TITLE: Cetylpyridinium chloride-containing mouthwashes reduce the infectivity of SARS-CoV-2 variants *in vitro*

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ABSTRACT (145)

Oral mouthwashes decrease the infectivity of several respiratory viruses including SARS-CoV-2. However, the precise agents with antiviral activity present in these oral rinses and their exact mechanism of action remain unknown. Here we show that Cetylpyridinium chloride (CPC), a quaternary ammonium compound present in many oral mouthwashes, reduces SARS-CoV-2 infectivity by inhibiting the viral fusion step with target cells after disrupting the integrity of the viral envelope. We also found that CPC-containing mouth rinses decreased more than a thousand times the infectivity of SARS-CoV-2 *in vitro,* while the corresponding vehicles had no effect. This activity was effective for different SARS-CoV-2 variants, including the B.1.1.7 variant, predominant in UK, also in the presence of sterilized saliva. CPC-containing mouth rinses could therefore represent a cost-effective measure to reduce SARS-CoV-2 infectivity in saliva, aiding to reduce viral transmission from infected individuals regardless of the variants they are infected with.

BACKGROUND

Several studies have shown the antiviral potential of mouthwashes, which decrease the infectivity of relevant airborne transmitted infectious viruses, such as influenza and distinct coronavirus, including SARS-CoV-2 [1–4]. If proven effective in the oral cavity, this antiviral strategy could represent a globally accessible and affordable measure that could easily be implemented worldwide to reduce the infectivity of SARS-CoV-2 in saliva and to cut the viral transmission chain. Indeed, higher viral loads found in index cases are associated with a significantly higher transmission rates among their contacts [5]. Thus, any intervention aimed at reducing viral loads in the saliva, exhaled as aerosols by infected individuals, could help decrease viral transmission and even prevent superspreading events. As mouthwashes are also available in oral spray formats, they represent a promising strategy for vulnerable populations such as the elderly.

Novel SARS-CoV-2 variants have appeared in different geographical areas, raising concerns about their higher viral transmission potential, the severity of the associated symptoms upon infection, and their ability to escape from pre-established neutralizing responses in individuals already vaccinated [6–9]. In this scenario, the documented broad antiviral efficacy of certain mouthwashes could be instrumental in tackling different SARS-CoV-2 variants and in reducing the impact of their transmission. Yet, and despite the universal applicability of this antiviral approach, and the diverse reports proving the activity of various oral products *in vitro*, we still don't know which are the individual active components present in these mouthwashes that exert the antiviral effect, and what is their precise mechanism of action. Moreover, we do not know if mouth rinses could be active against different SARS-CoV-2 variants.

Here we focused on the effect of Cetylpyridinium chloride (CPC), a quaternary ammonium compound used in many oral mouthwashes and breath sprays with broad antiseptic and microbicide activity. We compared the anti-SARS-CoV-2 activity of CPC and CPC-containing mouthwashes against their respective vehicles, and found that CPC-containing mouthwashes inhibit SARS-CoV-2 entry into target cells after disrupting the integrity of the viral membrane. CPC-containing mouth washes decreased more than a thousand times the infectivity of replication competent SARS-CoV-2 viruses, were active in the presence of sterilized saliva and effective against different SARS-CoV-2 variants.

MATERIAL & METHODS

Cell Cultures. Vero E6 cells (ATCC CRL-1586) were cultured in Dulbecco's modified Eagle medium, (DMEM) with 10% fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin (all from Invitrogen). HEK-293T overexpressing the human ACE2 were kindly provided by Integral Molecular Company and maintained in DMEM (Invitrogen) with 10% fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin, and 1 µg/ml of puromycin (all from Invitrogen).

Pseudovirus production. HIV-1 luciferase reporter pseudoviruses expressing SARS-CoV-2 Spike protein were generated using two plasmids. pNL4-3.Luc.R-.E- was obtained from the NIH AIDS repository. SARS-CoV-2.SctΔ19 was generated (Geneart) from the full protein sequence of SARS-CoV-2 spike with a deletion of the last 19 amino acids in C-terminal, human-codon optimized and inserted into pcDNA3.4-TOPO [10]. Spike plasmid was transfected with X-tremeGENE HP Transfection Reagent (Merck) into HEK-293T cells, and 24 hours later, cells were transfected with pNL4-3.Luc.R-.E-. Supernatants were harvested 48 hours later, filtered with 0.45 µm (Millex Millipore) and stored at -80ºC until use. Viruses were titrated in HEK-293T overexpressing human ACE2 to use an equal amount of fusogenic viruses. Pseudoviruses expressing the spike protein containing either the single D614G mutation or the full B.1.1.7 variant were generated and transfected as detailed before [11,12].

Pseudovirus assay. HEK-293T overexpressing the human ACE2 were used to test mouth rinses and their vehicles at the indicated concentrations. A constant pseudoviral titer was used to pulse cells in the presence of the mouth rinses. After 48h post-inoculation, cells were lysed with the Bright Glo Luciferase Assay system (Promega). Luminescence was measured with an EnSight Multimode Plate Reader (Perkin Elmer). To detect any associated cytotoxic effect, mouth rinse formulations were also mixed with media, and were equally cultured on cells but in the absence of virus. Cytotoxic effects of these products were measured 48h post-inoculation, using the CellTiter-Glo luminescent cell viability assay (Promega).

