Research Article

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Persistent Virucidal Activity in an Alcohol-Based Sanitizer Formulation (ProtecTeaV) for Potential Use against Norovirus

Widjaja Nicole[1], Dickinson Douglas[2], Shao Xueling[1] and Hsu Stephen[1*]

[1]Department of Oral Biology, Dental College of Georgia, Augusta University, USA.

[2]Camellix, LLC, 4106 Colben Blvd, Suite 101, Evans, GA, USA.

*Correspondence:
Stephen Hsu, Ph.D, Professor, Department of Oral Biology, Dental College of Georgia, Augusta University, US, Tel: (706) 721-4816; E-mail: shsu@augusta.edu.

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ABSTRACT

Background: *Norovirus is a major cause of acute gastroenteritis. Alcohol sanitization is ineffective, and currently used alcohol-based hand sanitizers are not recommended by the CDC for norovirus in healthcare settings. This study evaluated virucidal activity and surface persistence of a novel alcohol-based hand sanitizer formulation, ProtecTeaV, containing lipophilic epigallocatechin-3-gallate (EGCG-p) against a human norovirus surrogate.*

Methods: *Virucidal capacity against feline calicivirus (FCV) was tested using a standard 50% Tissue Culture Infective Dose (TCID50) suspension assay. Persistence of residual virucidal activity after application on a clean surface was determined through 12 hours. Controls included the formulation without EGCG-p, popular alcohol-based sanitizers, and antibacterial liquid hand soap (LHS). Statistical analysis employed one-way ANOVA (alpha=0.05).*

Results: *Suspension assays demonstrated that the ProtecTeaV formulation effectively reduced FCV viral infectivity >log_{10} 4 (10,000 fold). Surface applied residue activity remained strong (reduction of infectivity by > log_{10} 3) through 12 hours. In comparison, LHS did not show virucidal activity without washing with water, and other controls failed to reduce infectivity by more than log_{10} 3 (1,000 fold).*

Conclusion: *This non-toxic hand sanitizer/surface disinfectant demonstrated effective and prolonged virucidal activities against a norovirus surrogate. Therefore, the EGCG-p formulation is potentially a novel and effective approach to curtail norovirus outbreaks.*

Keywords

Norovirus, Sanitizer, Hand hygine, EGCG.

Introduction

Norovirus is estimated to be the most common cause of acute gastroenteritis in the world, with 685 million cases each year. The combined cost of global healthcare and lost productivity associated with norovirus is estimated at $60 billion (US CDC, Norovirus Worldwide). In the United States, norovirus affects 19-21 million annually, resulting in 91,000 emergency room visits, 56,000-71,000 hospitalizations and 570-800 deaths (US CDC, U.S. Trends and Outbreaks). The total of annual health care and social costs for norovirus disease in the US has been estimated at $2 billion (US CDC, Burden of Norovirus Illness and Outbreaks). One complication for sanitization efforts to prevent norovirus transmission is that the virus is resistant to inactivation by alcohol, the basis for many common hand sanitizers. Current CDC guidelines for hand hygiene to prevent norovirus transmission in healthcare settings remain with hand wash with soap and water, due to the lack of other effective methods. Therefore, there is an urgent need for an effective, environmentally friendly, non-toxic, and long-lasting strategy to combat alcohol-resistant viruses such as norovirus, and to reduce the burden of healthcare and social costs worldwide.

Previous studies have indicated that epigallocatechin-3-gallate (EGCG) and its lipid-soluble derivatives, especially EGCG-

palmitate (EGCG-p), could be strong candidates for the next generation of antiviral and virucidal materials [1-20]. EGCG is a major polyphenol component of extracts from dry leaves of the Camellia sinensis (tea) plant, and its antiviral activities have been studied widely. EGCG and lipophilic EGCG are classified as non-toxic food additives [21], and generally considered as a dietary supplement in the US according to the DSHEA (Dietary Supplement Health and Education Act of 1994). Due to their stability, global antiviral properties, high efficacy, persistency, and absence of known toxicity under normal doses, lipophilic EGCG compounds become the most suitable derivatives of EGCG for topical applications [18,20]. A group of lipophilic EGCG compounds has been tested, with promising results, in multiple *in vitro* and *in vivo* models against influenza A virus, herpes simplex 1 virus, and polio virus 1 [3,11,12,19,22].

