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Rapid *in vitro* virucidal activity of slightly acidic hypochlorous acid water toward aerosolized coronavirus in simulated human-dispersed droplets

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ABSTRACT

The virucidal activities were evaluated by spraying slightly acidic hypochlorous acid waters (SAHWs) containing various concentrations of free available chlorine - 100, 200, 300 and 500 ppm (SAHW-100, -200, -300 and -500, respectively) - toward aerosol of an avian coronavirus (infectious bronchitis virus: IBV). The viral solution was supplemented with 0.5% fetal bovine serum (FBS) to simulate normal human droplets generated by sneezing or coughing in a real-life scenario. The virus containing 0.5% FBS was sprayed and exposed to SAHWs for a few seconds in a closed chamber, before reaching the air sampler. The results showed that IBV exposed to SAHW-100 and -200 for a few seconds decreased by 0.21 $\log_{10}$ and 0.80 $\log_{10}$, respectively, compared to the pre-exposed samples to SAHWs as controls. On the other hand, reductions of 1.16 $\log_{10}$ and 1.67 $\log_{10}$ were achieved following the exposure to SAHW-300 and -500, respectively, within a few seconds. These results suggest that SAHWs have rapid *in vitro* virucidal activity toward aerosolized IBV. The findings obtained for IBV might basically be applicable in relation to SARS-CoV-2, given the resemblance between the two viruses. To prevent human-to-human transmissions by aerosols, the inactivation of viruses in the air by exposure to SAHWs for a few seconds seems to be an effective way.

1. Introduction

Dispersing aerosols through sneezing and coughing is one of the multiple routes of human-to-human transmissions for airborne infectious viruses, such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and influenza viruses (CDC, 2020; Cowling et al., 2013). To prevent human-to-human transmissions through aerosols, the inactivation of viruses in the air by sterilizing/disinfecting agents seems to be an effective way.

Hypochlorous acid water (HAW), a powerful oxidizing agent, is picked up as a candidate for the inactivation of viruses in aerosols in the air, due to its sterilizing effect and safety. Several products of HAW at various concentrations of free available chlorine (FAC) and pH values were produced by different manufacturing protocols. Although there are no established standardized methods for assessing the inactivation of aerosolized microorganisms by sterilizing agents, HAW was used for

experimental aerosol disinfection in some studies (Hao et al., 2013; Kurahashi et al., 2021; Zhao et al., 2014). These studies maintained that HAW may be a way to deal with infection via spatial spraying for a long period of time (more than 20 min) in a space where humans or animals are present. Alongside, though, it has also been concluded that peak exhalation speeds of droplets from a human sneeze can reach up to 10 to 30 m per second, creating a cloud that can span approximately 7 to 8 m (Bourouiba, 2020). The data in this research are of great value to prove that aerosols can move in the air at high speed and shortly reach humans or animals. Therefore, it would be essential to evaluate the virucidal activity of sterilizing agents toward aerosolized microorganisms for an abrupt duration. Our previous report evaluated the virucidal activity of slightly acidic hypochlorous acid waters (SAHWs) toward aerosolized Newcastle disease (ND) live vaccine (B1 strain) for a few seconds, using three-day-old conventional chicks *in vivo* experiments (Hakim et al., 2015). Thereupon no clinical signs were observed, and no virus was

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isolated from the group of chicks exposed to SAHW containing FAC at the rate of 100 parts per million (ppm). On the other hand, little is known about the virucidal activity of SAHWs toward aerosolized viruses for a short time through a quantitative evaluation test.

In the present experiments, the virucidal activities of sprayed SAHWs during a few seconds were evaluated toward aerosolized infectious bronchitis virus (IBV), which belongs to the genus *Gammacoronavirus* within the *Coronaviridae* family, in simulated human-dispersed droplets. We hereby provide a method to measure the inactivation of airborne viruses and prove that SAHW is capable of inactivating viruses in the air within a few seconds.

