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African swine fever viruses with two different genotypes, both of which occur in domestic pigs, are associated with ticks and adult warthogs, respectively, at a single geographical site

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The role of the ancestral sylvatic cycle of the African swine fever virus (ASFV) is not well understood in the endemic areas of eastern Africa. We therefore analysed the ASF infection status on samples collected from 51 free-ranging warthogs (*Phacocherus africanus*) and 1576 *Ornithodorus porcinus* ticks from 26 independent warthog burrows at a single ranch in Kenya. Abattoir samples from 83 domestic pigs without clinical symptoms, originating from specific locations with no recent reported ASF outbreaks were included in this study. All samples were derived from areas of central Kenya, where ASF outbreaks have been reported in the past. Infection with ASFV was confirmed in 22 % of *O. porcinus* pools, 3.22 % of adult warthog serum samples and 49 % of domestic pig serum samples by using *p72*-based PCR. All of the warthog sera were positive for anti-ASFV antibodies, investigated by using ELISA, but none of the domestic pig sera were positive. Twenty *O. porcinus*-, 12 domestic pig- and three warthog-derived viruses were genotyped at four polymorphic loci. The ASFV isolates from ticks and domestic pigs clustered within p72 genotype X. By contrast, ASF viruses genotyped directly from warthog sera, at same locality as the tick isolates, were within p72 genotype IX and genetically similar to viruses causing recent ASF outbreaks in Kenya and Uganda. This represents the first report of the co-existence of different ASFV genotypes in warthog burrow-associated ticks and adult wild warthogs. The data from this and earlier studies suggest transfer of viruses of at least two different p72 genotypes, from wild to domestic pigs in East Africa.

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INTRODUCTION

African swine fever (ASF) is a lethal, haemorrhagic disease of domestic pigs and has serious socio-economic implications, affecting the livelihoods of pig keepers in endemic countries of Africa. The disease also has adverse effects on both regional and international trade, and food security. Since there is no vaccine currently available, slaughter and quarantine are the only methods of control. ASF virus (ASFV), the causative agent of ASF, consists of a linear dsDNA molecule of 170–193 kb encoding between 151 and 167 genes depending on the specific isolate (Chapman *et al.*, 2008; de Villiers *et al.*, 2010). ASFV is the only member of the family *Asfarviridae*, genus *Asfivirus* and the only known DNA arbovirus (Dixon *et al.*, 2000, 2005). Virus epidemiology, distribution, pathology, economic impact and control strategies have recently been comprehensively reviewed (Costard *et al.*, 2009).

ASF was first described in Africa as the causal agent responsible for a lethal disease of domestic pigs in Kenya (Montgomery, 1921) and subsequently in 1928 in South Africa (De Kock, *et al.*, 1940). The association between warthogs (*Phacochoerus africanus*) and domestic pigs, and the occurrence of the virus in *Ornithodorus porcinus* ticks in warthog burrows was also first demonstrated in Kenya and subsequently observed in South Africa (Plowright *et al.*, 1969; reviewed by Penrith *et al.*, 2004a; Penrith, 2009). ASF first attracted major international attention in 1957 when it was transferred to Portugal from West Africa, subsequently spreading to Europe and to parts of Latin America between 1957 and 1990. Currently, ASF remains endemic within Europe in Sardinia (Italy) and is also present in the Caucasus and Russian Federation, following a more recent introduction to Georgia in 2007. The virus responsible for

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are HM745253–HM745392.

ASF in the Caucasus clusters with the South East African p72-genotype II (Rowlands *et al.*, 2008). In Africa, a great expansion of the disease has been observed since 1994 and the disease is now endemic in more than 20 sub-Saharan African countries (Penrith, 2009).

The epidemiology of ASF is complex and ASFV has been maintained in eastern and southern Africa for an unknown period of time, in an ancient sylvatic cycle involving soft ticks (genus *Ornithodorus*) and asymptomatic infected warthogs and bushpigs and red river hogs, (*Potamochoerus* spp.), although the role of the latter host is not fully defined (Haresnape *et al.*, 1988; Wilkinson *et al.*, 1988; Oura *et al.*, 1998; Kleiboeker & Scoles 2001; Bastos *et al.*, 2009). Two additional cycles have been described in endemic areas, namely a domestic pig–tick cycle, without warthog involvement, and a domestic pig–pig cycle in which the virus persists in domestic pigs in the absence of other vertebrate or invertebrate hosts (Penrith *et al.*, 2004a; Jori & Bastos, 2009). In some regions of South East Africa there is evidence that the disease can become endemic in populations of domestic pigs that have become resistant to the pathogenic effects of ASF (Penrith *et al.*, 2004b). In West Africa, virus transmission occurs in the absence of sylvatic host involvement and the majority of outbreaks are linked to the movement of infected pigs or pig products. These different epidemiological transmission patterns could be related to the genetic variability observed in eastern and southern African ASFV isolates that comprise 22 distinct p72 genotypes in contrast with high homogeneity in West African ASFV isolates that are classified in a single p72 genotype I (Lubisi *et al.*, 2005, 2007; Boshoff *et al.*, 2007; Gallardo *et al.*, 2009a).

Since the initial reports in 1921, Kenya has experienced recurrent outbreaks of disease caused by ASFV. An official reported ASF outbreak in Kenya occurred in 2001 in Thika district (Fig. 1). More recently, in 2006 an ASF outbreak was reported in the Kenya–Uganda border region and spread to central Kenya in regions adjacent to the city of Nairobi in 2007. Initial molecular analysis suggested that the 2006–2007 outbreaks were probably linked to cross-border movement of domestic pigs or pig products between Uganda and Kenya, without any evidence for

