In vitro primary porcine alveolar macrophage cell toxicity and African swine fever virus inactivation using five commercially supply compound disinfectants under various condition

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ABSTRACT. Efficacy of African swine fever virus (ASFV) inactivation using five commercially supply compound disinfectants was evaluated under various condition. Virucidal efficacy demonstrated that products A and E could inactivate at 1:800 within 1 min for both temperatures, while products B, C and D inactivated at 1:400. However, product D could inactivate at 1:800 when the exposure time was extended to 30 min and effected only 20°C. In addition, the cytotoxicity demonstrated that products A, B, C, D and E did not significantly affect to cell at 1:51,200, 1:12,800, 1:12,800, 1:25,600 and 1:12,800 dilution, respectively. In conclusion, these disinfectants could inactivate ASFV, however, the application of these products should be performed under safety precautions to prevent cytotoxicity in humans and animals.

KEY WORDS: African swine fever virus, commercially supply compound disinfectant, cytotoxicity, virucidal efficacy

African swine fever (ASF) was first identified and reported in East Africa in the early 1900s and ASF is listed as a "notifiable disease" by the World Organization for Animal Health (OIE), due to its high morbidity and mortality, resulting in substantial financial losses [5, 12, 15, 16]. ASF virus (ASFV) is a large, enveloped DNA-virus and its virion contains icosahedral capsid structural protein. The virus belongs to the *Asfarviridae* family and the *Asfivirus* genus [1, 4] and can persist in a pig farm environment, carcasses, and various swine products. The virus is transmitted to commercial pigs by vectors especially warthogs (*Phacochoerus africanus*), bush pigs (*Potamochoerus porcus* and *Potamochoerus larvatus*), and soft ticks (*Ornithodoros moubata*) [16] through transstadial and transovarial routes [6].

Concentrated biosecurity is mentioned in several research studies that could reduce the risk of infection via cleaning and disinfection [2]. ASFV can remain infectious for a long period of time in feces and blood. In the presence of organic materials, the virus might be even more stable and survive for longer [18]. Therefore, choosing a suitable disinfectant and applying it appropriately plays a crucial role in effective biosecurity. Some factors, including environmental conditions, contact time, pH and temperature ranges should be considered.

In the study, *in vitro* virucidal efficacy of five commercially supply compound disinfectants against ASFV, including host cell toxicity, were evaluated under various concentrations, exposure times and temperatures using primary porcine alveolar macrophage (PAM) culture.

Seven-week-old, ASFV seronegative and healthy pigs with polymerase chain reaction (PCR) negative for ASFV, porcine circovirus (PCV), classical swine fever virus (CSFV), and porcine respiratory and reproductive syndrome virus (PRRSV) were used for primary PAM cells preparation. A pig was euthanized and lung samples collected for PAM cells isolation. This *in vitro* study was approved by the Animal Welfare and Ethics Committee of Vietnam National University of Agriculture, Vietnam, and

all pigs were housed and used in an isolated area in the Biosecurity Animal Facility Centre of the Vietnam National University of Agriculture (VNUA), Hanoi, Vietnam (Ethics Approval number: VNUA-2021/05).

Primary PAM cells were cultured in a growth medium containing RPMI 1640 medium with 10% fetal calf serum and 1% penicillin-streptomycin solution (Gibco, Grand Island, NY, USA) into a 5% CO_2 incubator at 37°C. In addition, pig red blood cells (GE Healthcare, New York, NY, USA), were consumed and kept in a maintenance medium containing RPMI 1640 medium and 1% penicillin-streptomycin solution and stored at 4°C prior to testing. The ASFV, namely VNUA-ASFV-L01/HN/04/19, was propagated using primary PAM cells [21]. After harvesting, the virus was aliquoted and kept at −80°C until testing. In the present study, the viral concentration used at least 6.5 $\log_{10}$ hemadsorption (HAD_{50}/ml).