2. Materials and methods

2.1. Test virus

IBV strain M41 used in this study was provided by National Institute of Animal Health (Tsukuba, Ibaraki, Japan) and propagated in primary chicken kidney (CK) cells (Miyaoka et al., 2021a, 2021b). The viral supernatant of IBV was separated by centrifugation at 1750 x g for 15 min at 4 °C and then collected and preserved at -80 °C in one mL single-use tubes. For the aerosol disinfection test, the virus suspension was prepared around 10^7 plaque forming units (PFU)/mL in phosphate-buffered saline (PBS, pH 7.4) supplemented with 0.5% fetal bovine serum (FBS), in order to simulate human droplets, as previously described (Miyaoka et al., 2021a; Sattar et al., 1989).

2.2. Hypochlorous acid water

SAHWs with a pH of 5.2–5.8 and several concentrations of FAC, used in this study, were kindly provided by Nano-energy Co., Ltd. (Tokyo,

Japan). SAHWs containing FAC at the rates of 100, 200, 300, and 500 ppm were named SAHW-100, -200, -300 and -500, respectively, and kept sheltered from sunlight at room temperature (RT: 25 ± 2 °C), while thereafter closing the tube lid until the day of use. After preparation, each SAHW was used in this study within 3 months.

2.3. Experimental design

2.3.1. Experimental set up of a chamber

A virus in simulated human-dispersed droplets was aerosolized and exposed to SAHW in a closed chamber. Fig. 1A depicts a scheme of the experimental chamber used in this study to evaluate the virucidal activity of SAHWs toward aerosolized IBV in simulated human droplets for a few seconds. As a closed chamber, a plastic box with dimensions of W440 x D740 x H230 mm (around 75 L volume) purchased from a local market was placed in a biosafety level II cabinet to ensure the safety of the operator in case of leakage. The temperature in the biosafety level II cabinet was kept at RT. The closed chamber consisted of three areas, intended for: (1) aerosolization of IBV, (2) nebulization of SAHWs, and (3) aerosol sampling. The layout in the chamber and each distance from sampling area is presented in Fig. 1B. The first area included a sprayer, Aerial Mist, kindly prepared by Aelph Co., Ltd (Tokyo, Japan) and designed at the liquid aerosolization of 40 mL/hour with small aerosol particles (average size 26 μm in diameter; range 11 to 42 μm). The sprayer was connected to the chamber at a distance of 53 cm from an air sampler. The virus suspension containing 0.5% FBS was aerosolized by the sprayer, and the aerosol was let into the chamber. The second area contained two nebulizers (Omron Corp., Kyoto, Japan) that can release water at the liquid aerosolization form of 0.3 mL/min, each with small aerosol particles (size 5 μm in diameter), to inject SAHW into the chamber. Each nebulizer was placed on both sides faces of the chamber,
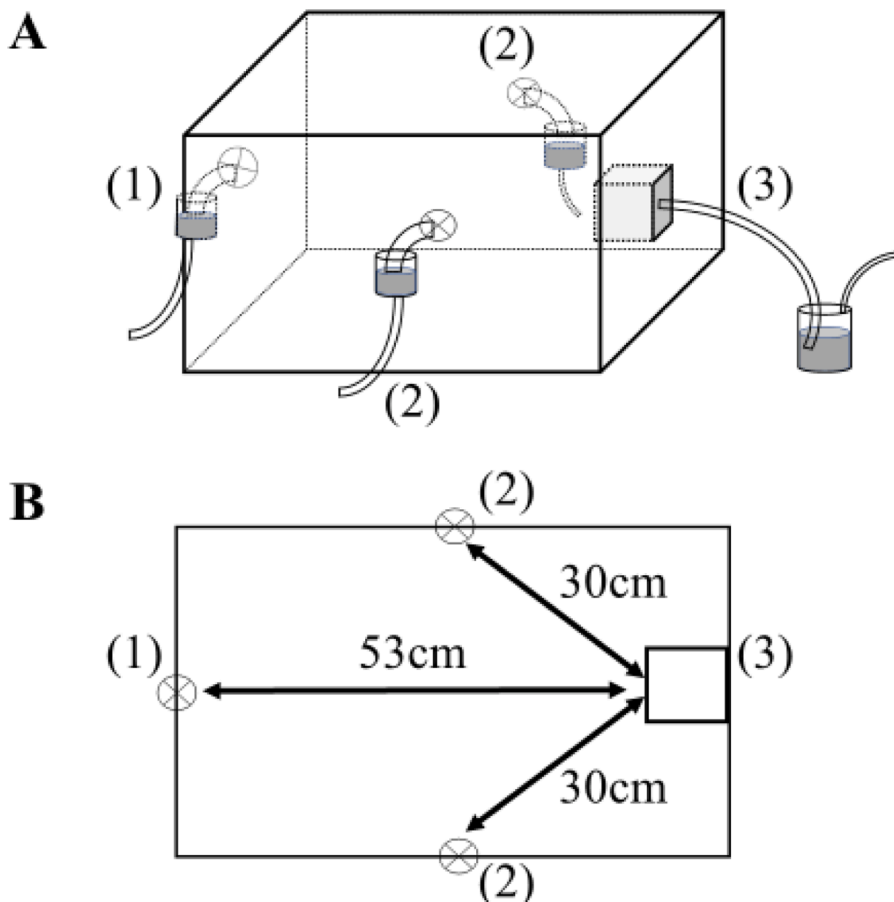
Fig. 1. A schematic diagram of the experimental chamber setting, as intended for utilizing three areas: (1) sprayer for IBV, (2) nebulizers for SAHWs and (3) air sampler. (A) The evaluation system of the virucidal activity of SAHWs toward aerosolized virus in simulated human droplets for a few seconds. The virus was injected into the chamber via the sprayer, exposed to SAHWs coming from the nebulizers, and then collected through sampling airflow using the air sampler. (B)The layout in the chamber. The arrow indicates each distance from the air sampler.

