Virucidal activity of CPC-containing oral rinses against SARS-CoV-2 variants and are active in the presence of human saliva

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Running title

Inactivation of SARS-CoV-2 with mouthwash.

Keywords

SARS-CoV-2, COVID-19, Mouthwash, Saliva, Oral Hygiene

Abstract

The role of human saliva in aerosol-based transmission of SARS-CoV-2 has highlighted the need to understand the potential of oral hygiene products to inactivate the virus. Here we examined the efficacy of mouthwashes containing cetylpyridinium chloride (CPC) or chlorhexidine (CHX) in inactivating SARS-CoV-2. After 30 seconds contact under standard aqueous conditions CPC mouthwashes achieved a ≥4.0$\log_{10}$ PFU/mL reduction in SARS-CoV-2 (USA-WA1/2020) titres whereas comparable products containing CHX achieved <2.0$\log_{10}$ PFU/mL reduction. Further testing with CPC mouthwashes demonstrated efficacy against multiple SARS-CoV-2 variants, with inactivation below the limit of detection observed against the Alpha (B.1.1.7), Beta (B.1.351) and Gamma (P.1) variants. Virucidal efficacy of CPC mouthwash was also observed in the presence of human saliva with the product delivering ≥4.0$\log_{10}$ PFU/mL reduction in SARS-CoV-2 titres after 30 seconds providing additional evidence for the virucidal efficacy of CPC mouthwashes under simulated physiological conditions. Together these data suggest CPC-based mouthwashes are effective at inactivating SARS-CoV-2 and further supports the use of mouthwash to mitigate the risk of transmission during dentistry procedures.

37 # Introduction

38 The high viral load of SARS-CoV-2 present in the saliva of infected individuals and their ease of
39 aerosolisation means that saliva is recognised as playing a key role in the transmission of SARS-CoV-2
40 [1-3]. The potential of the oral cavity to act as a viral reservoir is supported by the presence of the
41 angiotensin-converting enzyme 2 (ACE-2) receptor in oral gingival epithelia and salivary glands and
42 the infection of these tissues by SARS-CoV-2 *in vivo* [4], potentially aggravating systemic infection via
43 an oral-vasculo-pulmonary route [5].

44 The use of oral rinses or mouthwashes have been proposed by health organisations to mitigate
45 transmission of SARS-CoV-2 during dentistry procedures due to their demonstrated efficacy in
46 deactivating SARS-CoV-2 *in vitro* and *in vivo* [6-8]. The antimicrobial action of a mouthwash is
47 dependent on a combination of the active ingredients, their intrinsic efficacy, and their bioavailability
48 during use. Active ingredients used in mouthwashes include Quaternary Ammonium Compounds
49 (QACS) such as Dequalinium chloride, benzalkonium chloride, cetyl pyridinium chloride (CPC) and
50 chlorhexidine (CHX) which are believed function as antimicrobials via a stepwise process of charge
51 mediated attraction and destabilisation of the lipid envelop [9-11].

52 CPC is widely used in mouthwash formulations displaying substantive action against a range of oral
53 bacteria [12-14] and viruses, including SARS-CoV-2 [6, 15-17], whilst data on antiviral efficacy of CHX
54 against SARS-CoV-2 has been more varied [18-22]. All mouthwashes, regardless of composition, must
55 function *in situ* in the oral cavity, and hence must retain efficacy in the presence of human saliva,
56 overcoming any potential deactivation from salivary components [23-25].

57 To investigate the impact of formulation composition on efficacy we compared the *in vitro* virucidal
58 efficacy of mouthwashes containing 0.07% CPC and 0.2% CHX digluconate against a range of SARS-
59 CoV-2 variants. In addition, the efficacy of a representative CPC containing mouthwash was also
60 investigated in the presence of human saliva. Our findings suggest CPC mouthwashes offer potent

61 virucidal activity that is effective against all variants tested and which is maintained in the presence of
62 human saliva under simulated usage conditions.

