Complete genome analysis of African swine fever virus isolated from domestic pigs during the first ASF outbreaks in India.

Senthilkumar D[1], KATHERUKAMEM RAJUKUMAR[1], Venkatesh G[1], Fateh Singh[1], C. Tosh[1], Subbiah Kombiah[1], Chandan Dubey[1], AMITAV CHAKRAVARTY[2], Nagendra Nath Barman[3], and Vijendra Singh[1]

[1]National Institute of High Security Animal Diseases
[2]Government of Assam
[3]Assam Agricultural University College of Veterinary Science

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Abstract

African swine fever (ASF), considered as the most dreadful swine disease due to its very high mortality, emerged in India in 2020. The complete genome analysis of ASF viruses isolated during the first outbreaks in India showed a few unique non-synonymous mutations in MGF 369-11L, MGF 505-4R, K205R and B263R genes. Frame shiftsin the protein coding sequences were observed in DP60R, ASFV-G_ACD 00190, MGF 110-10-L- MGF110-14L fusion, MGF 360-14L and I267L genes of Indian ASFVs as compared to ASFV/Georgia/2007. Complete genome based phylogenetic analysis of p72-genotype-II viruses showed the clustering of Indian isolates with ASFV/Wuhan/2019 in a separate clade. Phylogenetic analysis of concatenated sequences of 14 open reading frames (ORF) having single nucleotide polymorphisms (SNP) showed distinct grouping of Indian ASFVs with other Asian ASFVs.Thisis the first complete genome characterization of ASF viruses isolated from domestic pigs in India. The resultsindicate that number of Tandem Repeat Sequence in the intergenic region between I73R and I329L genes, and the 14 ORFs with SNP reported in this study could be the genetic determinants to differentiate the closely related p72-genotype II viruses circulating in Asia.

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Dhanapal Senthilkumar[1*], Katherukamem Rajukumar[1], Govindarajulu Venkatesh[1], Fateh Singh[1], Chakradhar Tosh[1], Subbiah Kombiah[1], Chandan K. Dubey[1], Amitav Chakravarty[2], Nagendra N. Barman[3], Vijendra Pal Singh[1].

1. ICAR-National Institute of High Security Animal Diseases, Bhopal, India.
2. North Eastern Regional Disease Diagnostic Laboratory, Guwahati, India
3. College of Veterinary Science, Assam Agricultural University, Guwahati, India

*- Corresponding author- email: senvetpath@gmail.com

Abstract:

African swine fever (ASF), considered as the most dreadful swine disease due to its very high mortality, emerged in India in 2020. The complete genome analysis of ASF viruses isolated during the first outbreaks in India showed a few unique non-synonymous mutations in MGF 369-11L, MGF 505-4R, K205R and B263R genes. Frame shiftsin the protein coding sequences were observed in DP60R, ASFV-G_ACD 00190, MGF 110-10-L- MGF110-14L fusion, MGF 360-14L and I267L genes of Indian ASFVs as compared to ASFV/Georgia/2007. Complete genome based phylogenetic analysis of p72-genotype-II viruses showed the

clustering of Indian isolates with ASFV/Wuhan/2019 in a separate clade. Phylogenetic analysis of concatenated sequences of 14 open reading frames (ORF) having single nucleotide polymorphisms (SNP) showed distinct grouping of Indian ASFVs with other Asian ASFVs.Thisis the first complete genome characterization of ASF viruses isolated from domestic pigs in India. The resultsindicate that number of Tandem Repeat Sequence in the intergenic region between I73R and I329L genes, and the 14 ORFs with SNP reported in this study could be the genetic determinants to differentiate the closely related p72-genotype II viruses circulating in Asia.

