JOURNAL OF MEDICAL MICROBIOLOGY

RESEARCH ARTICLE

Anderson *et al.*, *Journal of Medical Microbiology* 2022;71:001508

DOI 10.1099/jmm.0.001508

MICROBIOLOGY SOCIETY

OPEN ACCESS

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

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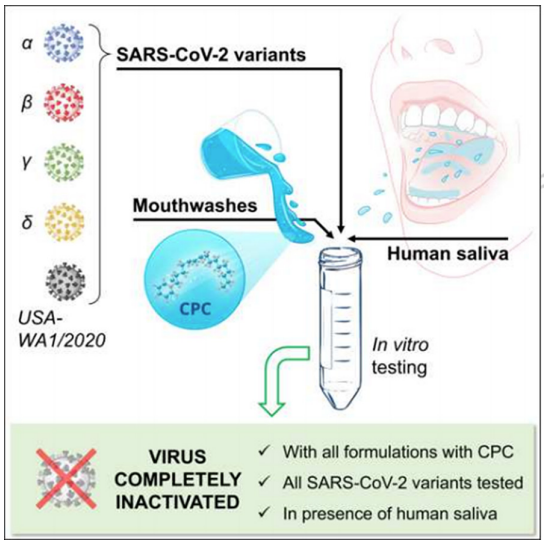

Graphical abstract

Inactivation of SARS-CoV-2 variants by oral mouthwashes containing 0.07% cetyl pyridinium chloride (CPC).

Abstract

Introduction. The importance of human saliva in aerosol-based transmission of SARS-CoV-2 is now widely recognized. However, little is known about the efficacy of virucidal mouthwash formulations against emergent SARS-CoV-2 variants of concern and in the presence of saliva.

Hypothesis. Mouthwashes containing virucidal actives will have similar inactivation effects against multiple SARS-CoV-2 variants of concern and will retain efficacy in the presence of human saliva.

Aim. To examine *in vitro* efficacy of mouthwash formulations to inactivate SARS-CoV-2 variants.

Received 26 October 2021; Accepted 06 January 2022; Published 18 February 2022

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Keywords: SARS-CoV-2; COVID-19; mouthwash; saliva; oral hygiene.

Abbreviations: ACE-2, angiotensin-converting enzyme 2; ASTM, American Society for Testing and Materials; CHX, chlorhexidine; CPC, cetyl pyridinium chloride; DMEM, Dulbecco's minimal essential medium; FBS, foetal bovine serum; LOD, limit of detection; p.f.u., plaque forming units; QACS, quaternary ammonium compounds.

Methodology. Inactivation of SARS-CoV-2 variants by mouthwash formulations in the presence or absence of human saliva was assayed using ASTM International Standard E1052-20 methodology.

Results. Appropriately formulated mouthwashes containing 0.07% cetylpyridinium chloride but not 0.2% chlorhexidine completely inactivated SARS-CoV-2 (USA-WA1/2020, Alpha, Beta, Gamma, Delta) up to the limit of detection in suspension assays. Tests using USA-WA1/2020 indicates that efficacy is maintained in the presence of human saliva.

Conclusions. Together these data suggest cetylpyridinium chloride-based mouthwashes are effective at inactivating SARS-CoV-2 variants. This indicates potential to reduce viral load in the oral cavity and mitigate transmission via salivary aerosols.

INTRODUCTION

The high viral load of SARS-CoV-2 present in the saliva of infected individuals [1] and in aerosol droplets formed passively during speech and breathing, and actively during coughing [2–4] have contributed to the recognition that saliva plays a key role in the transmission of SARS-CoV-2 [5–7]. The potential of the oral cavity to act as a viral reservoir is supported by the presence of the angiotensin-converting enzyme 2 (ACE-2) receptor in oral gingival epithelia and salivary glands and the infection of these tissues by SARS-CoV-2 *in vivo* [8], potentially aggravating systemic infection via an oral-vasculo-pulmonary route [9].

