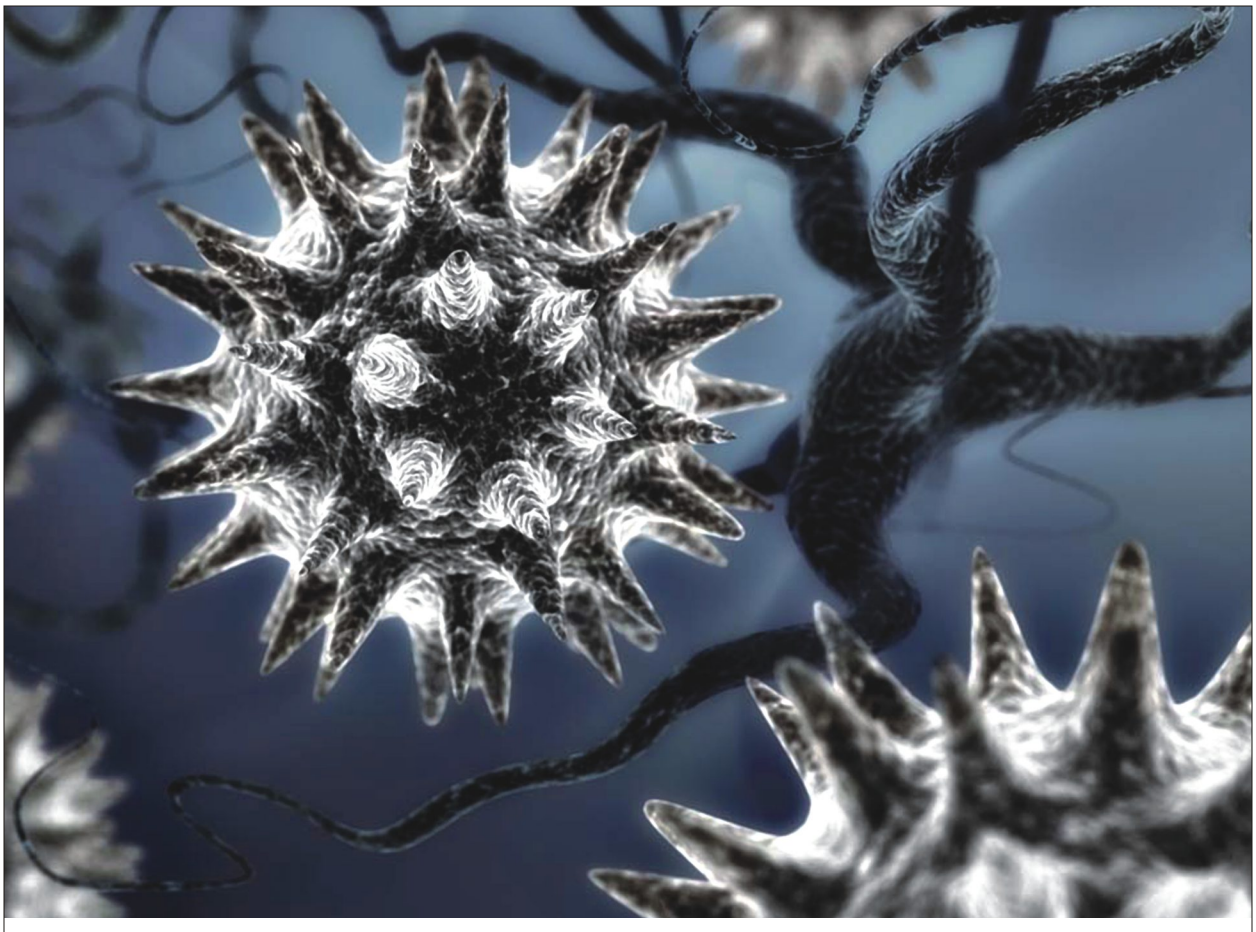

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Research Article
First Detection of African Swine Fever Virus Genotype IX at the Kenya Coast

