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Research article

Molecular characterization and identification of African Swine Fever virus isolates from emerging cases of infection in Central Luzon, Philippines

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Abstract

A study that evaluated the molecular characteristics to confirm the identity of the African Swine Fever (ASF) pathogen from emerging cases of infection in Central Luzon, Philippines was initiated. The study involved the collection of 125 blood samples from pigs during the four-month duration of the study when clinical signs of ASF was prevalent in the four out of seven provinces of Central Luzon, Philippines. This work included the amplification of the p72 gene in DNA samples through PCR and confirmation of the identity of the ASF pathogen through DNA sequencing. The DNA sequences of the p72 gene of the ASF pathogen are made up of 412 nucleotides and used as a basis in identifying the Philippine ASF pathogens as ASF virus PH/CL 4/2022, ASF virus PH/CL 3/2022, ASF virus PH/CL 2/2022 and ASF virus PH/CL 1/2022. Philippines ASF virus isolates showed 100% sequence similarity with sequences reported from Malaysia ASF isolate MVZT218/2021, Vietnam ASF isolate D/ASFV/VN/DN-VC/2019, and India ASF isolate ASF/MZ/IND/3 based on BLASTN homology search in the NCBI database. The results of the study revealed close phylogenetic relationships among ASF isolates from Vietnam, Malaysia, India and the Philippines.

Keywords: African Swine Fever, Central Luzon Philippines, Emerging ASF, P72 molecular characterization.

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INTRODUCTION

Unparalleled mortalities were evident in the local swine industry as an aftermath of the outbreaks of African swine fever (ASF). The impacts are reportedly multi-faceted and complicate effective and efficient management practices. Infections pose economic impacts in the Philippines where swine raising plays an important part in household livelihoods and as a pathway out of poverty (Perry and Grace, 2009). In situations where pig production contributes food, income and various social functions, the impacts of animal disease on poverty is critical (Delsart et al., 2020). Globalization plays a crucial role in the on-going geographic redistribution of pathogens, hosts and vectors and are reportedly precipitated by increased trade, traffic volumes and international passenger travel (Baker et al., 2022).

ASF is perhaps the most important animal health concern for animal raisers today. The animal disease is presently seen as a national concern as it has already spread to 16 regions in the country based on available data of the Philippine Department of Agriculture (DA) (https://www.woah.org/en/what-we-do/animal-health-and-welfare/disease-data-collection/). Recent report as of April 2023 cite ASF-related incursions in 16 regions like Bangsamoro Autonomous Region in Muslim Mindanao (BARMM), Cordillera Administrative Region, Ilocos Norte, Cagayan Valley, Central Luzon, Calabarzon, Mimaropa, Bicol, Western Visayas, Central Visayas, Eastern Visayas, Zamboanga peninsula, Northern Mindanao, Davao, Soccsksargen and Caraga. Records also describe that 54 provinces located within these regions which include Abra, Benguet, Ifugao, Kalinga, Mountain Province, Apayao, Pangasinan, Cagayan, Isabela, Nueva Vizcaya, Bataan, Bulacan, Nueva Ecija, Pampanga, Tarlac, Zambales, Aurora, Batangas, Cavite, Laguna, Quezon, Rizal, Marinduque, Camarines Sur, Sorsogon, Capiz, Iloilo, Guimaras, Cebu, Leyte, Northern Samar, Samar, Southern Leyte, Zamboanga del Norte, Zamboanga del Sur, Zamboanga Sibugay, Camiguin, Lanao del Norte, Misamis Oriental, Davao del Norte, Daval del Sur, Davao Oriental, Davao de Oro, Davao Occidental, North Cotabato, Sultan Kudarat, Sarangani, Agusan del Norte, Agusan del Sur, Surigao del Norte, Surigao del Sur, Dinagat Islands and Maguindanao del Sur; hitting a total of 460 towns within these provinces with ASF-stricken pig populations. There are, however, provinces that are still ASF-free and these are Ilocos Sur, La Union, Batanes, Quirino, Negros Occidental, Bohol, Negros Oriental, Eastern Samar, Biliran, Oriental Mindoro, Palawan, Romblon, Albay, Aklan, Antique, Bukidnon, Misamis Occidental and the National Capital Region. Local government units (LGUs) are mandated to report to local authorities any ASF outbreak to prevent its spread. A WOAH protocol provides a depopulation strategy of areas infected within the 500-meter radius affected by ASF. Infections when not properly checked lead to economic losses, mortality and morbidity resulting to poor productivity and farm performance. Infections in farms that remain undetected and untreated leads to the spread of infection in the entire herd making treatment difficult to handle. Effective monitoring and control of ASF may largely depend on an accurate and timely diagnosis as this strengthens the decision to rule out other infections that present clinical manifestations similar to those of ASF.

