1   **Brief Report: The Virucidal Efficacy of Oral Rinse Components Against SARS-CoV-2 In Vitro**

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24 **Abstract**

25 The ability of widely-available mouthwashes to inactivate SARS-CoV-2 in vitro was tested using a

26 protocol capable of detecting a 5-log10 reduction in infectivity, under conditions mimicking the

27 naso/oropharynx. During a 30 second exposure, two rinses containing cetylpyridinium-chloride and a

28 third with ethanol/ethyl lauroyl arginate eliminated live virus to EN14476 standards (>4-log10

29 reduction), while others with ethanol/essential oils and povidone-iodine (PVP-I) eliminated virus by 2-

30 3-log10. Chlorhexidine or ethanol alone displayed little or no ability to inactivate virus. Studies are

31 warranted to determine whether these formulations can inactivate virus in the human oropharynx *in*

32 *vivo*, and whether this might impact transmission risk.

33 Key words: SARS-CoV2, Lipids, Mouthwash

34 Word Count:100

Background

36 The lipid membranes of enveloped viruses are sensitive to disruption by lipidomimetic agents and

37 surfactants. Thus, we hypothesised that the SARS-CoV-2 virus would be susceptible to inactivation by

38 components in widely available mouthwashes, such as ethanol/essential oils, cetylpyridinium chloride

39 (CPC) and povidone-iodine (PVP-I) [1]. Indeed, mouthwashes have been empirically employed in

40 outbreaks in China during the current pandemic [2]. Two recent studies demonstrated that this

41 approach can work *in vitro* under conditions that mimic nasal/oral passages. First, several

42 formulations including dequalinium/benzalkonium chloride, PVP-I and ethanol/essential oils reduced

43 SARS-CoV-2 infectivity *in vitro* by up to 3-log10 [3]. Second, infectivity of the closely related HCoV-

44 229E coronavirus was reduced by 3-4-log10 using several agents including CPC, ethanol/essential oils

45 and PVP-I [4]. Inactivation of HCoV-229E by >3-log10 by CPC at 0.07% was also shown in a recent

46 preprint [5]. So far, only one of the products tested (Listerine Antiseptic, combining 26.9% alcohol

47 with essential oils) achieved the 4-log10 kill required to pass EN14476 as a virucidal [4]. Recent,

48 preliminary clinical studies have suggested that virucidal activity of oral rinses may occur in vivo

49 against SARS-CoV2 [6, 7].

50 Since only one of the *in vitro* studies has used the SARS-CoV-2 pathogen to date, here, we extended

51 this work by testing the virucidal activity against SARS-CoV-2 of a range of mouthwashes including CPC

52 (0.05-0.1%w/v Dentyl Dual Action, 0.05-0.1% w/v Dentyl Fresh Protect, 0.10% w/v SCD Max)

53 ethanol/essential oils (Listerine Cool Mint, 21.7% v/v ethanol), ethanol/ethyl lauroyl arginate

54 (Listerine Advanced Gum Treatment, 23% v/v ethanol), chlorhexidine (0.2% w/v; Corsodyl) and

55 povidone iodine (0.5% w/v; Videne). We found that three products had sufficient activity to pass

56 EN14476 against SARS-CoV2. We also investigated the contribution of ethanol to the observed

57 virucidal activity, to inform future studies as to which products are most likely to provide the greatest

58 benefit against SARS-CoV2, and to provide information as to how future virucidal formulations might

59 be optimised.

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Methods

62 Virucidal assays utilised VeroE6, a gift from the University of Glasgow/MRC Centre for Virology, UK.

63 The England2 strain of SARS-CoV2 was provided by Public Health England, and amplified in VeroE6

64 cells before being harvested from the supernatant. All cells were grown in DMEM containing 10 %

65 (v/v) FCS, and incubated at 37 °C in 5 % CO_2. Virucidal activity of mouthwash was studied in media

66 containing 100 µL mucin type I-S, 25 µL BSA Fraction V, and 35 µL yeast extract to mimic oral

67 secretions. 100 µL of this mixture was added to 100 µL of virus suspension, and 800 µL of the test-

68 product added. After 30 seconds, virucidal activity was neutralised by 10-fold serial dilution in ice-cold

