Sarkar et al | Article 11692
J Pure Appl Microbiol. 2026;20(2):1737-1745. doi: 10.22207/JPAM.20.2.64
Received: 21 April 2026 | Accepted: 18 May 2026
Published Online: 05 June 2026

P-ISSN: 0973-7510; E-ISSN: 2581-690X

RESEARCH ARTICLE OPEN ACCESS

Growth Kinetics of African Swine Fever Virus (Genotype II) Isolated from a Domestic Pig in Punjab, India

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Abstract

African swine fever (ASF), a deadly disease caused by African swine fever virus (ASFV), emerged in north-eastern India in 2020 and subsequently spread to several states, devastating swine herds due to the lack of commercial vaccines. We report the isolation, genetic characterization and growth kinetics of ASFV isolated from Punjab, India in primary porcine pulmonary alveolar macrophages (PAM). Partial sequencing of the B646L gene categorized the virus as genotype II, showing complete nucleotide identity with previously reported Indian genotype II isolates. Phylogenetic analysis placed the isolate amongst genotype II ASFVs circulating in Europe and the Asia-Pacific region since 2007. Multistep growth curves were constructed based on HAD_{50} titres and viral DNA copy numbers after infection of PAM cultures at 1.0 and 0.1 multiplicity of infection (MOI) observed up to 168 hrs post-infection (hpi). At 1.0 MOI, viral titres and genome copy numbers in the cell associated (CA) fraction peaked earlier (120-144 hpi) before declining, whereas the culture supernatant (CS) fraction showed a delayed but sustained increase, reaching maximum levels at 168 hpi. At 0.1 MOI, both HAD_{50} titres and viral genome copies increased progressively in CA and CS fractions, with higher peak levels observed in the CS fraction at later time points. By observing up to 168 hpi, this study reveals higher extracellular virus production with lower DNA copy to HAD ratio in PAM cultures infected at 0.1 MOI, demonstrating optimal duration for higher virus yield in cell culture supernatants, which would support efficient ASFV propagation, pathogenesis and vaccine development studies.

Keywords: African Swine Fever, African Swine Fever Virus, ASFV, Growth Kinetics, PAM

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Citation: Sarkar G, Rajukumar K, Senthilkumar D, et al. Growth Kinetics of African Swine Fever Virus (Genotype II) Isolated from A Domestic Pig in Punjab, India. *J Pure Appl Microbiol.* 2026;20(2):1737-1745. doi: 10.22207/JPAM.20.2.64.

INTRODUCTION

The African swine fever virus (ASFV) is the only DNA arbovirus classified within the *Asfarviridae* family, responsible for causing 100% mortality in all members of the pig family upon infection with virulent strains.[1,2] The first description of African swine fever (ASF) traces back nearly a century to 1921 in Kenya.[3] Since then, it has primarily been reported from different regions of Africa and occasionally from some European countries. However, following the 2007 Georgian outbreak, ASF rapidly spread to Eastern Europe, Asia and other geographical regions and became a grievous problem for the global pig industry.[4] ASF is a notifiable disease that savagely killed over half of the pig population of China within just one year of its spread, from 2018 to 2019.[5] Following China, ASF was reported in India on May 2020, leading to a similarly catastrophic situation in the Indian pig population. Molecular analysis of Indian ASFV isolates using E183L, B646L and B602L (central variable region) genes revealed their closed relatedness to highly pathogenic ASFV genotype II circulating in Europe and the Asia-Pacific region.[6] Whole-genome comparisons further suggested ASFV Wuhan/2019-1 from China as the nearest ancestor of Indian ASF viruses.[7] Currently, ASF outbreaks have been confirmed across several Indian states in both domesticated pigs and free-ranging wild boars,[8,9] raising concerns about its continued spread and economic consequences. In the absence of any therapeutic interventions or a globally accessible commercial vaccine, the ASF control measures are only bounded to early diagnosis followed by stamping out policy, strict sanitary and robust surveillance system.[10,11] Efforts to combat ASFV are currently focused on developing effective vaccines, with a particular emphasis on live-attenuated ASFV (LAV) strains. These LAVs have demonstrated substantial protection against homologous virulent strains, achieved through gene deletions that occur either naturally or via genetic modification.[12,13] In spite of these advancements, significant challenges remain, including scaling up production and ensuring cross-protection across diverse ASFV genotypes.

