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SHORT COMMUNICATION

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Inactivation of SARS-CoV-2 and HCoV-229E *in vitro* by ColdZyme® a medical device mouth spray against the common cold

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Abstract

Background: The coronavirus disease 2019 (COVID-19) pandemic calls for effective and safe treatments. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causing COVID-19 actively replicates in the throat, unlike SARS-CoV, and shows high pharyngeal viral shedding even in patients with mild symptoms of the disease. HCoV-229E is one of four coronaviruses causing the common cold. In this study, the efficacy of ColdZyme® (CZ-MD), a medical device mouth spray, was tested against SARS-CoV-2 and HCoV-229E *in vitro*. The CZ-MD provides a protective glycerol barrier containing cod trypsin as an ancillary component. Combined, these ingredients can inactivate common cold viruses in the throat and mouth. The CZ-MD is believed to act on the viral surface proteins that would perturb their entry pathway into cells. The efficacy and safety of the CZ-MD have been demonstrated in clinical trials on the common cold.

Method of Study: The ability of the CZ-MD to inactivate SARS-CoV-2 and HCoV-229E was tested using an *in vitro* virucidal suspension test (ASTM E1052).

Results: CZ-MD inactivated SARS-CoV-2 by 98.3% and HCoV-229E by 99.9%.

Conclusion: CZ-MD mouth spray can inactivate the respiratory coronaviruses SARS-CoV-2 and HCoV-229E *in vitro*. Although the *in vitro* results presented cannot be directly translated into clinical efficacy, the study indicates that CZ-MD might offer a protective barrier against SARS-CoV-2 and a decreased risk of COVID-19 transmission.

KEYWORDS

cell cultures, coronavirus, microbial cultures, respiratory tract

1 | INTRODUCTION

The coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has resulted in a global health crisis.[1] COVID-19 starts as an infection of the respiratory tract and active viral replication of SARS-CoV-2 in the throat has recently been confirmed.[2] Coronaviruses are divided into four subgroups where SARS-CoV-2 as well as SARS-CoV and Middle East respiratory syndrome-related coronavirus (MERS-CoV) are beta coronaviruses.[3] The alpha coronaviruses human coronavirus-229E (HCoV-229E) and

HCoV-NL63 and the beta coronaviruses HCoV-OC43 and HCoV-HKU1 are believed to cause about one-third of the common cold cases.[4] Treatments and vaccines against human coronavirus infections are lacking.[3]

ColdZyme® (CZ-MD) is a commercially available CE marked Class III medical device mouth spray against the common cold. It contains glycerol and minor amounts of purified cold-adapted trypsin[5] from Atlantic cod (*Gadus morhua*). CZ-MD creates a physical protective barrier on the mucus membrane (ClinicalTrials.gov Identifier: NCT03901846) against common cold viruses in the throat where SARS-CoV-2[2,6] and HCoV-229E[7] replicate. Viral particles become trapped in the barrier where they are subsequently inactivated.[8] Cod trypsin, an ancillary component within the barrier, aids in the viral inactivation process. The CZ-MD is believed to act on the viral surface proteins that would perturb their entry pathway into cells.[8] Clinical trials demonstrate the safety and efficacy of CZ-MD against the common cold.[9,10] Also, *in vitro* studies have shown inactivation of respiratory viruses such as rhinovirus (HRV), respiratory syncytial virus (RSV), and influenza by the CZ-MD.[8]

The entry of coronaviruses into host cells is mediated by the spike (S) glycoprotein that forms homotrimers protruding from the virus surface.[11] The S protein is frequently cleaved at the boundary between two functional subunits termed S_1 and S_2.[3] The S_1 subunit comprises the receptor-binding domain and contributes to the stabilization of the prefusion state and the S_2 subunit contains the fusion machinery.[3] SARS-CoV-2 and SARS-CoV use the ACE2 human cell receptor for cell entry.[12] The S protein is cleaved by host cell proteases such as furin, cathepsin, and transmembrane serine protease TMPRSS2.[3] Cleavage at the S_2' site is located upstream of the fusion peptide. This supposedly activates the S protein for membrane fusion involving irreversible conformational changes.[3] Therefore, entry of coronavirus into susceptible cells is a complex process that requires the concerted action of the S protein, receptor-binding, and proteolytic processing by host cell proteases to promote virus-cell fusion.[3]

