Laboratory diagnosis of a new outbreak of acute African swine fever in smallholder pig farms in Jos, Nigeria, in 2019

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Abstract

African swine fever is a highly contagious fatal infectious disease of pigs with a worldwide occurrence and economic importance. Two adult large white boars from two farms in Jos North, Plateau State-Nigeria kept under intensive management system were diagnosed of African swine fever between the last week of July and the first week of August 2019 at the Veterinary Teaching Hospital University of Jos. In both cases the farmers complained of sudden deaths of pigs. At post mortem examination carcasses grossly showed splenomegaly, hemorrhagic lymphadenitis, and hepatomegaly with severe congestion. The kidneys were enlarged and had generalized petechiae and blood clot in the pelvis.The heart was moderately enlarged.On microscopy, the spleen and lymph nodes, showed severe lymphocytic depletion, haemorrhage and severe haemosiderosis. The liver was severely congested with focal coagulative necrosis of the hepatocytes. The kidneys were severely congested and showed renal tubular necrosis with few tubular protein casts. Tissue samples were confirmed to be positive for ASFV by polymerase chain reaction and phylogenetic analysis revealed that the isolate belonged to genotype-I. Sequences obtained were compared and deposited in the GenBank and were accessioned MN888693 and MN888694. Keywords: African swine fever; laboratory; diagnosis; smallholder farms; pig; Nigeria

Introduction

African swine fever (ASF) is a highly contagious, fatal, economically important, transboundary, viral disease of pigs caused by a DNA virus belonging to the genus Asfavirus and in the family Asfaviridae (Murphy *et al* ., 1999; FAO, 2013; Igbokwe and Maduka, 2018). The first ASF outbreak in Nigeria was reported in 1998 at a farm located in Lagos (FAO, 1998; Odemuyiwa *et al* ., 2000). The disease has spread widely within the country and has become endemic resulting in huge economic losses in the pig industry (Majiyagbe, 1999; Luther, 2001; Costard *et al* ., 2009; Fadiga *et al* ., 2013; Igbokwe and Maduka, 2018). It is a very fatal disease that can cause up to 100% mortality in a naïve pig population (Costard *et al* ., 2009). Poor biosecurity, bad abattoir practices and extensive or free range pig farming systems led to extensive spread of the disease in the country (Owolodun *et al* ., 2010). African swine fever was previously thought to exist only within domestic pig population with humans and other formites potentiating its spread, however, Luther *et al* . (2007a,b) reported the existence of ASF virus in the bush pig (*Potamochoerus porcus*) and warthog

(*Phacochoerus aethiopicus*). It is therefore now a known fact that both domestic and wild pigs are susceptible to ASFV but the suids are hitherto said to be asymptomatic. The soft tick (Ornithodoros moubata) has been established as the primary reservoir of the virus (Luka *et al* ., 2016; Luka*et al* ., 2017a). Areas with high pig-related activities such as marketing, consumption, and farming, have higher prevalence compared with areas with less pig-related activities. Farm-gate buyers, marketing systems and transport of untested pigs within the country has greatly aided the circulation of the virus in the country (Fasina *et al* ., 2010) The disease is characterized by a febrile syndrome, erythema and cyanosis of the skin, anorexia, bloody diarrhea, abortion in pregnant sows, meningitis, interstitial pneumonia, high morbidity and mortality (Fasina *et al.* 2010; Igbokwe and Maduka, 2018). Diagnosis of ASF is based on history, clinical signs, lesions and laboratory confirmation by viral isolation, and molecular characterization. The report represents the investigation of an acute ASF outbreak (July-August, 2019) in pig farms in Jos, Nigeria.

Materials and Methods

History and clinical presentations

The two cases reported in this outbreak were presented to the Veterinary Teaching Hospital, University of Jos, two weeks apart i.e last week of July and first week of August, 2019. The chief complaint from both smallholder farmers was sudden high mortalities in their farms. The first farmer had about sixty pigs of varying ages and six of the pigs died before he was advised to dispose the rest of the pigs for slaughter, disinfect the farm and fallow it before restocking. The second farmer had four adult pigs and all died of the ASF. He was also advised to disinfect the farm and allow it to fallow before restocking. The clinical sings observed in both farms were sudden deaths with pigs in good body conditions, moderate multifocal areas of hyperemia on the skin and weakness in the infected pigs.

Postmortem examination (PM): The PM was carried out on two submitted carcasses and gross post-mortem lesions observed were identified and recorded.

Histopathology: Tissue samples of the affected organs (spleen, lymph nodes, liver, kidney and heart) were collected and fixed in 10% neutral buffered formalin. The samples were dehydrated in graded concentrations of alcohol, cleared in xylene, and impregnated in paraffin wax. Samples were subsequently incubated in vacuum oven at 60°C, embedded in plastic embedding rings, cut into 5 µm sections using a microtome, deparaffinized with xylene, rehydrated in graded concentrations of alcohol, stained with haematoxylin and eosin, and viewed under light microscope at X40 objective as outlined by Baker*et al* . (2000).

