ABSTRACT

The advent of the global pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) necessitates a thorough study of the stability and transmissibility in the environment. We characterized the stability of SARS-CoV-2 in three water matrices: fresh, tap, and seawater. The minimum infective dose of SARS-CoV-2 in Vero cells was confirmed to be 10³ PFU/mL. The stability of SARS-CoV-2 varied according to the water matrix: infective SARS-CoV-2 was undetectable after treatment with fresh water and seawater, but remained detectable for 2 days in tap water, when starting with an initial concentration of 10⁴ PFU/mL. When the starting concentration was increased to 10⁵ PFU/mL, a similar trend was observed. In addition, viral RNA persisted longer than infectious virus in all water matrices. This study was conducted in stagnant water containing a significantly high titer of virus, thus, human-to-human transmission of SARS-CoV-2 through the actual aquatic environment is expected to be rare.

Keywords: SARS-CoV-2; Coronavirus; Water Stability; Virus Inactivation

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which emerged in Wuhan, China, in late 2019, is the cause of a rapidly expanding, global pandemic. As a result, more than 7 million confirmed cases, with approximately 400,000 deaths, have been reported worldwide as of June 10, 2020.¹ SARS-CoV-2 was initially introduced into humans through contact with intermediate host animals: wild bats, natural reservoirs for SARS-CoV-2, and frequently sold in traditional Chinese markets.²,³ SARS-CoV-2 can be transmitted by respiratory droplets, aerosols, or contact. Contact can be direct (with an infected subject) or indirect (through hand-mediated transfer of the virus from contaminated fomites to the mouth, nose, or eyes).⁴ However, there is still limited information on the ability of SARS-CoV-2 to be transmitted through various types of water. Therefore, an analysis of SARS-CoV-2 stability in various water matrices will be helpful in establishing quarantine strategies to prevent this virus from spreading further. Herein, we compared the survival of SARS-CoV-2 in three type water: fresh water, tap water and seawater to identify the possibility of human-to-human transmission of SARS-CoV-2 through the aquatic environment in preparation for the summer swim season.-All experiments in this study were performed in triplicate.
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The authors have no potential conflicts of interest to disclose.

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SARS-CoV-2 (BetaCoV/Korea/KCDC03/2020) was 10-fold serially diluted with tap water or infection medium (Dulbecco’s minimal essential medium [Gibco, Grand Island, NY, USA] containing 2% fetal bovine serum [Gibco] and 1% penicillin/streptomycin [Gibco]). The samples diluted with tap water were mixed 1:1 with infection medium to prevent damage to cells during infection. Vero cells (ATCC® CCL-81™), prepared in 96-well plates, were infected with diluted SARS-CoV-2 (10^1–10^5 PFU/mL) and incubated at 37°C for 1 hour. After the removal of the solution, infection medium was added to each well. The infected cells were incubated at 37°C for 2–7 days and monitored for signs of cytopathic effect (CPE). In both infection medium and tap water samples, CPE was observed only in cells infected with SARS-CoV-2 titers of 10^3 PFU/mL or higher; CPE in cells infected with lower viral titers was not observed (Table 1). To confirm additional subcellular toxicity by water matrix itself, the tap water sample without SARS-CoV-2 was tested in the same method as for minimum infective dose (MID) experiment, and CPE was not observed (data not shown). Therefore, we determined 10^3 PFU/mL as the MID of SARS-CoV-2 in our model system.

We evaluated the stability of SARS-CoV-2 in 3 different types of water: fresh water (pH 7.38, 4.15 FTU; Inje-gun, Korea), tap water (pH 7.27, 0.03 FTU, residual chlorine 1.0 mg/L; www.kwater.or.kr), or seawater (pH 8, 1.45 FTU, salinity 32‰; Sokcho, Korea). To determine viral stability over time, virus was diluted to a concentration of 10^4 or 10^5 PFU/mL and to a final volume of 30 mL in water (fresh, tap, or seawater). Water-treated virus was then incubated at room temperature (23°C) for 6 days. Virus samples of 500 μL were harvested and filtered with 0.22 μm membrane daily. Vero cells prepared in 12-well plates were infected with 10-fold serial dilutions of the water-treated viral samples and then incubated at 37°C for 1 hour. After removal of medium, the plates were overlaid with minimal essential medium/agarose and were incubated at 37°C for three days. To visualize plaques, the cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet overnight. SARS-CoV-2 was more stable in tap water than in fresh water or seawater. Starting with a titer of 10^4 PFU/mL, SARS-CoV-2 was viable in tap water up to 3 days (Fig. 1A). However, in fresh water and seawater, viral viability was greatly reduced: the observed titer was significantly lower than the MID on day 0. Furthermore, no viable virus was measured after day 2 or day 1, respectively (Fig. 1A). Results followed a similar trend when using a starting titer of 10^5 PFU/mL: SARS-CoV-2 can remain viable for 6 days (tap water), 2 days (fresh water), and 1 day (seawater), respectively (Fig. 1B). In addition, the stability of SARS-CoV-2 was compared in filtered and unfiltered water matrices. Stability of SARS-CoV-2 was similar in filtered or unfiltered tap water (Supplementary Fig. 1). In contrast, SARS-CoV-2 in filtered fresh water or seawater was more significantly stable than that in unfiltered.

