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RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

Neisseria denitrificans restriction endonuclease digestion distinguishes African swine fever viruses of eastern from southern African origin circulating in Tanzania

Gerald Misinzo[1]*, Francisca B. Gwandu[1], Emiliana Z. Biseko[1], Neema B. Kulaya[1], Lawrence Mdimi[1], David E. Kwavi[1], Christopher D. Sikombe[1], Mariam Makange[1] and Michael J. Madege[2]

[1]Department of Veterinary Microbiology and Parasitology, Faculty of Veterinary Medicine, Sokoine University of Agriculture, Morogoro, Tanzania and [2]Zonal Veterinary Centre, Directorate of Veterinary Services, Ministry of Livestock and Fisheries Development, Arusha, Tanzania

Abstract

African swine fever (ASF) is a highly fatal hemorrhagic viral disease of domestic pigs caused by ASF virus (ASFV). Partial amplification of the *B646L* (p72) gene using PPA1/2 primers followed by *Neisseria denitrificans* (NdeI) restriction digestion of the PCR products differentiates eastern from southern African ASFV. ASFV circulating between 2010 and 2013 in eastern and southern Tanzania are of southern African origin. A suspected ASF outbreak was reported in northern Tanzania in 2013. Whether the ASFV circulating in eastern and southern Tanzania had spread to northern Tanzania in 2013 has not been determined. The aim of this study was to determine whether the ASFV circulating in eastern and southern Tanzania have genetic similarity to ASFV circulating in northern Tanzania. Partial amplification of the *B646L* (p72) gene using PPA1/2 primers was performed on ASFV circulating in eastern, southern and northern Tanzania followed by NdeI restriction digestion of the PCR products and nucleotide sequencing. PPA1/2 PCR products of ASFV circulating in eastern and southern Tanzania in 2013 were not cleaved by NdeI restriction endonuclease while those circulating in northern Tanzania were cleaved by NdeI restriction endonuclease. Nucleotide sequencing of PPA1/2 PCR products confirmed the absence of NdeI restriction site in ASFV circulating in eastern and southern Tanzania and its presence in ASFV circulating in northern Tanzania. ASFV in eastern and southern Tanzania is of southern Africa origin while ASFV in northern Tanzania is of eastern Africa origin indicating the co-circulation of two different strains of ASFV in Tanzania in 2013. NdeI restriction digestion can be used to distinguish between the two 2013 ASFV strains without the need for nucleotide sequencing.

Keywords: African swine fever; African swine fever virus; *Asfarviridae*; NdeI restriction endonuclease

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Introduction

African swine fever (ASF) is a viral hemorrhagic disease of domestic pigs that can cause mortalities of up to 100% (Penrith, 2009). ASF is caused by ASF virus (ASFV), a large enveloped double stranded DNA arbovirus (Dixon et al., 2005). In eastern and southern Africa, ASFV is maintained in a sylvatic cycle between warthogs and soft argasid ticks of the *Ornithodoros* spp (Jori et al., 2013). Infected *Ornithodoros* ticks that reside in burrows retain the virus for long periods and transmit ASFV to young warthogs leading to a transient viraemia (Penrith et al., 2004; Costard et al., 2009). *Ornithodoros* ticks get infected with ASFV after biting

***Corresponding author:** Gerald Misinzo, Department of Veterinary Microbiology and Parasitology, Faculty of Veterinary Medicine, Sokoine University of Agriculture, P.O. Box 3019, Morogoro, Tanzania. Tel: +255767058805. E-mail: gmisinzo@gmail.com; gmisinzo@suanet.ac.tz

viraemic warthogs. ASFV transmission from infected warthogs and/or ticks to domestic pigs occur through a tick bite, feeding contaminated warthog carcasses to domestic pigs or contact of domestic pigs with warthog feces (Costard et al., 2009). Once a domestic pig is infected with ASFV, the virus spreads within the domestic cycle between domestic pigs leading to an outbreak (Penrith et al., 2004).

Maintenance of ASFV within the sylvatic cycle and its occasional transmission between the sylvatic and domestic cycle causes ASFV genetic variation (Jori et al., 2013). The genetic variation between ASFV is mainly analyzed by nucleotide sequencing of the variable 3′-end of the *B646L* gene encoding the major capsid protein p72 (Bastos et al., 2003; Lubisi et al., 2005; Nix et al., 2006; Boshoff et al., 2007). To date, 22 ASFV genotypes have been identified in eastern and southern Africa, based on analysis of nucleotide sequence of the *B646L* (p72) gene. In addition, partial amplification of the *B646L* (p72) gene using PPA1 and PPA2 primers followed by *Neisseria denitrificans* (NdeI) restriction digestion of the PCR products differentiates eastern from southern ASFV (Gallardo et al., 2011). Eastern ASFV have the NdeI restriction site CA^TATG while this site is lacking in southern African ASFV (Gallardo et al., 2011).