Five commercially supply compound disinfectant products, whose compositions are listed in Table 1, were examined for cell toxicity testing and ASFV virucidal efficacy, and produced from manufacturers in Thailand. Prior to testing, each product was diluted in distilled water at concentrations of 1:200, 1:400 and 1:800 up to 1:51,200. The cytotoxicity of all disinfectant samples was evaluated using primary PAM cells at 1:200 to 1:51,200 of disinfectant dilutions. Briefly, each dilution of disinfectant was added to PAM cells and incubated for 1 hr before the maintenance medium was added and incubated in a CO_2 incubator. The cell viability was observed daily for five days under an inverted microscope, and the cutoff of cells viability was indicated when live cells clung to the bottom of the tissue culture microplate for more than 80% of the five-day culturing period, compared with cell control.

Each disinfectant was diluted, such as 1:200, 1:400 and 1:800 for the ASFV inactivation study. Briefly, equal volumes of each dilution were mixed with ASFV and then incubated at 20°C or 4°C (on ice) for indicating times, such as 1 min, 5 min or 30 min. After that, the mixture was diluted as a 10-fold serial dilution using the maintenance medium and inoculated into PAM cells for virus recovery. To these treatments was added the maintenance medium supplemented with 2% pig red blood cells and incubated into a 5% CO_2 incubator, as described [3, 8, 21]. A massive cytopathic effect, namely HAD or rosette formation, was observed using an inverted microscope twice a day for five days. The virus titer was calculated as HAD_{50}/ml following the Reed and Muench method [9, 13]. Each treatment and the virus control were tested in triplicates, and the titers were reported in mean with standard deviation (SD).

In the present study, the reduction factor (RF) was calculated using the following equation: $RF=t_{pc} -t_a$; where t_{pc} is the titer converted into an index in $\log_{10}$ of the virus control, and t_a is the titer converted into index in $\log_{10}$ of the recovered virus from the treated sample. ASFV inactivation was considered effective when RF was greater than or equal to 3 [11, 19, 20]. However, the statistically significant determination was classified as different when the P value was <0.05, using the one-way analysis of variance (ANOVA) *post hoc* test (SPSS, Armonk, NY, USA).

Cytotoxicity of five commercially supply compound disinfectants, using PAM cells and pig red blood cells under various concentrations, is shown in Table 2. The cutoff of PAM cells viability was indicated when live cells clung to the bottom of tissue culture microplate more than 80% of the five-day culturing period, equivalent to the control well. The results of the cytotoxic study indicated that, products A, B, C, D and E did not cause a significant cytotoxic effect to PAM cells at 1:51,200, 1:12,800, 1:12,800,

Table 1. Composition or main agent of each commercially supply compound disinfectants/cocktail disinfectant including manufacturers

Product name	Composition	Manufacturers
A	Glutaraldehyde 5.0% w/v Didecyl dimethyl ammonium chloride 18.0% w/v Ethanol 6.0% w/v Akyl polyglycoside 0.5% w/v Polyethoxylated propoxylated alkyl alcohol 4.00% w/v	Mixwell Marketing Co., Ltd., Bangkok, Thailand
B	Didecyl dimethyl ammonium chloride 5.50% w/v Polyethoxylated propoxylated alkyl alcohol 3.00% w/v Ethyl alcohol 6.0% w/v N,N-bis (3-aminopropyl) Dodecylamine 2.3% w/v Acetic acid 0.75% w/v EDTA, sodium salt 1.2% w/v Polyethylene glycol 3.0% w/v	Mixwell Marketing Co., Ltd.
C	Glutaraldehyde 2.3% w/v Didecyl dimethyl ammonium chloride 5.5% w/v Polyethoxylated propoxylated alkyl alcohol 3.0% w/v Ethanol 6.0% w/v	Mixwell Marketing Co., Ltd.
D	Alkyl dimethyl benzyl ammonium chloride 2.20% w/v Octyl decyl dimethyl ammonium chloride 1.65% w/v Dioctyl dimethyl ammonium chloride 0.66% w/v Didecyl dimethyl ammonium chloride 0.99% w/v Polyethoxylated Propoxylated alkyl alcohol 2.50% w/v Sodium metasilicate 0.50% w/v	Mixwell Marketing Co., Ltd.
E	Didecyl dimethyl ammonium chloride 12% w/v Alkyl dimethyl benzyl ammonium chloride 8% w/v	Animal Supplement & Pharmaceutical Co., Ltd., Pathumthani, Thailand

1:25,600 and 1:12,800 dilution, respectively.

