African Journal of Biotechnology Vol. 10(17), pp. 3488-3497, 25 April, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1439
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Genotyping of African swine fever virus (ASFV) isolates associated with disease outbreaks in Uganda in 2007

Carmina Gallardo[1]*, Anna R. Ademun[2], Raquel Nieto[1], Noelina Nantima[2], Marisa Arias[1], Elena Martín[1], Virginia Pelayo[1] and Richard P. Bishop[3]

[1]Centro de Investigación en Sanidad Animal, INIA. Valdeolmos, Madrid 28130, Spain.
[2]Ministry of Agriculture, Animal Industry and Fisheries. Entebbe, Uganda.
[3]International Livestock Research Institute (ILRI), P. O. Box 30709, Nairobi, Kenya.

Accepted 21 January, 2011

Samples from infected domestic pigs associated with an outbreak of African swine fever (ASF) in three districts of central Uganda in 2007 were confirmed as being infected with African swine fever virus (ASFV) using a *P72* gene-based polymerase chain reaction amplification (PCR) assay combined with restriction analysis. None of the sera collected from pigs with clinical symptoms were positive using the OIE serological prescribed tests. However, seven haemadsorbing viruses were isolated in macrophage culture and genotyped by partial *p72* and full length *p54*-gene sequencing. Four of these viruses were isolated directly from serum samples. All the viruses were classified within the domestic-pig cycle-associated *p72 and p54* genotype IX which also includes viruses responsible for ASF outbreaks in Kenya in 2006 and 2007 and Uganda in 2003. To define virus relationships at higher resolution, typing was performed by analysis of tetrameric amino acid repeat regions within the central variable region (CVR) of the *B602L* gene. Ugandan isolates sequences exhibited 100% identity to viruses isolated from outbreaks in Kenya in 2007. The identity was greater than the viruses obtained from an earlier outbreak in Kenya in 2006. This provides further evidence that genetically similar ASFV virus within *p72* Genotype IX may be circulating between Kenya and Uganda.

Key words*:* African swine fever virus (ASFV), restriction analysis, serological detection, genotyping, p72, p54, central variable region (CVR).

INTRODUCTION

African swine fever (ASF) is caused by a virus, ancestrally associated with argasid ticks and wild suids that can infect domestic pigs, frequently resulting in a rapidly lethal disease. However, depending on the combination of virus genotype and pig breed a range of outcomes varying from acute to chronic disease and apparently asymptomatic animals that are carriers of the virus can be observed. Virulent genotypes induce acute haemorrhagic disease, with symptoms including high fever, heamorrhages in the skin and internal organs and death within three to ten days.

ASFV is classified within the Asfarviridae family, genus *Asfivirus* (Dixon et al., 2000, 2005). It is highly contagious and can spread very rapidly in pig populations by direct or indirect contact. The disease has been reported in more than twenty African countries, where the virus is maintained either through a sylvatic cycle involving warthogs (*Phacochoerus aethiopicus*) and soft ticks in the genus *Ornithodoros* or in a domestic cycle that involves pigs of local breeds, with or without tick involvement (Haresnape and Wilkinson, 1989; Plowright and Pierce, 1969; Sanchez-Botija, 1963; Thomson, 1985). ASF is endemic in most of sub-Saharan Africa, including the island of Madagascar; the highest incidence of disease being recorded from the equator to the northern Transvaal in southern Africa. Disease outbreaks have also occurred in Europe, South America and the Caribbean. In 2007, it was introduced into Georgia, most probably through infected pig-meat that was unloaded from a ship, recycled and fed to local pigs and has since spread throughout the Caucasus and into southern Russia (Rowlands et al., 2008).

Uganda, situated in the eastern-central part of Africa, is divided into 80 districts and shares borders with Kenya,

*Corresponding author. E-mail: gallardo@inia.es

the Sudan, DRC, Rwanda and Tanzania. Currently, African swine fever is officially described as endemic in the whole country and outbreaks are reported sporadically, but without quantitative data on prevalence. Although, no new ASF outbreaks have been officially notified since 2004 (World Animal Health Organization-World Organisation for Animal Health (WHO-OIE), occurrence of hemorrhagic disease has been reported regularly to the Ugandan veterinary authorities by pig farmers. A more recent ASF outbreak occurred in Uganda-Kenyan border (Busia district) in 2007, placing a significant proportion of the Ugandan pig population at risk. In October 2007, an outbreak of virulent hemorrhagic disease was notified in three districts of Central Uganda, Mukono, Wakiso and Nakasongola. Following these outbreaks, blood, serum and tissue samples from pigs with clinical signs of hemorrhagic disease were collected for African swine fever diagnosis and molecular characterization.

We report here the diagnosis and genetic characterization of ASF viruses collected during outbreaks in Uganda in 2007. The genotyping involved sequencing the 3' end of the gene encoding the *p72* protein (Bastos et al., 2003; Boshoff et al., 2007; Lubisi et al., 2005) and the full length *p54*-gene (Gallardo et al., 2009) to place isolates into major subgroups, followed by higher resolution sub-typing through analysis of tandem repeat sequences (TRS) in the central variable region (CVR) of the ASFV *B602L* gene (Irusta et al., 1996; Lubisi et al., 2007; Nix et al., 2006; Phologane et al., 2005). The very close genetic similarity of isolates associated with recent disease outbreaks in both Uganda and Kenya emphasizes the value of molecular epidemiology for tracing the source and dynamics of ASF infections.

