Transboundary and Emerging Diseases

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SHORT COMMUNICATION

Genetic Characterization of African Swine Fever Viruses from a 2008 Outbreak in Tanzania

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Summary

Outbreaks of African swine fever (ASF) have been reported in the past from several countries in sub-Saharan Africa. The aim of this study was to genotype ASF viruses (ASFVs) from the 2008 outbreak in Morogoro and Dar es Salaam regions of Tanzania. Tissue samples from domestic pigs that died as a result of severe haemorrhagic disease were collected and analysed with PCR and genome sequencing methods using ASFV-specific primer sets. Nucleotide sequence data were obtained for the *B646L* (p72), *E183L* (p54) and the variable region of the *B602L* gene sequences. Phylogenetic analyses based on DNA sequences showed that the 2008 Tanzanian isolates belonged to p72 genotype XV and clustered together with those derived from the 2001 outbreak in Tanzania. Analysis of the tetrameric amino acid repeat regions within the variable region of the *B602L* gene showed that the repeat signature of the 2008 Tanzanian ASFV was unique and contained three novel tetramers (U = NIDT/NTDT and X = NTDI). Epidemiological investigation suggested that transportation of live pigs continues to play an active role in the epidemiology of ASF in Tanzania. It is recommended that future control of ASF spread in Tanzania should focus on the early detection and confirmation of the disease, prompt institution of quarantine measures, culling and proper disposal of infected and in-contact animals and decontamination of affected premises.

Introduction

African swine fever (ASF) is a highly contagious and fatal haemorrhagic viral disease of domestic pigs caused by African swine fever virus (ASFV). African swine fever virus is classified as the only member of the family Asfarviridae (Asfar, ASF and related viruses), genus *Asfivirus* (Dixon et al., 2005). African swine fever virus is a large, enveloped, complex icosahedral arbovirus containing a linear, covalently close-ended, double-stranded 2-deoxyribonucleic acid (DNA) genome varying from 170 to 190 kbp in length (Chapman et al., 2008; de Villiers et al., 2010). Variations in genome size are mainly because of insertions or deletions at the left (38–47 kbp) and the right (13–16 kbp) terminal regions of the ASFV genome (Tulman et al., 2009; de Villiers et al., 2010). In addition, variations in genome sizes are also associated with differences in the number of tandem repeats in the central variable region (CVR) within the central 125 kbp, a relatively conserved region of ASFV genome (Tulman et al., 2009).

The epidemiology of ASF in East Africa is complex because of the presence of sylvatic, domestic pig and pig-tick cycles. Several studies have described the heterogeneity of ASFV in Africa through genotyping (Bastos

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et al., 2003; Lubisi et al., 2005, 2009; Nix et al., 2006; Wambura et al., 2006; Boshoff et al., 2007; Gallardo et al., 2009). African swine fever virus genotyping is useful in understanding regional virus heterogeneity and establishing epidemiological links between outbreak viruses. Possible sources and spread of ASFV can be identified through genotyping, making it possible to prevent further introductions of ASF.

African swine fever virus genotyping strategies mostly involve partial (3′ end) and full length nucleotide sequence analysis of the B646L (coding for the p72 capsid protein) and E183L (coding for p54 envelope protein) genes, respectively, and CVR amino acid tetrameric repeats within the *B602L* gene (Gallardo et al., 2009). Using partial *B646L* (p72) gene sequencing, 22 ASFV genotypes (I–XXII) were described (Boshoff et al., 2007). Of these, 21 genotypes are present in Eastern and Southern Africa while ASFVs from Europe, South America, the Caribbean and West Africa (ESACWA) are aggregated within genotype I. Intragenotypic resolution of virus isolates into groups, and subtypes have been achieved by analysing the *E183L* (p54) and *B602L* genes (Lubisi et al., 2007; Gallardo et al., 2009). The *B602L* gene, in particular, is a highly discriminative genetic marker, and 31 subgroups of viruses which show variation in tetrameric amino acid repeats have been identified (Nix et al., 2006).

Several ASF outbreaks occurring sporadically have been reported in the past in Tanzania. Tanzanian ASFV isolates that have already been genotyped belong to genotypes X, XV and XVI (Lubisi et al., 2005; Wambura et al., 2006). The last reported outbreak of a haemorrhagic and fatal disease suspected to be ASF occurred in Tanzania in February 2008 in Morogoro and Dar es Salaam regions. The aim of this study was to genotype the ASFV that caused this outbreak to establish epidemiological links between the outbreaks in the two regions and the relatedness of the 2008 strains with previous Tanzanian isolates.

