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Detection of Novel Sequences Related to African Swine Fever Virus in Human Serum and Sewage

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Running title: Discovery of novel ASFV-like sequences in human serum

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ABSTRACT

The family *Asfarviridae* contains only a single virus species, African swine fever virus (ASFV). ASFV is a viral agent with significant economic impact due to its devastating effects on populations of domesticated pigs during outbreaks, but has not been reported to infect humans. We report here the discovery of novel viral sequences in human serum and sewage which are clearly related to the Asfarvirus family, but highly divergent from ASFV. Detection of these sequences suggests that greater genetic diversity may exist among Asfarviruses than previously thought, and raises the possibility that human infection by Asfarviruses may occur.

The family *Asfarviridae* contains a single double-stranded DNA (dsDNA) virus called African swine fever virus (ASFV), which is thought to have evolved from an ancestral virus common to the nucleocytoplasmic large DNA viruses including poxviruses, iridoviruses and phycodnaviruses (10, 11). ASFV infects ticks and swine but has not been reported to infect humans. ASFV infection of wild swine typically causes persistent infection with few symptoms (9, 17, 24, 25), but domesticated pigs can develop severe disease including acute hemorrhagic fever with near-100% mortality. As there is no vaccine and disease is contained by animal quarantine and slaughter, ASFV outbreaks can decimate pig populations and have significant economic impact—a 2007 outbreak in the former Soviet republic of Georgia resulted in the death and slaughter of over 80,000 pigs (20).

ASFV is endemic in sub-Saharan Africa but has also been introduced to countries in Europe, South America and the Caribbean (26). Characterization of various ASFV isolates has led to the identification of 22 genotypes based on sequence variation in the C-terminal portion of the B646L gene encoding the major capsid protein (2, 4, 14). Within this segment of B646L, approximately 14% of the nucleotide sites are variable amongst the ASFV isolates studied (2, 4). The ASFV genome has been completely sequenced for the Vero cell-adapted BA71V strain (29) and several wild isolates (5). Like many other large dsDNA viruses, ASFV encodes open reading frames (ORFs) with homology to cellular genes involved in DNA replication, transcription, repair and protein modification (29). ASFV also has an array of ORFs with potential function in modulating host cell function or immune response (7).

We report here the discovery of novel viral sequences in human serum from the Middle East and in sewage from Spain that have clear similarity to ASFV genes. Of the 36 sequences identified, 29 did not have significant overlap or nucleotide identity with any of the other sequences. These 29 sequences are similar to 18 different ASFV genes, with some sequences matching to different regions within the same ASFV genes. Sequence and phylogenetic analyses indicate that the novel viral sequences are most closely related to the Asfarvirus family, but are highly divergent from known ASFV strains. We therefore hypothesize that these viral sequences are derived from at least one novel virus in the *Asfarviridae* family, which we refer to herein as African Swine Fever-like virus (ASFLV).

Discovery of novel Asfarvirus-related viral sequences. We analyzed total nucleic acid extracted from human serum samples by 454 sequencing as an approach to identifying potential novel human pathogens. Serum samples were collected from patients with acute febrile illness (AFI) and from healthy volunteers (normal serum; NS) in the Middle East between 2002 and 2005, and stripped of identifying information before analysis to protect patient confidentiality. Total nucleic acid was extracted from 199 AFI and 200 NS samples, and reverse-transcribed to enable detection of both RNA and DNA viruses. Each sample was then amplified by sequence-independent PCR using a primer that incorporates a 6-nucleotide barcode unique to that sample. Amplicons from multiple samples were pooled and subjected to 454 pyrosequencing. Additional details on serum sample processing and sequence data analysis are provided in Supplemental Methods. From 1 AFI and 3 NS samples, we identified 6 novel viral sequences with no significant nucleotide

similarity to known viruses, but whose translated sequences had detectable sequence identity to several ASFV proteins (Table 1), as determined using tBLASTx (1).

ASFV-related sequences were also found in sewage collected from an urban wastewater treatment plant in Barcelona, Spain. Briefly, viral particles from the sewage samples were concentrated and separated into fractions by cesium chloride equilibrium gradient centrifugation. Fractions with high concentrations of virus, as determined by quantitative PCR for human adenovirus, were treated first with DNase I to degrade non-viral DNA not protected by viral capsid, and then with a lysis buffer to disrupt viral capsids and release viral nucleic acids. Total nucleic acid was extracted from these fractions and was reverse-transcribed and PCR-amplified prior to 454 sequencing as described above. Additional details on the processing of sewage samples are provided in Supplemental Methods. 15 ASFV-related sequences were identified in 5 sewage fractions (SF).

