










ORIGINAL ARTICLE

A Mouthwash with Cetylpyridinium Chloride is reducing salivary SARS-CoV-2 viral load in +COVID-19.

Un enjuague con Cloruro de Cetilpiridinio redujo la carga salival del virus SARS CoV-2 en pacientes con Covid-19

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ABSTRACT

Aim: The aim of this randomised, double-blind, placebo-controlled pilot clinical trial is to evaluate the capacity of a mouthwash to reduce SARS-CoV-2 viral load in the saliva of patients with COVID-19.

Methods: Twenty-three symptomatic SARS-CoV-2-positive outpatients were selected and randomised into two groups and registered at NTC 04563689. Both groups rinsed and gargled for one minute with either distilled water (Placebo) or with 0.05% Cetylpyridinium chloride (CPC) plus 0.12% Chlorhexidine (CHX) mouthwash (PERIOAID Intensive Care®). Saliva samples were collected before the use of placebo or mouthwash and after 15 minutes and 1 and 2 hours of either of the above treatment. A saliva sample was also taken five days after regular use of placebo or mouthwash twice daily. The virus was detected by qRT-PCR.

Results: A great heterogeneity in the viral load values was observed at baseline in both groups for nasopharyngeal and saliva samples. Most of the patients who used the mouthwash (8/12) had a significant decrease in baseline viral load after 15 min (greater than 99% reduction). This inhibitory effect was maintained for up to two hours in 10 of the 12 patients. At five days, SARS-CoV-2 RNA was detected in only 1 patient from the mouthwash group and in 5 from the placebo group.

Conclusions: This study points out that a CPC mouthwash can reduce the viral load in saliva of COVID-positive patients. This finding may be important in transmission control of SARS-CoV-2. Nevertheless, the clinical relevance of CPC mouthwash-reduction on SARS-CoV-2 shedding in saliva requires further study.

KEY WORDS

Antiviral mouthwash; SARS COV-2; Covid-19 patients; salivary viral load.

RESUMEN

Objetivo: El objetivo de este ensayo clínico piloto aleatorizado, doble ciego y controlado con placebo es evaluar la capacidad de un enjuague bucal para reducir la carga viral del SARS-CoV-2 en la saliva de pacientes con COVID-19.

Materiales y métodos: Veintitrés pacientes ambulatorios positivos para SARS-CoV-2 sintomáticos fueron seleccionados y aleatorizados en dos grupos y registrados en el NTC 04563689. Ambos grupos se enjuagaron y hicieron gárgaras durante un minuto con agua destilada (placebo) o con cloruro de cetilpiridinio al 0,05% (CPC) más enjuague bucal con Clorhexidina (CHX) al 0,12% (PERIOAID Intensive Care®). Se recolectaron muestras de saliva antes del uso de placebo o enjuague bucal y después de 15 minutos y 1 y 2 horas de cualquiera de los tratamientos anteriores. También se tomó una muestra de saliva cinco días después del uso regular de placebo o enjuague bucal dos veces al día. El virus fue detectado por qRT-PCR.

Resultados: Se demostró una gran heterogeneidad en los valores de carga viral al inicio del estudio en grupos ambos para muestras de nasofaringe y saliva. La mayoría de los pacientes que usaron el enjuague bucal (8/12) tuvieron una disminución significativa en la carga viral inicial después de 15 minutos (reducción superior al 99%). Este efecto inhibitor se mantuvo hasta dos horas en 10 de los 12 pacientes. A los cinco días, se detectó ARN del SARS-CoV-2 en solo 1 paciente del grupo de enjuague bucal y en 5 del grupo de placebo.

Conclusiones: Este señala que un enjuague bucal CPC puede reducir la carga viral en saliva de pacientes COVID positivos. Este hallazgo puede ser importante en el control de la transmisión del SARS-CoV-2. Sin embargo, la relevancia clínica de la reducción del enjuague bucal con CPC en la excreción de SARS-CoV-2 en la saliva requiere más estudios.

PALABRAS CLAVE

Enjuague bucal antiviral; SARS-CoV-2; pacientes con Covid-19; carga viral en saliva.

