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Establishment and utilization of an evaluation system for virucidal activity of disinfectants against a coronavirus with apparent applicability to SARS-CoV-2

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ABSTRACT. Decontamination of pathogens on surfaces of substances is very important for controlling infectious diseases. In the present experiments, we tested various disinfectants in aqueous phase as well as on plastic surface carrying a viral inoculum, through dropping and wiping decontamination techniques, comparatively, so as to evaluate virucidal efficacies of those disinfectants toward an avian coronavirus (infectious bronchitis virus: IBV). We regard this evaluation system applicable to SARS-CoV-2. The disinfectants evaluated were 0.17% food additive glade calcium hydroxide ($FdCa(OH)_2$) solution, sodium hypochlorite at 500 or 1,000 ppm of total chlorine (NaClO-500 or NaClO-1,000, respectively), NaClO at 500 ppm of total chlorine in 0.17% $FdCa(OH)_2$ (Mix-500) and quaternary ammonium compound (QAC) diluted 500-fold in water (QAC-500). In the suspension test, all solutions inactivated IBV inoculum that contained 5% fetal bovine serum (FBS) under detectable level within 30 sec. In the carrier test, all solutions, except NaClO-500, could inactivate IBV with 0.5% FBS on a carrier to undetectable level in the wiping-sheets and wiped-carriers. We thus conclude that suspension and carrier tests should be introduced to evaluate disinfectants for the field usage, and that this evaluation system is important and workable for resultful selection of the tested disinfectants against avian coronavirus and SARS-CoV-2 on surfaces, particularly on plastic fomite.

KEY WORDS: abiotic carrier, calcium hydroxide, quaternary ammonium compound, sodium hypochlorite

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In late 2019, a novel human coronavirus−severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a member of *Betacoronaviruses*, −emerged in China and has been declared a pandemic virus since 11 March 2020 [23]. Human-to-human transmissions through aerosol and fomite are plausible, and probably common, since SARS-CoV-2 can remain viable and infectious in aerosols for hours and on surfaces for days [22]. Chin *et al*. also reported the stability of SARS-CoV-2 in the environment [5]. It has also been suggested that the soles of medical staff shoes may function as carriers of nosocomial infection [8]. It is essential to disinfect the virus on nonporous surfaces, especially on steel and plastic, because these materials keep the virus infectious for 2 to 4 days [5, 22]. In Japan, the Ministry of Health, Labor and Welfare (MHLW) recommends using 70% alcohol for hand washing and sodium hypochlorite (NaClO) of chlorine concentration at 500–1,000 ppm for decontaminating the surfaces of substances.

A recent review on inactivation of human and animal coronaviruses on inanimate carriers with biocidal agents [11], presents several papers showing that NaClO of chlorine concentration at 1,000 ppm or 2,100 ppm could inactivate coronaviruses within 1 min on the carriers [6, 16].

It has been reported that decontamination of surfaces with dried inocula is invariably more difficult, as compared to contaminated suspension [13, 18]. In some experiments, it was also shown that pathogens in aqueous phase could be inactivated

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with disinfectants in short periods, such as 5 sec; however, pathogens on abiotic carriers or in feces could survive disinfectant treatments for considerably longer periods, namely, minutes or hours [1, 2, 9, 15, 20]. Particularly, the bactericidal activities of NaClO diminish in the presence of organic material loads [21]. Besides, it was elucidated that an alkaline agent, namely food additive grade calcium hydroxide ($FdCa(OH)_2$) has synergistic effects together with NaClO or quaternary ammonium compound (QAC), in terms of capacity for inactivating microorganisms in the presence of organic material contamination [1, 10, 21].

Here we used an avian coronavirus (infectious bronchitis virus: IBV) instead of the pandemic human coronavirus (SARS-CoV-2), so as to demonstrate an equivalent evaluation system for certain disinfectants applied through two techniques, assuming its applicability to both viruses. For the carrier tests, dropping and wiping disinfection techniques were compared. This evaluation system seems to be important and workable for resultful selection of the tested disinfectants against the pertinent viruses on surfaces, particularly (yet not solely) on plastic fomite.