The genome of the ASF virus reportedly varies as it encodes 168 different kinds of protein (Wang et al., 2021) and genes such as the C-terminal region of p72 (*B646L*) gene, p54 (complete *E183L*) gene, CD2v (variable region) of *EP402* gene, CVR (central variable region) of *B602L* gene and the full *MGF505-2R* gene have reportedly been used by researchers as the basis in classifying strains from different countries into 24 genotypes (Achenbach et al., 2017). Of the 24 genotypes reportedly found in Africa, only genotypes I and II have been associated with outbreaks. Genotype I reportedly spread to other European countries, Southern America and Caribbean islands while Genotype II reportedly spread from Georgia, Eastern Europe, Western Europe, Belgium and to Asian regions like China, Vietnam, Cambodia, Laos, and Korea (Ge et al., 2018).

The road to the discovery of an antiviral drug against ASF is still a long way to explore, although biosecurity measures when followed in animal production units remain as a protective panacea of swine against the virus. The challenge for ASF detection is quite huge and an initiative to define the species of the ASF pathogen under Philippine setting during emerging episodes of ASF is then necessary to verify and confirm the existence of the ASF pathogen. Research work that applies contemporary technics in molecular biology have been initiated in this laboratory to address the identity of the viral pathogen with p72 gene and other related genes (p54, p32) selected as targets for investigation. Studies on these areas may open avenues for research and development pursuits for the ASF viral pathogen and explore additional methodologies that enhance diagnostic capability and appropriate point-of-care applications for cases of ASF.

MATERIALS AND METHODS

Collection of Samples for Molecular Studies

Arrangement and permission to receive or collect blood samples from ASF-suspected animals were made with farm veterinary consultants who served as research collaborators in the months of January to April 2022 when emerging cases of ASF were grossly observed in four provinces of Central Luzon, Philippines. A total of 125 blood samples were obtained from 125 pigs raised under backyard conditions that manifested clinical signs of ASF marked by hemorrhagic spots in the abdomen and perineal region (purposive sampling). Blood sample collection involved withdrawal of 3 to 5 mL blood from the jugular vein which was undertaken by farm veterinarians and their animal technicians who received previous trainings on blood collection as a routine methodology during animal disease monitoring and outbreaks in backyard farms. The pertinent protocols described for the handling of animals used in research relative to the collection of 125 blood samples in this study are approved by the Central Luzon State University Ethics Review Committee Code No. 2022-130. The blood samples were temporarily held separately in tubes packed with ice and brought to the laboratory as samples for the subsequent studies.

Extraction of Viral DNA for PCR Amplification

DNA was extracted using a prescribed protocol (Norgen Biotek Corporation, Canada) that utilized a set of Wash and Elution buffers, Proteinase K application with alternate procedures of incubation at 55 ºC for 1 hr, centrifugation for 1 to 3 min at 5,200 x g and transfer in a spin column assembly. The purified genomic DNA was stored at -20˚C before PCR analysis. For the amplification of the p72 gene, the DNA samples were separately added to a reaction mixture that contained 9 µL nuclease-free water, 5.0 µL 1 x PCR buffer, 2 µL 2 µM $MgCl_2$, 2.5 µL 0.2 mM dNTPs, 2.5 µL 0.2 µM Forward and 2.5 µL 0.2 µM Reverse primers, 0.5 µL 0.625 U DNA polymerase and 1.0 µL 85 ng/µL DNA template, to make up for total volume of 25 µL.

A pair of primers (Forward, 5'- GGCACAAGTTCGGACATGT-3' and Reverse, 5'-GTACTGTAACGCAGCACAG-3') which were synthesized commercially (1st Base, Singapore) were used in the amplification of the p72 gene in the ASF DNA samples as described by other researchers (Aguero et al., 2003). The amplification of the p72 gene was carried out in a thermocycler (SimpliAmp™Thermal Cycler, ThermoFischer, USA) programmed to perform with an initial denaturation at 95ºC for 10 min, followed by 35 cycles of denaturation at 94ºC for 30 s; annealing at 54ºC for 30 s and extension at 72 ºC for 30 s, and a final extension at 72ºC for 7 min. The amplicon products were allowed to resolve in 1% agarose gel for 40 min at 220 V, stained with GelRed (Biotium Inc., USA), and visualized using UV transilluminator (Gel Doc XR+ System, USA).