Virus isolation: African swine fever virus (ASFV), was recovered from pooled tissues cultured on porcine leucocytes primary cell line and presumptively identified by the haemadsorption test, with pig erythrocytes on the inoculated cell culture plate incubated for one day at 37^{O}C in a CO_2 incubator as outlined by the standard operating procedure for the isolation of ASFV by the European Union Reference Laboratory for ASF (EURL-ASF, 2013).

Polymerase Chain Reaction (PCR) confirmation of ASFV: Genomic DNA was extracted from tissue samples (spleen, lymph node and kidney) using QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's specifications. Lyophilized freeze-dried E70 from the reference laboratory for ASF (CISA-INIA, Madrid, Spain) was used as control of this study. The presence of ASFV DNA was confirmed by the amplification of 278bp fragment of the VP72 gene (OIE, 2018) using the following primer pairs (i) p72 D [GTACTGTAACGCAGCACAG (forward)] and p72-U [GGCACAAGTTCGGACATGT (reverse)] and (ii) CVR-FL1 [TCG GCC TGA AGC TCA TTA G (forward)] and [CVR-FL2 CAG GAA ACT AAT GAT GTT CC (reverse)]. (OIE, 2008). The conditions for the PCR assay were as follows: 19 PCR buffer (50 mM KCl, 10 mM Tris–HCl), 2 mM $MgCl_2$, 0.4 µm concentration of primers, 0.2 mM dinucleotide triphosphates (dNTPs) and 2.5 U Taq polymerase in a total volume of 25 µl. The PCRs were performed in an Applied BioSystems® thermal cycler 9500 (Applied BioSystems, Waltham, MA, USA) withan initial denaturation at 94°C for 15 s, followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 62°C for 15 s and extensionat 72°C for 15 s; and a final extension step at 72degC for 5 min t. The PCR products were resolved by electrophoresis in a 1.5 % agarose gel and the ladder used was 100 bp. The

DNA amplicons were sequenced using Sanger's sequencing by LGC genomics (GmbH, Berlin Germany). The electropherographs of the sequenced genes were aligned, trimmed and deposited in the Genbank.