We reported previously that 50 μM EGCG-p completely blocks the infectivity of herpes simplex-1 virus (HSV-1) in Vero cells; this compound showed significantly higher efficacy than water-soluble EGCG [19]. The antiviral effect of lipophilic EGCG was further validated in a clinical trial treating herpes labialis caused by herpes simplex virus; it demonstrated high efficacy, without reported side effect [11,12]. Newly published data from hand sanitizer formulation testing demonstrated that, using 50% Tissue Culture Infective Dose (TCID50) assay methods, a novel alcohol-based sanitizer formulation containing EGCG-p resulted in a mean 6-$\log_{10}$-reduction of human poliovirus 1 (PV-1) infectivity (i.e., one million-fold reduction), exceeding by 100-fold the mandatory *in vitro* >4 $\log_{10}$-reduction of PV-1 infectivity for virucidal agents set by the US (EPA), EU, Canada and China [22]. In addition, ultrafiltration to remove the sanitizer prior to the infectivity assay revealed that the virucidal effect of ProtecTeaV (PT) formulation was associated with irreversible inactivation of PV-1, rather than a reversible inhibition mechanism [22].

Based on the *in vitro* and *in vivo* evidence, we hypothesized that surface-applied EGCG-p, either with or without ethanol, would provide a potent and persistent residual virucidal effect on the surface against nonenveloped viruses that are resistant to alcohol. The current study sought to determine if PT sanitizer formulations containing EGCG-p possess a persistent virucidal effect against a surrogate of human norovirus feline calicivirus (FCV) that is commonly used as a virucidal activity test standard. The US EPA requires data obtained from feline calicivirus (FCV) for claims of a virucidal disinfectant for hard surface use against norovirus.

Materials & Methods

FCV (VR-2057) and Fcwf cells (CRL-2787) were purchased from American Type Culture Collection (ATCC, Manassas, VA). EGCG-p was purchased from Camellix, LLC, Evans, GA. Carbopol Ultrez 20 gelling agent was obtained from Voyageur Soap and Candle Co (Surrey, BC Canada), and triethanolamine from Carolina Biological Supplies (Burlington, NC). Hanks Balanced Salt Solution (HBSS), and cell culture media EMEM were purchased from ATCC. Fetal bovine serum (FBS) was from Atlas Biologicals, Inc. (Fort Collins, CO). Other cell culture media were from Life Technologies Corporation (Carlsbad, CA). Cell culture plastic ware was purchased from Southern Labware Inc. (Cumming, GA). The 100% ethanol USP was from Augusta University Main Supplies (Augusta, GA).

ProtecTeaV (PT) formulations: The basic proprietary alcohol-based PT formulation (PT 0) contained 70% v/v ethanol USP, and the remaining 30% included deionized water and gelling agents (Ultrez 20 and triethanolamine). This v/v concentration is equivalent to 55.23% w/v of ethanol (using an ethanol density of 0.789). The basic PT formulation was used to prepare PT formulations containing different concentrations of EGCG-p; PT 0.1, 0.2 and 0.5 (% w/v).

Foaming formulations were also examined. The alcohol-free foaming formulations (Foam 0 and Foam 0.2) with and without EGCG-p were acquired from Gillons Inc., Chicago Ridge, IL. These proprietary alcohol-free formulations contain water, detergent, and 0.17% benzalkonium chloride.

A commercial hand sanitizer gel, PF (brand name not disclosed), was obtained from www.amazon.com. It contains 70% v/v ethyl alcohol plus proprietary amounts of isopropyl alcohol and polyquaternium-37, and is marketed with a claim of broad spectrum activity.

A second commercial hand sanitizer gel, PA (brand name not disclosed) was also commercially acquired from www.amazon.com. It contains 70% v/v ethyl alcohol, plus a proprietary amount of isopropyl alcohol.

A liquid hand soap (brand name not disclosed) was acquired from www.amazon.com, and tested for comparison. It contains sodium benzoate, citric acid, triclocarban, and detergent. The LHS was diluted 5-fold with deionized water before use to mimic hand wash concentration.

In addition to these test samples, 70% (v/v) alcohol was used as an experimental control.

Cell culture

Fcwf cells (from feline fetus) were maintained in Eagle's Minimum Essential Medium (EMEM) medium containing 10% FBS and 1% antibiotics (Penicillin Streptomycin Solution, 100 X, MediaTech, Inc. VA) at 37°C with 5% CO_2. Prior to confluency, the cells were harvested using 0.25% (w/v) trypsin – 0.53 mM EDTA (Life Technologies, CA), sub-cultured in 96-well tissue culture plates, and allowed to become 80-90% confluent before infection.

