

1 **Inactivation of SARS-CoV-2 in chlorinated swimming pool water**

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5

6 **Abstract**

7 SARS-CoV-2 transmission remains a global problem which exerts a significant direct cost to public
8 health. Additionally, other aspects of physical and mental health can be affected by limited access to
9 social and exercise venues as a result of lockdowns in the community or personal reluctance due to
10 safety concerns. Swimming pools have reopened in the UK as of April 12th, but the effect of swimming
11 pool water on inactivation of SARS-CoV-2 has not yet been directly demonstrated. Here we
12 demonstrate that water which adheres to UK swimming pool guidelines is sufficient to reduce SARS-
13 CoV-2 infectious titre by at least 3 orders of magnitude.

14 **Introduction**

15 SARS-CoV-2, the causative agent of the COVID-19 pandemic, continues to transmit globally and makes
16 quantifying the risks involved in different settings of great importance as societies attempt to return
17 to normal. The potential for waterborne transmission of SARS-CoV-2 in the context of public swimming
18 pools has not yet been investigated. Outbreaks of respiratory viruses such as adenoviruses, and
19 enteric viruses such as enteroviruses, Hepatitis A and noroviruses which can transmit by the faecal-
20 oral route are sometimes linked to swimming pools but often owe to improper maintenance of
21 chlorine levels (Bonadonna & La Rosa, 2019; WHO, 2000). In the UK, swimming pools are treated with
22 sodium hypochlorite to maintain a free chlorine level of 1.5-3 mg/l (ppm). The pH is also adjusted to
23 between 7.0 and 7.6 as the availability of active free chlorine decreases with increasing pH (PWTAG,
24 2020). Here, by treating SARS-CoV-2 with swimming pool water which conforms to UK guidelines we
25 demonstrate at least a 3-log₁₀ reduction in infectious titre.

26 **Results**

27 *Generating SARS-CoV-2 virus stocks suitable for inactivation testing*

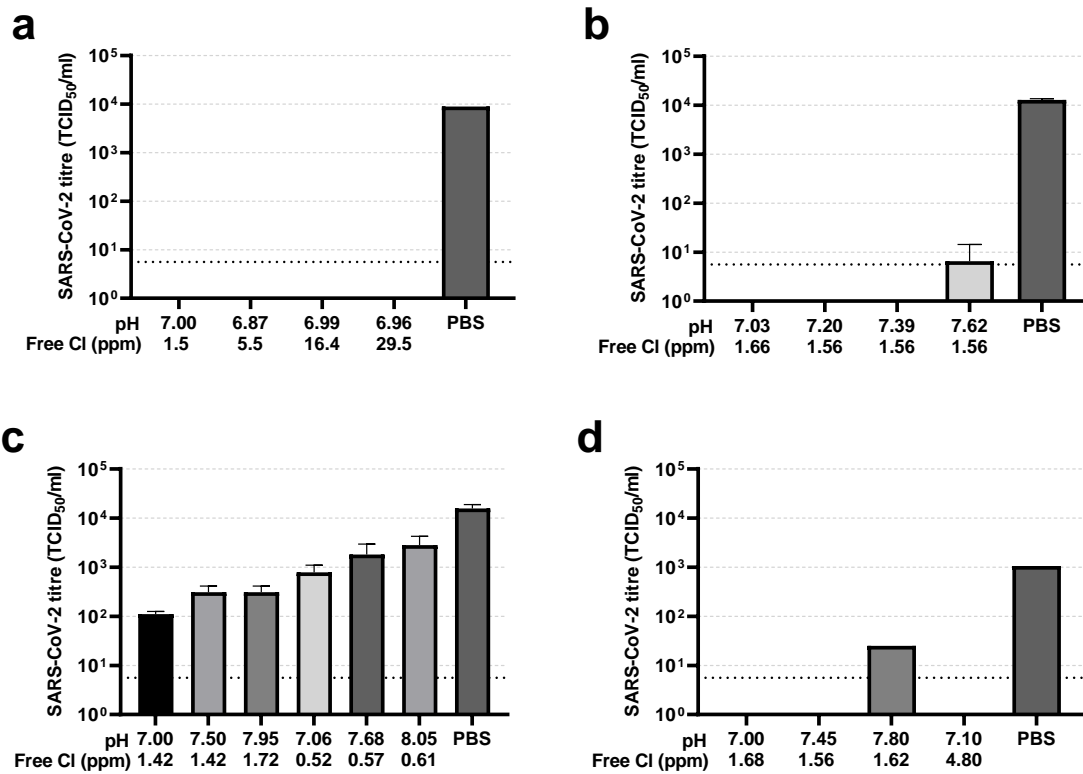
28 Virus stocks of SARS-CoV-2 for use in infectivity assays are generally generated by infection of a
29 permissive cell line such as Vero and harvesting of virus in highly buffered cell culture medium.
30 However, we observed in preliminary experiments that even a small amount of buffered medium was
31 able to quench the chlorine activity of water samples whereas with an unbuffered saline solution the
32 quenching was largely mitigated (not shown). The buffering capacity of the virus stock itself in cell
33 culture medium would make it difficult to observe inactivation at the desired free chlorine and pH
34 levels during testing. By infecting Caco-2 and Vero cells with a SARS-CoV-2 B.1 lineage virus at a
35 multiplicity of 0.01pfu/cell, extensively washing off and replacing the growth medium with saline
36 solution 24 hours before harvest at 3 days post-infection, we were able to generate stocks of infectious
37 virus with reduced buffering capacity. To further minimise the effect of the non-viral constituents of
38 the stock, such as cellular components which would exert a chlorine demand on the water samples
39 tested, a 1:100 dilution of virus in normal saline was used in all inactivation tests.

