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# Acidic electrolyzed water potently inactivates SARS-CoV-2 depending on the amount of free available chlorine contacting with the virus

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## ABSTRACT

Alcohol-based disinfectant shortage is a serious concern in the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic. Acidic electrolyzed water (EW) with a high concentration of free available chlorine (FAC) shows strong antimicrobial activity against bacteria, fungi, and viruses. Here, we assessed the SARS-CoV-2-inactivating efficacy of acidic EW for use as an alternative disinfectant. The quick virucidal effect of acidic EW depended on the concentrations of contained-FAC. The effect completely disappeared in acidic EW in which FAC was lost owing to long-time storage after generation. In addition, the virucidal activity increased proportionately with the volume of acidic EW mixed with the virus solution when the FAC concentration in EW was same. These findings suggest that the virucidal activity of acidic EW against SARS-CoV-2 depends on the amount of FAC contacting the virus.

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## 1. Introduction

Patients with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) were first reported in China in December 2019 [1]. On June 22, 2020, the World Health Organization (WHO) reported >8.8 million confirmed SARS-CoV-2 cases and >460,000 deaths worldwide [2]. It is fundamentally important to decrease human-to-human infection by enforcing strict, practically sustainable control measures such as providing a sufficient amount of antiviral disinfectant to people. However, the worldwide demand has led to a shortage of alcohol-based disinfectants that effectively inactivate SARS-CoV-2 [3].

Acidic electrolyzed water (EW) with a high concentration of free available chlorine (FAC) shows strong antimicrobial activity against bacteria, fungi, and viruses. EW does not harm humans compared to chlorine, so its application to agricultural and food industries is approved [4–6]. An EW generator simultaneously produces a large

amount of hypochlorous acid EW and strong alkaline water by electrolyzing water containing NaCl or KCl in an electrolysis chamber. Acidic EW has a virucidal effect against human immunodeficiency virus, hepatitis B virus, herpes simplex viruses, norovirus, influenza A virus, and food-and-mouth disease virus [7–12].

This study evaluated the virucidal effect of EW against SARS-CoV-2 in order to facilitate its wide usage as an alternative disinfectant and contribute to SARS-CoV-2 control.

## 2. Materials and methods

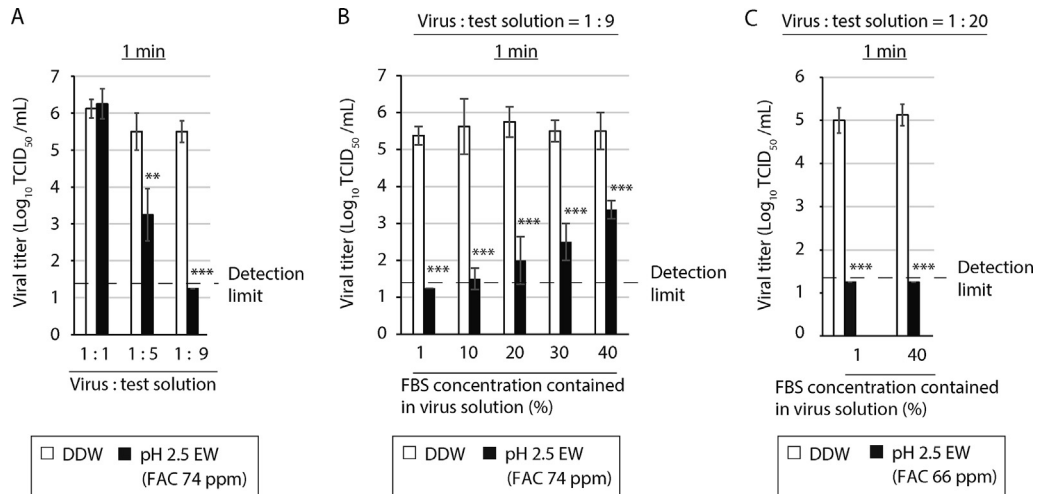
### 2.1. Test solutions

The pH 2.5 and FAC concentrations (66–109 ppm) of NaCl-free EW (Clean Refre; Act Co., Obihiro, Japan) was generated using a three-compartment Clean Fine electrolyzer (Act Co.). The difference in FAC concentrations was due to the difference in the production lot of EW. Each EW sample was stored in a tightly capped shade bottle at room temperature and used within 9 days. In addition, acidic EW samples (pH 2.7) with a low FAC concentrations (23 and 2 ppm) were prepared by leaving the fresh acidic EW for 17 and 31 days after generation, respectively, without closing the shade bottle cap. Double-distilled water (DDW) was used as a control.