FCV propagation

Fcwf cells ($10^5/cm^2$) were seeded in a 75 cm^2 tissue culture plate and grown for 24-48 hrs until the monolayer became 90% confluent. FCV at an MOI of 0.1 for infection was prepared by diluting a titered virus suspension in HBSS. The monolayer of Fcwf cells was washed briefly with HBSS prior to adding diluted virus in 3 ml HBSS. The flask was incubated in a cell culture

incubator for 1 hr with gentle rocking every 10 min to spread the virus evenly. EMEM (9 ml) was then added to the flask, and incubation continued for 24 hrs before observation for cytopathic effect (CPE). When >80% cells showed CPE, the flask was frozen at -80°C and thawed for two cycles, followed by centrifugation at 400 x g for 20 min. The supernatant was filtered using a 2 µm tube top filter (50 ml, Corning Inc., Corning, NY) and the virus was dispensed into cryogenic vials in 1 ml aliquots and stored at -80° C. Viral titer was determined by TCID50 assay.

TCID50 assay

To obtain a viral titer, 50 µl of virus suspension was added to 450 µl HBSS (i.e., 10^{-1} viral dilution). A series of 10-fold dilutions of this dilution up to 10^{-11} was made with HBSS. From each dilution, 100 µl was loaded in quadruplicate into a 96-well plate of Fcwf cells, followed by 1 hr incubation for viral absorption. The overlying liquid containing virus was then removed and replaced with EMEM. Incubation was continued for 24 hrs prior to observation for CPE. It took 4-7 days to complete the viral infection cycle when there was no new CPE emerging in the wells. The number of wells associated with CPE were entered into Reed & Muench calculation calculator software [23].

Suspension assay

Suspension tests for virucidal activity were performed according to the protocol described previously by Zhang et al. [22]. For each test formulation (PT0, PT0.1, PT0.2, PT0.5, Foam 0, Foam 0.2, PF, PA, LHS), 450 µl of the test sample was placed in a plastic centrifuge tube, 50 µl of FCV was added and mixed by shaking the tube for 60 sec. The test sample was then immediately neutralized by dilution with HBSS (i.e., 10^{-2} viral dilution), and a series of dilutions was made from this mix up to 10^{-8}, A TCID50 assay was performed on quadruplicate 100 µl aliquots from each dilution. A viral titer was simultaneously performed on the same plate with tested samples as untreated viral infectivity control.

Application of antiviral agents to an FCV contaminated hard surface

FCV (50 µl) was applied to a clean surface (plastic petri dish) and allowed to dry under a level 2 safety cabinet (approx. 30 min). Each test sample or control (450 µl) was then applied on the surface with a cell scraper to spread and allowed to dry (approx. 30 min). (After application and prior to drying, the LHS sample surface was washed 3 times with deionized water to mimic hand wash with soap). HBSS Buffer was used to collect surviving virus, followed by a TCID50 assay as described above, and the $\log_{10}$ fold reduction from each sample and from untreated control (dried virus with HBSS recovery) was calculated.

Persistent residue activity test after pre-application of antiviral agents to a clean surface (without following wash)

The purpose of this experiment was to determine the surface residue virucidal activity of the PT formulations in comparison to other products/samples. Each sample (450 µl) was applied to a clean hard surface (plastic Petri dish). (The surface applied with LHS was then washed three times with sterile deionized water to mimic hand washing.) All surfaces (without lid cover) were air dried under a level 2 safety cabinet for 1, 2, 4 and 12 hours. Subsequently, alcohol-containing samples (PT, PF) were collected from the surface by elution with 70% alcohol to a total volume of 450 µl. Non-alcohol samples (LHS, Foam 0 and Foam 0.2) were collected using HBSS in a total volume of 450 µl. To each collected sample, 50 µl of FCV suspension was added and mixed for 60 sec prior to TCID50 assays.

Persistent residue activity test after pre-application of test samples to clean surface followed by washing with water

Each sample (450 µl) was applied to a clean surface (plastic Petri dish). (The surface applied with LHS was also washed three times with sterile deionized water.) All samples were air dried under a level 2 safety cabinet for 1, 2, 4 and 12 hours, followed by washing three times with deionized water and then air drying, to mimic hand washing. Next, alcohol-containing samples (PT, PF) were collected from the surface using 70% alcohol to a total volume of 450 µl. Non-alcohol samples (LHS, Foam 0 and Foam 0.2) were collected with HBSS in a total volume of 450 µl. To each collected sample, 50 µl of FCV suspension was added and mixed for 60 sec prior to TCID50 assays.