63

64 Methods

65 Cell Culture and Viruses

66 Vero E6 cells (C1008: African green monkey kidney cells) obtained from Public Health England, were
67 maintained in Dulbecco's minimal essential medium (DMEM) with 10% foetal bovine serum (FBS) and
68 0.05mg/ml gentamicin. Cells were maintained at 37°C and 5% CO_2. Passage 5 of SARS-CoV-2 isolate
69 (USA-WA1/2020) and passage 2 or 3 of Alpha (hCoV-19/England/204820464/2020), Beta (hCoV-
70 19/South Africa/KRISP-EC-K005321/2020) and Gamma (hCoV-19/Japan/TY7-503/2021) obtained from
71 BEI Resources [26], were cultured in Vero E6 cells maintained in DMEM with 4% FBS and 0.05mg/ml
72 gentamicin at 37°C and 5% CO_2. 48 hours post inoculation, virus was harvested and stored at -80°C
73 until used.

74

75 Preparation of Saliva

76 Stimulated saliva was collected at Unilever Research Port Sunlight, with ethical approval from the
77 Unilever R&D Port Sunlight Independent Ethics Committee (GEN 022 13), collected during November
78 2019 from 11 donors over 2 days. The saliva was pooled and aliquoted into 250ml samples, and then
79 stored at -20°C until sterilisation. The saliva was sterilised using gamma irradiation (Systagenix, UK,
80 Cobolt 60 turntable, Dose rate 1.2 kGy/hr, minimum dose 32.1 kGy).

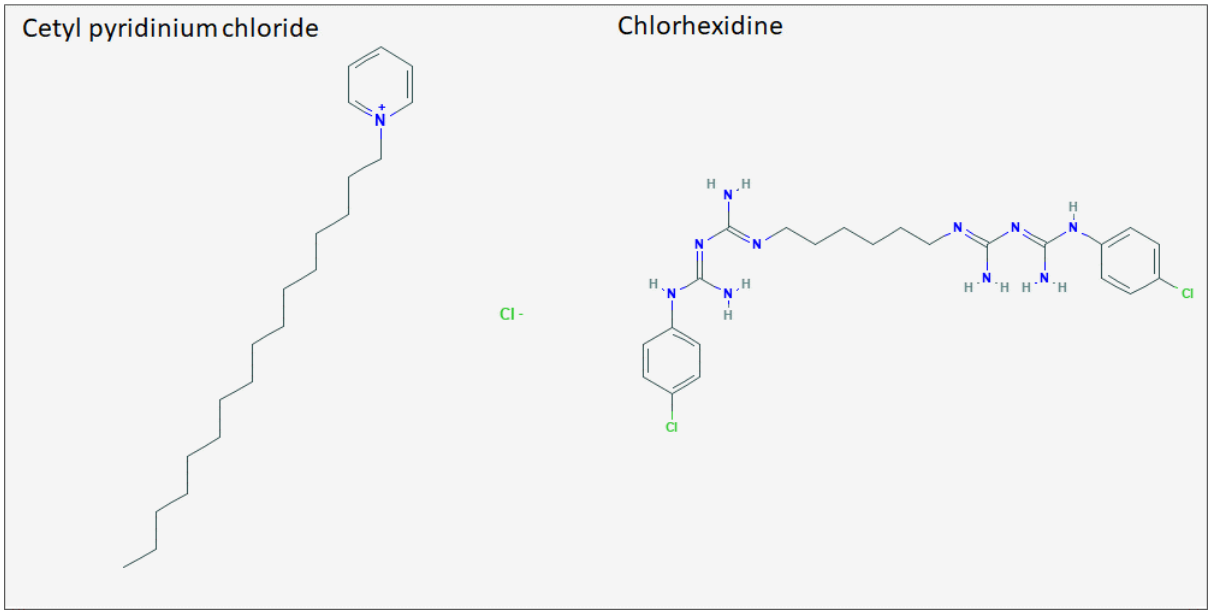
81

Virus Inactivation

Mouthwash formulations (Table 1 and Figure 1) were assessed following the ASTM International Standard E1052-20 [27]. SARS-CoV-2 titre was calculated for each experiment by plaque assays with the titre consistently >4.3$\log_{10}$ PFU/mL for USA-WA1/2020 and variants. Briefly, 900 µL of mouthwash formulation was added to 100 µL of virus suspension, containing 4% FBS and incubated for 30 seconds. After the 30 second incubation, 9mL of Dey and Engley neutralising broth (DE broth) was added and 25µL of the sample was transferred into a dilution series for quantification through a standard plaque assay as previously described .

Table 1. Mouthwash formulations examined for SARS-CoV-2 inactivation.