at a distance of 30 cm from an air sampler, respectively. SAHW was released from each nebulizer and let into the chamber for virus exposure. The third area includes an air sampler consisted of a Rayon-polyester sheet (100 mm x 100 mm, Alphas R 5, Iwatsuki Co., Ltd., Tokyo, Japan), air bypass tubes, a bottle filled with a sterilizing agent and a suction pump (SP40, MARKOS-MEFAR, Bovezzo, Italy) equipped with HEPA filter. As a sterilizing agent, a mixture of quaternary ammonium compound (Rontect®, Scientific Feed Laboratory Co., Ltd., Tokyo, Japan) diluted 500 times, with 0.17% food additive grade calcium hydroxide (Fine Co., Ltd., Tokyo, Japan) was used, for preventing contamination by bioaerosols toward a suction pump, which passed air samples; its virucidal effect has been previously described (Ito et al., 2018; Kabir et al., 2021; Miyaoka et al., 2021b). The bottle filled with the mixture was connected to the suction pump providing an airflow rate of 35 L/min via an air bypass tube, without interrupting the airflow through the chamber.

In order to prove that a sterilizing agent inactivated virus in the air, yet not on the air sampler, it is required to neutralize at once the virucidal activity of the sterilizing agent on the air sampler. In the present study, FBS treatment was performed toward the air sampler to neutralize the virucidal activity of SAHWs before the aerosol experiments. Based on results from the preliminary test (data not shown), the sampler was treated with FBS, described as follows. Firstly, the rayon sheet was soaked in 2 mL of FBS, and sterilized in a microwave oven for 3 min. The rayon sheet coating with FBS was subsequently connected to the bottle filled with the mixture via an air bypass tube without interrupting the sampling airflow by the suction pump, and then placed at the aerosol sampling area in the chamber. Secondly, three mL of FBS was released from the sprayer onto the sampler in the chamber, aspirated, and then captured on the sampler completely by means of sampling airflow for 3 min, which is sufficient to empty the chamber.