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Abstract

Background and Objective: African Swine Fever Virus (ASFV) causes a frequently lethal haemorrhagic disease in domestic pigs and is responsible for an ongoing global pig pandemic. The highly virulent genotype IX is the predominant strain in Kenya and Uganda but has previously not been detected further South than Central Kenya. This study was designed to assess the genotype of ASFV associated with outbreaks on the Kenya Coast. **Materials and Methods:** We used PCR amplification and sequencing of the C-terminal region of the p72 gene and the central variable region (CVR) of the B602L locus to genotype viruses from Western, Central and Coastal Kenya. The clinical symptoms of infected pigs were also assessed by a veterinarian. **Results:** This study investigated ASFV outbreaks in Kenya between 2010-2011 to determine associated genotypes and transmission routes. Outbreaks were all attributable to ASFV genotype IX and their temporal sequence suggested an origin in endemic regions of western Kenya and subsequent transmission to central and coastal districts through human activities. **Conclusion:** Confirmation of the occurrence of ASFV genotype IX associated with Clinical ASF at the Kenya Coast, a 500 km range extension, highlights the potential for dissemination of this highly virulent East African genotype outside Africa.

Key words: African Swine Fever, genotype IX, molecular detection, haemorrhagic, domestic swine, genetic investigation, veterinarian

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

African Swine Fever Virus (ASFV) causes African Swine Fever (ASF), a rapidly lethal hemorrhagic disease in domestic pigs and wild boar (*Sus scrofa*). Recent transmission of genotype II ASFV from Africa to Russia, Europe and China highlights the rapid, transboundary movement of ASFV[1]. ASF represents a constraint to the development of the pig industry globally, including Sub-Saharan Africa, where it is endemic. The first case of clinical ASF in domestic pigs was described in Kenya. Multiple ASF outbreaks have been reported in Kenya since 2001[2]. In May 2007, the Kenya Department of Veterinary Services (DVS), reported suspected outbreaks of ASF in Central, Rift Valley, Nyanza and Western provinces of the country to the OIE[3]. During the outbreaks, eleven haemadsorbing viruses were isolated[4] and found to be genetically closely related to viruses responsible for ASF outbreaks that occurred in Uganda in 2003 and subsequently in Western Kenya in 2006[2], suggesting the transboundary spread of infection through domestic pig to pig transmission. Further outbreaks occurred in Kenya between December, 2010 and February, 2011. The outbreak districts were Busia and Kakamega (Western Province), Kisumu (Nyanza Province), Kiambu and Thika (Central Province). Subsequently, an outbreak was reported from the Coast Province in September/October, 2011. The rapid spread of ASF between districts and extension to non-traditional ASF risk districts indicated that a detailed genetic investigation of these outbreaks and the transmission cycle was required. The technology for such studies is now well documented[3-5] and widely applied in African laboratories.

This study documents the genotype, clinical presentation, pathology, aspects of the epidemiology and transmission pathways of the ASF viruses responsible for the outbreaks in Kenya in 2010 and 2011.

MATERIALS AND METHODS

Study area: The samples analyzed in this study were obtained from January, 2010 to December, 2011 from the Western, Central and Coastal Regions of Kenya. The experimental analyses were performed at the International Livestock Research Institute in Kenya and the European Reference Laboratory in Spain (CISA-INIA).

Field Information: Information relayed from pig farmers to District Veterinary Officers (DVOs) initially identified outbreaks in Western, Central and Coastal Kenya. Affected farms were visited by the relevant DVOs as soon as possible after reports were received and observations were made on both clinically symptomatic and asymptomatic pigs, present within infected herds. This included selected information on the epidemiology (including sources of feed for the affected pig herds) the clinical presentation where animals were still alive and pathology, based on post-mortem for selected animals that had succumbed to the disease. The data indicated that pigs were dying in greater numbers than normal and within a short period. DVOs notified The Central Veterinary Laboratory at the Ministry of Livestock Health and Development.

Samples from suspected cases of ASF were stored at -80°C and subsequently analysed in the laboratory at the European Union (EU) Reference Laboratory, Valdeolmos Madrid, Spain and the International Livestock Research Institute (ILRI) Nairobi Kenya from April to June, 2013, using the OIE indirect antibody ELISA and immunoblotting, combined with viral detection using a p72-based PCR assay[3].

Sample collection: All live, dead and in-contact animals in a household were sampled for blood and/or tissues. Blood was collected from the subcutaneous abdominal or ear veins using BD Vacutainer® needles into 10 mL BD Vacutainer® serum tubes and 4.5 mL 15% BD EDTA vacutainer® tubes. Non-EDTA blood was allowed to clot and serum separated. A total of 61 domestic pig tissues, 31 sera and 22 EDTA blood samples from 15 pigs were shipped to the EU-ASFV reference laboratory at CISA-INIA, Valeomos Madrid on dry ice. Additional pig serum samples and tissues from 12 pigs sampled from Kisauni (Mombasa District) were sent to the laboratory at ILRI.