**Abbreviations:** DDW, double-distilled water; EW, electrolyzed water; FAC, free available chlorine; FBS, fetal bovine serum; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; TCID<sub>50</sub>, 50% tissue culture infective dose; WHO, World Health Organization.

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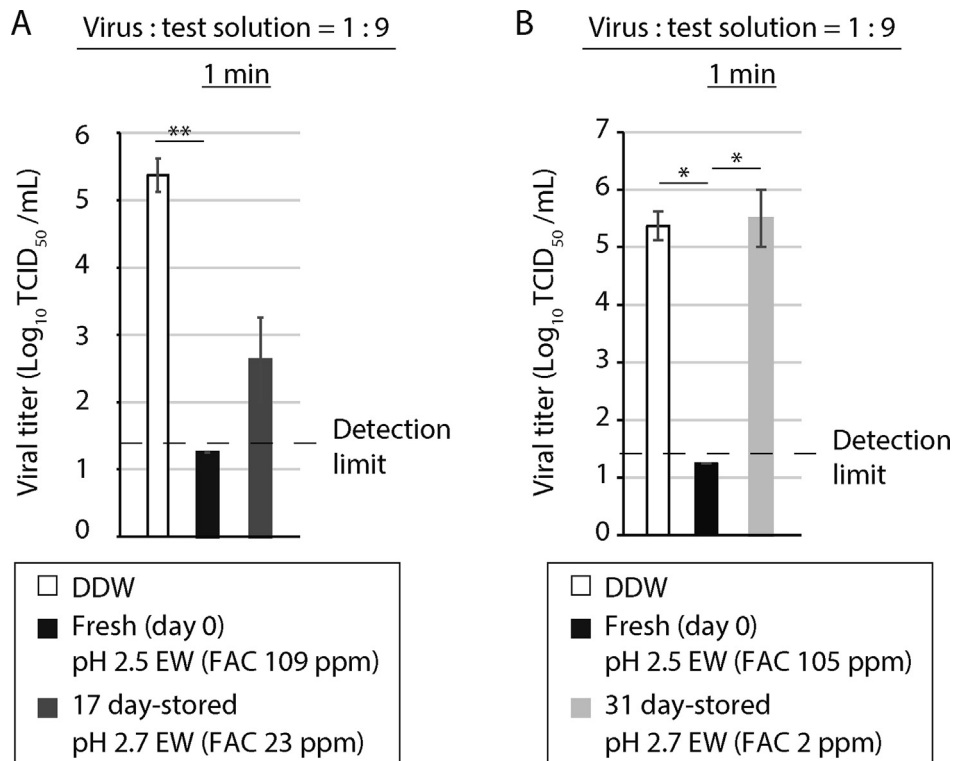


**Fig. 1.** Evaluation of SARS-CoV-2-inactivating activity of acidic EW. (A) 1% FBS-containing SARS-CoV-2 solution was mixed with DDW and acidic EW (pH 2.5, FAC 74 ppm) at virus:test solution ratios of 1:1, 1:5, and 1:9. The reaction time was 1 min. (B) 1%–40% FBS-containing SARS-CoV-2 solution was mixed with DDW and acidic EW (pH 2.5, FAC 74 ppm) at a 1:9 virus:test solution ratio. The reaction time was 1 min. (C) 1% and 40% FBS-containing SARS-CoV-2 solution was mixed with DDW and acidic EW (pH 2.5, FAC 66 ppm) at virus:test solution ratios of 1:20. The reaction time was 1 min. (A–C) Error bars indicate mean  $\pm$  SD ( $n = 4$  per group). Student's *t*-test was performed to analyze statistical significance between the DDW and acidic EW groups; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

## 2.2. Virus and cells

SARS-CoV-2 (JPN/TY/WK-521 strain) and VeroE6/TMPRSS2 cells [13] were obtained from the National Institute of Infectious Diseases (Tokyo, Japan). VeroE6/TMPRSS2 cells were inoculated with SARS-CoV-2 and then cultured in virus growth medium containing

Dulbecco's modified Eagle's medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 1% fetal bovine serum (FBS), 20 mM L-glutamine (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and 100  $\mu\text{g}/\text{mL}$  of kanamycin (Meiji Seika Pharma Co., Ltd., Tokyo, Japan).



**Fig. 2.** Comparison of the SARS-CoV-2-inactivating activity of acidic EWs with different FAC concentrations. (A) 1% FBS-containing SARS-CoV-2 solution was mixed with DDW, fresh (day 0) EW (pH 2.5, FAC 109 ppm), and 17 day-stored EW (pH 2.7, FAC 23 ppm) at a 1:9 virus:test solution ratio. The reaction time was 1 min. (B) 1% FBS-containing SARS-CoV-2 solution was mixed with DDW, fresh (day 0) EW (pH 2.5, FAC 105 ppm) and 31 day-stored EW (pH 2.7, FAC 2 ppm) at a 1:9 virus:test solution ratio. The reaction time was 1 min. (A, B) Error bars indicate mean  $\pm$  SD ( $n = 4$  per group). The Kruskal–Wallis test with Dunn's multiple comparison test was performed to analyze statistical significance among all the groups; \* $P < 0.05$ ; \*\* $P < 0.01$ .