2.3.2. Aerosol disinfection test

Fig. 2A shows a scheme of the spraying duration of IBV and SAHW in the aerosol experiments. Two nebulizers were run in parallel at the liquid aerosolization of 0.3 mL/min each for 3 min simultaneously, so as to equilibrate SAHW concentration in the chamber. After equilibration of SAHW, a volume of one mL of virus prepared in PBS supplemented with 0.5% FBS was aerosolized and sprayed completely for 3.5 min from the sprayer into the chamber. At the same time, the suction pump was run for sampling airflow to collect the aerosolized virus on the air sampler. Concurrently with the virus aerosolization for 3.5 min, SAHW was also released from the two nebulizers, aiming to be in contact with the aerosolized virus across airflow in the chamber. After complete viral aerosolization, SHAW was sprayed for 3 min, thus having contact with the virus-containing air in the chamber. The remained virus was thereby exposed to SAHW, and then captured on the sampler by means of sampling airflow. The bioaerosols that passed the sampler were collected in the bottle filled with the sterilizing mixture via air bypass tube in order to disinfect the virus, and the sterilized air was exhausted through HEPA filter to avoid any contamination of general space in the laboratory room.

After incubation, each rayon sheet was transferred into 2.0 mL of collecting liquid, namely a mixture of 0.7 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.2) + 30% FBS (Alam et al., 2018; Komura et al., 2019; Miyaoka et al., 2021a) in a stomacher bag (size 100 × 150 × 0.09 mm, capacity 80 mL; Organo Corporation, Tokyo, Japan). The captured viruses on the sheets were extracted, using BagMixer (MiniMix 100 W CC, Practical Japan Inc., Chiba, Japan) for 1 min, and the supernatant was decanted into a clean 1.5 mL microtube for infectivity assay.

2.3.3. Controls

In total, three controls were prepared during this study. As a first control, redistilled water (dW$_2$) was used instead of SAHWs in the aerosol disinfection test (dW$_2$ control). All the settings were the same as with the test, except for dW$_2$. A second control was prepared to measure the viral titer of the sprayed IBV in the chamber in each experiment. The virus suspension prepared in PBS containing 0.5% FBS was kept at RT in parallel along each experiment, and the concentrations of aerosolized virus in the chamber were measured (viral control). To verify whether SAHWs were neutralized immediately on the air sampler treated with FBS, a third control was prepared, while a scheme of the spraying duration of IBV and of SAHW for the control experiments is presented in Fig. 2B, as follows. Briefly, experimental chamber setting was performed as described above. Then SAHW was injected into the chamber by each nebulizer for 9.5 min, captured on the sampler treated with FBS through sampling airflow for 3 min, which is sufficient to empty the chamber. Following this, one mL of virus suspension prepared in PBS containing 0.5% FBS was sprayed by the sprayer and captured on the sampler
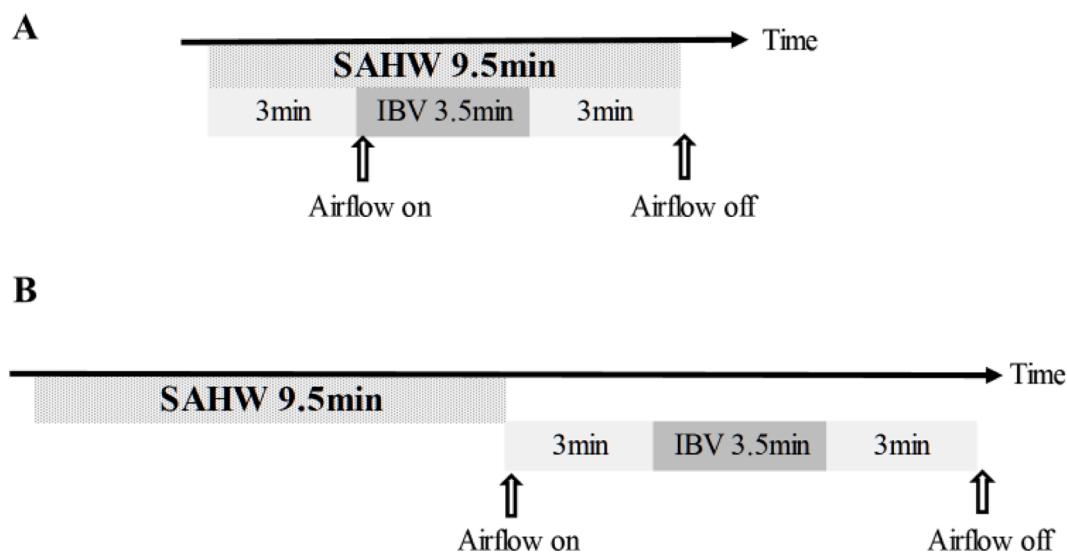

