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RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

Genotyping of African swine fever virus from a 2009 outbreak in Tanzania

Gerald Misinzo[1], Fatuma Jumapili[1], Makolo Ludosha[1], Eliakunda Mafie[1], Justinian Silialis[2], Rowenya Mushi[2], Wesley Viaene[3] and Jan Van Doorsselaere[3]

[1]Department of Veterinary Microbiology and Parasitology, Faculty of Veterinary Medicine, Sokoine University of Agriculture, Morogoro, Tanzania; [2]Veterinary Investigation Center, Arusha, Tanzania; [3]Higher Institute for Nursing and Biotechnology, Catholic University College, Roeselare, Belgium

Abstract

African swine fever is a highly contagious hemorrhagic disease of domestic pigs caused by African swine fever virus (ASFV) belonging to the *Asfarviridae* family. Twenty two ASFV genotypes (I - XXII) have been identified based on partial sequencing of the C-terminus of the major capsid protein p72 encoded by the *B646L* gene. Previously characterized, Tanzanian ASFV strains belong to genotypes X, XV and XVI. This study aimed at confirming and genotyping ASFV that caused an ASF outbreak in Longido district, Tanzania in the year 2009. Partial ASFV *B646L* (p72) gene amplification and sequencing was performed on lung, spleen, liver, heart and serum of a single pig that suffered from a hemorrhagic disease during the 2009 outbreak. Phylogenetic analysis of the Tanzanian 2009 ASFV strain grouped the virus within p72 genotype X and was 100% identical to some previously reported Kenyan ASFV strains, indicating cross-boundary distribution of this ASFV strain.

Keywords: African swine fever outbreaks; Asfarviridae; domestic pigs; genotyping; phylogenetic analysis

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Introduction

African swine fever virus (ASFV) belonging to the family *Asfarviridae* is an enveloped virus with a linear double stranded DNA genome (Dixon et al., 2005). ASFV causes a highly contagious and lethal haemorrhagic African swine fever (ASF) disease of domestic pigs. Since 2000, sporadic ASF outbreaks in Tanzania have been reported in Dar es Salaam and Kyela in 2001, Arusha in 2003, Kasulu, Kigoma and Kibondo in 2004, Mwanza in 2005 and Turiani, Morogoro and Dar es Salaam in 2008 (Lubisi et al., 2005; Wambura et al., 2006; Misinzo et al., 2011).

Genotyping is used to describe the heterogeneity and epidemiological links of ASFV (Bastos et al., 2003; Boshoff et al., 2007; Gallardo et al., 2009). Twenty two ASFV genotypes (I-XXII) have been identified based on partial sequencing of the C-terminus of the major capsid protein p72 encoded by the *B646L* gene (Bastos et al., 2003; Boshoff et al., 2007). Previous characterized Tanzanian ASFV strains belong to genotypes X, XV and XVI (Lubisi et al., 2005; Wambura et al., 2006; Misinzo et al., 2011).

In 2009, an outbreak of a haemorrhagic and fatal disease affecting domestic pigs suspected to be ASF occurred in Longido district in northern Tanzania. The aim of this study was to (i) confirm the ASF outbreak of 2009 in Tanzania by performing diagnostic polymerase chain reaction (PCR), (ii) genotype ASFV strains by sequencing the C3'- end of the *B646L* gene encoding the C-terminus of the major capsid protein p72 and (iii) determine the relationship of the 2009 strains with previous Tanzanian isolates.

