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Major Article

Evaluating the virucidal activity of four disinfectants against SARS-CoV-2

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Key Words:

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SARS-CoV-2

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Integrated cell culture-qPCR method

A B S T R A C T

Background: The recent COVID-19 pandemic highlights the need for efficacious virucidal products to limit the spread of SARS-CoV-2. Several studies have suggested that alcohol-based sanitizers and some disinfectants are effective. While virucidal activity data of low-level disinfectants are lacking and some conclusions are not clear yet.

Methods: We evaluated the virucidal activity of 2 quaternary ammonium compounds (QAC) disinfectants (MICRO-CHEM PLUS and FWD), W30 (an amphoteric surfactant), and Medical EtOH against SARS-CoV-2. Suspension tests covering different concentration and contact time were performed using the integrated cell culture-qPCR method.

Results: Each of disinfectants was effective at inactivating SARS-CoV-2. MCP and FWD are highly effective within 15 seconds. W30 is also efficient within 2 minutes at concentration of 1%. Consistent with previous report, our results also demonstrated that 38% ethanol was sufficient to completely inactivate virus, which proved the method used in this study is feasible.

Conclusions and Discussion: QAC disinfectants, MCP and FWD, are highly effective for the inactivation of SARS-CoV-2, which making them practical for use in health care setting and laboratories where prompt disinfection is important. The low-level disinfectant based on amphoteric surfactant, W30, which may present in commonly available household hygiene agents is also able to inactivate SARS-CoV-2.

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INTRODUCTION

An emergent pneumonia outbreak happened in the late December 2019. Researchers have quickly isolated a new virus from the patient and sequenced its genome.¹ The infectious agent of this disease was identified as a novel coronavirus, SARS-CoV-2, and the novel viral pneumonia was named as COVID-19 by WHO. The current COVID-19 pandemic in more than 250 countries has become a serious threat to the public health and economy worldwide.

The novel coronavirus belong to the family of Coronaviridae (β -CoV) together with SARS-CoV and MERS-CoV according to the

phylogenetic analysis based on the viral genome,^{1,2} which comprising large, single, plus-stranded RNA genome, but the nucleotide sequence similarity is less than 80% between SARS-CoV-2 and SARS-CoV (about 79%) or MERS-CoV (about 50%). Unlike SARS-CoV, SARS-CoV-2 seems to replicate efficiently in the upper airways during the incubation period, which is estimated to last up to 14 days.^{3,4} During the prodromal stage, asymptomatic and pre-symptomatic individuals release large amounts of viruses from infected cells.⁴ As a result, viral transmission is more effective with SARS-CoV-2 than with SARS-CoV. The rapidly increasing number of cases and evidence of human-to-human transmission also suggested that SARS-CoV-2 was more contagious than SARS-CoV and MERS-CoV.^{5,6}

SARS-CoV-2 is mainly transmitted through person-to-person close contact (<1.5–2.0 m), as well as by aerosol respiratory droplets smaller than 5 μ m of diameter.⁷ The transport of droplet aerosols generated by infected individuals is an issue of considerable concern and importance and has been taken into account to reduce the risk of infections.^{8,9} The common transmission routes include direct transmission (cough, sneeze, and droplet inhalation transmission) and

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indirect transmission. Among many sources of indirect transmission, there is the contamination of inert/inanimate surfaces and hands. SARS-CoV-2 may be transmitted via contact by touching contaminated surface, followed by touching mouth, nose or eyes.

Experimental studies have reported prolonged survival of SARS-CoV-2 on inanimate surfaces and objects under laboratory conditions (e.g., a large inoculum of 10^7 virus particles on a small surface), and the conclusion was that fomite transmission of SARS-CoV-2 is certainly plausible.¹⁰ The importance of surface-mediated transmission, particularly in light of the current outbreak, was demonstrated by Rawlinson et al.,¹¹ who used a DNA oligonucleotide surrogate for contaminated bodily fluid based on the cauliflower mosaic virus to determine how SARS-CoV-2 would spread within a clinical surface environment. The results showed that within 10 hours, the surrogate moved from the isolation room and transferred to 41% of all surfaces sampled. That study highlighted the role of surfaces as a reservoir of pathogens and the need to address requirements for surface cleaning.