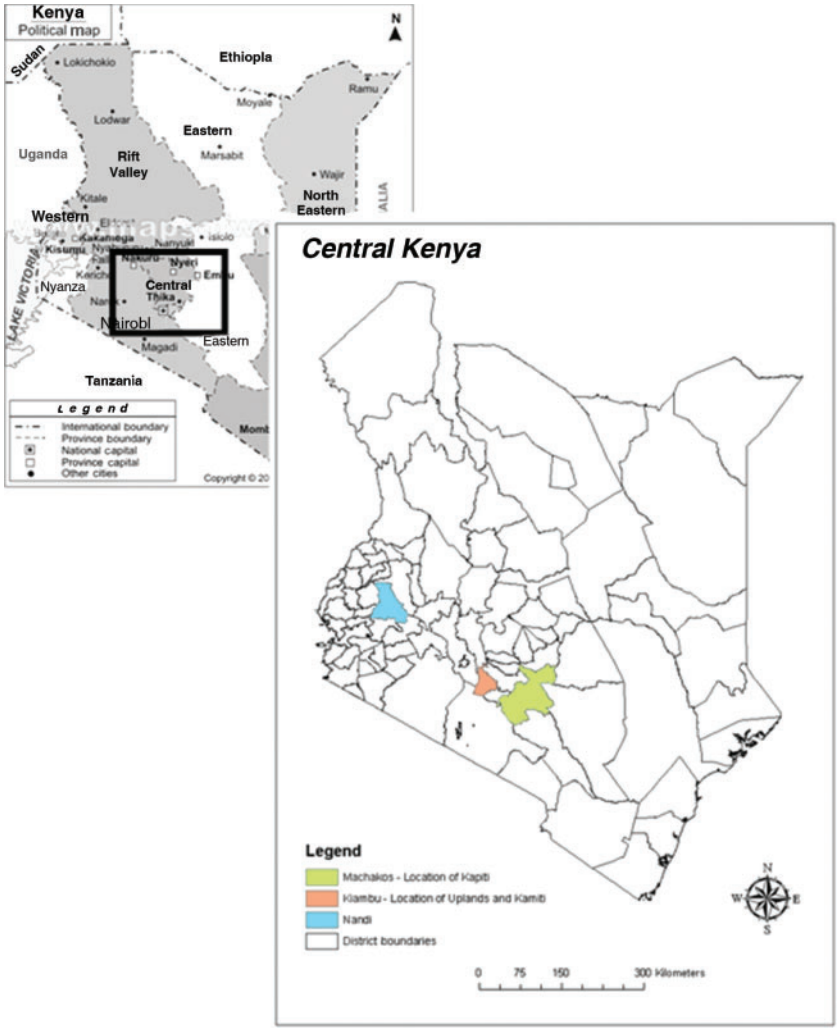

Fig. 1. Map of Kenya showing location of the study sites. Specific localities in central Kenya are indicated by colours.

direct involvement of the sylvatic cycle (Gallardo *et al.*, 2009a). Precise information relating to the significance of the sylvatic cycle for disease transmission in Kenya, and other parts of eastern Africa has not been published. Currently, information on the coexistence of domestic and sylvatic transmission cycles is based on studies conducted in Malawi and Mozambique (Haresnape *et al.*, 1985, 1988; Haresnape & Mamu, 1986; Penrith *et al.*, 2004a).

In order to investigate the possible role of the sylvatic cycle in the transmission of the disease in eastern Africa, we report the results of a survey conducted in central Kenya. The data indicate that ASF is present in seronegative domestic pigs, ticks inhabiting warthog burrows and seropositive adult warthogs. To develop a sequence database enabling more accurate assessment of the origins of past and future outbreaks of ASF in the region, we genotyped ASFV isolates obtained in this study. The genotyping strategy involved initial partial sequencing of the *p72*-gene to classify the ASFV isolates relative to the 22 currently known genotypes (Boshoff *et al.*, 2007). To increase resolution additional loci were sequenced, specifically the *E183L* and *CP204L* gene regions, encoding the p54 and p30 proteins (Gallardo *et al.*, 2009a; Rowlands *et al.*, 2008). The central variable region (CVR) within the ORF *B602L*, which has been found to be the most useful locus for differentiating closely related isolates and identifying virus subgroups within p72 (Bastos *et al.*, 2004; Nix *et al.*, 2006; Lubisi *et al.*, 2005, 2007; Phologane *et al.*, 2005; Gallardo *et al.*, 2009a; Owolodun *et al.*, 2010), was also sequenced. This study represents the first detailed molecular analysis of the role of the sylvatic cycle involving warthogs, *O. porcinus* ticks and domestic pigs in the epidemiology of ASF in East Africa.

RESULTS

Clinical and post-mortem examination of domestic pigs

Clinical examination of 83 domestic pigs at Farmer's Choice Nairobi slaughterhouse revealed no clinical signs of ASF. Further post-mortem examination of the carcasses showed no lesions indicative of disease. These results indicate that the animals were disease free.

Detection of ASFV DNA by PCR from field-collected samples

Detection of ASFV DNA from ticks. To detect ASFV DNA, 72 single ticks and 213 pooled ticks representing samples from different developmental stages collected during each of the three different samplings were initially screened by PCR using primers PPA1/2 (Aguero *et al.*, 2003). The proportion of positive-tick samples was 14 % (41 from a total of 285 pools and individual ticks analysed). Negative and positive results obtained by initial screening were confirmed by additional specific tick-PCR (Basto *et al.*, 2006). Primary amplicons were further assayed by using a nested PCR to maximize the probability of detecting ticks with low level ASFV infections. Using this method, the percentage of tick pools detected as containing virus increased to 22 % (62/285), as a result of amplicons being generated from 21 ticks that were initially observed as negative by using single-primer PCR (Table 1).

Detection of ASFV DNA from domestic pigs and warthogs

Of the 83 sera from domestic pigs collected from the Farmer's Choice Nairobi slaughterhouse in 2005, 41 (49 %) were confirmed as ASF positive by using the *p72*-gene based PCR followed by *Bsm*AI digestion (n=20 Kamiti; n=14 Uplands and n=7 Nandi). For ASFV DNA detection in warthog samples, PCR was performed on 51 whole-blood and serum sample pairs. The presence of ASFV DNA was confirmed in three adult warthog serum samples (6 %), all of them from the initial sampling in August 2008 (Table 2).

Isolation of ASFV from PCR-positive field samples from ticks, warthogs and domestic pigs

Attempts to isolate ASFV were performed by inoculation of cells derived from 62 nested PCR-positive-tick extracts, 41 PCR-positive domestic pigs and three PCR-positive warthogs

Table 1. Analysis of ASFV infection in different development stages of *O. porcinus* ticks from Kapiti Plains Estates collected in 2005, 2008 and 2009

Stage of development	No. ticks collected	Diagnostic PCR (Aguero *et al.*, 2003)		Specific-tick PCR (Basto *et al.*, 2006)		Isolation
		No. pools tested	No. positive (%)	No. pools tested	No. positive (%)	No. positive (%)*
N1–N3	891	116	15 (13)	116	19 (16)	7 (37)
N4–N5	485	80	9 (11)	80	18 (22)	4 (22)
Adult	200	89	17 (19)	89	25 (28)	9 (36)
Total	**1576**	**285/59**	**41 (14)**	**285**	**62 (22)**	**20 (32)**

*Percentage of positive isolations refer to the number of isolations in relation to the total number of positive ticks obtained by specific-tick PCR described by Basto *et al.* (2006) in each group.