Materials and Methods

Source and collection of samples

An outbreak of a haemorrhagic disease associated with high domestic pig mortalities was reported in 2009 to the Veterinary Investigation Center in Arusha. The

Corresponding author: Gerald Misinzo, Department of Veterinary Microbiology and Parasitology, Faculty of Veterinary Medicine, Sokoine University of Agriculture, Morogoro, Tanzania

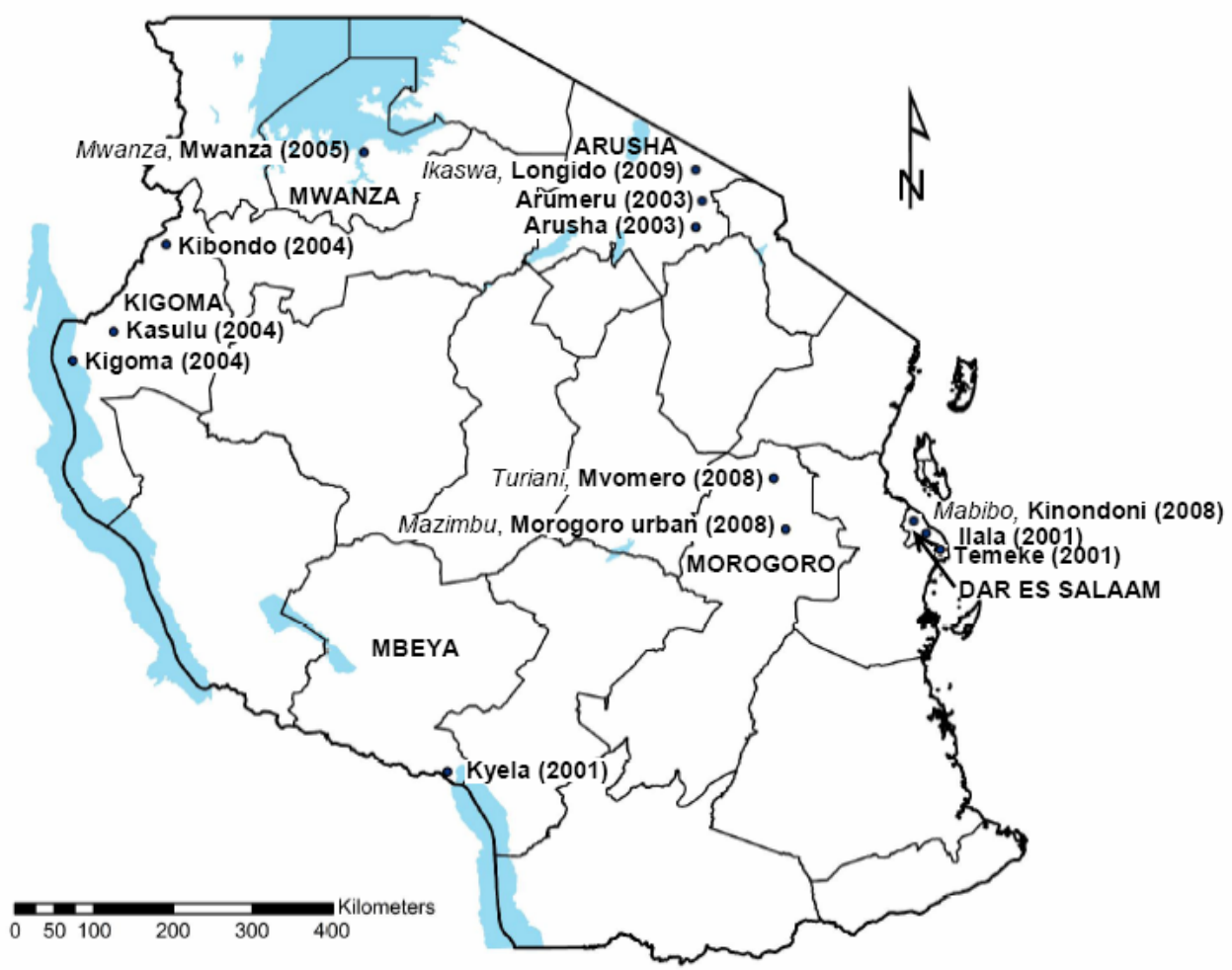

Fig. 1: Map of Tanzania showing the regions (indicated by capital letters) where ASF outbreaks have occurred in Tanzania since 2000, including the 2009 outbreak in Ikaswa, Longido, Arusha. Locations of ASF outbreaks (where available) are indicated in italics while districts are indicated with bold small letters. Adapted from Misinzo et al. 2011.