ASFV exhibits a narrow cell tropism, primarily replicating in cells including monocytes, blood-derived macrophages, and pulmonary alveolar macrophages of pigs. These cells have been crucial for studying ASFV isolation, virus-host interactions, and replication dynamics.[14,15] However, their use is limited by issues such as low reproducibility, batch-to-batch variability, expensive cell extraction, and ethical concerns related to animal welfare.[16,17] To overcome these challenges, there is a pressing need to identify suitable cell lines that can efficiently support ASFV replication. Such cell lines would facilitate the isolation, purification, and biological study of ASFV, ultimately aiding in the development of LAVs. However, continuous tissue culture passages can result in altered ASFV genome, affecting its biological properties in vitro and in vivo.[18-20]

Because of the challenges associated with the propagation of field-isolated ASFV strains in established cell lines, using porcine pulmonary alveolar macrophages (PAM) remains the preferred system for ASF virus isolation and propagation.[16,21] The growth characteristics of Indian ASFV isolates in cell culture systems remain unexplored. There is no report of systematic examination of the growth kinetics of Indian ASFV strains in cultured cells. In this study, we constructed and analysed the multistep growth curves of an Indian ASFV isolate to comprehensively evaluate its replication kinetics in PAM cultures.

MATERIALS AND METHODS

Cells

Porcine pulmonary alveolar macrophages (PAM) were harvested from four- to six-week-old piglets using an established protocol.[22] Harvested cells resuspended in cell freezing medium (RPMI-1640 containing 7% DMSO and 20% FBS) were cryopreserved in liquid nitrogen until use.

Virus isolation

Isolation of the ASFV strain Ind-NIHSAD/SW/22_1055 was done from a porcine blood sample obtained during a confirmed ASF outbreak in Punjab state as described previously with suitable modifications.[6] Briefly, 100 µl of growth medium [RPMI-1640 (Sigma, USA) containing 1X antibiotic-antimycotic solution (Sigma, USA)] and 10% FBS containing PAM revived from cryopreservation was added to each well of a cell

culture plate at a concentration of 2 × 10^5 cells per well. The plate was maintained overnight in a 37 °C incubator with 5% CO_2 environment. Plasma recovered from whole blood was treated with gentamicin (50 µg per ml concentration) for 45 min at 37 °C, and inoculated in PAM cells at final dilutions of 1/10 and 1/100 in triplicates. Each well was supplemented with 15 µL of freshly prepared 1% porcine erythrocyte suspension. The cells were placed in a carbon dioxide incubator at 37 °C and monitored for seven days for haemadsorption (HAD) or cytopathic effect. After two freeze-thaw cycles at the end of incubation, the culture supernatants were further passaged and propagated in PAM cells to get the virus stock (P5) used in this study. The titre of the ASF virus stock was estimated using the HAD_{50} assay and immunoperoxidase monolayer assay (IPMA), as per Reed and Muench method.[23]

ASFV B646L gene amplification, nucleotide sequencing and analysis

DNA extraction from the PAM cell lysate was done using a viral nucleic acid extraction kit (Genes2me, India) according to the manufacturer's protocol. Partial B646L gene fragment (478 bp, encoding the p72 capsid protein) was amplified by conventional PCR adapted from Bastos et al.[24] Each 25 µL PCR reaction contained 12.5 µL of Platinum PCR SuperMix High Fidelity (Invitrogen, USA), 1 µL of each primer [p72-F (5'-CTGCTCATGGTATCAATCTTATCGA-3') and p72-R (5'-GATACCACAAGATCRGCCGT-3')] at a final concentration of 10 µM, 2 µL of template DNA (~100 ng), and PCR grade water to 25 µL. The amplified products were resolved on 1.5% agarose gel and purified using a gel extraction Kit (QIAquick, Qiagen, USA). DNA concentration was measured using a BioSpectrometer (Eppendorf, Germany). BigDye Cycle Sequencing Kit v3.1 (Applied Biosystems, USA) was used for direct sequencing of the purified amplicons using ABI 3130 Genetic Analyzer (Applied Biosystems, USA).

Genetic relationship was inferred by aligning the ASFV sequence with representative GenBank sequences, using Clustal W. A neighbour-joining phylogenetic tree (Tamura–Nei model) was generated using MEGA 11 software. Bootstrap analysis (1,000 replicates) was done to assess the robustness of tree topology (https://www.megasoftware.net/).

