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Enhanced Sporicidal Activity of Alcohol and Epigallocatechin-Palmitate-Based Hand Hygiene Formulations Comprised of Plant-Derived Compounds

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Abstract

Pathogenic spore-forming bacteria pose high risks to healthcare settings, as well as in the food and beverage industries. We reported recently that novel alcohol-based formulations containing plant-derived compounds, including epigallocatechin-3-gallate-palmitate (EGCG-P), a green tea polyphenol ester, provide > 99.99% inactivation of bacterial spores within 60 sec. Based on recently published data from our group and others, we hypothesize that a combination of EGCG-P and alcohol formulated with other plant-derived ingredients would achieve high sporicidal efficacy against a wide spectrum of bacterial spores and can provide novel hand hygiene methods against bacterial spores without toxicity. The objectives of the current study were to optimize two novel formulations with combinations of glycerol, citric acid, and EGCG-P to increase sporicidal activity and explore the rapid inactivation mechanisms and suitability for sporicidal products with broad-spectrum activities against aerobic and anaerobic bacterial spores. Methods included suspension testing of two formulations against spores from *Bacillus cereus* and *Clostridium sporogenes*, quantification of spore germination, and scanning electron microscopy. The results demonstrated that these novel formulations were able to reduce spore germination by >99.999% after 30 sec exposure in suspension tests, and rapidly caused physical damage to the spores. Additional studies are

[#]Equal contribution to the project as co-authors.

warranted to determine the suitability of the novel formulations for future hand hygiene use.

Keywords

Alcohol, Hand Hygiene, Sporicidal, Bacterial Spores, Plant-Derived Compounds

1. Introduction

The current guidelines from United States Center for Disease Control and Prevention (CDC) and World Health Organization (WHO) for a hand hygiene method to prevent bacterial spore infection (and also norovirus infection) are still limited to hand wash with soap and water [1] [2]. This is based on evidence that the available hand hygiene products do not have sporicidal or virucidal activity (>4 log reduction of infectivity within 60 sec in a standard suspension test, or >2 log reduction of infectivity within 5 min in a human hand test: WHO guidelines attached) [1].

As of today, there is no established sporicidal test standard in the world for hand hygiene products, due to the lack of a reported nontoxic method with >4 log reduction (99.99% reduction) of spore germination under a 2 min-exposure. The European Union (EU) only has hand hygiene product standard/pass criteria for fungal spores, which requires >4 log reduction under 60 sec (EN 13624, EN 1650) [3] [4]. For surface and environmental cleaning sporicidal claims, EN 14347 and EN 13704 are European standards using *Bacillus subtilis* (*B. subtilis*) and *Clostridium sporogen*es (*C. sporogenes*) (but do not address *C. difficile*) for 60 min exposure time with pass criteria of >4 log reduction [5] [6] [7]. In the US, the Environmental Protection Agency (EPA) is the regulatory body for sporicidal disinfectant tests and claims using a protocol with SOP Number: MB-15-03, based on Association of Analytical Communities (AOAC) method 966.04, to test *B. subtilis*, or American Society for Testing and Materials (ASTM) method E2197-11 to test *B. subtilis* and *C. sporogenes* on a hard surface. The pass criteria are >4 log reduction in up to a 60 min-exposure [5]. Collectively, criteria for a sporicidal hand hygiene product might be expected to be a >4 log reduction in 30 - 60 sec against relevant bacterial spore species.

We previously tested the potential sporicidal activities of green tea polyphenols and their acyl esters. These plant-derived compounds in aqueous solutions exhibited sporicidal activities and demonstrated a capability to cause physical alteration of the spore coat after 2 h exposure [8]. However, the exposure time of 2 h used in that study is beyond the required exposure timeframe for hand hygiene use (30 - 120 sec). Since alcohol is a plant-derived bactericidal agent with low adverse effect on human skin [9], we next explored different alcohol-based formulations containing EGCG-P, together with other plant-derived compounds for delivery. The rationale for the formulations was based on the strong dena-

turing capability of alcohol combined with the physical destruction of the spore coat by EGCG-P. The combination of ingredients in these nontoxic formulations (F1, F2 and F3 do not contain benzalkonium chloride, a quaternary ammonium compounds (QAC)) reached the sporicidal range with 60 sec exposure [10].