We also identified an additional 15 ASFV-related sequences from our sequencing runs which could not be assigned to specific samples due to lack of a perfect sequence match with the barcode sequence. All of these unassignable sequences were identified in sequencing runs containing multiple samples including the sewage fractions which were found to contain ASFV-related sequences. We did not detect ASFV-related sequences in the other samples in these sequencing runs, and therefore speculate that these unassignable sequences are derived from the sewage samples within these runs that contained ASFV-like sequences. However,

as we cannot verify the specific sample source of these unassignable sequences, they are reported here as unassigned (UA) sequences.

A total of 36 novel ASFV-related sequences were identified, each of whose best-scoring tBLASTx matches were to the respective ASFV genes from various ASFV isolates (not shown). These sequences are listed in Table 1 and were positionally mapped to the complete genome for the ASFV BA71V strain [Fig. 1, (29)]. These novel viral sequences were similar not only to ASFV genes such as DNA polymerase and RNA polymerase, which are also conserved in other large, dsDNA virus families (10), but also to multiple ASFV genes including EP364R and M448R, which do not have significant similarity to genes in other viral families and are therefore, to date, specific to ASFV.

Novel Asfarvirus-related sequences are highly divergent from ASFV. The low amino acid identity between the novel viral sequences and the corresponding ASFV proteins (Table 1) suggested these sequences may belong to a genetically distinct virus rather than to a new isolate of ASFV. We therefore performed multiple sequence alignments to compare the DNA polymerase-like sequences from our samples to the corresponding sequences from ASFV isolates. Sequences SF-3, SF-6, SF-8.7 and UA.10 all mapped to the same region of ASFV DNA polymerase (Fig. 1) and were near-identical in nucleotide sequence where they overlapped (Supplemental Fig. 1). These sequences formed a 575-nucleotide consensus sequence which had 51% amino acid identity with ASFV DNA polymerase when compared by tBLASTx (e-value of $2e^{-26}$). Alignment of the regions of similarity between our novel DNA polymerase sequence and the corresponding

ASFV sequences showed that the ASFV sequences were highly conserved with each other, but the sequence from our samples was divergent except for small blocks of amino acids which were conserved with ASFV sequences (Fig. 2a). The same result was obtained using the NS-1.2 and NS-2.2 sequences, which are similar to ASFV RNA polymerase and topoisomerase II, respectively (Supplemental Fig. 2a and 2b). We also performed phylogenetic analysis using sequence SF-8.3 with similarity to the EP364R gene, which appears to be ASFV-specific. Sequences from ASFV isolates were again found to be highly conserved with each other, whereas the novel viral sequence was divergent (Fig. 2b). The same result was obtained using the UA.15 sequence with similarity to another ASFV-specific gene, M448R (Supplemental Fig. 2c).

Novel viral sequences are most closely related to the Asfarvirus family. As viruses in multiple dsDNA viral families encode homologs of DNA polymerase, RNA polymerase and topoisomerase II, we compared those sequences from our samples to corresponding ones from ASFV isolates as well as other dsDNA viruses from families including *Poxviridae*, *Iridoviridae*, *Phycodnaviridae*, *Herpesviridae*, and *Ascoviridae*. We also included in our analyses several sequences that had the most significant e-values following ASFV strains when the novel DNA and RNA polymerase sequences were queried against the nucleotide database by tBLASTx. The novel topoisomerase II sequence did not have any hits with significant e-values other than ASFV strains.

We found that our translated DNA polymerase consensus sequence formed a separate branch which was most closely related to the cluster containing the ASFV

isolates (Fig. 3a). The same result was observed for the RNA polymerase [Fig. 3b; NS-1.2] and topoisomerase II [Fig. 3c; NS-1.2] sequences. For all three proteins, grouping of the various dsDNA viruses was consistent with their classifications and with previously published studies using other sets of conserved genes (10, 18, 22).