Fig. 2. A scheme of the spraying duration of IBV and of SAHW. (A) A scheme of the spraying duration of IBV and of SAHW in the aerosol experiments. SAHWs were sprayed for 9.5 min (3 min before and 3 min after IBV spraying) in a chamber. Within 3.5 min, one mL volume of IBV was sprayed and exposed to SAHWs in the same space. (B) A scheme of the spraying duration of IBV and of SAHW in the control experiments. SAHWs were sprayed for 9.5 min (3 min before and 3 min after IBV spraying) in a chamber and aspirated completely; the same space was then sprayed with one mL volume of IBV.

through sampling airflow for 3.5 min. After complete viral aerosolization, additional sampling for 3 min was conducted to collect the virus remaining in the air within the chamber. This third control served as "the samples pre-exposed to SAHWs". Virus extraction from the sampler and treatment of bioaerosol which passed the sampler were performed as well, as described above.

2.4. Infectivity assay

All experiments were carried out in triplicates. Viral loads in samples exposed to SAHWs and control samples were quantified and measured by plaque assay on CK cells as previously described (Miyaoka et al., 2021a). The measured titer of each sample via the plaque assay was reported as $\log_{10}$ PFU/mL, and expressed as mean $\pm$ standard error (SE) in triplicates.

To investigate the virucidal activity toward the aerosolized virus in the air, yet not on the sampler, reduction factors (RF) derived from the difference between the viral titer of pre-exposed and exposed to SAHWs were calculated, and presented as $\log_{10}$ PFU/mL.

2.5. Statistical analysis

One-way analysis of variance (ANOVA) and post-hoc Tukey-Kramer tests were performed for comparison of samples (the controls and the samples exposed to SAHWs) to each other. P values < 0.05 were considered to be statistically significant.

3. Results

The virucidal activities of SAHWs at various concentrations of FAC (100–500 ppm) against aerosolized IBV in simulated human droplets are presented in Table 1 and Fig. 3. The aerosolized IBV was exposed to each SAHW for a few seconds in the closed chamber. The dW_2 control was used under the same condition as SAHWs. The mean viral titers and SE for aerosolized IBV picked up by the air sampler were the following: 3.33 $\pm$ 0.11, 3.02 $\pm$ 0.35, 2.87 $\pm$ 0.13 and 1.97 $\pm$ 0.26 ($\log_{10}$ PFU/mL), when exposed to SAHW-100, -200, -300 and -500, respectively. The variations were not significantly different in viral titer exposed to SAHW-100 and -200 compared to controls (samples pre-exposed to SAHWs and dW_2 control), whereas there were statistically significant differences in relation to the samples exposed to SAHW-300 and -500, as compared to the controls (P < 0.05).

Pre-exposed controls represent the effects of each SAHW toward virus captured within the sampler treated with FBS, so as to apply a correction for the effects of SAHW toward virus in the air. The mean titers and SE for aerosolized IBV in the samples pre-exposed to SAHWs were the following: 3.54 $\pm$ 0.23, 3.82 $\pm$ 0.10, 4.03 $\pm$ 0.27 and 3.64 $\pm$

0.27, when utilizing SAHW-100, -200, -300 and -500, respectively. The viral titers of samples pre-exposed to SAHWs were close to those of dW_2 control; statistically, the difference in the titer among these controls was not significant (P > 0.05).