disease started in Ikaswa village, Longido district of Arusha region in northern Tanzania (Fig. 1). Lung, spleen, liver, heart and serum were obtained from a clinically ill pig after performing routine postmortem examination. Samples were chilled on ice and transported to the laboratory and split in two portions. One portion was stored in the laboratory at -20 ºC until DNA extraction was performed while the other was archived at -80 ºC.

DNA extraction

Lung, spleen, liver and heart were homogenized and diluted 1 in 10 in RPMI-1640 basal cell culture medium (Invitrogen, Auckland, NZ). Afterwards, DNA was extracted directly from 150 µl homogenized tissue samples and serum using a commercial nucleic acid extraction kit (NucleoSpin, Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. Each extraction yielded 50 µl that was stored at -20 °C until PCR was performed.

PCR amplification

One µl of DNA was used in a single PCR reaction containing a high fidelity *Taq* polymerase (Superscript III Platinum One-Step qRT-PCR System, Invitrogen, Carlsbad, CA) with either PPA1/PPA2 (Aguero et al., 2003) or 72U/p72D (Bastos et al., 2003) to amplify the conserved and variable 3'-end of the *B646L* (p72) gene, respectively. Afterwards, agarose (Nippon Gene Co. Ltd, Toyama, Japan) gel electrophoresis of the PCR products and 100 bp DNA ladder (Promega, Madison, WI, USA) was performed using a Mupid-One electrophoresis unit (Advance Co. Ltd, Tokyo, Japan). Before casting, agarose gels were stained with GelRed nucleic acid stain (Phenix Research Products, Candler, NC, USA).