Keywords : African swine fever virus, complete genome, analysis, first report

Introduction :

African swine fever is a highly contagious haemorrhagic transboundary viral disease affecting domestic pigs and wild boars with case fatality rate up to 100%. The disease was reported in Kenya as early as 1921(Montgomery et al., 1921). After the transcontinental spread from Africa to Georgia in 2007, the disease has spread to other Caucasian and eastern European countries (Abrahantes et al., 2017). Since the report of ASF with the loss of over 7 million pigs (OIE report, 2021). At present, there is no treatment or vaccine available for this disease and it is controlled mainly by implementing strict biosecurity measures, rapid diagnosis and stamping out of the infected and in-contact pigs (Sánchez-Vizcaíno et al., 2015).

The disease is caused by ASF virus, a unique DNA virus belonging to the genus *Asfivirus* under the family *Asfarviridae* . The complete genome of the virus spans around 190kbp, encoding between 150 and 167 viral proteins depending on virus strains (Dixon et al., 2013). Based on sequence difference of B646L gene encoding p72 protein, ASFV strains were divided into 24 genotypes (Achenbach et al., 2017). Among genotype II viruses, four sub-clusters (IGR I-IV) were divided based on number of tandem repeat sequences (TRS) in the intergenic region between the open reading frames (ORFs) I73R and I329L (Mazur-Panasiuk et al., 2020). Sequence analyses have shown the presence of both IGR I and II variants in Vietnam (Tranet al., 2021) and the circulation of IGR I, II and III variants in Korea (Kimet al., 2020). Previous reports on whole genome sequence analysis have revealed SNP due to specific mutations in various ORF viz. K145R, MGF-505-5R, and O174L in the nucleotide sequences of ASFV reported from Poland as compared to Georgia 2007/1 strain (GA/2007) (Mazur-Panasiuk et al., 2019 & 2020).

In India, the disease was reported for the first time in May 2020 from outbreaks in Assam and Arunachal Pradesh states. Sequence analyses of partial B646L and E183L genes of Indian ASFV isolates showed complete identity with sequences of post-2007-p72-genotype II viruses reported from Georgia, Belgium, China, Vietnam etc., (Rajukumar et al., 2021). To further understand the genetic nature of Indian ASF viruses and the variations amongst the ASF viruses evolving in Asia, we report the complete genome sequence analysis of ASFV isolated from domestic pigs in India.

Materials and Methods:

Samples and virus:

African swine fever viruses isolated from diagnostic samples received at ICAR-National Institute of High Security animal diseases, Bhopal during the first outbreaks of ASF were used in this study (Rajukumar et al., 2021). Two ASFV isolates (second passage), from Arunachal Pradesh(IND/AR/SD-61/2020) and Assam(IND/AS/SD-02/2020), were further propagated for one more passage in porcine monocyte culture as described previously by Borca et al., 2020. Briefly, PBMCs of healthy pig were cultured in 10% fetal bovine serum containing RPMI-1640 medium (growth medium). After 48 hours of incubation at 37°C under 5% CO_2 concentration, monocytes wereharvestedin Dulbecco's PBSwith 1mM EDTA after removing unattached cells by thorough washing using PBS. Harvested monocytes were cultured in 24 well culture dishes at the concentration of $2x10^6$ cells per mL in growth medium. The monocytes were infected with supernatants obtained in the second passage at 1/10 dilution.Two wells were maintained as uninfected controls. One hundred µl of freshly prepared 1% swine RBCs in growth medium were added to each well and observed for Haemadsorption for up to 5 days post infection.

Viral genome enrichment:

The cell culture supernatant was harvested after three freeze-thaw cycles and centrifuged at 4000 RPM for 10 min. The clarified culture supernatants harvested at third passage were tested by real time PCR (Fernández-Pinero et al., 2013) and used for further process. The viral genome enrichment was carried out as described earlier, with minor modifications (Chapman et al., 2011). Briefly, cell supernatant containing virus was centrifuged at 118000 xg for 1h at 4°C. The pellet resuspended in equilibrating buffer (NaCl 10mM, Tris-HCl 10mM, EDTA 1mM) was treated with 75 units of DNase I for 1h at 37°C. The virus was pelleted over 20% sucrose in equilibrating buffer at 62000xg for 1.5 h. The pellet was treated with RNAse (40 µg/mL), proteinase K (200 µg/mL), and 1% sodium dodecyl sulfateand incubated for 18 h at 37°C. The genomic DNA was extracted from the enriched pellet by phenol-chloroform method (Sambrook and Russell, 2006).

