Development of Optimized Protocol for Culturing African Swine Fever Virus (ASFV) Field Isolates in MA104 Cells

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Abstract

I. Background: ASFV causes a highly contagious hemorrhagic disease with a high mortality rates in domestic pigs. The virus has been isolated across various cell lines, but identifying a cell line to develop an effective commercial vaccine has been challenging which a major obstacle to effective vaccine development is identifying a commercial cell line that is suitable for high-yield viral replication.

II. Methods and Results: The goal of this study was to identify a candidate commercial cell line for the replication of African swine fever virus (ASFV) by comparing several available cell lines with various medium factors. In the sensitivity test of cells, MA104 and MARC-145 had strong potential for ASFV replication. Next, MA104 cells were used to compare the adaptation of ASFV obtained from tissue homogenates and blood samples in various infectious media. At the 10th passage, the ASFV obtained from the blood sample had a significantly higher viral load than that obtained from the tissue sample ($P = 0.000$), exhibiting a mean Ct value = 20.39 ± 1.99 compared with 25.36 ± 2.11 . For blood samples, ASFV grew on infectious medium B more robustly than on infectious medium A ($P = 0.006$), corresponding to a Ct value = 19.58 ± 2.10 versus 21.20 ± 1.47 . ASFV originating from blood specimens continued to multiply gradually and peaked in the 15th passage, exhibiting a Ct value = 14.36 ± 0.22 in infectious medium B and a Ct value = 15.42 ± 0.14 in infectious medium A. However, there was no difference ($P = 0.062$) in ASFV growth between infectious media A and B when ASFV was cultured from tissue homogenates.

III. Conclusions: A model was developed to enhance ASFV replication through adaptation to MA104 cells and the lack of mutation in serial culture passages may serve to maintain the immunogenicity of ASFV isolates when they are developed as vaccine candidates.

1. Introduction

African swine fever virus (ASFV) is an enveloped icosahedral double-stranded DNA virus that belongs to the genus *Asfivirus* and family *Asfviridae* [1]. ASFV causes a highly contagious hemorrhagic disease with a high mortality rates in domestic pigs. The virus has been isolated across various cell lines, but identifying a cell line to develop an effective commercial vaccine has been challenging. Although many continuous cell lines, including Vero cells, are used for the propagation and titration of ASFV [2], the virus replicates most freely in a monocyte/macrophage lineage [3, 4]. Among the various types of macrophages assessed, pulmonary alveolar macrophages (PAMs) were suggested to be more susceptible to ASFV infection than bone marrow-derived macrophages or blood monocytes [5, 6]. The maturation stage of PAMs, which is relevant to the expression of surface molecules, may contribute to virus entry into the cell [7]. Despite the numerous advantages of PAMs, ethical constraints exist regarding these cells, as it is necessary to harvest large quantities to conduct a study. In addition, it is difficult to obtain consistent phenotypes of macrophages among different animals. Recently, a commercial cell type (MA104) was determined to be highly stable when used for the isolation of clinical samples [8].

In early 2019, ASFV invaded the pig population in northern Vietnam and was identified as genotype II, which was identical to the strain from China [9]. According to the history of this disease, pigs in Vietnam suffered the fastest spread of this virus; after only 9 months, 63/63 provinces announced the presence of the virus in pig herds, which caused heavy damage, threatening the stability of the pig herd in particular and the pork food production sector in general [10]. Therefore, research on the development of vaccines based on the prototype field virus is urgently required, but a major obstacle to effective vaccine development is identifying a commercial cell line that is suitable for high-yield viral replication. The goal of this study was to identify a candidate commercial cell line by comparing the levels of ASFV replication in several available cell lines with various medium factors.

