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Efficacy of EPA-registered disinfectants against two human norovirus surrogates and *Clostridioides difficile* endospores

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Abstract

Aims: To determine the efficacy of a panel of nine EPA-registered disinfectants against two human norovirus (HuNoV) surrogates (feline calicivirus [FCV] and Tulane virus [TuV]) and *Clostridioides difficile* endospores.

Methods and Results: Nine EPA-registered products, five of which contained H_2O_2 as active ingredient, were tested against infectious FCV, TuV and *C. difficile* endospores using two ASTM methods, a suspension and carrier test. Efficacy claims against FCV were confirmed for 8 of 9 products. The most efficacious product containing H_2O_2 as ingredient achieved a >5.1 log reduction of FCV and >3.1 log reduction of TuV after 5 min, and >6.0 log reduction of *C. difficile* endospores after 10 min. Of the five products containing H_2O_2, no strong correlation (R^2 = 0.25, p = 0.03) was observed between disinfection efficacy and H_2O_2 concentration. Addition of 0.025% ferrous sulphate to 1% H_2O_2 solution improved efficacy against FCV, TuV and *C. difficile*.

Conclusion: Disinfectants containing H_2O_2 are the most efficacious disinfection products against FCV, TuV and *C. difficile* endospores. Product formulation, rather than the concentration of H_2O_2 in a product, impacts the efficacy of a disinfection product.

Significance and Impact of Study: H_2O_2-based disinfectants are efficacious against surrogate viruses for HuNoV and *C. difficile* endospores.

Keywords

Clostridioides difficile endospores; disinfection efficacy; feline calicivirus; human norovirus surrogate; hydrogen peroxide; Tulane virus

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

INTRODUCTION

Healthcare-associated infections (HAIs) are linked to high morbidity, mortality and increased healthcare costs (Guh et al., 2020; Jump et al., 2018; Steele et al., 2020). For many years, reduction in HAIs has been a top priority of public health agencies in the United States (Guh et al., 2020; Steele et al., 2020). While progress has been made, a substantial burden from HAIs still persists. Many HAIs are transmitted through contact with contaminated surfaces, illustrating the importance of environmental disinfection as a strategy to prevent their spread (Centers for Disease Control and Prevention, 2003; Lopman et al., 2012). Disinfectants are essential tools for effective environmental disinfection. As a result, hundreds of disinfectants with an array of active ingredients and formulations are commercially available. The most common active ingredients used are chlorine, quaternary ammonium chemicals (QACs), alcohols and peroxides (Boyce, 2021), with each having limitations. For example, chlorine-based disinfectants are highly efficacious against bacteria and viruses because they are strong oxidizers, but they can damage surfaces when used at high concentrations or after prolonged use (Luijkx et al., 2004; Tyan et al., 2019). QACs and alcohols are less likely to damage surfaces but show weak efficacy against non-enveloped viruses (e.g. human norovirus [HuNoV]) and bacterial endospores (e.g. *Clostridioides difficile*) (Boyce, 2021). Peroxides, including accelerated hydrogen peroxide (H_2O_2), can denature viral proteins, but efficacy data from published studies are limited (Boyce, 2021).

To help users decide which disinfectant to use, the U.S. Environmental Protection Agency (EPA) maintains 15 individual lists of antimicrobials registered for use against specific pathogens (Environmental Protection Agency, 2020a). Two of these lists address two of the hardest-to-kill pathogens, HuNoV (List G) and *C. difficile* endospores (List K) (Environmental Protection Agency, 2020b; Environmental Protection Agency, 2020c). To be registered as effective against HuNoV, products must achieve a 4-log reduction of feline calicivirus (FCV), a surrogate for HuNoV, within 10 min (Environmental Protection Agency, 2018a). To be registered as effective against *C. difficile* endospores, products must achieve at least a 6-log reduction of *C. difficile* endospores within 10 min (Environmental Protection Agency, 2018b). HuNoV is difficult to culture in vitro which is why FCV is used to study it. Other surrogates, such as murine norovirus, sapovirus and Tulane virus (TuV), could be used but none, including FCV, perfectly mimics HuNoV (Cromeans et al., 2014; Park et al., 2010). TuV is a promising surrogate as it is more resistant to disinfectant activity than other HuNoV surrogates, including FCV (Cromeans et al., 2014). Hence, testing product efficacy against both FCV, required by EPA, and TuV could yield better estimates of disinfectant efficacy.

The EPA requires glass carrier tests be used to conduct efficacy tests (Environmental Protection Agency, 2000a; Environmental Protection Agency, 2020b). Results using glass coupons might not translate to other materials (e.g. brushed stainless steel and plastics). As stainless steel is widely used to construct surfaces in healthcare settings (Cheng et al., 2015; Kundrapu et al., 2012), ASTM E2197–17 (ASTM International, 2017), an alternative method, uses brushed stainless-steel coupons. At present, no published data are available comparing these two testing methods.

The aim of this study was to determine the efficacy of a panel of nine EPA-registered disinfectants against two HuNoV surrogate viruses (FCV and TuV) and *C. difficile* endospores, using two ASTM methods (E1052–11 and E2197–7). These findings can inform standard testing methods used to determine efficacy of disinfectants on hard non-porous surfaces.