COVID-19 has been particularly devastating, thus enhanced disinfection and other preventative measures against SARS-CoV-2 have been adopted worldwide to limit its spread. For example, WHO recommends cleaning surfaces with water, detergents and disinfectants usually effective to clean the environment,¹² because SARS-CoV-2 should be very susceptible to most cleaning agents as an enveloped virus. Some studies using other coronaviruses as the surrogate performed in the last decades have reported the effects of a number of disinfectants for the mitigation of the coronavirus: the ethanol at concentration >62%, isopropanol, povidone iodine, sodium hypochlorite and quaternary ammonium compounds combined with alcohol are effective for surface disinfection.¹³⁻¹⁶ In turn, hydrogen peroxide vapor, chlorine dioxide, ozone, and UV light could be applied to reduce viral load present in aerosols¹⁷⁻²⁰. And several recent studies have performed the in vitro evaluation of disinfection effectiveness against SARS-CoV-2 suggested that alcohol-based disinfectants such as ethanol and isopropanol, and some alcohol-free hand sanitizer and UV light are really effective against SARS-CoV-2.^{16,21-23}

Nevertheless, the disinfection data for SARS-CoV-2 are still limited as a novel virus and a biosafety level-3 (BSL-3) agent. We could draw conclusions about which disinfectants are effective against it from the studies using other coronaviruses speculatively, while even viruses within the same family can respond differently to a given disinfectant.²⁴ In addition, there is some divergence about whether some disinfectants work best against SARS-CoV-2. For instance, one prominent review article reported that benzalkonium chloride was probably “less effective” against SARS-CoV-2, which

was cited by the Centers for Disease Control (CDC) of the United States as a reason to avoid using benzalkonium chloride-based hand sanitizer products.^{25,26} At the same time, the Environmental Protection Agency (EPA) of the United States and Health Canada both list benzalkonium chloride product on their official list of disinfectants recommended for use against SARS-CoV-2.²⁷ More research is needed in this area.

In this study, we tested 2 quaternary ammonium compounds (QAC) (MICRO-CHEM PLUS and FWD), W30 and Medical EtOH against SARS-CoV-2 and performed a comparative inactivation analysis of these disinfectants. MICRO-CHEM PLUS Detergent Disinfectant (MCP) can be found on the EPA list and it claims that it can be used against SARS-CoV-2 when used in accordance with the directions for use against Norovirus on hard, nonporous surfaces. FWD is a novel dual quaternary ammonium biocide in the research and development stage, which was compared with MCP in our study. W30 is an amphoteric surfactant containing N-Alkyl aminopropyl glycine as the core ingredient, and can be used as biocidal product.

MATERIALS AND METHODS

Virucidal products tested

Four disinfectants were tested in this study (Table 1). Among them, Micro-Chem Plus (MCP, National Chemical Laboratories, Inc.) and Medical EtOH are 2 commercial, broad-spectrum disinfectants. Similar with MCP but more environmental friendly, FWD is also a dual quaternary ammonium compounds product which is still in the stage of research and development. W30 is a raw material on the basis of an amphoteric surfactant for use in biocidal products which core ingredient is. N-Alkyl aminopropyl glycine.

Cell culture and viral strains

Vero cells were cultured at 37°C with 5% CO₂ in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). SARS-CoV-2 (SARS-CoV-2 China/Wuhan/WIV04/201912) was propagated in Vero cells. All works involving infectious SARS-CoV-2 were performed in BSL-4 facility in National Biosafety Laboratory, Wuhan.

Determination of cytotoxicity

Most disinfectants destroy cell cultures and the mixtures of viruses and disinfectants must be diluted before testing. So

Table 1
Tested disinfectants list and the cell sensitivity to the disinfectants

Test products	Core biocidal agents	Concentrations (v/v)	Dilutions	Cytotoxicity
MCP	Dual quaternary ammonium compounds	5%	1:10	+
			1:100	+
			1:500	-
			1:1000	-
FWD	Dual quaternary ammonium compounds	5%	1:10	+
			1:100	+
			1:500	-
			1:1000	-
W30	N-Alkylaminopropyl Glycine	1%	1:10	+
			1:100	+
			1:1000	+
			1:2000	-
Medical EtOH	Ethanol	95%	1:10	+/-*
			1:100	-

“+” means cytotoxicity; “-” means no cytotoxicity.

