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Short communication

Efficient inactivation of African swine fever virus by ozonized water

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ABSTRACT

African swine fever virus (ASFV) is the causative agent of African swine fever (ASF), which is a devastating disease of domestic pigs and wild boar, causing significant economic losses to the pig industry worldwide. To evaluate the ability of ozonized water as a disinfectant to inactivate ASFV, ozonized water of different concentrations was tested, and the viral reduction was determined by infectivity assay on porcine primary alveolar macrophages. The results showed that 2 log10 (99 %) reduction in viral titer was observed when $10^{4.0}$ $TCID_{50}$/mL wild-type or reporter ASFV was inactivated with ozonized water as lower as 5 mg/L within 1 min at room temperature; while a viral reduction of approximately 2 log10 (99 %) was observed when $10^{5.0}$ $TCID_{50}$/mL wild-type or reporter ASFV was inactivated with 5 mg/L ozonized water within 1 min, and 3 log10 (99.9 %) virus was inactivated by 10 or 20 mg/L ozonized water within 3 or 1 min, respectively; furthermore, 5 mg/L ozonized water inactivated 2 log10 (99 %) reporter ASFV as higher as $10^{6.75}$ $TCID_{50}$/mL in 1 min, and a viral reduction of approximately 3 log10 (99.9 %) in reporter ASFV or 2 log10 (99 %) in wild-type virus was observed when inactivated with 10 mg/L ozonized water in 1 min; meanwhile, a viral reduction of 3 log10 (99.9 %) was observed when 20 mg/L ozonized water was applied to the wild-type ASFV of $10^{6.75}$ $TCID_{50}$/mL in 3 min. Overall, ozonized water can rapidly and efficiently inactivate ASFV, representing an effective disinfectant for ASF control.

1. Introduction

African swine fever (ASF) is an OIE (World Organization for Animal Health)-listed devastating disease of domestic pigs and wild boar, causing significant economic losses to the pig industry worldwide. The disease is caused by African swine fever virus (ASFV), a large, enveloped, double-stranded DNA virus belonging to the genus *Asfivirus* of the family *Asfarviridae*. The virus can infect pigs of all ages. Acute clinical forms of ASF are accompanied by hyperthermia, skin cyanosis and organ bleeding with a high mortality up to 100 % (Costard et al., 2013; Dixon et al., 2020).

ASF mainly exsisted in Africa and rarely introduced to other continents until 2007, when it was reported in Georgia (Rowlands et al., 2008). Then it rapidly spread across the Caucasus and into the Russian Federation and reached the European Union (EU) in 2014 (Gogin et al., 2013; World Organization for Animal Health (OIE, 2014). In August 2018, ASF emerged in China for the first time (Zhou et al., 2018) and then spread rapidly across all provinces and municipalities. And subsequently it spread to a number of Asia countries (World Organization for Animal Health (OIE, 2019). The continuous spread of the disease through Africa, Europe, Russian Federation and Asia countries, especially China, the biggest pig producer in the world, make the situation worsen. The worldwide prevalence of ASF has led to high socio-economic impacts on the global pig industry and trade (Dixon et al., 2020).

ASFV can be transmitted through different routes such as direct or indirect contact with infected pigs and their secretions, excretions, blood, tissues, pork and pork products, as well as contaminated transport vehicles, feed, water, personnel, etc. (Dixon et al., 2020; Luo et al., 2018). The survivability of ASFV in the environment and different biological matrices means that contaminated materials play an important role in transmission (Dixon et al., 2020; Petrini et al., 2019). As there are no effective vaccines and treatment available for ASF, quarantine and strict biosecurity measures are important for prevention and control of the disease. Considering the severe situation of ASF spreading in China and other countries, there is a pressing need to improve the

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Table 1
Evaluation of the cytotoxicity of ozonized water, neutralizer and tap water on PAMs.

Group	Treatment	ASFV titer (log10 $TCID_{50}$/mL)
1	(Ozonized water + ASFV) + Neutralizer	− ∞
2	(Ozonized water + ASFV) + Medium	− ∞
3	(Neutralizer + ASFV) + Medium	4.5
4	(Ozonized water + Neutralizer) + ASFV	4.41 ± 0.14
5	(Sterile tap water + ASFV) + Medium	4.66 ± 0.14
6	Medium only	− ∞

Table 2
Effects of ozonized water, neutralizer and tap water on the viability of PAMs.

	Incubation time (h)	Cell viability (%)
5 mg/L ozonized water	4	91.56 ± 2.04
	72	93.49 ± 4.28
10 mg/L ozonized water	4	86.38 ± 3.12
	72	89.29 ± 2.87
20 mg/L ozonized water	4	88.31 ± 4.42
	72	88.12 ± 4.75
Neutralizer	4	98.30 ± 6.05
	72	100.70 ± 6.93
Tap water	4	89.63 ± 4.52
	72	88.89 ± 6.07
Medium only	4	100.00 ± 1.72
	72	100.00 ± 0.75

Table 3
Stability of the ozonized water over time.

Starting concentration (mg/L)	Time (h)				
	24	48	72	96	120
5	3	1.16	0.56	0.27	0
10	4.9	1.66	0.52	0.34	0

control strategies for ASF. Effective disinfectants for inactivating ASFV are extremely essential for implementation of strict biosecurity measures.

