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https://dx.doi.org/10.4314/nvj.v43i3.3 **ORIGINAL ARTICLE**

DETECTION OF AFRICAN SWINE FEVER VIRUS GENOTYPE II IN DOMESTIC PIGS DURING A HEMORRHAGIC FEVER OUTBREAK IN OGUN STATE, NIGERIA

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SUMMARY

African swine fever outbreaks are ongoing in Europe and other parts of the world. In Nigeria, genotype I has been previously described, while genotype II was only reported recently. This study investigated a major disease outbreak in one of the largest pig farms in Nigeria, detected the etiology and determined the evolutionary history of African swine fever virus. Viral DNA was extracted and used to confirm the circulation of the African swine fever virus (ASFV) by Polymerase Chain Reaction, followed by the sequencing of the amplified products by Sanger's method. Phylogenetic analysis showed that all the ASFV detected in this study belong to genotype II and clustered with reference strains from the Euro-Asian region and Tanzania in East Africa. This being the second report of ASFV genotype II in quick succession from Nigeria, suggests there may have been an unrecognized indigenous circulation or re-emergence of previously contained, or introduction of ASFV genotype II into the country. This reinforces the need for active surveillance and biosecurity measures to prevent and control transmission.

Keywords: African swine fever virus, genotype II, domestic pig, outbreak, PCR, Nigeria

Running Title: Detection of ASFV in Ogun State.

INTRODUCTION

African Swine Fever (ASF) is a significant transboundary disease of global concern in animals (Beltran-Alcrudo *et al.,* 2019). Since the first description in early 1920 in Kenya, it has continued to spread and is now endemic in many other parts of the world (Montgomery, 1921) causing considerable economic losses in piggery production. Beyond the economic loss, outbreaks of ASF pose a significant challenge to food security with possible public health consequences. Presently, there are increasing reports of outbreaks in areas which have not previously recorded cases (FAO, 2021). African Swine Fever virus (ASFV) is the sole member of the family *Asfaviridae* and the only known arthropod-borne DNA virus. The ASF virion is between 260-300nm in diameter with a linear double stranded DNA genome contained inside an enveloped icosahedral capsid. The genetic material varies in length ranging from 170 to 193 kb and contains between 151 and 167 ORFs encoding about 100 polyproteins and 54 structural proteins. (Bastos *et al.,* 2003). The virus remains endemic in sub-Saharan Africa causing a very contagious hemorrhagic disease in the swine population. Mortality rates in affected domestic pigs can be variable in some cases, may approach up to 100% (Magadla *et al.,* 2016). Although maintenance and spread of ASF vary significantly between countries, it is known that contact with infected pigs, their food, contaminated materials, or vectors (Ornithodoros ticks) have facilitated the spread across most parts of Africa with the introduction into mainland Europe (Cwynar *et al.,* 2019). Presently, ASFV is classified into 24 genotypes (I to XXIV) based on partial sequencing of the B646L/p72 gene which encodes the major capsid protein. (Bastos *et al.,* 2003). Other viral proteins such as CVR and p54 have also been used for classification (Quembo *et al.,* 2018; Gaudreault *et al.,* 2020). Though, all genotypes are circulating in Africa, genotypes I and II have been found in other regions of the world with genotype II causing recent outbreaks in some European and Asian nations (Chang'a *et al.,* 2019; Sauter-Louis *et al.,* 2021). In 2007, there were claims of importation of ASFV II from Africa into Georgia and its continuous spread in Europe and parts of Asia had significant economic impact in swine population in the region (Sauter-Louis *et al.,* 2021). This transboundary spread is facilitated majorly when domestic pigs or their infected products are moved from place to place (Bosch *et al.,* 2016). Currently with no available vaccine or effective treatment option enhanced surveillance and biosecurity measures are effective in ASF control. In Nigeria, the first outbreak caused by ASFV II was reported in Lagos Nigeria in 2020 (Adedeji *et al.,* 2021). In the following year, another outbreak of viral hemorrhagic fever occurred among pigs in the Oke-aro farm settlement in Ogun State, Nigeria. We investigated this outbreak to determine the causal agent implicated.