Alcohol-free virus challenge of cells pre-incubated with EGCG-p (control for alcohol effect)

Fcwf cells were seeded in a 96-well tissue culture plate until the cell density reached 80-90%. EGCG-p was dissolved in dimethyl sulfoxide (Fisher Scientific, Hampton, NH) and diluted with EMEM to 0.001%, 0.002%, 0.005% and 0.01%. Cells were pre-incubated with aliquots of the different concentrations of EGCG-p for 1 hr, followed by HBSS wash prior to FCV challenge at different dilutions for 1 hr. After virus absorption, new medium without EGCG-p replaced the viral challenge and the plates were incubated for 24 hrs before observation for CPE.

Statistical analyses were performed using Prism 6.0 (GraphPad Software). For ANOVA, normality was assumed, and homogeneity of variance tested using the Brown-Forsythe test. Alpha was 0.05. Tukey's multiple comparisons test was used for one-way ANOVA group comparisons, and Sidak's multiple comparisons test for two-way ANOVA. One-sample t-tests (without Bonferroni correction for multiple tests) were used to compare group mean $\log_{10}$ reductions to fixed values (4.0 or 0.0).

Results

Feline calicivirus suspension test TCID50 assay results

As shown in Figure 1, all PT sanitizer formulations containing EGCG-p (from 0.1 to 0.5% w/v) demonstrated virucidal activity against FCV meeting or exceeding the international mandatory virucidal sanitizer standard for norovirus claims (>4 $\log_{10}$ reduction of FCV infectivity). The other sanitizer samples tested (PF, PA, PT 0, and 70% alcohol control) did not show virucidal activity that met this standard.

One-way ANOVA showed significant differences between the groups ($p<0.0001$). PA (1.76-$\log_{10}$ reduction) was not different

from 70% alcohol (0.45-$\log_{10}$; p=0.20), while PF (2.50-$\log_{10}$) showed a greater log reduction than alcohol (p=0.009). However, PF and PA showed no significant difference (p=0.86), suggesting a modest effect size with insufficient statistical power to detect a difference. LHS in this suspension assay showed minimal activity (0.42-$\log_{10}$ reduction), not significantly different from 70% alcohol (p=1.0) or PA (p=0.24), and significantly less reduction than PF (p=0.015). PT 0 (0% EGCG-p) was not significantly different from 70% alcohol (p=0.94), or from PF, PA, or LHS (p=0.097, 0.78 and 0.94 respectively). However, the $\log_{10}$ reductions with PT 0.1, 0.2 and 0.5 (4.63, 4.94 and 5.71 respectively) were all significantly greater than PT0, (<0.0001), 70% alcohol (p<0.0001), PA (p≥0.0001), PF (p<0.003), and LHS (p<0.0001). There were no significant differences between PT0.1, 0.2 and 0.5 in $\log_{10}$ reduction (p ≥ 0.20).

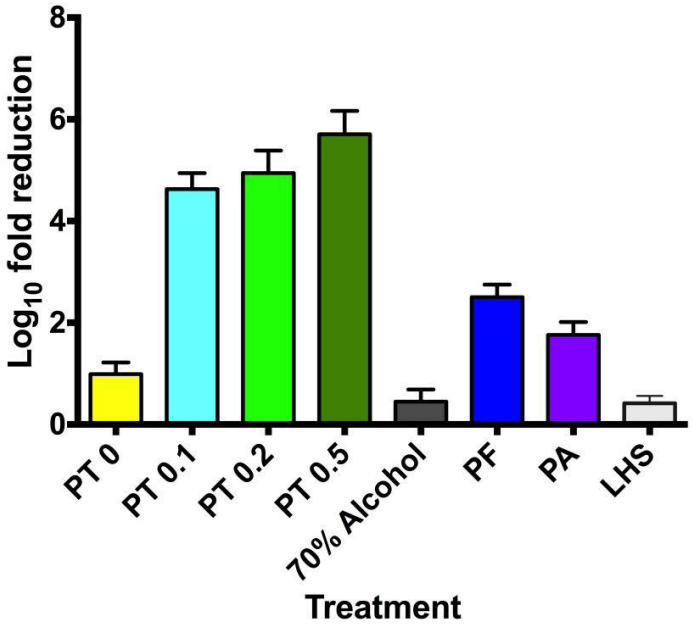

Figure 1: Feline calicivirus (FCV) suspension test and TCID50 assay of sanitizer efficacy. Bars show mean +SEM (n=3-6 replicate independent experiments). One-way ANOVA showed a highly significant difference between the groups (p<0.0001). Tukey's post hoc tests showed numerous differences between treatments, but not between PT 0.1, 0.2 and 0.5 (p ≥ 0.20), which all showed significantly greater log-fold reduction than all other treatments (p<0.003). Only PT formulations with EGCG-p achieved ≥ 4 $\log_{10}$ reduction (99.99% reduction of FCV infectivity).

