Intra-genotypic resolution of African swine fever viruses from an East African domestic pig cycle: a combined *p72* -CVR approach

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Abstract

Two of the 22 presently recognised African swine fever (ASF) virus *p72* genotypes are genetically homogeneous and are associated with domestic pig cycles. Of these, genotype VIII comprises just two *p72* variants, designated ‘a’ and ‘b’ in this study, and is confined to four East African countries where it has caused numerous outbreaks between 1961 and 2001. In order to resolve relationships within this homogeneous genotype, the central variable region (CVR) of the *9RL* open reading frame of 38 viruses was characterised and the resulting dataset complemented with seven published sequences. Phylogenetic analysis of the 45 taxa resulted in seven discrete amino acid CVR lineages (A–G). CVR lineage F, 84 amino acids in length and spanning a 40-year period, comprised 26 isolates from Malawi, Mozambique, Zambia and Zimbabwe. The second largest lineage (E), consisted of 10 viruses causing outbreaks over a 10-year period in Zambia, Malawi and Mozambique whilst the remaining five lineages were country-specific and represented by

four or less viruses with a maximum circulation period of three years. A combined *p72*-CVR analysis resulted in eight discrete lineages corresponding to eight unique *p72*-CVR combinations. One of these, b–F, appears to have arisen by convergent evolution or through an intra-genotypic recombination event, as the individual *p72* and CVR gene phylogenies are incongruent. This raises the possibility of intra-genotypic recombination in ASF viruses for the first time. However, given the repetitive nature of the CVR region, convergent evolution cannot be excluded and may be the more likely explanation.

Introduction

The genus *Asfivirus* and family *Asfarviridae* derive their names from a unique, large, enveloped, icosahedral arbovirus, *African swine fever virus* (ASFV) that is presently the sole representative of this family [*1*]. This virus has a linear, double-stranded DNA genome, varying from 170 to 190 kb in size, and is the causative agent of African swine fever (ASF), an often highly lethal, haemorrhagic domestic pig disease, that is endemic to sub-Saharan Africa. Here it occurs in one of three possible cycles with the virus being transmitted between (i) sylvatic hosts (vertebrate and invertebrate) and domestic pigs (ii) domestic pigs and the soft-shelled *Ornithodoros* ticks and lastly, (iii) domestic pigs alone [*2*]. The latter cycle is believed to occur independently of ticks and complicates disease control [*3*], which in the absence of a vaccine is reliant on strict biosecurity for prevention, and on stamping out in the event of an outbreak.

The epidemiological complexity of ASF was clearly demonstrated in East Africa, where characterisation of the *p72* gene coding for the major capsid and immuno-dominant protein, VP72, revealed the presence of 13 discrete genotypes in this region [*4*]. Of these, genotype VIII constituted a large, homogeneous group of viruses recovered solely from domestic pigs in Malawi, Mozambique and Zambia, over a 23 year period [*4*]. The low levels of intra-genotypic variation (0.1%) across this gene region precluded determination of the origin, subsequent spread or relatedness of these outbreaks. Previous RFLP studies of 17 viruses recovered from outbreaks in Malawi between 1982 and 1989 confirmed the presence of a central variable region (CVR) within the central 125 kb conserved region of the genome [*5*]. This length variation was subsequently shown to result from differences

in a tetrameric repeat region located within the late viral gene, *9RL* [*6*], also termed the *B602L* gene [*7*, *8*]. The protein, which varies in size in accordance with the number of repeats within the CVR is homogeneously distributed throughout the cytoplasm and is strongly recognised by convalescent swine serum [*6*]. Recent PCR-based studies directed at the CVR have revealed both size and sequence variability [*8*, *9*, *10*] for the limited number of genotype VIII viruses included in these studies. Although the CVR is not suitable for resolution of inter-genotypic relationships [*9*, *11*], it has proved useful for resolving epidemiological complexities at the country [*9*], genotype [*8*] and regional level [*11*]. A two-step approach is advocated for epidemiological studies [*9*], whereby viruses are first assigned to their *p72* genotype [*12*], prior to intra-genotypic resolution by CVR, and was followed in this study which represents the first comprehensive attempt to resolve the intra-genotypic relationships of 45 *p72* genotype VIII viruses causing outbreaks in East Africa over a 40 year period.