Virus isolation, titration and sequencing. SARS-CoV-2 was isolated in march 2020 from a nasopharyngeal swab as described in [13]. The virus was propagated for two passages

101 and a virus stock was prepared collecting the supernatant from Vero E6. Genomic
102 sequence was deposited at GISAID repository (http://gisaid.org) with accession ID
103 EPI_ISL_510689. Compared to Wuhan/Hu-1/2019 strain, this isolate has the following
104 point mutations: 376 D614G (Spike), R682L (Spike), C16X (NSP13) and other 12 in
105 NSP3 (M1376X, P1377X, 377 T1378X, T1379X, I1380X, A1381X, K1382X, N1383X,
106 T1384X, V1385X, K1386X, S1387X). The SARS-CoV-2 B.1.1.7 variant was identified
107 during routine sequencing of a clinical nasopharyngeal swab in Spain in January 2021,
108 deposited at GISAID with accession ID EPI_ISL_900493, and subsequently isolated on
109 Vero E6 cells.
110
111 ***Viral treatment with CPC-containing mouthwashes and nucleocapsid detection by***
112 ***ELISA***. SARS-CoV-2 B.1.1.7 variant and the D614G variant from March 2020 were
113 assayed with Vitis CPC Protect (Dentaid SL), with 2.063 mM of CPC. A total of 250 µl
114 of mouth rinse was mixed with 250 µl viruses for 2 minutes. Untreated viruses were
115 mixed with 250 µl of media for 2 minutes. Mixes with viruses were diluted in PBS and
116 filtered for 10 minutes at 1,000 xg in macrosept® advance centrifugal devices of 100 K
117 MWCO of exclusion (Pall Corporation) to wash away mouth rinses twice. Washed
118 viruses were resuspended in 1.5 ml of fresh media. The amount of SARS-CoV-2
119 nucleoprotein present in these supernatants was measured with a SARS-CoV-2
120 Nucleocapsid protein (NP) High-sensitivity Quantitative ELISA (ImmunoDiagnostics),
121 according to the manufacturer's protocol, but using a 1% BSA buffer instead of the assay
122 buffer of the kit, which contains a detergent to lyse viral membranes and release
123 nucleocapsid content.
124
125 ***Viral treatment with CPC and dynamic light scattering analysis***. 100 µl SARS-CoV-2
126 B.1.1.7 variant or the D614G variant from March 2020 were mixed with 100 µl of CPC
127 (10 mM) or with 100 µl of H_2O for 2 minutes. These samples were fixed with 1.2 ml of
128 paraformaldehyde 4% (Biotium) for 30 minutes. Particle size distributions of the viral
129 preparations and control vehicles were determined using a dynamic light scattering (DLS)
130 analyzer combined with noninvasive backscatter technology (Malvern Zetasizer;
131 Malvern Instruments, United Kingdom). Samples were measured without dilution. Three
132 different measurements were used to calculate the mean diameter and SD. The effective
133 electric charge on the viral surface was examined by measuring the zeta potential with an
134 electrophoretic mobility and light scattering (ESL) analyzer (Malvern Zetasizer). The

135 samples were placed into the cuvettes and measured without dilution. Three different
136 measurements were used to calculate the mean zeta potential and SD of the dispersed
137 system.
138
139 ***Antiviral activity***. Antiviral activity against SARS-CoV-2 D614G variant from March
140 2020 was tested against three oral formulations from Dentaid SL containing CPC: Vitis
141 Encias (with 1.47 mM of CPC), Perio Aid Intensive Care (with 1.47 mM of CPC plus
142 1.33 mM of Chlorhexidine) and Vitis CPC Protect (with 2.063 mM of CPC). Vehicles
143 containing the same formulation but without CPC were also tested in parallel. We also
144 assayed 10 mM of CPC diluted in distilled water. Of note, colorants were removed from
145 all formulations to avoid any interference with luciferase reactions. One ml of mouth
146 rinses or their corresponding vehicles were mixed with 1 ml of SARS-CoV-2 D614G
147 variant for 2 minutes. Virus was also mixed with 1 ml of media as positive control. After
148 2 minutes of incubation, mixes with viruses were diluted in PBS and filtered for 10
149 minutes at 1,000 xg in macrosept® advance centrifugal devices of 100 K MWCO of
150 exclusion (Pall Corporation) to wash away mouth rinses twice. Washed viruses were
151 resuspended in 2 ml of fresh media and titrated in triplicates on Vero E6 cells through 10
152 serial dilutions. After 3 days post infection, cells were assayed in a microscope for viral
153 induced cytopathic effect. To detect any associated cytotoxic effect, mouth rinse
154 formulations were also mixed with media, washed and centrifuged as previously
155 described, and were equally cultured on Vero E6, but in the absence of virus. Cytotoxic
156 effects of these products were measured 3 days after infection, using the CellTiter-Glo
157 luminescent cell viability assay (Promega). Luminescence was measured in a Fluoroskan
158 Ascent FL luminometer (ThermoFisher Scientific). The cytotoxicity obtained for each
159 compound tested determined the limit of detection of the assay.
160
161 Antiviral activity against SARS-CoV-2 B.1.1.7 variant along with the D614G variant
162 from March 2020 were assayed with Vitis CPC Protect (with 2.063 mM of CPC)
163 following the same procedure already described. This time, however, 250 µl of mouth
164 rinse was mixed with 250 µl of viruses for 1 or 2 minutes. Untreated viruses were mixed
165 with 250 µl of media and left for 2 minutes. Washed viruses were resuspended in 1.5 ml
166 of fresh media and titrated as previously described. Again, the cytotoxicity obtained for
167 this experiment was used to determine the limit of detection. Finally, antiviral activity
168 against SARS-CoV-2 B.1.1.7 variant in the presence or absence of saliva was assayed

