Adaptation of African Swine Virus in Non-Swine Cell Lines: A Preliminary Study for Vaccine Candidate

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Abstract

African Swine Fever (ASF) is among the most detrimental infectious viral diseases in pigs causing approximately 100% mortality. The disease was first reported about 83 years ago in Africa before spreading to Europe in 1957 and Asia in 2010. An adequate vaccine generally containing live attenuated virus isolates prepared in swine macrophages to control the disease is currently unavailable. Therefore, this study aimed to use murine neuroblastoma (N2a) cells, non-swine cell lines, to adapt African swine fever virus (ASFV) isolates for vaccine preparation. ASFV isolate called BL21 obtained from Bali and East Nusa Tenggara was previously propagated in swine macrophages. However, virus was currently adapted in the N2a cells to avoid unwanted issues associated with using swine macrophages, including microbial contamination, as well as technically laborious and ethical issues. The adapted BL21 was re-confirmed with quantitative polymerase chain reaction (q-PCR) and tested *in vivo* to examine the pathogenicity properties. The results showed that BL21 produced consistently and specifically positive q-PCR, killing experimental pigs with typical gross pathological changes of ASF. BL21 at a 10^{-3}/mL dilution adapted in N2a cells showed similar antigenic properties causing the death of nearly 50% N2a cells *in vitro* and terminating all *in vivo* experimental pigs. In conclusion, the BL21 isolate reported in this study could be used as a vaccine candidate after more attenuation and particularly to determine a lethal dose of 50% (LD50) for future investigations.

Keywords: African swine fever, murine neuroblastoma cells, N2a cells, non-swine cell lines, pigs, vaccine candidate

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INTRODUCTION

African swine fever (ASF) is a very detrimental viral disease of swine (wild/feral and domestic), leading to severe hemorrhagic disease with nearly 100% mortality and a serious socioeconomic impact globally (Dixon *et al.*, 2020; Ruiz-Saenz *et al.*, 2022; Chathuranga and Lee, 2023). This was first reported in Kenya in 1921 before spreading to some European countries by 1957 (Montgomery, 1921; Cwynar *et al.*, 2019). Subsequently, in the early part of 2020, virus spread into thirteen Asian countries (Blome *et al.*, 2020; Ackerman *et al.*, 2022). ASF outbreak first reported in 2019 killed approximately 42,000 pigs in North Sumatra province, Indonesia (Kementan, 2020), as well as 3.5 million pigs during the following years in Bali and East Nusa Tenggara (FAO, 2022). Due to adequate vaccine absence, the disease was only controlled in the two infected regions by implementing quarantine regulations, farmer education, biosecurity, and strict sanitary procedures. However, these types of control measures are not effective, leading to failed prevention of the disease. ASF has become a

major threat to the global pork industry and is considered a legally notifiable disease that should be reported (WOAH, 2023).

African Swine Fever Virus (ASFV) is among the most fatal virus strains in the family *Asfarviridae* that targets pigs as host tropism (Blome *et al.*, 2020; Borca *et al.*, 2021). This virus has a unique form characterized by a multilayer structure and icosahedral morphology, with large and double-stranded genomic DNA of different sizes ranging from 170–193 kb, as well as a complex genome encoding many viral proteins (Forth *et al.*, 2023). Phylogenetic analysis showed that ASFV circulating in some Asian countries and Russia were identical due to comprising similar antigenicity (Dharmayanti *et al.*, 2021). ASF cases reported in Bali and East Nusa Tenggara tend to be caused by genetically identical ASFV. The two provinces are under the supervision of Disease Investigation Centre Region VI (DIC-Denpasar) which investigated the first ASF outbreak.