Materials and methods

Viruses

A total of 38 viruses shown to constitute genotype VIII by *p72* genotyping [*4*] were selected for CVR typing. Seven additional viruses identified as genotype VIII in previous studies [*8*, *9*, *12*] and for which CVR data were available were included, bringing the total number of viruses used in this study to 45 (Table 1).

Table 1 Summary of the *p72* genotype VIII ASF viruses included in this study, arranged by CVR lineage

Isolate / Specimen name	**Country**	**Year**	**Number of AAs (Tets)**	**Aligned tetrameric repeat sequence**	**p72-CVR lineage**	**Genbank accession number**	**CVR sequence reference**
[1]LIL 20/1	Malawi	1983	124 (31)	AVSVSOVNAPNOPPNPOVNAPNOPPN-MQMJQ	a–A	AM259427	[*1*]
[2]Dowa	Malawi	1986	120 (30)	ANP- -OVNAPNOPPNPOVNAPNOPPNPMQMJQ	a–B	DQ874386	This study
[2]LIL 89/1	Malawi	1989	120 (30)	ANP--OVNAPNOPPNPOVNAPNOPPNPMQMJQ	a–B	DQ874387	This study
[2]ZON 88/1	Zambia	1988	124 (31)	AVSVSVSVSOVNAPNOPPG-PNOPPNPMQMJQ	a–C	DQ874388	This study
[2]CHG 88/1	Zambia	1988	116 (26)	AVSVSVS--OVNAPNOPPG-PNOPPNPMQMJQ	a–D	DQ874383	This study
[2]GUL 88/1	Zambia	1988	116 (26)	AVSVSVS--OVNAPNOPPG-PNOPPNPMQMJQ	a–D	DQ874385	This study
[2]KLI 88/2	Zambia	1988	116 (26)	AVSVSVS--OVNAPNOPPG-PNOPPNPMQMJQ	a–D	DQ874384	This study
[2]KAV 89/1	Zambia	1989	116 (26)	AVSVSVS--OVNAPNOPPG-PNOPPNPMQMJQ	a–D	DQ890169	This study

Isolate / Specimen name	Country	Year	Number of AAs (Tets)	Aligned tetrameric repeat sequence	p72-CVR lineage	Genbank accession number	CVR sequence reference
[2]KAL 88/1	Zambia	1988	92 (23)	AVSVSVS---VNAP-------NOPPNPMQMJQ	a–E	AY538732	[9]
[2]ZAW 88/1	Zambia	1988	92 (23)	AVSVSVS--OVNA-------PNOPPNPMQMJQ	a–E	DQ874377	This study
[2]CHK 89/2	Zambia	1989	92 (23)	AVSVSVS--OVNA-------PNOPPNPMQMJQ	a–E	DQ874379	This study
[2]MCH 89/3	Malawi	1989	92 (23)	AVSVSVS--OVNA-------PNOPPNPMQMJQ	a–E	DQ874380	This study
[2]KAC 91/2	Malawi	1991	92 (23)	AVSVSVS--OVNA-------PNOPPNPMQMJQ	a–E	DQ874381	This study
[2]SIY 91/2	Malawi	1991	92 (23)	AVSVSVS--OVNA-------PNOPPNPMQMJQ	a–E	DQ874382	This study
MOZ-A/98	Mozambique	1998	92 (23)	AVSVSVS–OVNA——-PNOPPNPMQMJQ	a–E	AY274461	[9]
MOZ-B/98	Mozambique	1998	92 (23)	AVSVSVS--OVNA-------PNOPPNPMQMJQ	a–E	AY274462	[9]
MOZ-C/98	Mozambique	1998	92 (23)	AVSVSVS–-OVNA-------PNOPPNPMQMJQ	a–E	AY274463	[9]
MOZ-62/98	Mozambique	1998	92 (23)	AVSVSVS--OVNA-------PNOPPNPMQMJQ	a–E	AY274464	[9]
[2]RHO 61/1	Zimbabwe	1961	84 (21)	AVSVS----OVNA-------PNOPPNPMQMJQ	b–F	DQ874356	This study
[2]Mchinji 075	Malawi	1987	84 (21)	AVSVS----OVNA-------PNOPPNPMQMJQ	a–F	DQ874359	This study
Malawi 78	Malawi	1978	84 (21)	AVSVS----OVNA-------PNOPPNPMQMJQ	a–F	AY274466	[9]
[2]ZOM 84/2	Malawi	1984	84 (21)	AVSVS----OVNA-------PNOPPNPMQMJQ	a–F	AY538731	[9]
[2]CHM 88/1	Zambia	1988	84 (21)	AVSVS----OVNA-------PNOPPNPMQMJQ	a–F	DQ874357	This study