69 DMEM (containing 10% FCS). Alternatively, in a modification of the methods of Mesiter [3], virus was

70 purified by size-exclusion chromatography (SEC) to prevent direct cytotoxic effects of the products on

71 the cell monolayer; 100 µL of the mixture was added to a microspin S-400 HR column, and centrifuged

72 for 2min at 700 x *g*. A 10-fold serial dilution was then made of the flow-through in DMEM containing

73 10% FCS. In a further modification to the methods of Meister *et al* [3], we titrated virus onto VeroE6

74 cells transduced with Lentivirus vectors expressing ACE2 and TMPRSS2 and drug selected, to enhance

75 virus entry (>1-log), generating a more sensitive test for virucidal activity. Titrations were performed

76 by plaque assay; serial dilutions were used to infect VeroE6/ACE2/TMPRSS2 cells for 1 h. Following

77 this, cells were overlaid with DMEM containing 2 % FCS, and 1.2 % Avicel®. After 72 h, the overlay was

78 removed, and the monolayer washed and fixed with 100% methanol. Monolayers were stained with

79 a solution of 2 5% (v/v) methanol and 0.5 % (w/v) Crystal Violet, then washed with water, and plaques

80 were enumerated.

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82   **Results**

83   In initial experiments, we examined the effect of mouthwashes on the VeroE6/ACE2/TMPRSS2

84   monolayers used to detect live virus, following serial dilution in DMEM. Four of the seven products

85   demonstrated toxicity to the monolayer, which was not eliminated until they were diluted at least

86   100-1000-fold, limiting the sensitivity of the assay to measure residual infectivity. We therefore used

87   size-exclusion chromatography (SEC) to rapidly purify virus away from the products. When virus was

88   purified on S-400 HR microspin columns, only minimal loss of infectivity was observed (Fig 1A),

89   however toxicity from the mouthwashes against the cell monolayer was virtually eliminated (Fig 1B).

90   SEC was therefore used for all assays from this point forwards. This approach also ensures that the

91   activity of the mouthwashes against the virus was rapidly stopped after the desired co-incubation

92   time. In comparison, our results suggest that 'stopping' the reaction by serial 10-fold dilution would

93   leave sufficient mouthwash activity to have continued biological activity against the virus. Combined

94   with the use of VeroE6/ACE2/TMPRSS2 cells for titration, which SARS-COV2 enters >1log more

95   efficiently than parental VeroE6, the assay was capable of detecting a 5-log10 decrease in virus titre.

96   This is more than sufficient to detect the 4-log10 reduction in activity specified by EN14476.

97   Having optimised a sensitive protocol for the detection of virucidal activity, we tested the ability of a

98   wide range of commercially available mouthwash formulations (Table 1) to reduce virus infectivity,

99   after a 30-second treatment. The mouthwashes demonstrated a wide spectrum of inactivation ability

100   (Fig 1C). Two Dentyl mouthwashes containing CPC, and Listerine Advanced (23 % ethanol) with ethyl

101   lauroyl arginate (LAE), a cationic surfactant, eradicated the virus completely, giving >5-log10 reduction

102   in viral titres. A moderate effect (~3-log fold reduction) was seen with the iodine containing product

103   (Videne), SCD Max (CPC and Sodium Citric Acid), and mouthwash containing 21 % v/v alcohol with

104   essential oils (Listerine Cool Mint) (Fig 1C). Ethanol alone at <23 % had no effect on virus infectivity,

105   thus the inclusion of essential oils (Listerine Cool Mint) or LAE (Listerine Advanced) appears to be

106   required for optimal efficacy. Lastly, chlorhexidine was relatively inactive (<2 log fold reduction).

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109   **Discussion**

110   Our data, using an assay that enabled us to detect up to 5-log10 reduction in SARS-CoV2 activity,

111   further support the accumulating evidence for high virucidal activity by widely-available mouthwashes

112   *in vitro*, against SARS-CoV2 and the related HCoV-229E coronavirus strain [3-5]. Here, three products

113   which contained either (i) 0.07-0.1 % CPC (Dentyl Dual Action, Dentyl Fresh Protect) or (ii) 23 % ethanol

114   with LAE (Listerine Advanced) provided the greatest level of inactivation, surpassing the level required

115   for EN14476.