The log RFs for aerosolized IBV between the titers of exposed and pre-exposed to SAHWs were the following: 0.21, 0.80, 1.16 and 1.67, when utilizing SAHW-100, -200, -300 and -500, respectively.

4. Discussion

Aerosols released through sneezing and coughing move in the air at high speed, and would shortly reach humans or animals (Bourouiba, 2020), whereas it is not clear what the virucidal activity of sterilizing agents is attained toward aerosolized virus during a few seconds – which might be insufficient for virus exposure – through a quantitative evaluation test. To our knowledge, this is the first study evaluating the virucidal activity of a sterilizing agent toward aerosolized viruses containing 0.5% FBS in simulated human droplets for a few seconds. We obtained that SAHW could cause a reduction of 0.21 $\log_{10}$ PFU/mL and 0.80 $\log_{10}$ PFU/mL due to virus exposures to SAHW-100 and -200, respectively, compared with that of pre-exposed to SAHW-100 and -200, respectively. Also, exposures to SAHW-300 and -500 inactivated the aerosolized virus by 1.16 $\log_{10}$ PFU/mL and 1.67 $\log_{10}$ PFU/mL, respectively. This study provides new information on utilizing SAHWs in a space where humans are present on practical usage, in order to prevent human-to-human transmissions by aerosols.

Besides studies using HAW products, recent investigations evaluated the virucidal activity of UV light, and of ozone gas toward airborne viruses, for establishing aerosol disinfection strategies (Dubuis et al., 2021, M.-E. 2020; Walker and Ko, 2007). Users need to understand that each aerosol disinfection strategy has advantages and disadvantages. For example, UV light and ozone gas are hazardous for humans, which make it difficult to utilize those measures for practical usage in a space where humans are present. HAW products, including SAHWs, revert to water after used (Adam et al., 2002), which minimizes the damage to human health and causes no environmental pollution. SAHW used in the present study – produced by ion-exchange resin using NaClO and purified water – is different from the method of mixing an acid with sodium hypochlorite. Thus, since this solution contains no sodium, it causes no metallic corrosion (Hakim et al., 2016), hence it can be utilized in a space where a metallic substance is present. From these points of view, the practical usage of SAHWs for efficient and safe aerosol disinfection is expected.

It is well known that the virucidal activity of HAW products, including SAHWs, is greatly reduced by the presence of organic materials (Bloomfield et al., 1991; Fukuzaki, 2006; Takeda et al., 2021, Y. 2020) – such as FBS or bovine mucin – which is its disadvantage in practical usage. In the present study, FBS was used to simulate human droplets in a real-life scenario (Miyaoka et al., 2021a; Sattar et al., 1989) and neutralize immediately the effects of SAHWs toward aerosolized viruses on the air sampler. Firstly, the rayon sheet, as the air sampler was soaked in 2 mL of FBS, sterilized in a microwave oven for 3 min, and then placed in the aerosol sampling area. Secondly, three mL of FBS was released from the sprayer to the sampler in the chamber, aspirated, and then completely captured via the sampler, through sampling airflow for 3 min. In the preliminary test, after treatment of the rayon sheet by soaking in 2 mL of FBS and sterilizing in a microwave oven for 3 min, the IBV was aerosolized and exposed to SAHW-500 in the chamber. Accordingly, the IBV titer exposed to SAHW-500 decreased by 2.09 $\log_{10}$ PFU/mL, as compared to that of dW_2, which was close to the results in the present study (a reduction of 2.17 $\log_{10}$ PFU/mL). On the other hand, the mean viral titer and SE in the samples pre-exposed to SAHW-500 were 3.24 $\pm$ 0.14 ($\log_{10}$ PFU/mL) in the preliminary test (data not shown), which was lower than the result in the present study (3.64 $\pm$ 0.27 $\log_{10}$ PFU/mL). These results demonstrate that the captured IBV was reduced by the effect of SAHW-500 on the sampler in

Table 1
Virucidal activity of SAHW toward aerosolized infectious bronchitis virus (IBV) containing 0.5% fetal bovine serum (FBS) for a few seconds.