Table 2. Analysis of ASFV infection in domestic pigs and warthogs on samples collected in central Kenya in 2005, 2008 and 2009, respectively

Origin	Town/ district	Date sampled	Species origin	No. blood samples collected	ASF genomic detection PCR	ASFV detection isolation*	ASF antibody detection
					No. positives (%)	No. positives (%)*	No. positives (%)
Farmer's Choice Nairobi slaughterhouse	Nandi	July 2005	Domestic pig	23	7 (30)	7 (100)	0 (0)
	Kamiti	July 2005	Domestic pig	30	20 (67)	14 (70)	0 (0)
	Uplands	July 2005	Domestic pig	30	14 (47)	3 (21)	0 (0)
Total domestic pigs				**83**	**41 (49)**	**24 (59)**	**0 (0)**
Kapiti Plains Estates	Machakos	August 2008	*P. africanus*	12	3 (25)	0 (0)	51 (100)
	Machakos	March 2009	*P. africanus*	39	0 (0)	0 (0)	51 (100)
Total warthogs				**51**	**3 (6)**	**0 (0)**	**100 (100)**

*Percentage of positive isolations refer to the number of isolations in relation to the total number of positive domestic pigs or warthogs obtained by PCR described by Aguero *et al.* (2003) in each group.

into porcine peripheral blood macrophages (PBM). Twenty ASFV tick-derived isolates and 24 ASFV domestic pig isolates with a haemadsorption pattern typical of ASF viruses were isolated after one to three passages in PBM cells. For ASFV isolated from ticks, the mean titre of virus was between 10^6 and 10^{12} 50 % haemadsorbing doses (HAD_{50}), whereas for ASFV from domestic pigs the maximum titre obtained was 10^4 HAD_{50}. No virus isolations were obtained after three passages from ticks that were negative in the initial PCR, but positive by using nested PCR. No virus could be isolated after three passages from PCR-positive warthog serum samples. No non-haemadsorbing ASFV strains were isolated from either ticks or domestic pigs.

Detection of ASF antibodies by using the OIE-prescribed serological test

Among the 83 domestic pig serum samples from domestic pigs collected from central Kenyan districts in 2005, ASFV antibody detection using the OIE-prescribed ELISA and immunoblotting assays revealed that none were positive for ASF. In contrast, all of the 51 warthog serum samples were positive for anti-ASF antibodies.

ASF molecular characterization

A total of 20 haemadsorbing ASFV tick (Tk) isolates, 12 haemadsorbing ASFV domestic pig (DP) isolates and three warthog (WH) serum samples that were ASFV positive were selected for genotyping (Table 3). In order to classify the Kenyan isolates characterized in this study into one of the 22 currently known p72 genotypes (Boshoff *et al.*, 2007), sequences obtained after amplification of the C-terminal end of the *p72* gene were compared with 276 sequences available in GenBank comprising representatives of each of the 22 p72 genotypes. Both neighbour-joining (NJ) trees generated using the UPGMA algorithm and minimum evolution (ME) trees inferred the same p72-phylogeny under which tick-derived and domestic pig Kenyan ASFV isolates were classified within p72 genotype X that comprises viruses from Rwanda, Burundi and Kenya. By contrast, the three adult warthog ASFV viruses were within p72 genotype IX. This genotype has previously been identified in domestic pig isolates associated with recent disease outbreaks. Genotype IX includes 15 isolates from western and eastern Uganda (UGA 1/95, Ug03H1–3, Ug03P4–6, UGA2003/1, Ug07.Wak1–4, Ug07.Mukono and Ug07.F7–8) from outbreaks in 1995, 2003 and 2007 (Bastos *et al.*, 2003; Gallardo *et al.*, 2009a; CISA, unpublished data) and 11 isolates from western and central Kenya (Ken06B1–5, Ken06.Bus, Ken06.Kis, Ken07Eld1–2, Ken07.Nak and Ken07.Kia) obtained from pigs associated with outbreaks during 2006–2007 (Gallardo *et al.*, 2009a) (Fig. 2).

Recent studies have demonstrated the value of *p54* and *p30* gene sequencing as additional, intermediate-resolution, methods for typing of ASF viruses (Gallardo *et al.*, 2009a; Rowlands *et al.*, 2008). A comparative sequence analysis of full-length *p54* and *p30* genes produced similar results to those obtained using *p72*, in that the warthog ASF viruses clustered separately from those recovered from ticks and domestic pigs. The warthog sequences were identical across the 478 bp C-terminal *p72*-gene, 558 bp full-length *p54*-gene and 567 bp full-length *p30*-gene. However, increased heterogeneity was observed in both

Table 3. Kenyan ASFV isolates selected for genotyping purposes obtained from ticks (Tk) and domestic pigs (DP) after virus isolation and warthogs (WH) whose nucleotide sequence was determined at four loci directly from serum samples

Isolate name	Source (district)	Date sampled	Host species	p72 genotype	*p72* gene GenBank accession no.
Ken05/Tk1	Kapiti Plains Estates (Machakos)	July 2005	Tick (*O. porcinus porcinus*)	X	HM745253
Ken05/Tk2	Kapiti Plains Estates (Machakos)	July 2005	Tick (*O. porcinus porcinus*)	X	HM745254
Ken05/Tk3	Kapiti Plains Estates (Machakos)	July 2005	Tick (*O. porcinus porcinus*)	X	HM745255
Ken05/Tk4	Kapiti Plains Estates (Machakos)	July 2005	Tick (*O. porcinus porcinus*)	X	HM745256
Ken05/Tk5	Kapiti Plains Estates (Machakos)	July 2005	Tick (*O. porcinus porcinus*)	X	HM745257
Ken05/Tk6	Kapiti Plains Estates (Machakos)	July 2005	Tick (*O. porcinus porcinus*)	X	HM745258
Ken05/Tk7	Kapiti Plains Estates (Machakos)	July 2005	Tick (*O. porcinus porcinus*)	X	HM745259
Ken05/Tk8	Kapiti Plains Estates (Machakos)	July 2005	Tick (*O. porcinus porcinus*)	X	HM745260
Ken05/Tk9	Kapiti Plains Estates (Machakos)	July 2005	Tick (*O. porcinus porcinus*)	X	HM745261
Ken05/Tk10	Kapiti Plains Estates (Machakos)	July 2005	Tick (*O. porcinus porcinus*)	X	HM745262
Ken05.DPk2	Farmer's Choice Nairobi slaughterhouse (Kamiti)	July 2005	Domestic pig	X	HM745263
Ken05.DPk16	Farmer's Choice Nairobi slaughterhouse (Kamiti)	July 2005	Domestic pig	X	HM745264
Ken05.DPk18	Farmer's Choice Nairobi slaughterhouse (Kamiti)	July 2005	Domestic pig	X	HM745265
Ken05.DPk21	Farmer's Choice Nairobi slaughterhouse (Kamiti)	July 2005	Domestic pig	X	HM745266
Ken05.DPk27	Farmer's Choice Nairobi slaughterhouse (Kamiti)	July 2005	Domestic pig	X	HM745267
Ken05.DPN2	Farmer's Choice Nairobi slaughterhouse (Nandi)	July 2005	Domestic pig	X	HM745268
Ken05.DPN15	Farmer's Choice Nairobi slaughterhouse (Nandi)	July 2005	Domestic pig	X	HM745269
Ken05.DPN23	Farmer's Choice Nairobi slaughterhouse (Nandi)	July 2005	Domestic pig	X	HM745270
Ken05.DPU1	Farmer's Choice Nairobi slaughterhouse (Uplands)	July 2005	Domestic pig	X	HM745271
Ken05.DPU2	Farmer's Choice Nairobi slaughterhouse (Uplands)	July 2005	Domestic pig	X	HM745272
Ken05.DPU11	Farmer's Choice Nairobi slaughterhouse (Uplands)	July 2005	Domestic pig	X	HM745273
Ken05.DPU22	Farmer's Choice Nairobi slaughterhouse (Uplands)	July 2005	Domestic pig	X	HM745274
Ken08WH/4	Kapiti Plains Estates (Machakos)	August 2008	Warthog (*P. africanus*)	IX	HM745285
Ken08WH/5	Kapiti Plains Estates (Machakos)	August 2008	Warthog (*P. africanus*)	IX	HM745286
Ken08WH/8	Kapiti Plains Estates (Machakos)	August 2008	Warthog (*P. africanus*)	IX	HM745287
Ken08Tk.2/1	Kapiti Plains Estates (Machakos)	August 2008	Tick (*O. porcinus porcinus*)	X	HM745275
Ken08Tk.2/3	Kapiti Plains Estates (Machakos)	August 2008	Tick (*O. porcinus porcinus*)	X	HM745276
Ken09Tk.13/1	Kapiti Plains Estates (Machakos)	March 2009	Tick (*O. porcinus porcinus*)	X	HM745277
Ken09Tk.13/2	Kapiti Plains Estates (Machakos)	March 2009	Tick (*O. porcinus porcinus*)	X	HM745278
Ken09Tk.15/4	Kapiti Plains Estates (Machakos)	March 2009	Tick (*O. porcinus porcinus*)	X	HM745279
Ken09Tk.15/6	Kapiti Plains Estates (Machakos)	March 2009	Tick (*O. porcinus porcinus*)	X	HM745280
Ken09Tk.19/2	Kapiti Plains Estates (Machakos)	March 2009	Tick (*O. porcinus porcinus*)	X	HM745281
Ken09Tk.19/7	Kapiti Plains Estates (Machakos)	March 2009	Tick (*O. porcinus porcinus*)	X	HM745282
Ken09Tk.19/11	Kapiti Plains Estates (Machakos)	March 2009	Tick (*O. porcinus porcinus*)	X	HM745283
Ken09Tk.20/5	Kapiti Plains Estates (Machakos)	March 2009	Tick (*O. porcinus porcinus*)	X	HM745284