40 *Inactivation of SARS-CoV-2 by chlorinated water*

41 Water was collected from swimming pools in volumes of up to 1 litre and transported to the laboratory
42 on the same day. The water was tested for free chlorine and pH levels upon arrival at the laboratory
43 and adjusted to a range of values. A 1:100 dilution of SARS-CoV-2 virus stock generated in Caco-2 cells
44 was then added to duplicate water samples in a total volume of 1 ml, incubated for 30 seconds at RT
45 before quenching the chlorine with a one-tenth volume of 10X cell culture medium. Residual virus
46 infectivity in the samples was then titrated on Vero cells by TCID₅₀ assay. In each experiment the same
47 virus stock was incubated for 30 seconds in PBS as a control.

48 Firstly, the effect of a range of increasing free chlorine levels in water starting at the minimum 1.5
49 ppm recommended in UK swimming pools were tested (Figure 1a). A low pH of approximately pH7

50 was used to give the best chance of observing virus inactivation as availability of free chlorine is
51 maximized at lower pH. Under these conditions no detectable virus infectivity remained
52 demonstrating at least a 3- \log_{10} reduction in infectious titre compared to the PBS control where
53 approximately 1×10^4 TCID₅₀/ml of virus was measured (Figure 1a). We next measured residual SARS-
54 CoV-2 infectivity in conditions with higher pH while keeping the free chlorine level at approximately
55 1.5 ppm. Inactivation was observed to undetectable levels in all conditions except at the elevated pH
56 of 7.62 at which low levels of virus infectivity were still observed at the threshold of detection of the
57 assay. This inactivation equated to 3- \log_{10} decreased infectivity compared to the control (Figure 1b).
58 To demonstrate the interaction between the variables of pH and free chlorine in causing inactivation
59 of SARS-CoV-2 infectivity, swimming pool water samples either at (1.42-1.72 ppm) or below (0.52-0.61
60 ppm) the UK recommended free chlorine levels were modified to pHs of approximately 7, 7.5 and 8.
61 This resulted in only partial inactivation of the virus infectivity and revealed the importance of both
62 chlorine levels and pH to achieve inactivation. (Figure 1c). Finally, we generated a further stock of the
63 SARS-CoV-2 lineage B.1 virus in unbuffered saline in Vero cells and tested it against water at 3 pH
64 levels at chlorine levels of 1.56 – 1.68 ppm. The new stock had a lower titre resulting in a yield of 1×10^3
65 TCID₅₀/ml from the PBS control condition. Nonetheless full inactivation equating to a greater than 2
66 \log_{10} drop in infectivity was observed at pH7.00 and pH7.45, and even at pH7.80 the infectivity was
67 decreased more than 50-fold (Figure 1d).



68

69 **Figure 1 - Exposure to chlorinated water inactivates SARS-CoV-2.** Water samples taken from a
70 swimming pool were modified in the laboratory to a range of pH and free chlorine values. A known
71 amount of infectious SARS-CoV-2 was added to duplicate water samples in a volume of 1 ml, incubated
72 for 30 seconds at RT and any remaining infectious virus then titrated by TCID₅₀ on Vero cells. Residual
73 virus titres are shown as the mean and SD of duplicate TCID₅₀/ml values. Successive experiments were
74 performed with varying free chlorine levels (a), varying pH (b), a range of both pH and free chlorine
75 levels (c), and an independent preparation of virus at a range of pH and chlorine levels (d). A PBS
76 control was included in each experiment to validate the infectivity of the virus input. Lower pH and
77 higher free chlorine levels resulted in greater inactivation of SARS-CoV-2. A pH of no more than 7.4
78 and free chlorine above 1.5 parts per million (ppm) resulted in at least a 3-log₁₀ reduction in viral titre.