As several additional novel sequences from our samples are similar to other ASFV genes that have also been reported to have homologs in other dsDNA virus families (10, 11), we performed similar phylogenetic analyses on these sequences. We found that 10 of the 11 sequences with similarity to ASFV genes B354L, C962R, D250R, EP1242L, B646L or B962L, were most closely related to, but distinct from, ASFV sequences (not shown), consistent with our results for the DNA polymerase, RNA polymerase and topoisomerase II sequences. The exception was sequence SF-9.1, which is similar to ASFV C962R but did not group with sequences from any of the dsDNA families in the phylogenetic analysis. SF-9.1 is 99% identical in nucleotide sequence over a 238-nucleotide overlap with sequence UA.6 (not shown), which is also similar to C962R. Two of the three nucleotide changes in SF-9.1 are insertions or deletions resulting in frameshifts that significantly altered portions of the SF-9.1 translated sequence relative to that of UA.6. It is therefore likely that the results of phylogenetic analysis for SF-9.1 differed from that of UA.6 because the frameshifts decreased the overall similarity of the SF-9.1 translated sequence to ASFV.

Discussion. The detection in this study of multiple viral sequences that are clearly related to, but phylogenetically distinct from, ASFV suggests that at least one additional member of the family *Asfarviridae* exists. The fact that these sequences

were identified in multiple 454 sequencing runs over a period of approximately 7 months, and that the first sequences identified—NS-1.2 and NS-2.2—were not detected in subsequent sequencing runs of different samples, strongly argues against the possibility that the detection of ASFV-like sequences in these samples is due to cross-contamination.

Since the ASFV-like sequence fragments were identified from two types of samples from different geographic regions, it is possible these sequences are derived from more than one virus in this family. In support of this possibility, sequences SF-10, UA.3 and UA.4 aligned to the same region in gene C475L of the reference ASFV genome, but had significantly different nucleotide sequences from each other. An alignment of the translated SF-10, UA.3 and UA.4 sequences with the corresponding segment of ASFV C475L showed only 28% identity between UA.3 and SF-10, and 71% identity between UA.3 and UA.4, in their respective regions of overlap (Supplemental Fig. 3). This suggests the possibility that our samples contained more than one Asfarvirus-related virus, although this cannot be conclusively determined since the regions of overlap between these sequences are short.

Although ASFV is not known to infect humans even where the virus is endemic, identification of ASFV-like sequences in serum from multiple human patients suggests human infection may occur. Further studies are underway to prospectively screen patient samples by PCR for presence of ASFV-like sequences using primers to the sequences reported here in order to assess prevalence and geographic distribution. These studies, in combination with serological analyses, will

be required to determine whether the ASFV-like virus is, in fact, a human virus and whether it is associated with human disease.

The finding of ASFV-like sequences in sewage from Spain indicates they are not geographically limited to the Middle East where the human patient specimens were obtained. Although it is unclear whether the source of the ASFV-like sequences in sewage is human or animal, this also suggests the virus may be fecally shed, and that screening of stool in addition to serum may be informative. Identification of additional samples containing ASFV-like sequences will be important for the key future goals of obtaining more sequence from the viral genome, determining if the virus can be cultured, and establishing a small animal model of infection.

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310 **FIGURE LEGENDS**

311 **Figure 1. Novel viral sequences with similarity to ASFV genes.** Novel viral
312 sequences were positionally mapped to the complete genome of the ASFV BA71V
313 strain (29) based on their amino acid sequence similarity to ASFV proteins. Each
314 novel viral sequence is represented by a black bar, and the ASFV genomic
315 nucleotide position to which it is similar is indicated below the bar.

316

317 **Figure 2. Novel viral sequences are divergent from ASFV. (A)** Translated
318 sequence from the novel DNA polymerase consensus sequence was aligned to
319 corresponding sequences from various ASFV isolates using AlignX (VectorNTI suite,
320 Invitrogen). Residues which are highly conserved are shaded in gray and those
321 which are divergent are in black. **(B)** Phylogenetic analysis of the translated SF-8.3
322 sequence and corresponding sequences from the EP364R gene of various ASFV
323 isolates was performed using the neighbor-joining method with 1000 bootstrap
324 replicates. Bootstrap values over 65% are shown. Sequences were aligned using
325 ClustalX (2.0) and phylogenetic trees were visualized using TreeView (16).
326 GenBank accession numbers for ASFV sequences are provided in Supplemental
327 Methods. Abbreviations: Benin, Benin 97/1 (5); Kenya, Kenya 1950; Malawi, Malawi
328 Lil-20/1 1983; Mkuzi, Mkuzi 1979; OURT, OURT 88/3 (5); Pretorisuskop,
329 Pretorisuskop/96/4; Tengani, Tengani 62.