MATERIALS AND METHODS

Virus propagation and assays

Crandell-Rees Feline Kidney (CRFK) cells (ATCC CCL-94) were cultured in Eagle′s modified essential medium (MEM; Gibco Life Technologies) containing 5% low-endotoxin heat-inactivated foetal bovine serum (FBS) (Seradigm, VWR International), 100 U L^{-1} penicillin (HyClone) and 100 mg L^{-1} streptomycin (HyClone). Ninety percent of confluent monolayers of CRFK cells were infected with FCV strain F9 (ATCC VR-782; American Type Culture Collection) at a multiplicity of infection (MOI) of 0.01 and held at 37°C for 2 days. FCV was then harvested from cell lysates by three freeze–thaw cycles followed by centrifugation for 10 min at 5000 *g* and 4°C. FCV stocks at ca. 10^8 plaque forming unit (PFU) L^{-1} were aliquoted and stored at −80°C. Infectious FCV was quantified by standard plaque assay as previously described (Buckley et al., 2018). To test for cell line permissiveness and contamination, FCV and PBS served as a positive control and negative control, respectively. CRFK cells were passaged less than 30 times.

LLC-MK2 cells (ATCC CCL-7) were cultured in Opti-MEM I reduced serum medium (Gibco Life Technologies) supplemented with 2% low-endotoxin heat-inactivated FBS, 100 U L^{-1} penicillin and 100 mg L^{-1} streptomycin. Ninety percent confluent monolayers of LLC-MK2 cells were infected with TuV, kindly provided by Dr. Jason Jiang (Cincinnati Children′s Hospital), at an MOI of 0.1 and held at 37°C for 2 days. TuV was harvested from cell lysates similar as for FCV. Infectious TuV titre was quantified by the median tissue culture infectious dose ($TCID_{50}$) assay as described with modifications (Tian et al., 2013). Twenty microliters of serially diluted viruses were added in each quantification well per column and eight wells used for each dilution. TuV stocks at ca. 10^7 $TCID_{50}$ L^{-1} were aliquoted and stored at −80°C. LLC-MK2 cells were passaged fewer than 30 times.

Preparation and purification of *C. difficile* endospores

C. difficile (ATCC 43593) was cultured on modified brain heart infusion agar plates containing 5 g L^{-1} yeast extract, 1 g L^{-1} cysteine and 1 g L^{-1} sodium taurocholate (BHIA/YE/CYS/T) and anaerobically incubated at 37°C for 7 days. All plates were sealed with parafilm (Pechiney) and incubated at ambient conditions for an additional 7 days. The agar plate was flooded with 5 ml of 0.01 M PBS with 0.1% Tween-80 and the colony mass was scraped from the agar plates using sterile cotton swabs. The cell suspension was washed five times by ice-cold sterile deionized (DI) water followed by centrifugation at 7000 *g* for 5 min at 4°C. Vegetative cells of *C. difficile* were removed by gradient centrifugation in 50% (w/v) sucrose solution (Edwards & McBride, 2016). The endospore suspension was washed three times with sterile ice-cold water after purification. Concentration of endospores was enumerated on BHIA/YE/CYS/T plates and the purity of prepared endospores confirmed via

endospore staining. The stock culture of *C. difficile* endospores at ca. 10^8 colony forming unit (CFU) L^{-1} was stored at 4°C for routine tests and at −80°C for long-term storage.

Candidate disinfectants

Selection criteria included the following: (1) ready-to-use (RTU), (2) non-chlorine-based, (3) commercially available and affordable for small businesses and (4) limited known health risks (Figure 1a, Table S1). Nine products selected from List K ($n = 64$) and List G ($n = 148$) met our criteria and were used for this study. The active ingredients, claimed contact times and pH of selected products are listed in Table 1. A sodium hypochlorite solution (1000 ppm) was also evaluated as a positive control in the carrier test.

As contact times listed on product labels for products on List G (i.e. efficacious against FCV, a surrogate of HuNoV) ranged from 30 s to 10 min (Table 1), 1 min was used to determine efficacy against FCV in the suspension test and three contact times – 1, 5 and 10 min were used in the quantitative carrier test. No products provided claims against TuV; therefore, 10 min was used in suspension test and 1, 5 and 10 min were used for TuV in the carrier test to compare FCV and TuV results. Only product D had a claimed contact time for *C. difficile* endospores, which was 10 min, so this contact time was used in both the suspension and the carrier test for all products.

Cytotoxicity and neutralization tests

Ingredient-specific neutralizers (Table 1) were evaluated for use with each product as previously described with modifications (Buckley et al., 2018). An additional ‘wash step’ was used to eliminate all residue cytotoxicity and antimicrobial activity. Briefly, mixtures of products and neutralizers were diluted by adding 3 ml PBS then it was concentrated via centrifugation using Amicon® Ultra-4 centrifugal 30 K MWCO filters (Millipore Sigma) at 4000 *g*, 4°C, repeated three times to remove disinfectant residue in the mixture. Following the wash step, undiluted, 10^{-1} and 10^{-2} diluted solutions of product mixtures were assayed by plaque assay for CRFK cells and $TCID_{50}$ assay for LLC-MK2 cells, as described above. Cytotoxicity against these cell lines was observed under an inverted microscope (Olympus CK2) and recorded at days 2 and 5. To test neutralization effect, 10 µl of either diluted FCV (ca. 10^4 PFU L^{-1}) or TuV (ca. 10^7 $TCID_{50}$ L^{-1}) stock was mixed with product-neutralizer solution and assayed as described above. As for *C. difficile*, 10 µl of endospores (ca. 10^4 CFU L^{-1}) was directly added to the product-neutralizer solution.

