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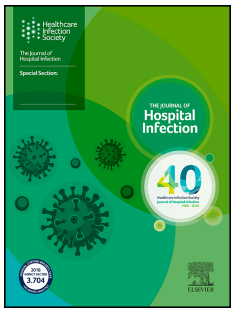

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Comparison of the *in vitro*-efficacy of different mouthwash solutions targeting SARS-CoV-2 based on the European Standard EN 14476

Katrin Steinhauer[1,2*], Toni Luise Meister [3], Daniel Todt [3,4], Adalbert Krawczyk [5,6], Lars Paßvogel[1], Britta Becker[7], Dajana Paulmann[7], Birte Bischoff[7], Stephanie Pfaender[3], Florian H H Brill[7] and Eike Steinmann[3]

[1]Department Research & Scientific Services, Schülke & Mayr GmbH, Norderstedt, Germany

[2]Faculty of Mechanical Engineering, Kiel University of Applied Sciences, Kiel, Germany

[3]Department of Molecular and Medical Virology, Ruhr University Bochum, 44801 Bochum, Germany

[4]European Virus Bioinformatics Center (EVBC), 07743 Jena, Germany

[5]Department of Infectious Diseases, West German Centre of Infectious Diseases, University Hospital Essen, University of Duisburg-Essen, Essen, Germany

[6]Institute for Virology, University Hospital Essen, University of Duisburg-Essen, Essen, Germany

[7]Dr. Brill + Partner GmbH Institut for Hygiene and Microbiology, Hamburg, Germany

Running title: *in vitro*-efficacy of mouthwash solutions targeting SARS-CoV-2 based on EN 14476

***For correspondence:**

Katrin Steinhauer

Schülke & Mayr GmbH

Research and Development

22840 Norderstedt

Germany

Tel: +49 40 52100557

Fax: +49 40 52100577

e-mail: katrin.steinhauer@schuelke-mayr.com

Abstract

The outbreak of the SARS-Cov-2 pandemic is triggering a global health emergency alert. Until vaccination becomes available, a bundle of effective preventive measures is desperately needed. Recent research is indicating the relevance of aerosols in the spread of SARS-CoV-2. Thus, in this study commercially available antiseptic mouthwashes based on the actives chlorhexidine digluconate (CHX) and octenidine dihydrochloride (OCT) were investigated regarding their efficacy against SARS-CoV-2 using the European Standard 14476. Based on the requirement of EN 14476 in which reduction of at least four decimal logarithms (log_{10}) of viral titer is requested to state efficacy, the OCT-based formulation was found to be effective within a contact time of only 15 sec against SARS-CoV-2. Based on this *in vitro*-data the OCT-mouthwash thus constitutes an interesting candidate for future clinical studies to prove its effectiveness in a potential prevention of SARS-CoV-2 transmission by aerosols.

Introduction

Coronaviruses are enveloped single-stranded RNA viruses and are characterized by club shaped spikes on the surface of the virion, prompting the name coronavirus due to the similarity in appearance to a solar corona [1]. Until the SARS-CoV outbreak in 2002, coronaviruses were thought to only cause mild self-limiting infections in humans but were known to cause a wide variety of infections in animals [1]. 17 years later, in December 2019, a novel coronavirus was identified as the causative agent of severe pneumonia in a cluster of patients [2], designated as SARS-CoV-2 due to its relatedness to severe acute respiratory syndrome coronavirus (SARS-CoV) [3]. Since then SARS-CoV-2 spread around the world thereby triggering a global health emergency alert. Thus, until vaccination becomes available a bundle of effective preventive measures is desperately needed.

In this context, recent publications suggest the use of antimicrobial mouthwashes as a preventive measure. This is based on the efficacy of antimicrobial mouthwashes to reduce the number of microorganisms in the oral cavity prompting a reduction of microorganisms in aerosols [4]. This is particularly interesting, as recent research indicates the relevance of aerosols also in the spread of SARS CoV-2 [5].

