In Vitro Evaluation of Eco-Proven as a Virucidal Against the SARS-COV-2 Virus Delta Variant

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Description of Materials

- Eco-Proven (100% concentration)
- SARS-CoV-2 virus stock (WA1/CDC isolate)
- SARS CoV 2 virus stock (Delta isolate)
- Artificial Saliva (Nanochemazone, Artifical saliva 6.8 pH, 100ml, Batch # NCZ-048-20C, CAS No 7732-18-5)

Summary

In a time-kill solution format, 0.1% Eco-Proven reduced the viral titer of both the SARS-CoV-2 Delta variant and WA1/CDC strains by 99.93%, following a 15 second exposure to Eco-Proven.

Description of Study:

Study Design

The overall goal of the study was to evaluate the efficacy of Eco-Proven (EP) to reduce the viral load of the SARS-CoV-2 Delta variant in a time-kill solution format. Briefly, EP was incubated for 15 or 30 seconds, with a salt solution mimicking human saliva, containing either the Delta variant or the Washington-1 strain (WA1) of Sars-CoV-2. After incubation, the mixture was diluted with phosphate-buffered saline (PBS) and the virus was plated in a plaque assay to determine the number of infectious viral particles remaining.

Virus Strain(s) and propagation

All virus propagation occurred in a BSL-3 laboratory setting at Colorado State University (Fort Collins, CO). Viruses B.1.617.2 “delta” and isolate USA-WA1/2020 were 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. Inoculation of Vero E6 cells with SARS-CV-2 was carried out directly in DMEM containing 1% FBS. Medium harvested from infected cells 3-4 days after inoculation was 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 isolates were mixed with artificial saliva at a viral titer of 10^4 pfu/ml or higher and mixed with EP at a ratio of 1:1 (virus/saliva: oral rinse) for 15, and 30 seconds. The final EP concentration at which the virus/saliva mixture was exposed was 0.1% EP. The reactions were then quenched by performing serial dilutions in PBS and a plaque assay was performed. Artificial saliva with no virus served as a negative control and virus with no oral rinse exposure served as a positive control. All plaque assays were performed in duplicate.

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 and each well was inoculated with 0.1 mL of the appropriate diluted sample. The plates were then rocked every 10-15 minutes for 45 minutes and 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).

Results

We examined the virucidal activity of EP oral against the WA1 isolate as well as the emerging Delta variant. The virus suspension was then exposed to EP at two time intervals, followed by a plaque assay to determine infectious viral titer.

Based on a previous study, EP showed virucidal effects in as short as 15 seconds. Therefore, this study only evaluated 15 second and 30 second time points. The virus/saliva mixture was exposed to the oral rinse for 15 and 30 seconds and immediately tested by a plaque assay to determine viral titers. Additionally, the virus titers of both WA-1 and Delta were determined. Lastly, virus titers of the virus/saliva mixture without EP exposure was also determined. No cytotoxicity was noted from the artificial saliva or EP, alone, on cell culture monolayers.

The WA1 virus/saliva mixture had a titer of 5.15×10^4 pfu/ml and the Delta virus/saliva mixture had a titer of 3.40×10^4 pfu/ml (**Table 1**). The WA-1virus/saliva mixture yielded a reduced virus titer of 3.5×10^1 pfu/ml after 15 seconds and 1.5×10^1 pfu/ml after 30 seconds exposure to EP. The Delta virus/saliva mixture also yielded reduced titers of 2.5×10^1 pfu/ml after 15 seconds and 7.5×10^1 pfu/ml after 30 seconds. EP was able to reduce the viral titers approximately 3 $\log_{10}$ when diluted with the virus/saliva mixture to a final concentration of 0.1x (**Figure 1**).

In conclusion, EP was effective in reducing viral titers against the new Delta variant by 99.93%. This reduction in viral titer of the Delta variant is the same that was observed for the original WA1 strain of Sars-CoV-2 in this experiment and two previous experiments. It should be noted that although there was a slight increase in the viral titer of the Delta variant (+0.015%), following a 30 second treatment with EP, this is likely due to statistical variation and the small number of replicates.

Table 1.

Treatment	Titer (pfu/ml)	Log10 Titer	Cytotoxicity?
Artificial saliva only	N/A	N/A	no
Virus stock- WA1	1.15E+05	5.06	no
Virus stock- delta	5.25E+05	5.72	no
virus+ saliva (WA1)	5.15E+04	4.71	no
virus + saliva (delta)	3.40E+04	4.53	no
EP alone	N/A	N/A	no
EP+ WA1- After 15 sec	3.50E+01	1.54	no
EP+ WA1- After 30 sec	1.50E+01	1.18	no
EP+ delta- After 15 sec	2.50E+01	1.40	no
EP+ delta- After 30 sec	7.50E+01	1.88	no

Figure 1. Virucidal effects of EcoProven against WA-1 and Delta strains of SARS-CoV2