MATERIALS AND METHODS

Sampling

During October 2007, the presence of virulent hemorrhagic disease suspected as being caused by ASFV was described by Uganda authorities in Central Uganda districts of Mukono, Wakiso and Nakasongola. Eighty four samples were collected by Ministry of Agriculture, Animal Industry and Fisheries (MAAIF) staff from pigs with clinical symptoms and submitted to the Animal Health Research Centre (CISA-INIA), Valdeolmos, Spain (EU ASF Reference Laboratory). Samples collected comprised sera from 39 pigs (Wakiso n = 13; Nakasangola n = 11; Mukono n = 15), EDTA blood samples from 39 pigs (Wakiso n = 11; Nakasangola n = 9; Mukono n = 19) and six tissue samples from one pig (Wakiso n = *6*). They were received at CISA-INIA on 23rd October 2007.

ASF diagnosis

ASF antibody detection

39 field sera were tested using OIE (2008) prescribed tests (conventional ELISA and Immunoblotting assays) using as antigen a lysate of monkey stable (MS) cells infected with ASF Spanish isolate E70MS48 and protein-A/HRPO as the reporter (OIE 2008).

Nucleic acid extraction and genomic DNA amplification

DNA was extracted directly from 35 sera, 39 blood samples and 10% suspensions of 6 ground tissues (from one animal) using a nucleic acid extraction kit (Nucleospin/ Machery-Nagel–Cultek) following the manufacturer's procedures. A polymerase chain reaction amplification (PCR) assay using the ASF diagnostic primers PPA1/PPA2, which generates an amplicon of 257 bp within the p72 protein (Aguero et al., 2003), was used to confirm the presence of ASFV DNA. The PCR products were analyzed by electrophoresis through 2% agarose gels visualized under UV light.

Restriction enzyme analysis

The *NdeI* restriction endonuclease (New England Biolabs) was used to analyze the DNA amplified by PCR. Briefly, 10 µl of the PCR sample was digested for 1 h at 37°C with 8 to 20 U of restriction enzyme in a total reaction volume of 20 µl in the appropriate restriction enzyme buffer. Aliquots of 10 µl of each reaction mixture were size fractionated on a 3% agarose gel and the restriction patterns were visualized under UV light.

Virus isolation

Primary leukocyte cultures were used for the isolation of samples recovered from naïve domestic pigs as previously described (Malmquist and Hay, 1960). Briefly, cells were seeded into 96-well tissue culture grade microtitre plates (200 µl; 300,000 cells per well) in homologous swine serum and incubated in a humidified atmosphere containing 5% CO_2 at 37°C. Three day cultures were infected at 1:10 dilution with serum or 10% suspensions of ground tissues supplemented with 5 µg/ml gentamycine sulphate (BioWhittaker) and incubated for 24 h at 37°C. After inoculation, a preparation of 1% homologous red blood cells (RBC) in buffered saline was added to each well. The plates were examined for haemadsorption over a 6 day period. The samples were blind passaged three times.

ASF molecular characterization

Genomic amplification and nucleotide sequencing

For molecular epidemiological analysis, DNA was extracted from cell cultures and three separate polymerase chain reactions (PCRs) were set up; for *p72* genotyping classification amplifying the C-terminal region of *p72* protein using the primers *p72*- U/D as previously described (Bastos et al., 2003) for *p54* genotyping amplifying the complete gene encoding the *p54* protein using the primers PPA722/PPA89 (Gallardo et al., 2009); for CVR sub-typing using the primer pairs ORF9L-F/9L-R to amplify the CVR located in the *B602L* gene (Nix et al., 2006). Amplicons of the expected size were excised, purified by Quiaex gel extraction (QIAGEN) and cloned into a pGEMT-Easy vector (Promega) according the manufacturer's instructions. The nucleotide sequence of the purified products was determined using SP6/T7 primers specific for the pGEMT vector using an automated 3730 genetic analyzer (Applied biosystems).

Sequence analysis

Analysis of sequence data was performed with Chromas (www.technelysium.com.au), BioEdit (www.mbio.ncsu.edu/ BioEdit/ BioEdit.html) and ClustalX version 1.83 (www.clustal.org). For the TRS, analyses including that of the CVR sequences and deduced amino acid sequences were manually aligned with gaps being

Table 1. ASFV Uganda isolates obtained from 2007 outbreak characterized in this study.

Isolate name	Id. sample	Kind of sample sequenced	Origin
UG07.Wak1	44a	Blood	SSEBUGWAWO mixed farm, Wakiso district Kinoni
UG07.Wak2	Wakiso 46	Sera	
UG07.Wak3	Wakiso 0656	Sera	
UG07.Wak4	1128 lymph node	Tissue	
UG07.Mukono	20	Blood	Katumba wamala farm, Mukono district
UG07.F7	63/Fattener 7 F7	Sera	Kinoni produce farm, Nakasongola district
UG07.F8	63/Fattener 8 F8	Sera	

inserted to optimize the alignment. Two datasets were generated for phylogenetic analyses conducted using MEGA version 4.0 (Kumar et al., 2001) (i) A *p72*-gene dataset comprising 73 taxa (404 characters) in which *p72* nucleotide sequences generated in this study from the Ugandan pig viruses were analyzed together with homologous sequences from at least two viruses representative of each of the 22 *p72* genotypes identified in a previous study (Boshoff et al., 2007), (ii) A *p54*-gene dataset comprising 155 taxa in which *p54* sequences generated in this study were compared with homologous sequences available in GenBank. Neighbor joining (NJ) and minimum evolution (ME) *p72* and *p54* trees were constructed employing the p-distance nucleotide substitution model as implemented in the MEGA v4.0 program. To determine the degree of statistical support for each node in the resulting *p72* and *p54* trees, data were re-sampled 1000 times using the bootstrap method.

RESULTS

ASF diagnosis

Among the 39 porcine serum samples received at CISA from the Central Ugandan Districts, ASFV antibody detection performed using the OIE-prescribed ELISA and immunoblotting (IB) assays revealed that, none of them were antibody-positive for ASF.