domestic pig and particularly tick ASFV isolates. This heterogeneity was highest within the *p54*-gene, which grouped the tick-derived ASFV isolates into six discrete clades (Fig. 3). The p54 sequence analyses demonstrated that the higher variability found in this protein was due to the presence of two arrays of amino acid repeats, RPATD and RPVAMN (Irusta *et al.*, 1996; Nix *et al.*, 2006), beginning at aa 106. These repeats vary in number among the ASFV isolates from ticks that were characterized (data not shown).

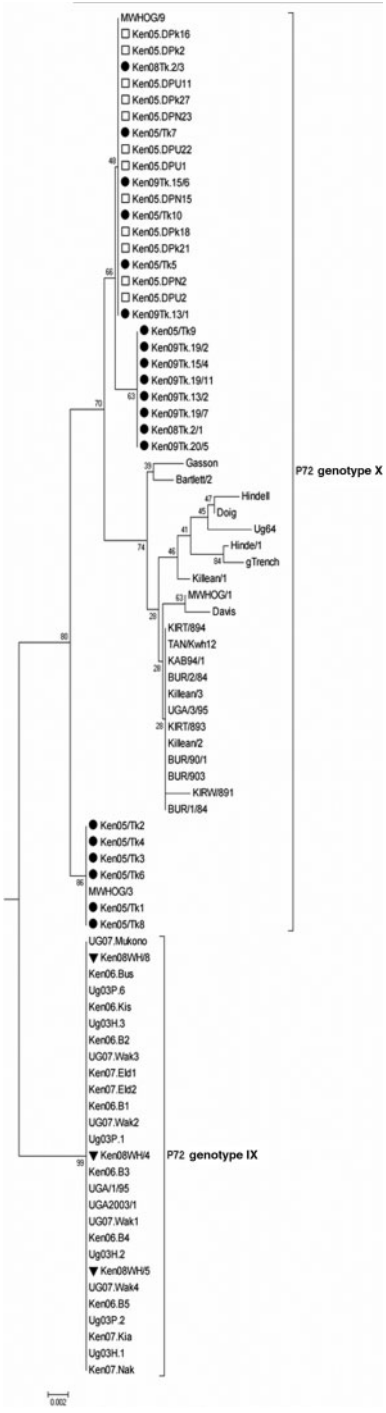

Fig. 2. Phylogenetic subtree based on the C-terminal end of the p72 protein of 20 Kenyan tick (●, Tk), 12 domestic pig (□, DP) and three warthog (▼, WH) ASF viruses characterized in this study, illustrating the genetic relationship with other ASF viruses belonging to ASFV p72 genotypes IX and X. A tree of 276 taxa was inferred using the ME method following initial application of an NJ algorithm. The percentage of replicate trees in which the associated taxa clustered together by bootstrap analysis (1000 replicates) is shown adjacent to the nodes. The robustness of the ME tree was tested using the Close-Neighbour-Interchange (CNI) algorithm at a search level of 1.

The analysis of tandem repeat sequences (TRS) within CVR of the *B602L*-gene has been demonstrated to provide the highest resolution discrimination of ASFV isolates, distinguishing between closely related ASFV isolates placed in a single p72 genotype and providing data that helps to precisely identify the source of outbreaks. Amplification of the CVR-generated amplicons of approximately 170 bp in domestic pigs and ticks Kenyan isolates, whereas the estimated size of the warthogs ASFV isolates was around 350 bp (data not shown). These PCR products were ligated into a pGEMT-Easy vector and 10 individual clones of each fragment were randomly selected for CVR-sequence analyses to detect intra-individual variation. The CVR-derived clones displaying around 100 % similarity. Minor heterogeneity related to unique amino acid mutation was observed in 2 of 10 clones selected from ASFV Kenya tick isolate Ken09Tk.20/5. Predominant sequences were selected for subtyping purposes. CVR-sequence analyses resulted in the identification of 22 different types of amino acid tetramers present in the three Kenyan warthog viruses. These different sequences were assigned code numbers according to the previously described system (Nix *et al.*, 2006) and are shown in Table 4. As in the case of the other genes, the CVR sequences identified from Kenyan warthog viruses were related to the CVR subgroup XXIV. The CVR subgroup XXIV with which the Kenya warthog viruses clustered also contain Ugandan isolates (UGA95/1, Ug03H1–3, Ug03P4–6, Ug07.Wak1–4, Ug07.Mukono and Ug07.F7–8) and Kenyan isolates (Ken06B1–5, Ken06.Bus, Ken06.Kis, Ken07Eld1–2, Ken07.Nak and Ken07.Kia) that are associated with recent disease outbreaks. When the tetrameric repeats located within the *B602L* gene from the three Kenyan warthog samples were compared with viruses included within the CVR subgroup XXIV (Nix *et al.*, 2006; Gallardo *et al.*, 2009a), the viral sequences from the Kenyan warthogs obtained in 2008 were shown to be identical to those obtained from viruses associated with the second domestic pig ASF outbreak that occurred in western Kenya in 2006 (Ken06.Bus) and to all isolates from subsequent outbreaks in Kenya and Uganda in 2007 (Gallardo *et al.*, 2009a; CISA, unpublished data).