330

331 **Figure 3. Phylogenetic analysis of novel viral sequences.** Translated novel viral
332 sequences similar to ASFV **(A)** DNA polymerase, **(B)** RNA polymerase and **(C)**

topoisomerase II were compared to corresponding sequences from dsDNA viruses and high-scoring non-viral BLAST matches as described in figure legend 2. Asfarviruses are shown in red, mimivirus in brown, poxviruses in purple, herpesviruses in grey, phycodnaviruses in green, ascoviruses in orange, and iridoviruses in blue. Non-viral BLAST matches with significant e-values for the DNA polymerase and RNA polymerase sequences are shown in black. Bootstrap values over 65% are shown. For **(A)**, virus intrafamily subclassifications are also shown where applicable. For **(B)** and **(C)**, virus families for which the corresponding RNA polymerase and topoisomerase II sequences could not be identified by BLAST were omitted from the analyses. GenBank accession numbers for the sequences analyzed are provided in Supplemental Methods. Abbreviations: AmEPV, Amsacta moorei entomopoxvirus; APMV, Acanthamoeba polyphaga mimivirus; ATCV1, Acanthocystis turfacea Chlorella virus 1; CIV, Chilo iridescent virus; CVM1, Chlorella virus Marburg 1; D. autotrophicum, Desulfobacterium autotrophicum; DpAV4, Diadromus pulchellus ascovirus 4; EBV, Epstein-Barr virus; EhV86, Emiliania huxleyi virus isolate 86; ESV, Ectocarpus siliculosus virus; FsV158, Feldmannia species virus isolate 158; G. anomala, Glugea anomala; H. andersenii, Hemiselmis andersenii; H. butylicus, Hyperthermus butylicus; HCMV, Human cytomegalovirus; HHV6, Human herpesvirus 6; HSV1, Herpes simplex virus 1; HSV2, Herpes simplex virus 2; HvAV3, Heliothis virescens ascovirus 3; I. scapularis, Ixodes scapularis; ISKNV, Infectious spleen and kidney necrosis virus; K. JI2008, Kabatana sp. JI2008; KSHV, Kaposi's sarcoma-associated herpesvirus; L. acerinae, Loma acerinae; LDV, Lymphocystis disease virus; LSDV, Lumpy skin disease virus; M. labreanum,

356 Methanocorpusculum labreanum; M. marisnigri, Methanoculleus marisnigri; MIV,
357 Mosquito (Aedes taeniorhynchus) iridescent virus; MsEPV, Melanoplus sanguinipes
358 entomopoxvirus; OSGIV, Orange-spotted grouper iridovirus; OsV5, Ostreococcus
359 virus 5; PBCV1, Paramecium bursaria Chlorella virus 1; S. islandicus, Sulfolobus
360 islandicus; SfAV1, Spodoptera frugiperda ascovirus 1; SGIV, Singapore grouper
361 iridovirus; TFV, Tiger frog virus; TnAV2c, Trichoplusia ni ascovirus 2c; VZV,
362 Varicella zoster virus; YMTV, Yaba monkey tumor virus.