169 with Vitis CPC Protect (with 2.063 mM of CPC). A total of 800 µl of mouth rinse or cell
170 media were mixed with 200 µl of SARS-CoV-2 B.1.1.7 for 30 seconds in the presence or
171 absence of 200 µl sterilized saliva obtained from a non-vaccinated donor with a negative
172 Panbio COVID-19 antigen test (Abbott) 2 days prior saliva donation. This saliva was
173 centrifuged for 20 minutes at 25,000 xg, and then sterilized first with a 0.45 µm and then
174 through a 0.22 µm filter device (Millex Millipore). After 30 seconds of incubation, mixes
175 with viruses were diluted in PBS and filtered for 10 minutes at 1,000 xg in macrosept®
176 advance centrifugal devices of 100 K MWCO of exclusion (Pall Corporation) to wash
177 away mouth rinses twice. Washed viruses were resuspended in 1.5 ml of fresh media and
178 titrated as previously described.
179
180
181 ***IC_{50} calculation***. Response curves of mouth rinses against pseudoviral entry were
182 adjusted to a non-linear fit regression model, calculated with a four-parameter logistic
183 curve with variable slope. Cells exposed to the pseudovirus in the absence of products
184 were used as positive controls of infection, and were set as 100% of viral fusion to
185 normalize data and calculate the percentage of viral entry inhibition. Cells not exposed to
186 the mouthwashes nor to the pseudovirus were used as positive controls of viability, and
187 were set as 100% to normalize data and calculate the percentage of cytopathic effect. All
188 analyses and figures were generated with the GraphPad Prism v8.0b Software.

189 **RESULTS**

190 We first tested the capacity of CPC-containing mouth rinses to inhibit SARS-CoV-2 entry
191 into target cells. We employed a luciferase-based assay, using a using a reporter lentivirus
192 pseudotyped with the spike protein of SARS-CoV-2, which allows the detection of viral
193 fusion with target HEK-293T cells expressing human ACE2 receptor. A constant
194 concentration of the reporter pseudovirus containing the SARS-CoV-2 original Spike
195 protein was mixed with increasing concentrations of the indicated CPC-containing mouth
196 rinses, or their corresponding vehicles, and added to the target cells. To control for any
197 mouthwash-induced cytotoxicity, target cells were also cultured with increasing
198 concentrations of the indicated products in the absence of pseudoviruses. By these means,
199 we calculated the concentration at which certain mouth rinses blocked viral entry and
200 achieved a 50% maximal inhibitory capacity (IC_{50}). CPC-containing mouth rinses were
201 able to inhibit viral fusion in a dose dependent manner (**Figure 1, A, C, E**, red lines) at
202 concentrations where no cytotoxic effects of the mouth rinses were observed (**Figure 1**,
203 **A, C, E**, grey lines). Of note, no obvious inhibitory activity was detected on vehicles
204 (**Figure 1**, **B, D, F**, red lines), clearly pointing to CPC as the antiviral compound
205 contained in the oral formulations. To confirm the specific antiviral activity of CPC, we
206 directly tested this compound resuspended in water, and found that it also inhibited
207 SARS-CoV-2 pseudoviral fusion and entry into target cells (**Figure 1G**). We also assayed
208 the capacity of CPC-containing mouth rinses to reduce the fusion of pseudoviruses
209 displaying different SARS-CoV-2 Spikes, including the D614G mutation and the full
210 B.1.1.7 variant, which has become predominant in UK, and whose higher transmissibility
211 and pathogenicity are a global concern [6]. Yet, a CPC-containing mouthwash was
212 equally effective in abrogating the entry of pseudoviruses expressing each spike these
213 variants (**Figure 1H**). These results indicate that CPC-containing mouth rinses are able
214 to block SARS-CoV-2 viral entry into target cells due to the activity of CPC, which is
215 efficacious against different variants.

216

217 To further understand if CPC-containing mouth rinses abrogated viral fusion by
218 disrupting the viral envelope of SARS-CoV-2, we next worked with two different SARS-
219 CoV-2 clinical isolate variants: the virus circulating in March 2020 in Spain containing
220 the D614G mutation and the B.1.1.7 variant originally detected in UK. Each SARS-CoV-
221 2 variant was mixed at a 1:1 volume ratio with a CPC-containing mouth rinse or left
222 untreated for 2 minutes. To remove the oral rinse, these samples were washed twice with