For ASFV detection, PCR was performed on 35 serum samples, 39 blood samples and also 6 individual tissues from one of the sampled animals. Following PCR amplification, a single major amplicon of approximately 260 bp was generated from 9 of the 35 sera, 17 of the 39 blood samples and all tissue samples examined. Virus isolation was performed using homogenenised pooled tissues and also sera and blood from each PCR positive animal. Seven ASFV strains with a haemadsorption pattern typical of virulent ASF viruses were isolated from the Ugandan samples after three passages in leukocytes (Table 1). In four cases these virus isolates were made directly from serum samples that were negative according to the OIE ELISA and IB (Table 1). DNA was extracted from the cell cultures and the presence of the ASF virus was confirmed using diagnostic PCR. The sequencing and compared with sequences available in the GenBank and with additional sequences obtained in a separate study from ASF isolates recovered during ASF outbreaks that occurred in 2003 and 2006 to 2007, in Uganda and Kenya, respectively (previously unpublished data held at CISA-INIA). Nucleotide sequence analysis of the genome region delimited by the PPA-1/2 primers revealed an *NdeI* restriction endonuclease site, which which could generate two fragments of 204 and 53 bp in length. *NdeI* digestion of the amplicons from the Ugandan ASFV isolates resulted in generation of the two expected specific restriction fragments (Figure 1). This site is conserved only in ASFV isolates obtained from eastern African countries, while in sequences from European, Caribbean, West and South African countries a mutation is present at bp 54 within the *NdeI* restriction site (Table 2).

ASF molecular characterization

Analysis of the *p72*-gene partial sequences from each of the seven Uganda ASFV isolates showed that, they were identical at the nucleotide level (results not shown). Comparison of these sequences to the 22 (I-XXII) currently known major *p72* genotypes (Boshoff et al., 2007) identified the Uganda 2007 sequences as falling within *p72* genotype IX (Figure 2).

Recent studies have demonstrated the value of *p54*-gene sequencing as an additional, intermediate-reso-lution, molecular epidemiological tool for typing of ASFV viruses (Gallardo et al., 2009). Amplification of the fragment containing the complete *p54*- gene from all the Ugandan isolates produced PCR products of ≈550 bp, which were identical in sequence. The sequences of the 7 Uganda isolates were compared with 155 *p54* ASFV sequences available in GenBank. As was the case with the p72-genotyping, the Uganda isolates were clustered together with Ugandan and Kenyan viruses obtained from domestic pigs (Figure 3).

In an attempt to discriminate genotypes at higher reso-amplified products were characterized by nucleotide lution, the CVR sequences of viruses from Uganda were

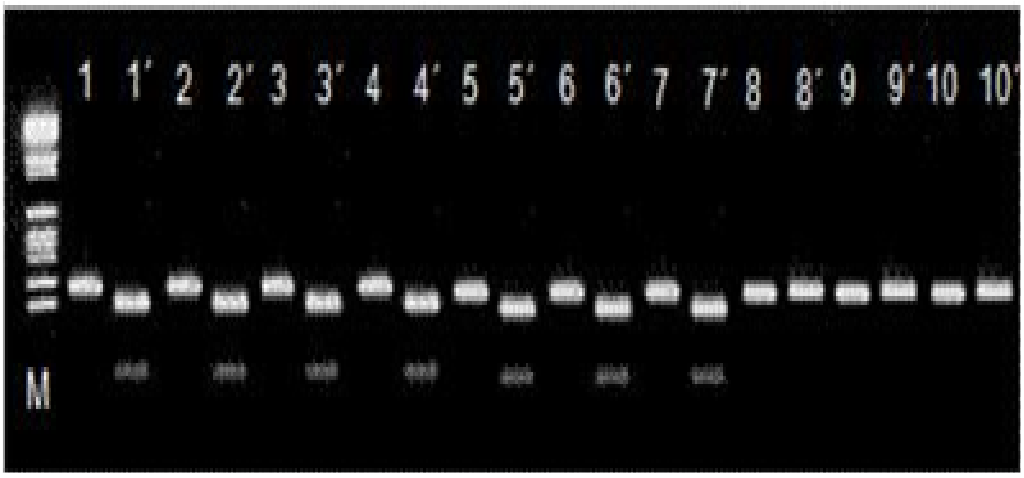

Figure 1. *NdeI* restriction endonuclease analysis of PPA1/2 amplicons derived from different representative ASFV isolates. Lanes 1 and 1´, UG07.Wak.1; 2 and 2´ UG07.Wak4; 3 and 3´, UG07.Mukono; 4 and 4´, UG07.F7; 5 and 5´, Ken06.Bus; 6 and 6´, Ken07.Eld1; 7 and 7´, Ug03H1; 8 and 8´, E70; 9 and 9´, Cam; 10 and 10´, Za. Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 show amplification products. Lanes 1´, 2´, 3´, 4´, 5´, 6´, 7´, 8´, 9´ and 10´ show amplification products after digestion with *NdeI*. Reaction conditions are described in the text. M, molecular weight marker VI.

Table 2. Partial nucleotide sequences showing residues 1 to 60 within the *p72*- gene PCR amplicon used for ASFV diagnosis. The *NdeI* restriction site conserved in Kenya and Uganda ASFV isolates is highlighted in grey.