Methodology: Antibody detection was performed on 32 serum samples through screening of sera using an indirect Enzyme-Linked Immunosorbent Assay (OIE-ELISA). ELISA and confirmatory immunoblotting assays were performed using a lysate of MS stable monkey kidney cell line (ECACC, 91070510) infected with ASFV E70MS48 as the antigen and protein-A conjugated to the enzyme horseradish peroxidase (HRP). Both procedures followed the protocols in the OIE Diagnostic Manual.

The DNA was extracted from 200 µL of 66 homogenized tissues (10% w/v), 32 sera and 22 blood domestic pig samples using a High Pure Viral Nucleic Acid Kit (Roche). Conventional and real-time PCRs were performed using both undiluted and diluted (1:10) DNA and the amplicons were validated using NdeI[5]. ASFV isolation and titration using the hemadsorption (HAD) assay were performed on Peripheral Blood Monocytes (PBMC) as previously described[4]. The PBMC were inoculated at

a multiplicity of infection (moi) of 1:10 with PCR-positive samples. Preparation of 1% homologous red blood cells in PBS was added and incubated at 37°C. The plates were examined for hemadsorption over 6 days. Titration was performed by inoculating limiting dilutions of samples into PBMC. The titer was estimated[6] and expressed as 50% hemadsorbing doses per millilitre.

PCR reaction: PCR was performed on nucleic acid extracted from ASFV positive samples using specific primers to amplify three regions of the ASFV genome, (i) 478 bp at the 3' end of the gene encoding the p72 protein using primers p72-U/D[7], (ii) the full-length p54-gene encoding the VP54 protein using primers PPA89/722[2] and (iii) 485 bp of Central Variable Region (CVR) within B602L gene using primers CVR1 and CVR2[5]. Amplicons of the predicted size were excised, purified and directly sequenced using an automated 3730 DNA analyzer.

Sequence analysis: Analysis of sequence data was performed using Chromas (www.technelysium.com.au), BioEdit (www.mbio.ncsu.edu/BioEdit/BioEdit.html) and ClustalX version 1.83 (www.clustal.org). For the tandem repeat sequences (TRS) within the CVR nucleotide sequence, deduced amino acid sequences were manually aligned with gaps being inserted to optimize the alignment. Phylogenetic analyses was performed using MEGA version 5.0[8].

RESULTS AND DISCUSSION

A summary of the outbreak reports in affected districts of Western, Central and Coastal Kenya is presented in Table 1. Western, Nyanza, Central and Rift Valley provinces had pig populations of 87,838, 27,612, 91,977 and 48,495 pigs, respectively (data provided to Dr. Macharia at the Ministry of Livestock Agriculture and Fisheries, by DVOs from the respective provinces). The population at the Kenya Coast was relatively small and estimated at approximately 400 pigs according to the Mombasa DVO. The pigs affected in Kondele, Kisumu East District, were free-ranging, whereas farms with confined pigs were not affected. An estimate of the population at risk was 1500 and the mortality was 80%. Two farms were investigated following ASF outbreaks in Kakamega District. One farm had 150 free-ranging pigs and the other had 50 pigs that were housed and therefore confined. The confined animals were fed swill from hotels in Kakamega town. The mortality was 99% in free-range animals and 68% in confined pigs. The pigs affected in Bumala Village, Busia District were free-ranging. The estimate of the pig population at risk of infection from the DVO was 655. Pigs affected in the peri-urban area of Kiambu town, in the central province were confined and fed in their stalls with commercial feeds. The pig population at risk in the district was estimated at 10,000. Two farms were investigated one with 5 pigs and the other with 6 pigs. Mortality was 55% on each farm. Two peri-urban informal settlements were investigated in Thika town, Central Province. The pigs affected were both free-ranging and confined. Mortality was 94%. At Kisauni in Mombasa province at the coast, pig production was peri-urban. The estimated pig population at risk was 250. The morbidity and mortality rates could not be estimated accurately, because the survey was performed after the active outbreak had finished. However, it was confirmed from blood, serum and tissue samples that ASFV was present in the tissues of deceased pigs and two virus isolates were obtained at ILRI. The major symptoms observed at all locations were a rapid onset of disease presenting as dullness, anorexia and a very high body temperature of 42°C. These initial symptoms were typically followed by dyspnoea, ataxia, recumbency and death. The duration of the disease was typically between 24-48 hrs until death. Post-mortem findings revealed carcasses in good body condition. Consistent findings from the post-mortem analyses included gastro-splenic and perianal lymph nodes exhibiting acute, diffuse and severe haemorrhage and mesenteric lymph nodes with acute, multifocal haemorrhage. The liver exhibited capsular, chronic and mild fibrosis. The primary diagnosis of ASF was made by p72-PCR amplification applied to templates extracted from blood and multiple tissues (liver, spleen, brain, lymph node, heart and lung). Tissues from two necropsies and three sera were confirmed as being positive for ASF in Kisumu District. Of the seven animals sampled in the Kakamega District, tissues from three animals were confirmed as positive for ASFV by both PCR and virus isolation and three were confirmed positive by indirect antibody ELISA and immunoblotting. The tissues collected from the two necropsies performed in Busia District were confirmed to be ASF positive by PCR. All four sera collected from surviving pigs were negative for ASF by indirect antibody ELISA, but positive for ASFV by virus detection using PCR. A pig sampled from one farm and two pigs sampled from a second farm in the Kiambu district were seronegative by indirect antibody ELISA but positive for ASFV using virus detection using PCR. Tissues from a pig necropsy in Thika District, were positive for ASFV by virus detection using PCR. Blood from six pigs had two samples that tested positive for ASFV using PCR, but sera from the same pigs were negative by indirect antibody ELISA. Among the fifteen pigs sampled from the coastal province, three were positive by PCR. All animals were seronegative using the