Application of antiviral agents to a contaminated hard surface

Results using FCV with six formulations tested on a hard surface are shown in Figure 2. Both PT 0.1 and LHS showed mean $\log_{10}$ reduction values meeting the international standard for norovirus claims (4.0 and 4.25 respectively). One-way ANOVA was used to compare the $\log_{10}$ reduction values for PF (2.38), PT 0 (2.38), PT 0.1 (4.25), and 70% alcohol (2.63) (LHS was excluded from this analysis as both replicates gave values of 4.0, and zero variance). There was a significant difference between samples (p=0.003). Tukey's multiple comparisons test showed that PT 0.1 gave a $\log_{10}$ reduction significantly greater than 70% alcohol, PF and PT 0 (p<0.008).

Both LHS experiments gave a 4.00 $\log_{10}$ reduction. In comparison to Figure 1, this reduction was likely due to the effect of water washing. This was not tested further. Foam 0 and 0.2 both gave fold reductions not significantly different from 0.00 (one sample t-test; p≥0.27). These results indicated that the PT 0.1 formulation met the EPA requirement for a disinfectant on a hard surface against norovirus.

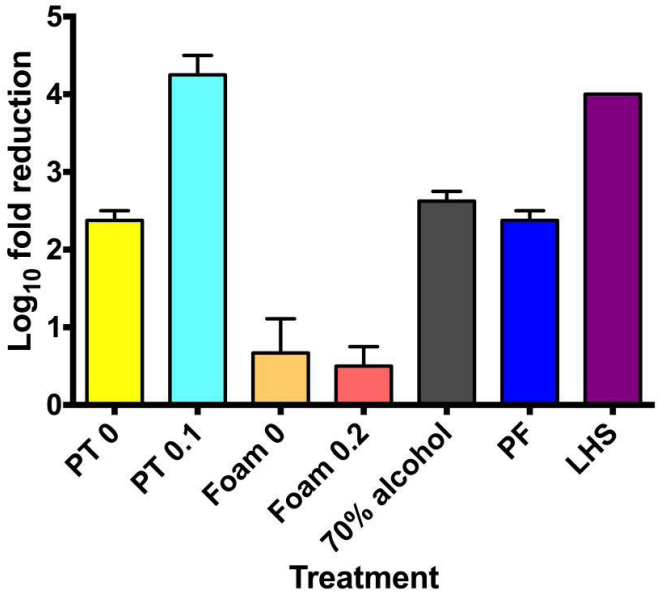

Figure 2: Virucidal test of treatments on FCV-contaminated hard surface. Mean and SEM are shown (n=2-3 independent replicate experiments; both LHS values for mean were identical). Liquid samples were applied to a dried FCV film on a hard surface and dried. The surface was washed and virus in the wash collected for TCID50 assay. The results showed only PT 0.1 led to a >4 $\log_{10}$ reduction. Excluding LHS and foam from the analysis, one-way ANOVA showed differences between the groups (p=0.003), with PT 0.1 (4.25-log0 fold reduction) showing a significantly greater reduction than PF 0 (p=0.005), PT o (p=0.005) or 70% alcohol (p=0.008). Comparison of the result of LHS treatment here to that seen in Figure 1 indicated that the >4 $\log_{10}$ reduction of FCV infectivity was due to washing with water 3 times.

Persistent residue activity test after pre-application of antiviral agents to a clean surface (without following wash)

Result of testing for persistence of virucidal activity after pre-application of sanitizers are shown in Figure 3. A two-way repeat measures (time) ANOVA showed no significant effect for time (p=0.32) or for interaction with treatment (p=0.98). However, treatment showed a significant effect (p=0.0003), as did matching (p<0.0001). Sidak's multiple comparisons test between treatments showed that PT 0.1 gave a significantly greater $\log_{10}$ reduction in TCID50 than all other sanitizer samples [PF (p=0.002), LHS (p=0.0006), Foam 0 (p=0.004), Foam 0.2 (p=0.003) and PT 0 (p=0.019)]. No other significant differences were observed (p>0.79). This result suggested that only PT 0.1 has persistent residue virucidal activity lasting for up to 12 hours after one application onto a hard surface, with a mean $\log_{10}$ reduction at 12 hrs of 3.56 ± 0.98 (sem; n=4).