Materials and Methods

Source and collection of samples

Outbreaks of severe haemorrhagic disease associated with high domestic pig mortalities were reported in February 2008 in two non-neighbouring regions, first in Mvomero district and then in Morogoro urban district of Morogoro region and later in Kinondoni district of Dar es Salaam region, (Fig. 1). Routine *post-mortem* examination of dead animals was performed. Spleen, kidney, liver, lymph node and whole blood were then collected from domestic pigs at Turiani (Mvomero District) and Mazimbu (Morogoro Urban District), both in Morogoro Region; and later from Mabibo (Kinondoni District) of Dar es

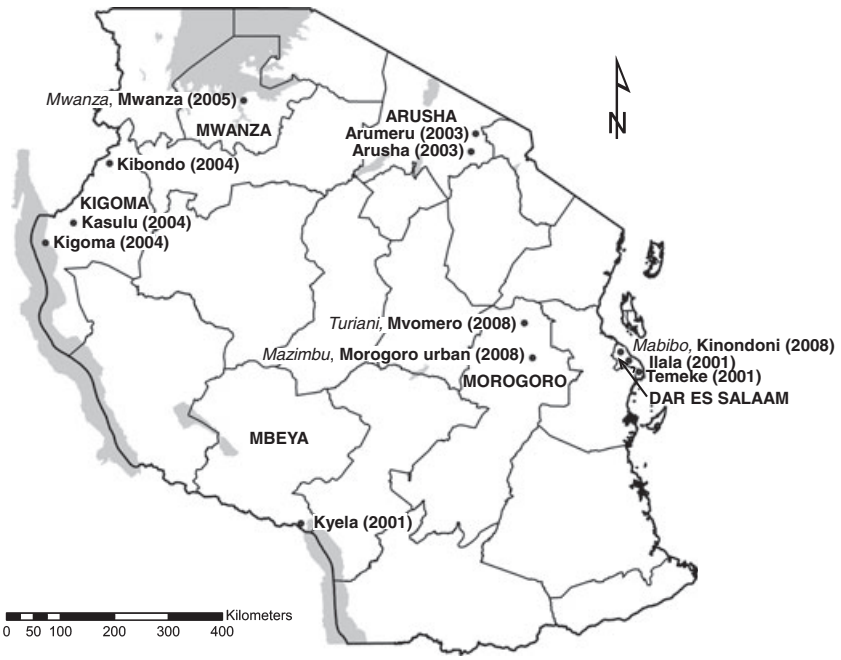

Fig. 1. Map of Tanzania showing the regions (indicated by capital letters) where ASF outbreaks have occurred in Tanzania since 2000. ASF outbreaks occurred in Dar es Salaam (Ilala and Temeke districts) and Mbeya (Kyela district) regions in 2001, Arusha region (Arusha and Arumeru Districts) in 2003, Kigoma region (Kasulu, Kigoma and Kibondo Districts) in 2004, Mwanza region (Mwanza City of Mwanza District) in 2005 and Morogoro (Turiani in Mvomero District and Mazimbu in Morogoro Urban District) and Dar es Salaam (Mabibo in Kinondoni District) regions in 2008. Locations of ASF outbreaks (where available) are indicated in italics while districts are indicated with bold small letters.

Salaam Region for laboratory confirmation of the tentative ASF diagnosis achieved by PCR and sequencing. Whole blood obtained from three pigs belonging to a single semi-intensive piggery unit at Turiani was collected and pooled. Spleen, kidney, liver, lymph node and whole blood from a single pig from a commercial pig farm in Mazimbu were also collected and pooled. In Mabibo, lymphoid organs from a single pig in a pre-slaughter handling yard were collected and pooled. All sampled pigs were Landrace and Large White crosses. Collected samples were stored at −20°C until DNA extraction was performed.