The efficacy of ASFV inactivation using five commercially supply compound disinfectants under various concentrations, temperatures and exposure times, is illustrated in Table 3. Products A and E could inactivate ASFV at 1:800 of dilution within 1 min at both temperatures, while products B, C and D inactivated the virus at 1:400. However, product D could inactivate the virus at 1:800 when the exposure time was extended to 30 min, but this effect was limited only at the incubated temperature of 20°C.

ASF has continued to spreading to several other countries, thus it is one of most dangerous threats to commercial pig industry

Table 2. The percentage of alive primary porcine alveolar macrophage (PAM) cells after incubation with a certain disinfectant of various concentrations

| Product name	Control	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200
A	100	0	0	0	0	0	0	0	30	90
B	100	0	0	0	0	0	0	85	100	100
C	100	0	0	0	0	0	0	85	100	100
D	100	0	0	0	0	0	0	70	100	100
E	100	0	0	0	0	0	0	80	100	100

Remarks: cutoff of cells viability was indicated when cell alive and cling to bottom more than 80% after 5 days culture, equivalent to the control well.

Table 3. The virus control and reduction factor ($\log_{10}$ HAD$_{50}$/ml) of African swine fever virus subsequent to treated by five commercially supply compound disinfectants/cocktail disinfectants under various concentrations, exposure times and temperatures

Product name	Temperature (C°)	Exposure time (min)	Virus control ($\log_{10}$ HAD$_{50}$/ml)	Mean ± SE of titer reduction ($\log_{10}$ HAD$_{50}$/ml) 1:200	1:400	1:800
A	4	1	6.78 ± 0.16	≥3.98 ± 0.16**	≥3.98 ± 0.16**	≥3.98 ± 0.16**
		5	6.78 ± 0.16	≥3.98 ± 0.16**	≥3.98 ± 0.16**	≥3.98 ± 0.16**
		30	6.80 ± 0.13	≥4.04 ± 0.08**	≥4.04 ± 0.08**	≥4.04 ± 0.08**
	20	1	6.74 ± 0.10	≥3.98 ± 0.10**	≥3.98 ± 0.10**	≥3.98 ± 0.10**
		5	6.74 ± 0.10	≥3.98 ± 0.10**	≥3.98 ± 0.10**	≥3.98 ± 0.10**
		30	6.84 ± 0.20	≥4.04 ± 0.20**	≥4.04 ± 0.20**	≥4.04 ± 0.20**
B	4	1	6.80 ± 0.00	≥4.00 ± 0.10**	≥4.00 ± 0.10**	2.55 ± 0.39
		5	6.84 ± 0.08	≥4.04 ± 0.08**	≥4.04 ± 0.08**	2.80 ± 0.33
		30	6.90 ± 0.09	≥4.10 ± 0.09**	≥4.10 ± 0.09**	2.75 ± 0.32
	20	1	6.72 ± 0.07	≥3.92 ± 0.17**	≥3.92 ± 0.17**	2.59 ± 0.23
		5	6.80 ± 0.18	≥4.00 ± 0.18**	≥4.00 ± 0.18**	2.90 ± 0.09
		30	6.78 ± 0.16	≥3.98 ± 0.16**	≥3.98 ± 0.16**	3.09 ± 0.08*
C	4	1	6.82 ± 0.15	≥4.02 ± 0.15**	3.90 ± 0.24*	3.00 ± 0.14*
		5	6.86 ± 0.10	≥4.06 ± 0.10**	3.62 ± 0.09*	2.97 ± 0.09
		30	6.86 ± 0.10	≥4.06 ± 0.10**	≥4.06 ± 0.10**	3.39 ± 0.10*
	20	1	6.80 ± 0.00	≥4.00 ± 0.00**	3.66 ± 0.48*	2.89 ± 0.19
		5	6.80 ± 0.13	≥3.87 ± 0.14**	3.67 ± 0.47*	2.84 ± 0.19
		30	6.80 ± 0.13	≥4.04 ± 0.08**	≥4.04 ± 0.08**	3.19 ± 0.12*
D	4	1	6.85 ± 0.14	≥4.05 ± 0.14**	3.17 ± 0.40*	2.24 ± 0.02
		5	6.88 ± 0.17	≥4.08 ± 0.17**	3.63 ± 0.50*	2.45 ± 0.13
		30	6.86 ± 0.10	≥4.06 ± 0.10**	≥4.06 ± 0.10**	2.90 ± 0.13
	20	1	6.70 ± 0.09	≥3.90 ± 0.09**	3.23 ± 0.66*	2.64 ± 0.20
		5	6.66 ± 0.03	≥3.86 ± 0.03**	3.64 ± 0.37*	2.85 ± 0.31
		30	6.80 ± 0.13	≥4.00 ± 0.13**	3.96 ± 0.07*	3.32 ± 0.29
E	4	1	6.78 ± 0.16	≥3.98 ± 0.16**	≥3.98 ± 0.16**	≥3.98 ± 0.16**
		5	6.78 ± 0.16	≥3.98 ± 0.16**	≥3.98 ± 0.16**	≥3.98 ± 0.16**
		30	6.84 ± 0.08	≥4.04 ± 0.08**	≥4.04 ± 0.08**	≥4.04 ± 0.08**
	20	1	6.70 ± 0.09	≥3.90 ± 0.09**	≥3.90 ± 0.09**	≥3.90 ± 0.09**
		5	6.70 ± 0.09	≥3.90 ± 0.09**	≥3.90 ± 0.09**	≥3.90 ± 0.09**
		30	6.80 ± 0.18	≥4.00 ± 0.18**	≥4.00 ± 0.18**	≥4.00 ± 0.18**