The use of oral rinses or mouthwashes have been proposed by health organizations to mitigate transmission of SARS-CoV-2 during dentistry procedures due to their demonstrated efficacy in inactivating SARS-CoV-2 *in vitro* and *in vivo* [10–12]. The antimicrobial action of a mouthwash is dependent on a combination of the active ingredients, their intrinsic efficacy and their bioavailability during use. Active ingredients used in mouthwashes include quaternary ammonium compounds (QACS) such as dequalinium chloride, benzalkonium chloride, cetyl pyridinium chloride (CPC) and chlorhexidine (CHX), which are believed to function as antimicrobials via a stepwise process of charge-mediated attraction and destabilisation of the bacterial lipid envelope [13–15].

CPC is widely used in mouthwash formulations displaying substantive action against a range of oral bacteria [16–18] and viruses, including SARS-CoV-2 [10, 19–21], whilst data on virucidal efficacy of CHX against SARS-CoV-2 has been more varied [22–26]. All mouthwashes, regardless of composition, must function *in situ* in the oral cavity, and hence must retain efficacy in the presence of human saliva, overcoming any potential deactivation from salivary components [27–29].

To investigate the impact of mouthwash composition on efficacy we compared the *in vitro* virucidal efficacy of mouthwashes containing 0.07% CPC and 0.2% CHX digluconate against a range of SARS-CoV-2 variants. In addition, the efficacy of a representative CPC containing mouthwash was also investigated in the presence of human saliva. Our findings suggest CPC mouthwashes offer potent virucidal activity that is effective against all variants tested and which is maintained in the presence of human saliva under simulated usage conditions.

METHODS

Cell culture and viruses

Vero E6 cells (C1008: African green monkey kidney cells) obtained from Public Health England, were maintained in Dulbecco's minimal essential medium (DMEM) with 10% foetal bovine serum (FBS) and 0.05 mg ml^{-1} gentamicin. Cells were maintained at 37 °C and 5% CO2. The following reagents were obtained through BEI Resources, NIAID, NIH: SARS-Related Coronavirus 2, Isolate USA-WA1/2020, NR-52281; SARS-Related Coronavirus 2, Isolate hCoV-19/England/204820464/2020, NR-54000, contributed by Bassam Hallis; SARS-Related Coronavirus 2, Isolate hCoV-19/South Africa/KRISP-EC-K005321/2020, NR-54008, contributed by Alex Sigal and Tulio de Oliveira; SARS-Related Coronavirus 2, Isolate hCoV-19/Japan/TY7-503/2021 (Brazil P.1), NR-54982, contributed by National Institute of Infectious Diseases. The viruses were obtained as passage 5 of SARS-CoV-2 isolate (USA-WA1/2020) and passage 2 or 3 of Alpha (hCoV-19/England/204820464/2020), Beta (hCoV-19/South Africa/KRISP-EC-K005321/2020) and Gamma (hCoV-19/Japan/TY7-503/2021). The Delta variant was passage 5 of a clinically isolated strain (SARS-CoV-2/human/GBR/Liv_273/2021, GenBank accession OK392641). Variants were cultured in Vero E6 cells maintained in DMEM with 4% FBS and 0.05 mg ml^{-1} gentamicin at 37 °C and 5% CO2. Then, 48 h post-inoculation, virus was harvested and stored at −80 °C until used. All SARS-CoV-2 work was conducted in a containment level 3 laboratory.

Preparation of saliva

Stimulated saliva was collected at Unilever Research Port Sunlight during November 2019 from six donors over 2 days. Healthy donors, three male, three female, age 18–60 years old, provided a stimulated daytime saliva sample for which they were given a piece of gum to chew (Wrigley's Turbulence) [30]. Subjects were given a maximum of five sterile 30 ml containers in which they were asked to provide a 20–25 ml sample of saliva per container. Subjects were requested to leave 30 min after eating or drinking before providing a saliva sample.