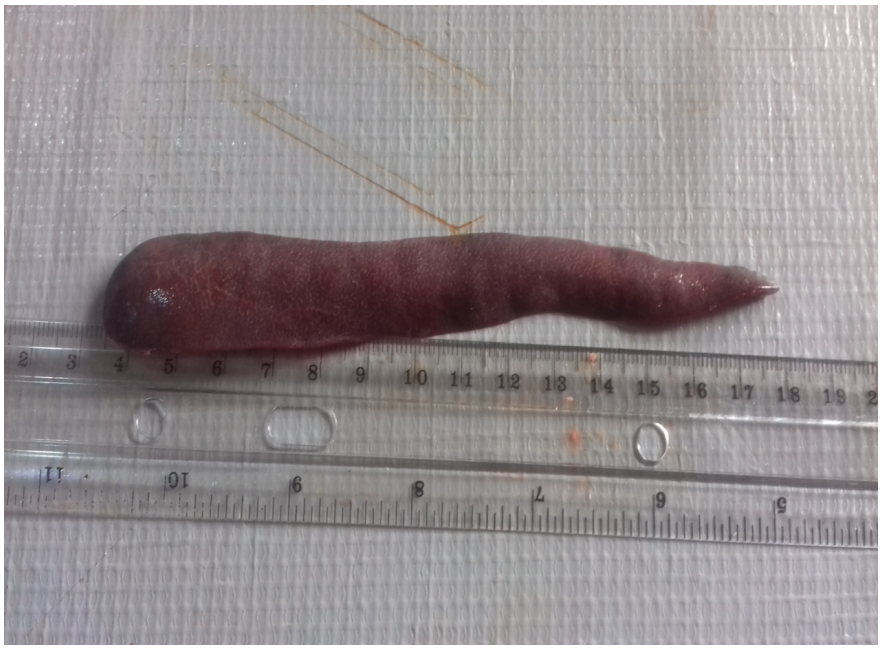
Results

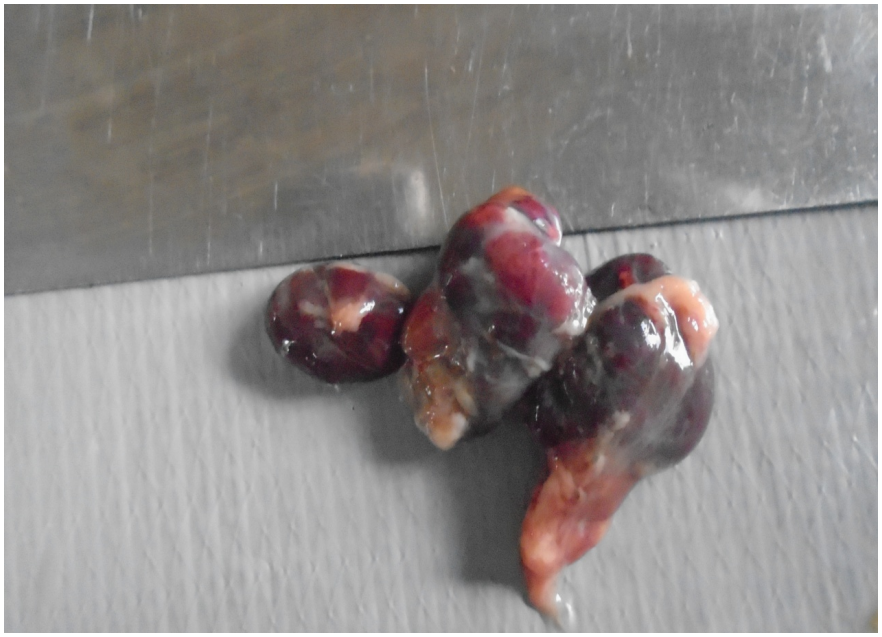
Postmortem examination revealed splenomegaly and severe congestion 2/2 (figure 1), enlarged and haemorrhagic lymphnodes 2/2 (figure 2), hepatomegaly and severe congestion 1/2 (figure 3a), white multi-focal patches on the liver 1/2 (figure 3b), globosed, severely congested, generalized cortical petechiae and severe blood clot in the pelvis 2/2 (figures 4a and b). Histopathological findings were thus: spleen: severe lymphocytes depletion (figure 5). Lymph node: severe lymphocytes depletion and severe hemosiderosis (figure 6). Liver: severe congestion with focal areas of coagulation necrosis (figure 7). Kidney: severe congestion and renal tubular and glomerular necrosis with few tubular proteins cast (figures 8a and b).

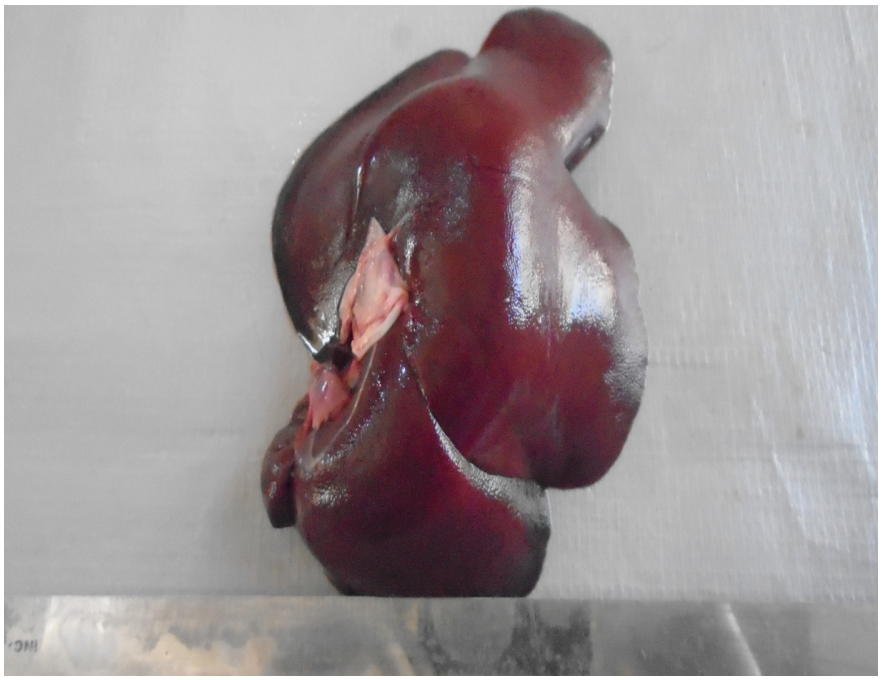
Viral isolation on the culture plate was revealed by haemadsorption. The presence of the virus typified by the attachment of large numbers of pig erythrocytes to the surface of infected cells thereby forming the rosette appearance (figure 9) as opposed to the negative wells which were not inoculated with the infected tissues (figure 10).

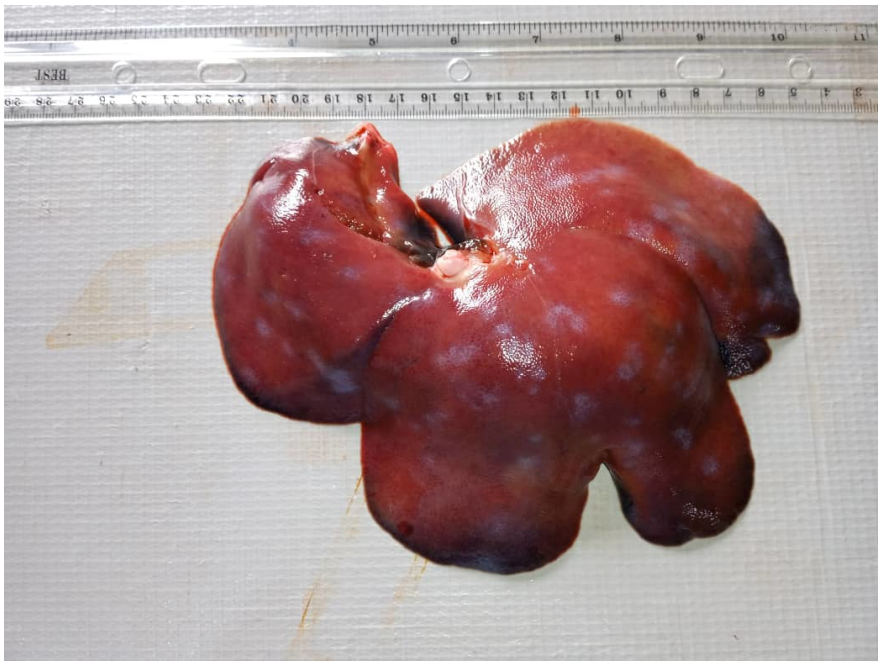
Gel picture (Figure 11) shows that DNA fragments of 278bp (confirming the presence of ASFV) were successfully extracted and amplified from the spleen, lymph node and kidney of the infected animals. Note that the size of the PCR products in lanes 1, 2, and 3, which were ASFV positive amplicons presented similar DNA fragment base pairs to the product of the positive control of ASFV in lane 4. The nucleotide sequences of ASFV generated from this study were deposited in the Genbank with accession numbers MN888963 and MN888964.