Next generation sequencing:

The genomic DNA extracted from the enriched virus pellet was tested for the presence ASFV genome by real time PCR. The quality and quantity of extracted nucleic acid was measured by nanospectrophotometer (Eppendorf, Germany).The complete genome sequencing of ASFV in the extracted DNA was performed by next generation sequencing commercially (Eurofins India Pvt. Ltd, Bengaluru, India). A sequencing library was constructed for each sample using the Nextera XT DNA library preparation kit (Illumina, San Diego, CA, USA) and 2 x 150 NextSeq500paired-end sequencing was performed on NextSeq500 sequencing system (Illumina, USA).Adapter and reads of quality less than Phred score 30 were trimmed by Trimmomatic v0.38 software (Bolger et al., 2014). *De-novo* assembly of adapter trimmed raw reads was carried out by using SPAdes 3.15.2 software (Bankevich et al., 2013).Blastn was performed to identify the closest available ASFV sequence using scaffold contigs of size less than 200 Kb. The trimmed reads were mapped against the closely matched ASFV genome (MN393476.1_Wuhan/2019-1) to generate the whole genome sequence of the Indian isolates using HISAT-2 software (Kimet al., 2015). Putative genes were annotated from assembled sequences using Genome Annotation Transfer Utility Software (Tcherepanov et al., 2006) and the complete genome sequences were submitted to Genbank with accession numbers OL692743 and OL692744.

Single nucleotide variant and phylogeneticanalyses:

The single nucleotide variant analyses were carried for the Indian ASFV isolates by comparing complete genome sequences ofothersviz. China/2018/AnhuiXCGQ (MK128995.1), ASFV Wuhan 2019-1 (MN393476.1), ASFV/pig/China/CAS19-01/2019 (MN172368.1), ASFV Georgia 2007/1 (NC_044959.2), ASFV/Timor-Leste/2019/1(MW396979.1) using mpileup utility of Samtools (v 0.1.18) from sorted BAM files (Li et al., 2009). The variants were filtered based on a minimum read depth of 100 and quality threshold of 25. The percentage of nucleotide identity values were generated from aligned sequences by using Sequence Manipulation Suite (https://www.bioinformatics.org/sms2/ident_sim.html).

Complete genome of Indian ASFV isolates were aligned with 33 additional genome sequences retrieved from NCBI Genbank database (Table 1) by multiple sequence alignment program MAFFT (v7.487) (Katoh et al., 2019). Maximum likelihood (ML) phylogenetic treesweregenerated from aligned sequences by using Randomized Axelerated Maximum Likelihood program (RAxML v1.0.0) software (Kozlov et al., 2019)undergeneral time reversible with gamma evolutionary model with 100 pseudo-replicates bootstrapping. Concatenated sequences of genes with single nucleotide polymorphism (SNP) of Indian ASFVs along with the selected P-72 genotype-II ASFVs were aligned and a maximum likelihood tree was constructed using Tamura–Nei parameter model (Tamura and Nei., 1993) in Mega X(1000 bootstrap iterations) software (Kumar et al., 2018). The Phylogenetic trees generated were visualized and annotated using iTOL v6 software (Letunic and Bork, 2021).

Results and Discussion:

African swine fever virus propagation and enrichment:

The two ASFV isolates IND/AR/SD-61/2020 and IND/AS/SD-02/2020propagated in porcine monocyte culture showed specific adsorption of porcine erythrocytes around the infected monocytes as rosette (Fig.1)

from 48 hours post infection. The concentration of genomic DNA extracted from enriched viral pellets was 9.6ng/µL and 11.2ng/µL with A260/280 ratio of around 1.8 and 1.7, respectively, for the two isolates.