Viral growth kinetics

Overnight grown PAM cultures were inoculated with the stock ASFV at 0.1 and 1.0 multiplicity of infection (MOI) in duplicate wells of a 24-well culture plate, and kept in an incubator set at 37 °C and 5% CO_2 tension. Cells were rinsed three times with PBS, after 2 hrs of incubation, to remove non-attached virus, and were then added to 500 µL of EMEM supplemented with 1x antibiotic-antimycotic solution and 10% FBS. Cells and the culture supernatants were harvested at 2, 24, 48, 72, 96, 120, 144 and 168 hours post infection (hpi) and stored at -80 °C until use. Sham control inoculated with medium alone was included in duplicate for each condition. Following three freeze-thaw cycles, ASFV titres were determined by HAD_{50} assay and quantitative real-time PCR (qPCR).

Determination of ASFV titres by HAD_{50} assay

Infectious ASFV particles in cellular fractions and culture supernatants were quantified using the HAD_{50} assay, a visual method where ASFV-infected cells form rosettes with swine red blood cells. The HAD_{50} titre was determined by endpoint titration in PAM cells, following the protocol of Rai et al.,[25] with suitable modifications. Fifty µL of serial ten-fold dilutions of each sample prepared in growth medium was distributed in triplicate in 96 well plates. Each well was then co-seeded with 50 µL of growth medium containing 3 × 10^4 PAM cells and placed in an incubator maintained at 37 °C and 5% CO_2 tension. After 24 hrs, each well was added with 15 µL of 1% swine RBC and incubated further for seven days. Plates were observed microscopically for appearance of HAD, and the HAD_{50} per mL titres were calculated using the Reed and Muench method.

qPCR for ASFV genome detection

For ASFV genome quantification, 200 µL of cell culture supernatants or cell homogenates was used to isolate total nucleic acid using SpinRNA viral RNA extraction Kit (Genes2me, India). The WOAH-recommended qPCR assay targeting the *B646L* gene[26] with

minor modifications was used to detect and quantify ASFV genomic DNA, using Premix ExTaq (Probe qPCR) master mix (Takara, Japan). Each 20 µL qPCR mix contained 0.5 µM of each primer [forward (5'-CTGCTCATGGTATCAATCTTATCGA-3') and reverse (5'-GATACCACAAGAT-CRGCCGT-3')], 0.15 µM of TaqMan probe 5'-FAM-CCACGGGAGGAATACCAACCCAGTG-TAMRA-3', 3 µL of template DNA and nuclease-free water to adjust the final volume. A CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA) was used to perform the amplification reaction using a cycling programme with an initial denaturation at 95 °C for 2 min, followed by 45 cycles of 95 °C for 15 s and 58 °C for 45 sec. A cycle threshold (Ct) value of <40 was considered as the cut-off for ASFV positivity.

Standard curve for ASFV *B646L* gene quantification

A 478 bp product of partial *B646L* gene (encoding C-terminal end of p72 protein), amplified using specific primers,[24] was cloned into the pGEM-T easy vector (Promega, USA). Recombinant plasmid (pGEM-T/B646L) was extracted using PureYield Plasmid Miniprep System (Promega, USA), and quantified using BioSpectrometer (Eppendorf, Germany). Serial ten-fold dilutions of the plasmid DNA were prepared and used as template in the qPCR assay. DNA copy number was estimated using the formula: CN (copies/ml) = $(6.02 \times 10^{23} \times C) / (n \times 1.096 \times 10^{-21})$, where C represents plasmid DNA concentration (g/mL) and n denotes the plasmid size (bp). Ct values were plotted against the logarithm of CN to construct a standard curve which was used to quantify the viral DNA load in test samples.

Data analysis

Statistical analysis of the multistep growth curves of the ASFV isolate Ind-NIHSAD/SW/22_1055 was carried out using GraphPad Prism (v10) software. Shapiro–Wilk test was used to assess data normality. Two-way ANOVA followed by Tukey's multiple comparison test was employed for analysing differences in viral titres (HAD_{50}/mL) and genome copy numbers (copies/mL) across different fractions and MOI. A P-value of less than 0.05 was considered statistically significant. Results are expressed as mean ± standard error (SE).

