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Case Report

MOLECULAR DETECTION OF AFRICAN SWINE FEVER VIRUS IN DOMESTIC PIGS IN BENUE STATE, NIGERIA

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ABSTRACT

A cross-sectional survey was carried out to investigate the presence of African swine fever virus (ASFV) in domestic pigs in Benue state. Reports have established the presence of the virus in two local government Areas in the state. As part of the technological advancements in sampling and diagnostic methodologies of ASFV in recent decades, this study is aimed at applying molecular tool to detect the presence of ASFV in six (6) local governments in Benue State, Nigeria. A population of 250 pigs where randomly selected out of which 175 pooled tissues (lymphnodes, lungs, liver and intestines), 45 (sera) and 30 (faecal) samples were collected between September 2022 to September 2023. Samples were analysed using the polymerase chain reaction technique. Results from PCR with expected amplicons of two hundred and fifty-seven base pair (257 bp) was demonstrated in 20 out of the 250 samples which gave 8.0% prevalence. This study confirmed the presence of ASFV in the tissue samples collected, while faecal and sera samples were negative for the virus. We recommend that control measures such as, test (using PCR) and slaughter be adopted with adequate compensation. The PCR provides higher sensitivity and specificity than alternative methods for antigen detection, such as the antigen Enzyme linked immunosorbent assay (ELISA) and the direct Fluorescent antibody test (FAT) (FAO 2000).

Keywords: African Swine Fiver Virus, Benue State, Molecular Detection, PCR, Domestic Pigs

INTRODUCTION

African swine fever virus (ASFV) remains the only DNA virus transmitted by arthropods. The virus is a large enveloped double-stranded DNA virus, the member of the family Asfarviridae within the genus Asfivirus. The length of the ASFV genome can vary remarkably from 170kbp to 194kbp, and the number of genes can vary from 150kbp to 167kbp, depending on the isolate (Yanez *et al*., 1995; Dixon *et al*., 2013; Portugal *et al*., 2015; Alonso *et al*., 2018).

It is highly contagious and transmission is by direct contact between infected and susceptible pigs or by contact with infectious secretions/excretion resulting in up 100% morbidity in exposed pigs herd with mortality varying from 0 and 100% depending on the strain, host, dose and the route of entry of the virus (Atuhaire *et al*., 2013). The virus can survive for 15 weeks in putrefied blood, 70 days in blood on wooden boards, 11 days in faeces at room temperature, 3 hours at 50°C, 18 months in pig blood at room temperature, 150 days in boned meat at 39°C and 140 days in salted dried hams (Vallee *et al*., 2001).

Polymerase chain reaction (PCR) is used to detect the ASFV genome in porcine samples (blood, organs etc.) and ticks. Small fragments of viral DNA are amplified by Polymerase chain reaction (PCR) to detectable quantities which provides a more sensitive, specific, and rapid alternative to virus isolation for the detection of ASFV.

The current genotyping of ASFV strains is based on partial nucleotide sequencing of the B646L gene, which encodes the major capsid p72 protein (Bastos *et al*., 2003; Boshoff *et al*., 2007). In total, 24 genotypes of ASFV have been determined worldwide (Bastos *et al*., 2003; Achenbach *et al*., 2017; Quembo *et al*., 2018).

The PCR provides higher sensitivity and specificity than alternative methods for antigen detection, such as the antigen

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enzyme-linked immunosorbent assay (ELISA) and the direct fluorescent antibody test (FAT) (FAO 2000).

Conventional PCR is recommended by the OIE in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (2016) have been fully validated over time and it is a useful tool for routine diagnosis of the diseases.

The aim of the study was to investigate the presence of ASFV in Benue State using PCR technique.

MATERIALS AND METHODS

STUDY AREA

Benue State is one of the North Central states in Nigeria. The State is divided into three zones namely; Zone C (Otukpo and Ado), Zone B (Makurdi and Gwer-West) and Zone A (Katsina-Ala and Konshisha) (Figure I). The study sites (Local government areas) where chosen using simple random sampling technique. Availability of Live pig markets/abattoirs/ slaughter slabs, insecurity and pig population were also considered in our choice.

Figure I: Map of Benue State showing the 6 Local Government Areas (Wikipedia 2020)

SAMPLE COLLECTION

The sample size for the research was calculated using method described by Thrustfield (2007). 2.8% (Asambe *et al*., 2018) was used to calculate serum sample (45 samples), 1.9% (Ayas *et al*., 2017) was used to calculate faecal sample size (30 samples), and 12.9% (Abwage *et al*., 2015) was used to calculate tissue sample size (175 pooled tissue samples).