Since 2000, sporadic ASF outbreaks in Tanzania have been reported in Turiani, Mazimbu and Temeke in 2008, Longido in 2009, Kyela, Ludewa, Chunya, Ileje, Mbarali, Rungwe, Tukuyu, Temeke, Kilolo, Iringa, Sumbawanga, Ifakara and Kilosa between 2010 and 2013 (Wambura et al., 2006; Misinzo et al., 2011; 2012a; 2012b). The 2010 to 2013 ASF outbreaks were confined within eastern and southern Tanzania and the ASFV circulating in this zone have been genetically characterized into genotype II (Misinzo et al., 2012b; Sikombe, 2013). Genotype II ASFV is prevalent in southern African countries including Mozambique, Malawi and Madagascar and has spread into Tanzania and the Caucasus region (Rowlands et al., 2008; Misinzo et al., 2012b; Uttenthal et al., 2013). While ASF outbreak was ongoing in eastern and southern Tanzania in 2013, another suspected ASF outbreak was reported in northern Tanzania in Arusha, Moshi, Rombo and Machame. Whether the ASFV circulating in eastern and southern Tanzania between 2010 and 2013 has spread to cause an outbreak in northern Tanzania in 2013 has not been determined. The aim of this study was to determine whether the ASFV circulating in eastern and southern Tanzania have any genetic similarity to ASFV that caused a new ASF outbreak in northern Tanzania based on partial amplification of the *B646L* (p72) gene using PPA1 and PPA2 primers followed by NdeI restriction digestion of the PCR products and nucleotide sequencing.

Materials and Methods

Study area and sampling

Archived spleen and lymph node from domestic pigs that died from ASF in 2008 at Mazimbu, Mabibo and Turiani, 2009 at Longido, 2010 at Kyela and Tukuyu, 2011 at Temeke, Ludewa and Chunya, and 2012 at Ifakara (Fig. 1) collected from previous studies were used (Misinzo et al., 2011, 2012a&b). Spleen and lymph node were collected in the present study from domestic pigs that died of a hemorrhagic disease in Iringa municipality in March 2013 and in Arusha, Machame, Moshi and Rombo between May and August 2013 (Fig. 1).

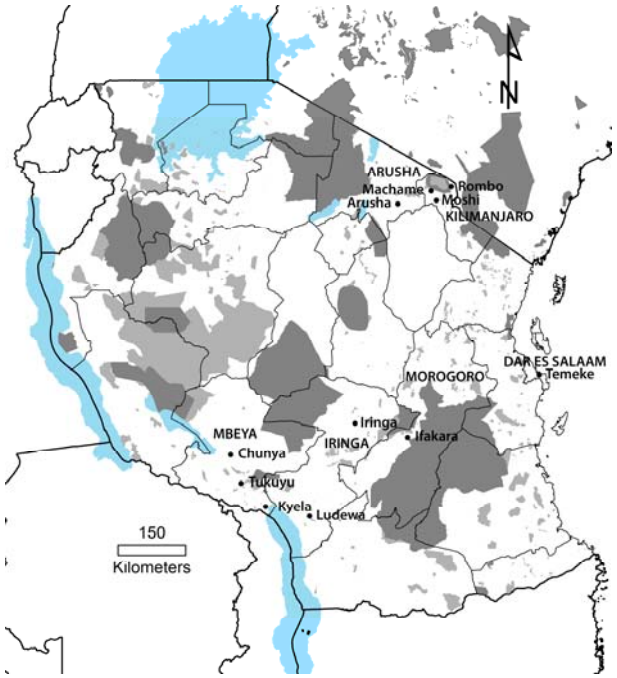

Fig. 1: Map of Tanzania showing the sampling locations for African swine fever virus (ASFV) between 2010 and 2013. (Regions from which samples were obtained are indicated in all caps, lakes in light blue, national parks and game reserves in dark grey and forests in light gray).