RESULTS

Isolation and confirmation of the ASFV Ind-NIHSAD/SW/22_1055 isolate

Isolation of the ASFV strain Ind-NIHSAD/SW/22_1055 in PAM cells was confirmed by conventional and real-time PCR and by the appearance of rosette-shaped HAD of porcine erythrocytes which is a distinctive phenotypic characteristic of ASFV, mediated by CD2v protein of the virus[27] (Figure 1). The HAD property was observed in PAM cells starting from 48 hpi during each passage. The titre of the virus at 5th passage was estimated to be $10^{6.25}$ HAD_{50}/ml.

Genetic characterization of the ASFV isolate

A 478 bp fragment of the *B646L* gene, which encodes the p72 protein (C-terminal end) of the virus was amplified and sequenced. GenBank accession number of the generated nucleotide sequence is PZ221713. Sequence analysis revealed that the ASFV isolated from Punjab state shared 100% nucleotide and amino acid sequence homology with Indian ASFV reported previously from the north-eastern states of India. The partial *B646L* gene based phylogenetic tree showed that the ASFV isolate reported in this study grouped with other ASFV (genotype II) reported from Europe and Asia (Figure 2).

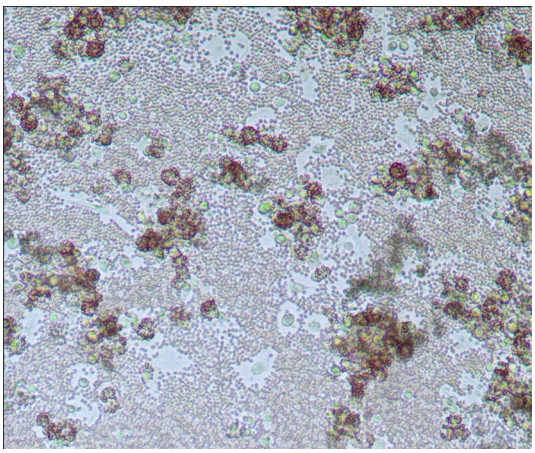

Figure 1. ASFV infected PAM culture showing haemadsorption of porcine erythrocytes, 72 hpi, passage two

ASFV growth kinetics in PAM cells

To understand the replication kinetics of the Indian ASFV field isolate Ind-NIHSAD/SW/22_1055 in PAM cultures, multistep growth curves were generated at 0.1 and 1 MOI. Presence of the virus in cell-associated (CA) and cell supernatant (CS) fractions were systematically analysed at various intervals up to 7 days. Infection of PAM cultures with the ASFV isolate induced characteristic cytopathic effects (CPE), including cytoplasmic vacuolization, cell rounding with clustering (5-15 cells), membrane blebbing, detachment and lysis, started between 48 hpi and 72 hpi, shortly after the rosette-shaped HAD formation, and progressively intensified from 72 hpi onwards. CPE progression was earlier and rapid in PAM cells infected at higher MOI. Infectivity titres (HAD_{50}/mL) as well as viral DNA copy