Isolate / Specimen name	Country	Year	Number of AAs (Tets)	Aligned tetrameric repeat sequence	p72-CVR lineage	Genbank accession number	CVR sequence reference
[2]NKZ 88/1	Zambia	1988	84 (21)	AVSVS----OVNA-------PNOPPNPMQMJQ	a–F	DQ874360	This study
[2]PHW 88/1	Zambia	1988	84 (21)	AVSVS----OVNA----PNOPPNPMQMJQ	a–F	DQ874361	This study
[2]YEL 88/4	Zambia	1988	84 (21)	AVSVS----OVNA-------PNOPPNPMQMJQ	a–F	DQ874362	This study
[2]CHJ 89/1	Zambia	1989	84 (21)	AVSVS----OVNA-------PNOPPNPMQMJQ	a–F	DQ874363	This study
[2]DED 89/1	Malawi	1989	84 (21)	AVSVS----OVNA-------PNOPPNPMQMJQ	a–F	DQ874364	This study
[2]JON 89/1	Zambia	1989	84 (21)	AVSVS----OVNA-------PNOPPNPMQMJQ	a–F	AY538728	[*9*]
[2]KANA 89/1	Zambia	1989	84 (21)	AVSVS----OVNA-------PNOPPNPMQMJQ	a–F	DQ874365	This study
[2]MAN 89/2	Zambia	1989	84 (21)	AVSVS----OVNA-------PNOPPNPMQMJQ	a–F	DQ874358	This study
[2]MCH 89/1	Malawi	1989	84 (21)	AVSVS----OVNA-------PNOPPNPMQMJQ	a–F	DQ874366	This study
[2]MPI 89/1	Zambia	1989	84 (21)	AVSVS----OVNA-------PNOPPNPMQMJQ	a–F	DQ874367	This study
[2]MPO 89/1	Zambia	1989	84 (21)	AVSVS----OVNA-------PNOPPNPMQMJQ	a–F	DQ874368	This study
[2]TEN 89/1	Zambia	1989	84 (21)	AVSVS----OVNA-------PNOPPNPMQMJQ	a–F	DQ874369	This study
[2]TMB 89/1	Zambia	1989	84 (21)	AVSVS----OVNA-------PNOPPNPMQMJQ	a–F	DQ874370	This study
[2]LIL 90/1	Malawi	1990	84 (21)	AVSVS----OVNA-------PNOPPNPMQMJQ	a–F	DQ874371	This study
[2]NDA 90/1	Malawi	1990	84 (21)	AVSVS----OVNA-------PNOPPNPMQMJQ	b–F	AY538730	[*9*]