The number of amino acid tetramer repeats among ASFV tick isolates varied between eight and nine, and 20 isolates from ticks whose CVR sequence was determined could be separated into four subgroups with tetramer repeat arrays closely related to the XXV CVR subgroup described by Nix *et al.* (2006), which also contains the UGA95/3 virus (GenBank accession no. AM259420). The most abundant tetrameric repeat sequence AABNAaBBA (see Table 4) was identified in a high proportion of the tick isolates together with the Kenyan domestic pig isolates, resulting in both being placed in the CVR subgroup XXVa. The ASFV Ken09Tk.20/5 isolate was classified into a single discrete CVR subgroup (XXVb), presenting a minor change due to the absence of a single internally located tetrameric repeat type 'a' (CVST). The remaining ASFV Ken05Tk/3–6 and Ken05Tk/2–8 isolates (CVR subgroup XXVc and XXVd,

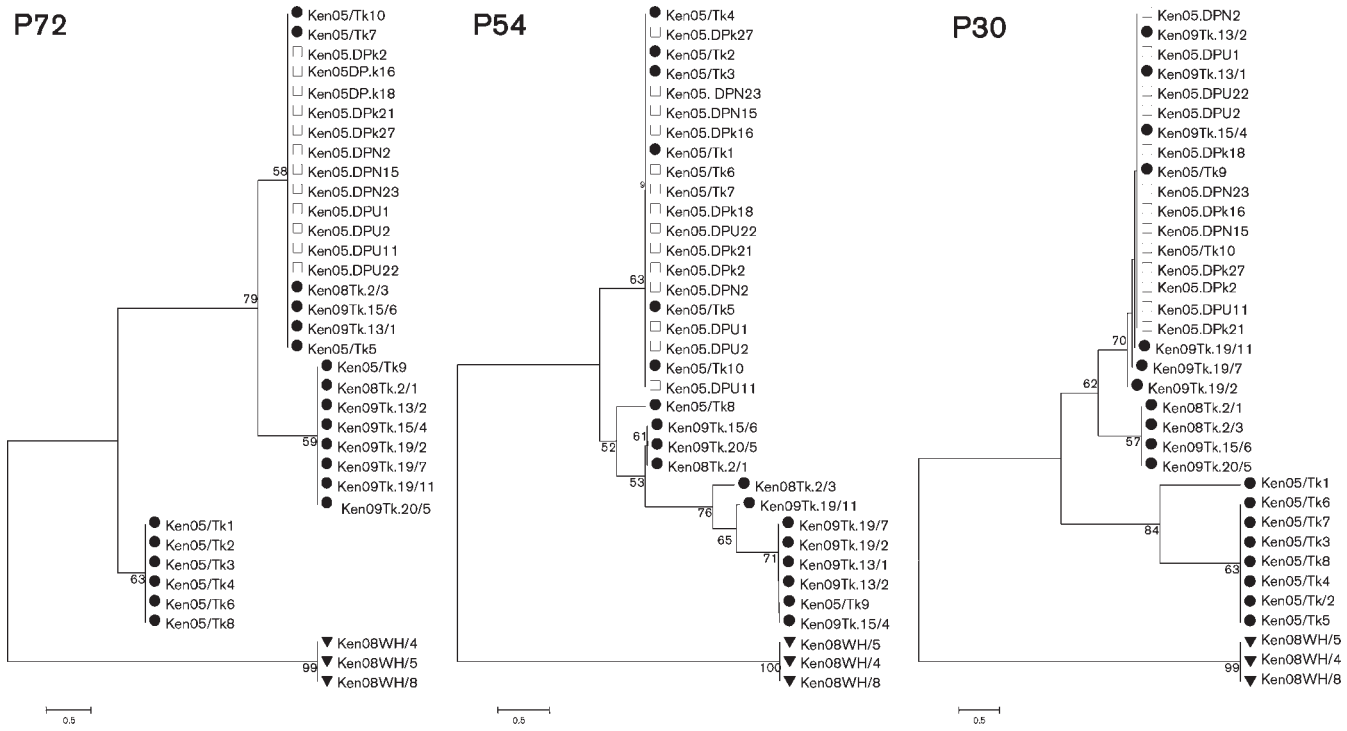

Fig. 3. Comparative phylogenetic trees of the C-terminal end of p72 (P72), the full-length *p54*-gene (P54) and the full-length *p30*-gene (P30) generated using sequences from 36 Kenyan ASFV isolates analysed in this study. The evolutionary history was inferred using the ME method. The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The ME trees were further analysed using the CNI algorithm at a search level of 1. An NJ algorithm was used to generate the initial trees using 1000 replicates. Bootstrap values >50 % are indicated next to the relevant node. ●, Indicates ASF tick-derived viruses; □, indicates ASF domestic pig-derived viruses and ▼, indicates ASF warthog-derived viruses characterized in this study.

respectively) containing a unique pattern of tetramer repeats AAANAaBBA and BNAANABAA not present in the other isolates characterized (Table 4).

DISCUSSION

In this study, we have discovered substantial differences at multiple loci in the genotype of viruses that are prominent at different phases of the warthog–tick sylvatic cycle of ASFV, suggesting that transfers of at least two different viruses into domestic pigs have occurred from the warthog–tick sylvatic cycle to domestic pigs in East Africa. It is also possible, although perhaps less likely, that one or more of these viruses may have transferred from domestic pigs to warthogs. Viruses from both p72 genotypes IX and X, occur in parallel at Kapiti Plains ranch in adult warthogs and ticks, respectively. Sequence analysis of the C-terminal end of *p72*-gene (Bastos *et al.*, 2003) placed the domestic pigs and Kenyan (Kapiti) tick ASFV isolates within genotype X, a sylvatic cycle-associated genotype that comprises viruses recovered from both domestic and wild pigs, and the arthropod tick vector from Rwanda, Burundi, Tanzania and Kenya. ASFV genotype X has historically been associated with ASF in domestic pigs in Kenya in 1950 (Zsak *et al.*, 2005; de Villiers *et al.*, 2010), but in this study was only isolated from ticks and asymptomatic domestic pigs, suggesting possible evolution of decreased viral virulence. Surprisingly, given the current paradigm that the tick–warthog sylvatic cycle is thought to be primarily maintained by the interaction of ticks and neonatal warthogs in the burrows, the genotypes of viruses in the ticks and adult warthogs differed. The p72 genotype of the Kapiti tick isolates was type X, whereas the three ASF viruses from warthogs that were genotyped by direct PCR amplification from serum samples were placed in a different p72 genotype, number IX. This genotype also contains virus isolates associated with recent Ugandan and Kenyan ASF outbreaks between 2003 and 2007 (Gallardo *et al.*, 2009a). However, current theory suggests that direct transfer of ASFV from adult warthogs to domestic pigs is unlikely (Thomson, 1985; reviewed by Penrith *et al.*, 2004a; Bastos *et al.*, 2004). The question of how the genotype IX virus is maintained in the warthog population is unclear since this p72 type was not detected in ticks from 26 different burrows at various locations on the ranch, and the adult warthog is typically regarded as a terminal host for ASFV

Table 4. Amino acid sequence of the tetrameric repeats that constitute the CVR of the *B602L* gene identified in viruses belonging to p72 genotype IX and p72 genotype X

A, CAST; a, CVST; B, CADT, CADI; N, NVDT. Dashes indicate gaps introduced manually to enable similarities between sequences to be more easily visualized. Sequences generated in this study are indicated in bold.