Table 1: Summary of the outbreak reports in affected districts of Western, Central and Coastal Kenya

	Location						
Reporting date	District	Administrative location	Total animals at risk per location	Number of animals affected	Number of dead animals	Crude morbidity proportion (%)	Crude mortality proportion (%)
22/12/2010	Kisumu East	Kondele	1500	1000	800	67	53
23/12/2010	Kakamega	Municipality	200	200	182	100	91
18/01/2011	Busia	Bumala	655	655	590	100	90
28/01/2010	Kiambu	Municipality	10000	11	6	0	0
30/01/2011	Thika	Makongeni	200	100	100	50	50
30/09/2011	Kisauni	Kisauni	250	15	2	6	0.8

indirect antibody ELISA but either blood or serum was positive for ASFV virus using p72 PCR. Tissues were collected following post-mortem from two pigs from the Kisauni District. In one pig five tissues tested ASFV positive by PCR and virus isolates were generated in porcine PBMC using ground-up spleen tissue from this and one other animal.

The virus sequences were identical across 478 bp of the C-terminal p72-gene and the 558 bp p54-gene. The p72 sequences were compared with 80 sequences in GenBank including representatives of the 22 (I-XXII) described p72[9,10]. The nine Kenyan ASFV isolates, including the two Kisauni coastal isolates (Ken11/Kisauni.P52 and Ken11/Kisauni.P13) clustered within genotype IX[11] together with isolates from 13 Ugandan pigs associated with outbreaks in 1995, 2003 and 2007[2]. The 14 isolates from Western and Central Kenya associated with outbreaks in 2006-2007 also clustered within p72 genotype IX. The two Kisauni p72 and p54 sequences were submitted to GenBank with accession numbers (KJ626191 and KJ626192) and (KJ626193 and KJ626194), respectively. The sequence of amino acid tetramer repeats (AAABNABBNABBaaBBNABNaBA) within the CVR of the B602L gene were all related to CVR subgroup XXIV[2] isolated from earlier outbreaks in Uganda and Kenya. Differences were primarily in the number of tetrameric amino acid repeats. The B602L gene of the Kenyan viruses, including the Kisauni isolates, were identical to those associated with Ugandan 2003 outbreaks but differed from the 2009 outbreaks in Congo and the 2006-2007 outbreaks in Western and Central Kenya[2,10]. The inferred amino acid sequences of the Kenyan 2010-2011 viruses exhibited a minor sequence change to the insert within a single internally located tetrameric repeat (CADT) which was present in viruses from Uganda in 2003 and Kenya in 2007.

To briefly summarize the data obtained in this study, the Kenyan ASFV isolates genotyped, including the two Kisauni coastal isolates (Ken11/Kisauni.P52 and Ken11/Kisauni.P13) clustered within genotype p72 genotype IX[7,11], Fourteen isolates from Western and Central Kenya together with additional viruses associated with outbreaks in 2006-2007 and 2013 were also classified in genotypeIX[2] together with isolates from 13 ASFV isolated from clinically reacting pigs from Uganda in 1995, 2003 and 2007[2].

