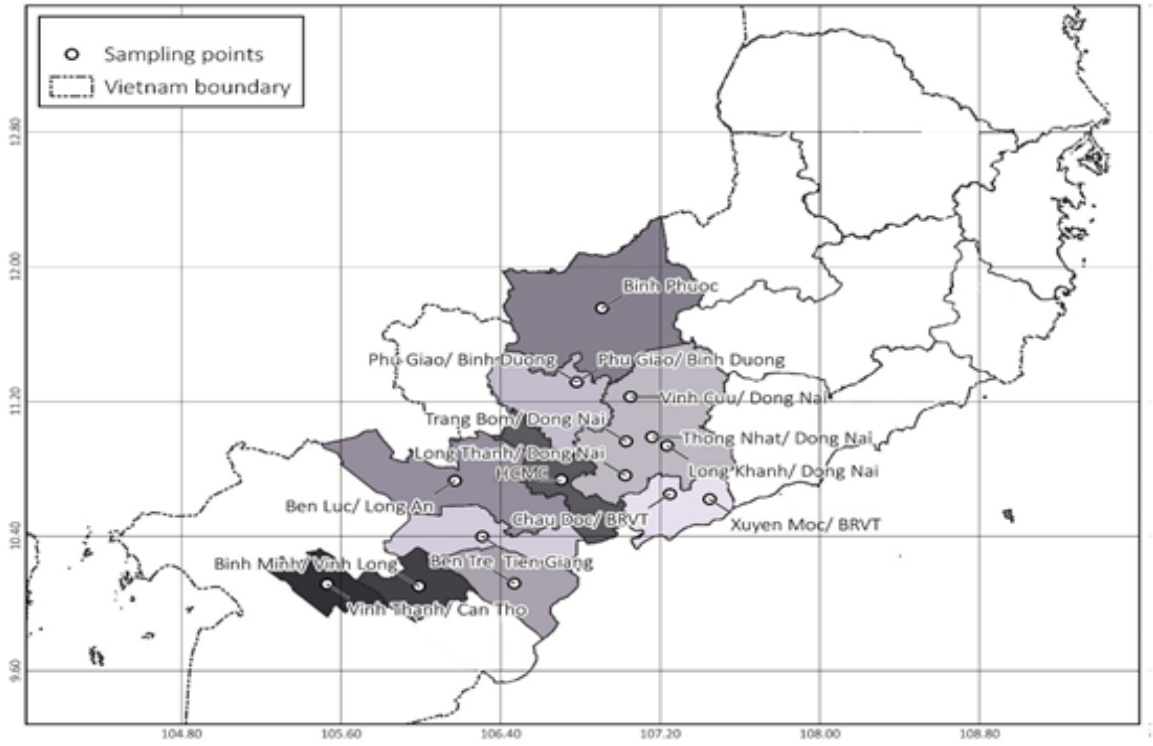

Figure 1. Map of Vietnam showing locations where field strains were obtained and genotyped.

Table 2. Summary of African swine fever viruses used in this study

ASFV strains	District/province	Sampling date	Sample obtained from each pig	Host
VN/HCMC/2019-ASF1	Binh Tan/HCMC	July, 2019	S	DP
VN/BRVT/2019-ASF1	Chau Doc/BRVT	August, 2019	S	DP
VN/BRVT/2019-ASF2	Xuyen Moc/BRVT	November, 2019	S	DP
VN/BD/2019-ASF1	Phu Giao/Binh Duong	September, 2019	S, Sp, K, L, LN	DP
VN/BD/2019-ASF2	Phu Giao/Binh Duong	July, 2019	S, Sp, K, L, LN	CP
VN/BD/2020-ASF3	Phu Giao/Binh Duong	July, 2020	S	DP
VN/BD/2020-ASF4	Phu Giao/Binh Duong	June, 2020	S	CP
VN/BP/2019-ASF1	Binh Phuoc	June, 2019	S	DP
VN/DN/2019-ASF1	Trang Bom/Dong Nai	June, 2020	S	CP
VN/DN/2019-ASF2	Vinh Cuu/Dong Nai	June, 2019	S	DP
VN/DN/2019-ASF3	Long Thanh/Dong Nai	August, 2019	S	DP
VN/DN/2020-ASF4	Long Khanh/Dong Nai	May, 2020	S	CP
VN/DN/2020-ASF5	Thong Nhat/Dong Nai	July, 2020	S	DP
VN/BenTre/2020-ASF1	Ben Tre	December, 2020	WB	DP
VN/CT/2019-ASF1	Vinh Thanh/Can Tho	July, 2019	S	DP
VN/LA/2019-ASF1	Ben Luc/Long An	September, 2019	S, Sp, K, L, LN	DP
VN/LA/2019-ASF2	Ben Luc/Long An	October, 2019	S, Sp, K, L, LN	DP
VN/LA/2019-ASF3	Ben Luc/Long An	September, 2019	S	DP
VN/TG/2020-ASF1	Tien Giang	July, 2020	S, Sp, K, L, LN	DP
VN/VL/2019-ASF1	Binh Minh/Vinh Long	August, 2019	S	DP