Complete genome sequence analysis:

Raw reads obtained from next generation sequencing of DNA extracted from enriched viral pellets were between 7,197,372 and 7,377,167 for the two samples. The size of assembled complete genome of IND/AS/SD-02/2020 and IND/AR/SD-61/2020 ASFV isolates was 190517 bp and 190572 bp, respectively. The average coverage depth of the assembled sequences of the two ASFV isolates were 801x and 978x.

The assembled sequences had GC content of around 38.4% including inverted terminal repeats (ITR) at 5' (1547 & 1596 bp) and 3' (1135&1144 bp)) ends. The complete genome sequences were annotated to 219 putative genes as compared to 194 genes in ASFV Georgia/2007. Global alignment of the complete genome sequences showed nucleotide identity of 99.96% among the two Indian isolates (IND/AS/SD-02/2020 and IND/AR/SD-61/2020). With other genotype-2 African swine fever viruses such as Georgia/2007 and Wuhan 2019-1, the nucleotide identity was 99.73%to 99.75% and 99.95%to99.98%, respectively.

On aligning the complete genome sequences of Indian isolates with ASFV Georgia/2007, IND/AS/SD-02/2020 had 43 single nucleotide insertions (19A, 18T, 2C, 4G), and 34 single nucleotide deletions (15A, 16T, 1C, 2G) at different genomic coordinates.IND/AR/SD-61/2020 had 33 single nucleotide insertions (13A, 14T, 3C, 3G), 26 single nucleotides deletions (11A, 14T, 1G) and one double nucleotide deletion (CC) at various positions (Fig. 2) (Supplementary table S1). Due to insertion/deletion, frame shifts were observed in the Indian viruses in the protein coding sequences of DP60R, ASFV-G_ACD 00190, MGF 110-10-L- MGF110-14L fusion, MGF 360-14L and I267L genes as compared to Georgia/2007. Both sequences of Indian ASFVs had insertion of one extra TRS (TATATAGGAA) in the intergenic region between I73R and I329L (Fig. 3), showing that Indian isolates belong to IGR II cluster of genotype II reported from China, Vietnam, Korea *etc* ., (Kim et al., 2019; Tran et al., 2021).

Single nucleotide variant analysis of sequences of Indian isolates with Georgia/2007 showed 70 nucleotide dissimilarity (29 in 5' ITR, 26 in 3' ITR, 1 to 2 in intergenic regions, 13 to 14 in genic region) (Supplementary table S2). SNP analysis among important Genotype II ASF viruses showed 19 non-synonymous SNP, 5 synonymous SNP in 14 different ORFs (Fig. 4). Synonymous SNP in 5 ORFs (MGF_110_1L, ASFVACD_-320, MGF 505-4R, C717R, B263R) and non-Synonymous SNP in 14 ORFs (MGF110-1L, MGF110-4L, MGF360-10L, MGF360-11L, MGF505-4R, MGF 505-9R, K205R, C717R, B263R, O174L, NP419L, E199L, MGF 360-21R) were observed. Out of the 14 ORFs, MGF110-1L, MGF110-4L, MGF505-4R, C717R, O174L, B263R, E199L showed more than one mutation. Among 19 non-synonymous SNP, four were unique mutations present only in the Indian isolates. Two SNP in B263R and MGF505-4R genes were unique to SD-61/2020, one in MGF 360-11L gene was unique to SD-02/2020 isolate and one SNP in K205R gene was observed in both the isolates (Fig. 4).

Complete genome based phylogenetic analysis of Indian isolates with 33 additional ASFV sequences retrieved from Genbank showed their clustering under clade 2.2.2 with other p72-genotype-II ASFV reported from Georgia, Tanzania, China, Vietnam, Poland, Ukraine, East Timor leste*etc* . between 2007 and 2020 (Fig. 5). Further phylogenetic analysis of only p72-genotype-II viruses showed the clustering of Indian isolates with ASFV/Wuhan/2019 in a separate clade (Fig. 6). Phylogenetic analysis of concatenated sequence of the 14 ORFs having SNP showed two distinct groups within genotype-II viruses, one with Tanzania/Rukwa/2017/1 and the other with Georgia/2007. Within the latter group, Indian isolates along with ASFV reported from other Asian countries reported during 2018-2020 formed a group distinct from Georgia/2007 (Fig. 7). Thus, our results showed the significance of the 14 ORFs in understanding the evolution of ASFV in Asian countries and their divergence from prototype Georgia/2007 ASFV. This might have implications on cross protectivity by the attenuated genotype-II vaccines, if derived from the Georgia/2007 isolate.