The ideal disinfectant should have the advantages of fast and high efficiency, low toxicity, broad antimicrobial spectrum and stable nature (Bicknell and Jain, 2001). Ozone (O_3), also known as superoxide, is an allotrope of oxygen (O_2). Ozone is a special odor gas at room temperature and soluble in water. Ozonized water has strong oxidation ability, broad spectrum of sterilization and disinfection, and fast sterilization and disinfection functions, which is stable and non-toxic and harmless. Furthermore, oxygen is generated after decomposition, without any residue and side effects, so it is widely used in food industry and medical areas (Wang, 2004; Ye, 2008). Previous studies have shown that ozonized water can inactivate RNA viruses, such as vesicular stomatitis virus (Zhang et al., 1998), Norwalk virus (Shin and Sobsey, 2003), poliovirus 1 (Shin and Sobsey, 2003), severe acute respiratory syndrome coronavirus (Zhang et al., 2004) and porcine epidemic diarrhea virus (Guo et al., 2016), indicating that ozonized water has a good inactivation ability for these viruses. So far, there is no report regarding the inactivation effect of ozonized water on DNA viruses.

In view of the urgent need for ASF biosecurity prevention and control in China, in this study we evaluated the inactivation effects of ozonized water produced by electrolytic ozonize generator on ASFV.

2. Materials and methods

2.1. Preparation of cells and viruses

Primary porcine alveolar macrophages (PAMs) were prepared from 3-week-old specific-pathogen-free (SPF) pigs and maintained in RPMI 1640 medium (Gibco, USA) containing 10 % fetal bovine serum (FBS) (Gibco, USA), 200 mg/mL streptomycin and 200 IU/mL penicillin. PAMs (10^6 cells/mL) were seeded into 6-well plates with 2 mL per well or 96-well plates with 100 μL per well and incubated at 37 ˚C in a humidified incubator with 5 % CO_2. Following tests were conducted after cell adherence (4–6 h or overnight). All experiments with ASFV in this study were performed in the biosafety level 3 (BSL-3) facilities in

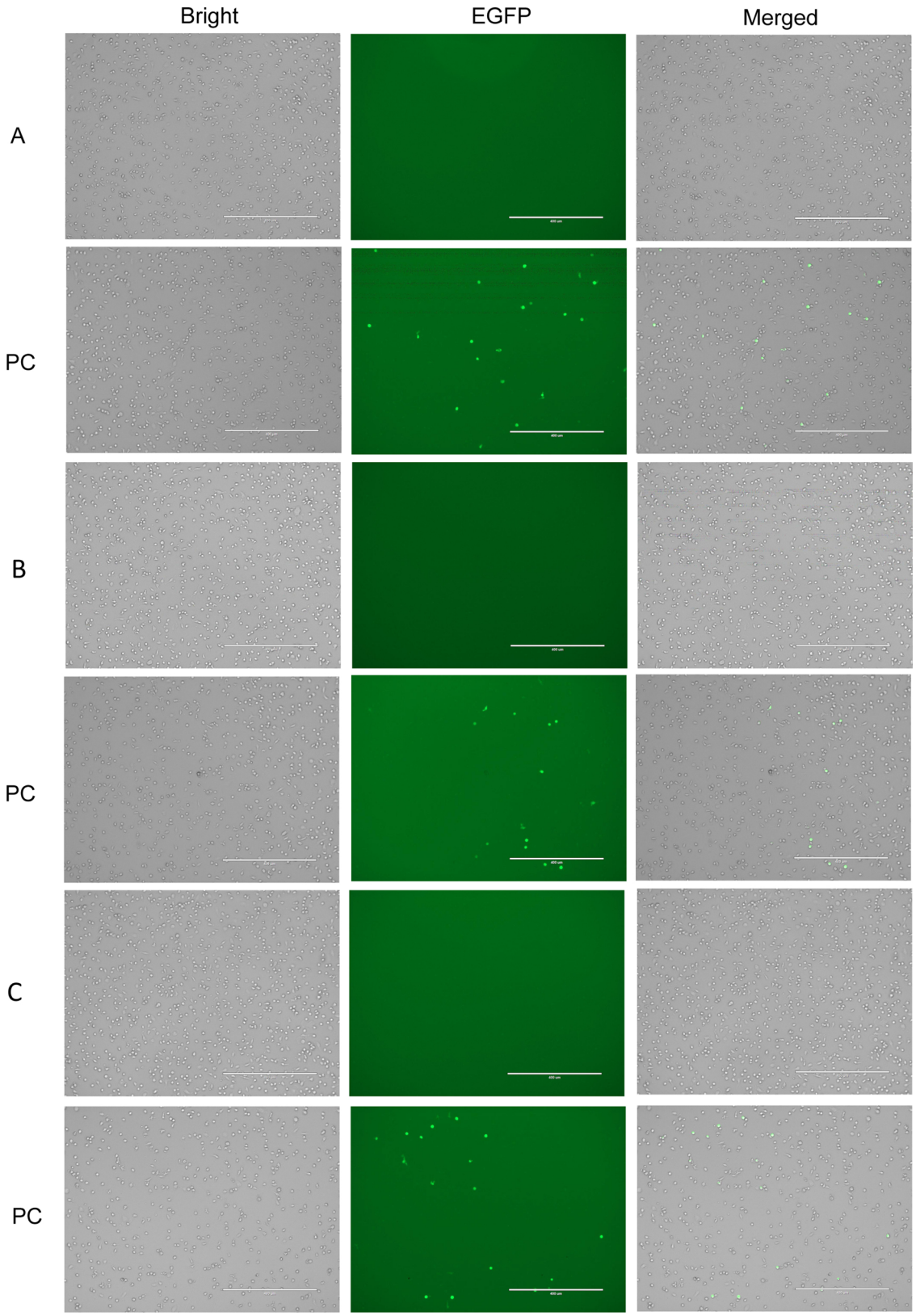

Fig. 1. Inactivation of $10^{4.0}$ $TCID_{50}$/mL reporter ASFV by ozonized water. $10^{4.0}$ $TCID_{50}$/mL ASFV was inactivated with 5 mg/L ozonized water for 1 (A) or 3 min (B) or 10 mg/L ozonized water for 1 min (C), and stopped by the neutralizer and the resulting viral titer was determined by infectivity assay on PAMs. PC, positive control.