CLINICAL RELEVANCE

SARS CoV-2 is responsible for Covid-19 that has been associated with severe respiratory syndrome and multiple organ disease involvement that affected 645 million people and caused more than 6 million deaths worldwide. Initial viral replication seems to occur mostly at epithelial cells from oral, salivary glands pharyngeal and nasal area from which the virus disseminates to the lungs and the other body organs. Therefore, using mouthwashes with antiviral capacity might represent an interesting approach to prevent and control early infection and to reduce clinical complications. This pilot clinical study demonstrated that twice use for 1 minute of one mouthwash containing Cetylpyridinium Chloride at 0.05% is able to reduce salivary SARS CoV-2 consistently.

INTRODUCTION

Since the beginning of the SARS-CoV-2 virus pandemic, the World Health Organization has pointed out that this virus is transmitted from person to person, mainly via respiratory droplets, when a patient breath, cough, sneeze, or even talk or sing.¹⁻² The saliva of symptomatic and asymptomatic individuals affected by Coronavirus Disease 2019 (COVID-19) can hold a high load of this virus. In addition, accumulating evidence suggests that during the first week of infection, and shortly before symptoms appear, the viral load in saliva can reach values of 10^6 - 10^8 copies/ml,³⁻⁵ moreover, it is in this pre-symptomatic stage that infected subjects may spread SARS COV-2 at their contacts.⁶⁻⁸ Additionally, asymptomatic individuals represent a key population group in the spread of the disease, since certain authors consider them to be the “main driving source” of this pandemic.⁸⁻⁹ These facts could likely make up the set of reasons that explain the apparently easy transmission and high incidence of this disease.²

For this reason, and in order to limit the transmission of the virus, the principal public health measures implemented worldwide include hand washing, masks wearing, facial and ocular protection, social distancing and vaccination. Although the regular use of mouthwashes is a less studied preventive measure, it has enormous potential for public health, since reducing the salivary viral load in COVID-positive individuals would help control the spread of the virus.⁹

Pre-pandemic *in vitro* studies have shown that some of the molecules used in commercially available mouthwashes may have antiviral activity. Already in the initial stages of pandemic it was suggested that probably chlorhexidine gluconate (CHX), povidone iodine (PVI), Hydrogen

Peroxide, essential oils and Cetylpyridinium Chloride (CPC) could be effective against SARS-CoV-2.¹⁰⁻¹¹

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That is why currently 18 clinical trials are being carried out covering this topic. (data from the ClinicalTrials.Gov data base consulted on May 12, 2021). The products being assessed contain beta-cyclodextrin-Citrox, Povidone Iodine (PVI), Chlorhexidine Gluconate (CHX), Hydrogen Peroxide, Cetylpyridinium Chloride (CPC), Ethanol-essential oils, and Sodium Hypochlorite at diverse concentrations for mouthwash use for one minute and following up with gargling. Moreover, in some RCTs the use of nasal spray has been included. For all these studies the primary outcome variable is the reduction of salivary SARS-CoV-2 viral load, although a few included a subsequent nasal sampling. Nearly two thousand Covid-19 positives are being enrolled in those trials, although the results are still unknown.

CPC is a quaternary ammonium present in various mouthwashes and toothpastes. Prior to the appearance of the SARS-CoV-2 virus, several studies had shown that this molecule has potent *in vitro* antiviral activity on lipid-enveloped viruses, including coronaviruses by exploding the viral envelope and then impeding infection of targeted cells.¹²⁻¹³ CPC also have antibacterial activity by altering and disrupting bacterial cell membrane and capsule.

Therefore, this clinical trial explores the capacity of a 0.05% Cetylpyridinium chloride (CPC) plus 0.12% Chlorhexidine (CHX) mouthwash to reduce the salivary viral load of patients with confirmed SARS-CoV-2 and compared them with a group using to double distilled water mouthwash as placebo.

MATERIALS AND METHODS

Sample collection

Patient recruitment for this study was done between July to September 2020. Symptomatic SARS-CoV-2-positive outpatients aged > 18 years were considered for enrolment. The protocol for this randomised, double-blind, placebo-controlled clinical trial was approved by the University of Valle IRB (IRB approval number: 009-020). SARS-CoV-

2-positive individuals diagnosed by RT-PCR of respiratory secretions were contacted the same day of their positive result and invited to participate in the study and registered with the number NTC 04563689.