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

Treatment	Code
Proprietary commercially available formulation containing 0.07% CPC with flavour and mix of herbal extracts (0% ethanol)	MW-A
Proprietary commercially available formulation containing 0.07% CPC with flavour (0% ethanol)	MW-B
0.2% CHX digluconate with flavour (7% ethanol)	MW-C
70% ethanol in distilled water	Positive control
Distilled water	Negative control

CHX, Chlorhexidine; CPC, cetyl pyridinium chloride.

The collected samples were stored overnight at 4 °C. A second collection from the same subjects was performed on the following day and the saliva from day 1 and day 2 was pooled together, decanted into bottles and stored at −80 °C prior to gamma irradiation (Systagenix, UK, Cobolt 60 turntable, dose rate 1.2 kGy/h, minimum dose 32.1 kGy) sufficient to achieve sterilization [31]. After sterilization saliva was stored in sealed bottles at 4 °C prior to testing.

Virus inactivation by different mouthwash formulations

Mouthwash formulations (Table 1 and Fig. 1) were assessed following the ASTM International Standard E1052-20 [32]. Briefly, 900 µl of mouthwash formulation was added to 100 µl of virus suspension (SARS-CoV-2, USA-WA1/2020), containing 4% FBS and incubated for 30 s. After the 30 s incubation, 9 ml of Dey and Engley neutralizing broth (DE broth) was added and 25 µl of the sample was transferred into a dilution series for quantification. The viral inoculum was quantified before each experiment. All experiments were carried out in triplicates. SARS-CoV-2 titre was calculated using standard plaque assays as previously described [33]. Briefly, samples were serially diluted in DMEM containing 2% FBS and incubated with Vero cells for 1 h. After incubation, an overlay with DMEM 2% FBS, 0.1% gentamycin and 0.4% agarose was applied to the cells. The cells were allowed to incubate for 72 h at 37 °C with 5% CO_2. After incubation, the cells were fixed with 10% formalin solution, and stained with 0.25% Crystal Violet.