Quantitative suspension test

Efficacy was first tested using ASTM standard E1052–11 (ASTM International, 2011) with several modifications (Figure 1b). Briefly, 10 µl of FCV, TuV, or *C. difficile* endospores was each mixed separately with 90 µl of undiluted disinfectant in a 1.5 ml centrifuge tube at room temperature for a designated contact time. Contact times were 1 min for FCV, 10 min for TuV and 10 min for *C. difficile* endospores. PBS was used as a negative control. Mixtures were neutralized by adding 900 µl of neutralizer (Table 1) then washed using Amicon® Ultra-4 centrifugal 30 K MWCO filters for both FCV and TuV, as described above. After removal of product residue, the retentate was collected and assayed with CRFK cells and LLC-MK2 cells for FCV and TuV, respectively (Figure 1b). Without using

centrifugal filters, *C. difficile* endospores were collected directly by centrifugation after neutralization and enumerated as described above.

Quantitative carrier test

Efficacy of the nine products was tested using ASTM standard E2197–17 with modifications (ASTM International, 2017). A sodium hypochlorite solution (1000 ppm) (Clorox) was used as a positive control. Briefly, each coupon of brushed stainless-steel (Muzeen & Blythe Ltd.) disk (1 cm in diameter) placed in a 24-well plate (Corning) was inoculated with 10 μl of one stock suspension of FCV, TuV or *C. difficile* endospores and dried for 1.5 h inside a biological safety cabinet set at room temperature (20–25°C) with 30%–50% relative humidity. Dried disks were then incubated with 90 μl of each disinfectant, whereas control disks only received 90 μl of appropriate neutralizers. After the designated contact time (1, 5 and 10 min for FCV and TuV, and 10 min for *C. difficile*), 900 μl of respective neutralizing broth (Table 1) was pipetted into each well to neutralize biocidal activity of disinfectant and to facilitate elution of virus or endospores from coupons. Samples were then assayed as described above. As for *C. difficile* endospores, coupons were first sonicated for 15 s at 40 kHz in a sonication bath (FS110; Fisher Scientific International) after neutralization then pipetted up and down 10 times to remove endospores from carrier coupons. Endospore suspensions were collected and enumerated as described above. Neutralization verification and cytotoxicity elimination were conducted as described in the ASTM standard (ASTM International, 2017). 'Efficacious' was defined as a 4-log reduction of FCV and a 6-log reduction of *C. difficile* endospores on hard non-porous surfaces (Environmental Protection Agency, 2018a; Environmental Protection Agency, 2018b). As TuV is not recognized by EPA as a target agent, 'Efficacious' was defined as a 3-log reduction of general viral surrogates was used (Environmental Protection Agency, 2018a).

Inactivation kinetics determination of four products against *C. difficile* endospores in suspension test

To determine whether concentration of active ingredients was correlated with efficacy of H_2O_2-based disinfectants, *D*-values of four products (*A*, *C*, *D* and *E*) that significantly inactivated *C. difficile* endospores were compared. The *D*-value, which indicates contact time needed to achieve a 1-log reduction of microorganism, was calculated from the inactivation kinetic curve using the following equation:

$$D = \frac{t}{\log_{10}\frac{N_0}{N_d}}$$

where *D* means *D*-value (min) at ambient conditions, N_0 indicates endospore population in the positive endospore control and N_d indicates surviving endospore population after a contact time of *t* (min). To accurately calculate *D*-values, log reductions for each of those products at five contact times were collected in suspension tests. When considering different inactivation rates, contact times for products *A* and *D* were 1, 2, 3, 4 and 5 min, while longer contact times of 5, 10, 15, 20 and 25 min were used to test products *C* and *E*.

Determination of synergistic effect of hydrogen peroxide and ferrous sulphate

Only H_2O_2-based products presented strong antimicrobial activity against FCV, TuV and *C. difficile* endospores in either suspension or carrier tests. Therefore, the efficacy of H_2O_2 (Honeywell, NC, USA) against FCV, TuV and *C. difficile* endospores was tested with the addition of $FeSO_4$ to H_2O_2-based products, known as the Fenton reaction. The Fenton reaction catalyses H_2O_2 to produce more hydroxyl radicals to oxidize proteins in microbial structures. This was done to better understand the effect of H_2O_2-based formulations against both HuNoV surrogates and *C. difficile* endospores. Hydrogen peroxide solutions of 0.5, 1, 3 and 5% (w/v) were prepared by diluting the concentrated H_2O_2 solution (50%) in deionized (DI) water and the pH adjusted to 2.90 ± 0.05 with 1 M citric acid. To determine the impact of the Fenton reaction, 0.025% (w/v) $FeSO_4$ was added into 1% (w/v) H_2O_2 solution compared to 0.025% (w/v) $FeSO_4$ in DI water, which was used as a negative control.