Virus primarily targets monocyte or macrophage lineages present in the bone marrow, lymphoid organs, or peripheral blood. However, the preparation of cells from pigs for *in vitro* studies was considered less practical due to being time-consuming, expensive, impossible for persistent sustenance in tissue culture, unsuitable for large-scale vaccine production, and associated with ethical issues (Franzoni, 2022; Liu *et al.*, 2023). Many *in vitro* studies using macrophages to observe virus-host interactions detected that cells responded differently to infection initiated by adapted ASFV (Montoya *et al.*, 2021; Bulu *et al*., 2023). In a recent investigation, locally isolated ASFV was propagated in swine macrophages but the preparation of cells was laborious (Tenaya *et al.*, 2023[b]). This condition becomes an obstacle to providing ASF vaccine because suitable cell lines are limited for a safe vaccine. ASFV adapted to non-porcine cells, such as monkey or human continuous cell lines, experienced genetic instability and loss of immunogenicity (Franzoni, 2022). Conclusive results have not been obtained from some porcine continuous cell lines applied as an alternative to primary macrophages. Many types of cell lines are investigated for isolating and propagation of ASFV (Franzoni, 2022), but the most suitable cells need to be adjusted as issues are often associated with the procurement of certain cells. A porcine kidney-derived clonal cell line PK1-C5 and macrophage-derived cell line IPKM recently found to be highly susceptible to ASFV (Borca *et al*., 2023; Cao *et al.*, 2024) are not yet available commercially. The use of stable cell lines to replace primary cell cultures was considered a priority in developing ASF vaccine (Muñoz-Pérez *et al*., 2021), leading to an attempt of obtaining reasonably feasible cell lines for this study. The most common cell lines used for viral studies include murine neuroblastoma (N2a) cells, which are quite safe for adapting rabies virus (Tenaya *et al*., 2023[a]). Cells are considered effective for use due being rapid, reliable, and easy to differentiate (Noviantari and Khariri, 2020). Data regarding the use of N2a cells for culturing ASFV are currently unvailable. Therefore, this study aimed to use N2a cells, non-swine cell lines, for adapting ASFV to prepare a vaccine. This was the first time to use cells for adapting ASFV because procurement of other commercial cell lines was difficult.

MATERIALS AND METHODS

Ethical Approval

The experimental animal used in this study was approved by the ethical commission of Udayana University with Letter No. B/217/UN14.2.9/PT.01.04/2022.

Study Period and Location

The study was conducted from April to November 2023 at the Laboratory of Immunology, Udayana University and the Disease Investigation Center VI of Denpasar, Bali.

Preparation of N2a Cells

The N2a cells used were provided by the Australian Animal Health Laboratory (AAHL, Gelong, Australia). Moreover, all tissue culture works were conducted in a type II biocontainment safety facility (Disease Investigation Centre

Reginal VI Denpasar-Bali) that implemented the International Standard for Laboratory Testing methodology (ISO/SNI 17015:2018), coded as LP:123 IDN. The preparation of cells was performed according to a previously published method (Tenaya *et al.,* 2023[a]) with minor modifications. An ampule of N2a stock was obtained from Liquid Nitrogen (LN) storage, thawed, diluted with 10 mL of Dulbecco's modified eagle medium (DMEM Cat#12491015 Thermo Fisher Scientific, Massachusetts, USA), and centrifuged at 500*g* for 5 minutes at 5ºC. After aspirating the supernatant, the cell pellet was resuspended in 10 mL of complete DMEM (CDMEM) supplemented with 2 g/mL of gentamicin, 0.3 g/mL of amphotericin B, and 0.1 mL of heat-inactivated fetal calf serum to obtain approximately 6.0×10^5 cells/mL. Cell suspension was placed in a 25 cm^2 cell culture flask (Cat# 3100-025x, Iwaki, Japan), then incubated at 37°C in 7% CO_2 and checked daily for contamination. When log phase cell growth was observed, cells were harvested after dissociation using 1 mL of 0.25% trypsin (Gibco Cat#2062476, Canada), resuspended in DMEM, and centrifuged 500*g* for 5 minutes at 5ºC. The supernatant was re-aspirated and the cell pellet was resuspended in 25 mL CDMEM containing not less than 6.0×10^5 cells/mL. Cell suspensions were re-subcultured into six of the 75 cm^2 cell culture flasks (Cat#3100-075x, Iwaki, Japan), and incubated as before until log phase cell growths were observed and ready to be infected with ASFV isolates.