The samples were collected between September 2022 and September 2023 (Table I). The samples were collected from different breeds including crosses of Large White, Landrace, Hampshire, Duroc and locals under free ranged, semi-intensive and intensively managed systems. The age of the pigs sampled ranged from less than 3 months to approximately 9 months and above.

Five (5) ml of blood samples were obtained into plain tubes from the cranial vena cava using sterile syringes and needles and transported in ice-packs to the laboratory. Sera were decanted into centrifuge tubes after keeping at room temperature for 60 minutes and centrifuging at 704 rpm for 20 minutes to remove the remaining clot/red blood cells and other insoluble materials and then stored at -20 °C.

Table I: Consistent for PCR.

Pipetting step	Master Mix Reagent	1x reaction Volume (50 µl)	½ reaction (25 µl)
1	Nuclease free H2O	17µl	8.5 µl
2	2X Master mix	25.0µl	12.5 µl
3	Primer (PPA-1) sequence 5'-AGTTATGGGAAACCCGACCC-3' (forward primer)	3.0µl	1.5 µl
4	Primer (PPA-2) sequence 5'-CCCTGAATCGGAGCATCCT-3' (reverse primer)	3.0µl	1.5 µl
5	DNA Extract	2.0µl	1 µl
Total volume		25.0 µl	50.0µl

Faecal samples were collected by inserting a gloved finger into the rectum while in young piglets a swab stick was inserted into the rectum and rotated to scoop the faeces and put into a sterile container, labeled, placed in icepacks.

Approximately 5g and 1cm3 of each of the tissues analyzed were collected post slaughter using sterile scissors for cutting and forceps for holding were inserted into a container containing a prepared virus transport media (VTM), labeled, and placed in ice-packs.

All samples were transported to the Biotechnology Center of the National Veterinary Research Institute Vom (NVRI) laboratory in cold boxes where they were stored at -4°C until processed for molecular detection of ASFV using PCR.

SAMPLE PREPARATION

A total of forty-five (45) sera and one hundred and seventy-five (175) groups of tissue samples (livers, spleens, lungs, lymph nodes and intestines) and 30 faecal samples were collected.

Tissues Samples: Pool tissues were cut into smaller pieces weighing up to 4 gram and put into a mortar. Sterile glass was added to the pooled tissues and crushed using a pestle until fully homogenized.

1000 µl of PBS (phosphate buffered saline) was added to the homogenized tissues using a pipette. The homogenized tissue samples was transferred into a 1.5ml centrifuge tube and vortexed, it was then spinned down by centrifuging at 10000 rpm for 2 minutes. The supernatant was put into a 1.5ml centrifuge tube thereafter for DNA Extraction.

Faecal Samples: About 3gm of faeces was added to 1000 µl of PBS (phosphate buffered saline), centrifuged at 1000rpm

for 2 minutes. The supernatant was put into1.5ml centrifuge tube thereafter for DNA Extraction

Serum Sample: Pipetted out serum into a 1.5ml centrifuge tube and taken for DNA Extraction.

LABORATORY ANALYSIS

DNA EXTRACTION

DNA was extracted directly from homogenized tissue, faecal samples as well as serum using a nucleic acid extraction kit (Qiagen purification kit) following the manufacturers procedures.

POLYMERASE CHAIN REACTION (PCR)

A PCR assay using the ASF diagnostic primers PPA1 [5´-AGTTATGGGAAACCCGACCC-3´], PPA2 [5´-CCCTGAATCGGAGCATCCT-3´] which generate an amplicon of 257 bp within the p72 gene (Aguero *et al*., 2003) was used to confirm the presence of ASFV DNA. For the amplification of DNA, 23µl of the previously prepared PCR mix (PCR master mix) was added to 2µl of the DNA template in a 0.2ml reaction tube. A similar preparation was made for the positive (Spain '70) and negative controls (nuclease free distilled water) templates. The reaction mixture was treated as follows: (i) incubated for 10 min at 95°C; (ii) subjected to 40 cycles of PCR, with 1 cycle consisting of 15 s at 95°C, 30 s at 62°C, and 30 s at 72°C; and (iii) incubated for 7 min at 72°C.

GEL ELECTROPHORESIS

PREPARATION OF 1.5% AGAROSE GEL

• Weigh out 1.5g of agarose powder
• Add 100mls of (1 x) TBE/TAE buffer and agitate
• Heat up in a microwave oven at maximum heat for 3 minutes
• Cool solution under tap water/ or swirl gently at room temperature
• Add 5 µl of ethidium bromide
• Pour cool-gel in gel-casting tray with attached combs, place at room temperature until full polymerization takes place (15 minutes)
• Retract combs

The PCR products (amplicons) with a DNA marker (ladder) will be added to one lane on each side of the prepared 1.5% agarose gel with wells produced by combs and visualized under UV light in a GEL DOCUMENTATION SYSTEM. The results will only be taken as valid if all the negative controls showed no bands while the positive controls displayed bands at the 257bp region of the DNA marker.