Treatment	Code
0.07% CPC with flavour and mix of herbal extracts	MW-A
0.07% CPC with flavour	MW-B
0.2% CHX digluconate with flavour	MW-C
70% ethanol in distilled water	Control



94 **Figure 1.** Structures of cetyl pyrinium chloride (CPC) and chlorhexidine (CHX).

95

96 To assess if human saliva alters the effectiveness of CPC mouthwash, 800 µL of MW-B mouthwash
97 formula was added to 100 µL of human saliva mixed with 100 µL of SARS-CoV-2 (USA-WA1/2020)
98 inoculum. The solution was incubated for 30 seconds and 9 mL of DE broth added. As a control, 800
99 µL of MW-B mouthwash formula was added to 100 µL of sterile water and 100 µL of virus inoculum,
100 with 9 mL of DE broth added after 30 seconds of incubation. Experiments were carried out in duplicate.

101

102 Saliva, neutralisation, and cytotoxicity assays

103 To assess if human saliva has inherent antiviral action against SARS-CoV-2 (USA-WA1/2020), 100 µL
104 of virus inoculum was added to either 800 µL of sterile water and 100 µL of irradiated human saliva
105 (dilute saliva), or 900µL of irradiated human saliva (neat saliva) for a 5-minute incubation. After 5
106 minutes had elapsed, a 25 µL sample was placed into a dilution series. Neutralisation controls were
107 carried out by adding 9 mL of DE broth to 900 µL of mouthwash formula. To this, 100 µL of virus
108 suspension was added for 30 seconds and 25 µL removed to a dilution series and a standard plaque
109 assay preformed. To determine the cytotoxicity of the mouthwashes, 100 µL of 4% DMEM was
110 added to 900 µL of test mouthwash formula for 30 seconds. To this 9 mL of DE broth was added and
111 25 µL placed into dilution series for a standard plaque assay. 25 µL samples from each condition
112 were serial diluted 10-fold to be quantified through a standard plaque assay. Plaques were counted
113 to determine viral titre. All experiments were carried out in triplicate.

114

115 # Results

116 ## Comparison of CPC and CHX containing mouthwashes

117 We tested the ability of CPC and CHX to inactivate SARS-CoV-2 (USA-WA1/2020). Following a 30
118 second incubation in the presence of the test mouthwashes a reduction in viral titre of ≥4.0$\log_{10}$
119 PFU/mL was observed with MW-A and MW-B and of <2.0 $\log_{10}$ PFU/mL for MW-C. No reduction of
120 viral titre occurred in the water control and however complete inactivation was observed by the 70%
121 ethanol control. All treatments were effectively neutralised by the addition of DE broth (Figure 2).
122 Cytotoxicity assays were able to determine the limit of detection (LOD) for this assay. The LOD is the
123 point at which Vero E6 cell death is due to cytotoxicity of mouthwashes rather than SARS-CoV-2. For
124 all three mouthwashes presented here, cytopathic effect was observed at 2.0$\log_{10}$ PFU/mL.

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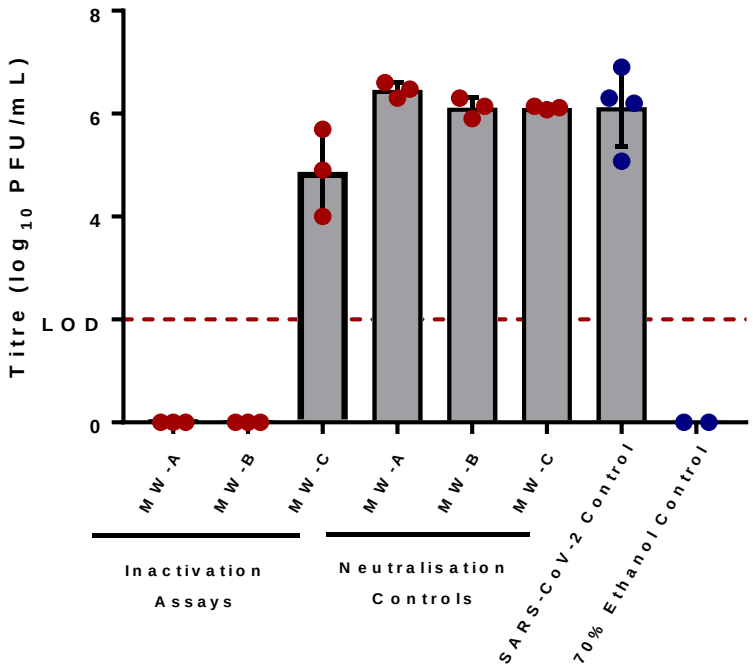