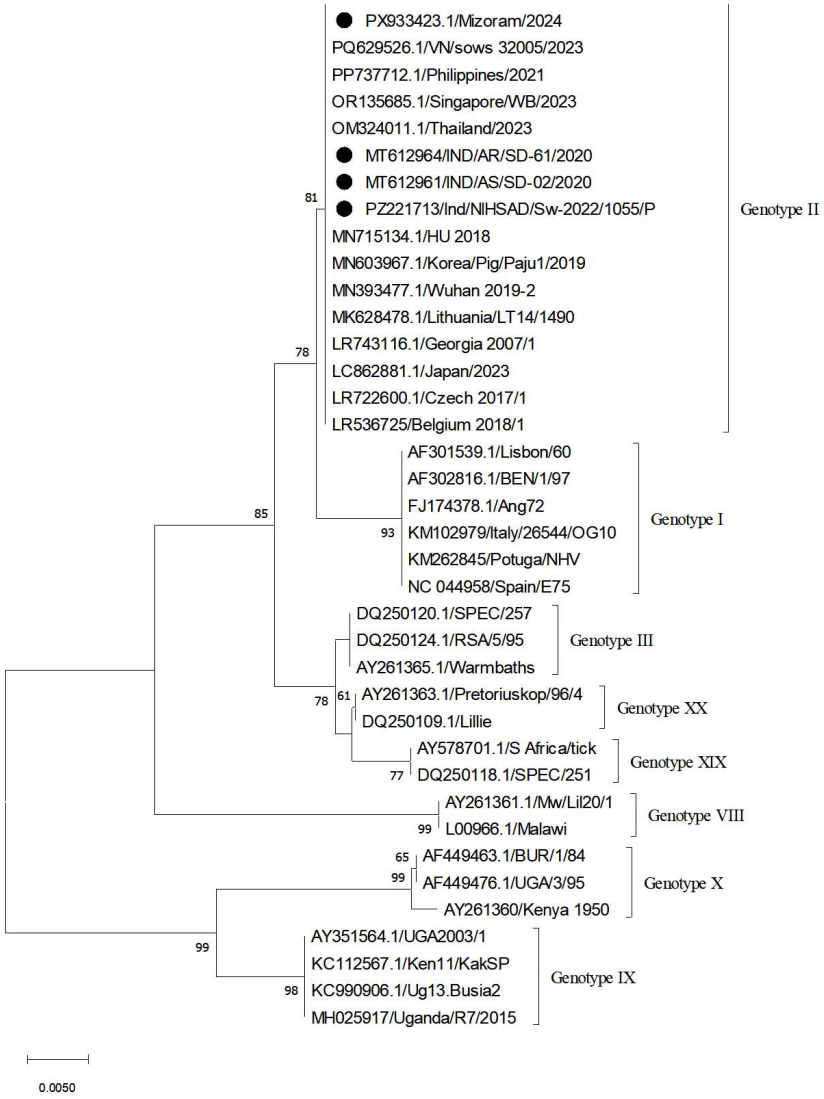

Figure 2. ASFV partial B646L gene based phylogenetic tree showing grouping of Indian isolates with other genotype II viruses reported across the world. Nodes show bootstrap values ≥60%. Substitutions per site is indicated by the scale bar

numbers (copies/mL) were estimated (Figures 3 and 4).

At 1 MOI, virus titre in CA fraction of PAM cell culture, as quantified using HAD assay was 1.78×10^3 HAD_{50}/mL at 24 hpi which peaked at 144 hpi with a mean titre of $1.39 \times 10^5 \pm 3.89 \times 10^4$ HAD_{50}/mL followed by a decline by 168 hpi. In the CS fraction, HAD_{50} titers began at $3.21 \times 10^2 \pm 0.5 \times 10^1$ HAD_{50}/mL at 24 hpi and reached a peak of 1.78×10^5 HAD_{50}/mL at 168 hpi, surpassing corresponding levels in CA at this endpoint.

At 1 MOI, evaluation of viral DNA copy numbers by qPCR showed that the CA fraction contained a mean of $9.45 \times 10^5 \pm 1.07 \times 10^5$ copies/mL at 24 hpi, showed maximum of $9.50 \times 10^7 \pm 1.72 \times 10^7$ copies/mL at 120 hpi, then declined to $4.13 \times 10^7 \pm 6.44 \times 10^6$ copies/mL at 168 hpi. Genome copies in the CS fractions were estimated to be $2.11 \times 10^5 \pm 2.90 \times 10^4$ copies/mL at 24 hpi which peaked to $8.55 \times 10^7 \pm 1.79 \times 10^7$ copies/mL at 168 hpi without any decline at previous time points.

At 0.1 MOI, HAD_{50} titre of CA fraction was estimated to be $4.4 \times 10^1 \pm 1.2 \times 10^1$ HAD_{50}/mL at 24 hpi which reached a maximum of at $1.17 \times 10^5 \pm 6.08 \times 10^4$ by 168 hpi, whereas CS HAD_{50} titres started at $1.6 \times 10 \pm 1.6 \times 10$ HAD_{50}/mL at 24 hpi and attained a peak of $2.47 \times 10^5 \pm 6.92 \times 10^4$ HAD_{50}/mL at 168 hpi.

At 0.1 MOI, ASFV DNA copy numbers in CA fraction advanced from $2.89 \times 10^4 \pm 1.52 \times 10^4$ copies/mL at 24 hpi to $2.41 \times 10^7 \pm 4.12 \times 10^6$ copies/mL at 168 hpi, while copies in CS progressed from $1.10 \times 10^4 \pm 2.59 \times 10^3$ copies/mL at 24 hpi to a peak of $1.18 \times 10^8 \pm 5.64 \times 10^7$ copies/mL at 168 hpi.