363 **Table 1. Novel viral sequences with similarity to ASFV.**

Sample	Sequence	Total reads[a]	ASFV gene[b]	E-value[c]	% Identity[d]	Gene description (references)	Accession[e]
Human Acute Febrile Illness (AFI) & Normal Serum (NS)	AFI-1	23224	G1211R	$1e^{-10}$	64	DNA polymerase (15, 19)	FJ957903
	NS-1.1	83028	B354L	$3e^{-11}$	42	ATPase (10)	FJ957904
	NS-1.2	14737	NP1450L	$2e^{-13}$	45	RNA polymerase, largest subunit (27)	FJ957905
	NS-2.1	42635	M1249L	$2e^{-10}$	36	unknown	FJ957906
	NS-2.2	3870	P1192R	$5e^{-12}$	37	topoisomerase II (3, 8)	FJ957907
	NS-3	2602	C962R	$7e^{-12}$	38	primase or ATPase (10, 12)	FJ957908
Sewage Fractions (SF)	SF-3[f, g]	78573	G1211R	$2e^{-26}$	51	DNA polymerase (15, 19)	FJ957909
	SF-6[f, g]	6633	G1211R	$1e^{-25}$	51	DNA polymerase (15, 19)	FJ957910
	SF-8.1[h]	33028	D250R	$4e^{-14}$	60	NTP pyrophosphohydrolase (10)	FJ957911
	SF-8.2	33028	EP1242L	$1e^{-19}$	45	RNA polymerase, subunit 2 (27)	FJ957912
	SF-8.3	33028	EP364R	$5e^{-9}$	39	nuclease (11)	FJ957913
	SF-8.4[i]	33028	EP424R	$5e^{-9}$	58	RNA methyltransferase (11)	FJ957914
	SF-8.5	33028	F1055L	$3e^{-18}$	32	helicase (23)	FJ957915
	SF-8.6[f]	33028	F1055L	$6e^{-8}$	50	helicase (23)	FJ957916
	SF-8.7[g]	33028	G1211R	$5e^{-23}$	49	DNA polymerase (15, 19)	FJ957917
	SF-8.8	33028	G1340L	$1e^{-8}$	27	unknown	FJ957918
	SF-8.9[j]	33028	H339R	$5e^{-13}$	44	unknown	FJ957919
	SF-9.1[k]	13968	C962R	$4e^{-10}$	40	primase or ATPase (10, 12)	FJ957920
	SF-9.2	13968	CP2475L	$5e^{-10}$	41	220kDa polyprotein (21)	FJ957921
	SF-9.3[i]	78307	EP424R	$2e^{-11}$	44	RNA methyltransferase (11)	FJ957922
	SF-10	16843	C475L	$9e^{-14}$	46	polyadenylate polymerase (6)	FJ957923
Unassigned (UA)	UA.1[f]	N/A	B646L	$1e^{-11}$	44	major capsid protein (13)	FJ957924
	UA.2[f]	N/A	B962L	$7e^{-37}$	50	helicase (28)	FJ957925
	UA.3	N/A	C475L	$1e^{-10}$	31	polyadenylate polymerase (6)	FJ957926
	UA.4	N/A	C475L	$8e^{-9}$	37	polyadenylate polymerase (6)	FJ957927
	UA.5	N/A	C962R	$6e^{-38}$	38	primase or ATPase (10, 12)	FJ957928
	UA.6[k]	N/A	C962R	$1e^{-17}$	32	primase or ATPase (10, 12)	FJ957929
	UA.7[f]	N/A	CP2475L	$2e^{-15}$	30	220kDa polyprotein (21)	FJ957930
	UA.8[h]	N/A	D250R	$5e^{-14}$	60	NTP pyrophosphohydrolase (10)	FJ957931
	UA.9[f]	N/A	EP1242L	$1e^{-20}$	44	RNA polymerase, subunit 2 (27)	FJ957932
	UA.10[f, g]	N/A	G1211R	$2e^{-26}$	51	DNA polymerase (15, 19)	FJ957933
	UA.11	N/A	G1211R	$1e^{-15}$	41	DNA polymerase (15, 19)	FJ957934

	UA.12	N/A	G1340L	$6e^{-15}$	32	unknown	FJ957935
	UA.13	N/A	G1340L	$1e^{-28}$	50	unknown	FJ957936
	UA.14[j]	N/A	H339R	$8e^{-12}$	35	unknown	FJ957937
	UA.15	N/A	M448R	$2e^{-9}$	34	unknown	FJ957938

[a]The total number of reads generated from each sample is given for each sequence whose parent sample is known. For samples that were sequenced in more than one sequencing run, the total number of reads is given for the run in which the indicated novel sequence was identified.

[b]ASFV genes to which the novel viral sequences have sequence similarity, as determined using tBLASTx (1). ASFV genes are listed using nomenclature for the BA71V strain (29).