The purpose of the current study was to determine whether combinations of plant-derived compounds (*i.e.* a natural formulation) could provide greater than 5 log reduction of spore germination in alcohol-based formulations containing EGCG-P, and the potential fast-acting mechanism of these formulations against both aerobic and anaerobic spores.

2. Materials and Methods

2.1. Chemicals, Reagents and Cultures

EGCG-P was purchased from Camellix, LLC (Evans, GA), and dissolved in 100% ethanol (Fisher Scientific, Hampton, NH) as a 10% stock prior to formulation. Glycerol (99.7%) USP Kosher was purchased from ChemWorld.com. Ultrez 20 was supplied by Voyageur Soap and Candle Company (Surrey, BC, Canada). Triethanolamine (TEA) was provided by Carolina Biological Supply Company, and citric acid (100% anhydrous fine granules) was purchased from HaleFresh.com. *B. cereus* and *C. sporogenes* were obtained from Carolina Biological Supply Co., Item#154870A, and Item#154995A, respectively.

2.2. Formulations

To further increase the efficacy of F1 and F2 described previously [10], adjustments to the formulations were made for the current study. The current F1 formulation (cF1 hereafter) is in a hand sanitizer gel form (pH > 6.0) that contains 78% ethanol, 0.2% epigallocatechin-3-gallate-palmitates (EGCG-P), glycerol, water, gelling agent, and triethanolamine (polymerization catalyst). The current F2 formulation (cF2 hereafter) is in hand rub form that contains 80% ethanol, 0.2% EGCG-P, citric acid and water (pH > 3.0).

2.3. Bacterial Spore Enrichment and Purification

For *B. cereus*, the methods were described previously [10]. For *C. sporogenes*, the cells were grown in reinforced clostridial medium (RCM) (BD#218081) and supplemented with 5% yeast extract and 2% agar. After inoculating 100 µL of *C. sporogenes* onto the plate, parafilmed, and placed it in a vacuum-sealed bag. The bag was filled with argon gas to remove any remaining oxygen. After incubating at 37˚C for 10 days, the *C. sporogenes* spores were harvested with the same procedure as reported previously [8].

2.4. Suspension and Neutralization Assay

Purified endospores from *B. cereus* were mixed for 30 sec with cF1 or cF2 at a 1:9 ratio between spores and the alcohol-based formulations, prior to neutralization of the reaction by immediate 10× dilution in phosphate buffer saline (PBS).

The serial 10× dilutions of the neutralized mixes were plated out in triplicate in 100 mm Petri dishes with nutrient agar, and incubated at 37˚C for 24 h. The CFU of spore germination was counted, and the % of inhibition and $\log_{10}$ reduction calculated. Untreated spore samples (*i.e.* suspended in PBS for 30 sec) were used as control. Three independent experiments were carried out and the mean and standard deviation of the results calculated. The $\log_{10}$ (fold) reduction was calculated with the following equation: Log reduction = $\log_{10}$ (CFU control/CFU treated).

To determine if a 10× dilution of cF1 and cF2 by PBS completely neutralized the sporicidal activity of the formulations, neutralization tests were performed to validate the test conditions by 30 sec exposure of the spores with 10× diluted cF1 and cF2, followed by serial 10× dilution, plating, incubation and CFU calculation as described in suspension test.

2.5. Scanning Electron Microscopy (SEM)

Purified spores of *B. cereus* and *C. sporogenes* were exposed to cF1 or cF2 for 60 sec or 5 min (*B. cereus* only) prior to neutralization by dilution in PBS, as described under suspension tests. Untreated spores were mixed with PBS for 60 sec as control. Once the treatment was completed, 100 µL of the samples were dispensed and vacuum filtered using 0.2 µm polycarbonate membrane (EMD Millipore Isopore #GTTP01300) with a Nalgene filter unit (Fisher Scientific #09-740-23A). Samples were rinsed with PBS (pH 7.2) or 0.1 mol·l^{-1} sodium cacodylate buffer [$Na(CH_3)_2AsO_2 \cdot 3H_2O$] three times for 5 min each; fixed with 2.5% glutaraldehyde in 0.1 mol·l^{-1} cacodylate buffer for 30 min at room temperature. The samples underwent a dehydration series and were immersed in ethanol, providing additional fixation, followed by drying with liquid CO_2 at 1072 psi and 31˚C in Denton Critical Point Dryer. Samples were mounted on a stub and coated with a thin layer of copper metal film using Denton IV Sputter Coater. Images were captured with a Hitachi S-3400N Scanning Electron Microscope.

