The In Vitro Virucidal Effects of Mouthwashes on SARS-CoV-2

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Abstract: Oral antiseptic mouthwashes have been widely used for their antibacterial activity. As a result of the SARS-CoV-2 pandemic, the antiviral properties of these oral antiseptics have been aggressively studied. To demonstrate the direct antiviral activity of mouthwashes against SARS-CoV-2, this review will focus on the in vitro virucidal effects of these mouthwashes. Knowledge of the type, concentration, and exposure time of available mouthwashes can provide insights into effective protocols for their clinical use. With an understanding of the characteristics of each oral antiseptic mouthwash, proper mouthwash selection against SARS-CoV-2 may become a useful adjunct to personal protective equipment.

Keywords: SARS-CoV-2; COVID-19; mouthwashes; virucidal; antiviral; oral rinses; hygiene; CHX; CPC; PVP

1. Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is responsible for the coronavirus disease 2019 (COVID-19) pandemic. SARS-CoV-2 is a single stranded enveloped RNA virus [1]. Its exponential spread, resulting in significant morbidity and mortality [2]. The SARS-CoV-2 viral load is highest in the oropharynx, nasopharynx, and nasal cavity [3]. This is due to the high angiotensin-converting enzyme 2 (ACE2) receptors in the respiratory epithelium. The ACE2 receptors are used by the virus on first entry into the body [4]. Since the mouth is part of the oropharynx, contaminated saliva can contribute to the spread of SARS-CoV-2 [5,6]. SARS-CoV-2 can spread via respiratory droplets, saliva, dental procedures that produce aerosolized viral particles [7], and physical contact. Dental providers are at risk of exposure to high levels of contamination with SARS-CoV-2 while performing aerosol-producing dental procedures [8].

SARS-CoV-2 shedding is the highest during the initial stages of disease and occurs in the upper respiratory tract [9,10]. SARS-CoV-2-infected symptomatic patients, as well as asymptomatic and pre-symptomatic SARS-CoV-2-infected individuals can spread the virus [11]. Social distancing and preventative measures, including personal hygiene and frequent disinfection of high-touch surfaces, are some important interventions to reduce person-to-person transmission. Cross-infection control guidelines need to stay abreast of the constantly mutating SARS-CoV-2 [12] and, likewise, new vaccines need to constantly evolve to continue to be effective [13]. Thus, strategies to curb the spread may involve proper hand washing and oral disinfection with mouthwashes [7].
2. Oral Antiseptic Mouthwashes

Oral antiseptic mouthwashes have been used widely for their antibacterial activity. Due to the COVID-19 pandemic, the antiviral properties of these oral antiseptics have been further investigated for infection control (Table 1). Commercially available mouthwashes contain active ingredients such as povidone iodine or polyvinylpyrrolidone iodine (PVP-I), cetylpyridinium chloride (CPC), chlorhexidine gluconate (CHX), dipotassium oxalate, stabilized hypochlorous acid, hydrogen peroxide ($\text{H}_2\text{O}_2$), eucalyptol, thymol, menthol, sodium fluoride, and zinc fluoride [14].

| Study | Contact Time | Active Ingredient | Viral Titer (Median) | % Viral Kill | LRV | Compared to Control |
|-------|--------------|-------------------|---------------------|-------------|-----|---------------------|
| Anderson et al., 2020 [15] | 30 s | PVP-I 0.5% | >0.67 | 3.63 | 4.33 |
| | 30 s | PVP-I 1.0% | >0.67 | 3.63 | 4.33 |
| Bidra et al., 2020 [16] | 15 s | PVP-I 0.5% | <0.67 | 1.8 | 2.5 |
| | 15 s | PVP-I 1.0% | >0.67 | 1.8 | 2.5 |
| | 30 s | PVP-I 0.5% | <0.67 | 1.8 | 2.5 |
| | 30 s | PVP-I 1.0% | >0.67 | 1.8 | 2.5 |
| Bidra et al., 2020 [17] | 15 s | PVP-I 0.5% | <0.67 | 3.33 | 3.0 |
| | 15 s | PVP-I 1.0% | >0.67 | 3.33 | 3.0 |
| | 30 s | PVP-I 0.5% | <0.67 | 3.33 | 3.0 |
| | 30 s | PVP-I 1.0% | >0.67 | 3.33 | 3.0 |
| Davies et al., 2021 [14] | PBS | 0.2% CHX (formulation contains ethanol with 0.2% CHX (alcohol-free formulation)) 1.4% dipotassium oxalate (alcohol-free formulation) Eucalyptol, thymol, menthol, sodium fluoride, zinc fluoride 0.01–0.02% stabilised hypochlorous acid 1.5% H$\text{O}_2$ 0.5% PVP-I (surfactant-free) | NR | NR | ≥4.1 |
| Hassandarvish et al., 2020 [18] | Distilled water | Bovine serum albumin group | NR | NR | ≥5 |