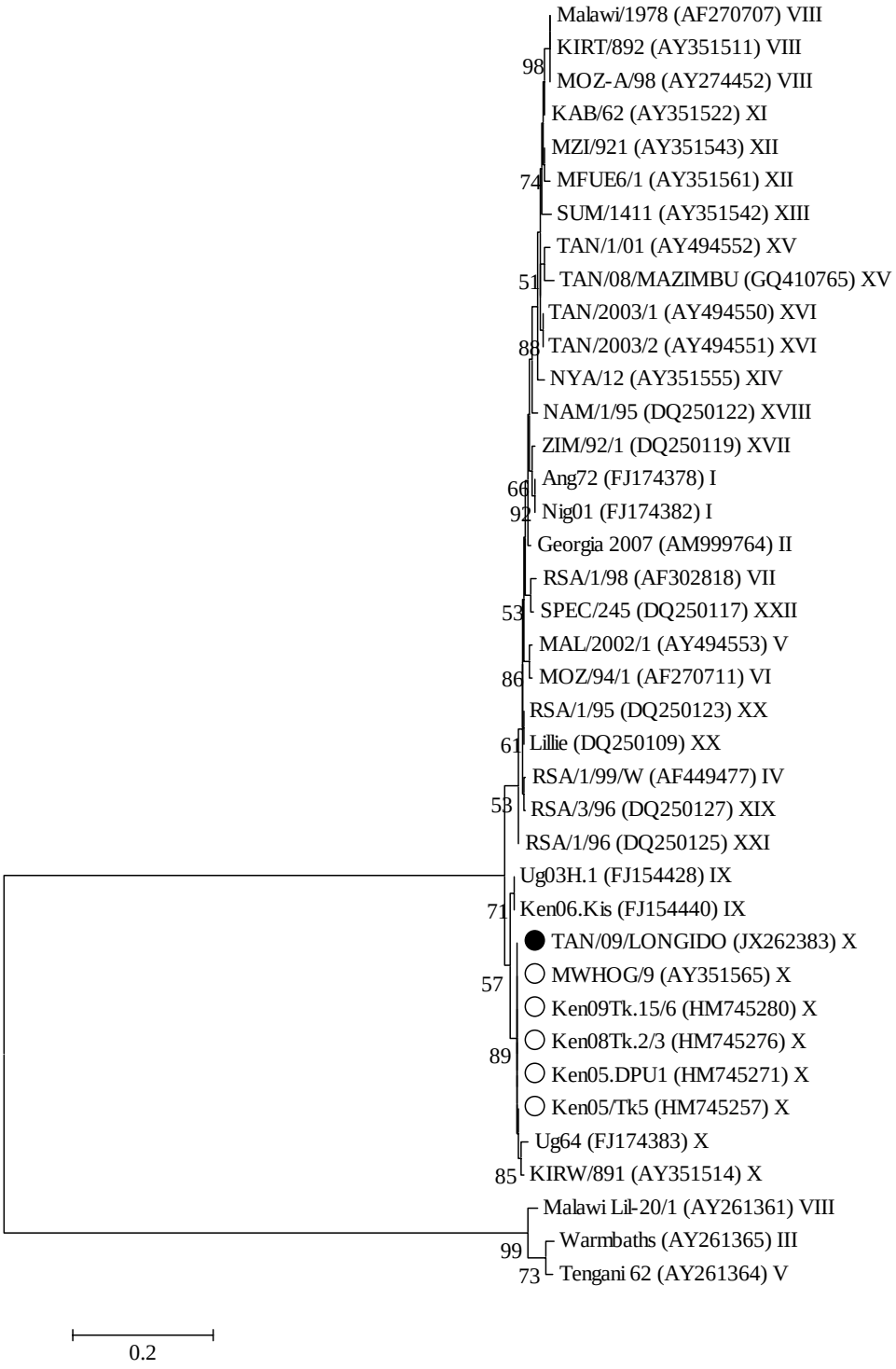

Fig. 2: Neighbour-joining phylogram depicting the relationship of the TAN/09/Longido ASFV isolate with representatives of the 22 genotypes (Boshoff et al. 2007; Gallardo et al. 2009) based on partial *B646L* (p72) gene sequences. ASFV isolates belonging to genotype X previously isolated in Kenya (Lubisi et al., 2005; Gallardo et al., 2011) are marked with a round empty spot (○) while ASFV TAN/09/Longido isolate reported in this study is marked with a round black spot (●). Numbers indicate the bootstrap values (1000 replicates), and only the values above 50% are shown in the figure. The accession number of each ASFV isolate is shown in parenthesis followed by its genotype in capital Roman numeral.

DNA sequencing

PCR fragments were cut from agarose gels and purified using a Qiaquick gel extraction kit (Qiagen Benelux, Venlo, The Netherlands). Purified DNA fragments were used for automated dideoxynucleotide cycle sequencing with a Big Dye Terminator Cycle sequencing kit V1.1 (Applied Biosystem, Foster City, USA). Products from cycle sequencing reaction were purified by ethanol precipitation and separated by capillary electrophoresis on an ABI Prism 310 Genetic Analyser (Applied Biosystem, Foster City, USA).

Phylogenetic analysis

ASFV nucleotide sequences representing the 22 *B646L* (p72) genotypes (Boshoff et al. 2007; Gallardo et al. 2009) were obtained from GenBank database and added to Mega 5 (Tamura et al. 2011). The ASFV *B646L* (p72) nucleotide sequence obtained from this study was also added to Mega 5. Using Mega 5, the *B646L* (p72) nucleotide sequences were aligned using ClustalW followed by phylogenetic reconstruction using neighbour-joining statistical method with 1000 bootstrap replications to test phylogeny.

Results

Post-mortem findings

Post-mortem findings included severe congestion of the skin especially on medial and lateral sides of the pinna, fore limbs above the carpal joint, facial region, scrotum and mammary glands. Other lesions included darkening and enlargement of the spleen and severe hemorrhages of mesenteric lymph nodes.