116   The ability of CPC-containing mouthwashes to reduce viral infectivity is in line with studies

117   using other enveloped viruses; similar microemulsion formulations of CPC (with a similar isopropyl

118   myristate microemulsion as Dentyl Dual Action) have been demonstrated to exhibit both inherent

119   antimicrobial activity [8] and specific activity against Herpes Simplex virus [9], and other mouthwash

120   formulations containing 0.07 % CPC have been reported to reduce infectivity of seasonal

121   coronaviruses by 3-4-log10 [4]. In our study, SARS-CoV2 was even more sensitive to the CPC-

122   containing mouthwashes than in these previous reports using hCoV-229E; this may reflect the

123   cumulative activity of additional components in these formulations. The SCD Max (containing CPC at

124   a higher concentration) only resulted in a 3-log10 reduction in infectivity, and suggests that the exact

125   formulation is important; thus, individual mouthwash formulations should be empirically tested for

126   antiviral activity, rather than basing decisions on the 'major' antimicrobial component.

127   Listerine (Cool Mint, Ultra, Antiseptic formulations) was recently shown by others to have

128   virucidal activity towards both SARS-CoV2 or HCoV-229E. Meyers *et al* showed >4 log10 reduction in

129   titres for Listerine Antiseptic and 3-4 log10 reductions for Listerine Ultra against HCoV-229E [4], while

130   Meister *et al* found either >2 or >3 log10 reductions for Listerine Cool Mint against 3 separate SARS-

131   CoV2 strains isolated from patients [3, 4]. Here, the greater sensitivity of our assay revealed that while

132   Listerine Cool Mint reduced infectivity by 3-logs, Listerine Advanced was superior, and was capable of

133   totally inactivating SARS-CoV2, reducing infectivity by >5-log10. As with the CPC-containing products,

134   the importance of formulation was evident in alcohol-containing preparations; alcohol alone at the

135   same concentration as in Listerine Cool Mint or Advanced had minimal impact, indicating that the

136   essential oils and LAE in these formulations significantly augment antiviral activity. Whilst poorly-

137   defined chemically, the antiviral activity of essential oils present in the Dentyl Products and Listerine

138   products (a mixture of plant-derived monoterpenes and phytoretinoids) has been previously

139   extensively described [10], albeit not in the context of SARS-CoV2. Moreover, the addition of the

140   cationic surfactant LAE, which is recognised to exhibit antiviral activity *in vitro* [11], resulted in a >2-

141 log increase in activity compared to the Listerine Cool Mint product; resulting in a product that gave
142 an equal level of kill to the CPC-containing products.

143   Two very preliminary studies using very small numbers of patients with COVID19 have
144 suggested that mouthwashes including PVP-I [6] and chlorhexidine [7] may reduce SARS-CoV2 loads
145 *in vivo*. However, these studies used qPCR, which does not determine whether the detected virus is
146 infectious, the numbers of patients studied was extremely low, and the impact was variable. While
147 these studies demonstrate a potential for reducing the level of SARS-CoV2 in the oropharynx, it is
148 important to note that *in vivo* studies are currently lacking into how effective such an approach might
149 be *in vivo* at reducing viral titre and transmission. It is critical to determine how quickly virus shedding
150 from actively infected cells in both the upper and lower respiratory tract replenishes live virus in the
151 oral cavity after treatment. Our *in vitro* data identifies products with high activity, and indicates that
152 investigating the duration of their effects *in vivo* against live virus load, and defining potential effects
153 on reducing the risk of virus exposure within the clinical setting (for example when performing clinical
154 examinations of the oropharynx, or visiting vulnerable elderly populations/patients), in well-designed,
155 randomised controlled trials is warranted. Importantly the anti-viral mechanisms of action for oral
156 rinses are dependent on the lipid composition of the viral envelope and its sensitivity to surfactants
157 and membrane disrupting agents. Since this membrane derives from host cell membranes, it is
158 unlikely to be altered by virus mutation.