DNA extraction

Spleen and lymph node pieces weighing approximately 1 g obtained from the same animal were pooled into a petri dish containing phosphate-buffered saline and macerated using sterile scalpel blades. Afterwards, the tissue homogenate was centrifuged and DNA was extracted from the supernatant using QIAamp® kits (Qiagen, Hilden, Germany) following manufacturer's instruction. Obtained DNA was frozen at -20 ºC until use.

DNA amplification

Partial amplification of the ASFV *B646L* (p72) gene was performed using PPA1 and PPA2 primers as previously described (Aguero et al., 2003). A DNA polymerase present in an AccuPower® PCR premix (Bioneer, Daejeon, Republic of Korea) was used for DNA amplification using a GeneAmp® PCR systems 9700 (Applied Biosystems, Foster City, CA). Afterwards, PCR

products were electrophoresed in a 2% agarose gel mixed with GelRed® nucleic acid stain (Phenix Research Products, Candler, NC) before visualization and imaging using a BioDoc-It® imaging system (UVP, Upland, CA).

Restriction digestion of PCR products

PCR products obtained after partial amplification of ASFV *B646L* (p72) gene using PPA1 and PPA2 primers were incubated with FastDigest® NdeI restriction endonuclease (Thermo Scientific, Pittsburgh, PA) for 1 hour at 37ºC in a GeneAmp® PCR systems 9700 (Applied Biosystems, Foster City, CA). Afterwards, digested PCR products were electrophoresed in a 2% agarose gel mixed with GelRed® nucleic acid stain (Phenix Research Products, Candler, NC) before visualization and imaging using a BioDoc-It® imaging system (UVP, Upland, CA).

Nucleotide sequencing of PCR products

PCR products obtained after partial amplification of ASFV *B646L* (p72) gene using PPA1 and PPA2 primers were purified from agarose gels using a NucleoSpin® gel and PCR clean-up kit (Macherey-Nagel, Düren, Germany). The gel purified PCR fragments were sequenced using a dideoxynucleotide cycle sequencing reaction performed using PPA1 or PPA2 primers using Big Dye® Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems, Foster City, CA). Sequencing PCR products were purified by ethanol precipitation and separated on a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA). Obtained nucleotide sequences were submitted to GenBank and provided with accession numbers.

Alignment of nucleotide sequences

Nucleotide sequences were checked for correctness using Sequence Scanner v1.0 software (Applied Biosystems, Foster City, CA) and only good quality chromatograms were selected. The nucleotide sequence obtained using PPA1 primers and the reverse complement nucleotide sequence obtained using PPA2 primers from each of the samples were merged to obtain a single sequence delimited by PPA1 and PPA2 primers sequences. The nucleotide sequences from each of the samples were aligned using Molecular Evolutionary Genetics Analysis (MEGA) version 5 (Tamura et al., 2011) in order to observe the presence or absence of NdeI restriction site in ASFV sequences.

Results

Postmortem findings

Postmortem examination was performed in dead pigs in Iringa, Machame, Moshi, Rombo and Arusha. Postmortem findings in examined pigs included congestion of the skin, splenomegaly and hemorrhagic lymph nodes especially the mesenteric and gastrohepatic lymph nodes (Fig. 2).

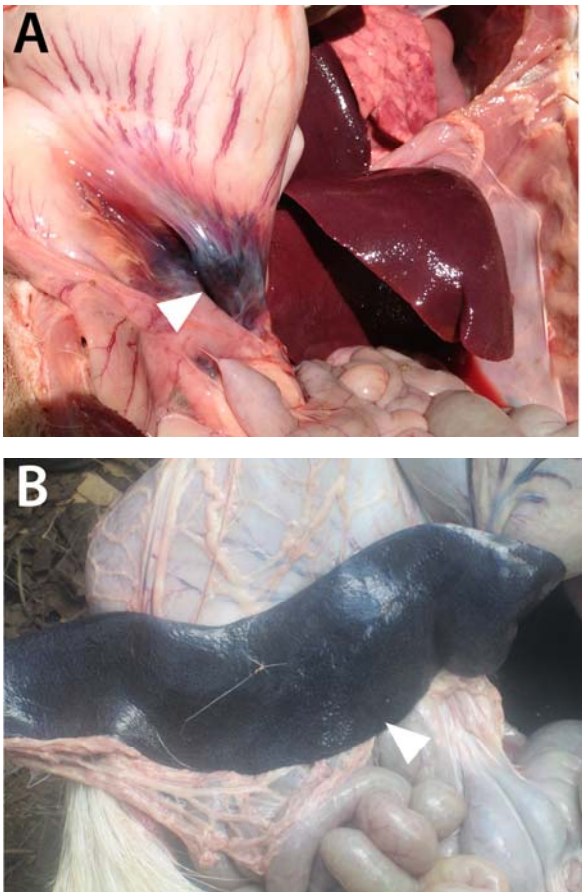

Fig. 2: Postmortem finding in pigs with African swine fever. (A) A hemorrhagic gastrohepatic lymph node (arrow head) and (B) enlargement of the spleen (arrow head) observed in pigs that had died from African swine fever.