Identification of ASF Local Isolates Based on DNA Sequences

Out of the 125 DNA extracts only 90 samples exhibited positive amplification of the p72 gene with the use of the pair of primers mentioned. These samples were labelled from 1 to 90 and only those samples with labels/numbers divisible by 9 (Sample numbers: 9, 18, 27, 36, 45, 54, 63, 72, 81 and 90) were randomly selected to come up with 10 representative samples for amplification. The amplified products of the p72 gene were purified before DNA sequencing in a sequencing service provider (1st Base, Singapore). DNA sequences were evaluated and quality-trimmed using a software (SnapGene Software,www.snapgene.com) before analysis using Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi) as described by other researchers to validate the identity of the isolates (Altschul et al., 1990).

Phylogenetic Profile of ASF Local Isolates

Some of the homologous sequences with the ASF virus p72 (B646L) gene in the NCBI BLASTN result were taken and used in the analysis of the phylogenetic profiles relative with those of ASF virus p72 (B646L) from other countries with reported cases of ASF such as Malaysia, India and Vietnam. Annotated sequences with significant homology were included for multiple sequence alignment. A total of 20 sequences with equal length of 406 bp were aligned using the MUSCLE alignment program in a MEGA X software (https://www.megasoftware.net).

Neighbor-Joining (NJ) method with Kimura 2-paramater distance correction and 1000 bootstrap values were used for phylogenetic analysis using the MEGA X Software as described by other researchers (Kimura, 1980; Felsenstein, 1985; Saitou and Nei, 1987; Kumar et al., 2018; Stecher et al., 2020).

RESULTS

Genetic Characteristics of ASF Local Isolates

The amplified products of the major capsid protein (p72) of ten (10) representative samples of ASF virus isolated from ASF-stricken pigs in swine production areas of Central Luzon, Philippines is shown in Figure 1. The products amplified by the primers during PCR exhibited a molecular weight of 400 bp (Figure 1). One representative sample (Lane 3), however, was apparently not efficiently amplified through PCR that used the pair of primers described.

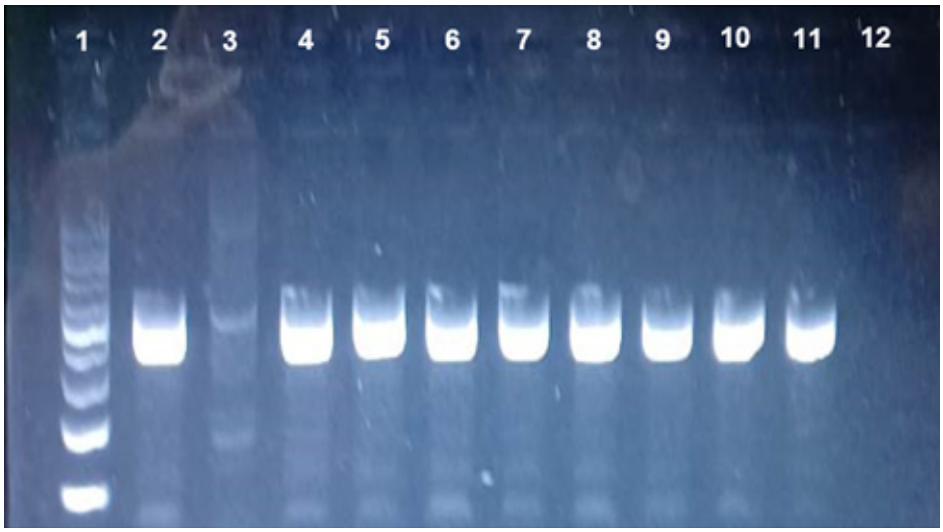

Figure 1 Amplicons of the p72 gene (representative samples) of ASF pathogens obtained from ASF-stricken pigs in backyard swine production areas of Central Luzon, Philippines. Lane 1 (100 bp DNA Ladder); Lane 2 (Isolate 9), Lane 3 (Isolate 18), Lane 4 (Isolate 27), Lane 5 (Isolate 36), Lane 6 (Isolate 45), Lane 7 (Isolate 54), Lane 8 (Isolate 63), Lane 9 (Isolate 72), Lane 10 (Isolate 81), Lane 11 (Isolate 90), Lane 12 (Nuclease-free water). Isolate 9 (PH/CL 4/22), Isolate 36 (PH/CL 3/22), Isolate 63 (PH/CL /22), Isolate 90 (PH/CL 1/22) amplicon products were used as samples for DNA sequencing.