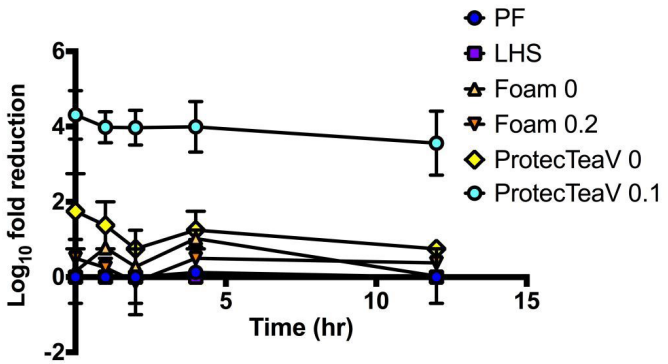

Figure 3: Persistent residue virucidal activity on hard surface. Elutable virucidal activity was determined after drying surfaces for up to 12 hrs. Results for $\log_{10}$ infectivity reduction for each sample at different time points are shown (mean ± SEM, n=2-4 independent replicate experiments for each sanitizer). There was no evident trend in the $\log_{10}$ fold reduction over time for any treatment other than PT 0.1, which showed a consistent fold reduction of around 4-fold through 4 hrs, declining to 3.56 ± 0.98-fold (n=4) at the 12 h time point.

Persistent residue activity test after pre-application of test samples to clean surface followed by washing with water

After water rinsing all surface applied sanitizer samples (not just LHS) to mimic hand washing after application, the results showed that all except PT 0.1 gave a consistent modest $\log_{10}$ fold reduction in the TCID50 assay (Figure 4A). Excluding PT 0.1, non-linear regression fit to a linear model gave r2 goodness of fit values of ≥ 0.27, and an F-test for the null hypothesis of slope = 0 did not give significant p values ($p \geq 0.12$), consistent with a minimal (linear) relationship between fold-reduction and time. Excluding PT 0.1, no significant differences were found by an F-test (p=0.14) between the y-intercepts (i.e., initial fold-reduction after washing) due to different treatments (Figure 4B).

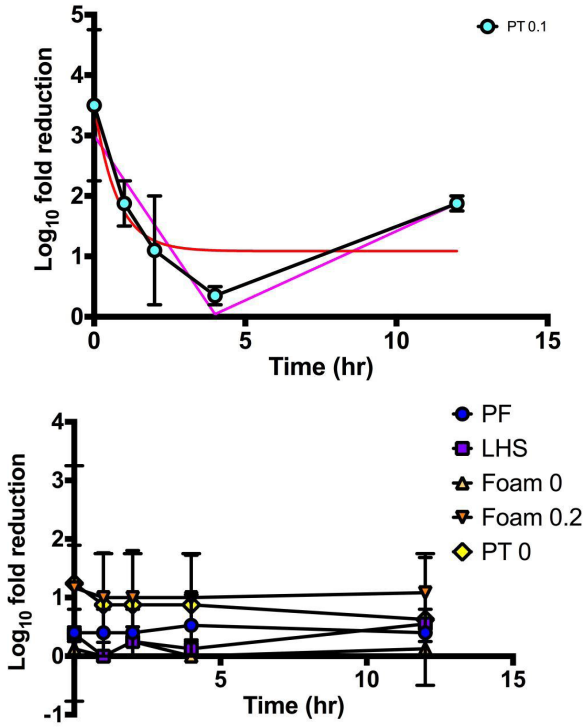

Figure 4: Residue virucidal activity test of samples after washing 3 times with water. A (upper): Mean and SEM of $\log_{10}$ reduction of PT 0.1 applied on a hard surface, dried, washed 3 times with water at indicated time points, and remaining material collected by elution for TCID50 assay against FCV. The red curve showed a non-linear regression fit of PT 0.1 values to an exponential decay model, the purple curve to a two-line segmental linear regression model. B (Lower): Mean and SEM of $\log_{10}$ reduction of other samples applied to hard surface, dried, washed 3 times with water at indicated time points, and collected for TCID50 assay against FCV. Only modest activity on the surface remained after washing, and there was no significant relationship between activity and time.

In contrast, PT 0.1 showed a significantly better fit to an exponential decay than a linear model (r2 0.53 versus, 0.04; F-test, p=0.030). The y-intercept for PT 0.1 (non-linear regression, exponential decay model) was 3.52 (95% CI 1.79-5.26). These results supported an initial retention of a substantial proportion of PT 0.1 antiviral activity even after handwashing once. However, washing with water did diminish the virucidal activity of PT 0.1 (<4 $\log_{10}$ Reduction).