Adaptation of ASFV in N2a Cells

The ASFV isolate named BL21 previously grown in swine macrophages (Tenaya *et al.,* 2023[b]) was used as a source of ASFV candidates. There were 14 isolates of the BL21 originating from 4 districts in Bali and 3 districts in East Nusa Tenggara, but only two were used in this study, including one representing Bali (Denpasar BL21a4) and another representing East Nusa Tenggara (Kupang City BL21b2). The two virus isolates were adapted in N2a cells (Table 1) and rapidly thawed from LN storage. After aspirating the supernatant, BL21a4 and BL21b2 were separately inserted aseptically into each of three different cell culture flasks containing log phase cell growth prepared according to the previous procedure. Both virus isolates in the flaks were gently agitated for even viral-cell contact, then incubated at 37°C for 30 minutes to allow viral absorption before carefully aspirating the supernatant containing the non-absorbed virus. Consecutively 20 mL of new CDMEM was added into each flask and incubated until cytotoxic effects (CPEs) appeared. Culture supernatants of the infected cells showing the strongest CPEs were collected and re-confirmed with Quantitative Polymerase Chain Reaction (q-PCR) before storage in LN for further study.

Isolation of ASFV-DNAs and q-PCR Analysis

The procedure for isolating ASFV DNAs and conducting q-PCR analysis was based on the previously reported work (Tenaya *et al.*, 2023[b]) with minor modifications. The DNA samples from the two different isolates (BL21a4 and BL21b2) were separately extracted with a DNeasy® blood and tissue isolation kit (Cat# 69504, Qiagen, UK), then used immediately or stored at −20°C until tested. A q-PCR test was based on the TaqMan PCR (Thermo Fisher Scientific) using a set of primers, i.e. forward (5' CTGCTCATGGTATCAATCTTATCGA '3) and reverse (5' GATACCACAAGATCRGCCGT '3), with probe (6FAM CCACGGGAGGAATACCAACCCAGT TAMRA). The target amplification of the primers is virus DNA with the conserved regions at the 3'-end of VP72 gen (King *et al.,* 2003). The composition of the master mix was 5.0 µL of PCR%-free water, 1 mL of primer forward (18 mg), 1 mL of primer reverse (18 mg), 12 µL of TaqMan universal, Primer Probe (5 mg), and 5 mL of DNA samples. The PCR products were amplified for 45 cycles with 4 conditions including denaturation at 50°C for 2 minutes, amplification at 95°C for 1 minutes, and extension at 95°C for 15 s followed by holding for 15 minutes until the fluorescence signal was measured using an ABI Prism 7200 sequence detection system (Applied Biosystems, USA).

A Preliminary *In vivo* Study of BL21a4 and BL21b2 in Pigs

The *in vivo* study was conducted using a published method (Yamada *et al.*, 2020) with modifications in Six 8-week-old ASFV-negative pigs, divided into three groups, each containing two pigs. The first group was injected intramuscularly with 1 mL of 10^{-3}/mL tissue culture infectious dose 50% (TCID50) of BL21a4 that caused nearly 50% CPEs on the infected N2a cells. The second group was injected with a similar preparation using 1 mL TCID50 of BL21b2 tissue culture supernatant. The third group was used as a negative control only injected with tissue culture supernatant containing no ASFV. Each group was housed separately in controlled cages and observed daily to record the development of clinical symptoms.

Data Analysis

The reported data were descriptively presented in the form of tables and images specifically for RT-PCR analysis and tissue cultures test. The prominent enlargement of the spleen found in the ASFV-infected pigs observed in this study was a significant and characteristic feature of pathological changes originating from the infection. This was not compared with a normal feature which was about 5 times smaller than the relatively similar body size of pigs, hence no statistical analysis was applied.

RESULTS AND DISCUSSION

The non-swine cells used showed a typical performance of N2a cells such as "triangle-shaped cells" firmly attached to the bottom of the tissue culture flask. On day 5 of the culture stages, there was rapid growth to form healthy monolayer cell lines without any microbial contaminations, suggesting the N2a cells were successfully propagated (Figure 1A). N2a cells applied for the first time in this study have not been reported for adaptation of ASFV, generally used to grow rabies virus strains (Zhao *et al.,* 2022; Tenaya *et al.*, 2023[a]; Li *et al.,* 2024). The main reason for using these cells for ASFV adaption was due to the difficulty in procuring other commercial cell lines. Although swine macrophages had been developed in the lab, the N2a cells were not consecutively used due to being laborious to prepare, time-consuming, expensive, and unsuitable for vaccine large-scale production, and associated with ethical or animal welfare issues (Purnama *et al*., 2020). ASFV was previously hypothesized to be capable of infecting many cells different from the types reported. Similarly, the rabies virus could infect N2a, BHK-21, and HEK-293 cell lines, as well as other cell types (Noviantari and Khariri, 2020). The adaptation of the N2a cells in this study following the tissue culture standard procedure (Segeritz and Vallier, 2017) was successful, as shown in Figure 1A.