Confirmatory diagnosis of ASF using PCR

Confirmation of ASF in collected samples was performed by PCR using PPA1 and PPA2 primers. A PCR band of 257 base pairs was observed after performing agarose gel electrophoresis in samples obtained from animals that were positive for ASFV. A single band from a positive animal from Mazimbu, Mabibo, Turiani, Longido, Kyela, Tukuyu, Temeke, Ludewa, Chunya, Ifakara, Iringa, Arusha, Machame, Moshi and Rombo were selected for NdeI restriction digestion and nucleotide sequencing.

NdeI restriction digestion of PCR products

PCR products from samples positive for ASFV were incubated with NdeI restriction endonuclease (Fig. 3). PCR products from samples obtained from Mazimbu, Mabibo, Turiani, Longido, Arusha, Machame, Moshi and Rombo were digested with NdeI restriction endonuclease while PCR products from samples obtained from Kyela, Tukuyu, Temeke, Ludewa, Chunya, Ifakara, and Iringa could not be digested (Fig. 3). PCR products that were

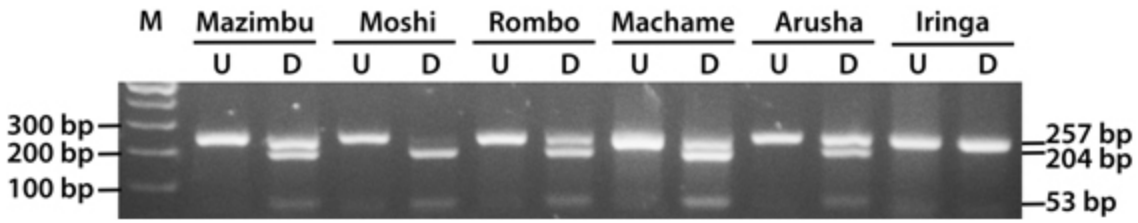

Fig. 3: Agarose gel electrophoresis of African swine fever virus (ASFV) PPA1/2 PCR products before and after NdeI restriction digestion. ASFV from Mazimbu collected in 2008 and those collected in 2013 from locations in northern Tanzania including Moshi, Rombo, Machame and Arusha and from Iringa in southern Tanzania were amplified using PPA1/2 primers. PCR products were either digested (D) or not digested (U) using NdeI restriction endonuclease. Undigested PCR products have the molecular weight of 257 bp that resulted into two fragments of 204 and 53 bp after digestion. In all but one case (Moshi), the original 257 bp PCR product is also visualized in digested PCR fragments due to incomplete cleavege by NdeI restriction endonuclease.

digested with NdeI restriction endonuclease produced two fragments of unequal size indicating the presence of a single restriction site within the PCR products.

Nucleotide sequencing of PCR products

To confirm the presence or absence of NdeI restriction site in ASFV-positive samples, PCR products before endonuclease digestion were sequenced. The ASFV strains were named according to sampling location and the year of collection (Table 1). The nucleotide sequences were submitted to GenBank and accession numbers provided (Table 1). The nucleotide sequences were aligned to view presence or absence of the NdeI restriction site (CA^TATG) in the ASFV strains. ASFV strains with NdeI restriction sites were digested with NdeI restriction endonuclease while ASFV strains lacking the NdeI restriction site (containing CATTTG instead of CA^TATG) were not digested with NdeI restriction endonuclease (Table 1).