*A slight cytotoxic effects caused by medical ethanol was observed, but it disappeared after dilution was discarded and cells were overlaid with fresh DMEM with 2% FBS medium for overnight.

cytotoxic effects were firstly assessed in Vero cells using medium (DMEM with 2% FBS) and disinfectant but without addition of virus to ensure that diluted disinfectants was not cytotoxic. Briefly, the test products were serially diluted, and aliquots of 1 mL from each sample were inoculated into cells. Following for 1 hour incubation at 37°C, dilutions were discarded and cells were overlaid with fresh medium. The cells were observed for cytotoxic effects for the same incubation time which was later used for the suspension tests (Table 1).

Inactivation assay using quantitative suspension test

A suspension test method was used for inactivation assay in this study.²⁸ For each of the inactivation experiments, equal volume of disinfectant with different concentration and virus stock were mixed. Immediately after the specific contact time at room temperature (RT), the virus-disinfectant mixture was diluted with medium to avoid extension of the effective incubation period and eliminated the toxicity of disinfectant according to above cytotoxicity assay results. Then some diluted virus-disinfectant mixture was taken out to infect Vero cells. The CPE was observed after 3-4 days postinfection and supernatant were harvest. The viral RNA was extracted from the supernatant and using quantitative RT-PCR (qPCR) to assess virus titer. At the same time, other diluted virus-disinfectant mixture was taken out to perform plaque assay to determine the residual infectivity, if possible. For each experiment, virus control containing medium instead of disinfectant was included.

Extraction of viral RNA and qRT-PCR

Viral RNA was isolated using the QIAamp 96 Virus QIAcube HT kit (QIAGEN) and used as a template for the amplification of selected genes by real-time qPCR using Detection Kit for novel Coronavirus (2019-nCoV) (PCR-Fluorescence Probing) (Da An Gene Co. Ltd) according to the manufacturer’s instructions. The standard curve for virus titres calculation was established using the virus stock with known titres (Fig 1). The virus stock was serial diluted (1:10) and viral RNAs were extracted from these serial dilutions. Ct values of the range of dilutions covering 8 gradients were used to draw the standard curve. The corresponding virus titre was calculated based on the standard curve.

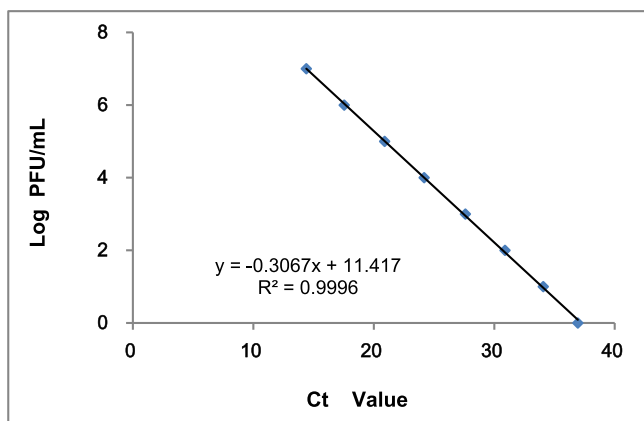


Fig 1. Q-PCR standard curve for SRAS-CoV-2. The virus stock was serial diluted (1:10) and viral RNAs were extracted from these serial dilutions. Ct values of the range of dilutions covering 8 gradients were used to draw the standard curve. The corresponding virus titre was calculated based on the standard curve.

Plaque assay

Plaque assay was performed as follows: Virus samples were serially diluted 10-fold in medium and layered on the cells cultured for 12-24 hours in triplicates. Following for 1 hour incubation at 37°C, cells were overlaid with CMC medium (2% FBS with 1% Carboxy Methyl Cellulose). Cells were cultured for 72-96 hours at 37°C to allow plaque formation. Once plaques were established, the supernatant was discarded and cells were fixed with 4% paraformaldehyde for 1 hour to inactivate infectious virus. Cells were stained with 1% crystal violet, and plaques were visualized and counted.

Calculation of the reduction factor

The virucidal activity was determined by the difference of the logarithmic titre of the virus control minus the logarithmic titre of the test virus, reduction factor (RF). The Log10 titre and its standard deviation (SD) were calculated as well as the variance of the RF. RF of ≥4 was regarded as evidence of sufficient virucidal activity.²⁸

RESULTS

Full results of suspension tests are listed in Table 2. Each of disinfectants tested was effective at inactivating SARS-CoV-2.