MATERIALS AND METHODS

Study area

This outbreak investigation was conducted on the 20th of July 2021 after a report of sudden death resulting from hemorrhagic fever at Oke-aro farm settlement was made to the Veterinary Department of the Ministry of Agriculture Ogun State, Nigeria. Oke-Aro piggery, though located in Ogun state was established 2 decades ago by the Lagos State Ministry of Agriculture with support from the

National Directorate of Employment. The farm is the largest piggery in Africa with over 5000 pens. It seats on over 30 hectares of land located in Giwa/ Oke-Aro

Sample collection and processing

Specimen collected included blood (5ml), spleen and liver of five dead pigs from different units of the farm settlement during the outbreak. Pig 1 (adult mixed breed female), Pig 2 (adult mixed breed female), pig 3 (adult male, Large White breed), Pig 4 (adult mixed-breed female), and Pig 5 (adult male large White breed). The pigs were monitored and had anorexia, with fever of over 39^{o}C, hemorrhage and they became recumbent and died. The samples were immediately transported at +4^{o}C to the Molecular Virology Unit in the Department of Virology, College of Medicine, the University of Ibadan where laboratory screening was carried out. Spleen and liver samples were macerated using sterile mortar and pestle in preparation for DNA extraction.

DNA Extraction

Viral DNA in blood, spleen, and liver specimen were extracted using the DNA extraction kit Jena Biosciences® (Germany). About 300 µl volume of the lysis buffer was added to tissue homogenate (200 µl) thereafter RNAse (2 µl) inhibitor was added and properly vortexed for 30 seconds. Subsequently, proteinase K (8µl) was added to digest the tissue which was then placed on the heating block set at 60^{o}C for 30 minutes. Following 5 minutes of cooling on the work bench, 300 µl binding buffer was added and centrifuged for another 5mins at 10,000g. The supernatant was washed twice and spin dried. Finally, 50 µl of viral DNA was eluted into a new Eppendorf tube and kept at -4^{o}C for the PCR.

Molecular detection using conventional Polymerase Chain Reaction.

Nucleic acid targets from the VP72 gene were amplified by conventional PCR in a 25µl volume reaction of 20UM sense and antisense primer each, dNTPs (2mM), 5x buffer for PCR, 5X Taq Polymerase (Jena Biosciences®, Germany) and extracted DNA (5 µl). The VP72 gene was amplified with sense primer ASF: 5'-GGCATCAGGAGGAGCTTTTTGTC-3' (position 3-25) and antisense ASR: 5'-TACTGTAACCAGCACAGCTGAACC-3' (position 1936-1912). For amplification of the gene, 5µl of Taq Polymerase (Jena Biosciences™, Germany) was used in the PCR reaction mix. An initial 10 cycles of 94^{o}C for 15s, 65^{o}C for 30s and 68^{o}C for 2.5 min was followed by another 25 cycles with the extension step increased by 10s per cycle on an Applied Biosystems Themal cycler (USA). For each PCR run, 5µl PCR grade water was added as negative control in place of the extracted DNA. Electrophoreses of PCR products was carried out on 1.5% agarose gel stained with gel red and visualized using a transilluminator.

Sequencing and phylogenetic analysis

Amplified PCR product of 1,900bp with an intensity of over 15ng (www.jenabioscience.com/molecularbiology/dna-ladders/linear-scale-dna-ladders/m-203-mid-range-dna-ladder) were used for the Sanger sequencing. Reaction products were analyzed on an automated DNA sequencer ABI Prism 3700 (Applied Biosystems, FC, California). Chromas v 2.6.2 for windows

licensed by Technelysium Pty Ltd on www.technelysium.com.au was used to trim low-quality reads from sequences. The sequences were aligned with references sequences in GenBank. Pairwise and multiple genomic alignments were done with MEGA 7 and ClustalW (1.6) alignment programs (Kumar *et al.*, 2016). The Maximum Composite Likelihood method was used to determine the evolutionary relationship in units of the number per site of base substitutions (Tamura *et al.*, 2004).