Table 1. In-vitro studies on the virucidal effects of mouthwash on SARS-CoV-2.
Table 1. Cont.

| Study | Control | Contact Time | Active Ingredient | Viral Titer (Median) | % Viral Kill | LRV Compared to Control |
|-------|---------|--------------|-------------------|---------------------|-------------|-------------------------|
| Kariva et al., 2021 [19] | 0.5% sodium thiosulfate | 30 s | PVP 0.47% | >99.94 | >3.2 | NR |
| | | 30 s | PVP 0.23% | >99.93 | >3.1 | NR |
| | | 30 s | PVP 0.35% | >99.92 | >3.1 | NR |
| | | 30 s | PVP 0.45% | >99.91 | >3.2 | NR |
| | | 60 s | PVP 0.47% | >99.90 | >3.2 | NR |
| | | 60 s | PVP 0.23% | >99.89 | >3.1 | NR |
| | | 60 s | PVP 0.35% | >99.88 | >3.1 | NR |
| | | 60 s | PVP 0.45% | >99.87 | >3.0 | NR |
| Koch-Heier et al., 2021 [20] | Infection medium control | NR | 0.05% CPC and 1.5% H₂O₂, 0.1% CHX, 0.05% PPC, and 0.00% F (fluoride), without ethanol | NR | NR | NR |
| Komine et al., 2021 [21] | Medium control | 30 s | H₂O₂ | <3.00 | <0.67 | >99.999 |
| | | 30 s | CHX | <3.00 | <0.67 | >99.999 |
| Meister et al., 2020 [22] | Medium control (BetaCoV/Germany/Ulm/01/2020) | 30 s | Ethanol and essential oils | <3.00 | <0.67 | >99.999 |
| | | 30 s | Polyaminopropyl biguanide | <3.00 | <0.67 | >99.999 |
| Muscovitz and Mendonchall, 2020 [23] | Water (Negative control) | 30 s | 1.5% H₂O₂ | <3.00 | <0.67 | >99.999 |
| | | 30 s | 0.2% PVP-I | <3.00 | <0.67 | >99.999 |
| | | 30 s | 0.12% CHX | <3.00 | <0.67 | >99.999 |
| Pelletier et al., 2021 [24] | Water | 60 s | 1.5% PVP-I | <0.67 | 4.63 | NA |
| Santos et al., 2021 [25] | Viral solution and cellular system (Positive Control) | 30 s | 0.1% anionic phthalocyanine derivate (APD) | <0.67 | 4.63 | NA |
| | | 60 s | Virus control | <0.67 | 4.63 | NA |
| | Cellular system only (Negative Control) | 30 s | | <0.67 | 4.63 | NA |
| Shet et al., 2022 [26] | Water (negative control) | 30 s | 1.5% PVP-I | <0.67 | 4.63 | NR |
| | | 60 s | 0.5% PVP-I | <0.67 | 4.63 | NR |
| | | 60 s | Negative control | <0.67 | 4.63 | NR |
| Shewale et al., 2021 [27] | PBS | 30 s | Stabilized chlorine dioxide | <0.67 | 98.4 | NR |
| | | 60 s | Ultra sensitive rinse | <0.67 | 98.4 | NR |