Statistical analysis

Four replicates of 10-fold serial dilutions of each product were tested in two independent experiments. Log reductions were calculated by $\log_{10}(N_0/N_d)$, where N_d is the average microbial population from the treatment samples and N_0 is the average microbial population from each control sample. Statistical analysis was performed using a one-way multiple-comparison *t*-test to determine the relationship between contact time and log reduction. All results were expressed as mean ± standard deviation. Statistical significance was defined as a *p*-value of <0.01 to establish a more conservative estimate of efficacy. Statistical analyses were conducted using GraphPad Prism 6.01 (GraphPad Software, Inc.).

RESULTS

Quantitative suspension test

Cytotoxicity of each product was eliminated with an ingredient-specific neutralizer (Table 1) before efficacy testing began. Although 5% FBS initially did not neutralize product I, the wash step using centrifugal filters eliminated all remaining cytotoxicity (<1 log reduction of viruses and *C. difficile* endospores).

After a 1 min contact time, the four H_2O_2-based products (A-D) and one ethanol-based product (I) achieved a 5.1, 4.1, 5.0, >5.4 and 5.2 log reduction of FCV, respectively, whereas the remaining four products (E-H) achieved a 2.7, 2.2, 0.3 and 1.9 log reduction of FCV (Table S2). Six products (A–D, H and I) achieved a 3.8, 3.4, 3.8, 3.9, 3.8 and 4.3 log reduction of TuV after 10 min contact time, respectively, whereas products E, F and G achieved a 2.5, 0.2 and 1.8 log reduction, respectively. Only product D listed a 10-min contact time against *C. difficile* endospores on its label (Table 1), but products A and D both showed a >6.0 log reduction of *C. difficile* endospores after 10 min. All other products were not efficacious against *C. difficile* endospores.

Quantitative carrier test

Seven of nine products (i.e. A–D, F, H and I) were efficacious against FCV, all achieving a >5.1 log reduction after 5 min (Figure 2). Although product E was not efficacious against

FCV after 5 min, it was efficacious after 10 min which was in agreement with the label claim (Table S3). Sodium hypochlorite solution (1000 ppm) and product D were efficacious against TuV (≥3-log reduction after 5 min) (Figure 2). As for *C. difficile* endospores, four products (A, C, D and E) showed sporicidal activity, but only product D was considered efficacious (≥6-log reduction of *C. difficile* endospores after 10 min) (Figure 2).

Inactivation kinetics against *C. difficile* endospores in suspension test

Because only four products (A, C, D and E) inactivated *C. difficile* endospores in the carrier test, inactivation kinetics of those four products were determined based on a suspension test to clearly illustrate relationships between concentration and efficacy. Products A and D achieved a >6.0 log reduction of *C. difficile* endospores in 4 and 3 min, respectively (Figure 3), whereas products C and E failed to achieve 6.0-log reduction by 25 min. When compared with D-values of 0.7 and 0.4 min for products A (0.5% H_2O_2) and D (3.13% H_2O_2), respectively, product E had a higher H_2O_2 concentration (5%) but a higher D-value (5.6 min) against *C. difficile* endospores. Although product C had a H_2O_2 concentration (1.4%) higher than product A, the D-value (6.2 min) for product C was greater than that of product A.

Synergistic effect of hydrogen peroxide and ferrous sulphate

Laboratory-prepared solutions of 3% H_2O_2 and 5% H_2O_2 achieved a 3.9- and 4.1-log reduction of FCV after 1 min (Figure 4) in a suspension test, with both solutions also achieving a 1.6 and 1.8 log reduction of TuV after 10 min and a 1.5 and 2.1 log reduction of *C. difficile* endospores after 10 min, respectively. At lower concentrations of H_2O_2 (≤1%), the efficacy was diminished for FCV (<2.1 log after 1 min), TuV and *C. difficile* endospores (0.7 and 0.3 log after 10 min, respectively).

The efficacies of five commercial H_2O_2-based disinfectants (products A–E) were compared with laboratory-prepared H_2O_2 solutions. Except for product E, four H_2O_2-based products (A–D) showed higher antiviral activity (additional ≥1.5-log reduction of FCV or TuV) than pure H_2O_2 solutions with equivalent concentrations (Figure 4). Three of these five products (products A, C and D) were more efficacious against *C. difficile* endospores than pure H_2O_2 solutions. Products A and D with lower concentrations of H_2O_2 achieved a >6.0 log reduction of *C. difficile* endospores as compared with a 2.0-log reduction by a 5% H_2O_2 solution. Product C containing 1.4% H_2O_2 achieved a 1.4 log reduction of *C. difficile* endospores while 1% H_2O_2 solution only achieved a 0.3 log reduction. Surprisingly, product E with 5% H_2O_2 had lower activity against FCV and *C. difficile* than 5% pure H_2O_2 solution. Overall, no strong correlation ($R^2 = 0.25$, $p = 0.03$) between log reduction and H_2O_2 concentration was observed.

Ferrous sulphate at 0.025% had a minimal effect on FCV, TuV and *C. difficile* endospores with ≤0.2 log reduction after 1, 10 and 10 min, respectively (Figure 5). Addition of 0.025% ferrous sulphate to 1% H_2O_2 solution improved efficacy against FCV, TuV and *C. difficile* resulting in additional 1.4, 0.4 and 0.9 log reduction, respectively.