127

128 **Figure 2.** Mouthwash formulas were tested for antiviral action against SARS-CoV-2. Mouthwashes
129 were incubated with SARS-CoV-2 inoculum for 30 seconds. Both MW-A and MW-B reduced to below
130 the limit of detection (LOD), while MW-C reduced viral titre by 1.26$\log_{10}$ PFU/mL compared to the
131 water control. LOD = 2.0$\log_{10}$ PFU/mL. Viral titres recovered from the water control averaged

132 6.12$\log_{10}$ PFU/mL, while viral titre recovered from neutralisation controls were within 1.0$\log_{10}$
133 PFU/mL indicating all antiviral activity occurred within 30 seconds of incubation. LOD (2.0$\log_{10}$
134 PFU/mL) is shown across the graph with a dotted red line. Error bars represent standard deviation,
135 while red dots are experimental data values and blue dots control values.
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137

138 ## Inactivation of SARS-CoV-2 Variants by Test Products

139 We also tested the ability of CPC and CHX to inactivate SARS-CoV-2 variants of concern, Alpha, Beta
140 and Gamma. Following the 30 second incubation of Alpha with MW-A and MW-B an average reduction
141 of 3.11$\log_{10}$ PFU/mL to below the LOD was seen. Incubation of Beta with test products saw an average
142 reduction of 4.1$\log_{10}$ PFU/mL, whilst Gamma saw an average reduction of 3.36$\log_{10}$ PFU/mL, both to
143 below the LOD (Table 1). In assays carried out with the variants, no reduction was seen in the water
144 control and reduction below the LOD was seen in the 70% ethanol control. The ability to achieve a
145 4.0$\log_{10}$ PFU/mL in the variant assays was dependent on titres of SARS-CoV-2 variants following
146 standard propagation methods.

147 **Table 1.** Mouthwash formulas that were proven to work against SARS-CoV-2 (USA-WA1/2020) were
148 then tested against Alpha, Beta, and Gamma variants of SARS-CoV-2. Both MW-A and MW-B were
149 able to reduce the viral titre of all three variants to below the limit of detection (2.0$\log_{10}$ PFU/mL)
150 within 30 seconds.
151

		Average Titre (Log_{10} PFU/mL) Reduction		
Variant	**Pango lineage**	**MW-A**	**MW-B**	**70% Ethanol**
Alpha	**B.1.1.7**	3.11	3.11	3.11
Beta	**B.1.351**	4.11	4.11	4.11
Gamma	**P.1**	3.36	3.36	3.36

152

153 ## Testing in presence of human saliva

154 Under normal usage mouthwashes must be functional in the presence of human saliva, hence
155 investigations were undertaken to assess whether saliva displays any measurable endogenous
156 antiviral activity against SARS-CoV-2 (USA-WA1/2020) or whether it acts as an inhibitory "soil"
157 quenching the antiviral function of mouthwash formulations. The endogenous antiviral activity of neat
158 and dilute human saliva was measured over a contact time of 5 minutes (Figure 3A) during which no

159 significant reduction in viral load was observed compared to the water control. Viral titres of 5.70$\log_{10}$

160 PFU/mL, 5.61$\log_{10}$ PFU/mL and 5.45$\log_{10}$ PFU/mL were recovered from the neat saliva, dilute saliva

161 incubation and the water control, respectively. It is essential that mouthwashes maintain efficacy in

162 the presence of human saliva. To investigate this, we examined if the antiviral efficacy of MW-B was

163 altered by saliva. We found that MW-B was still capable of inactivation of SARS-CoV-2 to below the