3. Results

A pilot study was conducted to improve the antimicrobial activity of F1 (hand sanitizer formulation) and F2 (hand rub formulation). Glycerol was added as an emollient to reduce the irritant effect of alcohol to the skin, rather than as an antimicrobial agent. It was found that when glycerol was added to F1 within a certain concentration range, the antimicrobial activity was significantly improved (data not shown, patent pending). Accordingly, we incorporated glycerol at an appropriate concentration in F1 to make cF1. For F2 improvement, it was found the antimicrobial activity increased significantly with the addition of citric acid within a certain concentration range. This was not due to the lowered pH because other pH reducing acids were not able to increase the antimicrobial activity of F2 (data not shown, patent pending). In fact, alcohol-based formulations containing different acids, with pH < 1, failed to reduce bacterial spore germina-

tion by >2.5 log after 5 min exposure [11]. In addition, this low concentration of citric acid did not lead to a pH drop to <3.3. Thus, it is possible the combination of citric acid and EGCG-P results in a synergistic effect in the alcohol-based formulation now referred to as cF2.

3.1. Suspension Tests

To determine if the sporicidal activity of cF1 and cF2 was enhanced with the combination of alcohol, EGCG-P and other plant-derived compounds, purified spores of *B. cereus* were exposed to cF1 and cF2 for 30 sec before the reactions were stopped by neutralization. Untreated spores (control) were incubated with PBS for 30 sec. The log reduction of CFUs was evaluated by analysis of variance (ANOVA). As shown in **Figure 1**, both cF1 and cF2 rapidly inactivated the spore germination capacity ($p = 0.004$). A highly significant difference between groups was found ($p < 0.005$). cF1 treatment resulted in 5.4 ± 0.12 log reduction of germination from *B. cereus* spores, while cF2 caused 5.84 ± 0.39 log reduction of *B. cereus* spore germination (the average log number for the control is 11.58). One sample t-test comparison showed that neutralized cF1 (cF1-N) and cF2 (cF2-N) failed to inactivate the spores, with only 0.12 ± 0.32 and 0.17 ± 0.35 log reduction respectively ($p < 0.001$). These results demonstrate that cF1 and cF2 rapidly inactivated *B. cereus* spores in the suspension tests, and the efficacy of the alcohol-based formulations were significantly improved by combination of plant-derived compounds, with both cF1 and cF2 capable of reducing the germination of *B. cereus* spores by >5 $\log_{10}$ (>99.999%).

3.2. SEM Validation Tests

Figure 2 shows two types of bacterial spores (*Bacillus cereus* and *Clostridium sporogenes*) treated with cF1 and cF2 for 60 sec in comparison with untreated control. In comparison to the untreated control, cF1-treated spores of *B. cereus* (upper row) exhibited an altered morphology with a reduction in size and porous spore coats. Additionally, there was a film-like appearance between the spores. The cF2-treated spores show a complete destruction of spore structures. Similarly, morphology changes were apparent after treatments of spores of *C. sporogenes* (lower row). Untreated control spores had a uniform spherical morphology. The cF1-treated spores were misshapen and clustered, and the surface was damaged while cF2-treated spores were shriveled and exhibited collapsed spore coats. These structural alterations suggested that both cF1 and cF2 were able to rapidly alter the spore morphology, damage the coat of the spores and lead to inactivation of these bacterial spores by collapsing the spore coats and by allowing ethanol to penetrate the outer coat of the spores.

To further understand the destructive capacity of the alcohol-based formulations, spores of *B. cereus* were treated with cF1 and cF2 for 5 min prior to SEM observation and imaging. **Figure 3** shows the result comparing 60 sec and 5 min treatments of the spores. The cF1-treated spores demonstrated broken spore

coats with debris on the filter surface after 5 min suspension treatment, suggesting a complete fragmentation of the spores. The 5 min cF2-treated spores demonstrated additional morphological alterations compared to the 60 sec treatment, with reduced size and collapsed spore coats.