* Virus inactivation regarded as effective when reduction factor was greater than or equal to 3 and indicated to be statistically significant ($P<0.05$). ** Indicates that recovery virus could not be detected in that condition so the reduction factor was showed ≥ mean ± standard deviation and also significantly inactivating effective.
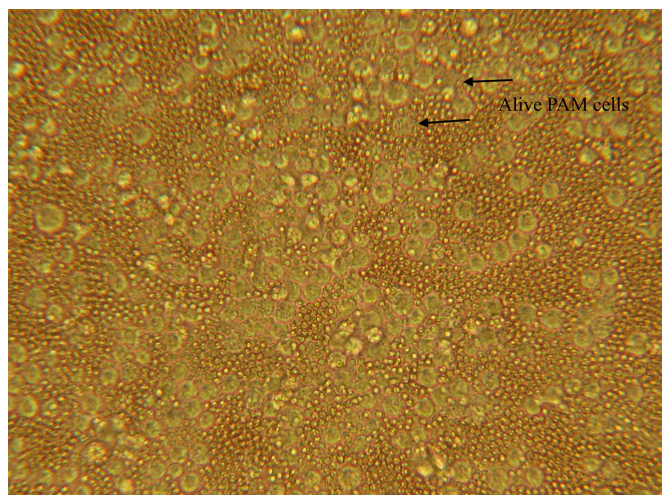
Fig. 1. Representative microscopic image (magnification 200×) of alive porcine alveolar macrophage cells or non-hemadsorption.
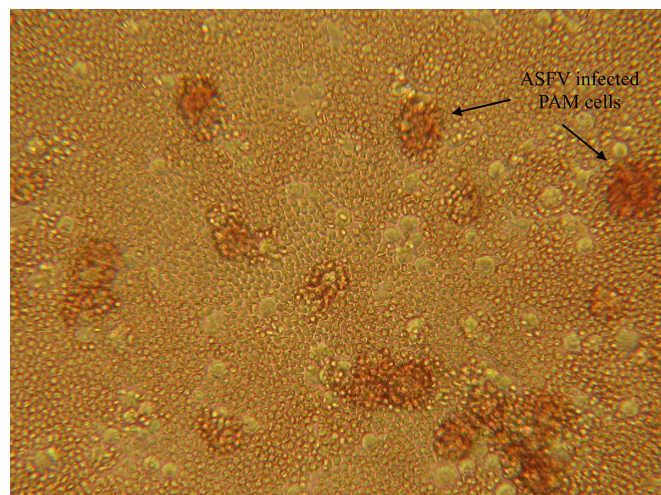
Fig. 2. Representative microscopic image (magnification 200×) of African swine fever virus infected porcine alveolar macrophage cells that showed hemadsorption.

due to an absence of an available, effective vaccine and treatment. Recently, commercial pig farms in China and Southeast Asian countries have been attacked by ASF. More than half of the world's pig population has been destroyed by these virus outbreaks [16], therefore, appropriate biosecurity measures, including a disinfection strategy, play an important role in disease prevention. Disinfectant selection and appropriated use are crucial for effective biosecurity to control the disease, so the efficacy of disinfectants must be evaluated before their application in real-world situations. In the present study, five commercially supply compound disinfectants were evaluated *in vitro* for host cell toxicity and virucidal efficacy against ASFV under various concentrations, exposure timings, and temperatures.