Statistical analysis revealed that in PAM cells infected with ASFV at 1.0 or 0.1 MOI, HAD_{50} titre in CA fraction was significantly higher at 120

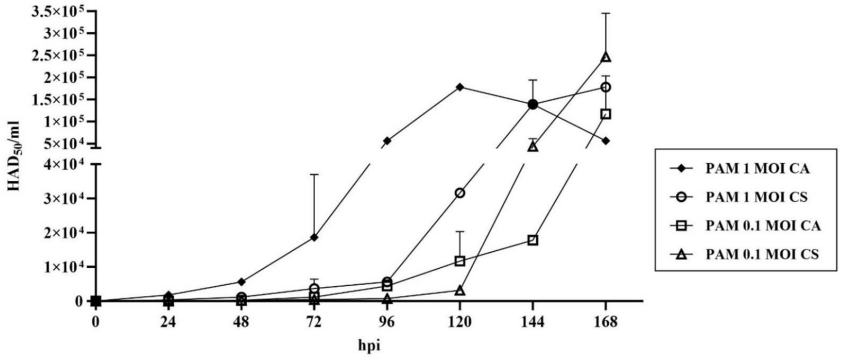

Figure 3. HAD_{50} titres estimated at different intervals in PAM cultures infected with ASF virus at 1.0 and 0.1 MOI

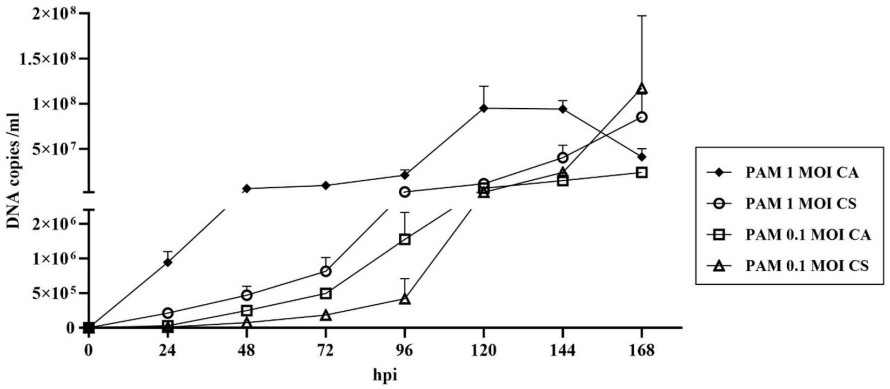

Figure 4. DNA copy numbers estimated at different intervals in PAM cultures infected with ASF virus at 1.0 and 0.1 MOI

hpi ($P < 0.0001$) and 144 hpi ($P < 0.001$), but lower at 168 hpi ($P < 0.05$) at 1.0 MOI as compared CS fractions. No significant differences were observed at other time points. The CS HAD_{50} titres were significantly higher ($P < 0.01$) at 168 hpi at 0.1 MOI as compared to 1.0 MOI.

In PAM cells infected with ASFV, CA genome copy numbers were significantly higher at from 24 hpi to 72 hpi ($P < 0.05$) at 1.0 MOI as compared to 0.1 MOI. Although cell supernatant (CS) fractions showed no statistically significant differences across various time points, the viral copy numbers were found to be higher at 0.1 MOI than at 1.0 MOI at 168 hpi.

To assess ASFV particle infectivity and to know the proportion of infectious virions and defective genomes, we quantified both HAD_{50} titers and qPCR genome copies, calculating their ratios (copies per HAD_{50}). At 1.0 MOI, ratios ranged from 374 (96 hpi) to 1,104 (48 hpi) for CA fractions and from 328 (72 hpi) to 656 (24 hpi) for CS fractions. At 0.1 MOI, ratios ranged from 257 (168 hpi) to 1123 (48 hpi) for CA fractions, and from 210 (24 hpi) to 653 (120 hpi) for CS fractions.

DISCUSSION

As ASF remains a major threat to the pig industry globally, understanding the replication behaviour of ASFV isolated in various parts of the world remains a priority in virology research. Replication of the ASF virus is predominantly restricted to monocyte/macrophage lineages, with PAM cultures serving as the ideal method for virus isolation and propagation of field isolates due to their physiological relevance.[28] This study provides the first detailed characterization of multistep growth kinetics of an Indian ASFV genotype II isolate in PAM cultures infected at low and high MOI, revealing robust infectivity and genome replication consistent with highly virulent strains.