DP: Domestic pig; CP: Convalescent pig; WB: whole blood; S: serum, LN: lymph node; Sp: pleen; L: lung; K: kidney; WB: whole blood.

Genomic DNA Purification System (Promega, USA). PCR amplification for the genomic characterization of ASFV was performed to amplify the variable 3'-end of the B646L gene, whole gene E183L encoding the p54 protein, and the CP204L (p30) gene using primers that showed in Table 1.

All PCR amplifications were performed using kit MyTaq TM HS Mix, 2x (Bioline, USA). Reactions contained 12 µL MyTaq TM HS Mix, 5 µL DNA, 1 µL of 10 µM Forward primer, 1 µL of 10 µM Reverse primer, and 6 µL free-DNA water in a total reaction volume of 25 µL. The positive control DNA was given by Veterinary Hospital Laboratory of Nong Lam University (Vietnam). DNA extracts were substituted by nuclease free water in negative control reaction. Thermocycling condition for PCR detection of ASFV included a 10 min initial denaturation step of 95°C, followed by 40 cycles of 15 sec at 95°C, 30 sec at 62°C, and 30 sec at 72°C with a 5-min elongation step at 72°C. Thermocycling condition for amplification of the p72, p54, and p30 genes, the initial denaturation at 95°C for 5 min, followed by 40 of 30 sec at 95°C, 30 sec at 55°C, and 1 min at 72°C, and a final elongation at 72°C for 10 min. Afterward, PCR products were mixed with GelRed nucleic acid stain (Merck, Germany) and electrophoresed in a 1% agarose gel electrophoresis (Invitrogen, Thermo Fisher Scientific, USA) using 1 Kb Plus ladder (Invitrogen, Thermo Fisher Scientific, USA) to indicate the sizes of amplification products.

2.3. Genetic sequence and analysis

The PCR products of 3 gene regions (p72, p54, and p30) were purified using the Top-PURER PCR/GEL DNA PURIFICATION kit (ABT, Vietnam) and were sent to sequencing laboratory (Macrogen, Korea). Both sequences from the forward strand and the reverse complement sequences were manually overlapped to obtain a single sequence. The nucleotide sequences of the B646L, E183L and CP204L genes of the collected ASFV strains were compared with previous Vietnamese strains and with other sequences published available in GenBank (NCBI) (Table 3). The nucleotide sequences of three defined genes obtained were aligned using the CLUSTAL W package and phylogenetic analyses were conducted according to the maximum likelihood approach with a bootstrap value of 1000 in the MEGA X software (https://www.megasoftware.net).

3. Results

3.1. Confirmation of ASF using PCR

The 20 pigs included sows, fattening pigs, and nursing pigs that showed specifically pathological and clinical signs of ASF, including fever (41 - 42°C), loss of appetite, vomiting, skin hemorrhages, bloody discharge from the nose, and joint swelling. PCR revealed a band size of 257 bp, hence 20 pigs tested positive for ASF viral DNA (gel not shown).