RESULTS

The detection of ASFV was molecularly confirmed by the presence of 257 bp of the virus, as shown in Figure II.

The details of all the 250 pigs used for this study and the results of PCR for ASF in Benue State are as presented in Tables II and III. There were 9(3.6%) males and 11(4.4%) females' pigs that tested positive. 9(3.6%) local pigs, 7(2.8%) crossed and 4 (1.6%) exotic pigs were positive. 4(1.6%) pigs between the ages of 0-7months,7(2.8%) pigs between the ages of 7-18months while 4(3.6%) pigs over 18 months were positive. 13(5.2%) pigs with loose faeces were positive while 7(2.8%) had firm faeces. 4(1.6%) pigs had the presence of ticks on them and tested positive while 16(6.4%) had no ticks but still tested positive. 13(5.2%) pigs tested positive during the wet season while 7(2.8%) pigs tested positive in the dry season.15(6.0%) pigs consuming swilled feed were positive while 5(2.0%) pigs tested positive consuming processed feed. Only 20 pigs that represented 8.0% of the total samples in Benue State tested positive while the remaining 230 that represented 92.0%% were negative.

Table II; Distribution of ASFV DNA in Pigs in Benue State, Nigeria (September 2022 to September, 2023).

VARIABLE	NO SAMPLED (%)	ASF STATUS POSITIVE PCR (%)
SEX		
Male	90(36%)	9(3.6%)
Female	160(64%)	11(4.4%)
BREED		
Local	77(30.8%)	9(3.6%)
Cross	97(38.8%)	7(2.8%)
Exotic	76(30.4%)	4(1.6%)
AGE		
0-7 months	106(42.4%)	4(1.6%)
7-18 months	44(17.6%)	7(2.8%)
>18 months	100(40.0%)	9(3.6%)
NATURE OF FAECES		
Loose	63(25.2)	13(5.2%)
Firm	187(74.8%)	7(2.8%)
PRESENCE OF SOFT TICKS ON THE PIGS		
Present	30(12.0%)	4(1.6%)
Absent	220(88.0%)	16(6.4%)
HUSBANDRY OF PIGS		
Extensive	110(44.0%)	10(4.0%)
Semi-intensive	111(44.4%)	7(2.8%)
Intensive	29(11.6%)	3(1.2%)

DISCUSSION

In this study, ASFV DNA was detected in 20 (8.0%) out of the 250 total samples collected from 6 local government areas of Benue state in tissue samples at slaughter slabs/abattoir and absent in faeces and serum of the pigs at piggeries using Polymerase chain reaction (PCR) technique. This finding is in agreement with reports by Asambe *et al*. (2018),

seroprevalence of 2.8% in Benue State, which is further delineated into two categories, i.e, a prevalences of 7 (1.7%) observed in piggeries and 6 (13.6%) were observed in Makurdi slaughter slabs. Fasina *et al*., 2012 suggested that abattoir/slaughter samples yielded more positive result which could be attributed to farmers' practices such as, during an active outbreak, farmers often will not report to authorities but will rapidly sell off pigs before they die of African Swine Fever disease and another habit of culling unthrifty and sick animals for slaughter or even culling animals in early sickness.

Table III; Showing Distribution of ASFV DNA in pigs in Benue State, Nigeria (September 2022 to September, 2023).

VARIABLE	NO SAMPLED (%)	ASF STATUS POSITIVE PCR (%)
SOURCE OF SAMPLE		
Abbattoir/Slaughter slabs	175(70%)	20(8.0%)
Piggery	75(30%)	0(0.0%)
PRESENCE OF ABBATTOIR IN PIG COMMUNITIES		
YES	227(90.8%)	18(7.2%)
NO	23(9.2%)	2(0.8%)
SEASON OF SAMPLING		
Wet (May-Oct.)	130(52.0%)	13(5.2%)
Dry (Nov.-April)	120(48.0%)	7(2.8%)
GEOGRAPHICAL LOCATION		
Otukpo	41(16.4%)	1(0.4%)
Okpokwu	42(16.8%)	1(0.4%)
Makurdi	42(16.8%)	7(2.8%)
Guma	42(16.8%)	5(2.0%)
Vandeikya	41(16.4%)	4(1.6%)
Ushongo	42(16.8%)	2(0.8%)
TYPE OF FEED		
Swill	150(60.0%)	15(6.0%)
Processed	100 (40.0%)	5(2.0%)

The study also revealed that apparently healthy domestic pigs indicate the presence of ASFV DNA. The endemic nature of the disease in the Benue state as well as adjoining neighbouring states and movement of pigs from northwest to some north central states may have been responsible for the spread. These results has corroborated the findings of Oluwole & Omitogun (2014) where ASFV genome was detected in Nigerian indigenous pigs infected with ASFV without showing any clinical symptoms.