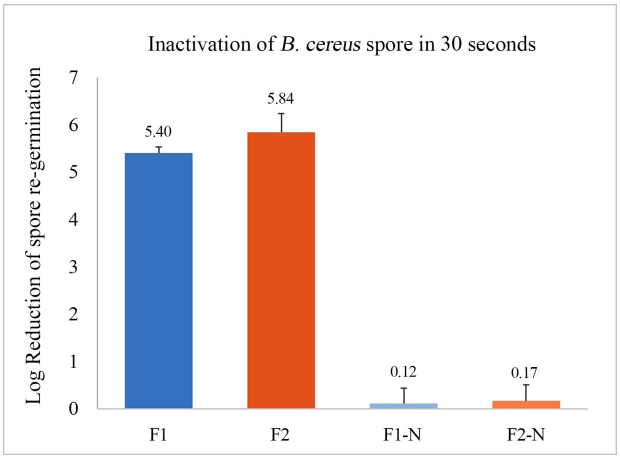

Figure 1. Log_{10} reduction of spore re-germination by cF1 & cF2 after a 30 sec incubation. cF1-N and cF2-N refer to the formulations neutralized with PBS (1:9 v/v). Mean are shown with standard deviation (n = 3). Log_{10} reduction by cF1 and F2, compared to untreated control, was statistically significant ($p < 0.005$).

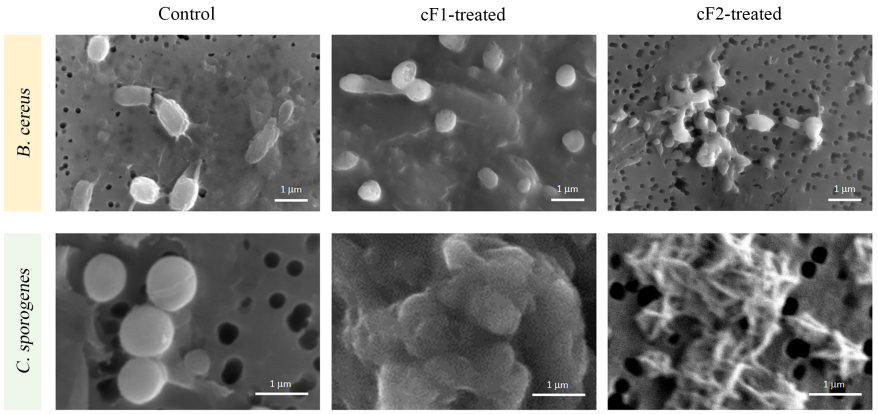

Figure 2. Morphological alterations of spores treated with cF1 and cF2. Left column: untreated spores of *B. cereus* (upper) and *C. sporogenes* (lower); Center column: spores of *B. cereus* (upper) and *C. sporogenes* (lower) treated with cF1 in suspension assay for 60 sec; Right column: spores of *B. cereus* (upper) and *C. sporogenes* (lower) treated with cF2 in suspension assay for 60 sec.

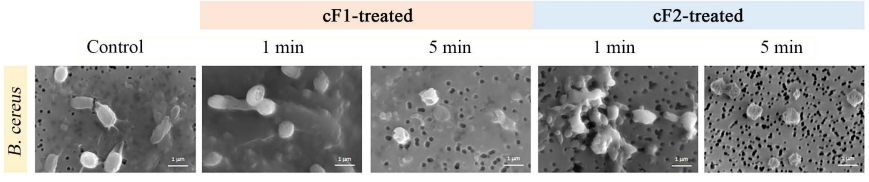

Figure 3. Morphological alterations of spores treated with cF1 and cF2 for 1 min and 5 min.