Table 4
Inactivation of $10^{4.0}$ $TCID_{50}$/mL ASFV by ozonized water.

Virus	Time (min)	5 mg/L		10 mg/L		20 mg/L	
		Viral titer (log10 $TCID_{50}$/mL)	Log10 reduction value	Viral titer (log10 $TCID_{50}$/mL)	Log10 reduction value	Viral titer (log10 $TCID_{50}$/mL)	Log10 reduction value
Reporter ASFV	1	$-\infty$	2.0	$-\infty$	2.0	$-\infty$	2.0
	3	$-\infty$	2.0	$-\infty$	2.0	$-\infty$	2.0
	6	$-\infty$	2.0	$-\infty$	2.0	$-\infty$	2.0
	10	$-\infty$	2.0	$-\infty$	2.0	$-\infty$	2.0
WT-ASFV	1	$-\infty$	2.0	$-\infty$	2.0	$-\infty$	2.0
	3	$-\infty$	2.0	$-\infty$	2.0	$-\infty$	2.0
	6	$-\infty$	2.0	$-\infty$	2.0	$-\infty$	2.0
	10	$-\infty$	2.0	$-\infty$	2.0	$-\infty$	2.0

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The wild-type ASFV SY18 strain (WT-ASFV) or the reporter ASFV (ASFV-ΔMGF-EGFP, with deletion of the MGF gene and introduction of the EGFP reporter based on the SY18 strain) (Zhou et al., 2018) was inoculated into PAMs (6-well plates) and incubated at 37 ˚C for 72–96 h. After two freeze-thaw cycles, the cell cultures were centrifuged at 2000 $\times g$ for 10 min to remove cells and other debris and the supernatant were collected and stored at −80 ˚C. The viral titer was determined by the method of Reed and Muench (Reed and Muench, 1938).

2.2. Preparation of ozonized water

Ozonized water was prepared by using an electrolytic ozonize generator produced by Hangzhou Qingwei Science and Technology Co. Ltd. Firstly, tap water was put into a non-conductive container (a glass beaker), and the electrical conductivity was measured using a conductivity meter and adjusted to be 400–800 μs/cm. Subsequently, electrode was inserted into the water and energized. After stirring for 2–4 min, the Ozonize concentration in water was determined by the iodometric method (Shi et al., 2007). The stability of ozonized water (0–120 h) was determined.

2.3. Cytotoxicity assay

To evaluate the cytotoxicity of ozonized water, neutralizer and neutralizing products on cells, reporter ASFV ($10^{6.75}$ $TCID_{50}$/mL) were incubated at room temperature with 20 mg/L of ozonized water (1:9, v/v) for 10 min, following by addition of neutralizer (PBS containing 5 g/L sodium thiosulfate and 10 % FBS) (Group 1 in Table 1) or RPMI 1640 medium (Group 2) (1:9, v/v). After 10-min incubation at room temperature, PAMs (in 96-well plates) were inoculated with the 100-μL mixtures and incubated at 37 ˚C for 3 days with 5% CO_2. The same procedures were performed for Groups 3–5 and medium only (Group 6) as a negative control (Table 1). Also, different concentrations of ozonized water (5, 10 and 20 mg/L), neutralizer or tap water were separately tested without ASFV. The effect of these treatments on the cells was determined using Cell Counting Kit-8 (CCK-8) (Dojindo) according to the manufacturer's instructions and the Technical Specification for Disinfection (The Ministry of Health of the People's Republic of China, 2017) (Table 2). At the same time, the pH values of ozonized water and neutralizer were determined, which were 7.39 ± 0.09 and 8.24 ± 0.04, respectively. Meanwhile, the inactivation effect of ozonized water on the reporter ASFV was evaluated by infectivity assays on PAMs and the viral titer was determined as described previously (Reed and Muench, 1938; Li and Yang, 2012). Average values and standard deviations of three independent experiments were calculated.

2.4. Evaluation of inactivation effect of ozonized water against ASFV

WT-ASFV or reporter ASFV ($10^{4.0}$, $10^{5.0}$ and $10^{6.75}$ $TCID_{50}$/mL) were incubated at room temperature (20−25 ˚C) with different concentrations of ozonized water (5, 10 and 20 mg/L) (1:9, v/v), respectively, for 1, 3, 6 or 10 min. Then neutralizer (9:1, v/v) was added. After 10-min incubation at room temperature, PAMs (in 96-well plates) were inoculated with the mixtures and incubated at 37 ˚C with 5 % CO_2. The fluorescence and cell viability were observed at 3–4 days post-inoculation (dpi) for reporter ASFV group. For WT-ASFV group, the cells were fixed with 4 % paraformaldehyde for 20 min. Subsequently, anti-ASFV p72 monoclonal antibody 1:1000 diluted with PBS containing 5% bovine serum albumin (BSA) was applied as the first antibody on the fixed plates and incubated for 2 h at 37 ˚C. After six washes with PBST, Alexa-Fluor® 488 conjugated goat anti-mouse IgG (Invitrogen) 1:500 diluted in PBS-BSA was further applied as a secondary antibody and incubated for 1 h at 37 ˚C. The plates were washed six times with PBST and mounted by adding 30 μL of a 50 % glycerol-PBS (v/v) solution. The plates were examined under an EVOS Cell Imaging System (Thermo Fisher Scientific, USA). Finally, viral titers were determined and the inactivation effect of ozonized water on ASFV was evaluated. Average values and standard deviations of three independent experiments were calculated.

3. Results

3.1. Ozonized water was relatively stable over time

The stability of ozonized water was assessed by determination of the concentration over time (0–120 h). The results showed that the half-life of the ozone water was approximately 24 h and which was completely degraded within 96–120 h (Table 3).