**Strain**

| Strain | 1 | 2 | 3 |
|--------|---|---|---|
| Medium control (BetaCoV/Germany/Ulm/01/2020) | 0.50 | 0.56 | 0.50 |
| Medium control (BetaCoV/Germany/Ulm/02/2020) | 0.50 | 0.56 | 0.50 |

**LRV**

| Strain | 1 | 2 | 3 |
|--------|---|---|---|
| Medium control (BetaCoV/Germany/Ulm/01/2020) | 0.50 | 0.56 | 0.50 |
| Medium control (BetaCoV/Germany/Ulm/02/2020) | 0.50 | 0.56 | 0.50 |

**Median % Viral Kill**

| Strain | 1 | 2 | 3 |
|--------|---|---|---|
| Medium control (BetaCoV/Germany/Ulm/01/2020) | 0.50 | 0.56 | 0.50 |
| Medium control (BetaCoV/Germany/Ulm/02/2020) | 0.50 | 0.56 | 0.50 |

**Virucidal**

| Strain | 1 | 2 | 3 |
|--------|---|---|---|
| Medium control (BetaCoV/Germany/Ulm/01/2020) | 0.50 | 0.56 | 0.50 |
| Medium control (BetaCoV/Germany/Ulm/02/2020) | 0.50 | 0.56 | 0.50 |

**No effect**

| Strain | 1 | 2 | 3 |
|--------|---|---|---|
| Medium control (BetaCoV/Germany/Ulm/01/2020) | 0.50 | 0.56 | 0.50 |
| Medium control (BetaCoV/Germany/Ulm/02/2020) | 0.50 | 0.56 | 0.50 |
Table 1. Cont.

| Study                  | Control                                      | Contact Time | Active Ingredient               | Viral Titer (Median) | % Viral Kill | LRV Compared to Control |
|------------------------|----------------------------------------------|--------------|----------------------------------|----------------------|--------------|-------------------------|
| Steinhauer et al., 2021 [28] | Validation control (EN14476 protocol)        | 300 s        | 0.1% CHX (80% conc)             | 0.15 s               | NR           | ≥4.38                   |
|                        |                                              | 600 s        | 0.2% CHX (80% conc)             | 0.30 s               | NR           | ≥4.38                   |
|                        |                                              | 15 s         | 0.1% Octenidine dihydrochloride (OCT) (80% conc) | 0.15 s               | 0.81         | ≥4.38                   |
|                        |                                              | 30 s         | 0.1% Octenidine dihydrochloride (OCT) (20% conc) | 0.30 s               | 0.4          | ≥4.38                   |
|                        |                                              | 60 s         | 0.1% Octenidine dihydrochloride (OCT) (20% conc) | 0.60 s               | 0.4          | ≥4.38                   |
| Tiong et al., 2021 [29] | Cell culture medium (EN14476:2013/FprA1:2015 protocol) | Clean 30 s   | 0.12% CHX                       | 0.5                  | 4.0          | 5.0                     |
|                        |                                              | Clean 30 s   | 0.05% CPC and 0.05% SF          | 0.5                  | 4.0          | 5.0                     |
|                        |                                              | Dirty 30 s   | 0.05% Thymol                    | 0.5                  | 5.0          | 5.0                     |
|                        |                                              | Dirty 60 s   | 0.1% Hexetidine and 9% Ethanol   | 0.75                 | 5.0          | 5.0                     |
|                        |                                              | Dirty 60 s   | 2% NaCl                         | 5.0                  | 5.0          | 5.0                     |
|                        |                                              | 60 s         | 0.05% Thymol                    | 5.0                  |              |                          |

NR: not reported. NA: not applicable.