4. Discussion

Unlike surface disinfectant and device disinfectant products with virucidal or sporicidal activity, hand hygiene products are not allowed to contain toxic chemicals, since they are not suitable for human use. Another difference between hand hygiene products and surface/device disinfectants is that hand hygiene products must be able to rapidly inactivate the pathogenic organisms (within 30 - 120 sec vs. 60 min or longer). The Food and Drug Administration (FDA) regulates hand hygiene products by issuing monographs for approved active ingredients. Some alcohols such as ethanol and isopropanol have been recognized as bactericidal agents that can be used in hand hygiene products to reduce bacterial infections. The current FDA monograph for over-the-counter hand hygiene products allows only ethanol, isopropanol, and benzalkonium chloride as the active ingredients [12]. However, alcohol is known to be relatively ineffective for sporicidal purposes. According to current regulations covering surgical hand hygiene products (mostly alcohol-based products), they must be bactericidal, fungicidal (against yeasts), and virucidal against enveloped viruses, but they are not required to have sporicidal, mycobactericidal, or virucidal activity against non-enveloped viruses [9]. Thus, nosocomial infections (NIs), or healthcare associated infections (HAIs) caused by bacterial spores or non-enveloped viruses are not well-controlled with currently used methods. In fact, HAIs remain one of the major causes of death in most countries [13].

During the 2020 coronavirus pandemic, hand hygiene was essential to help protect the population from infection by the virus. In fact, Coronaviridae, an enveloped virus family, is sensitive to most hand hygiene products containing alcohol. Unfortunately, alcohol-based hand hygiene products are not effective against non-enveloped viruses such as norovirus, or against bacterial spores [14] [15]. Therefore, there is an urgent need for alcohol-based hand hygiene products with broad-spectrum activities against non-enveloped viruses and bacterial spores.

Alcohols, especially ethanol and isopropanol, are able to rapidly denature most biomolecules in microorganisms. However, pathogenic bacterial spores are not sensitive to these alcohols, or several commonly used ingredients in hand hygiene products. As a matter of fact, ethanol, isopropanol, and n-propanol do not possess significant sporicidal activity; nor do other agents such as Clorisan, chlorohexidine, and plain soap [9]. Other agents possessing bactericidal but not sporicidal activity include organic acids/esters, phenols, quaternary ammonium compounds (QACs), biguanides and organomercurials [16].

Hand hygiene products typically contain multiple ingredients to provide a combination of antimicrobial activity with cosmetic functions such as emollients, and fluid properties to facilitate use, such as viscous gels. There is an increased public desire for products with a "natural" connotation. In a previously published study, we tested the sporicidal activity of alcohol-based formulations comprised of plant-derived ingredients, including EGCG-P, either in a hand sanitizer formulation (F1) or in liquid hand rub formulations (F2), using *B. cereus*

as the test organism. We found that F1 exhibited a significantly higher sporicidal efficacy in comparison to 80% v/v ethanol alone [10], reducing *B. cereus* spore germination by 3.94 $\log_{10}$, close to the desired 4 $\log_{10}$ reduction. F2 with 100% plant-based ingredients gave the highest average sporicidal activity, with a 5.58 log reduction after a 60 sec exposure. To the best of our knowledge, this was the first observation that a formulation (F2) with 100% plant-derived ingredients was able to reduce bacterial spore germination by more than 5 log after 60 sec-exposure [10].

The current study was designed to determine whether improved plant-derived formulations of cF1 and cF2 (for hand sanitizer gel and hand rub use, respectively) have higher *in vitro* efficacies with shorter exposure time, and if 60 sec exposure results in physical damage to the spore coats of two bacterial spore species. Results from the suspension tests demonstrated that both cF1 and cF2 possessed strong sporicidal activity, with >5 log reduction of spore germination at a 30 sec exposure (**Figure 1**). These results indicated that when certain plant-derived compounds are formulated with alcohol (ethanol), the alcohol-based formulations became sporicidal, instead of sporostatic. This finding provides important evidence to support the possibility of developing novel alcohol-based sporicidal products for hand hygiene use. However, it is not known what mechanisms and the role glycerol or citric acid play lead to the significant increase in sporicidal activity found in cF1 and cF2, which warrant further exploration. Another important observation was the damage caused by the formulations to spore coats of both aerobic and anaerobic bacterial spores, as shown in **Figure 2**. This result demonstrated the rapid inactivation of the spores was associated with physical damage of spore coats, which could lead to permeability of alcohol into the core of the spores causing denature of intrinsic biomolecules. This rapid structural alteration of the spore coats has not been reported previously for nontoxic hand hygiene or disinfectant formulations. When exposure time was increased to 5 min, damage to the spore structure became more apparent, as shown in **Figure 3**, which further validated the potential mechanism for the sporicidal activity.