Isolate	P72 genotype	Country of origin	*3´→ 5´ nucleotide sequences from* p72*- gene PCR diagnosis product*	P72*-based diagnosis Genbank accession no.*	*Reference*
E70	I	Spain	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATTTGGTT	AY578692	Zsak et al. (2005)
E75	I	Spain	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATTTGGTT	AY578693	Zsak et al. (2005)
Ba71V	I	Spain	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATTTGGTT	U18466	Complete genome
Haiti	I	Haiti	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATTTGGTT	AY578695	Zsak et al. (2005)
Tengani62	V	Malawi	AAGTACGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATTTGGTG	AY578704	Zsak et al. (2005)
M1	III	South Africa	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACGCATTTGGTG	AY578699	Zsak et al. (2005)
Za	I	DRC*	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATTTGGTT	AY578708	Zsak et al. (2005)
Mk	I	South Africa	AGTTATGGGAAACCCGATCCCGAACCCACTTT GAGTCAAATCGAAGAAACACATTTGGTG	AY578700	Zsak et al. (2005)
Wart	IV	Namibia	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATTTGGTG	AY578706	Zsak et al. (2005)
Cam	I	Cameroon	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATTTGGTT	AY578689	Zsak et al. (2005)
Vic	I	Zimbabwe	AGTTATGGGAAACCCGATCCCGAACCCACTTT GAGTCAAATCGAAGAAACACATTTGGTG	AY578705	Zsak et al. (2005)
Wb	III	South Africa	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATTTGGTG	AY578707	Zsak et al. (2005)
Cro1.2	IV	South Africa	AGTTATGGGAAACCTGACCCCGAACCCACTTT GAGTCAAATCGAAGAAACACATTTGGTG	AY578690	Zsak et al. (2005)
Cro 3.5	IV	South Africa	AGTTATGGGAAACCTGACCCCGAACCCACTTT GAGTCAAATCGAAGAAACACATTTGGTG	AY578691	Zsak et al. (2005)

Table 2. Continued.

F6	III	South Africa	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATTTGGTG	AY578694	Zsak et al. (2005)
K1	III	South Africa	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATTTGGTG	AY578696	Zsak et al. (2005)
O1	III	South Africa	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATTTGGTT	AY578701	Zsak et al. (2005)
Pr4	XX	South Africa	AGTTATGGGAAACCTGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATTTGGTG	AY578702	Zsak et al. (2005)
Pr5	XX	South Africa	AGTTATGGGAAACCTGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATTTGGTG	AY578703	Zsak et al. (2005)
Ug03H.1	IX	Uganda	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATATGGTG	GQ916933	This study
Ug03P.4	IX	Uganda	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATATGGTG	GQ916934	This study
UG07.Wak1	IX	Uganda	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATATGGTG	GQ916926	This study
UG07.Wak2	IX	Uganda	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATATGGTG	GQ916927	This study
UG07.Wak3	IX	Uganda	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATATGGTG	GQ916928	This study
UG07.Wak4	IX	Uganda	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATATGGTG	GQ916929	This study
UG07.Mukono	IX	Uganda	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATATGGTG	GQ916930	This study
UG07.F7	IX	Uganda	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATATGGTG	GQ916931	This study
UG07.F8	IX	Uganda	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATATGGTG	GQ916932	This study
Kenya1950	X	Kenya	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATATGGTG	AY261360	Complete genome
Ken06.B1	IX	Kenya	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATATGGTG	GQ916935	This study
Ken06.B2	IX	Kenya	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATATGGTG	GQ916936	This study
Ken06.B3	IX	Kenya	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATATGGTG	GQ916937	This study
Ken06.B4	IX	Kenya	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATATGGTG	GQ916938	This study
Ken06.B5	IX	Kenya	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATATGGTG	GQ916939	This study
Ken06.Bus	IX	Kenya	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATATGGTG	GQ916940	This study
Ken06.Kis	IX	Kenya	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATATGGTG	GQ916941	This study
Ken07.Eld1	IX	Kenya	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATATGGTG	GQ916942	This study
Ken07.Eld2	IX	Kenya	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATATGGTG	GQ916943	This study
Ken07.Kia	IX	Kenya	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATATGGTG	GQ916944	This study
Ken07.Nak	IX	Kenya	AGTTATGGGAAACCCGACCCCGAACCCACTT TGAGTCAAATCGAAGAAACACATATGGTG	GQ916945	This study

determined. Amplification of the CVR fragment generated PCR products of approximately 350 b from all Ugandan isolates. As in the case of the other genes, the Uganda isolates characterized in this study clustered with the