In contrast to SARS-CoV, active SARS-CoV-2 virus replication in the upper respiratory tract has been demonstrated.[2] Based on the article, SARS-CoV-2 virus shedding in the throat was shown to be high during the first week of symptoms. The presence of a polybasic furin-type cleavage site at the S_1–S_2 junction in the SARS-CoV-2 spike protein, not present in SARS-CoV, could explain the extension of tissue tropism of SARS-CoV-2 to the throat.[2]

Here we present research demonstrating the *in vitro* efficacy of a medical device mouth spray (CZ-MD) against SARS-CoV-2 and HCoV-229E. The results indicate that the CZ-MD may be active against a variety of coronaviruses *in vivo*.

2 | MATERIALS AND METHODS

2.1 | CZ-MD mouth spray

CZ-MD solution contained glycerol, water, cod trypsin, ethanol, calcium chloride, Tris, and menthol. Two lots were evaluated.

2.2 | Laboratory

Testing according to the ASTM International E1052-11 method, "Standard Test Method to Assess the Activity of Microbicides against Viruses in Suspension" was carried out by an independent testing laboratory under good laboratory practice conditions; Microbac Laboratories Inc., 105 Carpenter Drive, Sterling, VA.

2.3 | Cells and virus strains

Challenge viruses: SARS-CoV-2, strain USA-WA1/2020, Source: BEI Resources NR-52281, containing 5% fetal bovine serum (FBS) and HCoV, strain 229E, ATCC VR-740, without serum.

Host cells and culture media used: Vero E6 cells ATCC CRL-1586 (for SARS-CoV-2) in minimum essential medium (MEM) + 10% FBS and MRC-5 cells ATCC CCL-171 (for HCoV-229E) in MEM + 20% FBS.

2.4 | Viral inactivation test

Two lots of CZ-MD were evaluated against a challenge virus in suspension. For each run, a 1.2-ml aliquot of each lot of CZ-MD was mixed with 1.5 ml of buffer and 0.3 ml of the challenge virus suspension (each virus was tested independently) and mixed thoroughly by vortexing. The buffer used in the test for HCoV-229E was phosphate buffer (1X PB) without sodium chloride, pH 7.5 and for SARS-CoV-2 was 20 mM Tris, 1 mM $CaCl_2$, pH 8.2. The buffers were preheated at 36°C for SARS-CoV-2 and 37°C for HCoV-229E. The reaction mixtures were incubated at 36°C for SARS-CoV-2 and 37°C for HCoV-229E for 20 min. An aliquot or the entirety of the reaction mixture was immediately mixed with an equal volume of neutralizer (SARS-2: MEM + 10% newborn calf serum [NCS] and HCoV-229E: MEM + 10% FBS) after incubation. To assay for infectious virus, the quenched sample was serially diluted with medium (SARS-2: MEM + 2% NCS and HCoV-229E: MEM + 2% FBS) in 10-fold increments and inoculated onto host cells. Inoculated plates were incubated at 36 ± 2°C in 5 ± 3% CO_2 for 9 days for SARS-CoV-2 and 33 ± 2°C in 5 ± 3% CO_2 for 6 days for HCoV-229E. The cultures were scored for viral infection by determining the viral-induced cytopathic effect (CPE) after incubation.

By adding the viral titer ($\log_{10}$TCID50/ml) to the $\log_{10}$ (the volume of the reaction mixture in ml times the volume correction) the viral load ($\log_{10}$TCID50) was calculated. The volume correction accounted for the neutralization of the sample postcontact time. The virus units ($\log_{10}$ TCID50) recovered after incubating the virus in the medium before inoculation (virus recovery control, see Section 2.5) represent the input load. The virus units ($\log_{10}$TCID50) recovered after mixing and incubating the virus in presence of CZ-MD represent the output load. To calculate the $\log_{10}$ reduction factor the output viral load ($\log_{10}$) was subtracted from the input viral load ($\log_{10}$). The percent inactivation was calculated using the formula $\left(1\text{-}(1/10^{\log \text{red}})\right) \times 100\%$ where log reduction (log red) is the $\log_{10}$ reduction factor. The tests were done in duplicate for each CZ-MD lot and in duplicate for the viral recovery control.