In order to develop sporicidal hand hygiene products, additional work must be performed. To test the potential broad-spectrum of sporicidal activity, *Bacillus subtilis* (*B. subtilis*) and *Clostridium difficile* (*C. difficile*) need to be used. If these *in vitro* tests produced satisfactory results, clinical efficacy tests will be required to determine the *in vivo* efficacy using ASTM E-1174 method (for claims in the United States and Health Canada) according to efficacy criteria of the FDA's Tentative Final Monograph (TFM) [17] [18]. This standard requires a 2-$\log_{10}$ reduction of the indicator organism on each hand within 5 min after the first use, and a 3-$\log_{10}$ reduction of the indicator organism on each hand within 5 min after the tenth use. For claims in the European Union, similar clinical tests are needed using EN 1499 [19] or EN 1500 [20] methods. Again, as noted above, there are no current criteria specifically regulating test standards and efficacy for sporicidal hand hygiene products [21].

In conclusion, a strong and rapid-action sporicidal activity of the alcohol-based

formulations containing plant-derived ingredients was achieved. The mechanism of action of the formulations could depend on the plant-derived components to initially alter the structure of spore coats, and allow alcohol to penetrate into the matrix of the spore, leading to a rapid and irreversible inactivation. Since similar nontoxic formulations have been tested for their virucidal and bactericidal activities [15] [22], these alcohol-based formulations could be used to develop various sporicidal hand/skin hygiene products with comprehensive germicidal activities (bactericidal, sporicidal, fungicidal, and virucidal), pending future research and development efforts.

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Conflicts of Interest

The authors declare no conflict of interest.

References

[1] Boyce, J.M., Pittet, D. and Healthcare Infection Control Practices Advisory Committee. Society for Healthcare Epidemiology of America. Association for Professionals in Infection Control. Infectious Diseases Society of America. Hand Hygiene Task, F. (2002) Guideline for Hand Hygiene in Health-Care Settings: Recommendations of the Healthcare Infection Control Practices Advisory Committee and the HICPAC/SHEA/APIC/IDSA Hand Hygiene Task Force. *Infection Control & Hospital Epidemiology*, **23**, S3-S40. https://doi.org/10.1086/503164

[2] WHO (2009) WHO Guidelines on Hand Hygiene in Health Care: First Global Patient Safety Challenge Clean Care Is Safer Care. Geneva.

[3] European Committee for Standartization, B.S.I. (2013) BS EN 13624. Chemical Disinfectants and Antiseptics. Quantitative Suspension Test for the Evaluation of Fungicidal or Yeasticidal Activity in the Medical Area. Test Method and Requirements (Phase 2, Step 1). London.

[4] European Committee for Standartization, B.S.I. (2019) BS EN 1650. Chemical Disinfectants and Antiseptics. Quantitative Suspension Test for the Evaluation of Fungicidal or Yeasticidal Activity of Chemical Disinfectants and Antiseptics Used in Food, Industrial, Domestic and Institutional Areas. Test Method and Requirements (Phase 2, Step 1). London.

[5] Wesgate, R., Rauwel, G., Criquelion, J. and Maillard, J.Y. (2016) Impact of Standard Test Protocols on Sporicidal Efficacy. *Journal of Hospital Infection*, **93**, 256-262. https://doi.org/10.1016/j.jhin.2016.03.018

[6] European Committee for Standartization, B.S.I. (2002) BS EN 13704. Chemical Disinfectants—Quantitative Suspension Test for the Evaluation of Sporicidal Activity of Chemical Disinfectants Used in Food, Industrial, Domestic and Institutional

Areas—Test Method and Requirements (Phase 2, Step 1). London.

[7] European Committee for Standartization, B.S.I. (2005) BS EN 14347. Chemical Disinfectants and Antiseptics. Basic Sporicidal Activity. Test Method and Requirements (Phase 1, Step 1). London.