3.2. Nucleotide and amino acid sequence analyis

3.2.1. Sequence alignment

Nucleotide and amino acid sequences of partial p72, full-length p54, and p30 of the 20 strains shared 100% sequence identity with each other. They shared 98.63% - 100% nucleotide and amino acid identity with the partial p72, full-length p54, and p30 sequences of genotype II reference isolates. The amino acid sequence alignment revealed that 20 strains shared 96.42% - 100% p72 sequence identity, 100% p54 sequence identity, and 99.42% - 100% p30 sequence identity within p72 genotype II. Interestingly, nucleotide and amino acid sequences of partial p72, full-length p54, and p30 of CN201801 (MH735140) and Jilin/2018 (MK189456) ASFV strains from China shared 100% sequence identity with 20 strains in this study (Figure 2).

3.2.2. Phylogenetic analysis

P72 phylogenetic tree: The p72 genotyping PCR results indicated a band size of 478 bp from all the samples using primers P72U/P72D (gel not shown). Phylogenetic analysis and comparison of these sequences to other isolates of known genotypes identified the 20 sequences belonging to B646L genotype II as shown in Figure 2A.

P54 phylogenetic tree: A band size of 676 bp was amplified using primers PPA722/PPA89 (gel not shown). Analysis of whole-length p54 sequences confirmed that the circulating virus belongs to genotype II (Figure 2B).

P30 phylogenetic tree: PCR amplification of CP204L gene from each of 20 field strains pro-

Table 3. References of ASFV isolates from Vietnam and over the world from Genbank

Isolates	Year of isolation	Countries	P72 genotype	P72 gene Genbank accession number	P54 genotype	P54 gene Genbank accession number	P30 gene Genbank accession number
Hanoi/2019	2019	Vietnam	II	MT332151	II	MT166692	MT166692
NgheAn/2019	2019	Vietnam	II	MT180393	II	MT180393	MT180393
VN/Pig/NA/1393	2020	Vietnam	II	MN711740	-	NA	NA
CN201801	2018	China	II	MH722357	II	MH735140	MH735141
Wuhan/2019	2019	China	II	MN393476	II	MN393476	MN393476
Jilin/2018	2018	China	II	MK189456	-	-	MK214680
Pol18	2018	Poland	II	MT847621	II	MT847621	MT847621
Georgia_2007/1	2007	Georgia	II	NC_044959	-	-	NC_044959
Etalle/2018	2018	Belgium	II	MK543947	II	MK543947	MK543947
LT14/1490	2014	Lithuania	II	MK628478	II	MK628478	MK628478
ZAM/14/Chipata	2014	Zambia	II	LC174751	-	NA	LC213611
OURT_88/3	1989	Portugal	I	AM712240	Ic	AM712240	AM712240
Li20/1	1983	Malawi	VIII	AY261361	VIII	AY261361	AY261361
Kenya-1950	1950	Kenya	X	AY261360	X	AY261360	AY261360
MZUKI/1979	1979	South Africa	I	AY261362	Ib	AY261362	AY261362
Warthog	NK	Namibia	IV	AY261366	IVa	AY261366	AY261366
KAB/62	1983	Zambia	XI	AY351522	XI	EU874331	EU874289
SUM 14/11	1983	Zambia	XIII	AY351542	XIII	EU874357	EU874287
TMB 89/1	1989	Zambia	VIII	AY351557	-	NA	JQ76886
SPEC/53	NK	South Africa	XXI	DQ250111	XXI	KC662384	NA
Ken05/Tk1	2014	Kenya	X	KM111294	X	KM111294	KM111294
Ken06Bus	2014	Kenya	IX	KM111295	IX	KM111295	KM111295
ZAM/13/Kalomo	NK	Zambia	XIV	LC174752	XIV	LC174761	LC213612
ZAM/14/Kasempa	NK	Zambia	XIV	LC174753	XIV	LC174762	NA
R8	NK	Uganda	IX	MH025916	IX	MH025916	MH025916
RSA_2/2008	2008	South_Africa	XXII	MN336500	XXII	MN336500	MN336500
RSA_2/2004	2004	South_Africa	XX	MN641877	XX	MN641877	MN641877
Liv13/33		France	I	MN913970	Ia	MN913970	MN913970
RSA W1	1999	South_Africa	IV	MN641876	IVb	MN641876	MN641876