79 Discussion

80 Swimming pools have reopened in the UK as of April 12th 2021 and therefore present locations of
81 possible COVID-19 transmission. The likelihood of transmission events occurring in shared areas such
82 as changing rooms can be minimised with social distancing and hygiene measures around the pool but
83 different variables affect any risk associated with time spent in the water. Chlorination of swimming
84 pool water has been used for decades to mitigate any onwards transmission of pathogens between
85 swimmers. However, since the causative agent of COVID-19, the betacoronavirus named SARS-CoV-2,
86 only emerged in late 2019, inactivation of SARS-CoV-2 by chlorinated water has not yet been directly
87 demonstrated. Since viruses cannot replicate outside of a host, a transmission event via swimming
88 pool water would require that virus emitted directly from a bather reached another at a sufficient
89 infectious dose. Firstly, emitted virus will be greatly diluted before this occurs, potentially below a
90 minimal infectious dose. In addition, if chlorinated water is directly viricidal against SARS-CoV-2, the
91 likelihood of infectious virus being transmitted in swimming pool water will be further lowered.
92 Demonstrating this may be important in increasing public confidence in returning to pools. Here we
93 demonstrate that inactivation of SARS-CoV-2 in chlorinated swimming pool water is dependent on
94 free chlorine and pH levels with increased inactivation at higher free chlorine and lower pH. We show
95 that 30 seconds contact time at RT with water of a pH of no more than 7.4 and free chlorine above 1.5
96 mg/l (ppm) resulted in at least a 3- \log_{10} reduction in viral titre (Figure 1). These levels are within the
97 recommendations for swimming pools in the UK of at least 1.5 ppm free chlorine, although pH
98 guidelines allow a pH of 7.0-7.6 and we found here that some residual virus was detected after
99 treatment with water above pH7.4 even when at least 1.5 ppm free chlorine was present.

100 A limitation of this study is that we did not test survival of SARS-CoV-2 contained within mucus or
101 saliva mixed with swimming pool water. Further we were only able to test reduction of a virus stock
102 with infectivity around 10^4 TCID₅₀/ml due to the limited replication of SARS-CoV-2 in the laboratory
103 and the need use a minimal volume of virus material during testing. Nonetheless, the viral challenge

104 we presented equates to approximately 10^8 genomes, (with a Ct value of 23) which is in excess of the
105 amount of virus typically detected in the upper respiratory tract of asymptomatic people, with an
106 average Ct of 31.15 (Ra et al., 2021). The route by which any residual virus in swimming pool water
107 might infect another swimmer is not clear. SARS-CoV-2 is transmitted in the air and also by direct
108 inoculation. There is also a potential faecal-oral route of transmission for SARS-CoV-2 (Guo et al.,
109 2021). Our findings on the susceptibility of SARS-CoV-2 to inactivation by swimming pool water
110 underscore the importance for those who maintain swimming pools to adhere to UK guidelines for
111 chlorination, and this should give confidence in the safety of bathers when in the water. Finally, we
112 stress that swimmers should continue to adhere to locally recommended social distancing rules both
113 in and out of the water.

114

115 **Methods**

116 *Cells and viruses*

117 African green monkey kidney (Vero) cells (Nuvonis Technologies) were maintained in OptiPRO SFM
118 (Life Technologies) containing 2X GlutaMAX (Gibco). Human epithelial colorectal adenocarcinoma
119 (Caco-2) cells were maintained in DMEM, 20% FCS, 1% NEAA, 1% P/S. SARS-CoV-2 lineage B.1 isolate
120 hCoV-19/England/IC19/2020 (EPI_ISL_475572) was diluted in cell growth medium and used to infect
121 confluent cells at a multiplicity of 0.01 pfu/cell and incubated at 37°C, 5% CO₂. Growth medium was
122 removed 2 days post infection, the cell sheet washed twice with saline solution (ddH₂O, 0.9% NaCl)
123 and replaced with saline solution. After a further 24 hrs virus supernatant was harvested and clarified
124 by centrifugation.

125 *Water samples*

126 Swimming pool water samples were collected from pools in London, UK and tested upon arrival at the
127 laboratory. Free chlorine and pH levels were tested using a MD 100 photometer (Lovibond) to the

128 manufacturer's instructions for tests in Figure 1a-c and a PoolTest 25 (Palintest) for the test in Figure
129 1d. Chlorine levels of the water samples were increased by addition of sodium hypochlorite and pH
130 was increased by addition of sodium carbonate or decreased using sodium bisulphate before
131 retesting. Inactivation experiments were performed within 30 minutes of water sample preparation
132 to minimise decay of chlorine levels.

133 *Inactivation testing and titration of residual virus by TCID₅₀ assay*

134 Treatment of SARS-CoV-2 with water samples was carried out as described in the text. In short 10ul of
135 virus stock was added to 990ul of water sample, incubated for 30 seconds at RT before addition of
136 110ul of 10X MEM. Titration of residual virus was performed by TCID₅₀ assay on Vero cells using
137 cytopathic effect as the readout for infectious virus. In short, a half-log₁₀ dilution series of each sample
138 was performed and 4 replicates of each dilution transferred to 96-well plates of Vero cells, incubated
139 for 1 hr at 37°C, 5% CO₂ and replaced with cell growth medium. After 4 days, cells were stained with
140 crystal violet and scored for either an intact, stained cell sheet or the absence of cells due to virus-
141 induced cytopathic effect. For each condition, the Spearman-Kärber method was used to calculate the
142 50% tissue culture infectious dose (TCID₅₀) of the residual virus.

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