Conclusions:

Complete genome analyses showed the continuous evolution of genotype-II ASFV (Georgia/2007) with ac-

cumulation of SNPs, deletions and insertions through various regions in the genome of Indian ASFV isolated in 2020. The nucleotide identity and phylogenetic analysis indicated that ASFV/Wuhan/2019-1 could be the closest ancestor to the Indian viruses. The Indian isolates had unique SNP with predicted amino acid transitions viz., E294G in MGF 369-11L, K225E in MGF 505-4R, R188K in K205R and V168A in B263R genes. Significance of these unique SNP and frame shifts in DP60R, ASFV-G_ACD 00190, MGF 110-10-L-MGF110-14L fusion, MGF 360-14L and I267L genes in altering the virulence, replication and other properties of virus needs to be elucidated. Thus, our study is the first complete genome characterization of ASF viruses isolated from domestic pigs in India and provides important insights on their genetic relatedness/divergence with other ASF viruses of p72-genotype-II. The study also indicates that number of TRS in the intergenic region between I73R and I329L genes, and the 14 ORFs with SNP reported could be the genetic determinants to differentiate closely related p72-genotype II ASF viruses circulating in Asia.

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Conflict of Interest Statement

Authors do not have any conflict of interest

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Figure legends:

Fig. 1: Adsorption of porcine Red blood cells over African swine fever virus-infected monocytes in porcine monocyte culture (Magnification 100x).

Fig. 2: Insertion/deletion of nucleotides at various position in sequences of Indian ASFVs as compared to Georgia/2007 ASFV. [?]- indicates nucleotide deletion.

Fig. 3: Insertion of one extra TRS in the intergenic region between I73R and I329L in Indian isolates as compared to Georgia/2007 ASFV.

Fig. 4: Synonymous and non-synonymous SNPs across the genome of selected P-72 genotype-II ASFVs as compared to Indian ASFVs. * - indicates Synonymous SNPs, Unique SNPs are indicated in red colour.

Fig.5: ML tree generated from complete genome sequences (MAFFT aligned) using GTR plus gamma model in RAxML-NG showing relationship between Indian and other ASFVs. The branch length indicates the number of expected substitutions per site.

Fig. 6: Maximum-Likelihood phylogenetic tree generated using GTR plus gamma model in RAxML-NG showing relationship between Indian and other p72 genotype II full length ASFV sequences. The branch length indicates the no. of expected substitutions per site (tree rooted at mid-point).

Fig. 7: Maximum likelihood tree constructed based on concatenated sequences of genes with SNPs using Tamura–Nei parameter model of Mega X (1000 bootstrap iterations) showed clustering of Indian viruses with other Asian p72 genotype-II ASFVs (bootstrap values > 60 is shown over the nodes).