| Tested solutions | Viral titer in the air | | | |
	Virus	Control [a]	Exposed [b]	Pre-exposed [c]	RF [d]
SAHW-100	IBV	5.93±0.16	3.33±0.11	3.54±0.23	0.21
SAHW-200		($\log_{10}$PFU/ml)	3.02±0.35	3.82±0.10	0.80
SAHW-300			2.87±0.13	4.03±0.27	1.16
SAHW-500			1.97±0.26	3.64±0.27	1.67
dW_2			4.14±0.11	NT [e]	NC [f]

[a] Viral titer of 1 ml virus sprayed into a chamber in each experiment.
[b] Aerosolized IBV was exposed to SAHW (100, 200, 300 and 500 ppm) or dW_2.
[c] Each tested solution was sprayed in a chamber and aspirated completely; the same space was then sprayed with IBV.
[d] Log reduction factor between the exposed and pre-exposed viral titer to SAHWs.
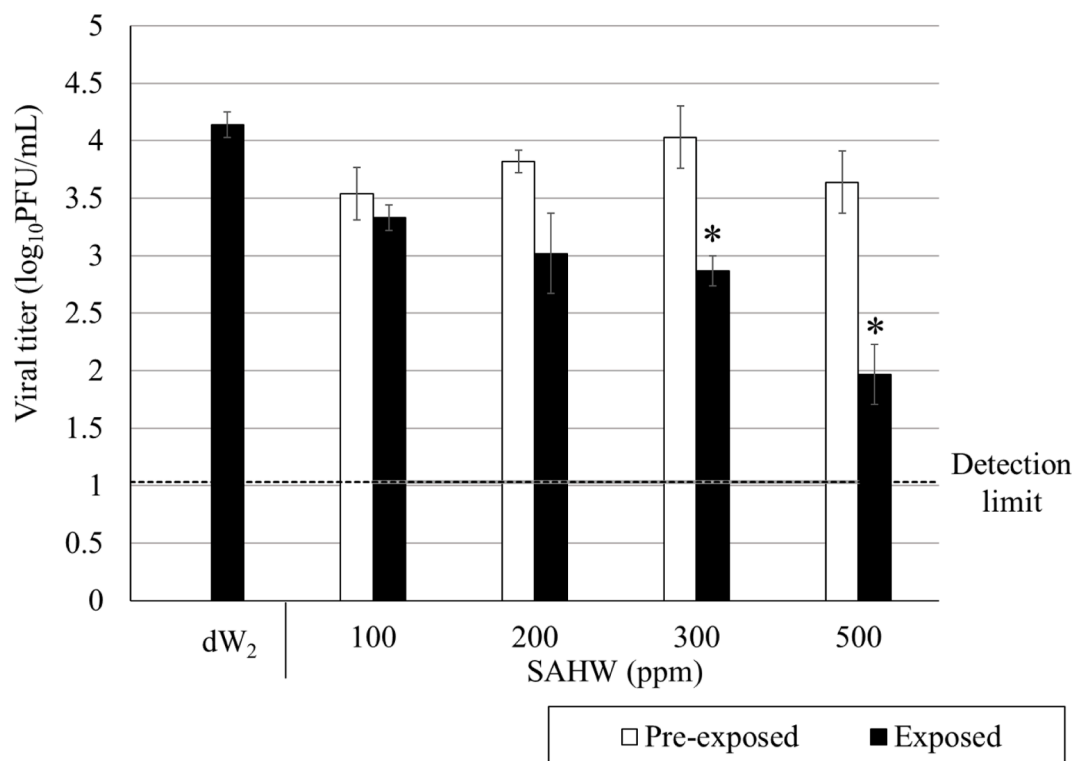[e] Not tested.
[f] Not calculated.