DNA extraction, PCR amplification and sequencing

DNA was extracted directly from the samples using NucleoSpin columns (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. Four sets of primers were used to amplify specific regions of ASFV by PCR including the ASF diagnosis primers PPA1/PPA2 that generate a 257-bp amplicon (Aguero et al., 2003), p72U/p72D (Bastos et al., 2003), PPA89/PPA722 (Gallardo et al., 2009) and ORF9R-L/ORF9R-F (Nix et al., 2006) that target the conserved part of the *B646L* (p72) gene, variable 3′ end of the *B646L* (p72) gene, complete *E183L* (p54) gene and the CVR of the *B602L* gene, respectively, following previously described protocols. PCR products were treated with exonuclease I (New England Biolabs, Ipswich, MA, USA) and Antarctic phosphatase (New England Biolabs) and subsequently used for dideoxynucleotide cycle sequencing. Alternatively, PCR fragments were purified from agarose gels using a Qiaquick gel extraction kit (Qiagen Benelux, Venlo, The Netherlands). Cycle sequencing was performed with a Big Dye Terminator Cycle sequencing kit V1.1 (Applied Biosystem, Foster City, CA, USA). Products from cycle sequencing reaction were purified by ethanol precipitation and separated on an ABI Prism 310 Genetic Analyser (Applied Biosystem).

Phylogenetic analysis

African swine fever virus DNA sequences were analysed and compiled using BlastN (http://www.ncbi.nlm.nih.gov) and Sixframe and ClustalW (http://workbench.sdsc.edu). Table 2 provides a summary of the isolates and sequences that were used to construct phylogenetic trees. These isolates have been described in Boshoff et al. (2007) and Gallardo et al. (2009) and are representatives of the 22 (I–XXII) different p72 genotypes.

Phylogenetic relationships among sequences (using 35 p72 and 16 p54 gene sequences, independently) were analysed as described by Tripathi and Sowdhamini (2006). ClustalW alignments were visually inspected, and sequences were trimmed at the beginning and end and were adjusted to maximize positional homology. No gaps were observed for the 404-bp p72 nucleotide sequences used for alignment. For the p54 alignment, a selection of isolates for which the p54 genes were available from Genbank were used. Gaps were deleted, and the sequences were 573 bp in length. These alignments were used as datasets in Phylip v3.67. Bootstrapping was performed 1000 times using Seqboot. Pairwise distances between sequences were determined with Dnadist. Neighbour-joining (NJ) trees were calculated with Neighbour. Majority-rule consensus trees were obtained with Consense and visualized with Drawgram.

Results and Discussion

Post-mortem findings included severe congestion of the skin, petechiation of the kidney cortex and severe haemorrhage in lymph nodes. The epidemiological units affected, total domestic pigs susceptible populations, cases and mortalities for the 2008 ASF outbreak in Tanzania are shown in Table 1. All three pooled samples obtained from domestic pigs that died of a severe haemorrhagic disease within each of the locations (Turiani, Mazimbu and Mabibo) were ASFV positive on diagnostic PCR employing PPA1 and PPA2 primers targeting a conserved region of the *B646L* (p72) gene (Aguero et al., 2003). The results confirmed an ASF diagnosis for the disease that affected domestic pigs in the two regions of Tanzania in February 2008. African swine fever viruses from Turiani, Mazimbu and Mabibo are designated as TAN/08/Turiani, TAN/08/Mazimbu and TAN/08/Mabibo, respectively.

Partial and complete nucleotide amplification and sequencing of the variable 3′ end of the B646L (p72) and E183L (p54) and CVR of the *B602L* genes were performed

Table 1. Cases and mortalities resulting from an African swine fever outbreak in domestic pigs at different locations in Tanzania in February 2008

Location	Epidemiological unit	Susceptible	Cases	Deaths	Slaughtered	References
Turiani	Village	2000	120	117	24	OIE, 2008
Mabibo	Pre-slaughter handling yard	300	87	22	101	OIE, 2008
Mazimbu	Intensive pig farm	205	104	97	108	This study

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Table 2. Summary of the African swine fever virus (ASFV) isolates used for the construction of phylogenetic trees based on partial *B646L* (p72) gene sequences. ASFV isolates used for the construction of phylogenetic trees based on full length E183L (p54) gene sequences are indicated with an asterisk