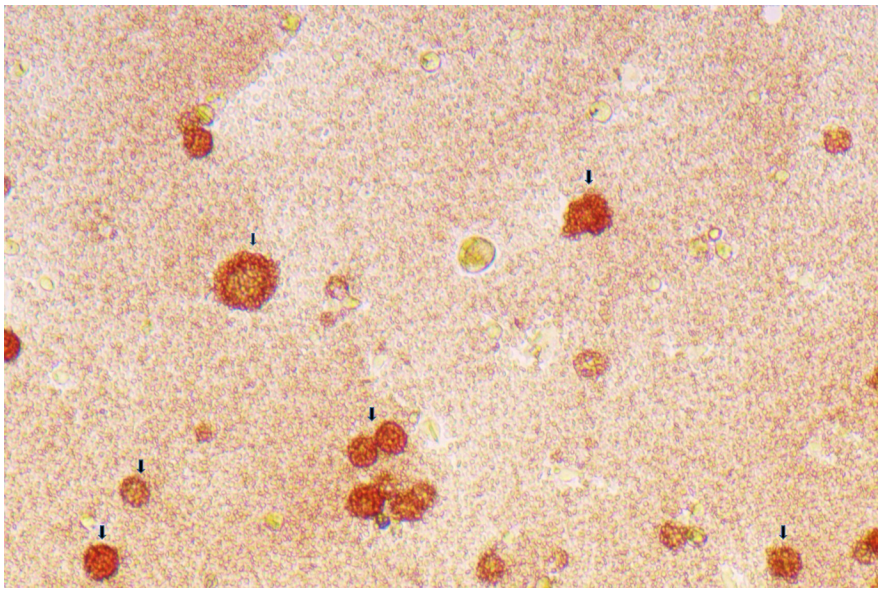
Table 1: List of additional complete genome sequences of ASFV used for genetic analyses.

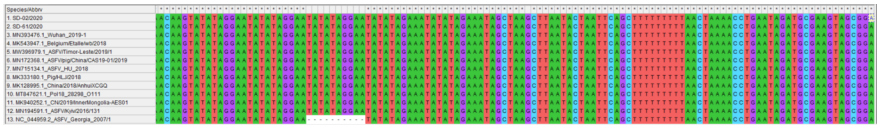
S. no.	Isolate	Country	Host	Year of submission/report	P72 genotype	Genbank Accession Number	Reference
1.	BA71V	Spain	Vero cells	1967	I	U18466.2	Yanez et al., 1995
2.	ASFV Wuhan 2019-1	China	Domestic Pig	2019	II	MN393476.1	Unpublished
3.	**Belgium/Etalle/wb/2018**	Belgium	Wild Boar	2018	II	MK543947.1	Gilliaux et al., 2019
4.	**ASFV_-Hanoi_-2019**	Vietnam	Pig	2020	II	MT166692.1	Unpublished
5.	**ASFV_-HU_-2018**	Hungary	Wild Boar	2019	II	MN715134.1	Olasz et al., 2019
6.	**Pig/HLJ/2018**	China	Pig/dried blood pig feed samples	2018	II	MK333180.1	Wen et al., 2019
7.	**China/2018/AnhuiXCGQ**	China	Domestic pig	2019	II	MK128995.1	Bao et al., 2019

S. no.	Isolate	Country	Host	Year of submission/report	P72 genotype	Genbank Accession Number	Reference
8.	**CN/2019/Inner Mongolia-AES01**	Mangolia	Wild boar	2020	II	MK940252.1	Unpublished
9.	**ASFV/Timor-Leste/2019/1**	Timor-Leste	Domestic pig	2019	II	MW396979.1	Unpublished
10.	**ASFV/Kyiv/2016/131**	Ukraine	Domestic pig	2016	II	MN194591.1	Kovalenko et al., 2019
11.	**Pol18_-28298_-O111**	Poland	Domestic pig	2017/2019	II	MT847621.1	Mazur-Panasiuk et al., 2020
12.	Tanzania/Rukwa/2017/1	Tanzania	Domestic pig	2017	II	LR813622.1	Unpublished
13.	**ASFV Georgia 2007/1**	Georgia	Domestic pig	2007	II	NC_044959.2	Chapman et al., 2011