Isolate / Specimen name	Country	Year	Number of AAs (Tets)	Aligned tetrameric repeat sequence	p72-CVR lineage	Genbank accession number	CVR sequence reference
[2]THY 90/1	Malawi	1990	84 (21)	AVSVS----OVNA-------PNOPPNPMQMJQ	a–F	DQ874372	This study
[2]BAN 91/1	Malawi	1991	84 (21)	AVSVS----OVNA-------PNOPPNPMQMJQ	a–F	DQ874373	This study
[2]DED 91/1	Malawi	1991	84 (21)	AVSVS----OVNA-------PNOPPNPMQMJQ	a–F	DQ874374	This study
[2]NGE 92/1	Malawi	1992	84 (21)	AVSVS----OVNA-------PNOPPNPMQMJQ	a–F	DQ874375	This study
[2]SAL 92/1	Malawi	1992	84 (21)	AVSVS----OVNA-------PNOPPNPMQMJQ	a–F	DQ874376	This study
MOZ 01/01	Mozambique	2001	84 (21)	AVSVS----OVNA-------PNOPPNPMQMJQ	a–F	DQ874377	This study
[2]Dedza	Malawi	1986	56 (14)	AVS----------NOPPNPMQMJQ	a–G	AY538729	[*9*]

AAs: Amino acid; Tets: Tetramers; [1]Tick collected from a domestic pig; [2]Isolates provided by the Institute for Animal Health (IAH), Pribright, UK

Unique single letter codes for each tetrameric repeat was assigned as follows: A = CAST; V = NADT; S = SAST; O = NASI; N = NVDT; P = NAST; M = NADI; Q = NANT; J = NANI, G = NVGT. Gaps/indels, denoted by a '-' and were inserted for alignment purposes

CVR amplification, sequence determination and alignment

Viral DNA was extracted as described previously [*12*] and used as template for amplification with CVR with primers CVR-FL1 (5′TCG GCC TGA AGC TCA TTA G3′) and CVR-FL2 (5′CAG GAA ACT AAT GAT GTT CC3′) that bind in the regions flanking the repeat region [*9*]. All reactions were performed in a final volume of 50 µl containing 200 µM dNTPs (Roche), 0.4 µM of each primer, 2.5 U of *Taq* DNA polymerase (Roche) and 3 µl of DNA extract. Thermal cycling was performed as previously described [*9*, *10*] with the resulting products being sized against a 100 bp molecular weight marker (Promega) following agarose gel electrophoresis. Amplicons were excised and purified from the gel by means of a Nucleospin kit (Machery-Nagel) and used as template for cycle sequencing with Big Dye version 3.1 (Applied Biosystems) with each of the PCR primers. Precipitated products were run on an ABI Prism 310 Genetic Analyser (Applied Biosystems) using a 47 cm capillary, visualised and edited in CHROMAS 1.43 [*13*], before being exported to the DAPSA alignment programme [*14*]. Forward and reverse sequences were aligned and the deduced amino acid (aa) sequences determined from the resulting full-length sequence. Amino acids were grouped into tetrameric repeats and manually aligned, before being converted to single letter tetramer codes (Table 1), which permitted further optimisation of the alignment.

Phylogenetic analyses

Three datasets were generated for parsimony analyses, namely a (i) *p72* gene dataset (404 characters), (ii) a CVR dataset (128 characters) and (iii) a combined *p72*-CVR (532

characters). For the CVR dataset, the aligned tetramers were used to infer the phylogeny, with gaps inserted for alignment purposes being treated as 21st character states. For neighbour-joining (NJ) analyses, the CVR dataset (ii) and CVR portion of the combined dataset (iii) was coded using a simple indel coding method [*15*] in order to preserve and utilise information contained in the gap regions that are usually deleted in either a pairwise or complete manner when performing a phenetic analysis. Between 1,000 and 10,000 bootstrap replicates were performed in PAUP* [*16*] in order to assess nodal support and a genotype V virus (MOZ/1960) from Mozambique [*9*] was used as an outgroup in all the analyses. Prior to performing a combined p72-CVR analysis, an incongruence length difference (ILD) test [*17*], known as the partition-homogeneity test (PHT) in PAUP* was performed with 1,000 replicates on the *p72* and CVR data partitions.