3. In vitro Studies assessing Virucidal Activity

To evaluate the disinfecting activity of mouthwashes, the EN14476 standard methods were utilized for the virus time–kill assay [30]. Against SARS-CoV-2, the evaluated mouthwashes were tested undiluted and 50% diluted, at contact times of 15, 30 and 60 s, and under clean (0.3 g/L bovine serum albumin (BSA)) or dirty (0.3 g/L BSA + 3 mL/L human erythrocytes) conditions. Viral activity was immediately neutralized at the specific contact times to ensure that there were no sequelae. The 10-fold serial dilutions were incubated with Vero E6 cells for 72 h until cytopathic effects were observed. The Spearman–Kärber method [31,32] was used to determine the viral titers. Based on the European Chemicals Agency (ECHA) guidelines [33], virucidal activity is calculated as the reduction in viral titer compared to control. The log reduction value (LRV) of each mouthwash (Table 1) was compared to the negative control (water).

4. Safety of Antiseptic Mouthwashes

For an antiseptic to be used safely as a mouthwash in the oral cavity, cytotoxicity assays were performed to evaluate the lowest concentration at which the mouthwash was non-cytotoxic to human cells. Mouthwash dilutions were added to confluent monolayers of Vero E6 cell culture and incubated for 72 h before measuring the cell viability to determine the concentration at which no cytotoxic effects were observed on human cells. This also needs to be taken into consideration when assessing virucidal activity via the time–kill assay [30]. The Vero E6 cell lines are commonly used to isolate, propagate, and study SARS-CoV-2. The Vero lineage was extracted and isolated from African green monkey kidney epithelial cells [34]. The Vero E6 is a clone derived from Vero 76. The advantages of the Vero E6 cells are that they support high titres of viral replication [35–39], due their high ACE2 receptor expression [40] and the lack of interferon-producing activity [41].

PVP-I at a 5% concentration has proven safe for oral use [42–44]. With 6 months of 5% PVP-I use, the thyroid-stimulating hormone was shown to slightly increase with no indication of thyroid disease [45]. Mouthwash absorption at concentrations of 0.2% to 0.5% iodine is minimal and below the daily 150 µg iodine intake for a healthy adult. Furthermore, no taste change or teeth discoloration were reported in the studies [46]. Importantly, PVP-I substantivity has been reported to be as long as 4 h [47]. Contraindications for PVP-I include anaphylactic allergy to iodine, active thyroid disease, pregnancy, and radioactive iodine therapy [48–51]. There was some cytotoxicity reported for H₂O₂ and CHX [23].
5. Effectiveness of Oral Antiseptic Mouthwashes

Virucidal activity of a mouthwash (Table 1) can be reported as the log reduction value (LRV), which compares the reduction in viral titers to viral control. A $\geq 4\log_{10}$ reduction in viral titers corresponds to a $\geq 99.99\%$ kill, which indicates rapid virucidal activity. The exposure time was tested at 15 or 30 s; the 30 s exposure time was mandated by the European Chemicals Agency (ECHA) guidelines [33]. The Centers for Disease Control and Prevention (CDC) has suggested the use of PVP-I, chlorhexidine gluconate, cetylpyridinium chloride, or essential oils as possible options for an antiviral mouth rinse [52].

SARS-CoV-2 can be effectively inactivated in 30 s by most commercially available mouthwashes [22]. The most effective active ingredients were povidone–iodine [18], cetylpyridinium chloride, hexetidine, benzalkonium chloride, and essential oils [22].

PVP-I is a complex of povidone and iodine. It has been used for over 60 years in healthcare because of its broad-spectrum antimicrobial properties and safety profile [30,53]. SARS-CoV-2 can be completely inactivated by PVP-I in-vitro at concentrations of 0.5%, 1.25%, or 1.5%, in as little as 15 s [16]. It can completely inactivate SARS-CoV-2 at a concentration as minimal as 0.5% and a contact time of as minimal as 15 s [17]. Other in vitro studies reported PVP-I solutions of 0.23% inactivated SARS-CoV-2 in as little as 15 s [19,30]. In Japan, PVP-I at a 0.23% concentration has been recommended by the Japanese Ministry of Health, Labor and Welfare and is routinely used for daily gargling to prevent upper respiratory tract infections and COVID-19 [19,30,54]. Commercial mouthwashes containing 0.01–0.02% hypochlorous acid or 0.58% PVP-I effectively inactivated SARS-CoV-2 [14]. Iotech International formula 100-S displayed the highest virucidal activity compared to other mouthwashes; it inactivated SARS-CoV-2 completely. The 100 ppm molecular iodine mouthwash is unique in iodine chemistry [55].