Identification of ASF Local Isolates Based on DNA Sequences

A summary of partial p72 gene DNA sequences of the identified ASF pathogens is shown in Table 1. Data show that the p72 gene of the four (4) representative isolates had 412 nucleotide bases and all the aligned sequences directly match the sequences of the p72 gene of ASF isolates previously reported in the NCBI database. These data altogether served as the basis in the identification of the Philippine ASF pathogens as ASF virus PH/CL 4/2022, ASF virus PH/CL 3/2022, ASF virus PH/CL 2/2022 and ASF virus PH/CL 1/2022. It is noteworthy that the afore-mentioned ASF Philippines isolates have been annotated (Accession numbers OQ525895, OQ525894, OQ525893 and OQ525892) as members of the serogroup 8, genotype 2 of ASF viruses.

Table 1 GenBank Accession Number and partial nucleotide sequences of p72 gene from ASF pathogens isolated from Central Luzon, Philippines.

Sample	Accession Number	Sequence
ASF virus PH/CL 4/2022	OQ525895	GATAGAGATACAGCTCTTCCAGACGCATGTTCATC-TATATCTGATATTAGCCCCGTTACGTATCCGATCACAT-
ASF virus PH/CL 3/2022	OQ525894	TACCTATTATTAAAAACATTTCCGTAACTGCTCATGGTAT-CAATCTTATCGATAAATTTCCATCAAAGTTCTGCAGCTCT-
ASF virus PH/CL 2/2022	OQ525893	TACATACCCTTCCACTACGGAGGCAATGCGATTAAAAC-CCCCGATGATCCGGGTGCGATGATGATTACCTTTGCTTT-
ASF virus P H/CL 1/2022	OQ525892	GAAGCCACGGGAGGAATACCAACCCAGTGGTCATATTA-ACGTATCCAGAGCAAGAGAATTTTATATTAGTTGGGA-CACGGATTACGTGGGGTCTATCACTACGGCTGATCTTGT-GGTATCGGCATCTGCTATTAACTTTCTTCTTCTTCAGAAC-GGTTCAGCTGTGCTGCGTTACAGTACAG

BLASTN search for homologous nucleotide sequences in the NCBI database which utilized the above sequences was equally important in confirming the identity of the ASF pathogens generated in this study. A summary of the homology scores, % identity and E-values of the p72 gene of other ASF viral isolates reported in the NCBI is shown in Table 2. No nucleotide polymorphisms were observed in the analysis of the p72 (B646L) gene sequences of the Philippine local isolates and those of the foreign ASF virus isolates listed. These data provided affirmation on the identity of the collected samples in the study as African swine fever pathogen based on the partial sequence of the p72 (B646L) gene which are homologous with those samples reported in other countries.

Table 2 Summary of ASF viral isolates reported in the NCBI from other countries which showed significant homology with the Philippine isolates.

Homology	Country	Size (bases)	Identity (%)	E-value	Accession No.
African swine fever virus isolate MVZT218/2021 P72 (B646L) gene, partial cds	Malaysia	414	100	0.0	MZ605374.1
African swine fever virus isolate D/ASFV/VN/DN-VC/2019 P72 (B646L) gene, partial cds	Vietnam	481	100	0.0	MW828879.1
African swine fever virus isolate ASF/MZ/IND/3 major capsid protein p72 (P72) gene, partial cds	India	482	100	0.0	MZ063773.1

Phylogenetic Profile of ASF Local Isolates

The homologous nucleotide sequences of the p72 gene were likewise useful in phylogenetic analysis to confirm the genetic relationship of the ASF pathogens examined and those isolates reported in other countries. Data in Figure 2 represents a close phylogenetic relationship among Vietnam ASF isolates (Accession numbers MW828879, MW828865, MW828863, MW825017, MW828854, MW825048, MW825038, MW825057, MW828879, MW828865, MW828863, MW825017, MW828854, MW825048, MW825038, MW825057); Malaysian ASF isolate (Accession number MZ605374); India ASF isolate (Accession number MZ063772) and the Philippine ASF isolates (PH/CL 4/2022, PH/CL 3/2022, PH/CL 2/2022 and PH/CL 1/2022, with Accession numbers OQ525895, OQ525894, OQ525893 and OQ525892, respectively.