We also used quantitative real-time polymerase chain reaction (qRT-PCR) to assess viral RNA levels in the varied types of water and confirmed that viral RNA levels were reduced concomitantly with the reduction of SARS-CoV-2 activity. Viral RNA was extracted from a 100 μL aliquot of the water-treated virus (QIAamp Viral RNA Mini Kit; QIAGEN, Hilden, Germany) using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Viral RNA was quantified by qRT-PCR (Cepheid GeneXpert Systems; Cepheid, Sunnyvale, CA, USA) using custom-designed primers and probes for SARS-CoV-2 (Table 2). Viral RNA levels were reduced concomitantly with the reduction of SARS-CoV-2 activity. Viral RNA was extracted from a 100 μL aliquot of the water-treated virus (QIAamp Viral RNA Mini Kit; QIAGEN, Hilden, Germany) using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Viral RNA was quantified by qRT-PCR (Cepheid GeneXpert Systems; Cepheid, Sunnyvale, CA, USA) using custom-designed primers and probes for SARS-CoV-2 (Table 2). Viral RNA levels were reduced concomitantly with the reduction of SARS-CoV-2 activity.

**Table 1. Determination of minimum infectious dose in Vero cells**

| Viral titer, PFU/mL | Media         | Tap water |
|---------------------|---------------|-----------|
| 10^5                | +             | +         |
| 10^4                | +             |           |
| 10^3                | +             | +         |
| 10^2                | −             | −         |
| 10^1                | −             | −         |

*Positive for cytopathic effect; Negative for cytopathic effect.

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Extracted RNA was reverse-transcribed and viral N3 primers were used for qRT-PCR (SuperScript IV; Thermo Fisher Scientific, Waltham, MA, USA). Primers used in this study were previously described by the Centers for Disease Control and Prevention (CDC).

6 To quantify viral RNA, the N3 gene was cloned into a pGEM-T Easy vector and N3 RNA was synthesized using the T7 promoter (pGEM-T Easy Vector, RiboMAXTM Large Scale RNA Production System-T7; Promega, Madison, WI, USA).

7 At a starting concentration of 10^4 PFU/mL, SARS-CoV-2 RNA levels were markedly reduced over the time course of the experiment (Fig. 1C). RNA levels dropped 99.26% between day 0 and day 5 (195,934 copies to 1,442 copies) in fresh water. In seawater the trend was similar: RNA levels were reduced by 99.21% between day 0 and day 5 (from 149,330 copies to 1,173 copies). No viral RNA was detectable after 5 days in either fresh or seawater. Notably, SARS-CoV-2 RNA was more stable in tap water: it was detectable until day 6 (Fig. 1D). These results demonstrate that viral RNA was detected longer than infectious virus under the same conditions.

The CDC reported SARS-CoV-2 was detectable in nasal mucus and sputum on surfaces for 24 hours.8 The virus also remained viable and infectious in aerosols for hours and on contaminated plastic and stainless steel surfaces for 72 hours.9 However, a quantitative

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Fig. 1. Stability of SARS-CoV-2 in 3 types of water. SARS-CoV-2, at (A) 10^4 PFU/mL or (B) 10^5 PFU/mL, was incubated in fresh water, tap water, and seawater at room temperature (23°C) for 6 days. Samples were harvested and filtered daily; viral titer was determined by plaque assay. Viral RNA copy number in daily samples, (C) starting application of 10^4 PFU/mL or (D) 10^5 PFU/mL, was also determined by real-time polymerase chain reaction. All values are expressed as the mean of each cohort, and the error bar indicates the standard error of the mean.

SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2.
analysis of the time required for complete inactivation of SARS-CoV-2 under varied water conditions has not been reported. Here, we present the stability of SARS-CoV-2 in three different water matrices and quantitatively document the time required for inactivation.

SARS-CoV-2 quickly lost activity as soon as it was introduced in fresh water or seawater (Fig. 1). On the other hand, SARS-CoV-2 was more stable in tap water: active virus was detectable longer in comparison to fresh water and seawater (Fig. IA and B). This trend was also observed when examining viral RNA levels (Fig. IC and D). In contrast, SARS-CoV-2 in filtered fresh water or seawater was more significantly stable than that in unfiltered. The results of this study indicate that SARS-CoV-2 is sensitive to fresh water and seawater environments that including diverse microbiota. The presence of predatory microbes in water has been reported to inactive viruses through protease and nuclease activity.10,11

Even after SARS-CoV-2 particles were inactivated, viral RNA still remained detectable (up to 5 or 6 days) in all 3 types of water tested (Fig. 1). Our observations are consistent with reports that viral RNA tends to be detectable longer than active virus in sputum and nasal mucus.8 It is expected that viral RNA was continuously detected because the nucleic acid remained after the virus was inactivated.

Notably, this study was conducted in stagnant water containing a significantly high titer of virus to observe that the starting concentration of SARS-CoV-2 decreased overtime in water matrices. These experimental conditions are more favorable for the virus than actual conditions in the natural environment. There is little chance that more than 10⁴ PFU/mL of SARS-CoV-2 has been exposed in actual aquatic environments. Therefore, while possible, human-to-human transmission of SARS-CoV-2 through the aquatic environment is expected to be rare.

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SUPPLEMENTARY MATERIAL

Supplementary Fig. 1
Stability of SARS-CoV-2 in filtered or unfiltered 3 types of water. SARS-CoV-2 at 10⁴ PFU/mL was (A) incubated in filtered or (B) unfiltered fresh water, tap water, and seawater at room temperature (23°C) for 3 days. Samples were harvested and viral titer was determined by plaque assay. All values are expressed as the mean of each cohort, and the error bar indicates the standard error of the mean.

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