The Spearman–Karber formula or Poisson distribution when no virus was detected was used to calculate the titer of the virus (log_{10}T-CID50/ml).

2.5 | Controls

The controls included were as previously described.[8] The buffers described in the viral inactivation test were used in the controls where buffer was used, HCoV-229E test: 1X PB without sodium chloride, pH 7.5 and SARS-CoV-2 test: 20 mM Tris, 1 mM $CaCl_2$, pH 8.2. The controls were done at the same time as the test samples.

3 | RESULTS

The virus inactivating ability of the CZ-MD solution against SARS-CoV-2 and HCoV-229E was determined as described under Section 2 (Table 1).

The 50% tissue culture infectious dose (TCID50) endpoint assay was used to titrate samples from each incubation using the appropriate host cell system for each virus, see Section 2. The tests were done in duplicate for each CZ-MD lot and in duplicate for the viral recovery control where the mean is reported for the results (Table 1).

The results show that CZ-MD inactivated both viruses with a log_{10} reduction of 1.76 or 98.3% inactivation of SARS-CoV-2 and with a log_{10} reduction of 2.88 or 99.9% inactivation of HCoV-229E (Table 1).

All the controls met the criteria for a valid test. There was no cytotoxicity detected at any dilution or cell line tested. In the cell viability control wells, virus was not detected, the cells remained viable and the media was sterile. Enough virus was recovered for the virus recovery control and the appropriate titer was used in the experiment based on the viral stock titer control (6.30 log_{10}TCID50/ml for SARS-CoV-2 and 6.20 log_{10}TCID50/ml for HCoV-229E) for each assay. Viral-induced CPE was distinguishable from uninfected cells. For the reference product control 2000 ppm NaOCl was used as a test substance which showed a log reduction of ≥4.45 for SARS-CoV-2 and ≥4.24 for HCoV-229E (Table 1). For the neutralizer effectiveness/viral interference control, virus was detected in all wells.

4 | DISCUSSION

Based on the results presented in this study, CZ-MD was found to inactivate SARS-CoV-2 (98.3%) and HCoV-229E (99.9%) *in vitro* (Table 1). The incubation time was based on results from a clinical study on the duration of the CZ-MD barrier in the mouth and throat (ClinicalTrials.gov Identifier: NCT03901846). No cytotoxicity was observed for CZ-MD at the dilutions tested.

The CZ-MD forms a physical barrier in the oral cavity against common cold viruses. It contains glycerol and a minor amount of cod trypsin that combined form a protective barrier which reduces the ability of the viruses to infect. SARS-CoV-2 and HCoV-229E belong to different coronavirus subgroups indicating that the CZ-MD can be effective against a variety of coronaviruses.[3,4] The findings are in line with previous *in vitro* studies on CZ-MD showing inactivation of common cold viruses such as HRV, RSV, and influenza.[8]

There is a lack of treatment options against coronaviruses infecting humans such as SARS-CoV-2, HCoV-229E, and other common cold coronaviruses. Coronaviruses cause about one-third of the common cold cases.[4] The efficacy and safety of CZ-MD against the common cold have been demonstrated in clinical trials.[9,10] Furthermore, active SARS-CoV-2 replication in the upper respiratory tract was demonstrated in patients suffering from COVID-19 with high virus shedding.[2] The severity of COVID-19 has been linked to a high oral load of the SARS-CoV-2 virus.[6] Therefore, reduction in the oral viral load might be associated with milder symptoms. Also, the reduced viral load would lead to lower viral shedding with less risk of transmission. This knowledge and the *in vitro* efficacy of CZ-MD against SARS-CoV-2 and HCoV-229E support the use of the CZ-MD barrier for protection against SARS-CoV-2 causing the COVID-19 pandemic.