223 PBS by ultrafiltration using a macrosept® centrifugal device. Collected viruses were
224 assayed with an ELISA that detects SARS-CoV-2 nucleocapsid, but in absence of the
225 detergent used to lyse the viruses in the conventional protocol (**Figure 2A**). In the absence
226 of the lysis buffer, viruses treated with CPC-containing mouthwash were detected to a
227 much higher extent than untreated viruses, regardless of the variant tested (**Figure 2B**).
228 Of note, addition of the ELISA assay buffer containing detergents in untreated viruses
229 increased nucleoprotein detection (**Figure 2C**), but not to the extent of CPC-containing
230 mouthwashes. In addition, we measured the impact of CPC treatment on the zeta potential
231 and size distribution of viral particle s(**Figure 2D** and **Table 1**). Each viral variant was
232 treated with CPC 10 mM at a 1 to 1 volume ratio for 2 minutes, fixed with
233 paraformaldehyde and analyzed by two light scattering methods. Using electrophoretic
234 light scattering (ELS), we found that upon CPC treatment, the zeta potential of untreated
235 viruses that was originally electronegative increased exponentially (**Table 1**). Moreover,
236 this treatment broadened the viral size distribution in both types of SARS-CoV-2 variants
237 (**Figure 2D** and **Table 1**), while CPC or paraformaldehyde alone had a narrower and
238 smaller distinctive profile (**Figure 2D** and **Table 1**), as detected with dynamic light
239 scattering (DLS). The complementary approaches of ELISA and ELS/DLS analyses point
240 out to the effective disruption of viral membranes and loss of electrostatic repulsion,
241 resulting in the emulsion and aggregation of the viral membrane lipids.
242
243 Next, we tested the capacity of CPC to reduce the infectivity of the clinical isolate of
244 SARS-CoV-2 D614G variant from March 2020. A 1 to 1 volume ratio of SARS-CoV-2
245 was mixed with CPC, CPC-containing mouth rinses or their vehicles for 2 minutes and
246 washed twice with PBS to remove the formulations by ultrafiltration using a macrosept®
247 centrifugal device. Collected viruses were titrated on Vero E6 cells to calculate the Tissue
248 Culture Infectious Dose 50% ($TCID_{50}$) per ml after each of the treatments. While water
249 used to dilute CPC had no effect on SARS-CoV-2 infectivity (**Figure 3A**), high doses of
250 CPC effectively suppressed viral infection on Vero E6 (**Figure 3A**). Analogously, 2
251 minutes treatment with CPC-containing mouthwashes decreased about 1,000 times the
252 $TCID_{50}$/ml of SARS-CoV-2, while vehicles had no impact on SARS-CoV-2 infectivity
253 when compared to untreated virus (**Figure 3A**). To control for the presence of mouthwash
254 remaining in the viral preparations that could induce cytotoxic effects, the indicated
255 products were washed in ultrafiltration centrifugal devices, but in the absence of SARS-
256 CoV-2, and equally cultured with target cells for three days. By these means we

257 confirmed that the observed SARS-CoV-2 induced cytopathic effect was effectively
258 inhibited at concentrations where the CPC-containing mouthwashes that could possibly
259 remain after filtration were not toxic for the cells. Similar inhibition was observed when
260 viral stocks were treated with a 10 fold excess volume of CPC-containing mouthwashes
261 for 2 minutes. Thus, CPC exerts an antiviral activity against replicative competent SARS-
262 CoV-2 and CPC-containing mouthwashes have the capacity to reduce 1,000 times the
263 infectivity of a viral stock when treated at least at a 1:1 volume ratio for 2 minutes.
264
265 To confirm if CPC could reduce the infectivity of different clinical isolates of SARS-
266 CoV-2, we performed and additional experiment including the B.1.1.7 SARS-CoV-2
267 variant along the D614G circulating variant in March 2020 (**Figure 3B**). In this
268 experiment, we also tested a 1 minute treatment as mouthwashes are usually
269 recommended to be used for 1 minute. After treatment, viruses were titrated as described
270 above, controlling again for the possible presence of mouthwash remaining in the viral
271 preparations that could induce cytotoxic effects. Once again, we could see a reduction of
272 infectivity above 1,000 times regardless of the variant employed or the duration of
273 exposure (**Figure 3B**).
274
275 Finally, we tested the capacity of a CPC-containing mouth rinse to exert its activity in the
276 presence of sterilized saliva and using a very restrictive treatment duration of 30 seconds.
277 A 1 to 10 volume ratio of the B.1.1.7 SARS-CoV-2 variant was mixed with CPC-
278 containing mouth rinse or media, in the presence or absence of a 1 volume ratio of
279 sterilized saliva. Viruses were washed twice with PBS to remove the formulations by
280 ultrafiltration, and assayed on Vero E6 as previously described. Treatment for 30 seconds
281 with CPC-containing mouthwashes decreased 10 fold the $TCID_{50}$/ml of the B.1.1.7
282 SARS-CoV-2 variant as compared to untreated virus (**Figure 3C**). Moreover, the
283 presence of absence of saliva did not alter the inhibition (**Figure 3C**), showing that the
284 CPC-containing mouth rinse has the same antiviral activity in the presence or absence of
285 saliva. Collectively, these results support the potential effectiveness of CPC-containing
286 mouth rinses to decrease viral loads in the oral cavity of infected individuals, regardless
287 of the SARS-CoV-2 variant they are infected with.
288

DISCUSSION

Here we have shown that CPC has an antiviral activity against different variants of SARS-CoV-2, and that this compound exerts its activity by blocking viral entry or by inhibiting viral fusion on target cells. CPC acts by disrupting the integrity of the viral envelope, as previously shown for influenza virus [3], which equally affects distinct SARS-CoV-2 variants. Our results indicate that CPC destabilizes the membrane of the different variants, as detected with the ELISA, via electrostatic interactions where the cationic amino groups of CPC cover the negatively charged viral membranes, as detected by the shift in zeta potential. Surfactant CPC activity triggers viral membrane aggregation and colloidal stabilization of solubilized viral membranes that tend to fuse with oppositely charged CPC-bound membranes, increasing size distribution of treated viruses by DLS analysis. This mechanism has therefore the potential to reduce viral infectivity regardless of the variant tested.

