In-Vitro Evaluation of EcoProven Anti-Viral Mouthwash as a Virucidal Against SARS-COV-2 Virus (Report)

Description of Materials

- EcoProven Anti-Viral Mouthwash (100x concentration)
- SARS CoV 2 virus stock (WA-1/CDC isolate)
- Artificial Saliva (Nanochemazone, Artifical saliva 6.8 pH, 100ml, Batch # NCZ-048-20C, CAS No 7732-18-5)
- Plaque assays for detection of infectious virus

Description of Study:

Study Design

The overall goal of the study was to evaluate the efficacy of EcoProven Anti-Viral Mouthwash to reduce the viral load of SARS-CoV-2 in the oral cavity. To test this, the candidate oral rinse will be evaluated *in vitro* using artificial saliva spiked with SARS-CoV-2 and followed by plaque assay for the detection of infectious viral particles after exposure to the oral rinse.

Virus Strain(s) and propagation

All virus propagation occurred in a BSL-3 laboratory setting. Virus (isolate USA-WA1/2020) was amplified in Vero C1008 (Vero E6) cell culture. Vero E6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with glucose, L-glutamine, sodium pyruvate, 5% fetal bovine serum (FBS) and antibiotics. Vero E6 cells with SARS-CoV-2 were inoculated directly in DMEM containing 1% FBS. Following inoculation, the tedium was harvested from infected cells 3-4 days after inoculation, clarified by centrifugation, supplemented with FBS to 10% and frozen to -80°C in aliquots. The virus titer was determined using a standard double overlay plaque assay (see below).

Virus inactivation with oral rinse

Virus was mixed with artificial saliva at a viral titer of 10^4 pfu/ml or higher (1:1 ratio). Eco Proven oral rinse was diluted to 0.1x in sterile PBS and the the virus suspension was mixed with the oral rinse at final ratio of 1:10 (virus/ saliva: oral rinse) for 15, 30, 60 or 120 seconds. The reactions were then quenched by performing serial dilutions in phosphate-buffered saline (PBS) and plated on a plaque assay. Artificial saliva with no virus served as a negative control. Virus with no oral rinse exposure served as a positive control. Oral rinse was serially diluted and plated on a plaque assay to determine virotoxic effects of the oral rinse and to determine limit of detection. All plaque assays were performed in triplicate.

Virus Titration and Quantification

Plaque assays were used to quantify infectious virus in the artificial saliva suspension before and after exposure to the oral rinse. Briefly, all samples were serially diluted 10-fold in PBS. Confluent Vero E6 cell monolayers were grown in 6-well tissue culture plates. The growth media was removed from the cell monolayers and washed with PBS immediately prior to inoculation. Each well was inoculated with 0.1 mL of the appropriate diluted sample. The plates were then rocked every 0-15 minutes for 45 minutes and then overlaid with 0.5%agarose, in media with 7.5% bicarbonate and incubated for 1 day at 37°C, 5%CO_2. A second overlay with neutral red dye was added at 24 hours and plaques were counted at 48-72 hours post-plating. Viral titers are reported as the $\log_{10}$ pfu per mL. Samples are considered negative for infectious virus if viral titers reached the limit of detection (LOD). The theoretical limit of detection was calculated using the following equation:

$$LOD = \log [1/ (N \times V)]$$

where N is the number of replicates per sample at the lowest dilution tested; V is the volume used for viral enumeration (volume inoculated/well in mL).

A total of 16 Vero plates were used for the study. Samples were serially diluted in PBS then plated at 10^0 to 10^{-3} in triplicates.

Results

We examined the virucidal activity of EcoProven oral rinse against one strain of SARS-CoV-2 in vitro. A suspension of virus and artificial saliva was used to mimic oral cavity secretions. The virus suspension was exposed to the oral rinse at multiple time intervals followed by a plaque assay to determine infectious viral titer.

EcoProven oral rinse was first tested on cell culture to determine the level of cytotoxicity. The oral rinse was cytotoxic at 1x concentration. Following 2-fold dilutions in PBS, it was determined the oral rinse was no longer cytotoxic at a 0.1x dilution. Therefore, a stock of 0.1x oral rinse was prepared for the experiment.

The virus/saliva mixture (1:1) had a titer of 1.13 x 10^6 pfu/ml (see Appendix A) and no cytotoxicity was noted from the artificial saliva alone. Following exposure to the oral rinse, no infectious virus was detected,above the LOD, for all tested time points. There was a reduction of greater than 6 $\log_{10}$ pfu/ml of virus (**Figure 1**). No other oral rinse products were tested as a comparison in this study. In summary, EcoProven can efficiently inactivate SARS-CoV-2 *in vitro*, following a 15 second exposure time.

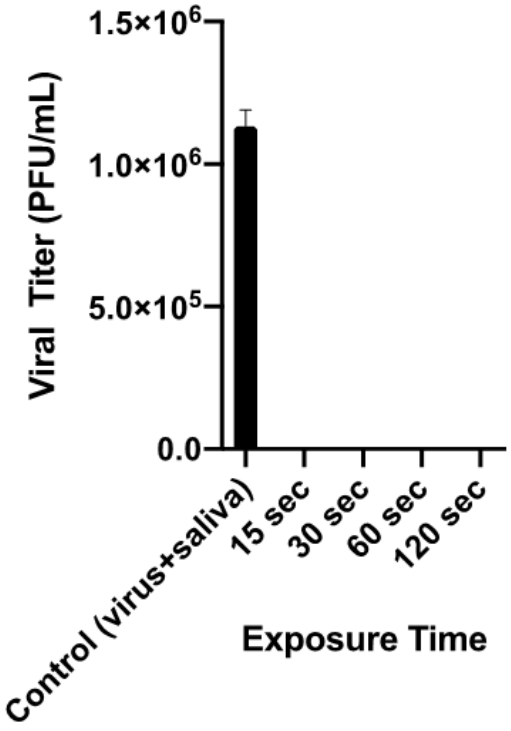

Figure 1. Evaluation of EcoProven Anti-Viral Mouthwash as a Virucidal against SARS-COV-2 In Vitro
The virus/saliva mixture was exposed to the oral rinse for 15, 30, 60 and 120 seconds then immediately tested by a plaque assay to determine viral titers. In addition, the virus titer of the virus/saliva mixture (1:1), without oral rinse exposure, was also determined.

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Appendix A

Treatment	Titer (pfu/ml)	Log10 Titer	Cytotoxicity?
EcoProven at 1x concentration	N/A	N/A	Yes
EcoProven at 0.1x concentration	N/A	N/A	No
Artificial saliva alone	N/A	N/A	No
Virus titer (stock virus)	2.20×10^6	6.3	No
Virus titer (1:1 of virus:saliva)	1.13×10^6	6.1	No
After 15 second exposure to EcoProven	0	<0.52	No
After 30 second exposure to EcoProven	0	<0.52	No
After 60 second exposure to EcoProven	0	<0.52	No
After 120 second exposure to EcoProven	0	<0.52	No
Saliva- negative control	0	<0.52	No

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