In the present experiments, we used contaminated suspension, and carrier tests were conducted to compare virucidal activities of disinfectants. For the carrier experiments, IBV was deposited on a plastic carrier, and the two following methods were compared to evaluate virudical efficacy of disinfectants toward the virus on the carrier. 1) 500 µl of each disinfectant was dropped to the virus on the carrier; hereafter dropping method. 2) a rayon sheet containing 500 µl of each disinfectant was used for wiping the carrier; hereafter wiping method.

MATERIALS AND METHODS

Virus

Coronaviridae, *Gammacoronavirus*, IBV strain M41, kindly supplied by National Institute of Animal Health (Tsukuba, Ibaraki, Japan), was propagated in primary chicken kidney cell (CKC) cultures, and titrated in plaque assay on CKC monolayers as described [19]. For virus growth, Eagle's minimum essential medium containing 0.3% tryptose phosphate broth (TPB), without fetal bovine serum (FBS), was used [19]. For evaluating decontamination in the presence of organic material, 0.5% or 5% FBS was added to the virus medium; these percentages were selected as the representative of normal human secretions, namely, 0.5% of bovine mucin [16], alongside with 5% as the imitation of field organic contamination [1]. Plaques were counted at 3 days post-inoculation. The titer was calculated as plaque forming units (PFU)/ml. Each test was carried out in triplicate; the titers are shown as mean ± SE. Inactivation was considered to be effective, if more than 1,000 times reduced virus titer was obtained [1, 12].

Disinfectants and blocking solution

$FdCa(OH)_2$ powder (Fine Co., Ltd., Tokyo, Japan), NaClO solution containing chlorine at 13% (Fujifilm Wako pure chemical Co., Ltd., Osaka, Japan) and QAC (Rontect®, Scientific Feed Laboratory Co., Ltd., Tokyo, Japan) were purchased. For making 0.17% $FdCa(OH)_2$ solution, 1.7 g of $FdCa(OH)_2$ powder was added to 1,000 ml of redistilled water (dW_2) and then centrifuged at 1,750 × g for 10 min at 4°C. The resulting supernatants were used as 0.17% $FdCa(OH)_2$ solution. NaClO solutions at 1,000 ppm or 500 ppm of total chlorine (NaClO-1,000 or NaClO-500, respectively) were prepared in dW_2, whereas for the mixed solution (NaClO-500 and 0.17% $FdCa(OH)_2$: Mix-500), NaClO was diluted at 500 ppm of total chlorine in 0.17% $FdCa(OH)_2$. The resultant solutions containing NaClO, namely NaClO-1,000, NaClO-500 and Mix-500, were each used for the experiments within 30 min after preparation. QAC was diluted 1:500 (QAC-500) with dW_2 to obtain a final concentration of 200 ppm didecyl-dimethylammonium chloride (DDAC), as recommended by the manufacturer.

A blocking solution containing 30% FBS in 0.7 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.2) was prepared for neutralizing the virus inactivation reaction by the disinfectants as described [1, 10].

Experimental designs

Suspension tests for evaluating the virucidal activities of the solutions against IBV in the aqueous phase: Suspension tests were performed at room temperature (RT: 25 ± 2°C) as previously described [1, 10]. Briefly, 400 µl of 0.17% $FdCa(OH)_2$, NaClO-1,000, NaClO-500, Mix-500 or QAC-500 were mixed with 100 µl of IBV containing 5% FBS in a microtube, respectively, then incubated for 30 sec. Following incubation, the virus inactivation was immediately stopped by adding 500 µl of the blocking solution. To ascertain the effect of the blocking solution, the test solutions were mixed with the blocking solution before the addition of virus (considered as 0 sec treatment and contact time). For the positive control, 100 µl of IBV was inoculated in 400 µl of dW_2 and at 30 sec 500 µl of the blocking solution was added. Then, the remaining virus was titrated after making serial 10-fold dilutions.