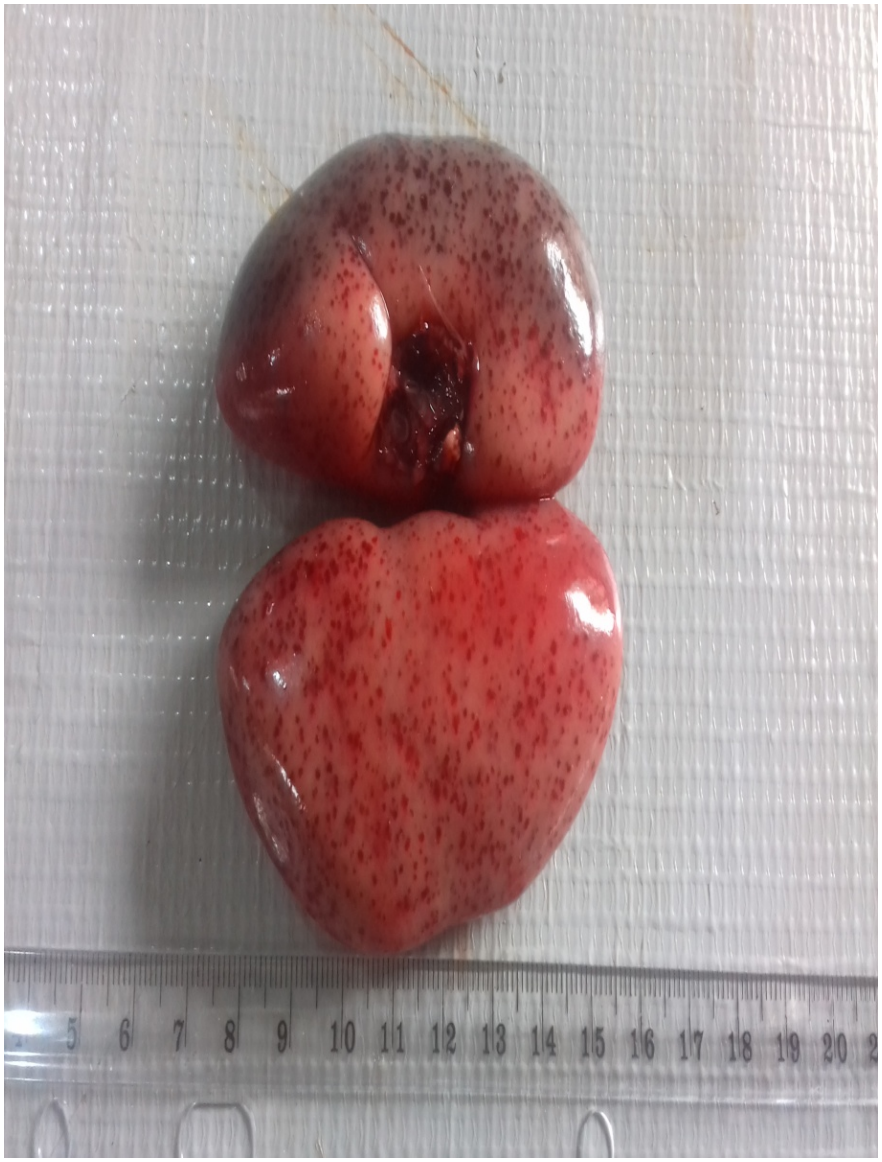
The result of phylogenetic analysis based on the p72 (B646L) gene is presented in figure 12. It shows the ancestral relatedness of the sequence from this study to other 53 sequences downloaded from the Genbank database, including those from Nigeria, different parts of Africa and beyond. Apparently, the sequence from this study failed to cluster with any other, including the ones from Nigeria. However, the closest relatives based on the phylogenetic analysis are sequences that make up the genotype-I ASFV group. These sequences were generated mainly from Nigeria, other countries (especially Western and Central Africa), and Europe. Consequently, the sequence from this study was considered to also belong to genotype-I ASFV.