3.2. Both the neutralizer and ozonized water did not affect cell viability

The cytotoxicity assay showed that over 86 % to near 100 % cell livability could be achieved following addition of different

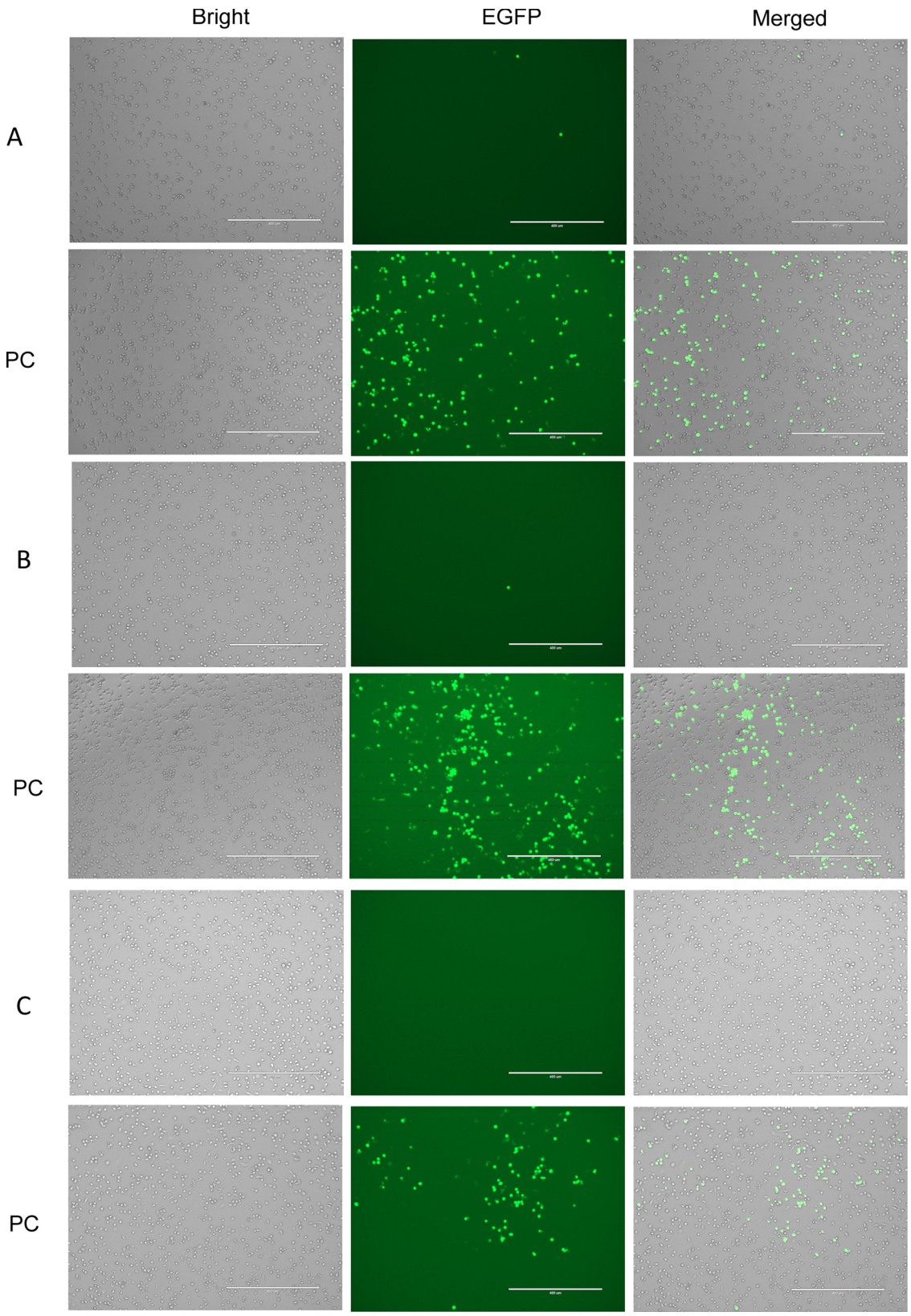

Fig. 2. Inactivation of $10^{5.0}$ $TCID_{50}$/mL reporter ASFV by ozonized water. $10^{5.0}$ $TCID_{50}$/mL ASFV was incubated with 5 (A), 10 (B) or 20 (C) mg/L ozonized water for 1 min and stopped by the neutralizer and the resulting viral titer was determined by infectivity assay on PAMs. PC, positive control.

Table 5
Inactivation of $10^{5.0}$ $TCID_{50}$/mL ASFV by ozonized water.

Virus	Time (min)	5 mg/L		10 mg/L		20 mg/L	
		Viral titer (log10 $TCID_{50}$/mL)	Log10 reduction value	Viral titer (log10 $TCID_{50}$/mL)	Log10 reduction value	Viral titer (log10 $TCID_{50}$/mL)	Log10 reduction value
Reporter ASFV	1	0.75	2.25	0.50	2.50	$-\infty$	3.0
	3	0.58 ± 0.14	2.42 ± 0.14	$-\infty$	3.0	$-\infty$	3.0
	6	0.50	2.50	$-\infty$	3.0	$-\infty$	3.0
	10	0.50	2.50	$-\infty$	3.0	$-\infty$	3.0
WT-ASFV	1	1.0 ± 0.25	2.0 ± 0.25	0.50	2.50	$-\infty$	3.0
	3	0.75	2.25	$-\infty$	3.0	$-\infty$	3.0
	6	0.50	2.50	$-\infty$	3.0	$-\infty$	3.0
	10	0.50	2.50	$-\infty$	3.0	$-\infty$	3.0

concentrations of ozonized water (5, 10 and 20 mg/L), neutralizer or tap water from 4 to 72 h, and also the viral titers in treatment groups 3–5 were near to 4.75 log10 (1/100 of the starting titer), indicating that these treatments did not significantly affect cell viability (Tables 1 and 2).