Isolate	Country of origin	Host species	Year of outbreak	Town/Province	p72 gene Genbank accession number	p72 genotype	References
Ang72*	Angola	Pig	1972	Not known	FJ174378	I	Gallardo et al., 2009
Ba71V*	Spain	Vero cell adapted pig isolate	1971	Badajoz	FJ174390	1	Gallardo et al., 2009
Nig01*	Nigeria	Pig	2001	Not known	FJ174382	I	Gallardo et al., 2009
Georgia1/2007	Georgia	Pig	2007	Not known	AM999764	II	Unpublished
Warmbaths*	South Africa	Tick	Not known	Not known	AY261365	III	Unpublished
RSA/1/99/W	South Africa	Warthog	1999	Not known	AF449477	IV	Bastos et al., 2003
Tengani62*	Malawi	Pig	1962	Nsanje	AY261364	V	Unpublished
Mal/2002/1	Malawi	Pig	2002	Mpemba Quarantaine Camp	AY494553	V	Lubisi et al., 2005
MOZ/94/1	Mozambique	Pig	1994	Maputo	AF270711	VI	Bastos et al., 2003
RSA/1/98	South Africa	Warthog	1999	Not known	AF302818	VII	Bastos et al., 2003
MOZ/A-98	Mozambique	Pig	1998	Tete	AY274452	VIII	Bastos et al., 2004
MwLil 20/1*	Malawi	Tick	1983	Chalaswa	AY261361	VIII	Unpublished
Malawi1978	Malawi	Pig	1978	Not known	AF270707	VIII	Bastos et al., 2003
Kirt89/2	Tanzania	Tick	1989	Kiriwara	AY351511	VIII	Lubisi et al., 2005
Ug03H.1*	Uganda	Pig	2003	Hoima	FJ154428	IX	Gallardo et al., 2009
Ken06.Kis*	Kenia	Pig	2006	Not known	FJ154440	IX	Gallardo et al., 2009
Ug64*	Uganda	Pig	1964	Not known	FJ174383	X	Gallardo et al., 2009
Kirw89/1	Tanzania	Phaecochoerus Aethiopicus	1989	Kiriwira	AY351514	X	Lubisi et al., 2005
Kab/62*	Zambia	Tick	1983	Livingstone game park, South Zambia	AY351522	XI	Lubisi et al., 2005
MZI/92/1	Malawi	Pig	1992	Euthini, Mzinda District, North Malawi	AY351543	XII	Lubisi et al., 2005
MFUE6/1	Zambia	Tick	1982	Mfue, Luangera National Park	AY351561	XII	Lubisi et al., 2005
Sum/14/11*	Zambia	Tick	1983	Sumbu National Park	AY351542	XIII	Lubisi et al., 2005
NYA/1/2*	Zambia	Tick	1986	Kalumo	AY351555	XIV	Lubisi et al., 2005
Tan/1/01*	Tanzania	Pig	2001	Dar es Salaam	AY494552	XV	Lubisi et al., 2005
TAN/08/Mazimbu*	Tanzania	Pig	2008	Mazimbu	GQ410765	XV	This study
TAN/08/Mabibo*	Tanzania	Pig	2008	Mabibo	GQ410768	XV	This study
TAN/2003/1	Tanzania	Pig	2003	Arusha	AY494550	XVI	Lubisi et al., 2005
TAN/2003/2	Tanzania	Pig	2003	Arusha	AY494551	XVI	Lubisi et al., 2005
ZIM/92/1	Zimbabwe	Pig	1992	Gweru Midlands	DQ250119	XVII	Boshoff et al.,2007
NAM/1/95	Namibia	Pig	1995	Windhoek	DQ250122	XVIII	Boshoff et al., 2007
RSA/3/96	South Africa	Pig	1996	Pienaarsrivier	DQ250127	XIX	Boshoff et al., 2007
RSA/1/95	South Africa	Pig	1995	Hoedspruit	DQ250123	XX	Boshoff et al., 2007
Lillie*	South Africa	Tick	1979	Not known	DQ250109	XX	Boshoff et al., 2007
RSA/1/96	South Africa	Pig	1996	Gravelotte	DQ250125	XXI	Boshoff et al., 2007
SPEC/245	South Africa	Pig	1992	Louis Trichardt	DQ250117	XXII	Boshoff et al., 2007

on the 2008 Tanzanian isolates (with the exception of p72 and CVR for TAN/08/Turiani). Seven 2008 Tanzanian ASFV DNA sequences have been deposited in Genbank including GQ410765 (p72; TAN/08/Mazimbu), GQ410766 (p72; TAN/08/Mabibo), GQ410767 (p54; TAN/08/Mazimbu), GQ410768 (p54; TAN/08/Mabibo), GQ410771 (p54; TAN/08/Turiani), GQ410769 (B602L; TAN/08/Mazimbu) and GQ410770 (B602L; TAN/08/Mabibo). African swine fever