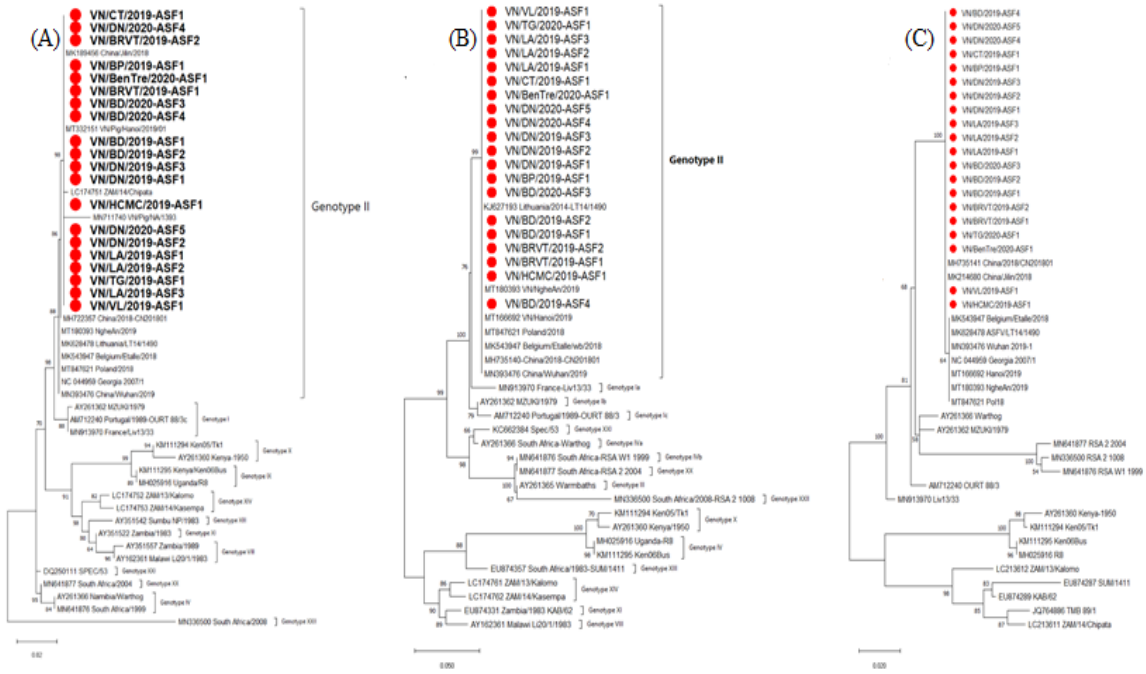

Figure 2. ASFV phylogenetic trees based on nucleotide sequences of genes encoding p72-p54-p30. (A) P72 genotype (B) P54 sub-group within p72 genotype, (C) P30 phylogenetic trees. The maximum likelihood approach was used for construction of phylogenetic trees in MEGA X software (https://www.megasoftware.net/). Numbers along branches indicate bootstrap values > 50% (1000 replicates). The red circles are ASFVs from this study. Scale bars indicate nucleotide substitutions per site.

duced a 521 bp band (gel not shown). All ASFVs collected from the 2019 - 2020 outbreaks in Southern Vietnam were indistinguishable in the p30 phylogeny. Except for one virus (ZAM14/Chipata, accession no. LC213611), all p72 genotype II ASFVs belonged to the same clade (Figure 2C).

4. Discussion

Phylogenetic analysis based on nucleotide sequences of the C-terminal of the B646L gene (p72), full-length E183L (p54) gene showed that all 20 Vietnamese ASFV field strains belonged to genotype II. The other analysis of p30 phylogeny showed that this genomic region could differentiate among closely related viruses (Simulundu et al., 2017). The findings are in contrast to those of a previous study in which p30 sequences obtained from ASFV collected from 10 different provinces over two years showed remarkable genetic stability and so could not obtain a finer level of discrimination among closely related viruses using this genomic region. The idea that illegal transportation of pork and pork products, triggering the outbreak in Southern Vietnam seems to support from this genetic analysis, which showed that the virus in this study was very similar in the comparison based on any of three selected gene fragments (B646L, E183L, and CP204L) to ASFVs which caused outbreaks in China during 2018 (CN201801 and Jilin/2018).