3.3. Ozonized water rapidly and effectively inactivated ASFV

Different concentrations of ozonized water were applied to $10^{4.0}$ $TCID_{50}$/mL WT-ASFV or reporter ASFV and incubated for 1, 3, 6 and 10 min, respectively, and then stopped by the neutralizer and the viral reductions were determined by infectivity assays on PAMs. As shown in Figs. 1 and 4 and Table 4, 2 log10 reduction (99 %) of either WT-ASFV or reporter ASFV was observed when inactivated with ozonized water as lower as 5 mg/L within 1 min, indicating that ozonized water can inactivate ASFV rapidly and efficiently.

Further, different concentrations of ozonized water (5, 10 and 20 mg/L) were applied to WT-ASFV or reporter ASFV of $10^{5.0}$ $TCID_{50}$/mL for 1, 3, 6 and 10 min, respectively. The results showed that a viral reduction of approximately 2 log10 (99 %) was observed when inactivated with 5 mg/L ozonized water within 1 min, and 3 log10 (99.9 %) virus was inactivated with 10 or 20 mg/L ozonized water within 3 min or 1 min, respectively (Figs. 2 and 5 and Table 5).

Different concentrations of ozonized water (5, 10 or 20 mg/L) were applied to $10^{6.75}$ $TCID_{50}$/mL WT-ASFV or reporter ASFV for 1, 3, 6 and 10 min, respectively. As shown Figs. 3 and 6 and Table 6, 5 mg/L ozonized water inactivated 2 log10 (99 %) reporter ASFV in 1 min, and a viral reduction of approximately 3 log10 (99.9 %) in reporter ASFV or 2 log10 (99 %) in wild-type virus was observed when inactivated with 10 mg/L ozonized water in 1 min; meanwhile, a viral reduction of 3 log10 (99.9 %) was observed when 20 mg/L ozonized water inactivated wild-type ASFV of $10^{6.75}$ $TCID_{50}$/mL in 3 min, indicating that ozonized water (5–20 mg/L) can also effectively inactivate the high dose of ASFV within a short time.

4. Discussion

The continuous spread of ASF worldwide has revealed big challenges for control. Thus, biosecurity measures are urgently needed to reduce the risk of the pathogen expansion and spread (Jancovich et al., 2018). Effective disinfectants are necessary for this issue.

As a strong oxide, ozone can destroy the structure of microorganisms such as bacteria and viruses in a short period of time, making it incapable of viability. There are many disinfectants that can kill microorganisms by oxidizing properties to achieve disinfection effects, including chlorine, bleaching powder, potassium permanganate, and so on. However, these disinfectants have shown slow antimicrobial ability and harmful to humans and animals. Ozone is different from other disinfectants, and excess ozone can be quickly decomposed into oxygen, which is non-toxic and harmless. High efficiency and rapid sterilization of microorganisms can be achieved by ozone disinfection in an instant. High concentration of ozonized water (> 40 mg/L) can be produced simply with an electrolysis method in several minutes. Ozonized water disinfection is not limited by space, region and temperature. It has been widely used in food production and processing fields such as dairy products, beverages, water, melons, pork and pork products.

Previous studies have shown that ozonized water directly destroys the nucleic acid (RNA or DNA) of the virus through oxidation, leading to the inactivation of the virus (Roy et al., 1981; Shin and Sobsey, 2003). This study evaluated the inactivation of ASFV by ozonized water for the first time at the cellular level. Our data showed that $10^{4.0}$ $TCID_{50}$/mL ASFV could be efficiently inactivated by 5 mg/L ozonized water within 1 min, and as high as $10^{6.75}$ $TCID_{50}$/mL ASFV could be inactivated by 5–20 mg/L ozonized water within 1 min, indicating that ozonized water can be used as an effective disinfectant for ASF control.

ASFV can be transmitted by direct or indirect contact between infected animals and/or other contaminated materials, such as drinking water and feed. Fomites such as clothing, transport trucks or feed supplies may act as a source of infection (Dixon et al., 2020). Ozonized water can be used for all-round disinfection of pig farms, as well as cleaning and disinfection of the environment and potential pollutants in the whole pig industry chain, such as slaughterhouses and meat processing plants. Ozonized water can be used by spraying, rinsing, soaking, wiping, etc., according to the environmental conditions. It can be used for air disinfection in offices and other areas by spraying or wiping; disinfection of drinking water; disinfection of food, clothes, boots, kitchen waste and other materials by soaking; disinfection of environments, transport vehicles, pig houses and others by rinsing. It is suggested that the epidemic areas can be disinfected with ozonized water of 5 mg/L or above, and that the non-epidemic area can be disinfected with ozonized water of 5 mg/L.