The PAM cells were prepared from the specific pathogen-free piglets and alive or normal PAM cells are shown in Fig. 1. Indicator or cytopathic effects or infected cell observation, is the ability to adsorb pig red blood cells around PAM cells, namely, hemadsorption (HAD) or rosette formation, as shown in Fig. 2. Several researchers have demonstrated that infected cells with wildtype of ASFV could absorb pig red blood cells, called hemadsorption [3, 7, 21] and Kouam *et al*. [10] and Revilla *et al*. [14] have described pig red blood cells around infected swine monocytes, namely rosettes formations, are both characteristic features of ASFV-infected cells. This characteristic has been successfully exploited to differentiate the ASFV from other agents which were consumed for recovered virus and virus titration.

In the present study, the disinfectant samples were produced by two manufacturers in Thailand and are commonly referred to as 'commercially supply compound disinfectants' or 'cocktail disinfectants' where each product contains multiple active ingredients (Table 1). The purpose of such designed commercially supply compound disinfectants/cocktail disinfectants is to enhance the synergistic effect of each main agent/ingredient which might increase the virucidal efficacy. Juszkiewicz *et al*. [8] reported the effectiveness of the chemical compounds used against ASFV in commercially available disinfectants, especially glutaraldehyde, quaternary ammonium compound and acetic acid, which showed effective percentages of 0.1%, 0.5% and 2%, respectively. Moreover, Shirai *et al*. [17], demonstrated that quaternary ammonium compound could inactivate enveloped viruses and African horse sickness virus at room temperature within 30 min at 0.003% of concentration. However, in the present study, it was found that all five products at 1:200 of dilution could affect ASFV more than a 4 $\log_{10}$ reduction at 30 min at both temperatures, where: (i) The tested concentration of glutaraldehyde in products A and C was 0.025% and 0.0115%, respectively, (ii) Quaternary ammonium compound in products A, B, C, D and E, as 0.09%, 0.0275%, 0.0275%, 0.0275% and 0.1%, respectively, (iii) Acetic acid in product B as 0.00375%. According to the possibility of the synergistic effect, the concentration of active ingredients in mixed/cocktail disinfectants was lower than single active ingredients of the referenced disinfectants [7, 8], which belong to common chemical reagents, especially glutaraldehyde, quaternary ammonium compound, acetic acid, and ethyl alcohol. In addition, all cocktail disinfectants possess a "Material Safety Data Sheet" and "Thai Registration Document" from the Department of Livestock Development, Ministry of Agriculture and Cooperatives, Thailand. Therefore, present commercially supply compound disinfectants are confirmed for safety and toxicity towards animals, humans and the environment.

The cytotoxicity testing demonstrated that all five products in the present study, had high cytotoxicity and must be diluted up to 1:12,800, so none of the disinfectant samples affected the PAM cells. These results correlated well with the study of Juszkiewicz *et al*. [7] which showed high cytotoxicity after using quaternary ammonium compound and glutaraldehyde. These results indicated that the present disinfectant products should not be used directly on humans or animals according to their cytotoxic effects. However, these products may be useful as an excellent disinfectant, especially on fomites, around pig houses, cases or trucks for biosecurity enhancement aiming to control ASF in pig farms. There are many disinfectants that cannot be used directly on people

or animals, but can be used to disinfect animal facilities.

The highlight of the present study is the virucidal efficacy of five commercially supply compound disinfectants. The trial outcome summarized that all commercially supply compound disinfectants could inactivate ASFV under various conditions, such as different concentrations, temperatures, and contact times. However, the application of these products should be performed under safety precautions to prevent cytotoxicity in humans and animals.

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