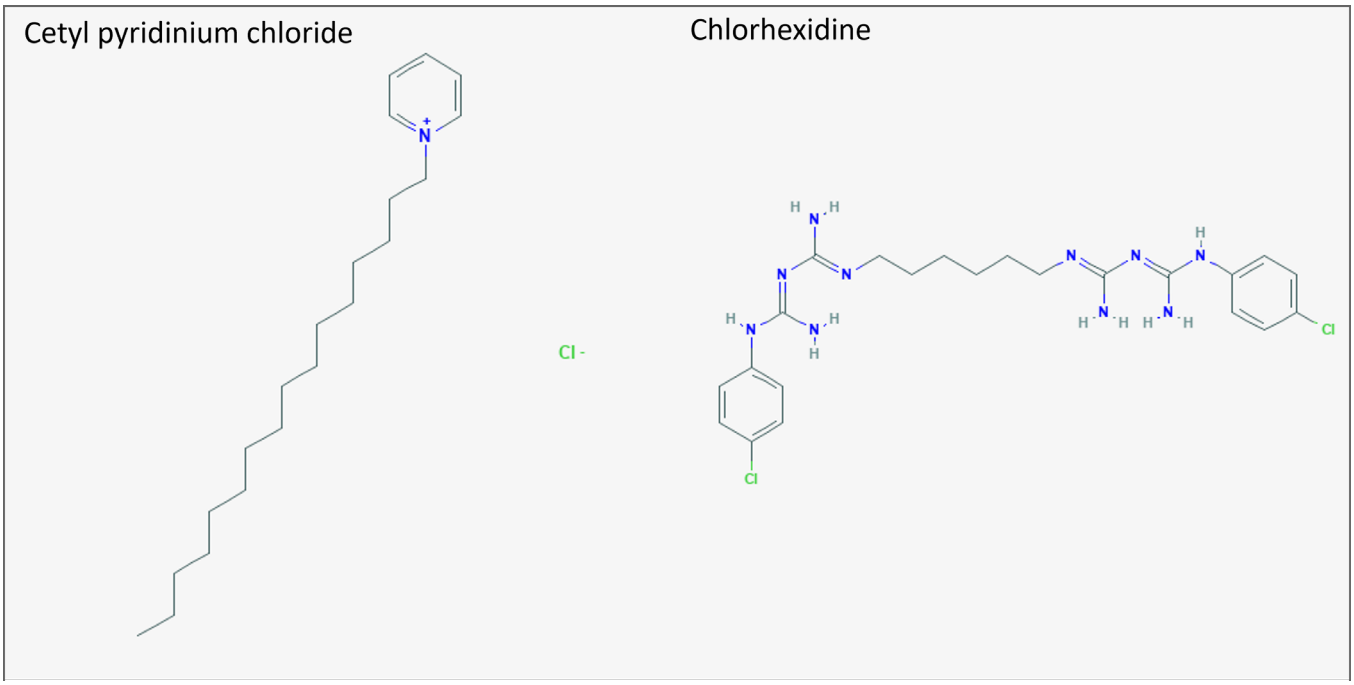

Fig. 1. Structures of cetylpyridinium chloride (CPC) and chlorhexidine (CHX).

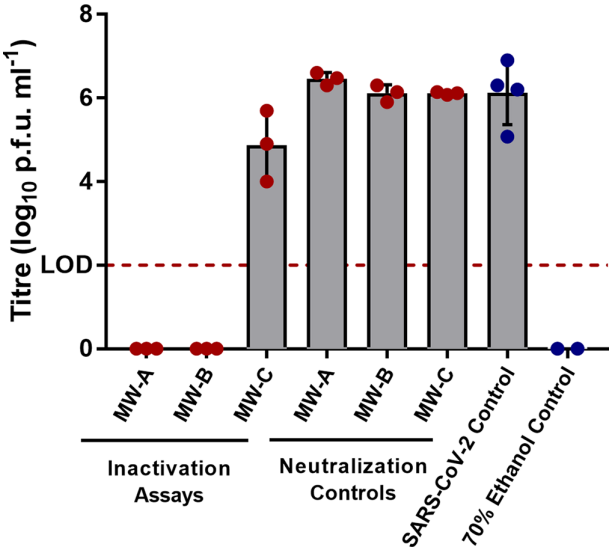

Fig. 2. Mouthwash formulas were tested for virucidal action against SARS-CoV-2 (USA-WA1/2020). Mouthwashes were incubated with SARS-CoV-2 inoculum for 30 s. Both MW-A and MW-B reduced to below the LOD, while MW-C reduced viral titre by 1.26log_{10} p.f.u. ml^{-1} compared to the water control. LOD=2.0log_{10} p.f.u. ml^{-1}. Viral titres recovered from the water control averaged 6.12log_{10} p.f.u. ml^{-1}, while viral titre recovered from neutralization controls were within 1.0log_{10} p.f.u. ml^{-1} indicating all virucidal activity occurred within 30 s of incubation. LOD (2.0log_{10} p.f.u. ml^{-1}) is shown across the graph with a dotted red line. Error bars represent standard deviation of triplicates, while red dots are experimental data values and blue dots control values.