virus from Morogoro (TAN/08/Mazimbu) and Dar es Salaam (TAN/08/Mabibo) regions was 100% identical in their *B646L* (p72), *E183L* (p54) and *B602L* nucleotide sequences. The p54 sequence from TAN/08/Turiani was 100% identical with the p54 sequences from the other 2008 isolates TAN/08/Mazimbu and TAN/08/Mabibo. Sequencing of the 257-bp PPA fragment from all three 2008 isolates showed 100% nucleotide identity and was different from other sequences in Genbank. These data strongly suggest that the disease incidents in 2008 were caused by closely related ASFVs. The fact that the disease was first reported at Turiani, later at Mazimbu and finally at Mabibo, as well as the similarity of the DNA sequences of all the outbreak viruses, indicates that in 2008 ASF spread within Tanzania from a common source. Records from the Ministry of Livestock Development and Fisheries show that the outbreak at Mabibo started in a pre-slaughter handling yard belonging to a trader who brought domestic pigs from Turiani (OIE, 2008). There is a possibility that ASF was introduced to the Dar es Salaam Region by the transport of live ASFV-infected domestic pigs for marketing purposes from the Morogoro region. Transport of live infected pigs has also been advanced as the cause of spread of ASF between two non-neighbouring regions of Mbeya and Dar es Salaam during the 2001 outbreak (Wambura et al., 2006; Fig. 1). Thus, future control of ASF spread in Tanzania should focus on the early detection and confirmation of the disease, prompt institution of quarantine measures, culling of infected and in-contact animals followed by disposal of carcasses and infected materials by deep burial or incineration and decontamination of premises.

Blast of *B646L* (p72) 2008 Tanzanian ASFV DNA sequences in Genbank showed highest (98%) nucleotide identity with p72 sequences of Tanzanian isolates TAN/1/01, TAN/2003/2 and TAN/2003/1. The 2008 Tanzanian ASFV *E183L* (p54) sequences showed 91% nucleotide identity with p54 sequences from TAN/02/3, TAN/03/1, and a Malawian isolate MZI/94/1 and 90% identity with isolates TAN/01/1 and Tanzania 87. These Blast results indicate that 2008 Tanzanian ASFV strains mainly resemble other Tanzanian viruses that caused ASF outbreaks in 2001 and 2003.

To determine the genetic relationship between the 2008 Tanzanian isolates and the 22 previously identified ASFV genotypes (I–XXII p72; Boshoff et al., 2007), phylogenetic trees were constructed by the neighbour-joining method using TAN/08/Mazimbu and TAN/08/Mabibo (Fig. 2). A comparable tree topology, as in Boshoff et al. (2007) and Gallardo et al. (2009), was obtained. Some genetic relationships were not supported by high bootstrap values. The 2008 isolates clustered under genotype XV (bootstrap value 0.59) together with TAN/1/01, isolated from a