Confirmation of ASF using PCR

Lung, spleen, liver, heart and serum obtained from a clinically ill pig were tested for the presence of ASFV using diagnostic PCR employing PPA1 and PPA2 primers that target a conserved region of the *B646L* (p72) gene. The lung, spleen and liver were ASFV positive while the heart and serum from the same animal tested negative. PCR amplicons for the positive samples were 257 nucleotides long upon sequencing.

Genotyping of ASFV

The variable part of the *B646L* (p72) gene was amplified and sequenced using p72U/p72D primers. The ASFV strain from Longido obtained in this study is designated as TAN/09/Longido. ASFV partial *B646L* (p72) gene nucleotide sequences from the lung, spleen and liver were 100% identical. The partial *B646L* (p72) gene sequence of TAN/09/Longido obtained in this study has been deposited in Genbank (accession number JX262383). Blast of *B646L* (p72) 2009 Tanzanian ASFV DNA sequences in Genbank showed 100% nucleotide identity with *B646L* (p72) genotype X ASFV isolated from a warthog *Phaecochoerus aethiopicus* in 1959 (Lubisi et al. 2005), domestic pigs in 2005 (Gallardo et al. 2011) and *Ornithodorus porcinus porcinus* ticks in 2005, 2008 and 2009 (Gallardo et al., 2011) in Kenya.

In order to determine the genetic relationship of TAN/09/Longido with other ASFV representing the 22 *B646L* (p72) genotypes and isolates with Kenyan isolates showing 100% nucleotide identity, phylogenetic trees were constructed with neighbour-joining method using partial *B646L* (p72) sequences. TAN/09/Longido ASFV strains clustered together with genotype X isolates, including the Kenyan isolates that showed 100% nucleotide identity with TAN/09/ Longido (Fig. 2).

Discussion

In this study, an outbreak of a hemorrhagic highly fatal disease that occurred in the year 2009 in Longido district of Arusha region in Tanzania was investigated. Based on post-mortem findings, diagnostic PCR and partial sequencing of the *B646L* (p72) gene performed in this study, the disease was confirmed to be ASF. The 2009 ASF outbreak in Tanzania follows other previously reported outbreaks in 2001, 2003, 2004, 2005 and 2008 (Lubisi et al., 2005; Wambura et al. 2006; Misinzo et al., 2011). Factors that lead to the sporadic emergence of ASF in Tanzania need to be investigated in order to prevent the disease from becoming endemic in the country.

The organs obtained from the pig with ASF have been archived for future virus isolation, testing of the haemadsorption properties of the virus and whole genome sequencing. The results presented in this study show that the lung, spleen and liver were ASFV positive while the heart and serum from the same animal tested negative. The positive results of lung, spleen and liver could be due to the presence of large numbers of macrophages within these organs. Macrophages are target cells of ASFV replication (Tulman et al., 2009).

Partial sequencing of the *B646L* (p72) gene of the TAN/09/Longido isolate that caused ASF in Longido showed that the virus isolate was 100% identical to other previously reported Kenyan ASFV isolated from a warthog *P. aethiopicus* in 1959 (Lubisi et al., 2005), domestic pigs in 2005 (Gallardo et al., 2011) and *O. porcinus porcinus* ticks in 2005, 2008 and 2009 (Gallardo et al., 2011). The high nucleotide identity between TAN/09/Longido and the Kenyan isolates indicates cross-boundary distribution of this ASFV isolate. Longido is located very close to the Tanzanian-Kenyan border, and is geographically in the vicinity of

Kapiti, where the 2009 Kenyan ASFV tick isolates were described (Gallardo et al., 2011). There is a need to perform a survey of ASFV in ticks and warthogs in Tanzania in order to understand the ASFV strains circulating within the country. This knowledge may help understanding the epidemiology and the control of ASF in the region.

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