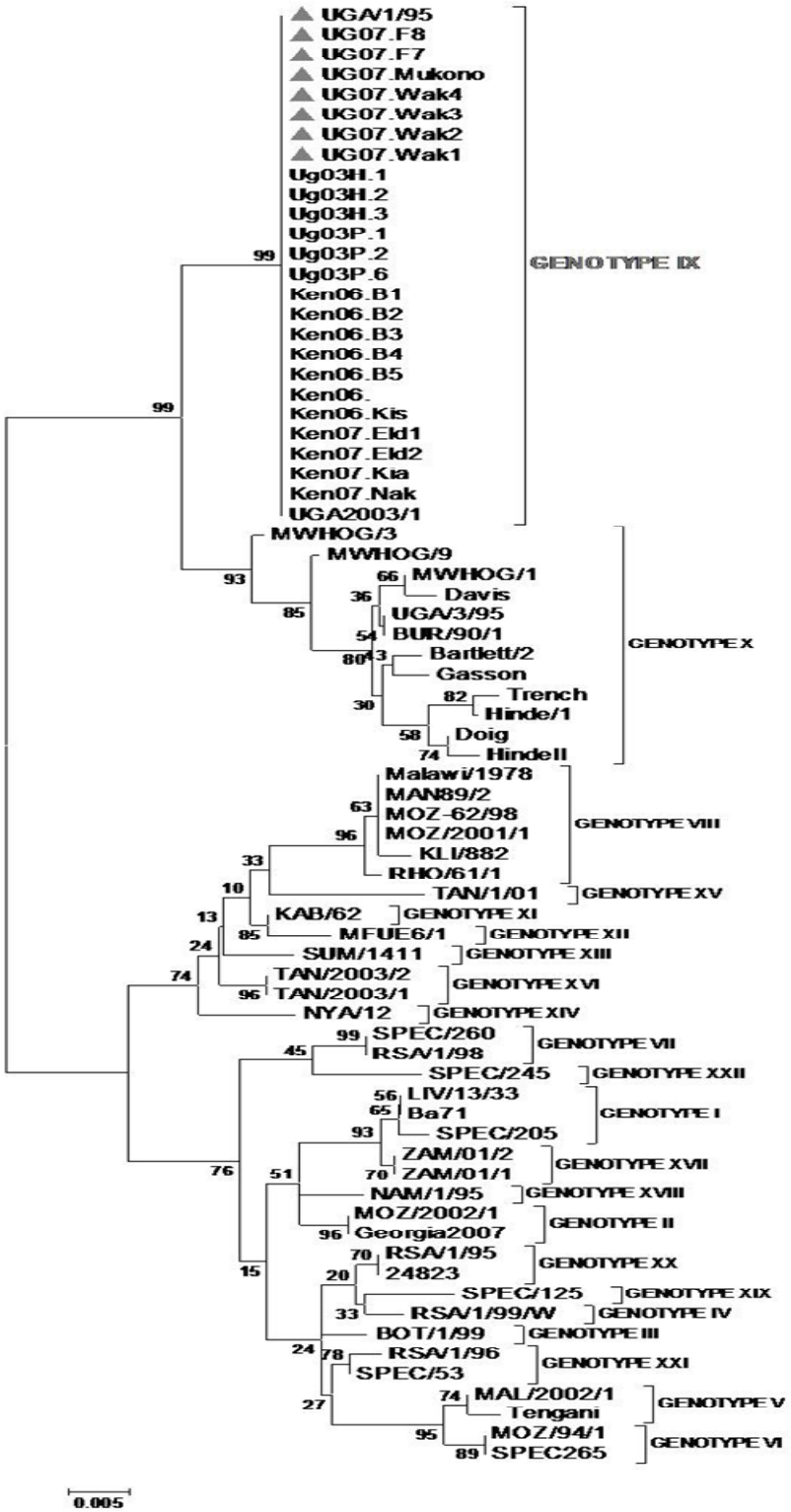

Figure 2. Minimum evolution (ME) tree representing the 22 p72 genotypes that distinguish African swine fever viruses (labelled I-XXII) illustrating the phylogenetic position of the Ugandan isolates from 2007. The optimal tree with the sum of branch lengths = 0.23816201 is shown. The percentage of replicate trees in which the associated taxa clustered together according to bootstrap analysis (1000 replicates) is marked adjacent to the nodes. The ME tree was searched using the Close-Neighbor- Interchange (CNI) algorithm at a search level of 1. A neighbor-joining algorithm was used to generate the initial tree. The seven Ugandan viruses characterized in this study are highlighted in grey (▲) within genotype IX (marked in grey).