A feature of ASF outbreaks in 2010-2011 was the rapid onset of clinical disease. The duration of the disease was frequently short, often with only 24-48 hrs elapsing before death. The rapid spread of the virus among pigs and the acute manifestation of the disease indicate that these genotype IX viruses represented highly virulent genotypes. Earlier studies in Kenya identified less virulent viruses resulting in infection without apparent clinical symptoms, however these clustered within the related, but distinct p72 genotype X[5]. Most of the affected farms contained pigs that ranged freely and could have come into contact with infected pigs or scavenged infected pork-containing waste. Where pigs were housed in sties, they were fed swill from hotels, which could have potentially contained pork waste from infected pigs. Farmers often sold sick pigs rapidly to butchers as soon as preliminary symptoms were observed, to avoid ASF-associated losses. This behaviour by farmers has recently been formally documented[12,13]. The transportation of infected pigs between farms for breeding or re-stocking presented an additional risk of the introduction of ASFV into previously clean farms, as observed in Kiambu District and at the Coast. No bio-security practices were implemented on affected farms and there was no barrier to workers and visitors carrying ASFV into these farms. One farm in Kondele, Kisumu was situated directly adjacent to a slaughter slab allowing free-range pigs to encounter infected blood and tissues at a high frequency. None of the slaughter slabs observed in the study was fenced.

The occurrence of both pig-keeping and ASFV on the Kenyan Coast is new and unexpected development since the majority of the population in this region are Muslims and do not consume pork. However, demand from the beach hotels, to whom most of the animals are sold, is high and pig-rearing is increasing in the coastal belt. Furthermore, pig keepers reported that animals were sometimes sold to commercial shipping lines. Given the recent history of the ship-mediated introduction of genotype II ASFV viruses to the Black Sea Region of Georgia, this constitutes a risk factor for transmission from Africa to other regions of the world. The

large ASFV DNA virus persists for several months, in both pig-derived products and the environment[14,15]. Pigs that suffer chronic infections have been shown to transmit ASF for one to two months after initial infection and viable ASFV may be recovered from tissues of infected pigs for approximately 6 months[16]. Genotyping of the outbreak viruses indicates a close genetic relationship between the outbreaks in Central and Coastal Kenya with a virus circulating in endemic Western regions. The timescale of the outbreaks is consistent with the spread of a conserved virus from the Kenya-Uganda border region during 2010-2011, to Central Kenya (Kiambu and Thika) and subsequently to the Coastal District (Kisauni). The Kisauni ASF outbreaks represent the first time that the ASFV has been reported on the Kenya Coast. The fact that the index farm in Kisauni had recently acquired six pigs from Thika in Central Kenya is consistent with the scenario of importation of the virus from endemic regions of Kenya. Currently, the total pig population of the coastal region is estimated at only 400 by the Mombasa DVO, compared to the total Kenyan population of about 440,000. However, given the high level of virulence of the genotype IX virus, the situation should be closely monitored by veterinary authorities to ensure that there is no onward transmission of genotype IX from East Africa to other parts of the world. The reality of this risk is amply demonstrated by the p72 genotype II virus that was inadvertently transported in a pork product from South-East Africa to the Caucasus in 2007[14-16] and subsequently to China, Vietnam and Mongolia[17,18]. Given the risk of future transmission of genotype IX ASFV, we recommend increased surveillance of pigs on the Kenya Coast by the government veterinary department for potential outbreaks of ASF.

CONCLUSION

The study highlighted for the first time the keeping of domestic pigs and associated with this, the incidence of African Swine Fever caused by the highly virulent East African genotype IX, likely resulting from the importation of ASFV infected pigs from central Kenya, occurs at the Kenya Coast. Given that many of these animals are sold to shipping lines and tourist hotels in the adjacent major port of Mombasa, there is a significant risk of transmission of this virus to countries outside Africa in future.

SIGNIFICANCE STATEMENT

The study documents the first outbreak of African Swine Fever on the Kenya Coast as a result of infection of domestic pigs with ASFV genotype IX through the introduction of infected pigs from Central Kenya. We highlight the potential risk of transmission of this highly virulent ASFV genotype to countries outside Africa for the benefit of the ASFV community, including both scientists working on these diseases and regulatory authorities concerned with the control of transboundary diseases of livestock.

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