The prevalence of 8.0% observed in this study is higher than 2.8 % of earlier reports conducted in South Kivu province of Congo, Uganda and Western Kenya (Etter *et al*., 2011; Gallardo *et al*., 2011; Atuhaire *et al*., 2013; Okoth *et al*., 2013; Bisimwa *et al*., 2020) from tissues and blood samples, and a higher prevalence of 33.3% reported by Luther *et al*. (2008) and 50.75% by Owolodun *et al*. (2007) in Plateau state and North central Nigeria using PCR on tissue samples of visceral lymph nodes, liver, spleen and kidneys.

African Swine Fever Virus is endemic in Benue state and adjoining states like Taraba, with seroprevalence of 12.9% as reported by study by Abwage *et al*. (2015). Neighbouring, Nasarawa state with prevalence of 1.9% as reported by Ayas *et al*. (2016). Similar molecular detection as well as seroprevalence has been carried out by Adenaike *et al*., (2023) in Benue state with a much higher prevalence of 42.86% from blood (PCR) and serum (ELISA).

Other reports by Adedeji *et al*., 2021; First-Time Presence of African Swine Fever Virus (ASFV) Genotype II in Lagos. Onoja *et al*. (2022); detection of ASFV Genotype II in pigs during haemorrhagic fever outbreak in Ogun State, Nigeria using blood and tissues samples. This is in agreement with our study where samples positive after analysis of ASFV are from tissues.

CONCLUSION

In conclusion, ASFV is circulating in domestic pigs in the six local government areas studied in Benue state. Older females with loose faeces, managed extensively around abattoir/slaughter slabs during the dry seasons while fed on swilled feeds were positive of ASFV. We recommend extensive pig management laws focusing on ensuring animal welfare by addressing housing, handling and environmental conditions. All swill feed to be given to pigs must be cooked and stirred for about 30 minutes. Veterinary attention should be called upon when signs like loose faeces are noticed in the herd. Abattoirs/slaughter slabs should be constructed away from human settlements and properly fenced to prevent entry of pigs and viscera/effluents are properly disposed.

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DECLARATIONS

Ethical approval was sought and granted by the animal care and use ethical committee of the college of Veterinary Medicine, JOSTUM (JOSTUM/CVM/ETHICS/2024/13) before the commencement of study.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest among them.

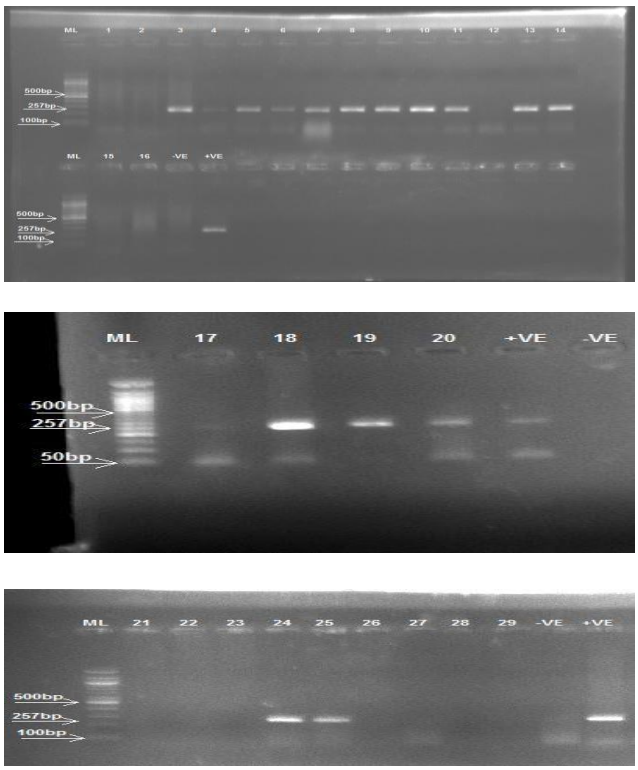

Figure II: Transcriptase Polymerase chain reaction (PCR) on agarose electrophoresis of amplicons of ASFV (257 bp) among pigs in Benue State Nigeria. Lane ML = 100 bp DNA ladder; lanes 3,4,5,6,7,8,9,10,11,13,14,16,18.19,20,24,25,30,31 and 32 = positive samples; lane -ve = negative control, and lane +ve = positive control.

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