Evaluating the virucidal activities of the test solutions toward IBV on contaminated carriers through the dropping technique: Carrier test with the dropping method was performed at RT as described previously [1]. In brief, an amount of 100 µl of IBV strain M41 containing 0.5% FBS was spotted on a plastic carrier coupon (around 5.0 cm × 5.0 cm) and subsequently spread by sterile glass spreader onto the carriers and air dried for 60 min inside the biological safety cabinet at RT (Fig. 1). Then 500 µl of each solution including dW_2 as the positive control was dropped on each carrier and incubated for 1 min. After incubation, the virucidal activities of the test solutions were blocked by placing the carrier into stomacher bags containing 2 ml of the blocking solution. Subsequently, each carrier surface was rubbed vigorously by finger over the stomacher bag to dislodge the virus from the carrier surfaces into fluids. The remaining viable virus in each sample, including the dW_2 control, was titrated on CKC cultures (PFU/ml).

Evaluating the virucidal activities of the test solutions toward IBV on contaminated carriers through the wiping technique: In the carrier test with the wiping method, the plastic plates with the spotted virus, as shown in the dropping technique, were wiped with a rayon-polyester sheets (5 cm × 5 cm, Alphase® 5, Iwatsuki Co., Ltd., Tokyo, Japan) folded into four pieces (2.5 cm × 2.5 cm) (Fig.

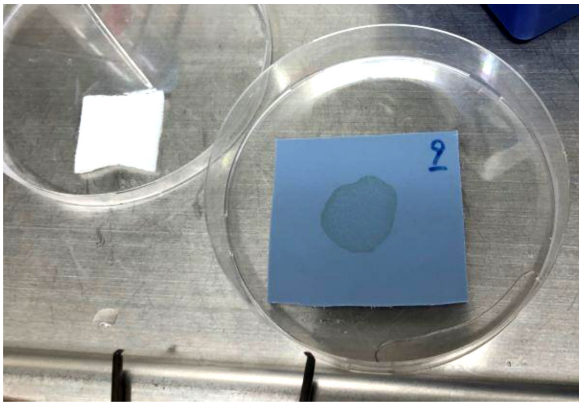

Fig. 1. Deposition of infectious bronchitis virus (IBV) for the carrier tests. One hundred microliter of IBV containing 0.5% fetal bovine serum was spotted on a plastic carrier coupon (around 5.0 cm × 5.0 cm) and subsequently spread by sterile glass spreader onto the carriers and air dried for 60 min inside the biological safety cabinet at room temperature. A rayon-polyester sheet folded into four pieces (2.5 cm × 2.5 cm) is shown in the left.

Table 1. Virucidal efficacy of the tested solutions toward infectious bronchitis virus (IBV) in aqueous phase containing 5% fetal bovine serum

Tested solutions	Viral titer [$\log_{10}$ (PFU/ml)] at different contact times		
	IBV control	0 sec	30 sec
$FdCa(OH)_2$[a]		6.005 ± 0.03	<1.398 ± 0
NaClO-1,000[b]		5.423 ± 0.03	<1.398 ± 0
NaClO-500[c]	6.012 ± 0.03	5.394 ± 0.03	<1.398 ± 0
Mix-500[d]		5.466 ± 0.07	<1.398 ± 0
QAC-500[e]		5.932 ± 0.02	<1.398 ± 0

[a] Food additive grade calcium hydroxide at 0.17% solution, [b] sodium hypochlorite solution at 1,000 ppm of total chlorine, [c] sodium hypochlorite solution at 500 ppm of total chlorine, [d] sodium hypochlorite solution at 500 ppm of total chlorine in 0.17% $FdCa(OH)_2$, [e] quaternary ammonium compound diluted 500-fold in redistilled water. Figures are shown as mean ± SE from replicated 3 times. Viral titer <1.398 $\log_{10}$ PFU (plaque forming units)/ml indicates the virus was inactivated to undetectable level.