2. Materials And Methods

2.1. Field sample and virus

The blood and visceral tissues of infected sows were collected from a farrow-to-finish, open-house family sow farm in southern Vietnam. The pathology in infected pigs was characteristic of the acute form, which was in keeping with the findings of previous reports [11]. Sows displayed anorexia, redness of skin, and high fever followed by sudden death, and growing pigs also showed clinical signs, including recumbency with a high fever (over 41°C) and dominant redness of skin followed by rapid death. The blood and fresh organ specimens were confirmed to be ASFV-positive by routine PCR, as recommended by the Office International des Epizooties (OIE, Paris, France). The DNA of the infected ASFV strain was amplified via PCR analyses, and the partial genetic segments encoding p72, p54 and p30 were sequenced using reference primers from previous reports [12–14], showing that the virus belonged to genotype II, which was entirely homologous to the first identified strain invading the Vietnamese pig population [9]. In addition, the ASFV sequences were submitted to GenBank (NCBI); they were named D/ASF/POT(TISSUE)/Vietnam/2019, D/ASF/POB(BLOOD)/Vietnam/2019) originating from fresh specimens and D/ASF/P1/Vietnam/2019, D/ASF/P5/Vietnam/2019, D/ASF/P10/Vietnam/2019 and D/ASF/P15/Vietnam/2019 from cultured viruses at passages 1, 5, 10, and 15, which had accession numbers as MW451088-92 for genetic sequences encoding p72, MW451094-98 for genetic sequences encoding p54, and MW451106-10 for genetic sequences encoding p30.

2.2. Cells

Cells were grown in Minimum Essential Eagle Medium-alpha modification (α -MEM) supplemented with 5% FBS, 100 U of penicillin/ml, and 100 μ g of streptomycin/ml. The cells were cultured in a humidified incubator with 5% CO₂ at 37°C. All cells (MA104, MARC-145, Vero, PK-15, PAM and BHK21) were provided by ChoongAng Vaccine Laboratories (CAVAC), Daejeon, 34055, Republic of Korea.

2.3. ASFV isolation and optimized protocol

The samples of infected pigs were determined ASFV-positive by RT-PCR and maintained in the laboratory. For tissue specimens, including spleen and lymph nodes were homogenized in PBS with 100 U of penicillin/ml and 100 μ g of streptomycin/ml, frozen and thawed three times and then clarified by

centrifugation at 12,000 *g* for 10 min at 4°C. The supernatants were collected, filtered through a 0.22 µm filter, and utilized to cell inoculation. The blood sample as well was collected and anticoagulated by EDTA from infected pigs then used for isolation.

In the first phase, an in vitro experiment was performed to detect ASFV-sensitive cell lines. Blood anticoagulated by EDTA from infected pigs had a Ct value = 20.21 (realtime PCR) and was used for isolation. Such cell lines as PAM, MARC-145, Vero, PK-15, MA104 and BHK21, described above, were used for the test. A mixing ratio of 50:50 diluted sample liquid and cells (1x10⁶ cell/ml) was employed, and cells were incubated for 3 days in 5% CO₂ at 37°C. After that step, the suspension was inoculated in 10 ml of the growth medium in a 25-ml flask. Infectious media (A and B) and α-MEM were used with individual cell lines to compare the adaptability of ASFV. Daily cell observation and viral load were identified by realtime PCR from 150 µl of the cultured supernatant and infected cells, and cells were subsequently stained to confirm the positivity of ASFV by immunocytochemistry (ICC) on day 7 after infection and incubation (passage 1). Next, the virus in the cultured fluid was subpassaged continuously 2 times (passages 2 and 3) in a similar manner.

After that step, MA104 cells were used to evaluate the progressive adaptation of ASFV. The infectious media (A and B) and two sample types (blood and tissue homogenate) from the same diseased sow were used to determine whether ASFV had different responses to the medium composition from different sample sources on MA104. A 50:50 diluted sample liquid and cells (1x10⁶ cell/ml) were mixed and incubated with 5% CO₂ at 37°C for 3 days, as described above. Next, the suspension was inoculated in 10 ml of the medium in a 25-ml flask. Daily cell observation and virus identification by realtime PCR from 150 µl of culture supernatant, as well as staining through ICC, confirmed the positivity of ASFV at 1, 3, 5 and 7 days. Next, the virus in the cultured fluid was continuously passaged further 14 times (passages 2 to 15) in a similar manner.