164 LOD in the presence of saliva, indicating that CPC retained efficacy despite the soil load.

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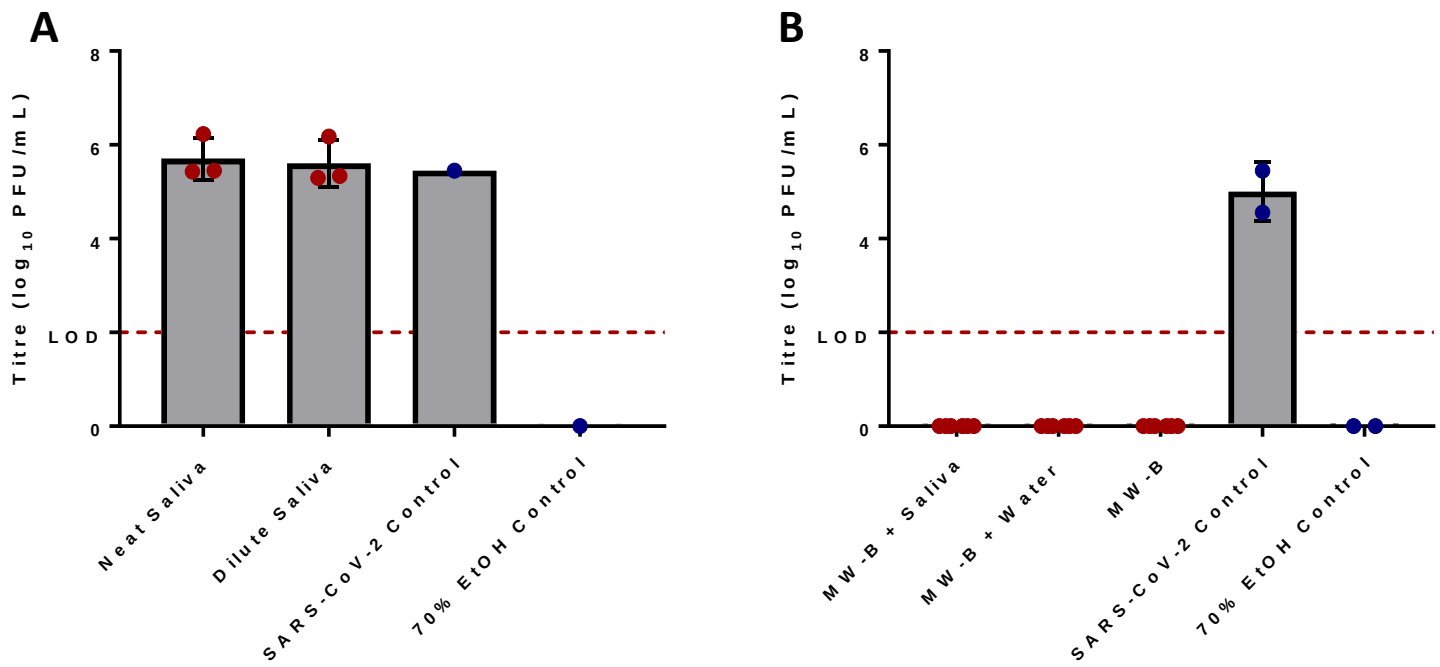

166 **Figure 3**. Irradiated human saliva has no effect upon the viral titre of SARS-CoV-2 as compared to the
167 water control after incubation with inoculum for 5 minutes. Neat saliva had a ratio of 8 parts water to
168 1-part irradiated human saliva to 1-part virus inoculum, while dilute saliva had a ratio 9 parts irradiated
169 human saliva to 1-part virus inoculum (A). Human saliva does not inhibit the antiviral activity of
170 mouthwash formulas proven to reduce the titre of SARS-CoV-2 (B). MW-B was able to reduce viral
171 titre to below the LOD both in the presence of irradiated human saliva and without. Human saliva was
172 added in a ratio of 8 parts MW-B to 1-part irradiated human saliva to 1-part virus inoculum. Limit of
173 detection (LOD) (2.0$\log_{10}$ PFU/mL) is shown across both graphs with a dotted red line. Error bars
174 represent standard deviation, while red dots are experimental data values and blue dots control
175 values.
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179 # Discussion

180 Our results confirm that mouthwash formulations containing 0.07% CPC, inactivate SARS-CoV-2 by up
181 to 99.99%, representing a value below the LOD after a contact time of 30 seconds. In contrast, within
182 the same experiment, a mouthwash containing CHX (0.2% chlorhexidine gluconate), exhibited poorer
183 virucidal activity against SARS-CoV-2. Our observations are consistent with others, where a number of
184 different CPC mouthwash formulations have been shown to effectively inactivate SARS-CoV-2 *in vitro*,
185 whereas CHX containing mouthwashes are reported to have modest ability to inactive SARS-CoV-2 [6,
186 19, 21]. The virucidal action of CPC mouthwash was maintained in the presence of whole human saliva,
187 consistent with human clinical trials which report that rinsing with CPC mouthwash can lower SARS-
188 CoV-2 salivary count for several hours after use [7, 28].