CPC is a quaternary ammonium compound with broad antiseptic and antimicrobial activity. It has been used in many oral mouthwashes and breath sprays. CPC is virucidal against enveloped viruses, including influenza and several coronaviruses [56,57]. CPC mouthwashes can effectively decrease the viral load of SARS-CoV-2-infected individuals regardless of the viral variant [58]. The CPC mouthwashes can retain their effectiveness in the presence or absence of saliva [58]. CPC and CPC-containing mouthwashes disrupt the viral membrane integrity and inhibit SARS-CoV-2 entry into human cells. This decreases the infectivity of SARS-CoV-2 and is effective against the viral variants [58]. Previous studies have also shown that CPC has substantivity, lasting 3–5 h in saliva [59]. Another study reported a 6 log reduction in virucidal activity in a solution containing CPC and D-limonene 0.2% [60].

Some mouthwash formulations containing CHX were reported to have limited effectiveness against SARS-CoV-2 [28]. One study reported that the virucidal activity of CHX-containing mouthwash against SARS-CoV-2 was slightly less than those of CPC and hexetidine. This is incongruent with another study, which showed log reduction factors ranging from 0.33 to 0.78 [22]. Other in vitro and in vivo studies reported that CHX at a concentration of 0.12–2% was effective against SARS-CoV-2 [61,62]. CHX may temporarily reduce SARS-CoV-2 viral load in COVID-19 patients [63].

H$_2$O$_2$ is low cost and easily accessible [64,65]. However, it has the potential for toxicity under routine use [64], which includes gastric and colon symptoms [66]. H$_2$O$_2$ can be inactivated by the catalase in the saliva [67], and there is a lack of clinical or in vitro data for its virucidal effects on SARS-CoV-2. H$_2$O$_2$ at 3.0% and 1.5% had minimal antiviral activity after 15 s or 30 s [16]. PVP-I has more virucidal activity than H$_2$O$_2$. [16]. Some studies reported that H$_2$O$_2$ and CHX alone had no virucidal effect against SARS-CoV-2 [20]. Commercial H$_2$O$_2$ oral rinses may have additional components that improve the virucidal activity.

Mouthwashes containing dequalinium chloride and benzalkonium chloride had virucidal activity against SARS-CoV-2 [22]. Delmopinol hydrochloride is a known cationic surfactant [22]. Thus, cationic surfactants may also be effective against SARS-CoV-2.
Mouthwashes with an anionic phthalocyanine derivative (APD) can adhere to the cellular components of microorganisms [68]. APD mouthwashes can produce an in vitro intense antiviral reaction resulting in 90% viral inactivation [25].

Stabilized chlorine dioxide is antiviral, antibacterial, anti-biofilm, antifungal, and oxidative on oral malodor compounds [69–71]. Diluted toothpaste slurry with stabilized 0.04% chloride dioxide, compared to 0.1% chloride dioxide in mouthwashes, showed comparable viral load reduction [27]. The amino acids and organic acids present in saliva quickly liberate the chloride dioxide from its stabilized form in the oral cavity [70]. The released chlorine dioxide exhibits antiviral and antimicrobial activity [69].

Ethanol at 70% (positive control) did not completely inactivate SARS-CoV-2 at 15 s of contact; 30 s of contact was required to inactivate the virus [20].

Salt water and thymol was not effective against SARS-CoV-2, even though a high concentration of NaCl (0.34 M) was used [29]. Salt water gargling has been a common home remedy to alleviate the symptoms of a cold and sore throat. Mucin in buffers with a high salt concentration (0.3 M) increased the mucin barrier function, blocking viral infection in vitro [29]. Increasing the concentration of sodium chloride (NaCl) can enhance the antiviral activity of epithelial cells via inhibition of RNA and DNA viral replications [72]. More elaborate studies are needed to evaluate the virucidal effect of different NaCl concentrations on SARS-CoV-2.