2.4. Realtime PCR

Detection of viral DNA in the cell culture supernatant was performed to evaluate virus production in the cells. Real-time polymerase chain reaction (qPCR) targeting p72 genes was performed on the infected culture supernatant of the cell line. The forward and reverse primers and probe were 5'-CTGCTCATGGTATCAATCTTATCGA-3' and 5'-GATACCACAAGATC(AG)GCCGT-3' and 5'-(FAM)-CCACGGGAGGAATACCAACCCAGTG-3'-(TAMRA), respectively ([15]; OIE recommendation). The results are presented as Ct values.

2.5. Immunocytochemistry (ICC) staining of cells in culture

Together with the realtime PCR, cells were specifically stained by immunocytochemistry, as previously described with only slight modifications [16], to determine the viral protein expressed by ASFV infection. At 7 dpi and under incubation at 37°C, the infected cells described above were fixed and permeabilized in 80% acetone for 30 min at -20°C. After fixation, immunocytochemistry was performed using commercially available monoclonal antibodies specific for the viral protein p30 (Humimmu, Salem, NH) to detect early protein synthesis.

2.5. Sequencing and genetic analysis in serial passages

DNA was extracted from blood and tissue homogenate before inoculation into cell culture (P0). Following passages 1 (P1), 5 (P5), 10 (P10), and 15 (P15), viral DNA from culture media was also obtained and subjected to comparative analysis of nucleotide and amino acid sequences through examination of amplified PCR products using reference primers from previous studies [12–14]. In this analysis, sequences encoding the major structural viral proteins such as p30, p54 and p72 were amplified, sequenced and analyzed using Mega X software. Bootstrap values were calculated based on 1000 replicates by the neighbor-joining method. The immunogenic proteins p30 and p54 were also further analyzed to determine whether there were any changes in the antigenic regions of ASFV among different culture passages and references [17, 18].

3. Results

The ASFV used in this study belonged to genotype II (Fig. 1), and the nucleotide sequences encoding p72, p54 and p30 were completely similar to those of the first ASFV strain previously published in northern Vietnam (Vietnam/Hanoi/2019), which was identified as originating in China.

The inoculation of ASFV into the PAM, MARC-145, Vero, PK15, MA104 and BHK21 cell lines, as monitored through 3 passages, showed that MA104 and MARC-145 have potential for ASFV replication (Table 1). With infectious medium B, in MA104 cells, the Ct value of ASFV DNA was 23.55, 22.14 and 19.95 at passages 1, 2 and 3, respectively, while in MARC-145 cells, the Ct value of ASFV DNA was 25.51, 26.31 and 25.37 at passages 1, 2, and 3, respectively. With infectious medium A, in MA104 cells, the Ct value of ASFV DNA was 23.87, 23.06, and 19.24 at passages 1, 2 and 3, respectively; whereas in MARC-145 cells, the Ct value of ASFV DNA was 23.81, 24.71 and 22.84 at passages 1, 2, and 3, respectively. Therefore, the findings showed that infectious media A and B are more suitable and optimal than α-MEM for the growth and adaptation of ASFV in this study (Table 1).

Table 1
The susceptibility of available cell lines to ASFV in various passages (pass#)

Media/Passage		PAM	MARC-145	Vero	PK-15	MA104	BHK21
Infectious medium B	Pass#1	na	25.51	na	na	23.55	na
	Pass#2	na	26.31	na	na	22.14	na
	Pass#3	na	25.37	na	na	19.95	na
	Mock	na	Neg	na	na	Neg	na
Infectious medium A	Pass#1	22.79	23.81	30.01	23.13	23.87	26.41
	Pass#2	29.19	24.71	34.43	29.34	23.06	Neg
	Pass#3	Neg	22.84	Neg	36.02	19.24	Neg
	Mock	Neg	Neg	Neg	Neg	Neg	Neg
α-MEM	Pass#1	26.75	30.08	33.12	25.02	28.18	30.42
	Pass#2	27.53	32.91	35.01	29.34	30.75	Neg
	Pass#3	Neg	36.23	Neg	33.48	Neg	Neg
	Mock	Neg	Neg	Neg	Neg	Neg	Neg
na, not available							