Our results show that MCP and FWD, QAC disinfectant, are highly effective at inactivating SARS-CoV-2 within 15 seconds of contact time and at very low concentration (Table 2 and Fig 2). The only difference is that FWD with concentration of 0.06% is unable to inactivate SARS-CoV-2, while MCP with the same concentration can inactivate the virus, but more than 8 minutes of contact time is necessary. Usually QAC disinfectants need to be diluted before use and they

Table 2
Reduction factors of four disinfectants against SRAS-CoV-2

Test products	Concentrations	Reduction factor					
		Contact time period					
		15 sec	30 sec	1 min	2 min	4 min	8 min
MCP	5%	>4	>4	>4	>4	>4	>4
	1.67% (5%/3)	>4	>4	>4	>4	>4	>4
	0.56% (5%/9)	>4	>4	>4	>4	>4	>4
	0.19% (5%/27)	1.46	3.23	>4	>4	>4	>4
	0.06% (5%/81)	0	0.1	0.41	1.47	3.03	>4
FWD	5%	>4	>4	>4	>4	>4	>4
	1.67% (5%/3)	>4	>4	>4	>4	>4	>4
	0.56% (5%/9)	>4	>4	>4	>4	>4	>4
	0.19% (5%/27)	0.11	>4	>4	>4	>4	>4
	0.06% (5%/81)	0	0	0	0	0	0
MCP-1W	5%	>4	>4	>4	>4	>4	>4
	1.67% (5%/3)	0	>4	>4	>4	>4	>4
	0.56% (5%/9)	0	0	>4	>4	>4	>4
	0.19% (5%/27)	0	0	0	>4	>4	>4
	0.06% (5%/81)	0	0	0	0	0	>4
FWD-1W	5%	>4	>4	>4	>4	>4	>4
	1.67% (5%/3)	0	1.91	>4	>4	>4	>4
	0.56% (5%/9)	0	0	>4	>4	>4	>4
	0.19% (5%/27)	0	0	0	>4	>4	>4
	0.06% (5%/81)	0	0	0	0	0	0
W30	1%	0	0	0	>4	>4	>4
	0.5%	0	0	0	0	1.85	>4
	0.25%	0	0	0	0	0	>4
	0.125%	0	0	0	0	0	0
Medical EtOH	100%	>4	>4	>4	>4	>4	>4
	80%	>4	>4	>4	>4	>4	>4
	60%	>4	>4	>4	>4	>4	>4
	40%	>4	>4	>4	>4	>4	>4
	20%	0.22	0.52	0.46	0.25	0.13	0.14