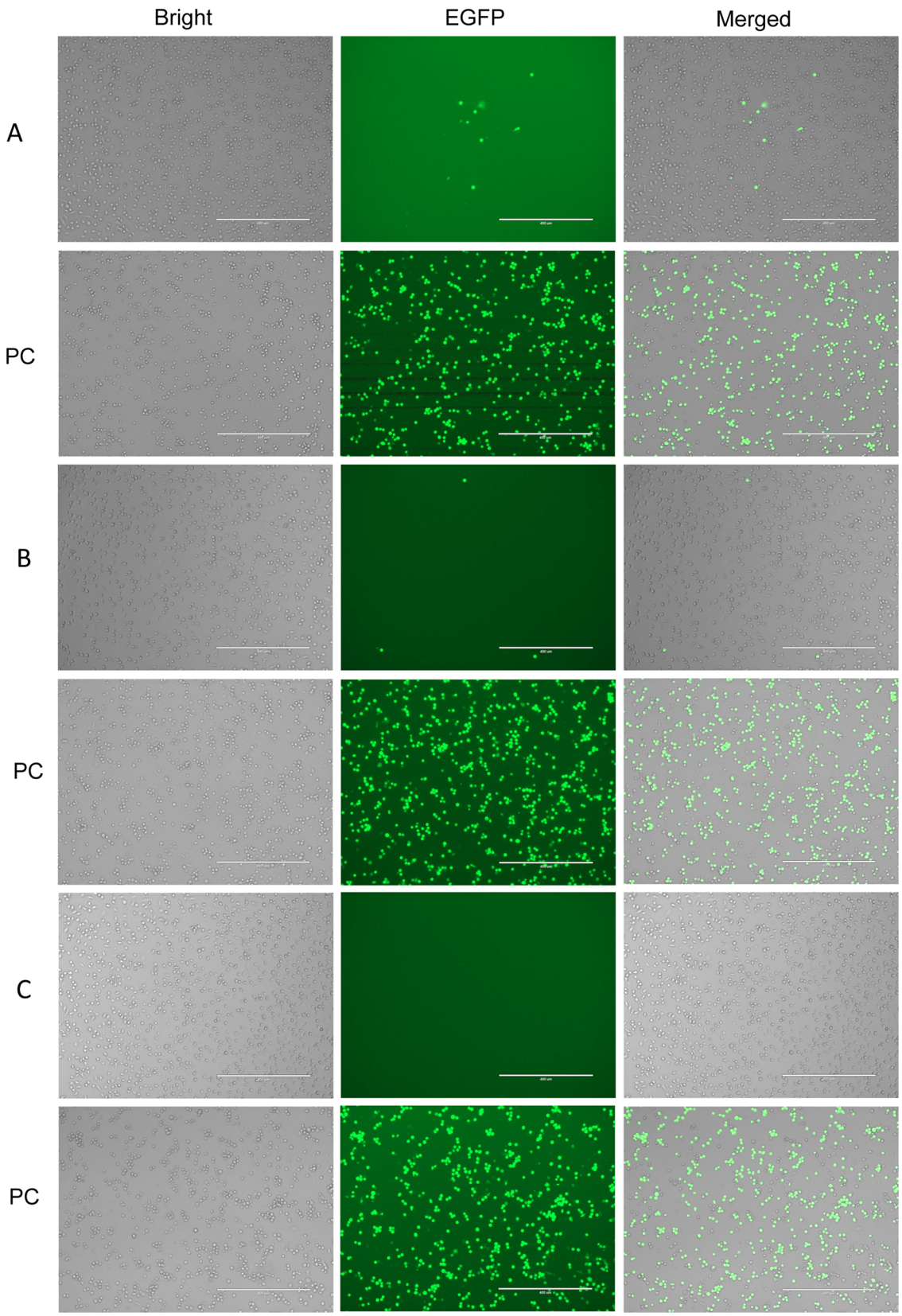

Fig. 3. Inactivation of $10^{6.75}$ $TCID_{50}$/mL reporter ASFV by ozonized water. $10^{6.75}$ $TCID_{50}$/mL ASFV was incubated with 5 (A), 10 (B) or 20 (C) mg/L ozonized water for 1 min and stopped by the neutralizer and the resulting viral titer was determined by infectivity assay on PAMs. PC, positive control.