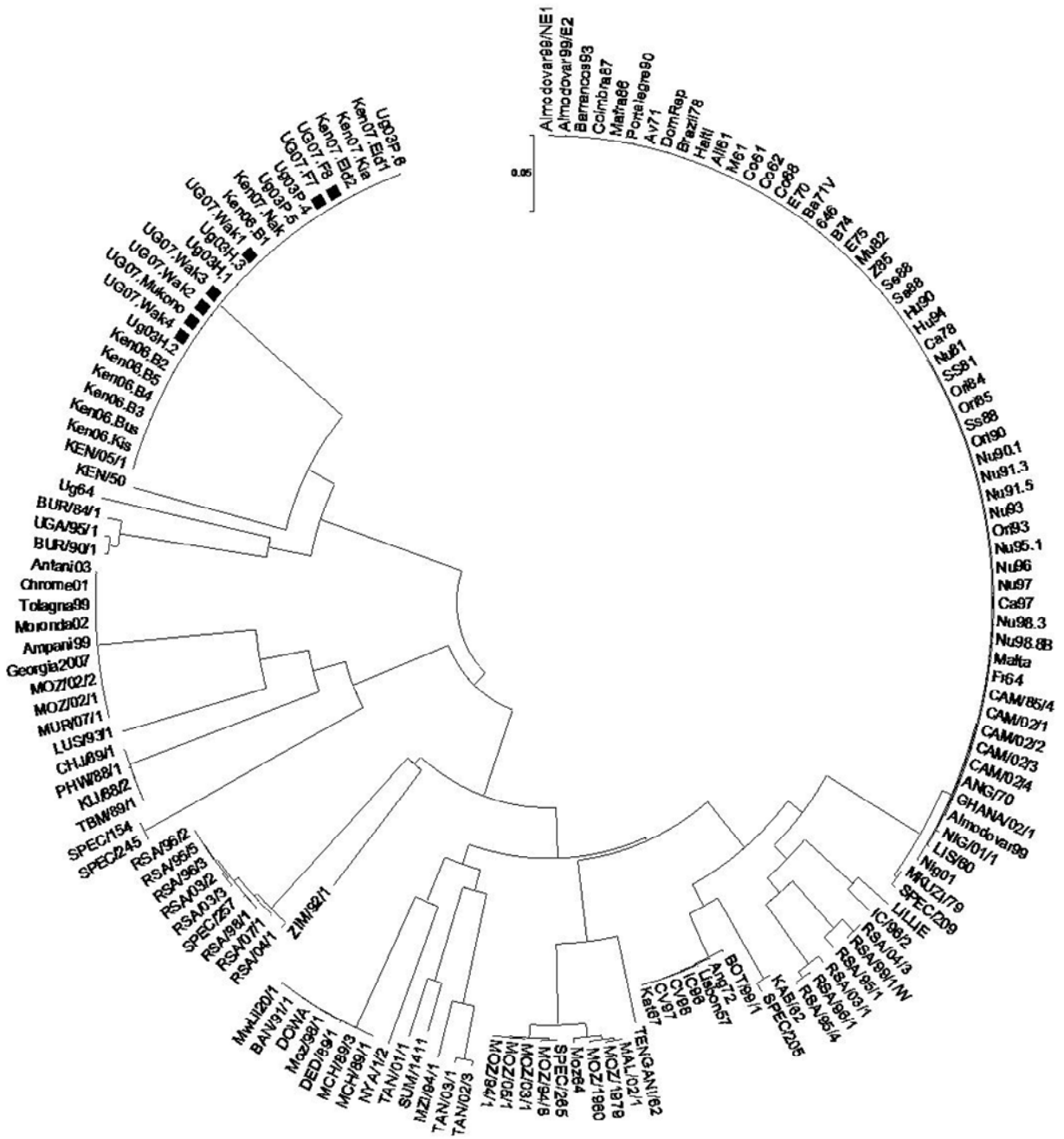

Figure 3. Minimum Evolution phylogenetic tree based on full length p54 gene sequences illustrating the position of Uganda 2007 isolates relative to other ASFV viruses. An unrooted minimum evolution tree was derived from alignments of complete *p54*-gene nucleotide sequences. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm at a search level of 1. A Neighbor-joining algorithm was used to generate the initial tree. The seven Ugandan viruses characterized in this study are highlighted in black (■).

Uganda isolates (UGA 95/1; Ug03H1-3, Ug03P4-6) and Kenya isolates (Ken06B1-5, Ken06.Bus, Ken06.Kis, Ken07Eld1-2, Ken07.Nak, Ken07.Kia) within CVR sub-group XXIV. Differences were identified mainly in the number of tetrameric amino acid repeats. When the tetrameric repeats within the *B602L* gene from the Uganda isolates were compared with viruses included within the CVR sub-group XXIV (Nix et al., 2006), the isolates from Uganda obtained in October 2007, contained a minor change due to the absence of a single internally located tetrameric repeat (CADT) present in the viruses recovered from earlier Uganda outbreaks that occurred in 2003 and in the Kenyan ASF Visolates obtained in May 2006 (Ken06.B1-5). However, they were identical to