### 2.3. Evaluation of virucidal activity of test solutions

Virus growth medium containing SARS-CoV-2 with 1%–40% (v/v) FBS, of which the viral titer was 5.75–7.25 log<sub>10</sub> 50% tissue culture infective dose (TCID<sub>50</sub>)/mL, was mixed with test solutions in virus:test solution ratios ranging from 1:1 to 1:20. Virus-containing mixtures were placed for 1 min at 25 °C and then inoculated into cells, and a tenfold serial dilution was performed. After incubation for 3 days, a cytopathic effect was observed, and TCID<sub>50</sub>/mL was calculated using the Behrens–Kärber method [14].

### 2.4. Statistical analysis

Student's *t*-test was performed to analyze statistically significant differences between the two groups. The Kruskal–Wallis test with Dunn's multiple comparison test was performed to analyze statistical significances among the three groups. *P* values < 0.05 were used to determine statistical significance.

## 3. Results and discussion

First, the SARS-CoV-2-inactivating activities of acidic EWs (pH 2.5, FAC 74 ppm) with different virus:acidic EW ratios were evaluated. We mixed 1% FBS-containing SARS-CoV-2 solution with DDW or acidic EW in virus:test solution ratios of 1:1, 1:5, and 1:9. After a 1-min reaction, the acidic EW potently inactivated SARS-CoV-2 using 9 times volume of SARS-CoV-2 solution, and the viral titer of acidic EW-treated SARS-CoV-2 solution was below the detection limit ( $\geq 99.99\%$  inactivation; decrease of  $\geq 4.25$  log<sub>10</sub> TCID<sub>50</sub>/mL). However, its activity decreased when using 5 times volume and was unrecognizable when using an equal volume (Fig. 1A). Next, to evaluate the effect of protein in the virus solution on the virucidal activity of acidic EW, 1%–40% FBS-containing SARS-CoV-2 solution was mixed with DDW or EW (pH 2.5, FAC 74 ppm) in a 1:9 virus:test solution ratio. After a 1 min-reaction, the SARS-CoV-2-inactivating ability of acidic EW decreased in proportion to the FBS concentration (Fig. 1B). On the other hand, at a 1:20 virus:test solution ratio, acidic EW (pH 2.5, FAC 66 ppm) potently inactivated the 40% FBS-containing SARS-CoV-2 solution (below the detection limit) (Fig. 1C).

Next, we compared the virucidal activities of fresh (day 0) EW (pH 2.5, FAC 109 ppm) and 17 day-stored EW (pH 2.7, FAC 23 ppm). As a result, the virucidal activity of the 17 day-stored EW was low compared with that of fresh (day 0) EW (Fig. 2A). Furthermore, we evaluated the virucidal activity of 31 day-stored EW (pH 2.7, FAC 2 ppm) and observed that its virucidal activity was completely lost (Fig. 2B).

In this study, the rapid virucidal effect of acidic EW depended on the FAC concentration, and the aged acidic EW in which FAC was almost lost did not show SARS-CoV-2-inactivating ability. This result shows that a mere acidic solution without FAC does not inactivate SARS-CoV-2 in a short period of time. Therefore, the FAC concentration is critical in the virus-inactivating ability of acidic EW. However, when the volume ratio of acidic EW to viral solution is low, even if with a high FAC concentration, such a small volume of EW was not able to inactivate SARS-CoV-2 sufficiently. These results suggest that the virucidal activity of acidic EW against SARS-CoV-2 depends on the amount of FAC contacting the virus. In addition, the presence of proteins in high concentrations prevents the virucidal effect of acidic EW. Our findings further suggest that acidic EW with high amounts of FAC is required to inactivate SARS-CoV-2 in a dirty surface containing many proteins. Therefore, we recommend using adequate volume of acidic EW with high FAC

concentrations for robust SARS-CoV-2 inactivation.

In conclusion, acidic EW with high amounts of FAC is an effective anti-SARS-CoV-2 disinfectant. An ample supply of such effective antiviral disinfectant with quick, potent SARS-CoV-2-inactivating ability can protect people at high risk of SARS-CoV-2 infection and prevent SARS-CoV-2 spread.

### Declaration of competing interest

The authors declared no conflict of interest.

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