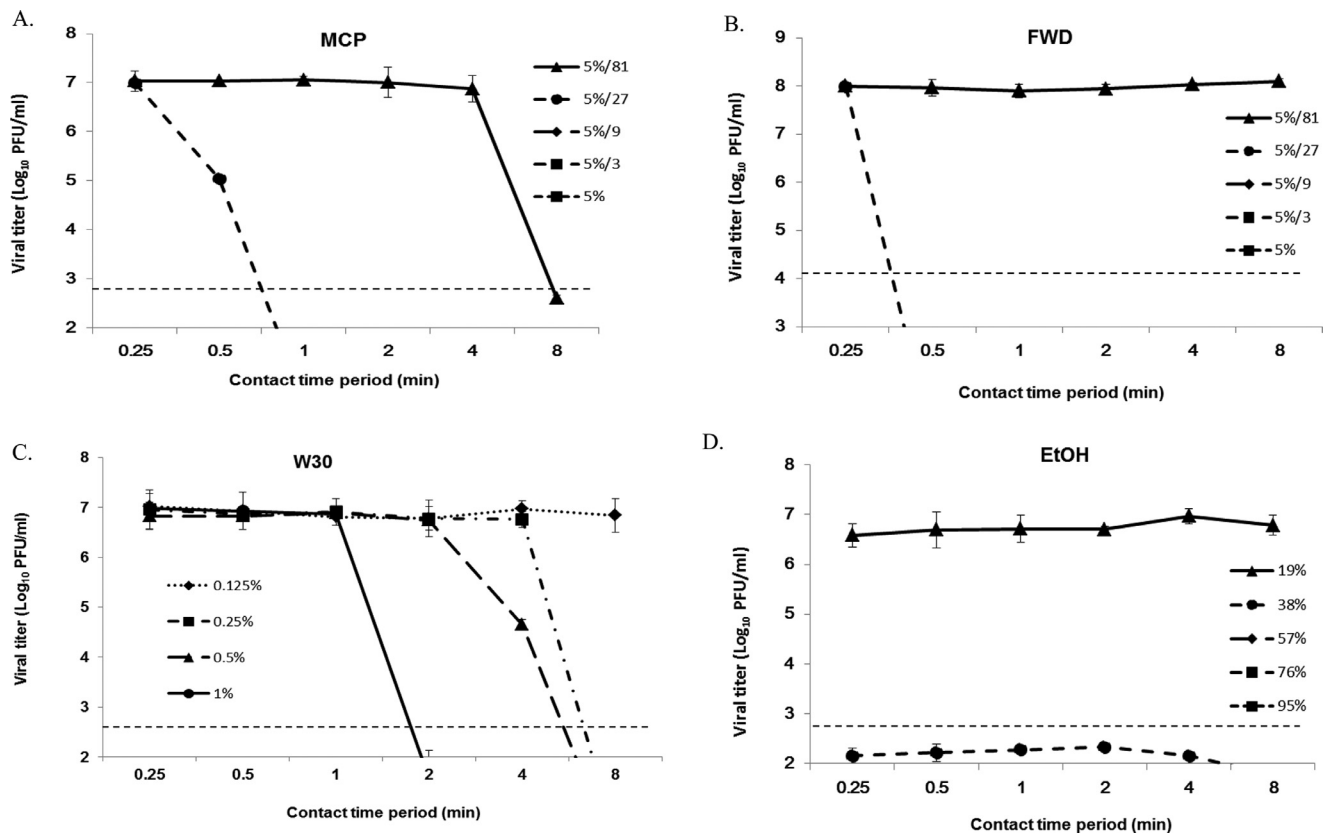


Fig 2. Virucidal activity of MCP, FWD, W30, and medical ethanol against SARS-CoV-2. The biocide concentrations ranged from about 0.06% to 5% (MCP, FWD, W30) or 20% to 100% (medicinal alcohol, 95%) with an exposure time from 15 seconds to 8 minutes. CPE of infected cells in each test was observed after 3 days postinfection. Efficacy of four disinfectants against SARS-CoV-2 was addressed by quantitative suspension assay and residual infectivity was determined by virus titer using quantitative RT-PCR (qPCR). (A) MCP. (B) FWD. (C) W30. (D) Medical ethanol.

may not be used up immediately. We wonder how about their virucidal activity after these QAC disinfectants are diluted and kept for a period of time at RT. Then the virucidal activity of MCP and FWD were tested after they were diluted and kept at RT for 1 week (MCP-1W and FWD-1W, Table 2). Our results showed that their virucidal effect was decreased a little, but they can still be effective against SARS-CoV-2 when we used the disinfectant with the same concentration and kept longer contact time. It is amazing that 0.06% MCP can still inactivate SARS-CoV-2 completely with more than 8 minutes of contact time even it has been kept for one week at RT. These results indicated that the virucidal activity against SARS-CoV-2 of MCP and FWD are very stable and enough contact time is important, which may be important to all QAC disinfectant.

Under the same conditions, W30 is likely not very effective against SARS-CoV-2 comparing with QAC disinfectants above 1% W30 is effective at inactivating SARS-CoV-2 within 2 minutes, which is much longer than QAC disinfectant (Table 2 and Fig 2). W30 with lower concentration need more contact time to inactivate the virus. But, as an amphoteric surfactant, it could be used in daily chemical products usually because of its lower toxicity to human. In this case, it should be contribute to household environmental cleaning and personal hygiene, particularly critical in the COVID-19 pandemic.

Most alcohols exhibit a broad spectrum of germicidal activity against bacteria, viruses, and fungi. And the degree of effect depends on the percentage concentrations of the alcohol and the physical properties of the target microorganism. The optimum bactericidal concentrations of alcohols range from 60% to 90% v/v solutions in

water but are generally ineffective against most microorganisms below 50% v/v.²⁹ A recent study has shown that >30% concentrations of ethanol or isopropanol were effective in inactivating SARS-CoV-2 within 30 seconds.¹⁶ Consistent with this report, our results showed that 38% ethanol was sufficient to completely inactivate virus immediately (within 15 seconds) (Table 2 and Fig 2).