RESULTS

Following the PCR run, amplified products were visualized on 20ng intensity gel electrophoresis. (Fig. 1) Sequencing was successfully carried out on the amplicons from each pig and direct submission of five sequence data was immediately made to the GenBank. Accession numbers OK340647-OK340651 were assigned for the nucleotide sequences obtained in this study. A BLAST search revealed the etiology of the ASFV causal agent of this outbreak was classical ASFV belonging to Genotype II.

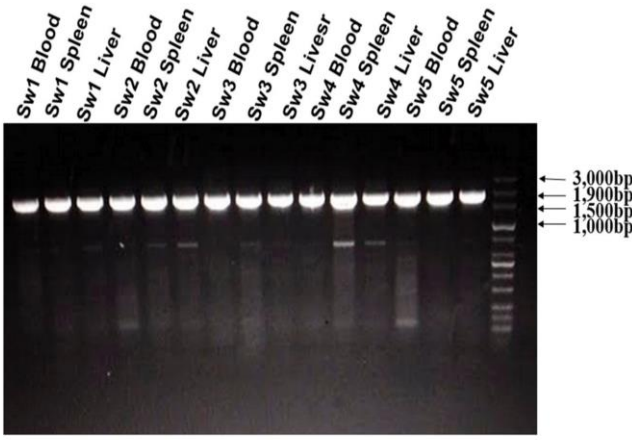

Fig. 1: Gel image of PCR positive ASF samples obtained from swine blood, spleen and liver

DISCUSSION

In the last decade, sub-Saharan Africa has witnessed the transmission of ASF to several countries, with an increase in re-infection in endemic nations and new introduction areas without any history of ASFV transmission. Yet, the ASF status in some countries is unknown because of poor reporting. (Penrith, 2020). The lack of cheap, rapid, and readily available diagnostic reagents makes the diagnosis and extensive epizootiological investigation of ASF difficult in many developing countries including Nigeria (Odemuyiwa *et al.*, 2000). In this present report, we identified the p72 gene by conventional PCR in all the specimens collected from each pig (Fig. 1) whereas previous studies performed DNA extraction on spleen samples (Adedeji *et al.*, 2021). The outbreak was extensive with over 70% mortality (Fig 2), affecting over 14 farms and spreading to other states including Oyo State and Edo State more than 100km far away (Onoja AB, personal communication). Although the spill over outbreaks were not investigated (to the best of our knowledge) as many farmers neither notified the relevant authorities nor their local veterinarian during the outbreak. We propose due to the proximity of the farms the possibility that these outbreaks may be linked with a common origin. Also, this outbreak and the spillovers may have begun in 2019 when genotype II was first isolated in Lagos State which shares a land boundary with Ogun State (Adedeji *et al*., 2021).

Fig. 2: Mortality resulting from ASFV in mixed breeds of pigs in Ogun State. (Dead Pigs)

In this study, phylogenetic analysis of the ASFV sequences suggests that ASFV II (Fig. 3) was responsible for the outbreak of viral hemorrhagic fever in the Oke-aro farm settlement. Using phylogeny, we could not ascertain if our isolates clustered with the reported ASFV II from Nigeria as different segments of the p72/B646L gene were amplified in both studies making sequences from both studies too divergent to be aligned. However, isolates from this study clustered with recent ASFV II causing major outbreaks in many parts of Europe, Asia, and Tanzania (Fig. 3).