Results

Individual gene phylogenies

Neighbour-joining and parsimony trees inferred from the homologous 404 nucleotides corresponding to the *C*-terminal end of the *p72* gene resulted in 2 lineages, designated ‘a’ and ‘b’ in Fig. 1 (i). Lineage ‘a’ comprised 43 isolates from Malawi, Mozambique and Zambia, spanning a 23 year period, while lineage ‘b’ contained two identical viruses from Malawi and Zimbabwe, sampled 29 years apart. The NJ tree inferred with the coded CVR dataset produced seven discrete and well-supported CVR lineages, designated A–G in Fig. 1 (ii). Virus representatives of *p72* lineage ‘a’ were found in all seven CVR lineages whilst the two *p72* lineage ‘b’ viruses clustered together with 24 *p72* lineage ‘a’ viruses within CVR lineage F.

Fig. 1 Neighbor-joining (NJ) trees depicting (i) the *p72* gene and (ii) CVR relationships of *p72* genotype VIII African swine fever viruses. The two major *p72* genotype VIII lineages are denoted ‘a’ and ‘b’ in Fig. 1 (i), whilst the seven major CVR lineages (A–G) are indicated in Fig 1 (ii). Bootstrap values next to each node are those >50 and obtained

following 1,000 replications with support obtained from neighbor-joining being followed by the bootstrap values from parsimony, given in brackets

Combined p72-CVR analysis

The p-value obtained from the PHT performed in PAUP* was 1, indicating that the *p72* and CVR data partitions did not differ significantly and could therefore be combined. Eight unique virus lineages were recovered on analysis of the combined dataset (Fig. 2), corresponding to eight unique *p72*-CVR combinations (Table 1), which is higher than the two and seven lineages recovered from individual gene analysis of *p72* and CVR, respectively. Lineages a–A, a–B, a–C and a–D formed a monophyletic lineage (designated I in Fig. 2) that had 83 and 97 % bootstrap support in NJ and MP, respectively. Lineages a–E, a–F and b–F formed a second monophyletic lineage (II, 95% bootstrap support), whilst Dedza (III), a virus recovered from an outbreak in Malawi in 1986 was basal to these two major lineages.

Fig. 2 Neighbor-joining tree obtained with the combined *p72* nucleotide and coded CVR data. Bootstrap values >50% obtained following 1,000 replicates with neighbor-joining are indicated next to each node and are followed where applicable by the bootstrap value obtained from parsimony in brackets. Each unique *p72*-CVR lineage is indicated by means of the letters used in Fig. 1(i) and 1 (ii), to assign the *p72* and CVR lineages (a–b and A–G), respectively. Each *p72*-CVR lineage that is represented by a single virus is given in brackets to the right of the virus name, whilst those lineages comprising two or more viruses are indicated to the right of the square bracket, together with the temporal and geographical range of that lineage

Length and sequence variation of the genotype VIII CVR lineages

Tetrameric repeats, NAST, NVDT, NADT and to a lesser extent SAST, were most abundant in genotype VIII viruses (Table 1). Lil 20/1, a virus recovered from a tick feeding on a domestic pig, was the sole member of lineage A and had a CVR of 124 aa. Two Malawian isolates (LIL 89/1 and Dowa) with a CVR 120 aa in length constituted lineage B, and had a field presence of 3 years (1986–1989). Lineage C was represented

by a single Zambian virus (ZON88/1) with a CVR equal in length to that of the lineage A tick virus (124 aa), but distinct in tetrameric repeat composition. Lineage D comprised solely of four Zambian isolates with a CVR length of 116 aa, and a 2-year field presence (1988–1989). Ten viruses from Zambia, Malawi and Mozambique, with CVR aa lengths of 92 and spanning a 10-year period (1988–1998), formed lineage E, whilst the largest CVR lineage (F), comprised 26 isolates from Zambia, Malawi and Mozambique recovered from outbreaks over a 23 year period and having a CVR of 84 aa. Lineage G represented by Dedza, a 1986 virus from Malawi, had a unique, and comparatively short CVR, 56 aa in length.