The infection of cells with both ASFV isolates BL21a4 of Denpasar and BL21b2 of Kupang City caused CPEs, as observed from the death of most cells. Some of the dead cells floated on the supernatant of the cloudy-looking culture and no microbial contaminations were detected (data not shown) (Figure 1B). However, negative cells in non-infected ASFV flask steadily showed healthy-looking cells (Figure 1C). The other five out of the seven total isolates listed in Table 1 were not adapted to the N2A cells because of the consideration that results identical to the observations from BL21a4 or BL21b2 might be produced. Re-confirmation of tissue culture supernatant of BL21a4 and BL21b2 with q-PCR showed consistently positive results with specific DNA amplifications using the same set of primers as previously reported. The BL21a4 of Denpasar origin and the BL21b2 from Kupang city generated higher DNA quantity as presented by corresponding lines at the same cycle values and were higher than BL21 isolated from macrophages-infected ASFV (Tenaya *et al.,* 2023[b]) (Figure 2). Comparative results of epidemiological origin, code of ASFV isolates, and q-PCR results are presented in Table 2. Furthermore, experimental pigs infected using both isolates showed acute disease progress with general clinical symptoms, including severe hemorrhages, lethargy, as well as decreased appetite for eating and drinking. Blood sample collection and body temperature measurement were not carried out during the experiment due to

biosecurity reasons for not disturbing the animals and only clinical observations were detected. At days 12–15 post-infection, all ASFV-infected pigs died with characteristic pathological changes of ASF, specifically spleen enlargement to nearly double the normal size (Figure 3), while the control animals survived until the experiment was terminated.

Table 1. Epidemiological origin and list of ASFV isolates stored in LN

Province	Districts	Isolates	Code isolates
Bali	Badung	2	BL21a.1
	Gianyar	3	BL21a.2
	Karangasem	1	BL21a.3
	Denpasar	3	BL21a.4
Subtotal	4	9	4
East Nusa Tenggara	Sikka	2	BL21b.1
	Kupang City	2	BL21b.2
	Belu	1	BL21b.3
Subtotal	3	5	3
Total	7	14	7

Table 2. Epidemiological origin, code of strong ASFV isolates, and q-PCR results

Provinces	Districts	Code isolates	Quantity of DNAs
Bali	Badung	BL21a.1	Not tested
	Gianyar	BL21a.2	Not tested
	Karangasem	BL21a.3	Not tested
	Denpasar	BL21a.4	Very strong (++++)
Subtotal	4	4	
East Nusa Tenggara	Sikka	BL21b.1	Not tested
	Kota Kupang	BL21b.2	Strong (+++)
	Belu	Bl21b.3	Not tested
Subtotal	3	3	
Total	7	7	

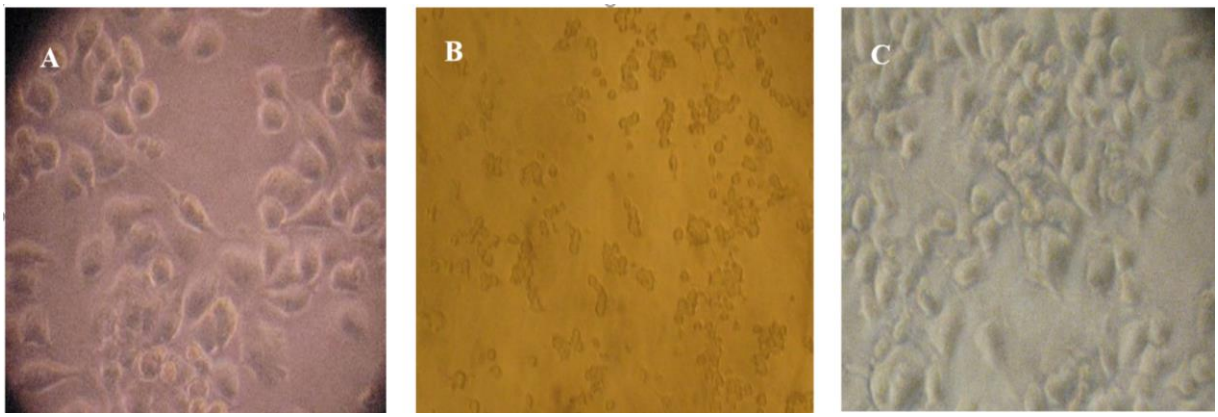

Figure 1. Preparation of N2a cells and an example adaptation/infection of BL21a4 in cells. (A) Normal N2a cells with a typical "triangle-shaped group of cells". (B) N2a cells infected with ASFV BL21a4 isolate caused CPEs (100×). (C) Negative N2a control cells at 5 days of culture (100×).