Virus inactivation by mouthwash in the presence of saliva

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

Saliva, neutralization, and cytotoxicity assays

To assess if human saliva has inherent virucidal action against SARS-CoV-2 (USA-WA1/2020) 100 µl of virus inoculum was added to either 800 µl of sterile water and 100 µl of irradiated human saliva (dilute saliva), or 900 µl of irradiated human saliva (neat saliva) for a 5 min incubation. After 5 min had elapsed, a 25 µl sample was placed into a dilution series. Neutralization and cytotoxicity assays were performed following the ASTM International Standard E1052-20 [32]. Briefly, neutralization controls were carried out by adding 9 ml of DE broth to 900 µl of mouthwash formula. To this, 100 µl of virus suspension was added for 30 s and 25 µl removed to a dilution series. Samples (25 µl) from each condition were serial diluted tenfold and quantified via a standard plaque assay. Plaques were counted to determine viral titre. To determine the cytotoxicity of the mouthwashes, 100 µl of 4% DMEM was added to 900 µl of test mouthwash formula for 30 s. To this 9 ml of DE broth was added and 25 µl placed into dilution series. Samples (25 µl) from each dilution were processed by standard plaque assay and cell viability was evaluated at day 3 post-inoculation. All experiments were carried out in triplicate.

RESULTS

Comparison of CPC- and CHX containing mouthwashes

We tested the ability of CPC and CHX to inactivate SARS-CoV-2 (USA-WA1/2020). Following a 30 s incubation in the presence of the test mouthwashes a reduction in viral titre of ≥4.0log_{10} p.f.u. ml^{-1} was observed with MW-A and MW-B and of <2.0 log_{10} p.f.u. ml^{-1} for MW-C. No reduction of viral titre occurred in the water control and however complete inactivation was observed by the 70% ethanol control. All treatments were effectively neutralized by the addition of DE broth (Fig. 2). Cytotoxicity assays showed no cytopathic effect of the mouthwashes on Vero E6 cells at the dilutions tested. The limit of detection (LOD of the assay was 2.0log_{10} p.f.u. ml^{-1}.

Table 2. Mouthwash formulas that were proven to work against SARS-CoV-2 (USA-WA1/2020) were then tested against Alpha, Beta, and Gamma and Delta variants of SARS-CoV-2. Both MW-A and MW-B were able to reduce the viral titre of all three variants to below the limit of detection (2.0$\log_{10}$ p.f.u. ml^{-1}) within 30 s

		Average titre (Log_{10} p.f.u. ml^{-1}) 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
Delta	**B.1.617.2**	4.52	4.52	4.52

p.f.u., plaque forming units.

Inactivation of SARS-CoV-2 variants by test products

We also tested the ability of CPC and CHX to inactivate SARS-CoV-2 variants of concern, Alpha, Beta, Gamma and Delta. Following the 30 s incubation of Alpha with MW-A and MW-B an average reduction of 3.11 $\log_{10}$ p.f.u. ml^{-1} to below the LOD was seen. Incubation of Beta with test products saw an average reduction of 4.1 $\log_{10}$ p.f.u. ml^{-1}, Gamma saw an average reduction of 3.36$\log_{10}$ p.f.u. ml^{-1}, and Delta saw an average reduction of 4.52 $\log_{10}$ p.f.u. ml^{-1}, all below the LOD (Table 2). In assays carried out with the variants, no reduction was seen in the water control and reduction below the LOD was seen in the 70% ethanol control. The ability to achieve a 4.0$\log_{10}$ p.f.u. ml^{-1} in the variant assays was dependent on titres of SARS-CoV-2 variants following standard propagation methods.

Testing in the presence of human saliva

Under normal usage mouthwashes must be functional in the presence of human saliva, hence investigations were undertaken to assess whether saliva displays any measurable endogenous virucidal activity against SARS-CoV-2 (USA-WA1/2020) and to assess whether saliva acts as an inhibitory 'soil' quenching the virucidal function of mouthwash formulations. The endogenous virucidal activity of neat and dilute human saliva was measured over a contact time of 5 min (Fig. 3a) during which no significant reduction in viral load was observed compared to the water control. Viral titres of 5.70$\log_{10}$ p.f.u. ml^{-1}, 5.61$\log_{10}$ p.f.u. ml^{-1} and