On the other hand, the analysis and comparison of the three independent regions of ASFV gene sequences in this study and other reports in Vietnam showed high stability of the ASFV genome (Le et al., 2019; Mai et al., 2021). However, the finding conflicted with the previous study in which p72 sequences obtained from ASFV collected from Nghe An province showed the presence of some nucleotide point substitution mutations (Tran et al., 2020). In general, the mutation rate of DNA viruses is lower than RNA viruses and ranges between 10^{-8} to 10^{-6} substitutions per nucleotide per cell infection (Sanjuan et al., 2010). Mowever, double-stranded DNA's evolutionary rate is difficult to estimate (Firth et al., 2010). The accurate proofreading of DNA polymerase and virus-encoded base excision DNA

repair system results in a low mutation rate in ASFV DNA. Our results may be rational to the previous description that ASFV is very stable and shows a very low mutation rate, which leads to low genetic variability in affected regions (Dixon et al., 2020). Interestingly, the virus collected from apparently convalescent pigs in a survey following this study had presented 100% of identity in the 3 ASFV genome regions of the 2019 - 2020 ASFV strains which induced an acute form of the infection for the 20 pigs. It could be assumed that after the first introduction of the disease in an area, increased numbers of subacute and subclinical infections could happen over time, and that mortality rates decrease. In such circumstances, the clinical manifestations are more variable, and recognition of the disease becomes problematic in the field, emphasizing the need for implementation of appropriate surveillance programs to control the disease. This study provides the phylogenetic information about ASFV strains circulating in Southern areas of Vietnam during 2019 - 2020, which will be useful for ASF control program in Vietnam and neighboring countries.

Vietnam shares a cross-border with China, and illegal transport of pigs and pig products frequently occurs in the border provinces of both countries (Kreindel et al., 2018). On the other hand, Vietnam imports pork and other pork products from different European countries, such as Poland, Russia. The exact route of the introduction of ASFV into Vietnam remains unknown. According to recent molecular studies, some different ASFV strains circulate in China (Ge et al., 2018; 2019). These strains may represent multiple introductions of ASFV into China or intra-epidemic genome variation caused by a genetic mutation. To trace the source and extend the knowledge of ASFV evolution and epidemiology in Vietnam, we analyzed nucleotide and amino acid sequences of three defined regions of the genome B646L, E183L, and CP204L from the tested ASFV. Because all nucleotide and amino acid sequences for each genome region from all pigs in various cases obtained from Vietnam were identical, this study concluded that the ASF outbreaks in Vietnam and the surrounding areas were probably due to a single introduction of the virus. The genetic analysis results also reflected the rapid spread of pathogens within six months, spread to 63 provinces throughout the country and from a single source after entering Vietnam. ASFV is a pathogen that spread slowly in nature or under strict supervision. A nationwide outbreak of ASF indicated that the control of the epidemic in Vietnam was quite loose and less stringent. Vietnam has many small and household-scale farms, typically poor biosecurity, outdated facilities, limited breeding techniques and low awareness of veterinary regulations, which were significant contributors to the introduction, spread, and circulation of the disease. Besides, a lot of slaughterhouses and fresh meat markets are located close to the livestock sector, increasing the difficulty of disease control in Vietnam.

5. Conclusions

The results revealed that ASFV circulating in Southern Vietnam during 2019 - 2020 outbreaks belong to genotype II. The combination of molecular data and epidemiologic findings have confirmed that the ASFVs detected in Southern regions of Vietnam most probably originated or showed the same genetic relatedness to ASFVs detected in Northern Vietnam during 2019 and China in 2018. Analysis of the three independent genes of ASFV in this study and previous studies in Vietnam showed high stability of ASFV genome.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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