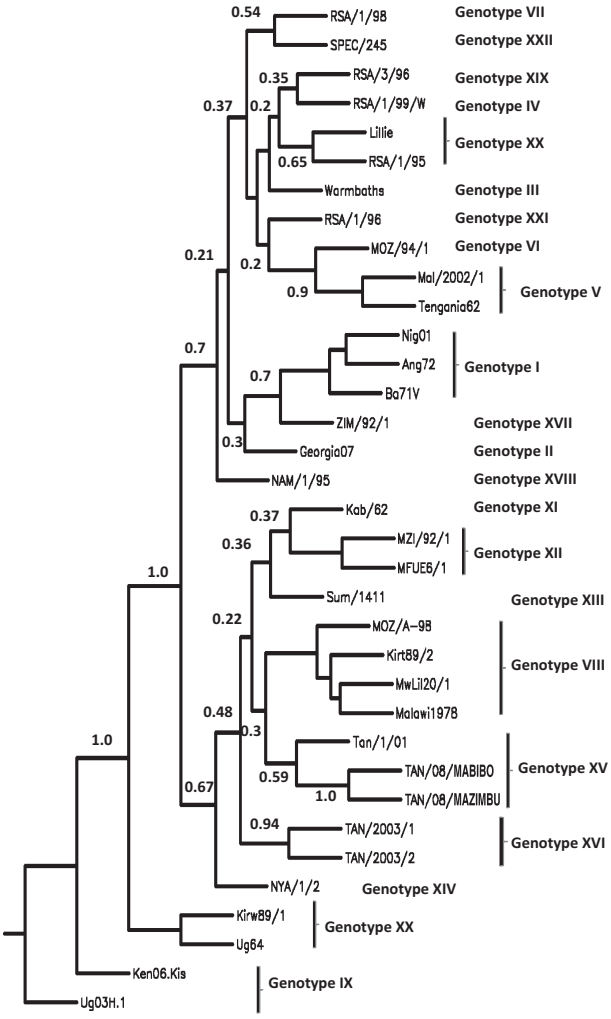

Fig. 2. Neighbour-joining phylogram depicting the relationship of the 2008 Tanzanian isolate with representatives of the 22 (I–XXII) genotypes (Boshoff et al., 2007; Gallardo et al.,2009) based on partial p72 gene sequences. Phylogeny was inferred following 1000 bootstrap replications.

domestic pig during the 2001 outbreak in Dar es Salaam (Wambura et al., 2006). In addition, a preliminary p54 NJ tree was constructed using p54 sequences from the isolates in Table 2 (for which p54 sequences were available). The 2008 Tanzanian isolates also clustered together with TAN/01/1 (bootstrap value of 0.85; phylogram not shown). These results indicate that the 2008 isolates are related to the 2001 outbreak viruses. It would, therefore, be worthwhile investigating these relationships with those of ASFVs circulating in warthogs and *Ornithodoros* ticks. That may establish a link between sylvatic and domestic pig ASF occurrence.

The *B602L* gene is a hypervariable region that contains twelve base pair repeats that encode four amino acids that

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vary in number and sequence (Gallardo et al., 2009). This region has previously been used as a marker for intragenotypic resolution of ASFV isolates (Gallardo et al., 2009). Amplicons of 430 bp were obtained by PCR using primers ORF9R-L/ORF9R-F for TAN/08/Mazimbu and TAN/08/Mabibo isolates, and an identical sequence was obtained. BlastP (v2.2.23) analysis using the deduced amino acid sequence showed 74% overall amino acid identity with the B602L amino acid sequence of the Malawian ASFV isolate MwLil 20/1 (isolated in 1983). The 2008 Tanzanian isolates had 31 tetrameric amino acid repeats. Tetrameric amino acid repeats that are present in known ASFV isolates include CAST/CVST/CTST (repeat code A), CADT/CTDT (B), GAST/GANT (C), CASM (D), CANT (F), CTNT (G), NEDT (M), NVDT/NVGT/NVNT (N), NANI/NADI/NASI (O), RAST (H), SAST (S), NVNT (T), NAST/NADT/NANT (V) and SADT/SVDT (W) (Nix et al., 2006; Boshoff et al., 2007; Lubisi et al., 2007). Analysis of the tetrameric amino acid repeats of TAN/08/Mazimbu revealed three tetramers that have not been described including NIDT and NTDT (defined with code U) and NTDI (defined with code X). More particularly, the tetramers NIDT and NTDT were abundant (nine of 31 tetramers). Although the overall percentage amino acid identity between MwLil 20/1 and the 2008 Tanzanian isolates is high, a distinct tetrameric repeat signature is observed for the 2008 Tanzanian isolates (AVUAVUVAVVUAVUVAVUVAVVUAVVUUUXV). The MwLil 20/1 signature is AVSVSOVNAVNOVVNVOVNANVOVVNOVOOV. Nix et al. (2006) distinguished 31 subgroups (which varied in sequence and number of tetrameric repeats). The signature of the 2008 Tanzanian isolates is distinct from all subgroups, and therefore, this would define a new subgroup. In this respect, it would be interesting to obtain CVR sequences from the outbreak strains from 2001, 2003, 2004 and 2005.

It can be concluded from the results obtained in this study that the ASFVs that caused the 2008 outbreak in the Morogoro and Dar es Salaam regions of Tanzania are identical and related to Tan/01/1, which are within genotype XV. Furthermore, pig transportation for marketing purposes continues to contribute to the spread of ASF outbreaks within Tanzania.

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