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160 **References**

161 1. O'Donnell VB, Thomas D, Stanton R, et al. Potential Role of Oral Rinses Targeting the Viral Lipid
162 Envelope in SARS-CoV-2 Infection. Function **2020**; 1.10.1093/function/zqaa002

163 2. Meng L, Hua F, Bian Z. Coronavirus Disease 2019 (COVID-19): Emerging and Future Challenges for
164 Dental and Oral Medicine. J Dent Res **2020**; 99:481-7.10.1177/0022034520914246

165 3. Meister TL, Bruggemann Y, Todt D, et al. Virucidal Efficacy of Different Oral Rinses Against Severe
166 Acute Respiratory Syndrome Coronavirus 2. The Journal of infectious diseases **2020**; 222:1289-
167 92.10.1093/infdis/jiaa471

168 4. Meyers C, Robison R, Milici J, et al. Lowering the transmission and spread of human coronavirus. J
169 Med Virol **2020**; n/a.10.1002/jmv.26514

7

170   5. Green A, Roberts G, Tobery T, Vincent C, Barili M, Jones C. In vitro assessment of the virucidal activity

171   of four mouthwashes containing Cetylpyridinium Chloride, ethanol, zinc and a mix of enzyme and

172   proteins against a human coronavirus. bioRxiv **2020**:2020.10.28.359257.10.1101/2020.10.28.359257

173   6. Martinez Lamas L, Diz Dios P, Perez Rodriguez MT, et al. Is povidone iodine mouthwash effective

174   against SARS-CoV-2? First in vivo tests. Oral Dis **2020**; n/a.10.1111/odi.13526

175   7. Yoon JG, Yoon J, Song JY, et al. Clinical Significance of a High SARS-CoV-2 Viral Load in the Saliva. J

176   Korean Med Sci **2020**; 35:e195.10.3346/jkms.2020.35.e195

177   8. Alkhatib MH, Aly MM, Rahbeni RA, Balamash KS. Antimicrobial Activity of Biocompatible

178   Microemulsions Against Aspergillus niger and Herpes Simplex Virus Type 2. Jundishapur J Microbiol

179   **2016**; 9:e37437.10.5812/jjm.37437

180   9. Shishu, Rajan S, Kamalpreet. Development of novel microemulsion-based topical formulations of

181   acyclovir for the treatment of cutaneous herpetic infections. AAPS PharmSciTech **2009**; 10:559-

182   65.10.1208/s12249-009-9242-1

183   10. Astani A, Reichling J, Schnitzler P. Comparative study on the antiviral activity of selected

184   monoterpenes derived from essential oils. Phytother Res **2010**; 24:673-9.10.1002/ptr.2955

185   11. Tripathy DB, Mishra A, Clark J, Farmer T. Synthesis, chemistry, physicochemical properties and

186   industrial applications of amino acid surfactants: A review. Comptes Rendus Chimie **2018**; 21:112-