Taken in consideration that for the study design there not were previous RCT with the use of mouthwashes to control salivary SARS-CoV-2 infection this was a pilot study without sample calculation. The study was explained to the patients, who signed informed consent. We confirm that this study was conducted in accordance with the Helsinki Declaration of 1964, and its subsequent modifications.

Patients did not consume food during the two hours prior to saliva sample collection. Saliva samples were taken by using falcon 45 ml sterile plastic tubes by 2 trained physicians that visited patients and approximate 2 ml of saliva each time was preserved with cold pack containers before delivering the same day to the laboratory.

Five samples of whole unstimulated saliva were taken from each patient at differing time points - the first one at baseline and 3 consecutive samples at 15 minutes, 1 hour, and 2 hours after an initial use of mouthwash or placebo (M/P), with a final sample taken after 5 days of regular use of M/P twice daily for one minute.

Patients were randomised into two groups; 8 females and 4 males received 0.05% Cetylpyridinium chloride (CPC) plus 0.12% Chlorhexidine (CHX) mouthwash (PERIO-AID Intensive Care®) and 6 females and 5 males received distilled water as placebo.

Viral copy number was estimated by the same qRT-PCR assay using a standard curve (ranging from 2×10^5 copies/ μ L to 1.95×10^2 copies/ μ L) prepared with a synthetic plasmid positive control (IDT, USA) at 4-fold dilutions. Limit of detection (LOD) of the assay is 49 copies/ μ L. Each of the assays was carried out with appropriate extraction and amplification controls.

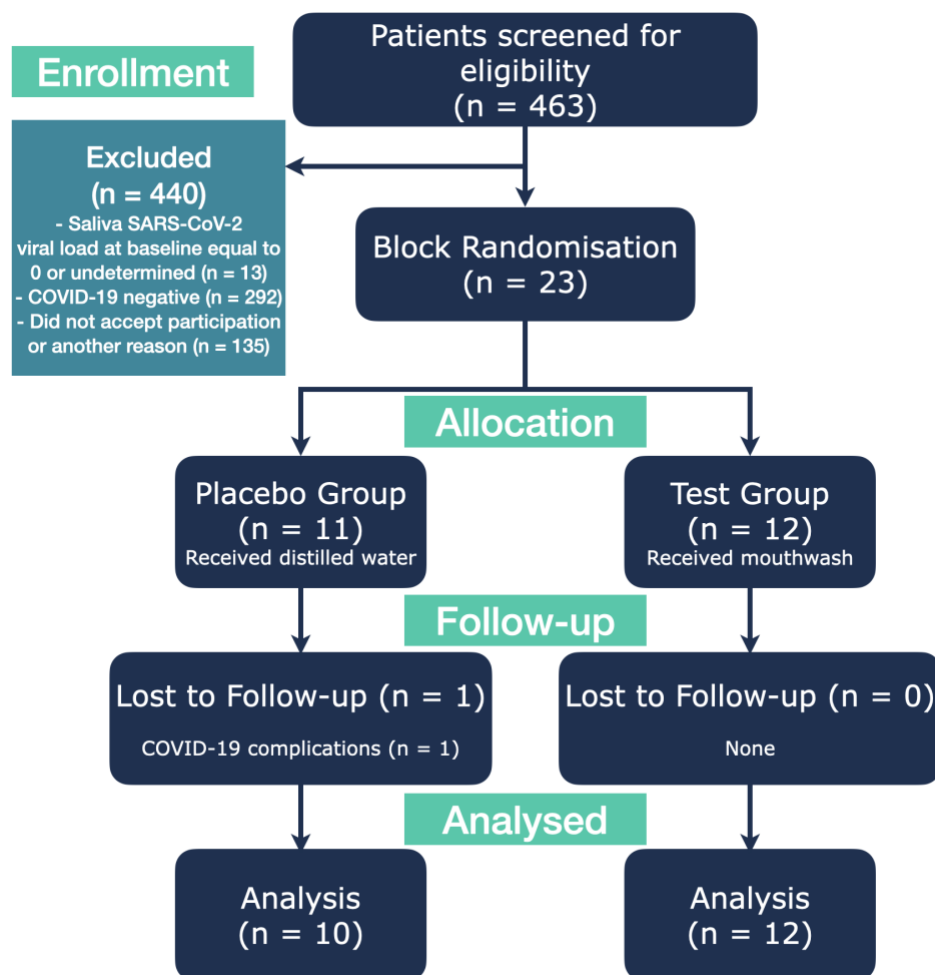


Figure 1. Study flow chart.