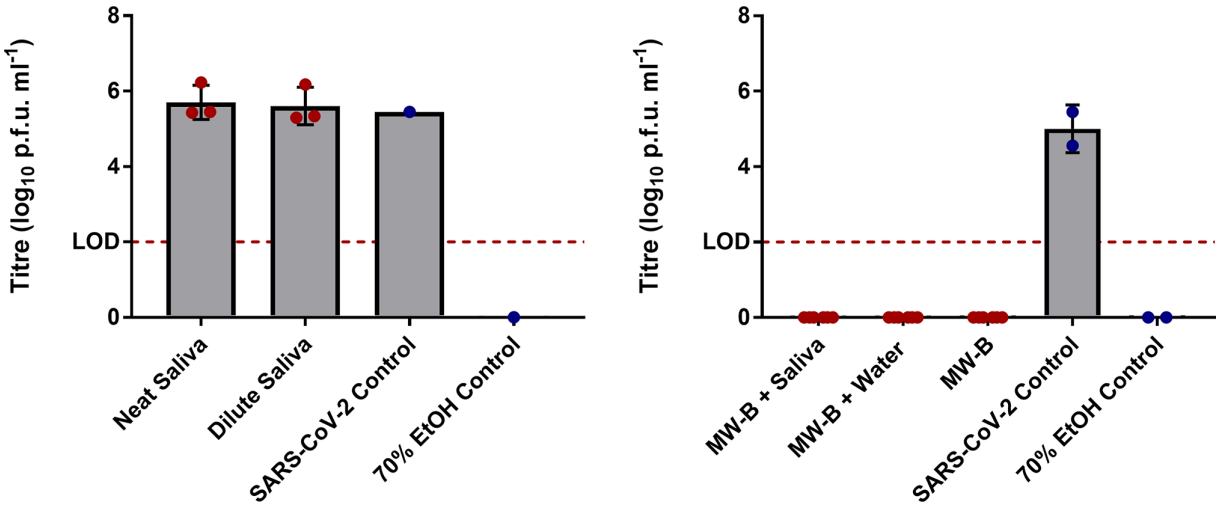

Fig. 3. Irradiated human saliva has no effect upon the viral titre of SARS-CoV-2 as compared to the water control after incubation with inoculum for 5 min. Neat saliva had a ratio of eight parts water to one-part irradiated human saliva to one-part virus inoculum, while dilute saliva had a ratio nine parts irradiated human saliva to one-part virus inoculum (a). Human saliva does not inhibit the virucidal activity of mouthwash formulas proven to reduce the titre of SARS-CoV-2. MW-B was able to reduce viral titre to below the LOD both in the presence of irradiated human saliva and without. Human saliva was added in a ratio of eight parts MW-B to one-part irradiated human saliva to one-part virus inoculum (b). LOD (2.0$\log_{10}$ p.f.u. ml^{-1}) is shown across both graphs with a dotted red line. Error bars represent standard deviation, while red dots are experimental data values and blue dots control values.

5.45log$_{10}$ p.f.u. ml^{-1} were recovered from the neat saliva, dilute saliva incubation and the water control, respectively. It is essential that mouthwashes maintain efficacy in the presence of human saliva. To investigate this, we examined if the virucidal efficacy of MW-B was altered by saliva. We found that MW-B was still capable of inactivation of SARS-CoV-2 to below the LOD in the presence of saliva, indicating that CPC retained efficacy despite the soil load (Fig. 3b).

DISCUSSION

Our results confirm that mouthwash formulations containing 0.07% CPC, inactivate SARS-CoV-2 by up to 99.99%, representing a value below the LOD after a contact time of 30 s. In contrast, a mouthwash containing CHX (0.2% chlorhexidine gluconate), exhibited poorer virucidal activity against SARS-CoV-2. Our observations are consistent with others, where multiple different CPC mouthwash formulations have been shown to effectively inactivate SARS-CoV-2 *in vitro*, whereas CHX containing mouthwashes are reported to have modest ability to inactive SARS-CoV-2 [10, 25, 34]. The virucidal action of CPC mouthwash was maintained in the presence of whole human saliva, consistent with human clinical trials, which report that rinsing with CPC mouthwash can lower SARS-CoV-2 salivary count for several hours after use [11, 35].