Fig. 3. Virucidal activity of SAHW toward aerosolized infectious bronchitis virus (IBV) containing 0.5% fetal bovine serum (FBS) for a few seconds. * Single asterisk indicates that values are significantly different between respective pre-exposed and exposed viral titers.
Dot line indicates detection limit (1.031 $\log_{10}$ PFU/mL) in the present study.

the preliminary test, indicating that the method of treatment with FBS toward the sampler in the preliminary test was not effective in neutralizing the effects of SAHWs immediately on the sampler. Moreover, it was shown that the effects of SAHWs toward aerosolized virus in the air were not interfered by FBS in this study after FBS in the air was aspirated completely through airflow and captured on the sampler. Additionally, in this study, the statistical difference in the titer among controls (the samples pre-exposed to SAHWs and dW_2 control) was not significant (P > 0.05); there were statistically significant differences among the samples exposed to SAHW-300 and -500 to the controls (P < 0.05). Taken together, it is reasonable to conclude that aerosolized IBV was inactivated by SAHWs in the air, within a few seconds. which was thus found to be sufficient to reach the virus from the sprayer to the sampler.

As an index to verify whether the inactivation of microorganisms by sterilizing agents is to be considered effective, log RF was used for the evaluation test of the agents. The guidelines (Anonymous, 2018) specify the effective level at least a 4-log RF, based on the results of the suspension or surface tests. On the other hand, there are no established standardized methods for assessing the inactivation of aerosolized microorganisms by sterilizing agents, which means that no index can be found to verify whether their inactivation by the aerosol disinfection is to be considered effective. As shown in Table 1, the log RF was increased with the increase in the concentration of FAC: more than 1 log reduction of viral titer exposed to SAHW-300, compared with that of pre-exposed to SAHW-300. Although several points bear on the effective level for aerosol disinfection, 1 log reduction of viral titer in the air may be considered to be effective.

Compared with the viral titer pre-exposed to SAHW-100, 38% of the aerosolized IBV was reduced by exposure to SAHW-100 for a few seconds. Our previous report showed that the virucidal activity of SAHWs toward aerosolized ND vaccine strain for a few seconds using three-day-old conventional chicks *in vivo* experiments was evaluated (Hakim et al., 2015). Briefly, in the aerosol infection experiment, in the group of chicks

exposed to 10 doses of aerosolized ND vaccine, two chicks of 5 were infected, whereas, in the group exposed to 25 doses of the vaccine, all 5 chicks were infected. Moreover, in the aerosol disinfection test with SAHWs, no virus was isolated from the SAHW-100 treatment group exposed to the aerosolized ND vaccine at 25 doses. These results show that the aerosolized ND vaccine exposed to SAHW-100 for a few seconds was reduced to less than 10 doses from 25 doses (more than 60% reduction). The reduction ratio with SAHW-100 in our previous study was higher than that in the present study (38%), which is considered to associate with the presence of FBS in this study and innate differences between the tested viruses (ND vaccine and IBV). Admittedly, a 38% reduction of virus in the air due to SAHW-100 exposure seems to be incomplete to prevent transmission by aerosols, nonetheless, the exposure of SAHW-100 would help reduce the aerosol infection risk by appreciably decreasing the number of viral particles and aerial contagion.

In summary, we have shown the virucidal activity of SAHW toward aerosolized IBV within a few seconds in the quantitative test. Furthermore, to our knowledge, this is the first report about the evaluation of a sterilizing agent toward aerosolized viruses within a few seconds interaction. Our findings provide, thus, new insight into the aerosol disinfection strategy using SAHW for a few seconds, which will help us prevent disease and control infection. Presumably, there are also implications for the disinfection of SARS-CoV-2, being too an airborne coronavirus.

CRediT authorship contribution statement

Yu Miyaoka: Investigation, Data curation, Formal analysis, Methodology, Writing – original draft. **Makiko Yamaguchi:** Investigation, Methodology. **Chisaki Kadota:** Investigation, Methodology. **Md. Amirul Hasan:** Investigation. **Md. Humayun Kabir:** Investigation. **Dany Shoham:** Writing – review & editing. **Harumi Murakami:** Methodology. **Kazuaki Takehara:** Conceptualization, Funding

acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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