MA104 cells were used to compare ASFV growth and adaptation from tissue homogenate and blood sample on various infectious media (Fig. 2). At the 10th passage, the ASFV obtained from the blood sample had a significantly higher viral load than that obtained from the tissue sample ($P = 0.000$), exhibiting a Ct value = 20.39 ± 1.99 compared with 25.36 ± 2.11 . With blood sample, ASFV grew on infectious medium B better than on infectious medium A ($P = 0.006$), corresponding to a Ct value = 19.58 ± 2.10 compared to 21.20 ± 1.47 ; ASFV originating from blood specimen continued to multiply gradually and peaked in the 15th passage with a Ct value = 14.36 ± 0.22 in infectious medium B and a Ct value = 15.42 ± 0.14 in infectious medium A. However, there was no difference ($P = 0.062$) in ASFV growth between infectious media A and B when cultured from tissue homogenate. The results showed that in MA104 cells, ASFV isolation from blood sample under infectious medium B condition promoted optimal growth of this virus (black line in Fig. 2; Fig. 3). The sequences of nucleotide/amino acid encoding the p72, p54 and p30 of the ASFV collected from MA104 cell supernatants through passages P0, P1, P5, P10 and P15 were 100% homologous when aligned. Moreover, antigenic regions in proteins p30 and p54 were highly conserved among the various ASFV culture passages (Table 2).

Table 2
Comparison of the p30 and p54 antigenic regions of the studied ASFVs with those of other reference strains

Virus strain	Genotype	p30		p54	
		61-DIVKSARIYAGQGYTEHQAQEEWNMILHVL-90	96-ESSASSENIIH-105	Grp1 (65–75) EDIQFINPYQD	Grp2a (93–107) ATTASVGKPV TGRF
D/ASF/POT/Vietnam/2019	II				
D/ASF/POB/Vietnam/2019	II				
D/ASF/P1/Vietnam/2019	II				
D/ASF/P5/Vietnam/2019	II				
D/ASF/P10/Vietnam/2019	II				
D/ASF/P15/Vietnam/2019	II				
MK543947/Belgium/Etalle/wb/2018	II				
MK628478/Lithuania/LT14/1490/2014	II				
MT180393/Vietnam/NgheAn/2019	II				
MT166692/ Vietnam/Hanoi/2019	II				
MN172368/China/CAS19-01/2019	II				
AM712240/Portugal/OURT88/3	I	... H.	... S. .		... A.
MH025916/Uganda/R8/2015	I	.. RN.. .. Q.. ..	.. TS. TSLES		.. GN.. . I. D..

4. Discussion

Previous studies of ASFV isolation used primary pulmonary alveolar macrophage (PAM) cells because of their high adaptation [1, 2, 4, 6]; however, this cell line can only be utilized in research and is not a candidate to grow large amounts of ASFV for vaccine production due to high costs. In addition, there is also an issue of animal ethics with the large-scale collection of primary cells; meanwhile, several other commercial cell lines exhibit no evidence of stable replication of the ASFV. Recently, a commercial cell line (MA104) was determined to be highly stable when used for the isolation of clinical samples [8]. Our results in this study appear to be in keeping with the findings of the study by Rai et al [8]; however, the COVID-19 pandemic interrupted some of the final steps necessary to obtain sufficient scientific information to enable publication. However, it should be noted that the results obtained by our study and previous work [8] demonstrate that MA104 is not only a stable cell line for the isolation of ASFV obtained from clinical samples but also exhibits strong potential as a candidate cell line for commercial vaccine development.

A notable finding of this study was that in the same MA104 cells, ASFV from various sample sources (blood and tissue homogenate) exhibited significant differences in adaptability and replication. In addition, modification of the medium composition in the maintenance and growth medium for the same MA104 cells also affected viral growth over 15 passages. The viral load was calculated via the Ct value of realtime PCR, and the ICC staining technique used a p30 polyclonal antibody. Therefore, medium components may play essential roles in enhancing the reproduction of the ASFV in viral culture. The results of this study showed that there was gradual adaptation and higher growth of ASFV in the serial subpassages, particularly after the 10th to 15th passages. Interestingly, it was noted in this study that ASFV isolated from blood on MA104 cells exhibited considerably better adaptation up to the 10th passage; in contrast, ASFV isolated from tissue sample exhibited slow adaptation, and it was terminated after the 10th passage.