Discussion

The usefulness of the *C*-terminal end of the *p72* gene for genotyping ASF viruses and for inferring broad epidemiological relationships is well established [*4*, *11*, *12*], particularly in those areas where a sylvatic cycle occurs. However, low levels of variation, principally within homogeneous pig and pig-tick associated genotypes, remains problematic and requires that an additional, more variable gene region be characterised in order to achieve optimal levels of resolution for epidemiological inferences. The value of a *p72*-CVR two-step approach has been demonstrated at a country, genotype and regional level [*8*, *9*, *11*] and its usefulness was confirmed in this study of genotype VIII viruses from East Africa, where seven CVR lineages were recovered from viruses that could previously only be resolved into two *p72* genotype VIII lineages [*4*, *12*]. This is, however, the first time that the genes have been combined in a single analysis in order to improve resolution, which in the case of genotype VIII led to the recovery of eight discrete *p72*-CVR lineages, which clustered within three main monophyletic lineages (I–III in Fig. 2).

Of the eight possible *p72*-CVR combinations, six occurred in Malawi, four in Zambia, two in Mozambique and only one was found in Zimbabwe. The two largest lineages, a–E and a–F have a trans-boundary distribution occurring in Malawi, Mozambique and Zambia. Lineage b–F was shared between Zimbabwe and Malawi, whilst the remaining five lineages appeared to be restricted to individual countries. Country-specific CVR sequences were identified in lineages a–C, a–D and a–B, with the former two from

Zambia having a unique NVGT tetramer, whilst Malawian isolates Lil 89/1 and Dowa which constituted lineage a–B, lacked the SAST tetramer which was present in all other genotype VIII viruses. Documentation of absence or presence of general and unique tetramers, respectively in an apparently country-specific manner may prove useful in future for determining the origins of genotype VIII trans-boundary infections.

Comparison of the individual gene analyses proved valuable in this study for identifying contradictory evolutionary histories of two genotype VIII viruses, NDA 90/1 and RHO 61/1 (*p72* lineage 'b'). Despite being distinct from all other viruses across the more conserved *p72 C*-terminal gene region, these viruses did not have a similarly distinct CVR lineage. Instead the CVR amino acid sequence was indistinguishable from that of 24 other *p72* lineage 'a' viruses. Given that *p72* is more conserved than CVR [*11*] and presumably operating under different selective pressures, and that the central conserved region in which both the *p72* gene and CVR of the *9RL* ORF are located [*7*] is inherited as a single genomic unit, the discrepancy between the *p72* and CVR phylogenies could only have arisen by recombination or convergent evolution. Recombination would require co-infection of a host with two different genotype VIII strains and exchange of their genetic material, whilst convergent evolution implies that CVR replication is sufficiently error prone that two unrelated lineages can independently converge on the same sequence. Both seem unlikely in the domestic pig host as individual co-infection could not be demonstrated in pigs simultaneously exposed to two different ASF viruses [*12*], and serial passage on pig cells confirmed stable inheritance of CVR [*6*]. The latter in vitro result was confirmed in vivo in this field study by the apparent long-term stability of the 26 identical CVR lineage F viruses, recovered from domestic pigs over a 40 year period. It is therefore not possible to determine which of the two explanations is more likely, but does for the first time raise the possibility of recombination in ASF viruses.
In conclusion, *p72* genotyping of ASFV previously revealed the complexity of ASF epidemiology in East Africa [*4*], whilst CVR gene characterisation performed in this study, improved resolution of genotype VIII viruses. Malawi was shown to have the highest number of genotype VIII virus variants, many of which were shared with neighbouring Mozambique and Zambia, pointing to Malawi being a possible reservoir of

infection, and to a need for regional collaboration in order to successfully control the disease. As the combined gene analysis recovered more lineages than the individual gene analyses, future studies should consider simultaneous analysis of these datasets, but only in those cases where statistical tests indicate that the data may be pooled. Individual gene analyses were however valuable for uncovering incongruence between the resulting phylogenies, which was attributed to either convergent evolution or to recombination. Although some data are available from domestic pigs, studies on co-infection and stability of CVR inheritance in the *Ornithodoros porcinus* invertebrate tick are presently lacking, and may provide new insights into this observed phylogenetic incongruence in genotype VIII viruses.

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