Although CPC-containing mouthwashes could protect the oral mucosa from infection, SARS-CoV-2 most likely infect cells via the upper respiratory tract. Thus, further strategies should consider the use of CPC in nasal sprays to fully achieve the prophylactic potential of this approach. Our results point to the utility of CPC-containing oral rinses to decrease viral load in saliva. We have showed that in a very restrictive experiment, where we mixed equal volumes of highly infectious SARS-CoV-2 viral variants with CPC-containing mouthwashes, these treatments reduced more than 1,000 times the $TCID_{50}$ while corresponding vehicles had no impact. Since virucidal activity with a CPC-containing oral rinse was equally effective when saliva was added, this suggest that CPC-containing mouthwashes will most likely be active in the oral cavity.

CPC-containing mouthwashes could be a cost-effective measure to reduce SARS-CoV-2 infectivity in saliva, aiding to reduce viral transmission from infected individuals. Performing oral washes for 1 to 2 minutes should be enough to effectively decrease the infectivity of viruses in the saliva, especially during the first 2 weeks after infection, when higher viral titers are detected and individuals are more contagious [14]. Several events where numerous people were infected at the same time, which are considered superspreading events, are related to activities where people were either talking, shouting or singing [15,16]. Indeed, viable viruses were isolated from the saliva of COVID-19 infected individuals [17], proving that exhaled saliva micro-droplets and aerosols are

323 infectious. Future work should address if CPC-containing mouth rinses are able to
324 decrease viral load and infectivity of viruses found in the oral cavity of SARS-CoV-2
325 infected individuals. If proven effective, CPC-containing mouthwashes should be active
326 against those variants that pose a threat to vaccine efficacy, that may increase
327 transmissibility rates, and could even worsen clinical outcome. While prior studies have
328 shown that CPC has an anti-bacterial activity that last for 3 to 5 hours in saliva [18],
329 forthcoming studies should address the duration of the CPC antiviral activity in the oral
330 cavity. This information will be key to effectively validate this approach as a mean to
331 maintain a reduced infectious capacity of SARS-CoV-2 in the saliva with this cost-
332 effective intervention.

333 **FIGURES**

334 ***Figure 1. Antiviral activity of CPC-containing mouthwashes inhibiting SARS-CoV-2***
335 ***entry***. Percentage of viral entry inhibition on target HEK-293T cells expressing ACE2
336 exposed to a fixed concentration of SARS-CoV-2 in the presence of increasing
337 concentrations of oral formulations (**A, C, E**), their vehicles (**B**, **D, F**) and CPC diluted
338 in water (**G**). Non-linear fit to a variable response curve from one experiment with two
339 replicates is shown (red lines), excluding data from drug concentrations with associated
340 toxicity. When calculated, the particular IC_{50} value of the graph is indicated. Cytotoxic
341 effect on HEK-293T cells expressing ACE2 cells exposed to increasing concentrations
342 of mouthwashes or vehicles in the absence of virus is also shown (grey lines). **H**.
343 Percentage of viral entry inhibition on target HEK-293T cells expressing ACE2 exposed
344 to a fixed concentration of SARS-CoV-2 variants (D614G and B.1.1.7) in the presence
345 of a final concentration of 3,9 µM of Vitis CPC Protect, which had no cell-associated
346 cytotoxicity.

347

348 ***Figure 2. ELISA and DLS analysis of SARS-CoV-2 variants treated with CPC.*** **A**.
349 Schematic representation of the expected outcome of an ELISA performed in the absence
350 of lysis buffer for untreated viruses (shaded in blue) and those treated with CPC-
351 containing mouth rinse (shaded in yellow) **B**. Amount of nucleocapsid measured after
352 250 µl of SARS-CoV-2 B.1.1.7 or the D614G variant isolated in March 2020 were left
353 untreated (grey bars) or mixed with 250 µl of CPC-containing mouth rinse (yellow bars)
354 for 2 minutes. Viruses were washed right after treatment by ultrafiltration to remove
355 mouthwashes and were assayed with an ELISA detecting SARS-CoV-2 nucleocapsid
356 performed in the absence of lysis buffer. **C**. Amount of nucleocapsid measured on
357 untreated viruses (grey bars) with the same ELISA in the absence (shaded in blue) or
358 presence (shaded in yellow) of the lysis assay buffer of the kit. **D**. Hydrodynamic size
359 (intensity averaged) of different SARS-CoV-2 variants (B.1.1.7 and D614G) in the
360 presence of CPC obtained by dynamic light scattering. Viruses were mixed at a 1:1
361 volume ratio with CPC (10 mM) or H_2O for 2 minutes and fixed with paraformaldehyde.

362

363 ***Figure 3. Infectivity of different SARS-CoV-2 variants treated with CPC-containing***
364 ***mouthwashes for different timeframes and with or without sterilized saliva***. **A**. One ml
365 of SARS-CoV-2 D614G variant isolated in March 2020 with $10^{5.8}$ $TCID_{50}$ was treated
366 with CPC (10 mM) or CPC-containing mouth rinses (2 mM) and their respective vehicles