Other cell lines, such as PAM, MARC-145, Vero and PK-15, have been described by previous studies on the adaptive capacity of ASFV [5–7]. In a recent study, several established porcine cell lines are compared to PAM in terms of ASFV infection and production, however the cells expressed low levels of specific receptors linked to the monocyte/macrophage lineage with low levels of viral infection [19]. Interestingly, according to the results of this study, MA104 cells exhibited the most robust ASFV replication, which was in keeping with the findings of a recent publication [8]. Therefore, further research to determine the best biological properties, MOI, variability, and optimal medium composition for the development of ASFV in MA104 cells may facilitate the development of an effective ASF vaccine. This study developed a model to optimize the adaptation of ASFV to MA104 cells. The virus showed high growth potential, but no mutations occurred in structural proteins (p30, p54, and p72), and no change was observed in the antigenic regions of proteins p30 and p54. This lack of change is important in increasing the probability of maintaining immunogenicity when developing a vaccine candidate.

The evidence of propagation of ASFV in the cells in this study was determined by two methods as realtime PCR (viral load exhibits according to the Ct value) and immunocytochemistry (ICC), and highly homologous results were obtained. Hemadsorption (HA) is often the criterion for determining the presence of ASFV in assays, but required good blood quality for testing is not always available in every laboratory; moreover, some strains of virulent ASFV may not possess erythrocytic adsorption properties [20–22] and the application of ICC in MA104 cells with clear backgrounds is more effective than employing this technique in primary macrophage cells with peroxidase activity [8].

Declarations

Declaration of Competing Interest

The authors declare that there is no conflict of interest

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Author contributions

HIK, DDT, JYL and TNT designed study. HVV, QTVL and TMT collected the samples. HIK, DDT, SCL, MHK, DTTN, TMT, ATVL and TTNN performed the viral isolation, molecular laboratory works. DDT, TTNN and NMN performed genetic analyses. JYL, SCL, TNT, MHK provided technical assistance. DDT and HIK wrote the initial draft of the manuscript. JYL and TNT revised the initial draft of the manuscript. All authors read and approved the final manuscript

Compliance with ethical standards

Conflict of interest

All authors have declare that no conflicts of interest exist

Ethical approval

Permission to collect the sample and conduct the lab analysis and study from Center for Veterinary Diagnostics, Regional Animal Health Office No. 6, Ho Chi Minh City, Department of Animal Health, Vietnam, under the acceptance and approval by Department of Animal Health, Vietnam (DAH) and the Ministry of Education and Training, Vietnam (MOET): 2867-QĐ BGDDT, dated 01/10/2020.

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Figures

Figure 1

Phylogenetic tree of three nucleotide sequences encoded p30, p54, and p72 of ASFV used for isolation. The red circles represent the ASFV sequences from tissue homogenates and blood prior culture (D/ASF/POT(TISSUE)/Vietnam/2019; D/ASF/POB(BLOOD)/Vietnam/2019), and from cultured viruses at passage 1 (D/ASF/P1/Vietnam/2019), 5 (D/ASF/P5/Vietnam/2019), 10 (D/ASF/P10/Vietnam/2019) and 15 (D/ASF/P15/Vietnam/2019).

Figure 2

The dynamics of ASFV load (Ct_value realtime PCR) in MA104 cells over various culture passages (Pass#1 to Pass#15). The black curve represents the increase in viral load in several culture passages, presented by decreasing Ct value.

Figure 3

MA104 cells infected and mock infected with ASFV on the infectious medium A (a_mock culture, b_passage 1, c_passage 5, d_passage 10, and e_passage 15 and on the infectious medium B (f_mock culture, g_passage 1, h_passage 5, i_passage 10, and j_passage 15). The presence of the virus was determined by using a monoclonal antibody that detects ASFV protein p30 visualized using an immunoperoxidase assay.