The difference in efficacy of CZ-MD against SARS-CoV-2 and HCoV-229E could be partly explained by the presence of 5% serum in the viral stock of SARS-CoV-2 compared to no serum in the viral stock of HCoV-229E. The serum is a complex mixture containing protease inhibitors and other factors that may affect the efficacy of CZ-MD against SARS-CoV-2. Another explanation for the difference might be the accessibility of trypsin specific sites in their S proteins. The spike protein S is responsible for the entry of coronaviruses into host cells.[3]

TABLE 1 Inactivation of SARS-CoV-2 and HCoV-229E by ColdZyme® (CZ-MD)

Virus	Sample	Input load log_{10} TCID50 (mean)[a]	Output load log_{10} TCID50 (mean ± *SD*)	log_{10} Reduction (mean)	Percent inactivation
SARS-CoV-2, strain USA-WA1/2020, BEI Resources NR-52281	CZ-MD	6.06	4.30 ± 0.21	1.76	98.3
	Reference agent (2000 ppm NaOCl)		≤ 1.61	≥4.45	
Human coronavirus (HCoV), strain 229E, ATCC VR-740	CZ-MD	5.55	2.67 ± 0.13	2.88	99.9
	Reference agent (2000 ppm NaOCl)		≤1.31	≥4.24	

Abbreviation: SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; TCID50, 50% tissue culture infectious dose.

[a]Mean of two experimental values.

Trypsin cleaves at arginine and lysine amino acid residues within proteins.[5] There are over 100 lysine and arginine residues present within SARS-CoV-2 S protein (GenBank QHD43416.1) and about 80 such residues in HCoV-229E S protein (GenBank ABB90529.1). These trypsin specific sites within the S proteins of SARS-CoV-2 and HCoV-229E may not be readily accessible due to a glycan shield and some of the lysine or arginine residues may be buried within the S protein.[13] On the other hand, based on the high number of potential trypsin sites in the S protein and the barrier function of CZ-MD, mutations in the S protein in coronaviruses infecting humans are unlikely to result in resistance to CZ-MD.

The entry of coronaviruses into susceptible cells requires receptor-binding of the S protein and its proteolytic processing by host cell proteases that occurs in a concerted action to promote virus-cell fusion.[3] Externally added trypsin is sometimes used in *in vitro* studies as a surrogate for more biologically relevant host cell proteases as these proteases have trypsin-like substrate specificity.[14,15] However, the *in vitro* study presented here clearly demonstrates that the CZ-MD (containing cod trypsin) inactivates SARS-CoV-2 and HCoV-229E. In addition, the *in vivo* topical application of CZ-MD as a mouth spray limits the activity of cod trypsin to the surface of the oral and throat mucous layer. The oral and throat mucus protects epithelial surfaces by trapping pathogens and foreign particles.[16] The activity of endogenous proteases and external proteases, such as those found in food, are under strict control to prevent unintended proteolysis at the cellular level.[16,17] Mucus membranes play a vital role in this anti-proteolytic process by preventing the penetration of negatively charged proteins, such as cod trypsin, through its layers of glycosylated proteins containing protease inhibitors.[16]

Although the *in vitro* results presented cannot be directly translated into clinical efficacy, the study indicates that CZ-MD might offer a protective barrier against coronaviruses such as SARS-CoV-2 and a decreased risk of COVID-19 transmission.

CONFLICT OF INTERESTS

Reynir Scheving and Bjarki Stefansson are employed at Zymetech. Ágústa Gudmundsdottir is Professor emeritus at the University of Iceland and partially employed at Zymetech. Fredrik Lindberg was employed at Enzymatica AB.

AUTHOR CONTRIBUTIONS

Bjarki Stefansson and Ágústa Gudmundsdottir designed the experiments. Bjarki Stefansson and Ágústa Gudmundsdottir wrote, reviewed, edited, and approved the manuscript. Reynir Scheving and Fredrik Lindberg reviewed, edited, and approved the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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