367 for 2 minutes at a 1:1 volume ratio. Untreated virus was used as positive control.
368 Infectivity of treated viruses washed right after treatment by ultrafiltration to remove
369 cytotoxic mouthwashes were assayed on Vero E6 cells 3 days post infection. In parallel,
370 we confirmed that the inhibitory effect was not due to any remaining cytotoxic effect of
371 the mouthwashes, as tested on Vero E6 cells exposed to the media left from washed mouth
372 rinses that were equally centrifuged in the absence of virus. The cytotoxicity obtained for
373 each compound tested determined the detection limit of the assay, which is represented
374 with a shaded red area. Of note, equivalent decrease in $TCID_{50}$ was obtained when viral
375 stocks were mixed at a 1 to 10 volume ratio with CPC-containing mouthwashes (data not
376 shown). **B**. 250 µl of SARS-CoV-2 B.1.1.7 with $10^{4.8}$ $TCID_{50}$ or 250 µl of SARS-CoV-2
377 D614G variant isolated in March 2020 was treated with a CPC-containing mouth rinse (2
378 mM) or left untreated for 1 to 2 minutes at a 1:1 ratio. Infectivity of treated viruses washed
379 right after treatment by ultrafiltration was performed as described in **A**. The cytotoxicity
380 obtained for this mouthwash determined the detection limit of the assay, which is
381 represented with a shaded red area. **C**. 200 µl of SARS-CoV-2 B.1.1.7 was treated with
382 a CPC-containing mouth rinse (2 mM) or left untreated for 30 seconds at a 1 to 10 volume
383 ratio in the presence or absence of 200 µl of sterilized saliva. Infectivity of treated viruses
384 washed right after treatment by ultrafiltration was performed as described in **A**. The
385 cytotoxicity obtained for this mouthwash determined the detection limit of the assay,
386 which is represented with a shaded red area.
387
388 **TABLES**
389 **Table 1.** Hydrodynamic size change (Peaks and intensity in %) of different SARS-CoV-
390 2 variants (B.1.1.7 and D614G) and increase in the average zeta potential (mV) of
391 different SARS-CoV-2 variants (B.1.1.7 and D614G) in the presence of CPC obtained by
392 dynamic light scattering. Viruses were mixed with CPC (10 mM) or H_2O for 2 minutes
393 and fixed with paraformaldehyde. The mean from three different acquisitions was used
394 to measure the mean and SD values represented.
395
396
397
398
399
400

TABLE 1

Sample	Mean particle size				Zeta Potential (mV)
	Peak 1 (nm)	Intensity (%)	Peak 2 (nm)	Intensity (%)	
CPC paraformaldehyde	5,8 ± 1,1	56	121,2 ± 19,3	44	15,5 ± 0,9
D614G + H_2O	16,1 ± 5,5	12	226,6 ± 63,7	82	-12,8 ± 1,8
D614G + CPC	260,4 ± 40,2	9	1780 ± 369,4	91	8,9 ± 0,6
B.1.1.7 + H_2O	9,2 ± 2,4	3	237 ± 72,3	84	-11,9 ± 1,4
B.1.1.7 + CPC	21,2 ± 2,51	9	1888 ± 231,2	91	7,87 ± 0,2

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453 **AUTHOR CONTRIBUTION**

454 Conceived and designed the experiments: R.L, V.B, J.G, B.C, N.I-U.

455 Performed experiments and provided pseudoviruses: J.M-B, D.P-Z, D.R-R, M.C-S, B.T,
456 E.P, J.B.

457 Analyzed and interpreted the data: J.M-B, D.P-Z, R.L, VB, D.R-R, M.C-S, B.T., E.P, J.B,
458 J.G, B.C, N.I-U.

459 Wrote the paper: J.M-B, D.P-Z, D.R-R, M.C-S, B.T, N.I-U.

460

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466

467 **COMPETING INTEREST**

468 R.L, VB. And J.G are researchers working for Dentaid Research Center. The authors
469 declare that no other competing interest exist. Unrelated to the submitted work, JB and
470 BC are founders and shareholders of AlbaJuna Therapeutics, S.L. BC is founder and
471 shareholder of AELIX Therapeutics, S.L.

472

473 **DATA AVAILABILITY** Data is available from corresponding author upon reasonable
474 request.