DISCUSSION

We determined the efficacy of a panel of nine EPA-registered disinfectants against two HuNoV surrogate viruses (FCV and TuV) and *C. difficile* endospores. First, we found that eight of the nine product claims could be verified via our testing methods, suggesting our methods were more conservative than those required by the EPA. Second, H_2O_2-based products presented strong disinfection efficacy against FCV, TuV and *C. difficile* endospores. Lastly, the production formulation, not just concentration of active ingredients, affects product efficacy.

Product claims for all nine disinfectants, except for product G, were verified against FCV (Environmental Protection Agency, 2020b). Product G was the only product not efficacious against FCV or TuV presumably due to differences in test conditions (inoculum volume and drying time) recommended by the EPA and ASTM testing methods (ASTM International, 2017; Environmental Protection Agency, 2000a; Environmental Protection Agency, 2000b). A likely explanation for this observation is that the differences in inoculum volume and drying time between the two methods led to differences in virus susceptibility to tested disinfectants. Viruses are more susceptible to disinfectant activity in suspension than when dried on carriers (Park et al., 2007). Moreover, our modified ASTM testing methods used smaller inoculum volume and longer drying time leading to more conservative estimates of disinfectant efficacy than what was reported on product claim labels. Neutralizing the disinfectants after the specified contact time to eliminate potential cytotoxicity is critical for an efficacy test when using a cell culture to measure reduction of viral infectivity. Cell death caused by potential cytotoxicity of disinfectants cannot be distinguished from the cytopathic effect caused by viral infectivity; thus, strong cytotoxicity can result in difficulties to estimate product efficacy (Geller et al., 2009). Our testing protocol which included a ‘wash step’ was designed to minimize the cytotoxicity of the disinfectants. In addition, concentrating disinfectant-treated viruses by ultrafiltration has been shown to maintain infectivity of SARS-CoV-2, a more sensitive virus than HuNoV, suggesting our testing method was more conservative than the EPA carrier methods (Welch et al., 2020).

Chlorine-based disinfectants show efficacy against *C. difficile* endospores due to their oxidation activity, whereas QACs and alcohols are ineffective against bacterial endospores and HuNoV (Boyce, 2021; Cromeans et al., 2014; Ha et al., 2016). In our study, only product D with H_2O_2 as the main active ingredient made a claim against both HuNoV and *C. difficile* endospores. The other three H_2O_2-based products (A, C and E), without any claim against TuV and *C. difficile* endospores, showed efficacy against FCV, TuV and *C. difficile* endospores in a suspension test. Moreover, two products (A and D) containing H_2O_2 were efficacious against FCV, TuV and *C. difficile* endospores, while disinfectants that contained other active ingredients (i.e. QACs and alcohols) were not efficacious against *C. difficile* endospores. Alcohol and QAC have a limited impact on the surface structure of bacterial endospores (Russell, 2001), whereas H_2O_2, which yields hydroxyl radicals, was reported to be toxic to some bacterial endospores and viral particles (Linley et al., 2012; Sugiura et al., 1982).

No strong correlation was found between the concentration of H_2O_2-based disinfectants and log reduction. Specifically, the D-values (>5 min) of two H_2O_2-based products (C and E) were greater than those of products A and D, which contained even lower concentrations of H_2O_2. These higher D-values may be explained by the interactions between active ingredients and inert ingredients (Cromeans et al., 2014), added to improve cleaning performance, aesthetics, formulation stability and hard water tolerance (Fraser et al., 2021). In addition to H_2O_2, accelerated hydrogen peroxide contains surfactants and other inert ingredients, which act synergistically to yield an efficacious disinfectant (Grascha & Battut, 2014; Ramirez & Omidbakhsh, 2014; Ramirez & Rochon, 2004; Watts et al., 2007). For example, H_2O_2 is commonly stabilized by organic ligands (e.g. citric acid and malonic acid) to prevent self-degradation (Watts et al., 2007). Adding ferrous ions to a H_2O_2 solution, known as Fenton reaction, enhances H_2O_2 reactivity (Cross et al., 2003; Hayyan et al., 2016; Nieto-Juarez et al., 2010; Polo et al., 2018; Tong et al., 2020). Production of hydroxyl radicals and hydroperoxyl radicals during the Fenton reaction is believed to cause cytotoxicity leading to DNA damage and protein denaturation (Nieto-Juarez et al., 2010; Tong et al., 2020). In our study, the inclusion of ferrous ions increased the efficacy of 1% stabilized H_2O_2 solution and resulted in an additional reduction of FCV, TuV, and *C. difficile* endospores.

In agreement with previously reported data (Cromeans et al., 2014), TuV was more resistant to disinfectants than FCV presumably due to differences in their viral capsid structures (Bailey et al., 2008). Preserving amino acid residue G329 of the S-P1 hinge region of FCV is critical to maintain its infectivity (Ossiboff et al., 2010). However, TuV has an isoleucine residue instead of glycine at this position, which is less impacted by oxidation (Dean et al., 1997; Yu et al., 2013). In addition, the structure of TuV virion is more similar to HuNoV than other genera in the family of *Caliciviridae* (Yu et al., 2013). Furthermore, like HuNoV, TuV utilizes histo-blood group antigens as binding ligands to infect cells (Tan et al., 2009).