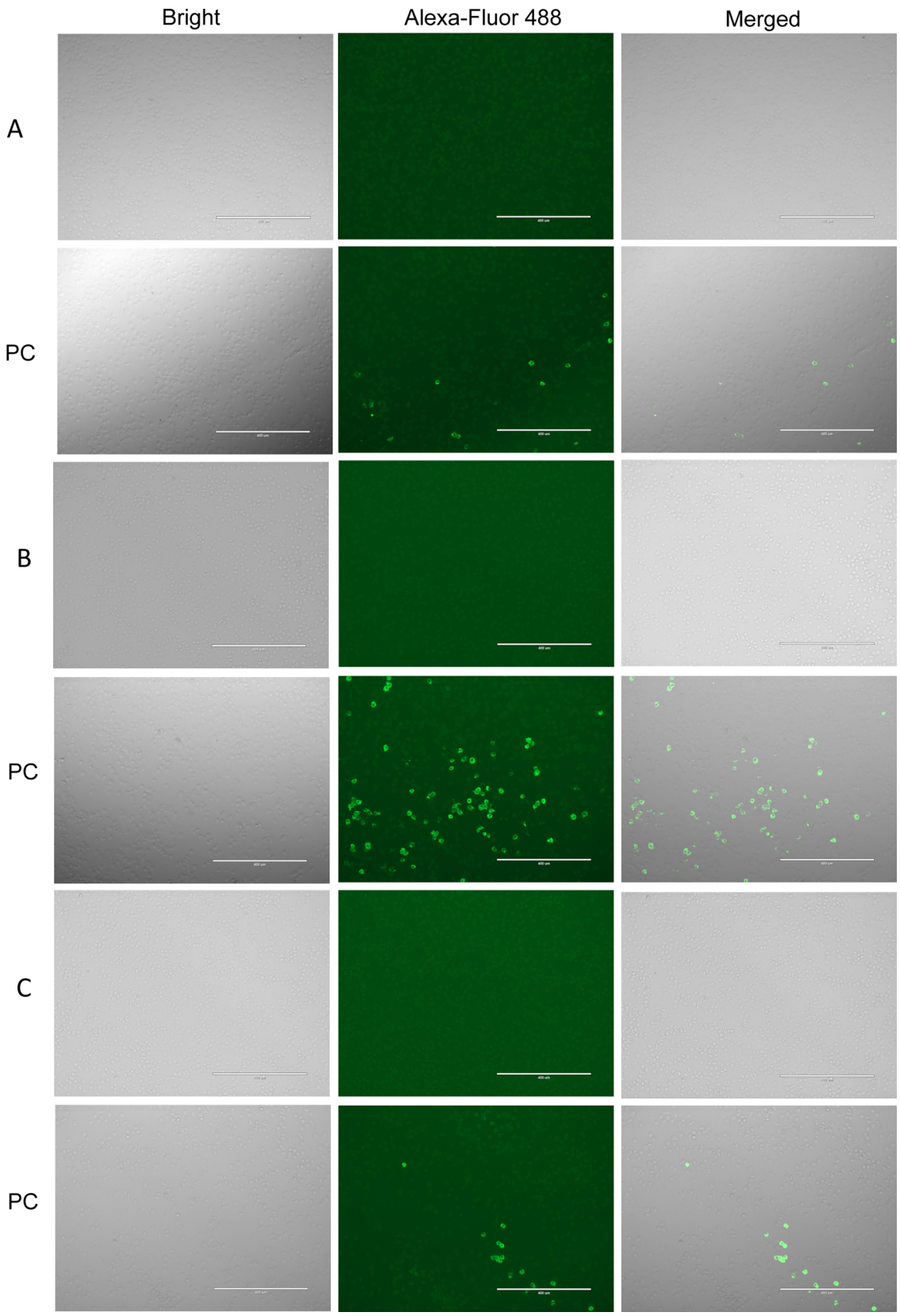

Fig. 4. Inactivation of $10^{4.0}$ $TCID_{50}$/mL wild-type ASFV by ozonized water. $10^{4.0}$ $TCID_{50}$/mL ASFV was inactivated with 5 mg/L ozonized water for 1 (A) or 3 min (B) or 10 mg/L ozonized water for 1 min (C), and stopped by the neutralizer and the resulting viral titer was determined by infectivity assay on PAMs. PC, positive control.

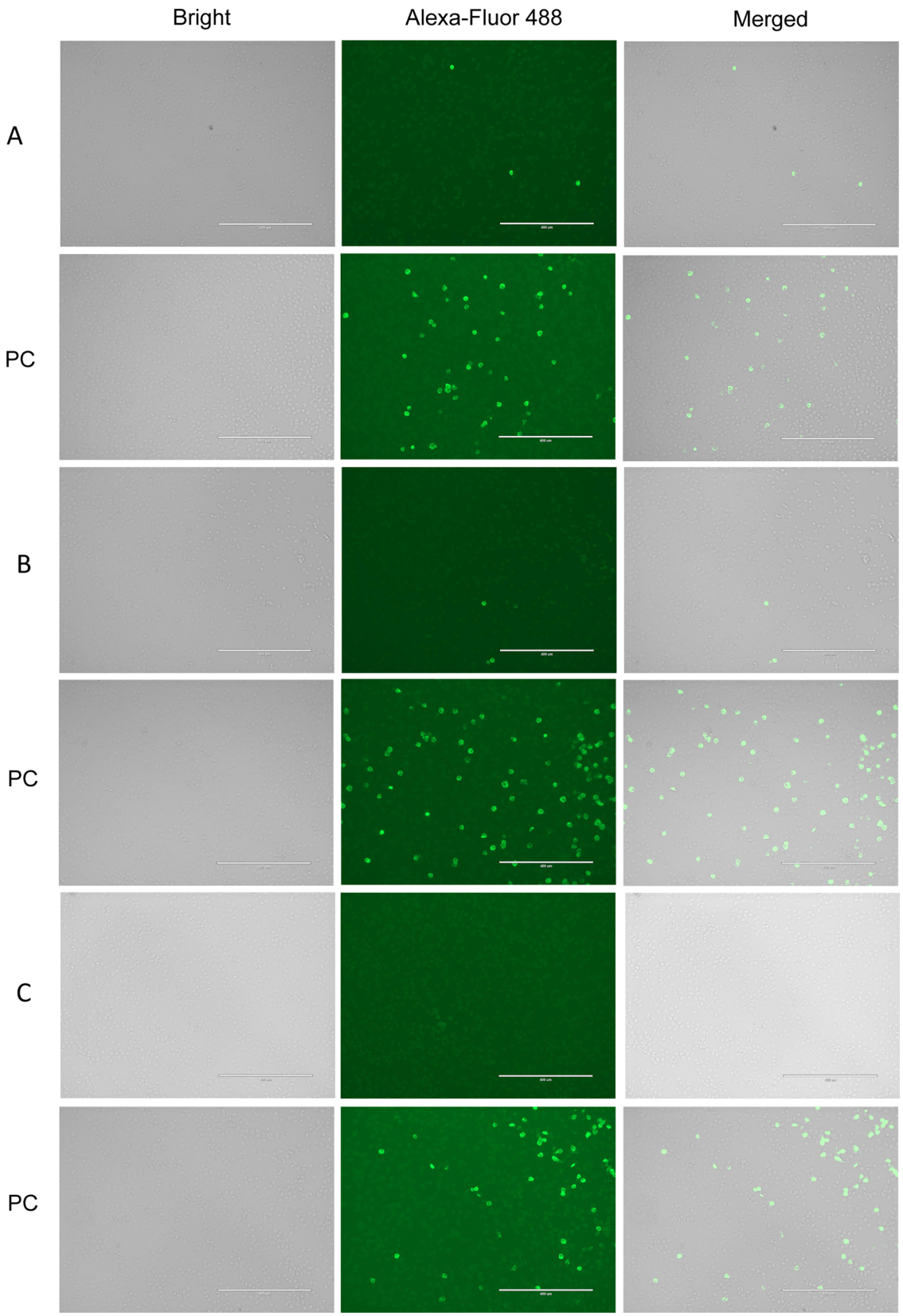

Fig. 5. Inactivation of $10^{5.0}$ $TCID_{50}$/mL wild-type ASFV by ozonized water. $10^{5.0}$ $TCID_{50}$/mL ASFV was incubated with 5 (A), 10 (B) or 20 (C) mg/L ozonized water for 1 min and stopped by the neutralizer and the resulting viral titer was determined by infectivity assay on PAMs. PC, positive control.