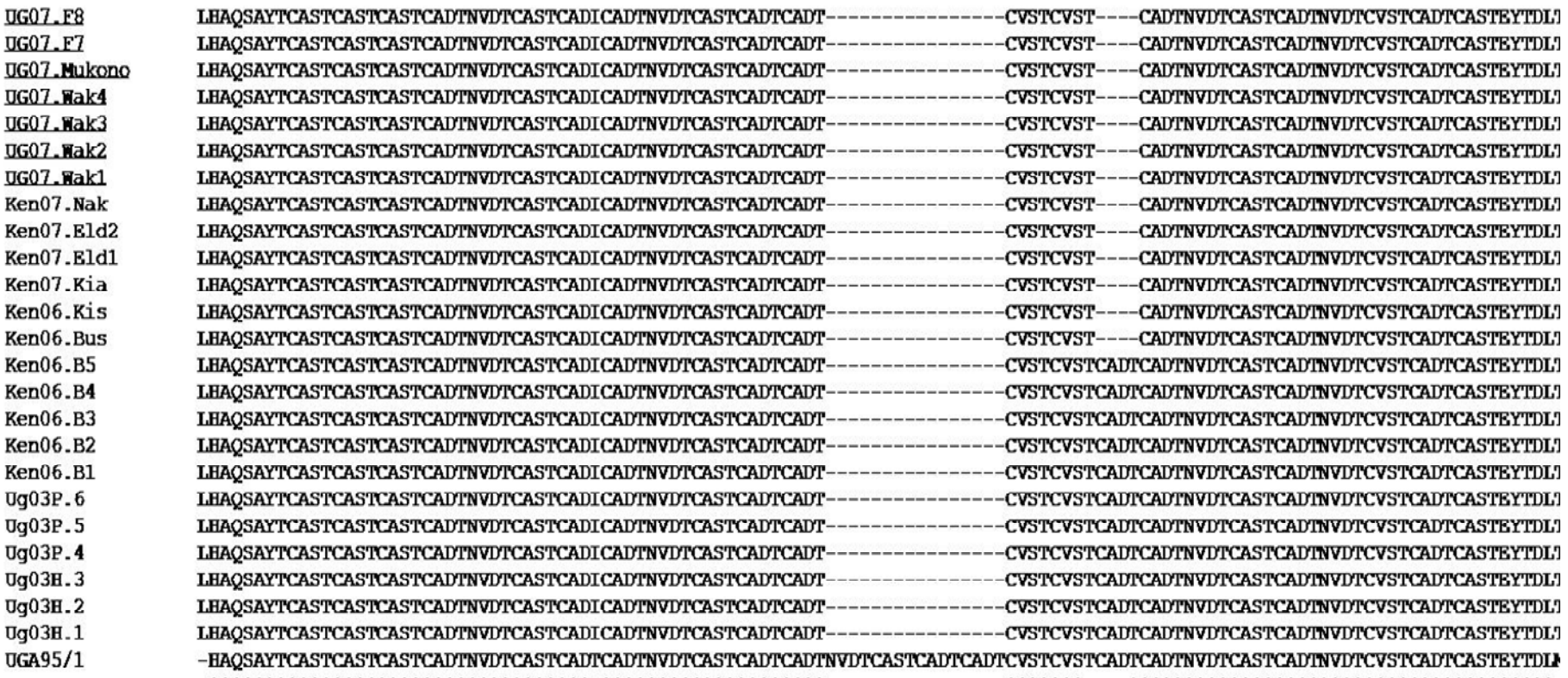

Figure 4. Amino acid sequence alignment of tetrameric tandem repeats within the central variable region (CVR) of gene *B602L* from Ugandan and Kenyan p72 group IX isolates associated with recent outbreaks. The isolates from the Ugandan 2007 outbreaks are underlined. The Ugandan 2007 isolate sequences determined in this study were aligned with additional Kenyan and Ugandan CVR sequences available in GenBank (UGA95/1, Accession No. CAJ90783; Ug03H1-3 and Ug03P4-6 Accession Nos FJ174339-44; Ken06.B1-B5, Ken06.Bus, Ken06.Kis, Ken07Eld1-2, Ken07.Nak, Ken07.Kia, Accession Nos FJ174329-39) belonging to *B602L* sub-group XXIV.

those obtained from the second outbreak that occurred in western Kenya in 2006 (Ken06.Bus) and to all isolates from subsequent outbreaks in the country (Figure 4).

DISCUSSION

Outbreaks of African swine fever have been reported in Uganda sporadically, but regularly in specific areas since 1996 and the pig industry has been very significantly affected. The last major outbreak, according to the OIE reports was in 2004 when an estimated 7,500 pigs were destroyed or slaughtered in twelve districts across the country. Since then, outbreaks of ASF have occurred on a regular basis in Uganda, causing serious losses to both smallholder pig keepers and commercial pig farms. At present, ASF is officially described as "present in the whole country but without quantitative data on the prevalence of the virus". In October 2007, a suspected African swine fever outbreak was reported by farmers in three central Uganda districts. Sampling in these areas in collaboration with the Uganda MAAIF resulted in confirmation of

gene based PCR and virus isolation at the CISA-INIA EU reference laboratory. Subsequent nucleotide sequence determi-nation of the PCR product allowed identification of a new *NdeI* restriction site, specific for a particular group of viruses belonging to east African countries. The res-triction fragment pattern enables the specificity of the amplicons to be validated within hours. However, the method does not permit unambiguous classification of virus isolates because of the high variability observed in east African ASVF isolates. Although, nucleotide sequencing of the *p72* amplicon would be more definitive, the technique is expensive, time consuming and less universally available in African laboratories. Restriction digestion with *Nde1* therefore, represents a useful rapid method for confirmation of the presence of ASFV following *p72* PCR.

Interestingly, no positive antibody response was detected using OIE-prescribed serological methods in any of the serum samples collected from these outbreaks. This data reconfirms previous studies that describe a low incidence of detectable serological response to ASFV infection in pigs from east African countries using both recombinant antigen and OIE-prescribed African swine fever serological methods (Perez-Filgueira et al., 2006). Since the OIE-prescribed methods are based on the use of total ASF virus as antigen that includes many viral proteins, a possible explanation for the unexpectedly low seropositive response with east African sera may reside in the immunogenetics of the indigenous pig populations in the region and is unlikely to be due to polymorphisms in immunodominant viral antigens. The area of host-virus interaction, including possible differences in both innate and acquired immune responses therefore, represents an important area for future research on African swine fever (ASF).

To characterize the viruses responsible for the out-break of ASF in Uganda in 2007 and identify genetic relationships with recent ASFV isolates from neighboring countries, the sequence of three regions of the ASFV genome was determined. Sequence analysis of the C-terminal end of *p72*-gene and complete *p54*-gene placed the Uganda isolates within genotype IX together with isolates from Kenya and Uganda. This is a domestic pig-associated genotype that contains 8 isolates from Western and Eastern Uganda (UGA 1/95; Ug03H1-3, Ug03P4-6, UGA2003/1), isolated from outbreaks in 1995 and 2003 (Bastos et al., 2003; Gallardo et al., 2009) and 11 isolates from Western ands Central Kenya (Ken06B1-5, Ken06.Bus, Ken06.Kis, Ken07Eld1-2, Ken07.Nak, Ken07.Kia) obtained from outbreaks in 2006 to 2007 (Gallardo et al., 2009). One additional genetic type of ASFV was also identified in this study as having occurred in Uganda; this is classified within *p72* genotype X that comprises viruses recovered from both domestic and wild pigs and the arthropod tick vector.

The data generated using three genes indicated that, African swine fever outbreaks occurring in the three viruses whilst the 1995 outbreak was caused by two different viruses belonging to p72 genotypes IX and X (UGA/1/95 and UGA/3/95) (Boshoff et al., 2007; Lubisi et al., 2005).

Sequence analysis of the central variable region (CVR) from the Ugandan isolates identified 22 different tetrameric amino acid units. The sequence of the CVR from the Ugandan isolates was identical to the sequence of 6 isolates responsible for the second wave of infections that occurred in Western and Central Kenya from October 2006 to January 2007, but was distinguishable from Kenyan isolates recovered from an earlier outbreak in May 2006 and from Ugandan viruses recovered from the outbreaks that occurred in Western and Eastern Uganda in 2003. A deletion of one tetrameric repeat that was observed in viruses recovered from the 2007 Ugandan outbreak was also present in viruses responsible for an outbreak that occurred in the Uganda-Kenya border district, subsequently spreading to central Kenya, in 2006 to 2007. This is consistent with a single origin for the Kenyan 2006 to 2007 and Ugandan 2007 outbreaks, if there is rapid cross-border movement of the virus. The data from the CVR analysis are consistent with the hypothesis that, ASFV virus exchange between the two countries has occurred on more than one occasion, although, an alternative explanation would be that identical mutations have occurred independently in the central variable region of two closely related virus lineages. Such information should be proved useful to veterinary authorities for better disease control and highlight the requirement for improved regulation and monitoring of domestic pig movement and the pork product trade.