Thus, in their review summarizing data for mouthwashes with chlorhexidine digluconate (CHX), cetylpyridinium chloride (CPC), povidone-iodine (PVP-I), and hydrogen peroxide (H_2O_2) Vergara-Beunaventura and Castro-Ruiz indicate an essential role of antiseptic mouthwashes to reduce SARS-CoV-2 viral load in dental practice. They undermine that research on this topic is urgently needed to verify the potential of antiseptic mouth rinses as a further preventive measure [6]. The aim of

our study was therefore, to directly compare commercially available antiseptic mouthwash formulations. The mouthwash formulations were based on the common antiseptic actives chlorhexidine digluconate (CHX) and octenidine dihydrochloride (OCT) and were investigated regarding their efficacy against the pandemic coronavirus SARS-CoV-2.

Material and Methods

Quantitative Suspension tests according to EN 14476

Quantitative suspensions tests were carried out as described in EN 14476 [7]. Briefly, efficacy against SARS CoV-2 [8] was studied using commercially available mouthwashes. A commercially available ready-to-use formulation designated formulation A (trade name: chlorhexamed fluid 0,1 %; 100 g contains: 0.1 g Chlorhexidine bis-(D-gluconate); GlaxoSmithKline Consumer Health GmbH & Co. KG, Germany) was used as one test formulation. In addition, a commercially available ready-to-use formulation designated formulation B (trade name: chlorhexamed forte alkoholfrei 0,2%; 100 g contains: 0.2 g Chlorhexidine bis-(D-gluconate); GlaxoSmithKline Consumer Health GmbH & Co. KG, Germany) was used. Formulation C used in this study was also a ready-to-use preparation (trade name: octenisept, (drug authorisation number: 32834.00.00) 100 g contains: 0.1 g octenidine dihydrochloride (CAS-number: 70775-75-6), 2 g phenoxyethanol; drug authorisation number: 32834.00.00). Concentrations and contact times used throughout this study are indicated. In reality organic soiling in the oral cavity can be considered quite diverse. Thus, for comparative reasons the standardized protocol of EN 14476 [7] was chosen for this *in-vitro* study under conditions of low organic soiling (0.3 g/L bovine serum albumin (BSA); “clean conditions”) to give a first indication of the virucidal efficacy of the tested formulations against SARS-CoV-2.

Data presented are based on at least two independent experiments. Validation controls as defined in EN 14476 [7] were found to be effective in all experiments indicating validity of presented data.

Results and Discussion

Data is presented in Figure 1. Figure 1 A shows SARS-CoV-2 reduction obtained for formulations A, B and C using end point titration. In these experiments the two formulations based on CHX (formulations A and B) were found to have only limited efficacy against SARS-CoV-2. Thus, at a concentration of 80% (v/v) formulation A containing 0.1 % CHX reduced the virus titer even at a prolonged contact time of 10 min by less than 1 $\log_{10}$. Formulation B containing 0.2 % CHX reduced SARS-CoV-2 within a contact time of 1 min as well as at a prolonged contact time of 5 min when tested at 80% (v/v) concentration also by less than 1 $\log_{10}$. No additional large volume plating (LVP) experiments were conducted for formulations A and B. For these formulations cytotoxic effects of the formulation were found to have no impact, which is indicated by the lower limit of quantification (LLOQ). This is well in line with data from screening experiments in our lab, where virus reduction titers were found to be not elevated due to less toxicity when both formulations were tested at a concentration of only 20% (v/v) (data not shown).

In contrast, when looking at the data for formulation C logarithmic reduction factors $\log_{10}$ were found to be 1 $\log_{10}$ higher (i.e. ≥ 3.02 $\log_{10}$) for the 20% (v/v) concentration of product C compared to the 80% (v/v) test concentration (i.e. ≥ 2.02 $\log_{10}$). This indicates, that the measuring window for product was diminished by cytotoxicity. Therefore, additional large volume plating (LVP) experiments to obtain a wider measuring window were conducted with formulation C. Data obtained using LVP are presented in figure 1 B. LVP-data indicate a reduction of SARS-CoV-2 titers by ≥ 4.38 $\log_{10}$ already within the shortest contact time of 15 sec for the OCT based

mouthwash (formulation C). This was found for both concentrations tested (80% (v/v) and 20% (v/v)).