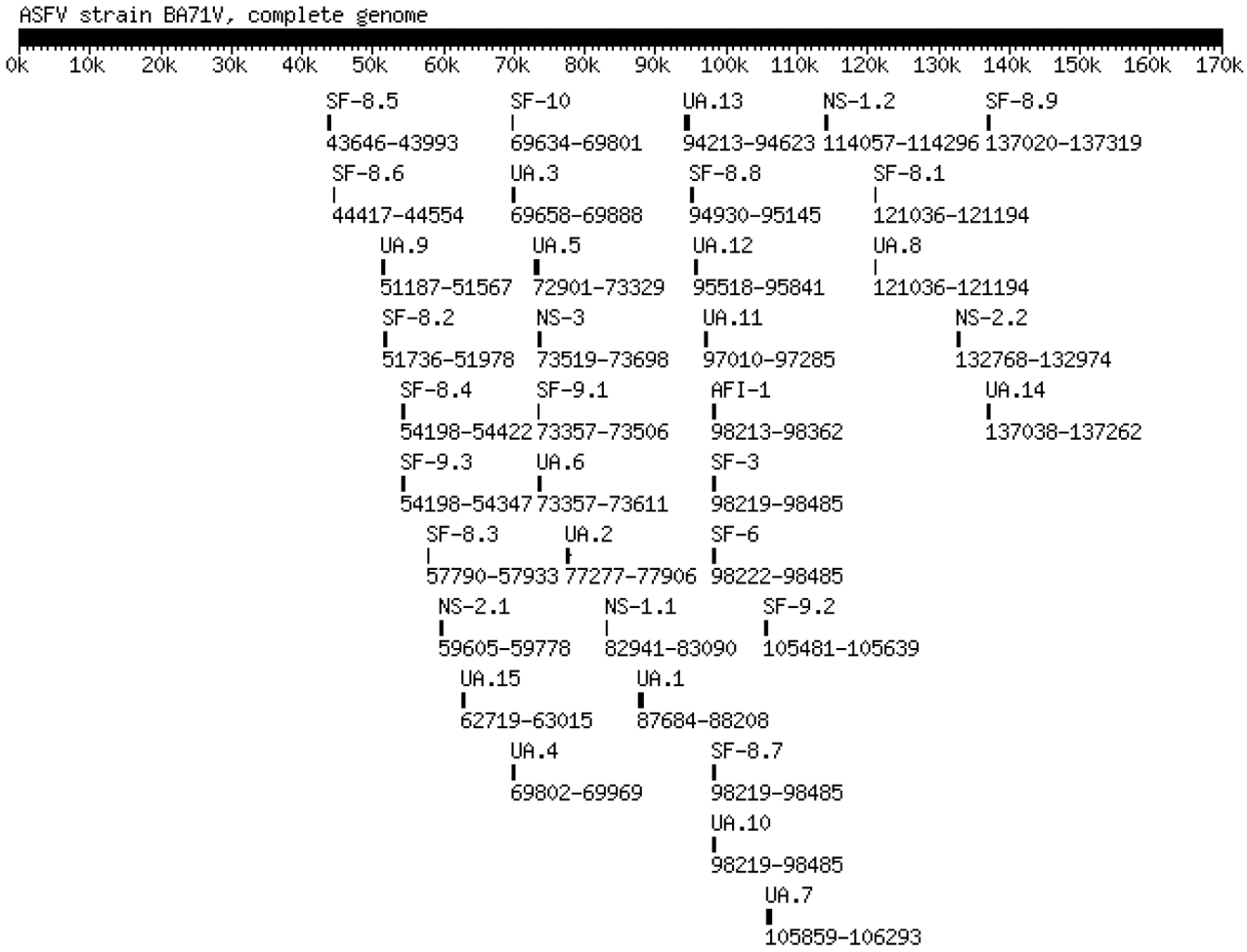
[c]E-value for tBLASTx comparison of the novel viral sequences to the ASFV BA71V complete genome (29).

[d]Percent amino acid identity between the novel viral sequences and corresponding sequences in the ASFV BA71V strain within the region of similarity identified using tBLASTx.

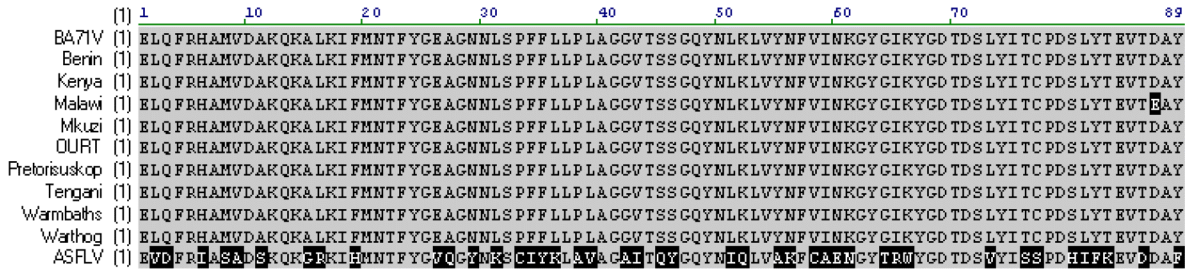
[e]GenBank accession numbers for each of the reported sequences.

[f]Sequence was assembled from two or more overlapping reads.

[g-k]Sets of sequences whose members were similar to the same regions of the ASFV genome and had significant overlap and nucleotide sequence identity with each other.



A



B