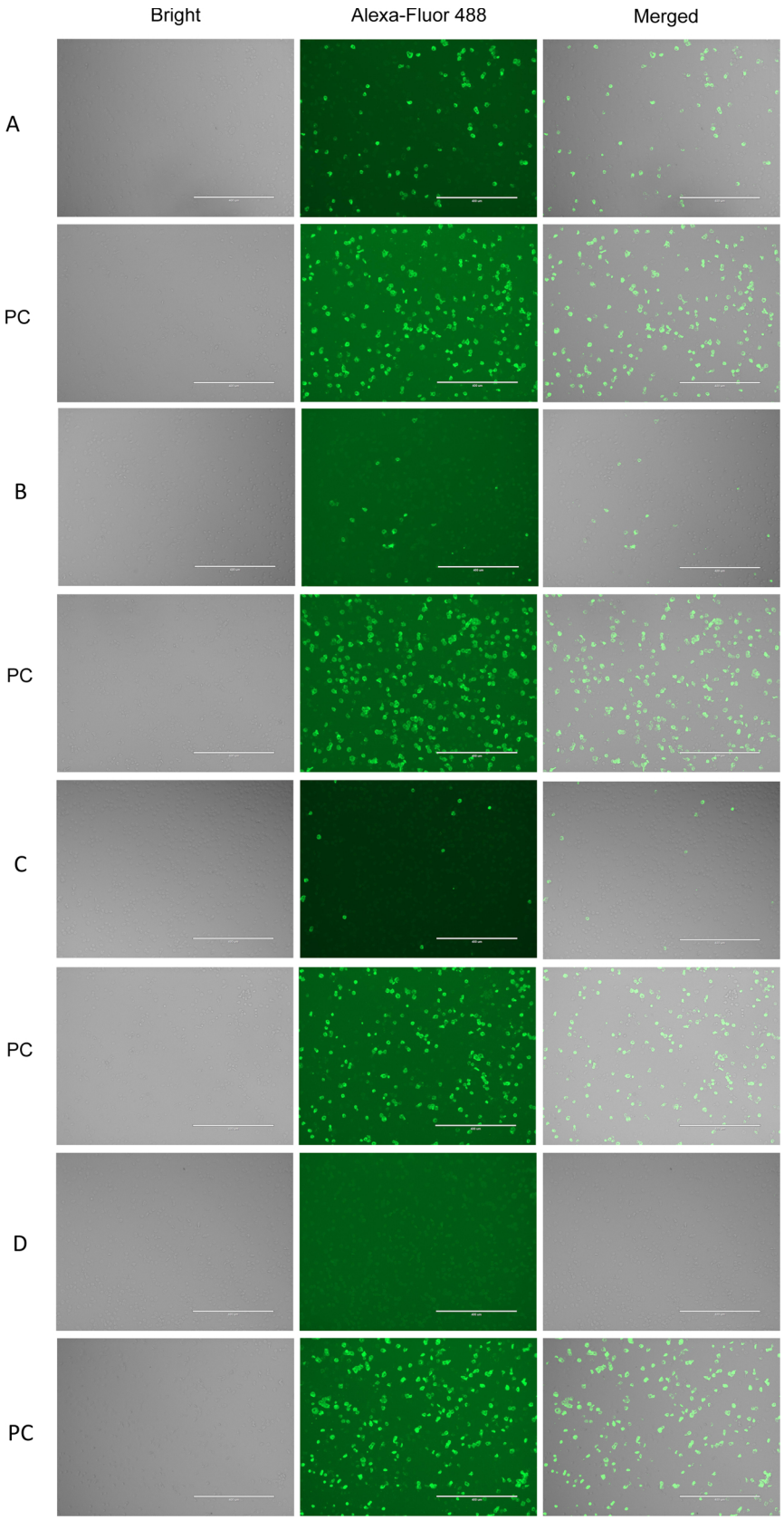

Fig. 6. Inactivation of $10^{6.75}$ $TCID_{50}$/mL wild-type ASFV by ozonized water. $10^{6.75}$ $TCID_{50}$/mL ASFV was incubated with 5 (A) or 10 (B) mg/L ozonized water for 1 min or 20 mg/L ozonized water for 1 min (C) and 6 min (D), and stopped by the neutralizer and the resulting viral titer was determined by infectivity assay on PAMs. PC, positive control.

Table 6
Inactivation of $10^{6.75}$ $TCID_{50}$/mL ASFV by ozonized water.

Virus	Time (min)	5 mg/L		10 mg/L		20 mg/L	
		Viral titer (log10 $TCID_{50}$/mL)	Log10 reduction value	Viral titer (log10 $TCID_{50}$/mL)	Log10 reduction value	Viral titer (log10 $TCID_{50}$/mL)	Log10 reduction value
Reporter ASFV	1	2.75 ± 0.25	2.0 ± 0.25	1.75	3.0	− ∞	4.75
	3	2.66 ± 0.14	2.09 ± 0.14	1.66 ± 0.14	3.09 ± 0.14	− ∞	4.75
	6	2.5 ± 0.25	2.25 ± 0.25	1.50	3.25	− ∞	4.75
	10	2.5 ± 0.25	2.25 ± 0.25	1.75	3.0	− ∞	4.75
WT-ASFV	1	3.25 ± 0.25	1.5 ± 0.25	2.75	2.0	2.25 ± 0.25	2.50 ± 0.25
	3	3.25	1.50	2.75 ± 0.25	2.0 ± 0.25	1.75 ± 0.25	3.0 ± 0.25
	6	3.0 ± 0.25	1.75 ± 0.25	2.75	2.0	− ∞	4.75
	10	3.0 ± 0.25	1.75 ± 0.25	2.75	2.0	− ∞	4.75

Acknowledgements

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