In their study on the stability of SARS Cov-2 at different environmental conditions Chin et al. [9] found no detectable virus when adding 15 µl viral solution (titre appr. 7-8 log unit of $TCID_{50}$ per mL) to 135 µl CHX solution (0,05%) after 5 min contact time. The detection limit for their experiments is stated to be 10^4 $TCID_{50}$/mL. Data with a lower limit of quantification would be desirable to assess the efficacy of the rather low concentration of CHX in the study of Chin et al. [9]. In our experiments having a lower limit of quantification we only found limited efficacy of even higher concentrations of CHX when using the standardized protocol of EN 14476.

Data presented in this study for the two CHX-based mouthwashes (formulations A and B) are well in line with data published by Meister et al. [10]. This is particularly true, as experiments conducted in our lab to directly compare the soiling conditions mimicking respiratory secretions used by Meister et al. [10] (i.e. 100 µl mucin type I-S, 25 µl BSA fraction V, and 35 µl yeast extract) with the clean conditions (i.e. 0.03% BSA) used in this study were found to give equivalent data for all three tested formulations (data not shown). Thus, in their investigation of different mouthwashes targeting SARS-CoV-2 Meister et al. [10] also found only a limited efficacy (i.e. < 1 log_{10}) of the two tested commercially available mouthwashes based on CHX . However, looking at the data for the OCT based mouthwashes, in the earlier study by Meister et. al. [10] only limited virucidal activity of the formulation tested (i.e. < 1 log_{10}) was found, whereas in this study the tested OCT based formulation (C) was found effective against SARS-CoV-2 within 15 sec (i.e. ≥ 4 log_{10}). This differing data

is likely to be explained by the use of two different OCT based formulations in the two studies. In the earlier study [10] a formulation containing OCT as the only active was used as compared to the OCT-based formulation (formulation C) used in this study which contained OCT in combination with phenoxyethanol (PE). Future experiments might help to elucidate the impact of the active phenoxyethanol in more detail, e.g. by direct comparison of formulations with and without OCT in the presence or absence of phenoxyethanol. In any case, this discrepancy indicates the value of pre-evaluating each individual formulation on the basis of EN 14476 when assessing the virucidal potential against SARS CoV-2. For this pre-evaluation the standard test surrogate virus modified vaccinia virus strain Ankara (MVA) to assess "virucidal activity against enveloped viruses" as defined in EN 14476 [7] has been found to be of value, as with this approach a non-pathogenic virus can be used in the lab to obtain reliable data regarding virucidal activity against enveloped viruses in general including SARS CoV-2.

In conclusion, in this *in vitro*-study virucidal efficacy against SARS-CoV-2 could be demonstrated for formulation C meeting the > 4 $\log_{10}$ requirement of EN 14476 [7] within a contact time of only 15 sec. This *in vitro*-data gives a good indication of the efficacy of the tested formulations using the standardized EN 14476 protocol in the presence of low organic soling. Clinical trial data will help to elucidate effectiveness against SARS CoV-2 under physiological conditions as organic soiling in the oral cavity can be considered more diverse in the field.

Thus, based on this *in vitro*-data the OCT-based commercially available formulation used in this study constitutes an interesting candidate for future clinical studies to prove its effectiveness in a potential prevention of SARS-CoV-2 as a mouthwash.

Clinical data aims to give use recommendations and will also help to elucidate practical use of the mouthwash (clinical environment and/or general prophylaxis).

Conflict of interest

The authors KS and LP are employees of Schülke & Mayr GmbH, Norderstedt, Germany.