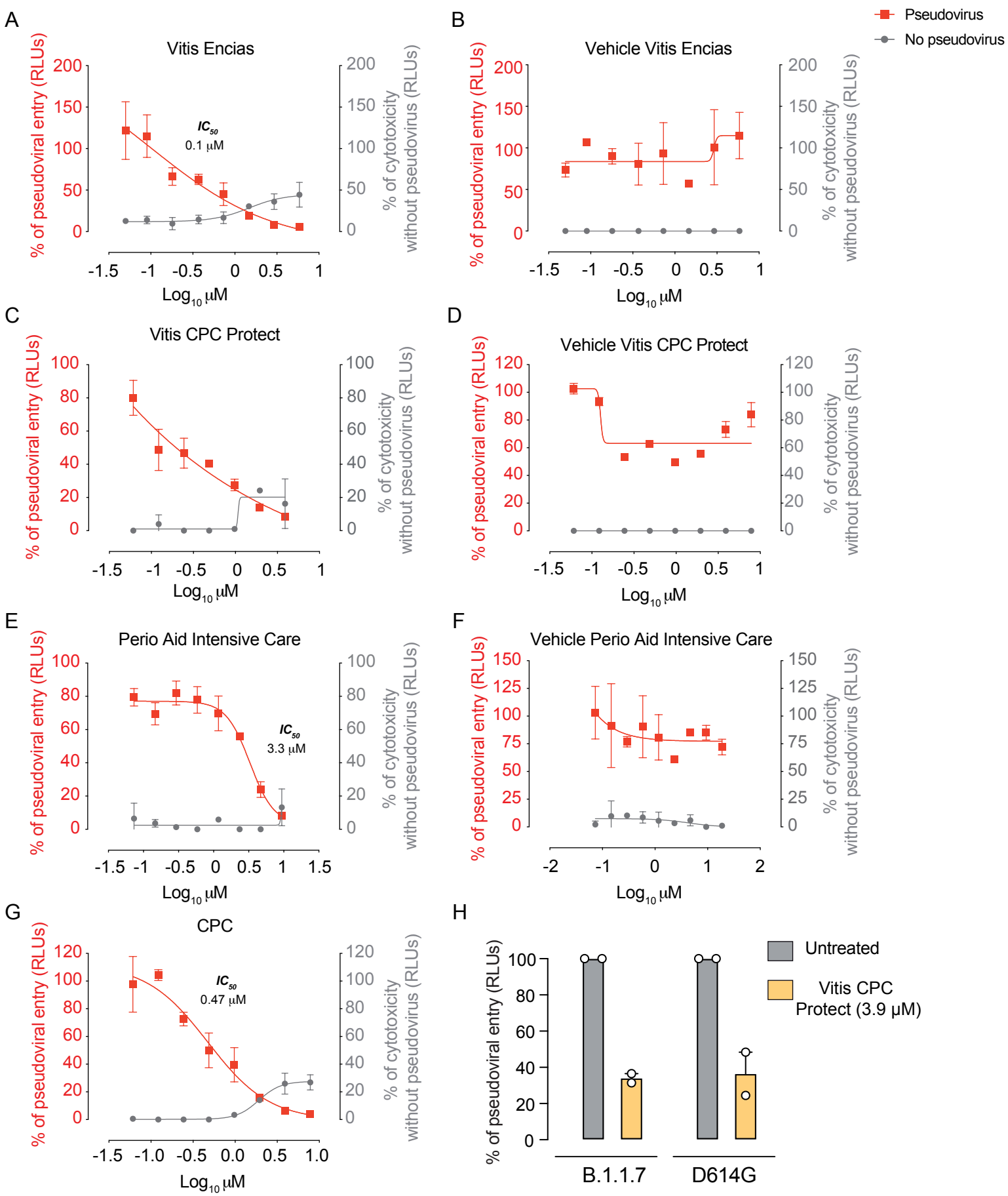
A
Vitis Encias
IC50
0.1 µM
% of pseudoviral entry (RLUs)
% of cytotoxicity without pseudovirus (RLUs)
Log10 µM
B
Vehicle Vitis Encias
Pseudovirus
No pseudovirus
C
Vitis CPC Protect
D
Vehicle Vitis CPC Protect
E
Perio Aid Intensive Care
IC50
3.3 µM
F
Vehicle Perio Aid Intensive Care
G
CPC
IC50
0.47 µM
H
Untreated
Vitis CPC Protect (3.9 µM)
B.1.1.7
D614G

Figure 1

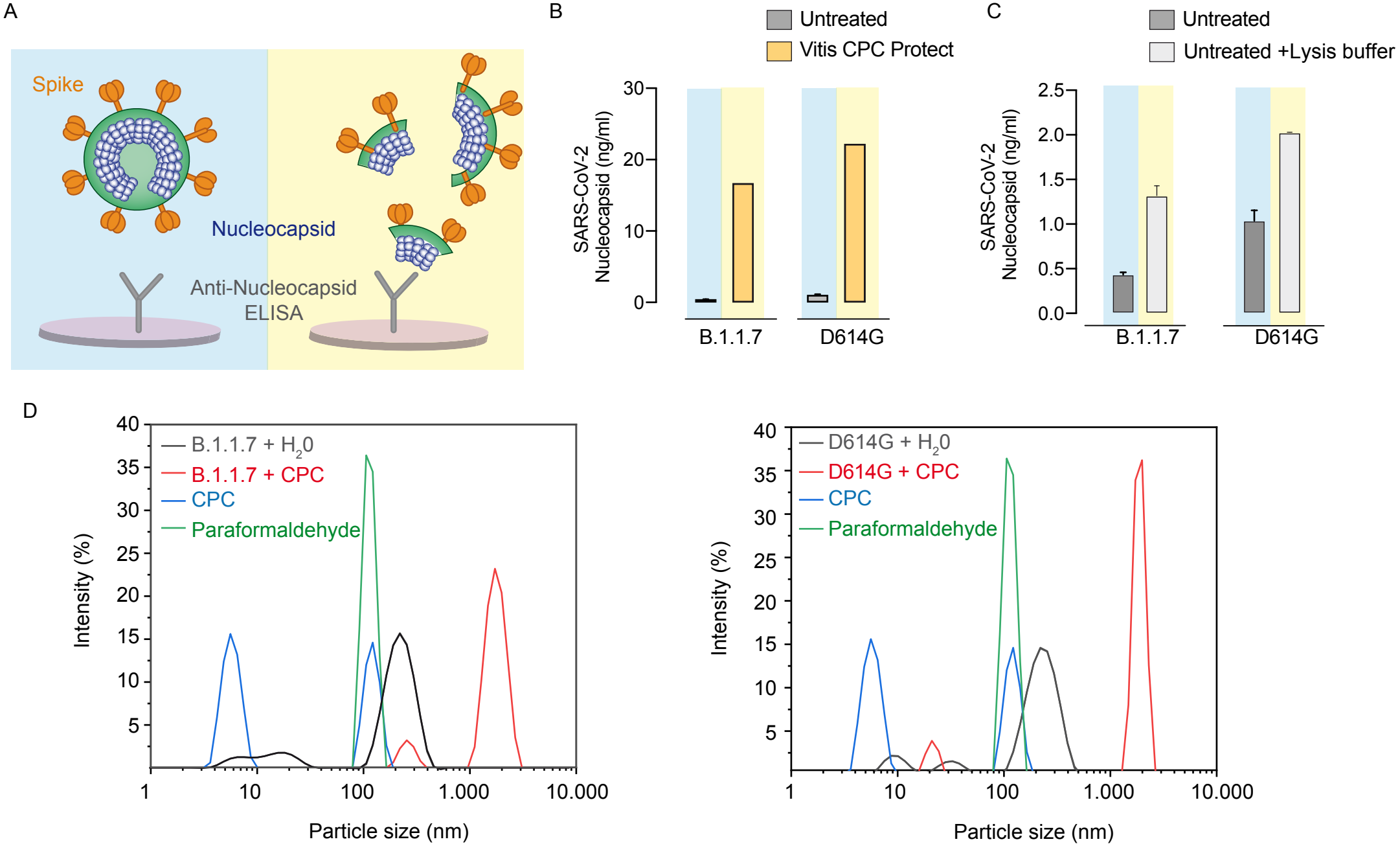
A
Spike
Nucleocapsid
Anti-Nucleocapsid
ELISA
B
Untreated
Vitis CPC Protect
SARS-CoV-2
Nucleocapsid (ng/ml)
30
20
10
0
B.1.1.7
D614G
C
Untreated
Untreated +Lysis buffer
SARS-CoV-2
Nucleocapsid (ng/ml)
2.5
2.0
1.5
1.0
0.5
0.0
B.1.1.7
D614G
D
B.1.1.7 + H2O
B.1.1.7 + CPC
CPC
Paraformaldehyde
Intensity (%)
Particle size (nm)
1
10
100
1.000
10.000
D614G + H2O
D614G + CPC
CPC
Paraformaldehyde
Intensity (%)
Particle size (nm)