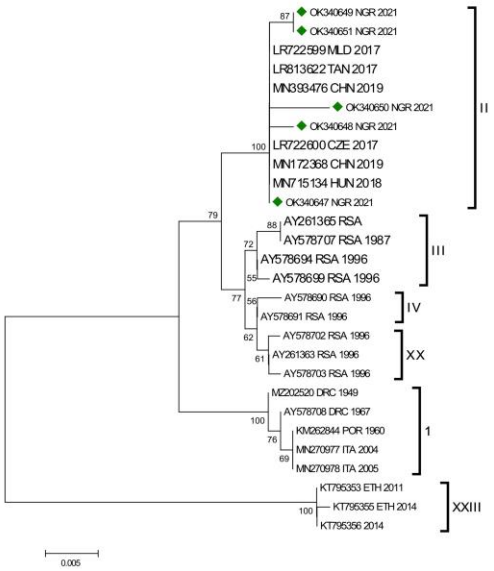

Fig 3 : Phylogenetic tree showing classification

African Swine fever virus genotype II had previously been reported in major outbreak among domestic pig in some East African countries including Rwanda, Zambia, Tanzania, and Malawi (Misinzo *et al*., 2012; Simulundu *et al*., 2017; 2018; Hakizimana *et al*., 2020) but there have not been many reports of this genotype in West Africa. Sequences from previous report from Nigeria and other west African countries clustered around ASTV I (Tizhe *et al.*, 2021) while ASTV II circulated in EuroAsia region (Quembo *et al.*, 2018; Penrith, 2020). Thus, we are providing more evidence of circulation of genotype II in Nigeria. Currently, there are 24 ASFV genotypes among which genotype II is of immense global significance. This is because of the ongoing pig pandemic spanning Eastern Europe to South-east Asia with huge losses of up to 25% of the global pig production (Njau *et al.*, 2021). The active spread of genotype II to several European countries is largely due to the movement of wild suids (Wozniakowski *et al.*, 2016; Nurmoja *et al.*, 2017; Frant *et al.*, 2020; Olsevskis *et al.*, 2020). In 2021 ASF was reported in a wild boar in eastern German (Mecklenburg-Vorpommern) close to another region with an initial report of over 2700 ASF cases in wild animals. (ProMED, 2021). Wild boar encroaching farm settlements have been reported to spread ASF in Germany, causing major loss of pork exports. German pork was banned by China and other pork buyers in 2020 after the cases of ASF was initially found in wild animals. Since wild boars are not found in or near Oke-aro farm settlement, we presume the investigated outbreak was caused by endemic transmission of ASFV among domestic pigs following direct contact between infected or from infected pig products. Nigeria is a leading producer of pigs on the African

continent creating job for many people. Thus, uncontrolled continuous outbreaks will cause enormous economic losses with a significant negative impact on food security. Notably, relevant authorities were not alerted in subsequent spill over outbreaks that followed the one reported in this study. This may suggest a non-existent or sub-optimal link between the local farmers and relevant authorities thus portraying a weak surveillance system. Weak animal health surveillance and biosecurity measures often lead to public health emergencies that undermines the concept of one health in any nation. As part of activities to prevent and control ASF in Nigeria, the Food and Agriculture (FAO) in partnership with the Federal Ministry of Agriculture and Rural Development (FMARD); and USAID project on the Strengthening Global Coordination of Animal Health Emergencies of International Concerns project) has empowered some field epidemiologists with skills and support for ASF management and containment (FAO, 2020). In 2015, researchers that determined the utilization of extension services among pig farmers in the Oke-Aro farm using multistage sampling technique showed that while ASF was a major disease affecting the farmers (Adeyanju and Akinwumi, 2015), yet information received by pig farmers was not relevant to their specific needs suggesting that extension services were needed to package innovations in a manner that met the specific needs of pig farmers. In conclusion, we confirm the circulation of ASFV genotype II in Ogun State, the second successive report in Nigeria and reinforce the presence of the strain in southwestern Nigeria. We provide genomic data for the development of countermeasures such as generic vaccines and therapeutic agents that will target specific viral epitopes. Furthermore, the study reinforces the need for effective biosecurity measures and increase collaboration, coordination, and pool interagency resources to strengthened surveillance to address the threat of ASF in Nigeria.

Authors' contributions

AOB and JAO conceived the idea, JAO and OIA collected the samples; AOB and IMI carried out molecular detection, IMI performed data analysis, AOB and IMI wrote the first draft; all authors read and approved the manuscript before submission.

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