Statistical analysis

Data were statistically analysed non-parametrically using the Mann-Whitney-Wilcoxon and Fisher tests. Being an exploratory RCT study, level of significance was established with alfa equal to 0.1 for virologic assessment.

RESULTS

A total of 463 patients suspicious for Covid-19 were screened for eligibility and 23 included as depicts Figure 1. One patient from the Placebo group was lost and a final sample size of twenty-two patients was analysed (Figure 1).

Table 1 depicted the demographics and the similarity of placebo and control groups in which not differences among important clinical and virologic variables were not determined. All enrolled subjects were symptomatic.

Viral load was observed to vary greatly at baseline between patients, however, it was found to be distributed equally between the two groups, and it was not related with the age or symptoms of the patients. The study was completed by all patients, except for one from the placebo group (PG) who was hospitalised on day four due to the worsening of COVID-19 symptoms. Figure 2 shows the individual viral load change *versus* baseline in both groups of patients.

The study was completed by all patients, except for one from the placebo group (PG) who was hospitalised on day four due to the worsening of COVID-19 symptoms. Figure 2 shows the individual viral load change *versus* baseline in both groups of patients.

Statistically significant differences were found in the mean change of viral load at 15 minutes *versus* baseline (p=0.093). The number of patients who had a decrease in viral load was significantly greater in the group that used

Table 1. Characteristics of included COVID – 19 positive patients.

| Variables | Placebo Group (n=10) | Test Group (n=12) | p-value* |
|---|----------------------|-------------------|----------|
| Males (n) | 4 (40) | 4 (33.33) | > 0.999 |
| Females (n) | 6 (60) | 8 (66.67) | > 0.999 |
| Males age (years) | 51 ± 18.7 | 38.8 ± 15 | 0.486 |
| Female age (years) | 32.7 ± 10.5 | 46.8 ± 23.7 | 0.357 |
| All age (years) | 40 ± 16.4 | 44.1 ± 20.9 | 0.833 |
| Previous contact with a positive case (n) | 6 (60) | 3 (25) | 0.192 |
| Symptomatic (n) | 10 (100) | 12 (100) | > 0.999 |
| Onset of symptoms until nasopharyngeal swab sample (days) | 3 ± 1.8 | 4.08 ± 2.4 | 0.293 |
| Onset of symptoms until saliva sample (days) | 4.70 ± 1.7 | 6.17 ± 2.3 | 0.169 |
| Baseline SARS-CoV-2 viral load in nasopharyngeal swab Log10 (copies/µl) | 5.83 ± 0.8 | 5.97 ± 1.2 | 0.923 |
| Baseline SARS-CoV-2 viral load in saliva Log10 (copies/µl) | 3.17 ± 0.9 | 3.73 ± 1.0 | 0.346 |

Quantitative values are reported as mean ± standard deviation and dichotomic values as number (percentage). Placebo Group received distilled water. Test Group received mouthwash containing Cetylpyridinium Chloride (CPC) 0.05% plus Chlorhexidine Gluconate (CHX) 0.12%.

* p-values < 0.05 were considered significant.

Mann-Whitney test and Fisher’s exact test.