Over the course of the global pandemic, several SARS-CoV-2 variants have emerged with mutations changing the amino acid sequence of the receptor-binding domain of the spike protein [36]. Four variants of concern, Alpha, Beta, Gamma and Delta [37], were effectively inactivated within 30 s by both 0.07% CPC mouthwashes, with a reduction in viral titre below the LOD and equivalent to the 70% ethanol control. As the CPC molecule disrupts the viral lipid envelope and the membrane is unchanged by mutations, our data supports the likely efficacy of CPC mouthwash in reducing viral load irrespective of the SARS-CoV-2 variant. It is interesting to note, as highlighted in Table 2, that SARS-CoV-2 variants displayed reproducible differences in titre. Although elucidating the exact mechanisms underpinning this observation are beyond the scope of this report, recent work [38] has suggested that natural variations in the SARS-CoV-2 viral accessory protein, ORF3a and non-structural protein, NSP2, may impact infectivity in laboratory models and we believe further investigation of such effects would assist the global response to the current pandemic.

Recently the oral cavity has been proposed to have a direct role in COVID-19 disease severity based on a proposed oral-vasculo-pulmonary infection route. Poor oral hygiene with plaque build-up, subsequent gingivitis and periodontitis facilitates direct entry of the virus via the oral gingival sulcus and periodontal pockets enabling infection of the circulatory system and lungs [9]. CPC mouthwashes with anti-plaque and virucidal activity against SARS-CoV-2 could have the potential to lower viral count and lessen the risk of severe lung disease in COVID-19 patients.

In conclusion, two mouthwashes containing 0.07% CPC were effective at inactivating SARS-CoV-2, within 30 s with greater than 4.0log$_{10}$ p.f.u. ml^{-1} reduction in viral titre. Moreover, virucidal activity of CPC was maintained in the presence of whole human saliva. Both 0.07% CPC mouthwashes were as effective as 70% ethanol against four variants of concern; Alpha, Beta, Gamma and Delta suggesting these CPC formulations possess virucidal action against all variants. In contrast, under the same experimental conditions, a mouthwash containing 0.2% chlorhexidine digluconate did not have substantial action against SARS-CoV-2 *in vitro*. Given the ongoing global pandemic, and the recognition of the significance of the oral cavity in infection, transmission and disease severity, daily use of an effective CPC mouthwash as part of a good oral hygiene routine, could be a low-cost and simple measure to reduce transmission risk.

Funding information
Funding for this work and was provided by Unilever.

Acknowledgements
Unilever funded this study and supplied mouthwash formulations. Carole Philpotts (Unilever) is thanked for her assistance in supply of saliva. E.I.P. and G.L.H. were supported by the EPSRC (V043811/1) and UKRI-BBSRC COVID rolling fund (BB/V017772/1). G.L.H. was also supported by the BBSRC (BB/T001240/1 and BB/V011278/1), a Royal Society Wolfson Fellowship (RSWF\R1\180013), the NIH (R21AI138074), the EPSRC (EP/V043811/1), the UKRI (20197 and 85336), and the NIHR (NIHR2000907). G.L.H. is affiliated to the National Institute for Health Research Health Protection Research Unit (NIHR HPRU) in Emerging and Zoonotic Infections at University of Liverpool in partnership with Public Health England (PHE), in collaboration with Liverpool School of Tropical Medicine and the University of Oxford. G.L.H. 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, Alpha (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, Beta (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, Gamma (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.

Conflicts of interest
A.G., S.M. and M.H. are employees of Unilever.

Ethical statement

Ethical approval for saliva collection from the Unilever R&D Port Sunlight Independent Ethics Committee (GEN 022 13). All individuals gave informed consent to participate in the study. Appropriate consent was obtained from all participants/subjects in this research.

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