1) containing 500 µl of each disinfectant for 30 sec. Each sheet was thereafter transferred into a stomacher bag containing 2.0 ml of blocking solution. The wiped plastic carrier was immediately put into a stomacher bag containing 2.5 ml of the blocking solution. Subsequently, each sheet and carrier was rubbed vigorously, as shown in the dropping technique. Then, the recovered virus was titrated on CKC cultures.

RESULTS

Suspension test for evaluating the virucidal activities of the solutions against IBV in the aqueous phase

As shown in Table 1, 0.17% $FdCa(OH)_2$ solution, NaClO-1,000, NaClO-500, Mix-500 and QAC-500 could inactivate IBV to undetectable level within 30 sec, even in the presence of 5% FBS. When the blocking solution was added before the addition of the virus (0 sec), the viral titer was similar to the positive control. Thus, the blocking solution could stop the virucidal activity of the tested solutions, when mixed together, at equal volume to the reaction medium.

Evaluating the virucidal activities of the test solutions toward IBV on the contaminated carriers with the dropping and wiping techniques

As shown in Table 2, 0.17% $FdCa(OH)_2$ solution resulted in more than 1,000 times reduced virus titer through the dropping technique, within 1 min. NaClO-1,000 and QAC-500 inactivated IBV to the undetectable level. NaClO-500 could not inactivate the virus higher than 1,000 times reduction, whereas Mix-500 did inactivate the virus to undetectable level. With the dropping method, the virus titer in the dW_2 was $10^{6.004}$ PFU/ml, and the original viral titer was $10^{6.472}$ PFU/ml (Table 2). The recovery ratio of IBV from the carrier in dW_2 was around 30%.

With the wiping method, no virus was detected within the sheets after wiping with 0.17% $FdCa(OH)_2$ solution, NaClO-1,000, Mix-500, or QAC-500. These data mean that 0.17% $FdCa(OH)_2$ solution, NaClO-1,000, Mix-500 and QAC-500 were capable of inactivating IBV within 30 sec to undetectable level. The infectious virus remained in NaClO-500 sheet (Table 2), but the virus titer underwent higher than 1,000 times reduction, and was even though considered effective [1, 12]. When the carrier was wiped with dW_2 sheet, the virus was detected within the sheet at $10^{5.4}$ PFU/ml. The recovery ratio of the virus from the sheet with dW_2 was around 20%.

Subsequent to wiping with disinfectant solutions including NaClO-500, no virus was detected on the carriers. With dW_2 wiping, the virus at $10^{2.9}$ PFU/ml was thereafter detected on the carriers. This means that wiping with dW_2 could remove the virus up to more than 99.9% from the contaminated carrier via a mechanical action, although the sheet contained large amount of infectious virus (around 20% of the original viral load).

DISCUSSION

As shown in Table 1, at 0 sec, 500 µl of blocking solution was added to the disinfectants before adding the virus, and the viral titer was similar to the virus control for each disinfectant, showing that an equal amount of blocking solution was capable of stopping the virucidal effect of the disinfectants. This means that the 4 times volumes of the blocking solution halted disinfectant

Table 2. Virucidal efficacies of the tested solutions toward infectious bronchitis virus (IBV) on a plastic carrier, applied through the dropping and the wiping methods

Tested solutions	Viral titer [$\log_{10}$ (PFU/ml)] on the carrier or in the sheet			
	IBV control	Dropping-carrier	Wiping-sheet	Wiped-carrier
$FdCa(OH)_2$[a]	6.472 ± 0.04	3.030 ± 0.04*	<1.796 ± 0	<1.796 ± 0
NaClO-1,000[b]		<1.796 ± 0	<1.796 ± 0	<1.796 ± 0
NaClO-500[c]		4.783 ± 0.05	2.515 ± 0.15*	<1.796 ± 0
Mix-500[d]		2.078 ± 0.23*	<1.796 ± 0	<1.796 ± 0
QAC-500[e]		<1.796 ± 0	<1.796 ± 0	<1.796 ± 0
dW_2		6.004 ± 0.04	5.449 ± 0.05	2.877 ± 0.22*