S. no.	Isolate	Country	Host	Year of submission/report	P72 genotype	Genbank Accession Number	Reference
14.	**ASFV/Primorsky 19/WB-6723**	Russia	wild boar	2019	II	MW306191.1	Mazloum et al., 2021
15.	**ASFV/pig/China/CAS19-01/2019**	China	Domestic pig	2019	II	MN172368.1	Jia et al., 2020
16.	Warmbaths	South Africa	Tick	N/A	III/I	AY261365	Unpublished
17.	Warthog	Namibia	Warthog	1980	IV	AY261366	Zsak et al. 2005
18.	Tengani 62	Malawi	Domestic pigs	1962	V/I	AY261364.1	Unpublished
19.	Malwailil 20/1	Malawi	Tick	1983	VIII	AY261361	Unpublished
20.	SPEC_57	South Africa	Tick	1985	VIII	MN394630	Unpublished
21.	R35	Uganda	Domestic pigs	2015	IX	MH025920	Unpublished
22.	Ken06.Bus	Kenya	Domestic pigs	2006	IX	KM111295.1	Bishop et al.
23.	N10	Uganda	Domestic pigs	2015	IX	MH025919	Unpublished
24.	Uvira B53	Democratic Republic of the Congo		2019	X	MT956648.1	Bisimwa et al., 2021
25.	Pretorisuskop/96/4 A	South Africa	Tick	1996	XX/I	AY261363	Zsak et al. 2005
26.	R8	Uganda	Domestic pigs	2015	IX	MH025916	Unpublished
27.	Zaire	Zaire	Domestic pigs	2020	XX	MN630494	unpublished
28.	RSA-2-2004	South Africa	Wild boar	2004	XX	MN641877	Unpublished
29.	RSA-2-2008	South Africa	Tick	2008	XXII	MN336500.3	unpublished
30.	Mkuzi 1979	South Africa	Tick	1979	I/XII	AY261362.1	Zsak et al. 2005

S. no.	Isolate	Country	Host	Year of submission/report	P72 genotype	Genbank Accession Number	Reference
31.	Kenya 1950	Kenya	Domestic pigs	1950	X	AY261360	Zsak et al. 2005
32.	Ken06.Bus	Kenya	Domestic pigs	2006	IX	NC_-044946	Unpublished
33.	Ken05/Tk1	Kenya	Tick	2005	IX/X	NC_-044945	Bishop et al., 2015





Gene name	MGF110-1L	MGF110-1L	MGF110-4L	MGF110-4L	MGF110-4L	ASFV G ACD 00320	MGF 360-10L	MGF 360-11L	MGF 505-4R	MGF 505-4R	MGF 505-4R	MGF 505-9R	K205R	C717R	C717R	B263R	B263R	O174L	O174L	O174L	NP419L	E199L	E199L	MGF 360-21R
Nucleotide position	325	590	325	141	185	33	986	881	673	692	1108	967	563	797	1410	503	564	199	224	328	1241	385	583	71
Amino acid Position (Variant <Indian isolates)	109 R<W	*	109 D<N	47 S<R	62 R<K	*	329 N<S	294 E<G	225K <E	231 L<P	*	323 K<E	188 R<K	266 G<V	*	168 V<A	*	67 S<P	75 S<F	110 S<P	414 N<S	129 V<I	195 I<F	24 P<H
IND/AS/SD-02/2020	T	A	A	G	A	A	G	G	G	C	C	G	A	T	C	T	T	C	C	C	G	A	T	A
IND/AR/SD-61/2020	T	A	A	G	A	A	G	A	A	C	C	G	A	T	C	C	T	C	C	C	G	A	T	A
Georgia/2007	T	G	A	G	A	A	A	A	G	C	C	A	G	T	C	T	T	C	C	C	A	A	A	A
Anhui XCGQ/2018	T	A	A	G	A	A	G	A	G	C	C	G	G	T	C	T	T	T	T	T	G	A	T	A
Wuhan/2019-1	T	A	A	G	A	A	G	A	G	C	C	G	G	G	C	T	T	C	C	C	G	A	T	C
Timor-Leste/2019	T	A	G	G	A	A	G	A	G	C	C	G	G	T	C	T	T	C	C	C	G	A	T	A
China/CAS19-01/2019	T	A	A	G	A	G	G	A	G	C	C	G	G	T	C	T	T	C	C	C	G	A	T	A
HU/2018	T	A	A	G	A	A	G	A	G	C	C	G	G	T	C	T	C	C	C	C	G	A	T	A
Tanzania/Rukwa/2017	A	G	-	T	G	A	A	A	G	T	T	A	G	T	T	T	T	C	C	C	A	G	A	-