Alcohol-free virus challenge of cells pre-incubated with EGCG-palmitate

As shown in Figure 5, pre-incubation of Fcwf cells with EGCG-p at concentrations ranging from 0.001% to 0.01% for 1 h in the absence of alcohol effectively reduced FCV infectivity by 10,000 fold or greater (>4 $\log_{10}$ reduction; not significantly different from 4-fold (one-sample t-test with Bonferroni correction of alpha=0.013 for four comparisons), indicating the effect of EGCG-p is independent of the presence of alcohol. There was no significant difference between the four concentrations tested for the fold reduction (p=0.43) at the power employed.

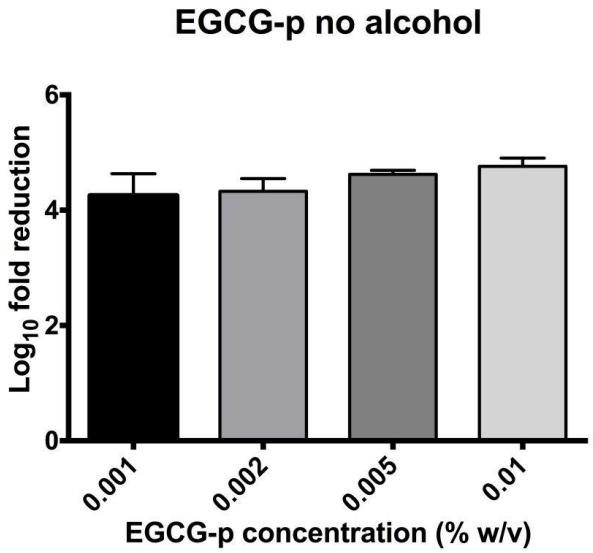

Figure 5: Inhibitory effect of EGCG-p in alcohol-free cell culture challenge. Bars represent TCID50 mean ± SEM (n=3) of $\log_{10}$ reduction for cells pre-incubated with different concentration of EGCG-p for 1 hr, washed with HBSS, and incubated with FCV for 1 hr. No statistical difference among the concentrations of EGCG-p (p=0.43). The $\log_{10}$ reduction was obtained by comparison to results with cells not pre-incubated with EGCG-p.

Discussion

The effectiveness of prevention methods in current use against viral infection, including vaccination, has been questioned, even for the prevention of influenza (an enveloped virus family) [24]. In a recent report by the CDC, the 2014-2015 laboratory-confirmed influenza-associated hospitalization rate among adults age 65 or older was the highest since 2005 [25]. Due to the relative ineffectiveness of the mismatched vaccine against H3N2 virus (approximately 10%-30% in effectiveness), a severe 2017-18 influenza seasons is in progress at the time of writing [26]. Thus, novel sanitizers/disinfectants with high virucidal capacity and persistent surface residue activity (in combination with other germicidal activities) could make a significant contribution to disease prevention, especially against alcohol-resistant nonenveloped viruses such as norovirus.

Nonenveloped viruses, including norovirus, rotavirus, enteroviruses, adenovirus, and hepatitis A virus, are major causes of human morbidity and mortality [27]. Unlike the enveloped viruses, nonenveloped viruses are resistant to alcohol sanitization, a widely used method for reducing infections. Norovirus, rotavirus and adenovirus were responsible for the majority of viral gastroenteritis cases observed among children in a large pediatric hospital setting [28]. Gastroenteritis caused by these viruses can be severe and costly to patients and healthcare providers [29]. Despite a 2011 CDC update to the Guidelines for the Prevention and Control of Norovirus Gastroenteritis Outbreaks in Healthcare Settings, the trend for norovirus outbreaks is not showing a positive change. Indeed, there was a sharp increase in norovirus outbreaks during 2013-2014, consistent with a lack of alternatives for personal sanitization other than hand washing (CDC NoroSTAT, 2009-2015). Research evidence demonstrates that currently available alcohol-based hand sanitizers are not effective against norovirus [30,31]. In fact, such sanitizers were found to be a risk factor for norovirus spread in comparison to soap and water wash in long-term care facilities [32]. However, hand washing with soap and water does not inactivate norovirus, but rather washes the virus off hands and into the waste water system [33]. It is known that norovirus can survive in water for 60 to 728 days, and it persists on various surfaces for prolonged periods of time [34]. An additional problem with current alcohol-based hand sanitizers is that alcohol evaporates in less than 20 seconds after each application, leaving the skin (or other surface) as a platform readily to transmit virus. Therefore, novel hand hygiene approaches that are more effective than hand washing with soap and water, as well as providing environmentally friendly surface disinfection, are in urgent need in order to reduce outbreaks and infections from norovirus and other nonenveloped (as well as enveloped) viruses [35].