Discussion

ASFV belonging to genotypes II, IX, X, XV and XVI, based on nucleotide sequence analysis of the *B646L* (p72) gene, are responsible for causing several ASF outbreaks in Tanzania between 2001 and 2013 (Wambura et al., 2006; Misinzo et al., 2011; 2012a&b). Of these ASFV genotypes, genotypes IX, X, XV and XVI are of eastern African origin while genotype II is of southern African origin (Lubisi et al., 2005). The incursion of genotype II ASFV into Tanzania in 2010 is believed to be through Kyela, a city at the Tanzania-Malawi border (Misinzo et al., 2012b). From Kyela, ASFV spread to regions in southern and eastern Tanzania including Mbeya, Iringa, Rukwa, Morogoro and Dar es Salaam (Misinzo et al., 2012b). The virus persistently circulated in eastern and southern Tanzania and the results obtained from this study confirm the circulation of ASFV in Iringa in the year 2013. While ASF outbreak in eastern and southern Tanzania was active, another ASF outbreak was reported in northern Tanzania in Kilimanjaro and Arusha regions. Due to the uncontrolled movement of pig and pig products within Tanzania, ASFV circulating in the eastern and southern Tanzania might have spread to northern Tanzania. In the present study, the genetic similarity of ASFV circulating in eastern and southern Tanzania to those circulating in northern Tanzania was studied using partial amplification of the *B646L* (p72) gene using PPA1 and PPA2 primers followed by NdeI restriction digestion of the PCR products and nucleotide sequencing. NdeI restriction digestion of ASFV PPA1/2 PCR products has been shown to distinguish eastern from southern African ASFV (Gallardo et al., 2011). Our results confirm that ASFV circulating in eastern and southern Tanzania are of southern Africa origin because they lack the NdeI restriction site while ASFV circulating in northern Tanzania are of eastern Africa origin as they have the NdeI restriction site. Furthermore, two different ASFV were circulating in Tanzania in 2013. In addition, NdeI restriction digestion of other ASFV showed that the 2008 and 2009 ASF outbreaks were caused by ASFV of eastern African origin.

In the present study, results from NdeI restriction digestion were confirmed by nucleotide sequencing of the PPA1/2 PCR products. PCR products that could be digested with NdeI restriction endonuclease possess the CA^TATG restriction site while ASFV that could not be digested with NdeI had CATTTG nucleotide sequence. The determination of the presence or absence of the NdeI restriction site in PPA1/2 PCR products using restriction digestion followed by agarose gel electrophoresis can be used in determining the origin of ASFV in the absence of nucleotide sequencing facilities as previously shown by Gallardo et al. (2011). In addition, when both ASFV of eastern and southern Africa origin are co-circulating, NdeI restriction digestion can be used in determining the type of ASFV in suspected samples. In conclusion, *NdeI* restriction endonuclease digestion distinguishes African swine fever viruses of eastern from those of southern African origin circulating in Tanzania.

Table 1: African swine fever viruses that have caused outbreaks between 2008 and 2013 in different locations within eastern, northern and southern Tanzania. The nucleotide sequence of *B646L* (p72) gene amplified using PPA1/2 primers at position 50 to 60 containing the NdeI restriction site CA^TATG (indicated in bold) are shown.

ASFV Strain	Accession number	Location	Zone	Year	Nucleotide sequence	NdeI restriction site
TAN/08/Mazimbu	KJ028027	Mazimbu	Eastern	2008	A**CATATG**GTG	Present
TAN/08/Mabibo	KJ028028	Mabibo	Eastern	2008	A**CATATG**GTG	Present
TAN/08/Turiani	KJ028029	Turiani	Eastern	2008	A**CATATG**GTG	Present
TAN/09/Longido	KJ028030	Longido	Northern	2009	A**CATATG**GTG	Present
TAN/10/Kyela	KJ028031	Kyela	Southern	2010	ACATTTGGTG	Absent
TAN/10/Tukuyu	KJ028032	Tukuyu	Southern	2010	ACATTTGGTG	Absent
TAN/11/Temeke	KJ028033	Temeke	Southern	2011	ACATTTGGTG	Absent
TAN/11/Ludewa	KJ028034	Ludewa	Southern	2011	ACATTTGGTG	Absent
TAN/11/Chunya	KJ028035	Chunya	Southern	2011	ACATTTGGTG	Absent
TAN/12/Ifakara	KJ028036	Ifakara	Eastern	2012	ACATTTGGTG	Absent
TAN/13/Iringa	KJ028037	Iringa	Southern	2013	ACATTTGGTG	Absent
TAN/13/Moshi	KF706352	Moshi	Northern	2013	A**CATATG**GTG	Present
TAN/13/Rombo	KF706353	Rombo	Northern	2013	A**CATATG**GTG	Present
TAN/13/Machame	KF706354	Machame	Northern	2013	A**CATATG**GTG	Present
TAN/13/Arusha	KF706355	Arusha	Northern	2013	A**CATATG**GTG	Present

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