The differences in the efficacy claims by the manufacturer and our data likely can be explained by the use of different testing methods. Though we conservatively estimated the efficacy of EPA-registered disinfectants on stainless-steel carriers, efficacy needs to be validated on other surfaces due to the effect of different surface characteristics (e.g. roughness and water absorbance). Although TuV was confirmed as a more conservative surrogate for HuNoV than FCV, ultimately our findings need to be validated using the recently reported human intestinal enteroid system for HuNoV (Costantini et al., 2018).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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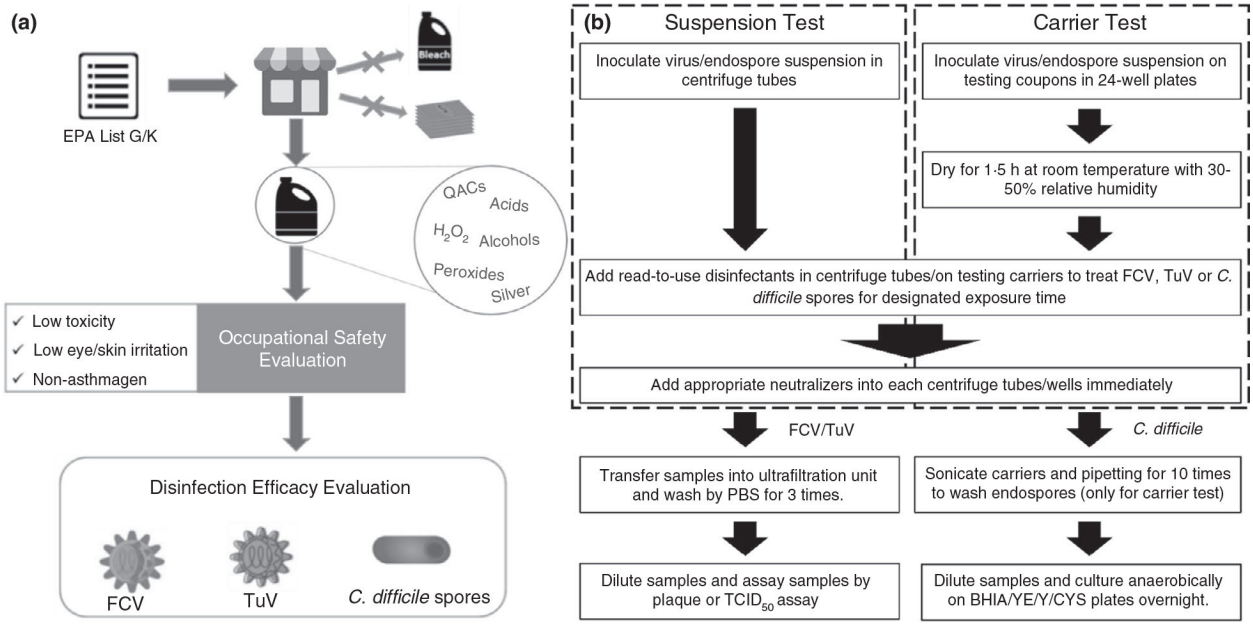

FIGURE 1.
Selection criteria of disinfectants (a) and workflow for suspension test and carrier test

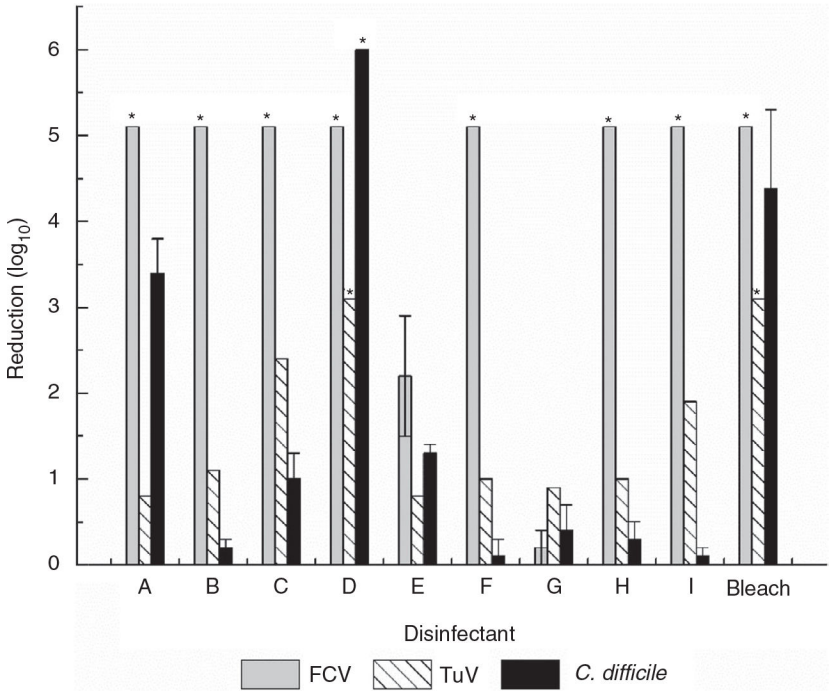

FIGURE 2.