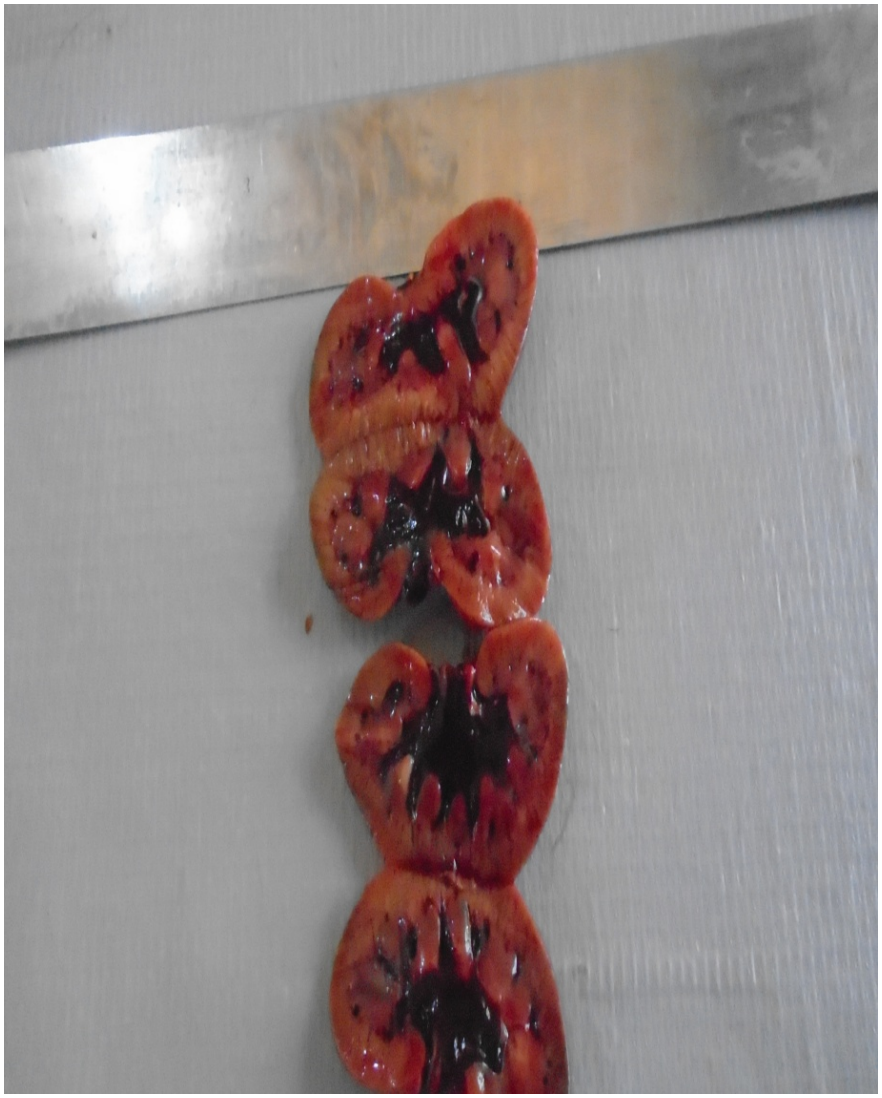


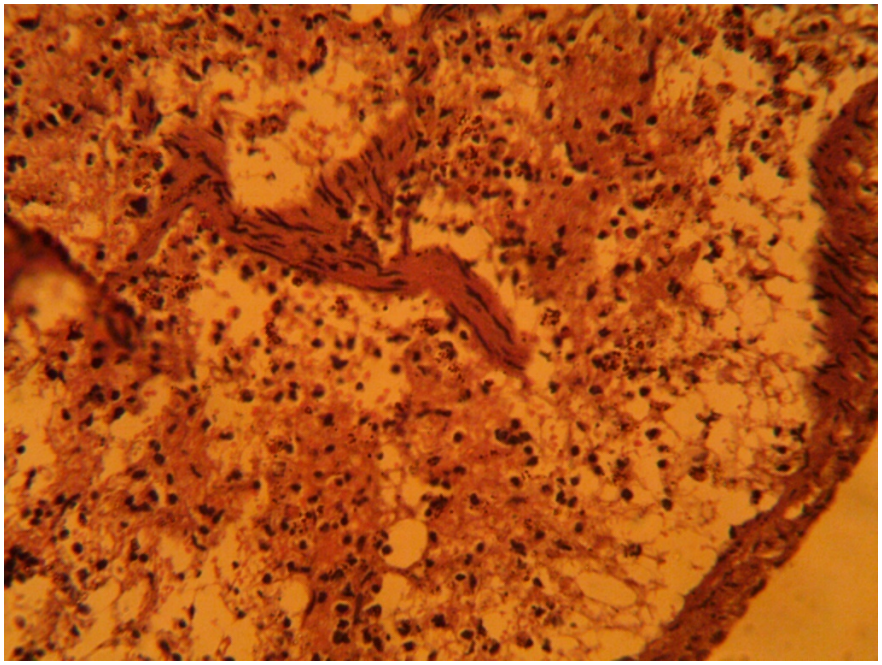


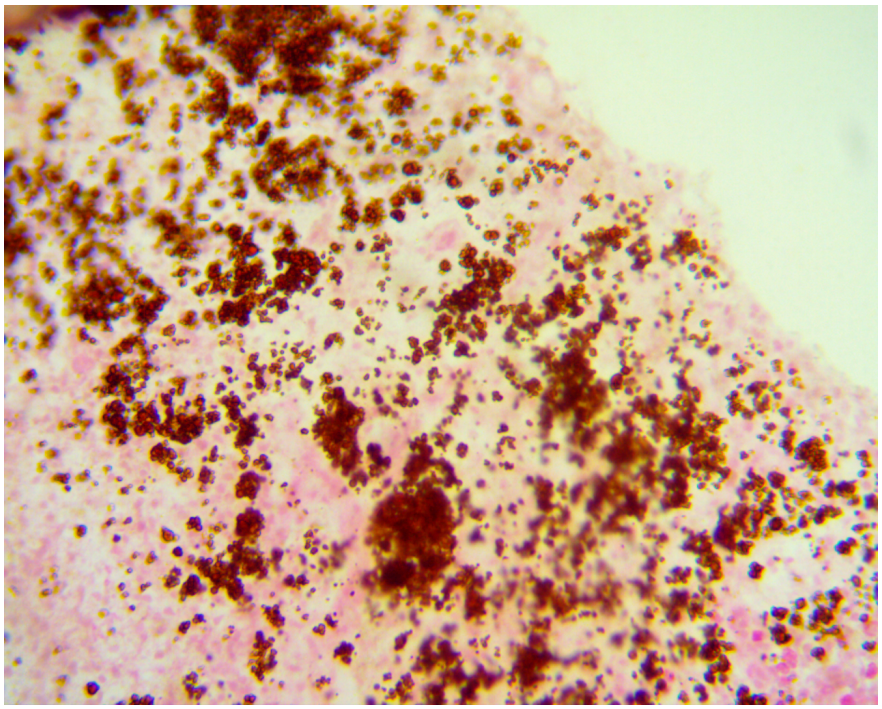


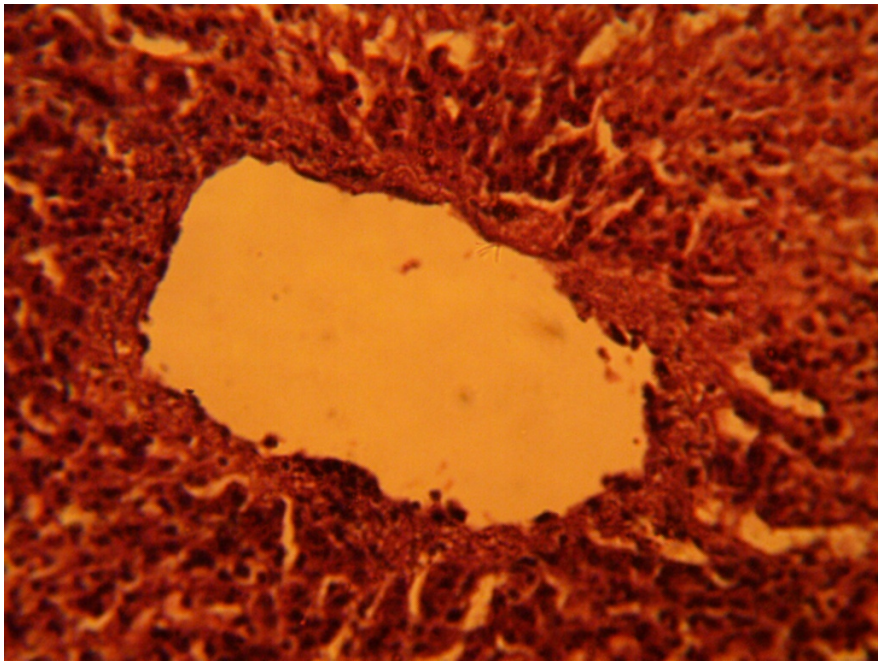


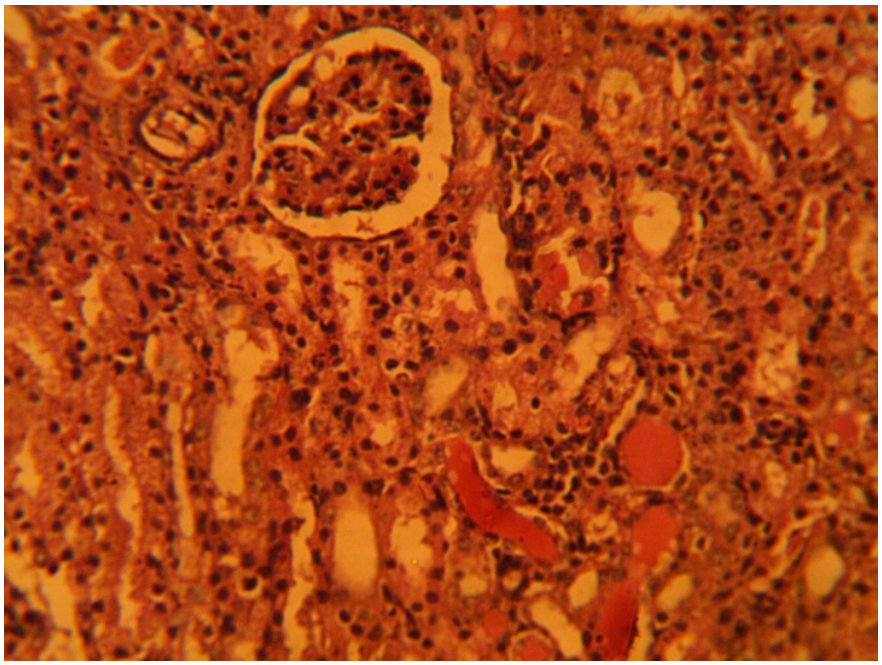


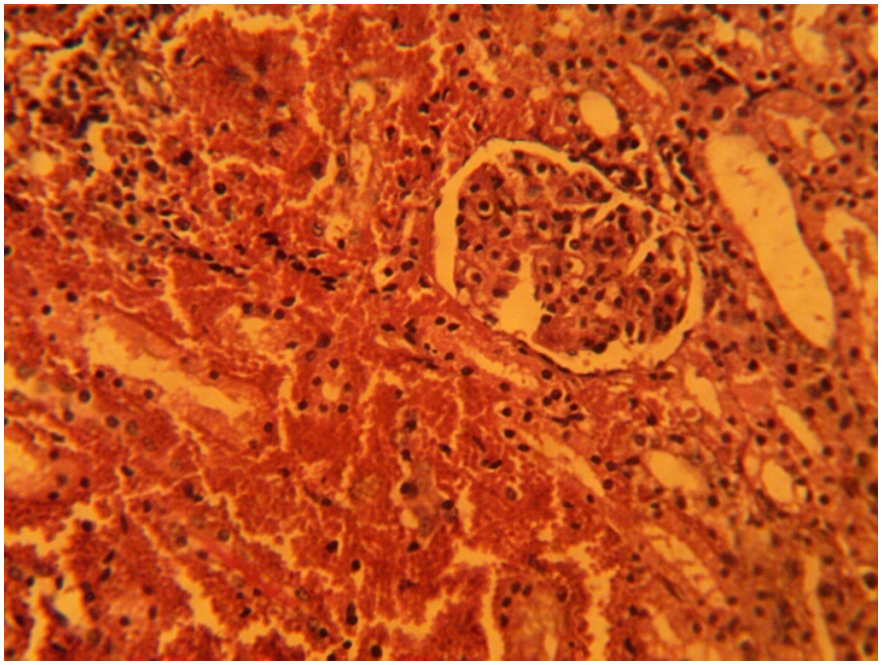


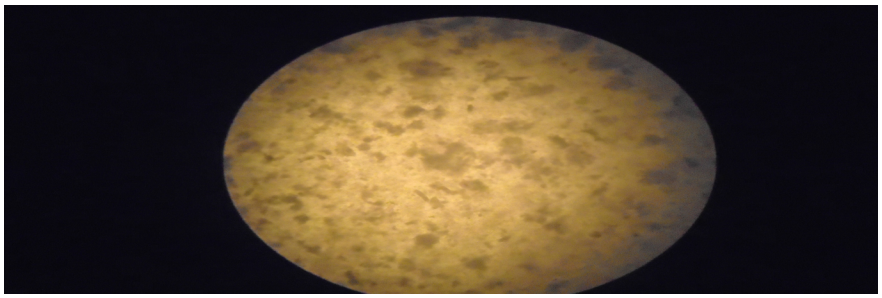


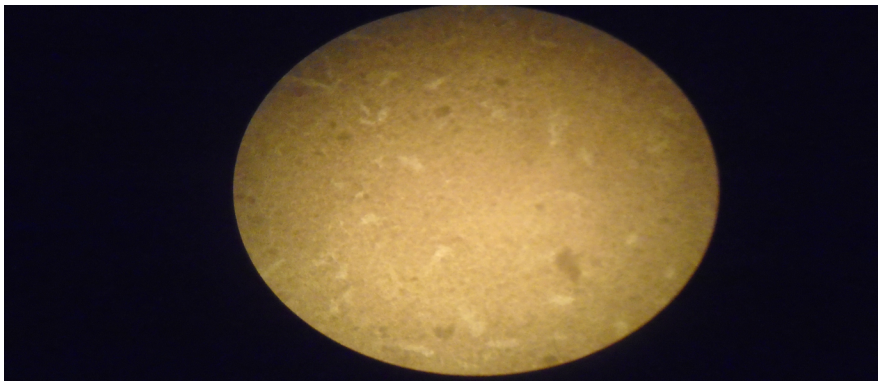


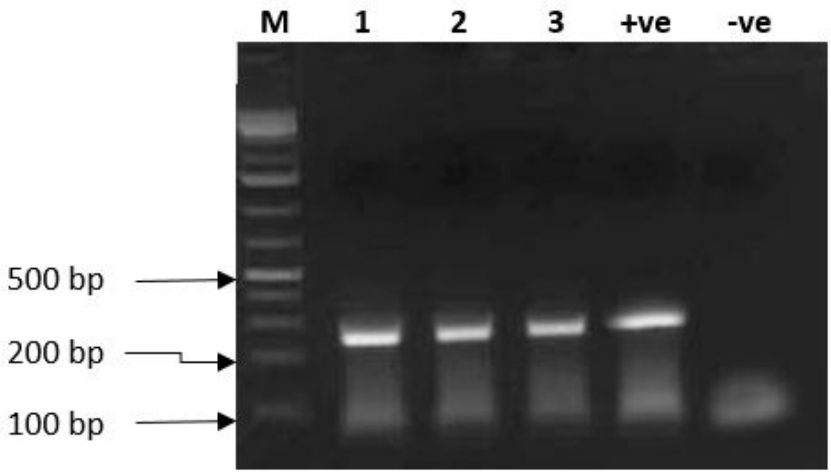












Discussion

Gross pathological lesions observed in the spleen were severe congestion, splenomegaly and severe depletion of lymphocytes. The lesions may be because of leucopoenia and lymphopoenia that were said to be occasioned by apoptosis, necrosis of the lymphocytes or due to activation and mobilization of the lymphocytes to fight the virus (Otesile *et al* ., 2005; Junt *et al* ., 2008; Ganowiak, 2012; Liao and Padera, 2013; Jubb *et al* ., 2016).

Similarly, the lymph nodes were severely enlarged and haemorrhagic likely due to severe haemorrhages and necrosis as reported previously by Jubb *et al* . (2016). Severe lymphocytes depletion and severe hemosiderosis was recorded in the lymph nodes histologically, which may be due to apoptosis, necrosis or severe erythrolysis (Ganowiak, 2012; Jubb *et al* ., 2016). Although moderate multifocal white patches on the liver has not been previously reported in ASF, liver in this study showed severe