Isolate	Country	p72 genotype	CVR amino acid sequence	No. repeats	CVR subgroup	CVR GenBank accession no.	Reference
Ug03H.1	Uganda	IX	AAABNABBNABB---aaBBNABNaBA	23	XXIV	FJ174339	Gallardo *et al.* (2009a)
Ug03P.4	Uganda	IX	AAABNABBNABB---aaBBNABNaBA	23		FJ174342	Gallardo *et al.* (2009a)
Ken06.B1	Kenya	IX	AAABNABBNABB---aaBBNABNaBA	23		FJ174329	Gallardo *et al.* (2009a)
Ken06.Bus	Kenya	IX	AAABNABBNABB---aa-BNABNaBA	22		FJ174334	Gallardo *et al.* (2009a)
Ken06.Kis	Kenya	IX	AAABNABBNABB---aa-BNABNaBA	22		FJ174337	Gallardo *et al.* (2009a)
Ken07.Eld1	Kenya	IX	AAABNABBNABB---aa-BNABNaBA	22		FJ174335	Gallardo *et al.* (2009a)
Ken07.Eld2	Kenya	IX	AAABNABBNABB---aa-BNABNaBA	22		FJ174336	Gallardo *et al.* (2009a)
Ken07.Kia	Kenya	IX	AAABNABBNABB---aa-BNABNaBA	22		FJ238539	Gallardo *et al.* (2009a)
Ken07.Nak	Kenya	IX	AAABNABBNABB---aa-BNABNaBA	22		FJ174338	Gallardo *et al.* (2009a)
UG07.Wak1	Uganda	IX	AAABNABBNABB---aa-BNABNaBA	22		GQ477152	CISA-unpublished
UG07.Wak2	Uganda	IX	AAABNABBNABB---aa-BNABNaBA	22		GQ477153	CISA-unpublished
UG07.Wak3	Uganda	IX	AAABNABBNABB---aa-BNABNaBA	22		GQ477154	CISA-unpublished
UG07.Wak4	Uganda	IX	AAABNABBNABB---aa-BNABNaBA	22		GQ477155	CISA-unpublished
UG07.Mukono	Uganda	IX	AAABNABBNABB---aa-BNABNaBA	22		GQ477156	CISA-unpublished
UG07.F7	Uganda	IX	AAABNABBNABB---aa-BNABNaBA	22		GQ477157	CISA-unpublished
UG07.F8	Uganda	IX	AAABNABBNABB---aa-BNABNaBA	22		GQ477158	CISA-unpublished
Ken08WH/4	Kenya	IX	AAABNABBNABB---aa-BNABNaBA	22		HM745320	This study
Ken08WH/5	Kenya	IX	AAABNABBNABB---aa-BNABNaBA	22		HM745321	This study
Ken08WH/8	Kenya	IX	AAABNABBNABB---aa-BNABNaBA	22		HM745322	This study
UGA95/1	Uganda	IX	AAABNABBNABBNABaa BBNABNaBA	26		AM259419.1	Nix *et al.* (2006)
HindII	Kenya	X	AAA---BNAAAAAAAAAAB-A	17	XXVI	AM259421.1	Nix *et al.* (2006)
Bur90/1	Burundi	X	AAA---BNAAAAAAAAAAB-A	17		AM259424.1	Nix *et al.* (2006)
Bur84/2	Burundi	X	AAA---BNAAAAAAAAAAB-A	17		AM259423.1	Nix *et al.* (2006)
Bur84/1	Burundi	X	AAA---BNAAAAAAAAAAB-A	17		AM259422.1	Nix *et al.* (2006)
Uga95/3	Uganda	X	AAAAAABN---------BABA	12	XXV	AM259420.1	Nix *et al.* (2006)
Ken05.DPk2	Kenya	X	AA----BNAa--------BBA	9	XXVa	HM745298	This study
Ken05.DPk16	Kenya	X	AA----BNAa--------BBA	9		HM745299	This study
Ken05.DPk18	Kenya	X	AA----BNAa--------BBA	9		HM745300	This study
Ken05.DPk21	Kenya	X	AA----BNAa--------BBA	9		HM745301	This study
Ken05.DPk27	Kenya	X	AA----BNAa--------BBA	9		HM745302	This study
Ken05.DPN2	Kenya	X	AA----BNAa--------BBA	9		HM745303	This study
Ken05.DPN15	Kenya	X	AA----BNAa--------BBA	9		HM745304	This study
Ken05.DPN23	Kenya	X	AA----BNAa--------BBA	9		HM745305	This study
Ken05.DPU1	Kenya	X	AA----BNAa--------BBA	9		HM745306	This study
Ken05.DPU2	Kenya	X	AA----BNAa--------BBA	9		HM745307	This study
Ken05.DPU11	Kenya	X	AA----BNAa--------BBA	9		HM745308	This study

Table 4. cont.

Isolate	Country	p72 genotype	CVR amino acid sequence	No. repeats	CVR subgroup	CVR GenBank accession no.	Reference
Ken05.DPU22	Kenya	X	AA----BNAa--------BBA	9		HM745309	This study
Ken05/Tk9	Kenya	X	AA----BNAa--------BBA	9		HM745296	This study
Ken05/Tk10	Kenya	X	AA----BNAa--------BBA	9		HM745297	This study
Ken05/Tk7	Kenya	X	AA----BNAa--------BBA	9		HM745294	This study
Ken05/Tk5	Kenya	X	AA----BNAa--------BBA	9		HM745292	This study
Ken05/Tk1	Kenya	X	AA----BNAa--------BBA	9		HM745288	This study
Ken05/Tk4	Kenya	X	AA----BNAa--------BBA	9		HM745291	This study
Ken08Tk.2/1	Kenya	X	AA----BNAa--------BBA	9		HM745310	This study
Ken08Tk.2/3	Kenya	X	AA----BNAa--------BBA	9		HM745311	This study
Ken09Tk.13/1	Kenya	X	AA----BNAa--------BBA	9		HM745312	This study
Ken09Tk.13/2	Kenya	X	AA----BNAa--------BBA	9		HM745313	This study
Ken09Tk.15/4	Kenya	X	AA----BNAa--------BBA	9		HM745314	This study
Ken09Tk.15/6	Kenya	X	AA----BNAa--------BBA	9		HM745315	This study
Ken09Tk.19/2	Kenya	X	AA----BNAa--------BBA	9		HM745316	This study
Ken09Tk.19/7	Kenya	X	AA----BNAa--------BBA	9		HM745317	This study
Ken09Tk.19/11	Kenya	X	AA----BNAa--------BBA	9		HM745318	This study
Ken09Tk.20/5	Kenya	X	AA----BNA--------–BBa	8	XXVb	HM745319	This study
Ken05/Tk3	Kenya	X	AAA----NAa--------BBA	9		HM745290	This study
Ken05/Tk6	Kenya	X	AAA----NAa--------BBA	9	XXVc	HM745293	This study
Ken05/Tk2	Kenya	X	------BNAANAB------AA	9	XXVd	HM745289	This study
Ken05/Tk8	Kenya	X	------BNAANAB------AA	9		HM745295	This study