Efficacy of nine EPA-registered disinfectants and bleach (1000 ppm) against FCV, TuV and *C. difficile* spores on stainless-steel carriers. Contact time for FCV, TuV and *C. difficile* spores was 5, 5 and 10 min, respectively. Error bars represent standard deviations from replicates in two independent experiments, and stars represent reaching limits of detection

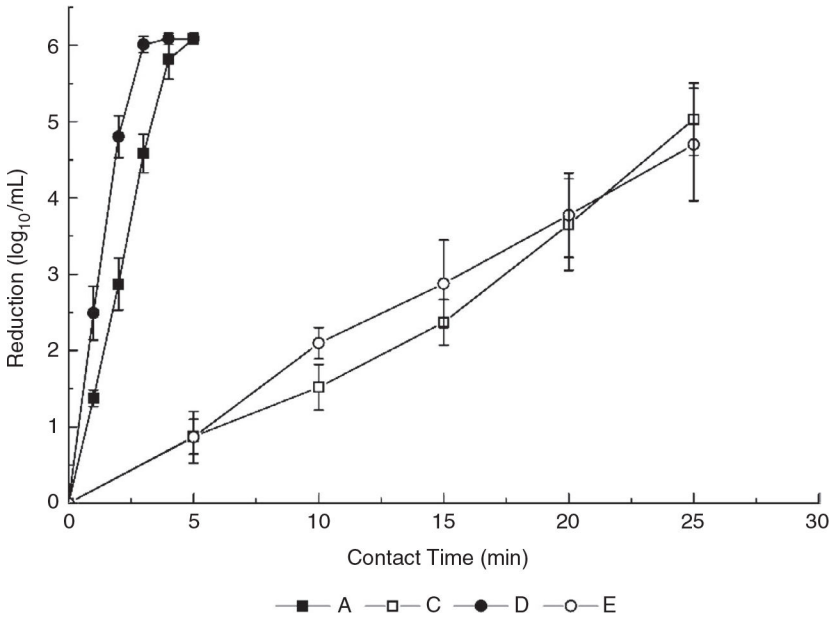

FIGURE 3.

Inactivation curves of products a (■), C (□), D (●) and E (○) against *C. difficile* spores. Error bars represent standard deviations from replicates in two independent experiments. Contact times were only for accurate calculation of D-values, not for comparison of disinfectant efficacies

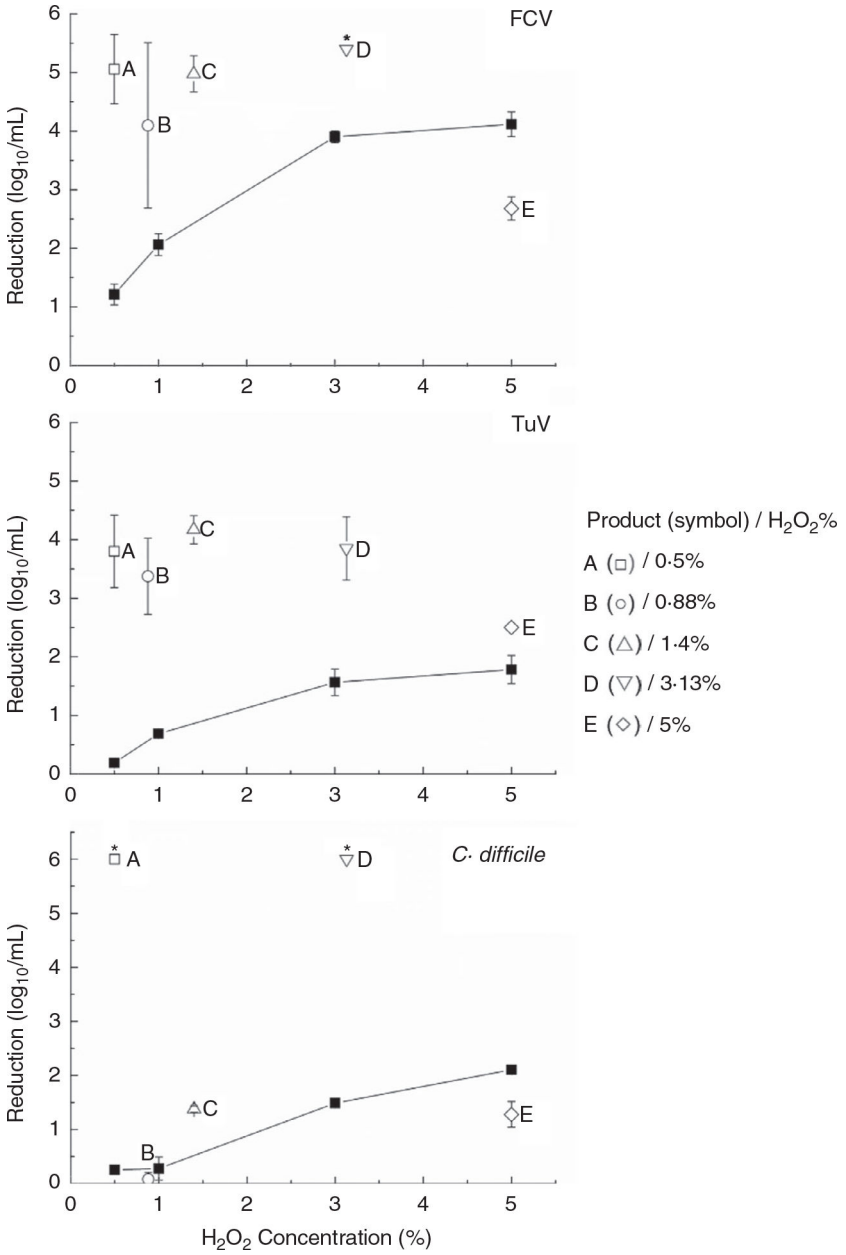

FIGURE 4.