ACKNOWLEDGEMENTS

The present work was partly supported under the grant ILRI2003-001 funded by the Spanish Ministerio de Innovacion y Ciencia and by the European Union through the network of excellence "EPIZONE" and also the ASFRISK project (Grant Agreement) 211691. We greatly appreciate the intellectual and practical contributions of our colleagues at Centro de Investigación en Sanidad Animal (CISA-INIA) and International Livestock Research Institute (ILRI). This ILRI publication number IL-201003.

REFERENCES

Aguero M, Fernández J, Romero L, Sánchez Mascaraque C, Arias M, Sánchez-Vizcaíno JM (2003). Highly Sensitive PCR Assay for Routine Diagnosis of African Swine Fever Virus in Clinical Samples. J. Clin. Microbiol. 41(9): 4431-4434.

Bastos ADS, Penrith ML, Cruciere C, Edrich JL, Hutchings G, Roger F, Couacy-Hymann E, Thomson GR (2003). Genotyping field strains of African swine fever virus by partial p72 gene characterisation. Arch. Virol. 148: 693-706.

Boshoff CI, Bastos AD, Gerber LJ, Vosloo W (2007). Genetic characterisation of African swine fever viruses from outbreaks in southern Africa (1973-1999)]. Vet. Microbiol. 121(1-2): 45-55.

Dixon LK, Costa JV, Escribano JM, Rock DL, Vinuela E, Wilkinson PJ (2000). Family Asfarviridae. In: Van Regenmortel MHV, Fauquel CM,

Bishop DHL (eds). Virus taxonomy, 7th Report of the ICTV, Academic Press, San Diego, pp. 159-165.
Dixon LK, Escribano JM, Martins C, Rock DL, Salas ML, Wilkinson PJ (2005). Asfarviridae. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (eds). Virus taxonomy, VIIIth Report of the ICTV, Elsevier/Academic Press, London, pp. 135-143.
Gallardo C, Mwaengo DM, Macharia JM, Arias M, Taracha EA, Soler A, Okoth E, Martín E, Kasiti J, Bishop RP (2009). Enhanced discrimination of African swine fever virus isolates through nucleotide sequencing of the p54, p72, and pB602L (CVR) genes. Virus Genes, Feb; 38(1): 85-95.
Haresnape JM, Wilkinson PJ (1989) A study of African swine fever virus-infected ticks (*Ornithodoros-Moubata*) collected from 3 villages in the ASF enzootic area of Malawi following an outbreak of the disease in domestic pigs. Epidemiol. Infect. 102: 507-522
Irusta PM, Borca MV, Kutish GF, Lu Z, Caler E, Carrillo C, Rock DL (1996). Amino acid tandem repeats within a late viral gene define the central variable region of African swine fever virus. Virology, 220: 20-27.
Kumar S, Tamura K, Jakobsen IB, Nei M (2001). MEGA2: molecular evolutionary genetics analysis software. Bioinformatics, 17(12): 1244-1245.
Lubisi BA, Bastos AD, Dwarka RM, Vosloo W (2005). Molecular epidemiology of African swine fever in East Africa. Arch. Virol. Dec; 150(12): 2439-2452.
Lubisi BA, Bastos AD, Dwarka RM, Vosloo W (2007). Intra-genotypic resolution of African swine fever viruses from an East African domestic pig cycle: a combined p72-CVR approach. Virus Genes, Dec. 35(3): 729-735.
Malmquist W, Hay D (1960). Haemadsorption and cytopathic effect produced by African swine fever virus in swine bone marrow and buffy coat cultures]. Am. J. Vet. Res. 21: 104-108.
Nix RJ, Gallardo C, Hutchings G, Blanco E, Dixon LK (2006). Molecular epidemiology of African swine fever virus studied by analysis of four variable genome regions. Arch. Virol. 151(12): 2475-2494.
OIE. July 2008, posting date. Chapter 2.8.1. African swine fever. *In* Manual of diagnostic tests and vaccines for terrestrial animals 2008. Office International des Epizooties, Paris, France. http://www.oie.int/eng/normes/mmanual/2008/pdf/2.08.01_ASF.pdf
Perez-Filgueira DMF Gonzalez-Camacho C, Gallardo P, Resino-Talavan E, Blanco E, Gomez-Casado C, Alonso JM, Escribano (2006). Optimization and validation of recombinant serological tests for African Swine Fever diagnosis based on detection of the p30 protein produced in *Trichoplusia ni larvae*. J. Clin. Microbiol. 11(44): 3114-3121.
Phologane SB, Bastos AD, Penrith ML (2005). Intra- and inter-genotypic size variation in the central variable region of the 9RL open reading frame of diverse African swine fever viruses. Virus Genes, 2005 Dec. 31(3): 357-360.
Plowright WPJ, Pierce MA (1969). African swine fever in ticks (*Ornithodoros moubata*, Murray) collected from animal burrows in Tanzania. Nature, 221: 1071-1073
Rowlands RJ, Michaud V, Heath L, Hutchings G, Oura C, Vosloo W, Dwarka R, Onashvili T, Albina E, Dixon LK (2008). African swine fever virus isolate, Georgia, Emerg. Infect. Dis. 2008 Dec. 14(12): 1870-1874. Sanchez Botija C (1963). Reservatoirios del virus de la peste porcina africana. Investigacion del virus de la PPA en los atropodos mediante prova de la hemadsorcion. Bull. Off. Int. Epiz. 60: 895-899.
Thomson GR (1985) The epidemiology of ASF: the role of free-living hosts in Africa. Onderstepoort J. Vet. Res. 52: 201-209.