[a] Food additive grade calcium hydroxide at 0.17% solution, [b] sodium hypochlorite solution at 1,000 ppm of total chlorine, [c] sodium hypochlorite solution at 500 ppm of total chlorine, [d] sodium hypochlorite solution at 500 ppm of total chlorine in 0.17% $FdCa(OH)_2$, [e] quaternary ammonium compound diluted 500-fold in redistilled water. Figures are shown as mean ± SE from replicated 3 times. *Single asterisk indicates effective viral reduction (>3 $\log_{10}$ plaque forming units (PFU)/ml). Viral titer <1.796 $\log_{10}$ PFU/ml indicates the virus was inactivated to undetectable level.

activity in the carrier tests.

All disinfectants could inactivate IBV in the suspension tests within 30 sec. Such a suspension test should be used as the first step for screening of disinfectants, while the second screening is required by means of carrier disinfection tests for field usage [14, 17, 18]. Sattar *et al*. used the concentration of 0.5% of bovine mucin, which is representative of the level of human mucin found in normal human secretions [16]. In the present study, 0.5% FBS was used to imitate mucin for the carrier tests.

Normally, pathogens will not be recovered from towels used for decontamination, because relatively large volumes of a neutralizer are required to properly immerse the entire towel for microbial recovery [17]. Becker *et al.* found that the wipes contained viruses after use, yet they did not use blocking solution to stop the virucidal activities of the disinfectants, and detected cytotoxicity of the disinfectants toward the host cells [3]. In the present study, the rayon-polyester sheet was used for wiping, and it was then transferred into the blocking solution immediately after wiping. The virus was recovered from the sheet to confirm the inactivation of the virus. We thus used 60 mm dishes for the plaque assay that allowed low detection limit as $10^{1.4}$ PFU/ml, without cytotoxicity of the disinfectants in the suspension tests (Table 1). For the carrier tests, 500 µl of disinfectant solutions and 2 ml of the blocking solution allowed detection limit as low as $10^{1.8}$ PFU/ml (Table 2).

It was previously demonstrated that pathogens on abiotic carriers or in feces are more resistant to disinfectants, as compared with the aqueous phase contamination [1, 2, 9, 15, 20]. When the disinfectant efficacies toward pathogens found on a table or a button of an elevator are appraised, the carrier test should be performed. It has been suggested that carrier tests which simulate practical conditions are required to evaluate disinfectants [14, 18]. Otherwise, we may not make the proper decisions concerning the effectiveness and usability of disinfectants toward pathogens deposited on fomites.

In the present study, dropping and wiping techniques were also compared to evaluate the efficacy of virucidal activities of disinfectants. To inactivate the virus on the carrier with the dropping method, more time or higher concentration than with the wiping method was required. Our hypothesis is that during wiping the virus particles were removed from the carrier into the disinfectant solution in the rayon-polyester sheets, and that this wiping step was essential for inactivation, because the wiping allowed virus particles on the carrier moving to the aqueous phase.

Even under organic contamination, namely with 5% FBS, the 0.17% $FdCa(OH)_2$ solution was capable of inactivating the virus in the aqueous phase within 30 sec. This fact means that it is safe to reuse the towel wiped on a surface and rinse it with a bucket of 0.17% $FdCa(OH)_2$ solution, in order to wipe a new area. NaClO-1,000, NaClO-500 and QAC-500 could also inactivate IBV inoculum containing 5% FBS, but it is widely known that NaClO and QAC lose their microbicidal activities in the presence of organic materials [4, 7].

At the level of field usage, one wipe (towel or sheet) should be used for a wide surface, while the accumulation of organic materials in the wipe must be considered. Notably, the 0.17% $FdCa(OH)_2$ solution showed stable virucidal efficacy for more than 3 months after preparation.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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