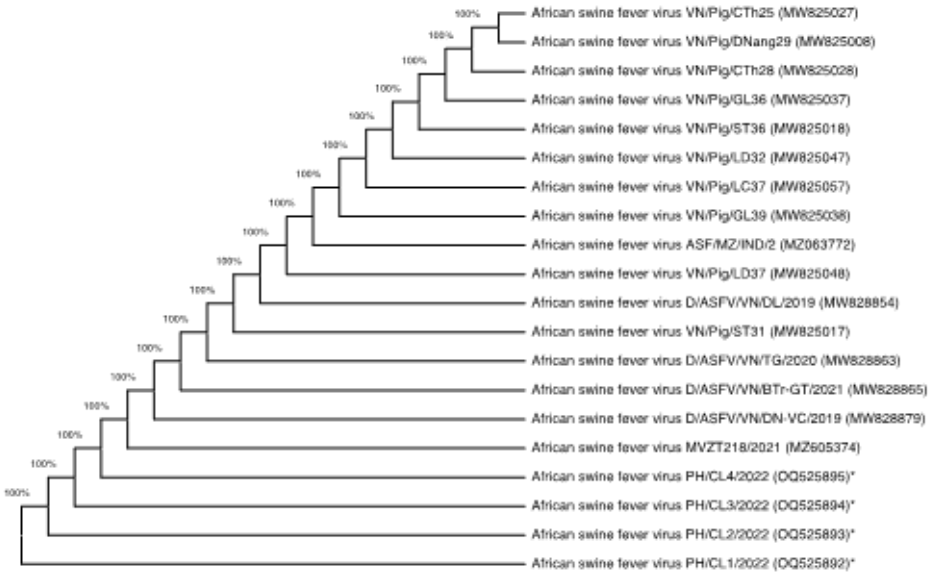

Figure 2 Unrooted Neighbor-Joining (NJ) tree of p72 (B646L) gene partial nucleotide sequences from ASF virus isolated from backyard hog farms in Central Luzon, Philippines (*). All the other sequences are reportedly obtained from Vietnam, Malaysia and India and are annotated in NCBI GenBank. Numbers at nodes represent bootstrap values from 1000 v esampled datasets while Kimura 2-parameter method was used for distance correction. The accession numbers are inside parentheses.

DISCUSSION

Attempts to study closely the nature of the ASF pathogen that caused the decimation of many hog populations in different parts of the Philippines were temporarily put on hold due to the limited access to animal production units responsible with the stringent implementation of biosafety and biosecurity measures during the time of the global pandemic. As the incursions of ASF still unpredictably emerge in various parts of the Philippines, incessant work that addresses the nature of ASF infection will remain undisrupted. With the application of recent protocols in molecular biology and related bioinformatic technics, genetic characteristics of the major capsid protein (p72) of the ASF

virus has been described. Results of a study conducted recently demonstrate that ASF pathogens collected from Philippine swine production areas possess a p72 gene which when analyzed were homologous and does not exhibit nucleotide polymorphisms divergent from ASF viral pathogens reported from other countries. Relevant data on homologous sequences, % identity and E-values are important as criteria for bioinformatic studies to validate the similarity of sequences of specific genes like p72 (Avila, 2021). Data in the study where E-values at 0.00 were obtained confirm an exact congruence of the query sequence and the reported sequences in GenBank.

A close similarity in the phylogenetic profiles of the identified Philippine ASF viral pathogens with the ASF virus reported in countries like Malaysia, India and Vietnam as indicated by the 100% bootstrap-values in the phylogenetic data was also demonstrated in the study. These findings strongly provide a substantial explanation which can be linked to the absence of nucleotide polymorphisms in the sequences of the p72 (B646L) genes of Philippine ASF samples and those samples reported from Malaysia (MZ605374), India (MZ063772) and Vietnam. The data at hand further strengthens the contention that the p72 (B646L) gene qualifies as an ideal molecular marker for the identification of the ASF pathogen. This raises the likelihood, however, that p72 is usually used in studies on ASF in Southeast Asian countries like India, Malaysia and Vietnam and that it is in these three countries where emerging and re-emerging cases of ASF prevail and regularly reported in the Genbank. As the present study relied mainly on the DNA sequences of ASF viral isolates reported in the GenBank, these data further explain the close phylogenetic relationships of ASF isolates in the three countries and that of the Philippines. Several groups of researchers have probably attempted to study the course of ASF infections and the nature of the pathogen in the local setting. The disease appears to have an unpredictable pattern, with episodes of remission in some places and provinces while it may present with aggravations in other places. No reports can describe an effective vaccine as a strategy for control. Implementation of intensified biosafety and biosecurity measures in farms remain as the primary measure for disease management and containment, with depopulation as an ultimate measure to protect potentially challenged swine populations.

CONCLUSIONS

The interest to strengthen the diagnostic capability of researchers and other stakeholders on ASF will probably capitalize on the data that presently highlights the identification of ASF pathogens recovered in the present study. Experiments are currently undertaken which relentlessly seek the possibility of defining a point-of-care diagnostic platform for ASF which can be used in field and clinical settings to be used by animal health practitioners in giving a right recommendation for ASF management and containment.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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