Here we report, for the first time, that alcohol-based PT formulations containing EGCG-p consistently demonstrated virucidal activity against FCV, a surrogate for human norovirus in laboratory testing, achieving a >4 $\log_{10}$ reduction of viral infectivity, either in suspension test (60 sec exposure of virus to the formulation prior to neutralization by dilution with HBSS) or in a contaminated hard surface application test (Figures 1 and 2, respectively). In contrast, commercially acquired name-brand alcohol-based hand sanitizers failed to demonstrate virucidal activity against FCV (<4 $\log_{10}$ reduction of viral infectivity in 60 sec suspension test), and antibacterial liquid hand soap (LHS) was effective only when the soap-virus mix on the surface was washed three times with water (compare Figures 1 and 2). As expected, LHS in a suspension assay without wash has a very small impact on FCV infectivity (0.42 $\log_{10}$ reduction after 60 sec exposure). This result was consistent with previous reports that hand wash with soap and water only washes virus away from the skin (and into the waste water system), with retained infectivity of contaminated waste water and systems for a prolong period [33,34].

The virucidal activity of PT formulations was not dependent on the presence of ethanol; pre-incubation of cells with even low concentrations of EGCG-p (0.01% or lower) effectively blocked FCV infection by more than 10,000 fold (Figure 5). Incubation of Fcwf cells with EMEM containing only 0.001% of EGCG-p for 1 hr (before removal) was able to reduce FCV infectivity (1 h viral challenge) by 4.25 $\log_{10}$ fold. The protective effect was not significantly different from an EGCG-p concentration of 0.01% (Figure 5; 4.77 $\log_{10}$ reduction). This result suggests that cells pre-coated with EGCG-p, even after removal of EGCG-p-containing medium, becomes highly resistant to FCV infection for a minimum of a 1 h period of FCV challenge. One potential explanation is interference by EGCG-p with viral binding to cell surface membrane receptors. The mechanism of such interference warrants further studies.

Within the statistical design limitations of this study, EGCG-p in alcohol-containing PT formulations did not show a significant difference in FCV antiviral activity at concentrations between 0.1 and 0.5 % w/v (Figure 1). Concentrations higher than 0.5% were not tested due to the effectiveness of this concentration range against a number of nonenveloped viruses (data not shown). Further evaluation of the apparent trend to a dose dependent effect seen here would require more replicates.

Importantly, after one application on a hard surface the PT formulation with 0.1% EGCG-p demonstrated a significant residue virucidal activity (Figure 2), and this activity was persistent for up to 12 h (Figure 3). Collection at different time points of dried PT 0.1 residues left on a hard surface (by elution using 70% v/v ethanol) consistently demonstrated virucidal activity against FCV in a suspension test (>3 $\log_{10}$ reduction), whereas other sanitizer samples showed low activity (<2 $\log_{10}$ reduction, Figure 3).

These results are consistent with our overall hypothesis. On the other hand, the alcohol-free foaming formulations (with EGCG-p and 0.17% benzalkonium chloride) tested in the present study did not possess any measurable virucidal activity against FCV (Figures 1 and 2). This could have been due to the detergent content and/or the insolubility of EGCG-p in a complete water-based formula that was only incubated with FCV for 60 sec. Thus, the virucidal activity of the EGCG-p compound could depend on its solubility in a given formulation. It will be important to develop future

alcohol-free sanitizing formulations with EGCG-p that take into account its lipid-soluble nature, in order to maintain the virucidal activity of this compound.

The characteristics of PT 0.1 described here would potentially enable the PT formulation to be not only virucidal, but also to provide sanitizing products with persistent effect against norovirus after surface application, and without harmful impact to human skin and environment. Further research and development into this application is therefore justified. Collectively, the evidence suggests that future alcohol-based and alcohol-free sanitizer and prevention products containing EGCG-p and with different methods for delivery can be developed, such as lotion and coating applications, or a nose drop/spray, which could be considered for prevention of viral entry into the cells.

Conclusion

In conclusion, the alcohol-based ProtecTeaV formulations with EGCG-p possess both virucidal activity and persistent surface residue virucidal activity against FCV. Along with previous data demonstrating virucidal activity against human polio virus type 1 (PV-1) (22), EGCG-p-containing formulations could have a broad-spectrum of virucidal and persistent activity against pathogenic viruses, according to international virucidal product standards for specific claims. Lipid-soluble derivatives of EGCG, such as EGCG-p, could be incorporated into novel global strategies of prevention against viral infections, in combination with vaccines, hand wash with water and soap, masks, and other effective measures.

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