[8] Ali, B., Lee, L.H., Laskar, N., Shaikh, N., Tahir, H., Hsu, S.D., Newby, R., Valsechi-Diaz, J. and Chu, T. (2017) Modified Green Tea Polyphenols, EGCG-S and LTP, Inhibit Endospore in Three Bacillus spp. *Advances in Microbiology*, **7**, 175-187. https://doi.org/10.4236/aim.2017.73014

[9] Kampf, G. and Kramer, A. (2004) Epidemiologic Background of Hand Hygiene and Evaluation of the Most Important Agents for Scrubs and Rubs. *Clinical Microbiology Reviews*, **17**, 863-893. https://doi.org/10.1128/CMR.17.4.863-893.2004

[10] Chu, T., Lee, L.H., Aponte, T., Lopez, S., Lalata, G., Herrera, G., Yussof, A., Dickinson, D. and Hsu, S.D. (2019) Sporicidal Activity of Novel Formulations Containing Lipophilic Epigallocatechin-3-Gallate and Natural Ingredients. *Microbiology & Infectious Diseases*, **3**, 1-6.

[11] Nerandzic, M.M., Sunkesula, V.C., C, T.S., Setlow, P. and Donskey, C.J. (2015) Unlocking the Sporicidal Potential of Ethanol: Induced Sporicidal Activity of Ethanol against *Clostridium difficile* and Bacillus Spores under Altered Physical and Chemical Conditions. *PLoS ONE*, **10**, e0132805. https://doi.org/10.1371/journal.pone.0132805

[12] Food and Drug Administration (2019) Safety and Effectiveness of Consumer Antiseptic Rubs; Topical Antimicrobial Drug Products for Over-the-Counter Human Use. 14847-14864.

[13] Haque, M., Sartelli, M., McKimm, J. and Abu Bakar, M. (2018) Health Care-Associated Infections: An Overview. *Infection and Drug Resistance*, **11**, 2321-2333. https://doi.org/10.2147/IDR.S177247

[14] Sasahara, T., Ae, R., Watanabe, M., Kimura, Y., Yonekawa, C., Hayashi, S. and Morisawa, Y. (2016) Contamination of Healthcare Workers' Hands with Bacterial Spores. *Journal of Infection and Chemotherapy*, **22**, 521-525. https://doi.org/10.1016/j.jiac.2016.04.007

[15] Widjaja, N., Dickinson, D., Shao, X. and Hsu, S. (2018) Persistent Virucidal Activity in an Alcohol-Based Sanitizer Formulation (ProtecTeaV) for Potential Use against Norovirus. *International Journal of Microbiology & Infectious Diseases*, **2**, 1-8. https://doi.org/10.33425/2639-9458.1029

[16] Russell, A.D. (1990) Bacterial Spores and Chemical Sporicidal Agents. *Clinical Microbiology Reviews*, **3**, 99-119. https://doi.org/10.1128/CMR.3.2.99

[17] (1994) Tentative Final Monograph for Healthcare Antiseptic Drug Products: Proposed Rule. In: Administration F.A.D., Ed., *Federal Register*, Washington DC, 31441-31452.

[18] Administration F.A.D. (2018) Summary: Safety and Effectiveness of Health Care Antiseptics; Topical Antimicrobial Drug Products for Over-the-Counter Human Use; Proposed Amendment of the Tentative Final Monograph; Reopening of Administrative Record (Proposed Rule). Washington DC.

[19] European Committee for Standartization B.S.I. (2013) BS EN 1499. Chemical Disinfectants and Antiseptics. Hygienic Handwash. Test Method and Requirements (Phase 2/Step 2). London.

[20] European Committee for Standartization B.S.I. (2013) BS EN 1500. Chemical Disinfectants and Antiseptics. Hygienic Handrub. Test Method and Requirements (Phase 2/Step 2). London.

[21] World Health Organization (2009) Methods to Evaluate the Antimicrobial Efficacy of Handrub and Handwash Agents and Formulations for Surgical Hand Preparation. WHO Guidelines on Hand Hygiene in Health Care: First Global Patient Safety Challenge Clean Care Is Safer Care. 25-29.

[22] Zhang, Q., Yang, Q., Yang, W., Wu, K., Wu, J., Dickinson, D. and Hsu, S. (2016) Virucidal Capacity of Novel ProtecTeaV Sanitizer Formulations Containing Lipophilic Epigallocatechin-3-Gallate (EGCG). *Journal of Antivirals*, *Antiretrovirals Research & Therapy*, **1**, 002.