Funding source

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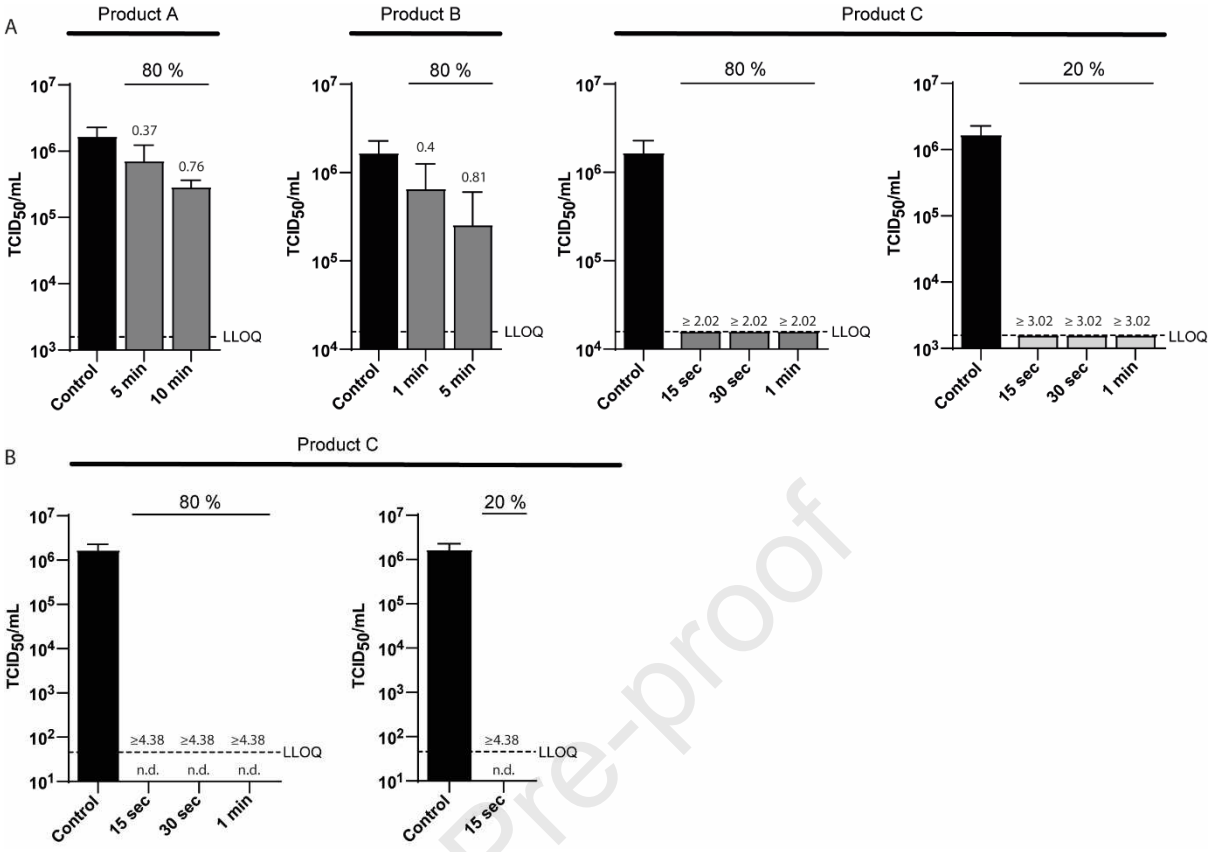

Figure 1

Legend Figure 1

Figure 1: Virucidal activity of oral rinses against SARS-CoV-2. SARS-CoV-2 was incubated with medium (control, black bar) or various oral rinses (Product A-C) for indicated concentrations (80 % and/or 20 %) and time periods (15 sec to 10 min). The cytotoxic effect was monitored using non-infected cells incubated with the different products, defined as lower limit of quantification (LLOQ). Log-reduction factors are indicated above the bars. In panal A viral titers were determined upon limited endpoint titration on Vero E6 cells. Tissue culture infectious dose 50 (TCID50/mL) was calculated according to Spearman-Kärber. Due to high cytotoxic effects diminishing the measuring window for product C large volume plating was performed to reduce cytotoxicity and evaluate the remaining titers below 10^4 (panel B). No remaining cytopathic effects were observed (n.d.). Data is reported as mean values with standard deviation from at least two independent experiments. Experiments were carried out according to EN 14476 under clean conditions.