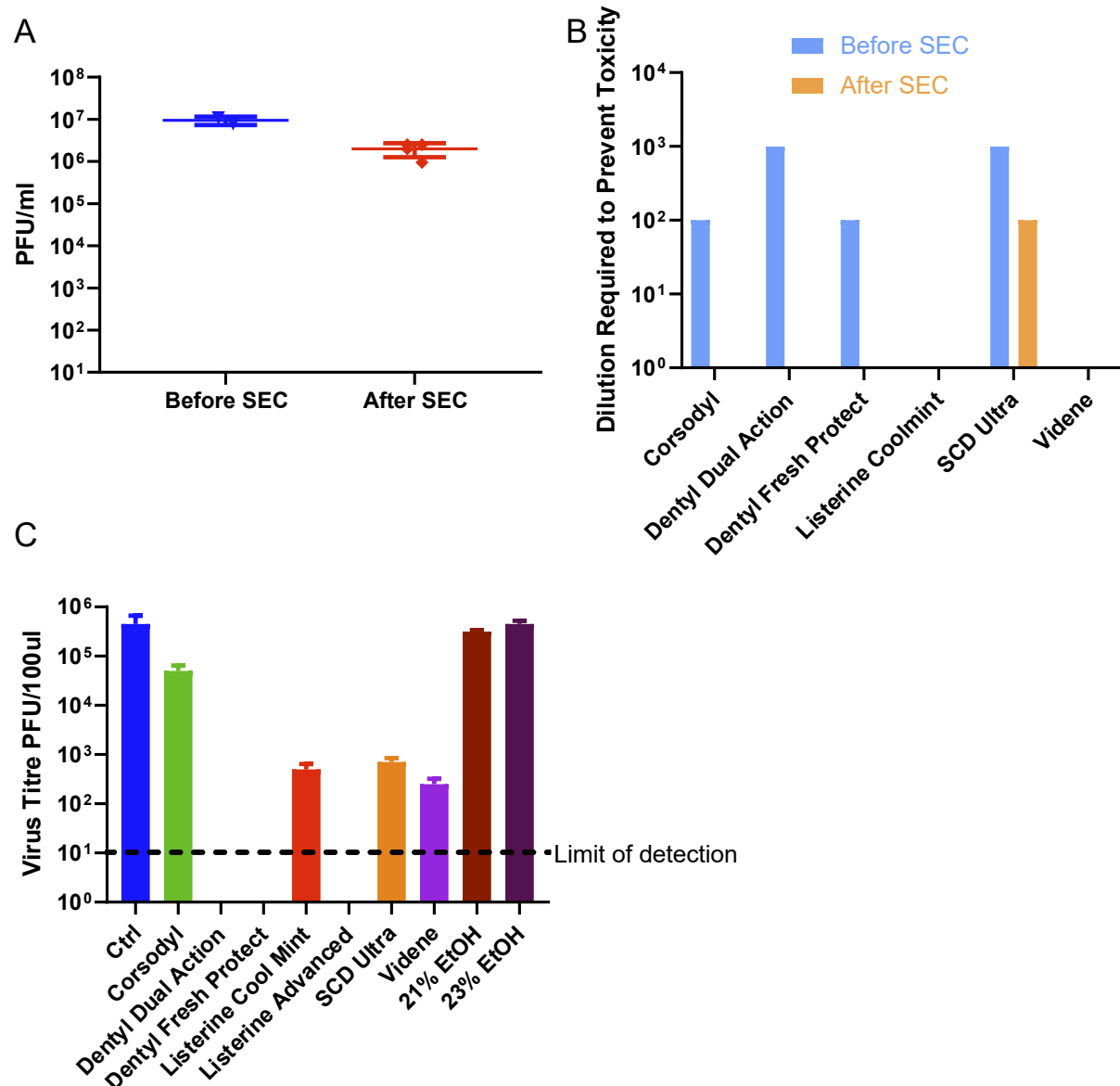187   30.10.1016/j.crci.2017.11.005

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Figure 1. (A) 100µl virus was purified through a S-400 HR spin column, and live virus measured by plaque assay on VeroE6/ACE2/TMPRSS2. Only minimal loss of virus titre was observed. (B) Mouthwashes were mixed with DMEM (instead of virus) and synthetic salivary secretions, then 100µl of the mixture was purified through a S-400 HR spin column, diluted by serial 10-fold dilution in DMEM/10, and titrated onto VeroE6/ACE2/TMPRSS2. After 72h, overlays were removed and monolayers were fixed and stained with crystal violet, then toxicity was scored based on visual inspection of monolayer integrity. (C) Virus was mixed with synthetic salivary secretions and mouthwash, then purified by SEC after 30 seconds, before being titrated by plaque assay on VeroE6/ACE2/TMPRSS2.

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204   Table 1

Product name	Active Ingredients
Corsodyl	7 % (v/v) ethanol, 0.2 % (w/v) chlorhexidine Other active ingredients: peppermint oil
Dentyl Dual Action	0.05 %-0.1 % (v/v) cetylpyridinium chloride (CPC), Other active ingredients: isopropyl myrisate, Mentha Arvensis extract
Dentyl Fresh Protect	0.05 %-0.1 % (v/v) cetylpyridinium ehloride (CPC) Other active ingredients: xylitol
Listerine Cool Mint	21 % (v/v) ethanol Other active ingredients: thymol 0.064 %, eucalyptol 0.092 %, methyl salicylate 0.060 % and menthol 0.042 %
Listerine Advanced Gum Treatment	23 % (v/v) ethanol, Other active ingredients: ethyl lauroyl arginate HCl (LAE) 0.147% w/w.
SCD Max	0.07-0.1 % (v/v) cetylpyridinium chloride (CPC) and sodium citric acid 0.05 % Other active ingredients: sodium monofluorophosphate.
Videne	7.5% iodinated povidone equivalent to 8.25 mg/ml iodine

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