Based on the data obtained above we compared the inactivation profiles of four disinfectants (Table 2). Except ethanol, QAC disinfectant (MCP and FWD) and amphoteric surfactant (W30) all exhibit the similar dose-dependent inactivating SARS-CoV-2 pattern. That is, the better inactivation effect are shown when the disinfectants are used at higher concentration and have longer exposure time.

In this study, we use the integrated cell culture-qPCR method to validate virus inactivation effect, not to detect the residue virus directly. The general process is as follows: we pretreat virus with disinfectants, and then inoculate host cells with the viruses-disinfectant mixture (containing inactivated viruses and infectious viruses). The noninfectious viruses are removed after the cells are washed several times. Subsequently, the cells are incubated for an optimized period to amplify the intracellular viruses. Finally, the nucleic acids of viruses are extracted from cell culture supernatant and used for qPCR with virus-specific primers to quantify the infectious virus after virus inactivation. The virucidal activity of ethanol against SARS-CoV-2 shown in our study was consistent with previous studies¹⁶ and demonstrated that this method is a feasible strategy. In addition, we also performed the plaque assay to calculate the residue virus and obtained the same results with the integrated cell culture-qPCR method (Fig 3). At the same time, we found that

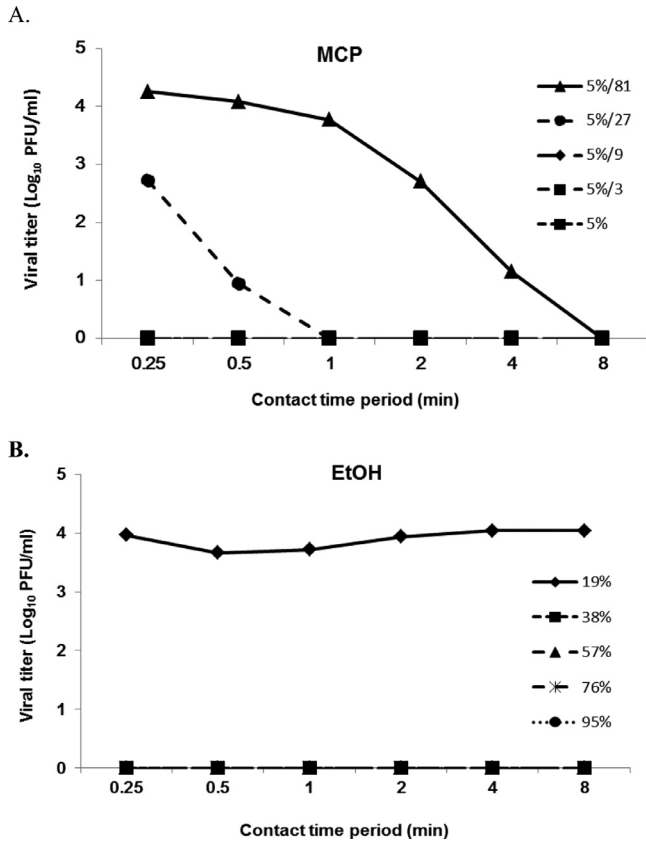


Fig. 3. Virucidal activity of MCP and medical ethanol against SARS-CoV-2. The biocide concentrations ranged from about 0.06% to 5% or 20% to 100% of medicinal alcohol (95%) with an exposure time from 15 seconds to 8 minutes. CPE of infected cells in each test was observed after virus incubation for different time with different final concentration of four disinfectants. The residual infectivity was determined by virus titer using plaque assay. (A) MCP. (B) Medical ethanol.

the primary results of inactivation effect could be easily obtained from CPE observation which was very close to the RF results (Supplementary Tables 1-4).

DISCUSSION

When a new pandemic occurs, a lot of health advices are given, but it should be noted that these methods may not be the most accurate and correct until sufficient knowledge are obtained. Due to the insufficient treatments and vaccine, prevent the spread of SARS-CoV-2 via droplets and contaminated surface is the primary intervention during the prevalence of COVID-19 in most parts of the world.