Efficacy of H_2O_2 against FCV, TuV and *C. difficile* spores at contact times of 1, 10 and 10 min, respectively. Solid squares indicate efficacy of laboratory prepared H_2O_2 solutions at various concentrations (0.5, 1, 3 and 5%), and open symbols indicate inactivation efficacy of 5 commercial H_2O_2-based disinfectants (a, b, c, d and e). Error bars represent standard deviation from replicates in two independent experiments, and stars represent reaching limits of detection

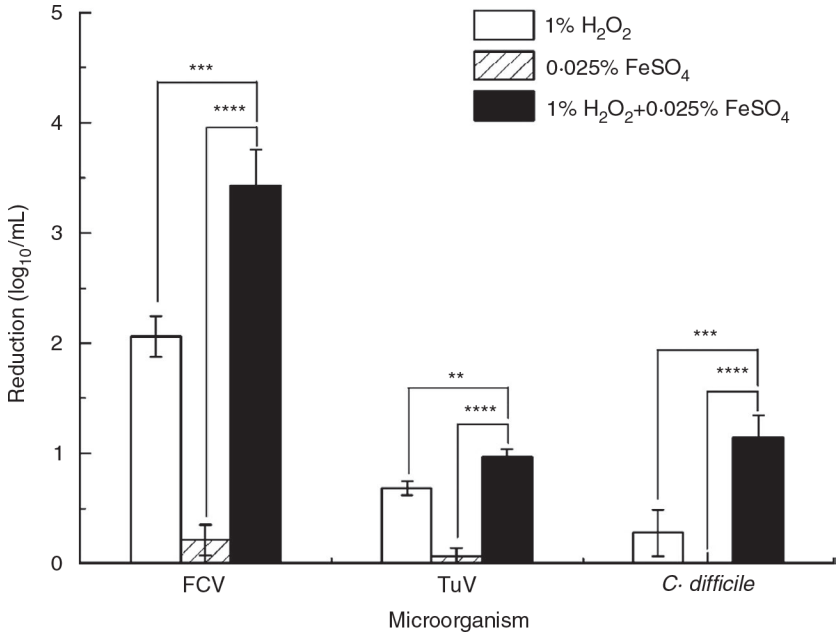

FIGURE 5.

Efficacy of H_2O_2 against FCV, TuV and *C. difficile* spores as affected by ferrous sulphate. White bars indicate efficacy of 1% H_2O_2, bars with slash pattern indicate efficacy of 0.025% $FeSO_4$ and black bars indicate efficacy of 1% H_2O_2 + 0.025% $FeSO_4$. The contact times for FCV, TuV and *C. difficile* spores were 1, 10 and 10 min, respectively. Error bars represent standard deviations from replicates in two independent experiments. The *p*-value among treatments for each micro-organism was ≤0.01 (**), ≤0.001 (***) and ≤0.0001 (****), respectively

TABLE 1

Active ingredients of selected disinfecting products and appropriate neutralizers

Product	Active ingredient	Label contact time (min)[a]		pH	Neutralizer (concentration)	Cytotoxicity/CPE after neutralization
		FCV	*C. difficile*			
A	0.5% hydrogen peroxide	1	NA	2.64	catalase (1300 U l^{-1})	_[b]
B	0.88% hydrogen peroxide	10	NA	2.85	catalase (1300 U l^{-1})	−
C	1.4% hydrogen peroxide	1	NA	2.38	catalase (1300 U l^{-1})	−
D	3.13% hydrogen peroxide/0.099% octanoic acid/0.05% peracetic acid	4	10	2.95	catalase (1300 U l^{-1})	−
E	5% hydrogen peroxide/0.005% silver	10	NA	3.07	catalase (1300 U l^{-1})	−
F	4.85% citric acid/0.003% silver	10	NA	1.79	FBS (5%)	−
G	0.2% chlorine dioxide/0.125% alkyl dimethyl benzyl ammonium chloride/0.125% alkyl dimethyl ethylbenzyl ammonium chloride	5	NA	8.68	FBS (5%) + sodium thiosulphate (0.1%)	−
H	15% isopropanol/7.5% ethanol/0.76% didecyldimethylammonium chloride	1	NA	12.17	FBS (5%) + sodium thiosulphate (0.1%)	−
I	29.4% ethanol	0.5	NA	13.07	FBS (5%)	+[c]

[a]Recommended contact time against FCV listed on product labels; NA represents ‘not available’.

[b]No cytotoxicity/CPE to both cell lines after neutralization, that is, <1 log reduction of viruses or spores in neutralization effectiveness treatments.

[c]Cytotoxicity of samples was finally neutralized by washing with centrifugal filters.