The isolation and genetic characterization of the ASFV field isolate described in this study confirmed its classification within genotype II, exhibiting 100% sequence identity in the B646L (p72) gene with other Indian ASFV isolates reported previously.[6,7,29] The phylogenetic clustering with Asian and European genotype II strains aligns with the ongoing spread of this dominant ASFV lineage, as documented in outbreaks across the Indian subcontinent since 2020, as well as in the Asia-Pacific region.

The ASFV isolate used in the current study consistently exhibited the distinctive rosette-shaped hemadsorption (HAD) phenomenon across all five passages in PAM cells, confirming preservation of its CD2v (EP402R)-mediated erythrocyte binding capacity. This hallmark phenotypic trait represents a critical virulence determinant conserved across genotype II ASFV strains reported from Europe (Georgia/2007 lineage), Asia (China/Wuhan), and Africa.[25,28] These findings underscore the Indian isolate's alignment with globally circulating highly pathogenic forms, achieving a titre of $10^{6.25}$ HAD_{50}/ml at passage five. Following HAD formation, infected PAM cultures displayed characteristic cytopathic effects including cytoplasmic vacuolization, cell clustering, membrane blebbing, and progressive lysis from 72 hpi onwards. These morphological changes precisely mirror CPE patterns documented for other genotype II isolates such as ASFV YZ-1-3 and Armenia07, as well as genotype I (Espana75) and genotype X (Kenya05/Tk-1) isolates in primary PAM cultures.[30,31] These CPE patterns directly correlated with MOI-dependent replication dynamics observed in multistep growth curves. At high MOI (1.0), rapid CPE progression drove early cell-associated (CA) titer peaks at 120-144 hpi followed by significant declines at 168 hpi, reflecting virus-induced lysis and progeny release into cell supernatants (CS). In contrast, low MOI (0.1) infections exhibited slower initial replication, but with sustained CA and CS titers through 168 hpi without decline, indicative of slower cell-to-cell spread and persistent extracellular virus production.

These growth patterns demonstrate accelerated intracellular replication and earlier cell lysis under high-MOI (1 MOI) conditions, evidenced by earlier peak CA HAD_{50} titers. No significant difference ($P < 0.05$) was observed between 1.0 and 0.1 MOI up to 96 hpi, aligning with previous studies involving a genotype II ASFV Congo-a virus and a genotype IX ASFV-Kenya-IX-1033.[32,33] These kinetics suggest low-inoculum infection favours persistent extracellular virion release, a replication strategy potentially critical

for ASFV transmission via oronasal secretions in swine. Most previous ASFV growth kinetic studies involving various ASFV isolates concluded analyses at 120 hpi or earlier,[32,34-36], potentially missing late-stage replication dynamics critical for virus yield optimization in vitro.

Analysis of viral DNA copies per HAD_{50} provided insight into virion infectivity in PAM cells. In the current study, viral DNA copy numbers were significantly higher up to 72 hpi in CA fractions of 1.0 MOI infected cells, with higher DNA copies per HAD_{50}. This suggests that both infectious and non-infectious forms of the ASF virus were produced during their replication, with a higher tendency for accumulation of non-infectious DNA in cellular fractions at higher MOI infection. Lower minimum ratios observed at 0.1 MOI means a slightly higher infectivity efficiency at lower MOI, which indicates less interference between viruses and defective virions. CS ratios were consistently lower as compared to the CA fractions, indicating a lower proportion of defective particles in supernatants.

CONCLUSION

By extending observations to 168 hpi, this study reveals higher extracellular virus production with lower DNA copy to HAD ratio in PAM cultures infected at 0.1 MOI, demonstrating optimal duration for higher virus yield in cell culture supernatants, and provides further insights into prolonged ASFV replication relevant for isolate propagation, pathogenesis and vaccine development studies.

ACKNOWLEDGMENTS

The authors are grateful to the Indian Council of Agricultural Research, New Delhi, and the Director, ICAR–National Institute of High Security Animal Diseases, Bhopal, for facilities and funding. The authors also appreciate the Department of Animal Husbandry, Punjab, for sharing samples from disease outbreak.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This study was supported by the National Agricultural Science Fund (NASF) of ICAR through grant number NASF/BGAM-9005/2022-23.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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