(Bastos, *et al.*, 2009; Jori & Bastos, 2009). However, the adult warthog and tick samples could not be directly cross referenced in this study, due to the fact that the burrows from which the adult warthogs originated are unknown. Since, due to the low viraemia in the blood we were only able to genotype viruses from three adult warthogs; it is possible that a more extensive survey might also reveal the presence of genotype IX in adult warthogs. The data generated using the *p54* and *p30* genes were consistent with the p72 genotyping information, indicating that the ASF viruses recovered from warthogs in central Kenya in 2009 were genetically very similar to those responsible for the outbreaks in Kenya and neighbouring Uganda between 2003 and 2007. The higher level of resolution of the viral discrimination using *p54*-gene sequencing was again illustrated by the separation of the Kenyan tick-derived viruses into six clusters as compared with three using p72.

Sequence analysis of the CVR of viruses from Kenyan warthog isolates identified 22 different tetrameric amino acid units. The sequence of the CVR from the warthog isolates was identical to that of six isolates associated with the second wave of infections that occurred in western and central Kenya from October 2006 to January 2007 and Uganda in 2007. However, the warthog CVR was distinguishable from Kenyan isolates recovered from an earlier outbreak in May 2006 and from Ugandan viruses from 2003 outbreaks, which were identical in their CVR sequences (Gallardo *et al.*, 2009a). One explanation that is consistent with the spread of the virus from western to central Kenya via domestic pigs is that the same CVR mutation has occurred more than once in both warthogs and domestic pigs. However, warthogs are present in the regions of central Kenya where disease outbreaks were recorded in 2007 and are also widespread throughout East Africa. Therefore, 'sylvatic cycle' transmission from warthogs to domestic pigs cannot be excluded and could play a role in future re-emergence of sporadic virulent outbreaks in the region.

A high level of CVR diversity was found in ASFV strains isolated from Kenyan ticks. The viruses were divided into four subgroups, which were similar to the previously defined CVR subgroup XXV containing the Ugandan ASF isolate (Uga95/3). This greater diversity is consistent with the tick acting as the primary host for long-term maintenance of the virus.

PCR detected viral DNA in 22 % of individual ticks and tick pools sampled, both before and after the Kenyan ASF outbreaks during 2006–2007, indicating that a significant proportion had the opportunity to feed on viraemic pigs. The nested *p72* PCR assay (Basto *et al.*, 2006) that we used can detect quantities of virus below the detection threshold of virus isolation in cell culture. Both PCR assays can detect DNA in viruses that are not replicating and virus was only isolated from 31 % of PCR-positive ticks or tick pools. However, based on our data alone, we cannot be sure regarding whether or not the ASF viral DNA that we detected in the ticks was derived from actively replicating virus. Viruses that were isolated from ticks exhibited the characteristic haemadsorption pattern of ASFV, and high virus titres.

We demonstrated a high prevalence of ASFV with p72 genotype IX in seronegative domestic pigs originating from several locations in which no disease had been reported and which did not exhibit clinical symptoms. The presence of the virus was confirmed by PCR in approximately 50 % of domestic pigs sampled, with a high percentage of positives from Kamiti district, near Nairobi. In 30 %, of the PCR-positive domestic pigs, virus was isolated directly from serum with titres ranging from 10^1 to 10^4 (HAD_{50}) ml^{-1}, a level sufficient to infect naive pigs or ticks. No anti-ASF antibodies were detected in the domestic pig serum samples tested using OIE-serological prescribed methods. This result supports previous studies documenting a low incidence of detectable serological responses to ASFV (Perez-Filgueira *et al.*, 2006; Gallardo *et al.*, 2009b). The data obtained from warthog serum samples in the same region of Kenya do not support the explanation that failure to detect antibodies is due to antigenic polymorphism, since all were positive, using the same serological techniques. This was despite the fact that the warthogs were infected with viruses of a different genotype to that used to generate the antigen extract used for serodiagnosis. An alternative explanation for the low seropositivity in East Africa may reside in the immunogenetics of the indigenous pig populations, rather than being due to polymorphisms in immunodominant viral antigens. However, it is possible that the apparent lack of a detectable antibody response is due to the fact that the strain present in the warthogs is different to that in the domestic pigs. It also cannot be formally excluded that the pigs sampled were at an early stage of infection by the virus and had not yet mounted an antibody response. Additional research is therefore required to clarify the situation, including experimental infection of local breeds of African domestic pigs and monitoring with these two strains and monitoring of the antibody response.

Serological and PCR results obtained from warthog samples collected from Kapiti Plains were similar to previous findings in ASFV-enzootic areas in southern Africa, demonstrating that adult warthogs are typically non-viraemic, but seropositive, although virus can usually be isolated only from lymph nodes (Penrith *et al.*, 2004a). It is currently thought that neonatal/young warthogs become infected by *Ornithodoros* ticks in warthog burrows early in life. The virus replicates in the warthog and produces a transient viraemia, which is sufficient for transmission to uninfected ticks that feed on the young warthogs, thus maintaining the cycle (Thomson, 1985; reviewed by Penrith *et al.*, 2004a). Older warthogs are probably persistently infected but, seldom viraemic, with the lymph nodes thought to be the most frequently infected tissue. Current understanding of the sylvatic cycle based primarily on southern African data suggest that large

numbers of *O. porcinus* infested warthog burrows play the key role in maintenance of the virus. By contrast adult warthogs have a low blood virus concentration and appear to represent a terminal host (Bastos *et al.*, 2009). The mechanism of ASFV transmission from the sylvatic cycle in eastern and southern African regions is most likely through feeding of infected ticks on pigs, since direct contact between infected warthogs and domestic pigs does not result in transmission (Heuschele & Coggins, 1969; Thomson, 1985), except in a single case (Detray, 1957). Interestingly, our study revealed that viruses present in the serum of adult warthogs were genetically distinct from those detected in ticks isolated from warthog burrows at the same location, but similar to those responsible for recent ASF outbreaks. As far as we are aware this is a novel observation.

The detection and molecular characterization of ASFV in warthogs, ticks and domestic pigs that we report suggest the concurrent existence of three different transmission cycles of ASF in Kenya; (i) domestic pig–pig transmission without tick involvement associated with the recent outbreak in western Kenya; (ii) domestic pig–warthog and (iii) domestic pig–tick–warthog sylvatic transmission role in the maintenance and potential re-emergence of the disease. The observation of segregation of different genotypes between ticks and adult warthogs is surprising and suggests that in future a more in depth survey of viruses present in warthogs and ticks at multiple locations in East Africa will be required to better understand the nature of the sylvatic transmission cycle of ASFV and the maintenance of the virus.