Several previous studies have performed the in vitro evaluation of disinfection effectiveness against SARS-CoV-2 and these studies all have suggested that alcohol-based disinfectants such as ethanol (EA) and isopropanol (IPA) are effective.^{16,21,22,30,31} Conversely, the disinfection effectiveness of low-level disinfectants in the limited number of studies, such as chlorhexidine gluconate and benzalkonium chloride, is not very unclear yet.^{23,25}

In this study, we evaluated virucidal efficacy of 2 QAC disinfectants, a kind of low-level disinfectant, against SARS-CoV-2 in detail. The first synthesis and recognition of their antimicrobial activity occurred almost 100 years ago, but it was not until after World War II that QACs came into widespread use. Today, they are used in numerous consumer products and in the food and health care industries for cleaning, sanitizing and disinfecting surfaces. Their low toxicity and

ability to be formulated for specific applications and target organisms help account for their widespread use. QACs are cationic detergents (surfactants or surface-active agents). They reduce surface tension and form micelles, allowing dispersion in a liquid. QACs are active ingredients in over 200 disinfectants currently recommended by the USA EPA to inactivate SARS-CoV-2.^{27,32} However, only a few detailed efficacy reports, including in vitro or in clinical trials, could be searched so far. Here we determined the virucidal effect of MCP (QAC disinfectant, approved by EPA officially) and a novel QAC disinfectant (FWD) in detail. Our results show that all of them are very effective at inactivating SARA-CoV-2.

The virucidal efficacy between MCP and FWD vary slightly at concentration of 0.06% because they are a little different in composition. As we known, QACs are classified on the basis of the nature of the R groups, which can include the number of nitrogen atoms, branching of the carbon chain, and the presence of aromatic groups. These variations can affect the antimicrobial activity of the QAC in terms of dose and action against different groups of microorganisms, and the length of the R groups can also greatly affect their antimicrobial activity.³³ In addition, the potential environmental impact of QACs, which may include disruption of wastewater treatment unit operations, proliferation of antibiotic resistance, formation of nitrosamine disinfection byproducts, and negative effects on biota of surface waters, should be considered. Exploration of potential technologies to minimize the environmental releases of QACs is highly warranted.³⁴ For example, MCP contains surfactants known as nonylphenol ethoxylates (NPE) that are considered highly toxic to the aquatic environment. FWD, a novel disinfectant which is still in the stage of research and development, is also based on dual quaternary ammonium compounds but lacks NPEs. The similar virucidal activity in our study indicated FWD could be considered as a very potential alternate for MCP.

W30, as another kind of low-level disinfectant based on amphoteric surfactant, is not likely to be very effective compared with QAC disinfectant and ethanol. However, surfactant has several advantages for skin disinfection. It is nontoxic, less irritating and non-flammable and can be used in many household cleaning/hygiene agents, such as hand soap and shampoo. We would like to highlight that this kind of amphoteric surfactant, such as W30, is also able to inactivate SARS-CoV-2. Thus household cleaning and skin hygiene using such products containing W30 may also act as a contributing factor to limit the spread of SARS-CoV-2, potentially reducing the need for the use of disinfectants in settings with limited availability.

One significant point of our study is that we use the integrated cell culture-qPCR method to validate virus inactivation effect of disinfectants. Even though traditional virus detection methods in the inactivation validation study utilize CPE and TCID₅₀ assay/plaque assay as the gold standard, there are still several disadvantages. For example, when the initial titre of virus used for inactivation effect test is low, RF >4 could not be reached or longer incubation times are needed. To find a more feasible strategy, we involved pretreatment of the virus with disinfectant and quantify the virus titres using qPCR. This method utilizes the host cell as an efficient tool to separate infectious and noninfectious viruses because only the living virus can inject its genome into the host cell for amplification. The cells were incubated for an optimized period to amplify the viruses, decrease the limit of quantitation and improve the sensitivity of detection.

We conclude that QAC disinfectants, MCP and FWD, are highly effective for the inactivation of SARS-CoV-2. Beyond merely inactivating the virus, QAC disinfectants act quickly, making them practical for use in health care setting and laboratories where prompt disinfection is important. In addition, the low-level disinfectant based on amphoteric surfactant, W30, which may present in commonly available household cleaning/hygiene agents is also able to inactivate SARS-CoV-2.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at <https://doi.org/10.1016/j.ajic.2021.10.035>.

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