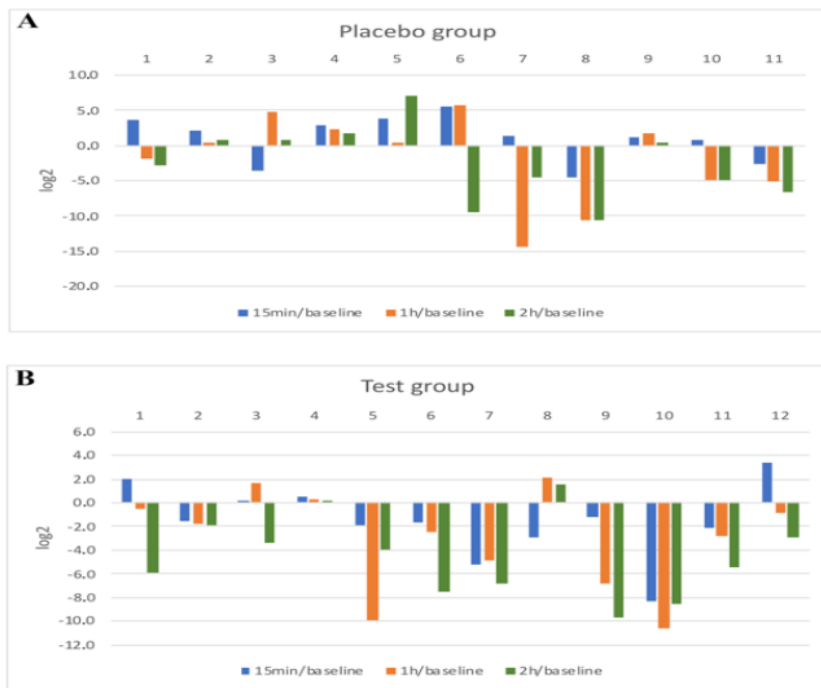


Figure 2. Relative proportions of individual viral load versus baseline in both groups of patients (log₂ transformed). A) Placebo group and B) test groups. Blue bars: 15 minutes/baseline, orange bars: 1 hour/baseline; Green bars: 2 hours/ baseline.

the mouthwash (MG) than in PG (8/12 vs 3/11, respectively) ($p=0.099$). Moreover, six patients in PG had an increase in viral load of up to two orders of magnitude. Besides, an important difference between responders for 2 hours post-rinsing was observed, but this did not reach a statistically significant value, due to the high viral load variability in the PG. After 5 days of using the study rinses twice daily, statistically significant differences were seen in the mean viral load, which was also lower in the MG versus PG ($p=0.025$). At that time point, SARS-CoV-2 virus was detected in the saliva of a single patient from the MG, while five PG patients showed salivary viral load. No safety concerns were reported regarding the products used.

DISCUSSION

In this randomised, double-blind, placebo-controlled clinical trial we have studied the reduction of viral load in the saliva of COVID-19-positive patients. The results (Figure 2) show that there is a different behaviour between the placebo group (PG) and the test group (MG). Although the salivary viral load at baseline varied greatly among the patients, probably due to the fact that they were in different stages of the disease, MG showed a decrease in viral load in most of the points sampled, and statistically significant differences were reached between both groups of patients at 15 minutes ($p = 0.093$) and 5 days ($p = 0.025$).

Furthermore, in most of the patients who received the mouthwash, there was viral load reduction of 2-3-fold log₂ with respect to baseline. That is equivalent to a reduction of more than 99% of virus. It should be noted that a large increase in viral load was observed in several PG patients after rinsing. This could be due to the release of viruses adhering to the epithelium of the oral mucosa and of the pharynx, and to the stimulation of saliva production. These events may be induced by the mechanical effect of rinsing and gargling. This viral load increment was not observed in the MG, possibly due to the expected viricidal activity and the substantivity of CPC.¹³⁻¹⁷

Before the start of the SARS-CoV-2 pandemic, there was evidence that the CPC molecule could inactivate lipid-enveloped viruses, including Coronaviruses. An initial study determined that the EC₅₀ for different strains of influenza virus was between approximately 10 μM and 25 μM. Through electron microscopy, the authors showed that CPC destabilised the envelope of this virus and that the use of a 0.1% CPC spray decreased morbidity and mortality in mice infected with Influenza virus.¹³ Another study analysed the antiviral capacity of a wide range of compounds on 4 different types of coronavirus, including MERS. Within a subgroup formed by 36 molecules with the highest activity, CPC ranked ninth, demonstrating a potent anti-coronavirus activity, with EC₅₀ values of

between 0.6 and 7.6 μM .¹⁴ Moreover, in a randomised, double-blind, placebo-controlled pilot study, a 0.1% CPC oral spray was used. The objective was to determine the possible preventive effect of CPC on upper airway infections caused by viruses (Influenza virus, respiratory syncytial virus, metapneumovirus, rhinovirus and adenovirus). The comparison between the placebo and experimental groups did not allow for finding significant differences in the ability to prevent upper respiratory infections, however, significant differences were observed in relation to the severity and duration of cough and sore throat.¹⁵

Recently, *in vitro* studies have shown that CPC has anti-SARS-CoV-2 activity, and different authors have described that oral hygiene products formulated with said antiseptic are capable of reducing the infectivity of the virus by between 3 and 4 orders of magnitude. High-throughput screening carried out with more than 5,600 compounds confirmed that CPC was among the six best compounds that inhibited the SARS-CoV-2 cytopathic effect, with EC_{50} values similar to those described by Shen et al, 2019 for MERS.¹⁶ Komine et al, 2021¹⁷ studied the ability of 10 commercial oral hygiene product formulations to decrease the infectivity of SARS-CoV-2.