METHODS

Study area and sampling collection. The ASF domestic pig survey was conducted in July 2005 at the Farmer's Choice Nairobi slaughterhouse. Eighty-three randomly selected animals were first subjected to routine clinical examination by a veterinarian and subsequent collection of blood samples through the ear/jugular vein. After slaughter, a comprehensive post-mortem examination by the abattoir veterinarian was conducted followed by tissue sampling. The animals were local breeds of domestic pigs from areas without reports of any clinical outbreak. These animals originated from three areas in western and central Kenya: Nandi (n=23), Kamiti (n=30) and Uplands (n=30) (Fig. 1).

The ASF tick and warthog survey study was conducted at the International Livestock Research Institute (ILRI) farm at Kapiti Plains Estate, a 13 000 ha ranch located in Machakos district of central Kenya, about 65 km south-east of Nairobi (Fig. 1). The first tick sample collection was performed in July 2005. A total of 378 ticks were collected from five independent warthog burrows. During 2008–2009, following ASF outbreaks in Kenya in 2006 and 2007, a second tick survey involving collection of 1198 specimens from 21 independent warthog burrows was conducted at Kapiti Plains Estate. Additionally, in August 2008 and March 2009, 51 whole-blood and serum samples were collected from adult warthogs that were part of an estimated population of 150 were also collected from the ranch. All the ticks were identified as soft ticks of the species *O. porcinus*.

Tick sample preparation. The ticks collected were classified according to the stage of development into 891 small nymphs (nymphal stages 1–3, N1–N3), large nymphs (stages 4 and 5, N4–N5) and adults (a mixture of males and females). Following identification, a total of 1576 ticks from 26 independent warthog's burrow were separated into groups comprising adult ticks (72 individual ticks and 17 pools), small nymphs (116 pools) and large nymphs (80 pools) that were ground in porcelain vessels with 1.5 ml cold PBS (1 ×) supplemented with 0.1 % of antibiotic (gentamicin sulphate). Suspensions were clarified by centrifugation (5000 ***g***, 5 min, 4 °C) and the supernatants were stored at −70 °C until further use.

Detection of ASFV DNA in field-collected tick and mammalian samples by PCR. Detection of ASFV DNA in field-collected samples used previously described protocols (Aguero *et al.*, 2003). Briefly, DNA was extracted from 200 µl of each tick supernatant, 83 domestic pig sera, 51 warthog sera and 51 blood warthog samples and recovered in a final volume of 50 µl using a High Pure Viral Nucleic Acid kit (Roche) following the manufacturer's instructions. A PCR assay using the ASF diagnostic primers PPA1/PPA2, which generates an amplicon of 257 bp within the gene encoding the p72 protein (Aguero *et al.*, 2003), was used to confirm the presence of ASFV DNA. The PCR products were size fractionated by electrophoresis through 2 % agarose gels and the specificity of the amplicons was confirmed by *Bsm*AI restriction enzyme digestion (Aguero *et al.* 2003). In order to eliminate the possibility of false-negatives, due to the presence of PCR inhibitors in DNA samples purified from tick supernatants, PCR-positive and -negative ticks were analysed by a more sensitive nested PCR protocol (Basto *et al.*, 2006) based on alternative primers from the VP72 gene designated 72ARs/72ARas. These primers were used in combination with internal primers 72Ns/72Nas to maximize the probability of detecting small amounts of ASFV DNA in tick samples.

Virus isolation and titration. Virus isolation and titration were performed by using a haemadsorption assay with minor modifications (Malmquist & Hay, 1960). Briefly, PBMs were seeded into 96-well tissue culture grade microtitre plates (200 µl; 300 000 cells per well) in homologous swine serum, and incubated at 95 % relative humidity with 5 % CO_2 at 37 °C. Three day cultures were infected with PCR-positive samples collected from the field (four wells per sample; 20 µl inoculum per well). After inoculation, a preparation of 1 % homologous red blood cells in buffered saline was added to each well and was incubated at 37 °C. The plates were examined for haemadsorption over a period of 6 days. Samples were blind passaged three times. Viral isolation in PBM cells showing cytopathic effects (CPE) and/or haemadsorption was confirmed by PCR using PPA1/2 primers (Aguero *et al.*, 2003) and *Nde*I enzyme restriction analyses (CISA, unpublished data). Titrations were performed by using a haemadsorption assay involving inoculation of limiting dilutions of supernatants from haemadsorbent positive field-collected samples into PBM. Titres were estimated using the method of Reed & Muench (1938) and expressed as HAD_{50} per sample.

Detection of antibodies against ASF in field-collected serum samples. Serum samples from 83 domestic pigs and 51 warthog field sera were tested using the OIE 2008 prescribed tests (OIE, 2008). This included the OIE indirect ELISA and immunoblotting assays, using as antigen a lysate of Monkey Stable cells infected with ASF Spanish isolate E70MS48 and protein-A conjugated to horseradish peroxidase as the reporter (OIE, 2008).

ASF molecular characterization. PCR amplification and nucleotide sequencing: for genetic characterization, PCR was performed on nucleic acid extracted from ASFV containing samples by using specific primers to amplify four independent regions from the ASFV genome: (i) 478 bp within the 3′ end of the p72 protein were

amplified using primers p72-U/D (Bastos *et al.*, 2003); (ii) the full-length *p54*-gene encoding the VP54 protein was amplified using the primers PPA89/722 (Gallardo *et al.*, 2009a); (iii) the full-length *p30*-gene encoding the VP30 protein was amplified using the primers p30R/F (Rowlands *et al.*, 2008); (iv) the primer pairs ORF9L-F/9L-R were used to amplify the CVR within *B602L* gene (Nix *et al.*, 2006). p72, p54 and p30 sequences were determined by direct sequencing of PCR amplified products. CVR amplicons of the predicted size were excised and purified by Qiaex gel extraction (Qiagen) and cloned into a pGEMT-Easy vector according the manufacturer's instructions. Ten clones from each sample were sequenced using primers specific for the pGEMT-Easy vector (SP6/T7) using an automated 3730 DNA analyser (Applied Biosystems).

Analysis of sequence data was performed with Chromas (www.technelysium.com.au), BioEdit (www.mbio.ncsu.edu/BioEdit/BioEdit.html) and CLUSTAL_X version 1.83 (www.clustal.org). For TRS analyses including that of the CVR-nucleotide sequence, deduced amino acid sequences were manually aligned with gaps being inserted to optimize the alignment. Two different datasets were employed for phylogenetic analyses conducted by using MEGA version 4 (Tamura *et al.*, 2007): (i) a *p72*-gene dataset comprising 276 taxa in which sequences generated in this study from Kenyan viruses were compared with ASFV sequences available in GenBank; (ii) a *p54-p30-p72* comparative dataset comprising 36 taxa corresponding to Kenya ASFV viruses characterized in this study. NJ and ME p72, p54 and p30 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 trees, data were resampled 1000 times using the bootstrap method.

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