189 Over the course of the global pandemic, several SARS-CoV-2 variants have emerged with mutations
190 changing the amino acid sequence of the receptor-binding domain of the spike protein [29]. Three
191 variants of concern; Alpha, Beta, and Gamma [30] were effectively inactivated within 30 seconds by
192 both 0.07% CPC mouthwashes, with a reduction in viral titre below the LOD and equivalent to the 70%
193 ethanol control. As the CPC molecule disrupts the viral lipid envelope and the membrane is unchanged
194 by mutations, our data supports the likely efficacy of CPC mouthwash in reducing viral load
195 irrespective of the SARS-CoV-2 variant. Recently the oral cavity has been proposed to have a direct
196 role in COVID-19 disease severity based on a proposed oral-vasculo-pulmonary infection route. Poor
197 oral hygiene with plaque build-up, subsequent gingivitis and periodontitis facilitates direct entry of
198 the virus via the oral gingival sulcus and periodontal pockets enabling infection of the circulatory
199 system and lungs [5]. CPC mouthwashes with anti-plaque and virucidal activity against SARS-CoV-2
200 could have the potential to lower viral count and lessen the risk of severe lung disease in COVID-19
201 patients.

202

203 In conclusion, two mouthwashes containing 0.07% CPC were effective at inactivating SARS-CoV-2,
204 within 30 seconds with greater than 4.0$\log_{10}$ PFU/ml reduction in viral titre. Moreover, virucidal
205 activity of CPC was maintained in the presence of whole human saliva. Both 0.07% CPC mouthwashes
206 were as effective as 70% ethanol against three variants of concern; Alpha, Beta and Gamma suggesting
207 these CPC formulations possess virucidal action against all variants. In contrast, under the same
208 experimental conditions, a mouthwash containing 0.2% chlorhexidine digluconate did not have
209 substantial action against SARS-CoV-2 *in vitro*. Given the ongoing global pandemic, and the recognition
210 of the significance of the oral cavity in infection, transmission, and disease severity, daily use of an
211 effective CPC mouthwash as part of a good oral hygiene routine, could be a low-cost and simple
212 measure to reduce transmission risk and potentially, lower the risk of developing severe forms of
213 COVID-19.

214

215 Declaration of Interests

216 AG, SM, and MH are employees of Unilever.

217

218 Acknowledgements

219 Unilever funded this study. EIP and GLH were supported by the EPSRC (V043811/1) and UKRI-BBSRC
220 COVID rolling fund (BB/V017772/1). GLH was also supported by the BBSRC (BB/T001240/1 and
221 BB/V011278/1), a Royal Society Wolfson Fellowship (RSWF\R1\180013), the NIH (R21AI138074), the
222 EPSRC (EP/V043811/1), the UKRI (20197 and 85336), and the NIHR (NIHR2000907). GLH is affiliated
223 to the National Institute for Health Research Health Protection Research Unit (NIHR HPRU) in
224 Emerging and Zoonotic Infections at University of Liverpool in partnership with Public Health England
225 (PHE), in collaboration with Liverpool School of Tropical Medicine and the University of Oxford. GLH is

based at LSTM. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, the Department of Health or Public Health England. USA-WA1/2020 was deposited by the Centre's for Disease Control and Prevention and obtained through BEI Resources. NIAID, NIH: SARS-Related Coronavirus 2, Isolate, NR-52281. B.1.1.7 (hCoV-19/England/204820464/2020) was obtained through BEI Resources, NIAID, NIH: SARS-Related Coronavirus 2, Isolate hCoV-19/England/204820464/2020, NR-54000, contributed by Bassam Hallis. B.1.351 (hCoV-19/South Africa/KRISP-EC-K005321/2020) was obtained through BEI Resources, NIAID, NIH: SARS-Related Coronavirus 2, Isolate hCoV-19/South Africa/KRISP-EC-K005321/2020, NR-54008, contributed by Alex Sigal and Tulio de Oliveira. P.1 (hCoV-19/Japan/TY7-503/2021) was obtained through BEI Resources, NIAID, NIH: SARS-Related Coronavirus 2, Isolate hCoV-19/Japan/TY7-503/2021 (Brazil P.1), NR-54982, contributed by National Institute of Infectious Diseases.

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