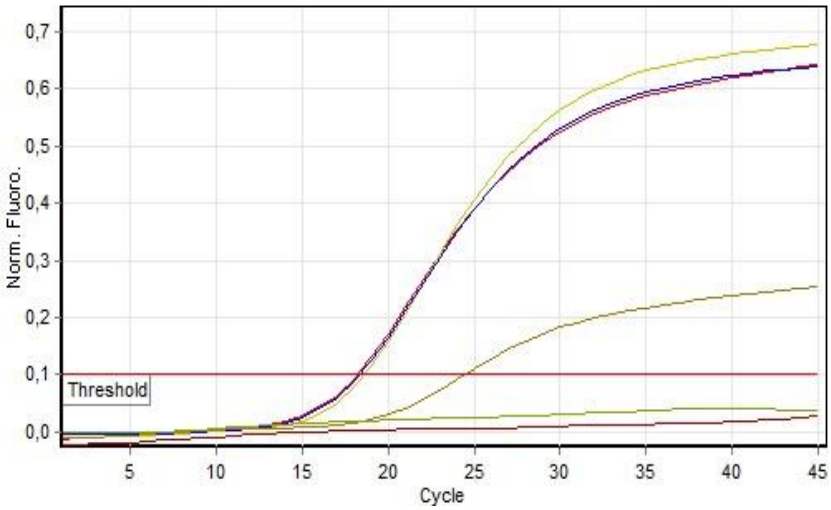

Figure 2. The q-PCR analysis of ASFV BL21 isolates adapted in N2a cells. The top line is DNA positive control, while the middle double lines represent DNAs of Bl21a4 (Bali) and Bl21b2 (East Nusa Tenggara), respectively. The lower line (above the Threshold line) is DNA isolated from macrophages-infected ASFV in the previous study and all negative controls are below the cycle threshold value. The CT value of BL21a4 and BL21b2 DNAs increased from 20 along with the value of the positive control, suggesting the content of ASFV-DNA concentration was significantly higher than those isolated from the previously published macrophage-infected ASFV (Tenaya *et al.*, 2023[b]).