6. Other Effects of Mouthwashes on SARS-CoV-2

In addition to the virucidal effects, the previously described mouthwashes may have inhibitory effects on the viral spike protein–ACE2 interaction, as well as transmembrane protease serine 2 (TMPRSS2) activity [73]. Disruption to the viral spike protein–ACE2 interaction and the TMPRSS2 activity can limit the entry of SAR-CoV-2 into the host cells and help prevent the spread of SARS-CoV-2 [74,75]. TMPRSS2 cleaves the viral spike protein and facilitates SARS-CoV-2 fusion to the host cell membrane [76].

Some active ingredients present in commercially available mouthwashes and toothpastes include sodium dodecyl sulfate (SDS), sodium N-lauroyl-N-methyltaurine (LMT), sodium tetradecene sulfonate (TDS), sodium N-lauroylsarcosinate (LSS), and copper gluconate (GCU). These active ingredients are reported to be effective against ACE2 and TMPRSS2, resulting in a highly preventive effect [73]. In addition, tranexamic acid (TXA) has inhibitory effects on TMPRSS2 protease activity [73].

7. Biocide Resistance of Mouthwashes

Long-term use of a sublethal dose of mouthwash may increase the bacterial minimum inhibitory concentration (MIC) and biocide resistance [77–81]. The MICs are lower for Gram-positive bacteria compared to Gram-negative bacteria; CHX has a greater affinity for the Gram-positive bacterial cell wall. Thus, prolonged use may increase risk of Gram-negative bacterial overgrowth.

The development of biocide resistance may lead to concomitant antibiotic cross-resistance [82]. Bacterial drug resistance was reported after frequent use of CPC [83]. Likewise, there is evidence of the development of bacterial resistance in response to low-level exposure to CHX [84]. Some mechanisms for bacterial resistance involve dysfunctional efflux pumps and cell membrane mutation [84].

However, studies reported that biocide resistance was to non-oral bacteria. There are limited reports on the long-term effects of low-concentration mouthwashes on oral bacteria. One study reported that the short-term use of CPC did not result in non-native bacterial colonization nor increased Gram-negative microorganisms [85].

The widespread use of mouthwashes may pose a potential risk of bacterial phenotypic adaptation, biocide resistance, and antibiotic cross-resistance. Further studies needed to investigate this potential risk and the underlying molecular mechanisms.
8. Limitations of In Vitro Studies

The effects of mouthwashes in vitro may not perform the same way in vivo; the human oral microenvironment is more complex. Substances such as saliva or teeth can act as viral carriers [86]. The presence of saliva may interfere with the effectiveness of the mouthwash in vivo. Saliva in the mouth with a flow rate of 5mL/min [87] can dilute the concentration of the mouthwash. In addition, bacteria in biofilms may exhibit a higher tolerance to antimicrobial mouthwashes [88]. In addition, the oral cavity maintains a mean temperature of 36.6 °C [89], while in vitro studies occur at ambient temperature. SARS-CoV-2 may be more stable at ambient temperature than at body temperature [90]. The temperature difference in the mouth and in vitro may contribute to some errors in outcomes. Furthermore, in vitro studies use standardized validated virucidal efficacy tests that are not representative of the in vivo antiviral effects in the oral cavity. The substantivity of the mouthwashes may also be different in the oral cavity compared to in vitro.

9. Conclusions

The broad-spectrum antibacterial and rapid virucidal activities against SARS-CoV-2, of the antiseptic mouthwashes discussed above suggests an important application in infection control. Randomized clinical trials comparing mouthwashes in COVID-19-positive patients are the next step in determining the ideal mouthwash and pre-procedural strategies to inactivate SARS-CoV-2 in the oral cavity in a clinical setting. PVP-I at 0.5–1.5% or CPC should be preferred over H₂O₂ or CHX. These virucidal mouthwashes with adequate substantivity can help reduce disease transmission and can be easily integrated into existing infection control protocols. The use of these mouthwashes can complement hygiene measures and curb the transmission of COVID-19 in the community.

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