Figure 2

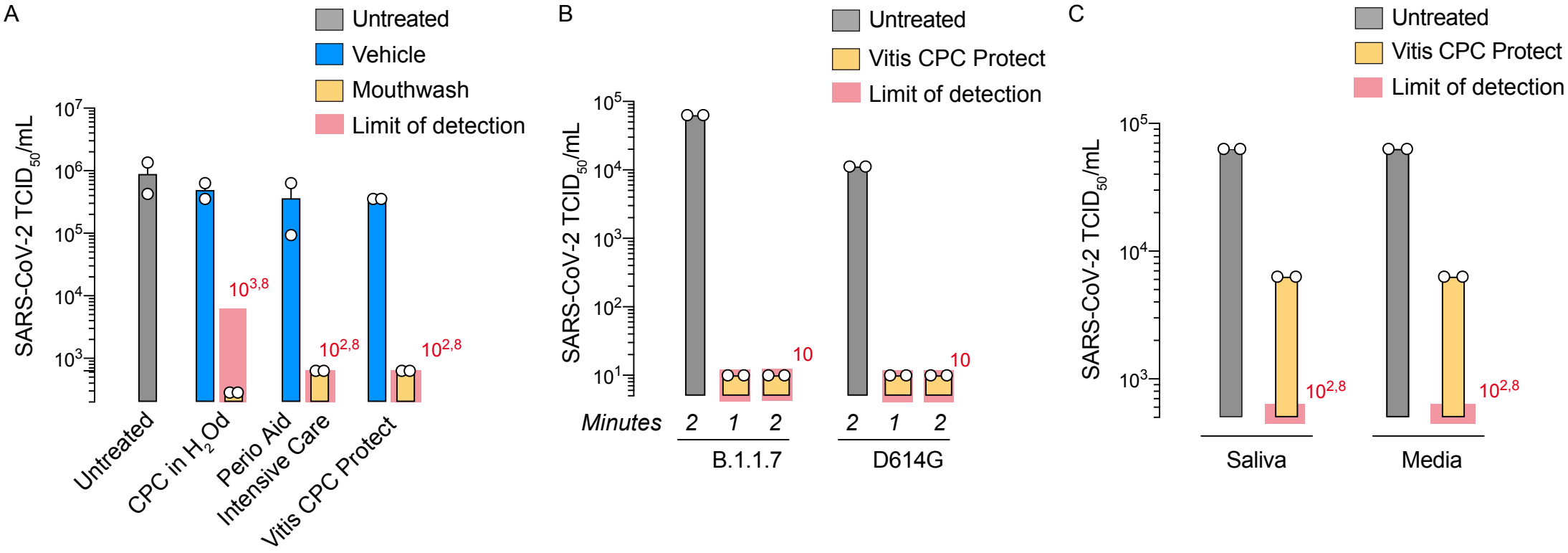
A
Untreated
Vehicle
Mouthwash
Limit of detection
SARS-CoV-2 TCID50/mL
10^7
10^6
10^5
10^4
10^3
10^3,8
10^2,8
10^2,8
Untreated
CPC in H2Od
Perio Aid
Intensive Care
Vitis CPC Protect
B
Untreated
Vitis CPC Protect
Limit of detection
SARS-CoV-2 TCID50/mL
10^5
10^4
10^3
10^2
10^1
10
10
Minutes
2 1 2
2 1 2
B.1.1.7
D614G
C
Untreated
Vitis CPC Protect
Limit of detection
SARS-CoV-2 TCID50/mL
10^5
10^4
10^3
10^2,8
10^2,8
Saliva
Media

Figure 3