All the products (sprays, toothpastes and mouthwashes) that contained CPC decreased the infectivity of SARS-CoV-2 by more than 4 orders of magnitude (more than 99.9%), however, that formulated with only CHX decreased the viral activity by 0.2 orders. The results obtained with products containing CHX are contradictory; some studies show that it has a low activity against SARS-CoV-2,¹⁸⁻¹⁹⁻²⁰ while others indicate that it can inhibit up to 99% of viral activity.²¹ These studies have also analysed the ability of hydrogen peroxide and PVI to inactivate SARS-CoV-2. They show that PVI inactivates SARS-CoV-2, decreasing its infectivity by approximately 4 orders of magnitude.¹⁸⁻²²⁻²³ However, in the case of hydrogen peroxide, the decrease in infectivity reached values close to 0.5-fold \log_{10} .¹⁸⁻²³ In *in vivo* studies, this molecule also did not cause a significant reduction in viral load in saliva.²⁴

Few *in vivo* studies have been published demonstrating the behaviour of mouthwashes against SARS-CoV-2 in saliva. All of them have been carried out with a low number of patients. Martínez-Lamas et al, 2020²⁵ observed a significant decrease of the virus in the saliva of 4 patients who used a 1% PVI rinse. Seneviratne et al, evaluated mouthwashes with CHX (0.2%), PVI (0.5%) and CPC (0.75%). Both the CPC and PVI rinses caused a reduction in viral load that was maintained for up to 6 hours after use of the products.²⁶

In summary, the different articles published so far show that some molecules present in mouthwashes are capable of inactivating the SARS-CoV-2 virus. CPC and PVI are those that have consistently shown the highest antiviral activity, both *in vitro* and *in vivo*.

The main limitations of our study are the low number of patients and the heterogeneity of baseline viral load values in saliva (probably due to the fact that the patients were in different disease stages). Furthermore, we should also mention that qRT-PCR is probably not the best tool for determining the inactivation of SARS-CoV-2 in saliva. Different studies indicate that the detection of the genetic material of the virus does not necessarily indicate the presence of viable infectious virions,²⁷⁻²⁸ a fact also described for SARS-CoV-1.²⁹ Considering that CPC would break the lipid envelope of SARS-CoV-2, it is likely that the qPCR technique would detect the viral RNA corresponding to viruses/virions that are not active.

Moreover, it has also been reported that the binding of the genomic RNA of the virus to the N protein would make the virus more stable and allow it to remain in the environment for longer.³⁰ Despite the limitations described, we have been able to observe a different behaviour of the viral load in saliva between the patients who received the mouthwash and those who received the placebo. In addition to this, this study has an important added value in terms of the information obtained so that we can design a more robust study in the future. This means that it is essential to enrol a greater number of patients, whose symptoms should not have been present for more than 4 days at the time of disease onset and possibly correlates within a high viral load at saliva and nasopharyngeal secretion. And finally, it is necessary to consider the application of a technique for detecting viral load in saliva that is more in line with the mechanism of action of CPC.

This RCP demonstrated that a CPC mouthwash can reduce the viral load in saliva of COVID-19-positive patients. Although over the course of the current pandemic the health authorities have not emphasised enough at oral hygiene as a preventive, mitigation and control measure, it is completely reasonable to think that controlling the viral load in saliva of infected individuals (symptomatic or asymptomatic) could be a fundamental strategy in reducing infection transmission and diminishing the complication of the already infected patients.

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CONFLICT OF INTEREST

Authors from Universidad del Valle and from ESE Centro do not have any interest conflict to declare however, the authors associated to Dentaïd Research Centre worked within the company that provided the mouthwashes used in this study.

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