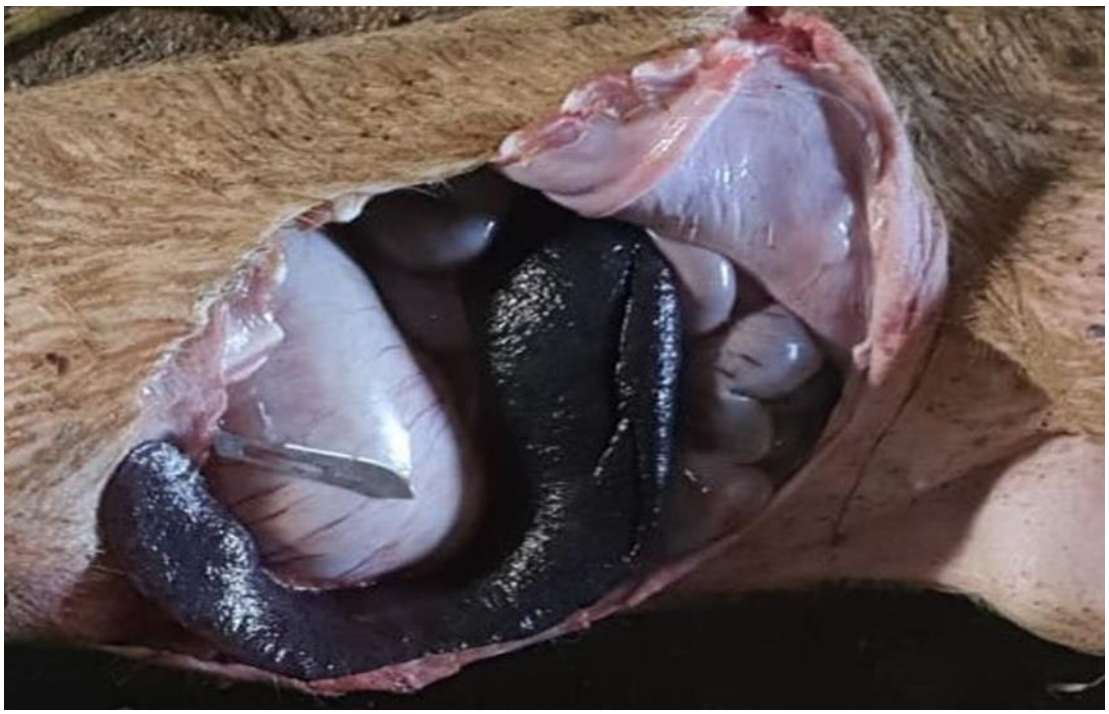

Figure 3. An experimental pig that died 12 days post-infection with BL21a4 showed classical clinical signs of ASF, a typical gross pathological change in the form of a prominently enlarged spleen.

In vitro and *in vivo* studies following infection of N2a cells with ASFV isolates of Bali and East Nusa Tenggara origins showed mutually supporting evidence. In tissue culture, both virus isolates in N2a cells caused CPEs as shown by serious cell damages similar to when swine macrophages were infected with ASFV (Tenaya *et al.*, 2023[b]). These data implied the supernatant

of the infected cells contained the propagated virus isolates and were positive with specific q-PCR test (Figure 2). The molecular evidence might signify the presence of BL21a4 and BL21b2 virus with highly stable genome in a suitable culture condition (Zheng *et al.,* 2023). There was no assurance that virus isolates proliferated naturally in cells because an electron microscopy study was not conducted to show cell-viral integration sites or morphological images, suggesting a need for further investigations. In other cases, the reduction of the non-target cells was not often related to the infection by various virus strains. For example, in equine immunodeficiency anemia virus (EIAV) infection, both CD4+ and CD8+ T-cell subsets in the blood reduced significantly during acute stages, although the main target cells were mature macrophages (Murakami *et al.,* 1999; Cook *et al.,* 2001). The reduction of the T-cell subsets might be due to an indirect effect of the infection or virus components (Murakami *et al.,* 1999). In a previous investigation regarding human immunodeficiency virus (HIV) infection, a phenomenon known as "bystander" depletion affected both uninfected and infected CD4+ T cells. This process led to the induction of cell-surface pro-apoptotic molecules, including Fas/FasL and undefined soluble mediators, triggering cell death (Ji *et al.,* 2007; Arokium *et al.,* 2009). Furthermore, a significant reduction of CD4+ cells during acute infection of Jembrana disease virus (JDV) in Bali cattle was not related to the target cells, namely mature-B and plasma cells (Desport *et al.,* 2009; Tenaya *et al.,* 2012).

All experimental pigs injected with 10^{-3}/mL of tissue culture supernatant of BL21a4 and BL21b2 died, presenting characteristic clinical and gross pathological changes related to ASFV infection (Figure 3). This viral concentration terminating nearly 50% of the N2a cells *in vitro* was primarily presumed to have killed 50% of the experimental pigs. Therefore, no correlation was found between the *in vitro* and *in vivo* studies conducted to determine the pathogenesis of BL21a4 and BL21b2, leading to the requirement of a lethal dose of 50% (LD50) of virus for future vaccine investigations. A limitation of the current study was the unavailability of monoclonal antibodies against the local ASFV isolate, signifying that immuno-histochemical exploration could be performed to identify virus presence in N2a-ASFV infected cells.

CONCLUSION

The results showed that the non-swine cell lines known as N2a cells could be substantially used for adapting ASFV isolates obtained from the two different epidemiological origins. The propagated virus isolates presented similar antigenic properties during *in vivo* and *in vitro* studies, but the LD50 should be determined for more vaccine investigations. Therefore, the N2a cell lines might be useful as an alternative media to grow ASFV, and more studies should be conducted to confirm viral and cell co-localization.

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AUTHORS' CONTRIBUTIONS

IWMT and KKA: Conceptualization and drafted the manuscript. IKS, IAPA, TKS and NMH: Prepared and worked cell culture. AAASSW, AAKS, IMS, INA, IPS: Conducted *in vivo* works and sample evaluation. IWW, NKAJ: Performed q-PCR analysis. NLD and IMD: Preparation of tables and figures. All authors have read and approve the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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