congestion and moderate enlargement with severe necrosis on histopathological evaluation. Previous reports on ASF documented congestion and necrosis as lesions associated with the acute form of the disease and adduced it to marked thrombocytopenia or disruption in blood clotting factors. (Oura , 2005; Otesile *et al* ., 2005; Jubb*et al* ., 2016). The kidney of pigs was severely congested with severe cortical petechiae which is a consistent finding previously reported in ASF and severe ecchymotic haemorrhages in the renal medulla which we observed for the first time in ASF. Histopathological examination revealed severe necrosis largely characterized by severe pyknosis and moderate renal tubular protein fat. Similar lesions were also observed in earlier reports on ASF and were attributed to be likely due to severe thrombocytopenia or disruption in blood clotting factors (Oura, 2005; Ganowiak, 2012; Jubb *et al* ., 2016). The heart on the other hand was observed to have mild focal congestion grossly with severe vascular congestion histomorphologically. The disease was reported by earlier researchers to cause cardiopathology ranging from hydropericardium, hemopericardium and fibrinous pericarditis whichwas said to be associated with the chronic form of the disease. The lesions so recorded in the heart were said to be likely caused by oxidative stress and ischaemia (Oura, 2005; Otesile *et al* ., 2005; Ganowiak, 2012; Jubb *et al* ., 2016; Semerjyan *et al* ., 2018).

The aetiological diagnosis of the disease was by virus culture and indentification via haemadsorption test characterized by erythrocytes attachment to the surface of the infected cells several hours post inoculation. This has been documented to be a reliable and effective method of ASFV isolation (Oura, 2005; Jubb *et al* ., 2016). Furthermore, the aetiological diagnosis was achieved by amplification of viral glycoprotein VP72 from the tissues of the infected pigs which among others, plays an important role in attaching to and entering target cells. Successful amplification of a 278bp of the glycoprotein by PCR has earlier been reported to be confirmatory of ASFV in a given clinical sample (Otesile *et al* ., 2005; Fernandez-pinero *et al* ., 2012; Jubb, 2016; Mwiine *et al* ., 2019).

Phylogenetic analysis based on the VP72 (b646L) gene sequences showed that the isolate from this study failed to cluster with others downloaded from the Genbank database, including the Nigerian isolates. This observation is inconsistent with earlier finding that Nigerian isolates always cluster together on the tree (Luka *et al* ., 2017b). The reason for the observed difference is not known for certain, however, it could be an outcome of continued evolution of the virus. It could also be possible that there are unreported strains of the virus circulating in the country, and this, therefore, highlights the need for more extensive studies to characterize the circulating strains of ASFV in the country as previously recommended (Luka *et al* ., 2017b). This, if done, would serve as baseline for formulating adequate and appropriate control strategies against ASF outbreaks, including vaccine production. Furthermore, the phylogenetic analysis revealed that the virus associated with the disease reported in this study belonged to genotype-I. This further corroborates the previous reorts (Owolodun*et al* ., 2020; Luka *et al* ., 2017b) on circulating viruses in Nigeria.

Data availability statement: The data that support the findings of this study as presented in the manuscript are available from the corresponding author, Dr. Tizhe E.V., upon reasonable and/or genuine request.

Ethical statement: Ethical clearance is not applicable in this study as the data were generated from the swine carcasses submitted to the Veterinary Teaching hospital, University of Jos, 2019 for diagnostic purpose.

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Competing interest: The authors declare that there is no competing interest.

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