



# Activity of Hospital Disinfectants against Vegetative Cells and Spores of *Clostridioides difficile* Embedded in Biofilms

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**ABSTRACT** *Clostridioides difficile* spores can survive in the environment in either mono- or mixed-species biofilms. However, no previous studies have investigated chemical disinfection of *C. difficile* spores embedded in biofilms. Thus, the purpose of this study was to assess the *in vitro* effectiveness of hospital disinfectants against *C. difficile* spores embedded within biofilms. Five unique *C. difficile* strains embedded in three different biofilm types grown for 72 or 120 h were exposed to seven different hospital disinfectants. *C. difficile* abundance [as log(number of CFU/milliliter)] was calculated after manufacturer-determined contact times along with biofilm biomass and microscopy. The primary analysis compared differences between *C. difficile* vegetative cell and spore counts as well as amounts of biomass after exposure to disinfectants. *C. difficile* vegetative cells and spores were recovered from biofilms regardless of the type of biofilm growth or biofilm growth time. No disinfectant was able to completely eliminate *C. difficile* from the biofilms. Overall, Clorox, ortho-phthalaldehyde (OPA), and Virex were most effective at killing *C. difficile* spores regardless of biofilm age, ribotype, or wash conditions (whether biofilms are washed or unwashed) ( $P = 0.001$ , each). Clorox and OPA were also effective at killing total vegetative cell growth ( $P = 0.001$ , each), but Virex was found to be ineffective against vegetative cell growth in biofilms ( $P = 0.77$ ). Clorox and Virex were most effective in reducing biomass, followed by Nixall, OPA, and Vital Oxide. No disinfectant was able to completely eliminate *C. difficile* embedded within biofilms although differences among disinfectants were noted. Future research will be required to determine methods to eradicate this persistor reservoir.

**KEYWORDS** *in vitro* study, anaerobic infections, biofilm, environmental decontamination, ribotype 027

*Clostridioides difficile* is a Gram-positive, obligate, anaerobic, spore-forming bacterium and the most common health care-acquired infection in the United States (1). Spores can be transmitted via symptomatic and asymptomatic carriers to the environment or via health care personnel (2, 3). To break the transmission cycle, hospitals and health care institutions commonly use chemical sporicidal agents for environmental surface cleaning (4, 5). Current guidelines of the United Kingdom Department of Health recommend the use of chlorine-based disinfectants to reduce *C. difficile* spore levels in clinical settings (5, 6). As per the Infectious Diseases Society of America (IDSA), daily cleaning with a sporicidal agent in conjunction with other modalities is recommended in the case of an outbreak, hyperendemic settings, or a high rate of repeated infection (7). However, clinical studies investigating chlorine-based disinfectants found that no disinfectant tested achieved adequate disinfection within the labeled determined contact time in either a clean or dirty environment (6). The reasons for this lack of efficacy are unclear but may relate to the presence of biofilms.

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*In vitro* studies that evaluate the efficacy of sporicidal disinfectants generally test the compound against planktonic bacteria or spores (8). However, it is likely that *C. difficile* survives in the abiotic environment in either mono- or mixed-species biofilms (9). Biofilm formation by *C. difficile* was first reported in 2012 (10, 11). Although biofilm production can vary between strains, *C. difficile* biofilms have been shown to form a complex multilayered protein, containing DNA and polysaccharide, in the gut as well as on abiotic surfaces (9, 12, 13). The exopolysaccharide (EPS) matrix provides an anaerobic scaffold that supports both vegetative cell growth and spores (10). Multiple variables influence biofilm formation, including virulence-associated proteins (cwp84, flagella, transcription factors, and SpoA) and the quorum sensing regulator LuxS (9, 11). Using mixed-species biofilms, a coinfection model of *Fingoldia magna* and *C. difficile* enhanced biofilm formation of both bacteria (11), and another polymicrobial biofilm model described *C. difficile* spores embedded in the biofilm (14).

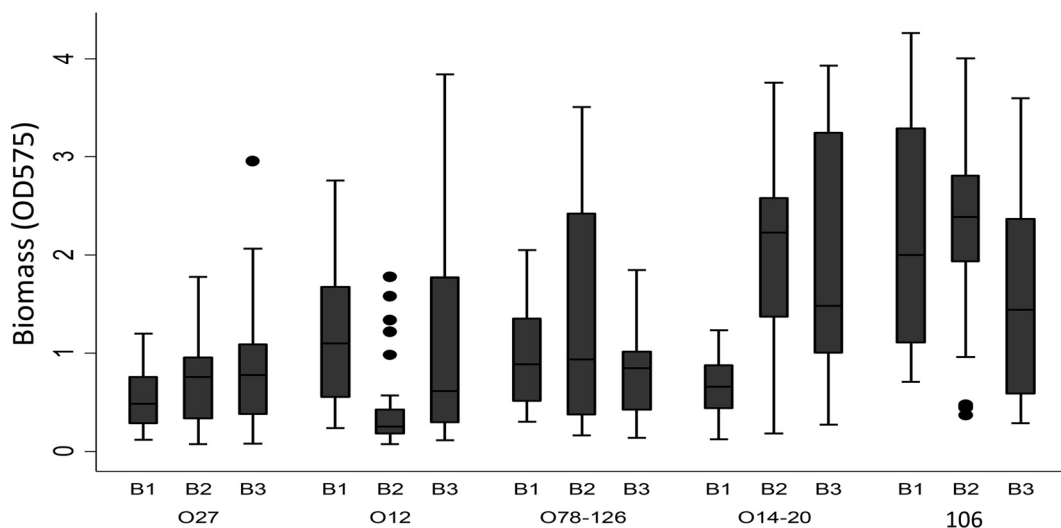
Most sporicidal disinfectants are not as effective against biofilm embedded spores (15–18). Microorganisms in biofilm may be up to 1,000-fold more resistant to disinfectants than their planktonic counterparts. For example, spores of *Bacillus cereus* embedded in a biofilm are highly resistant to cleaning procedures (19). After 6 days of exposure to chemical disinfection, *Bacillus* spores could still be observed within a biofilm using microslicing techniques (20). *Penicillium brevicompactum* spores were also resistant to chlorine disinfection when embedded within a biofilm but were killed as free spores (21). Biofilm properties that affect the efficacy of chemical disinfectants include the age of the biofilm and cell density (22, 23). Despite this known association on spores and biofilms, few studies have investigated chemical disinfection of *C. difficile* spores embedded in biofilms. Thus, the purpose of this study was to assess the *in vitro* effectiveness of hospital-based disinfectants against *C. difficile* spores within a mono- or mixed-species biofilm at various stages of biofilm development.

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## RESULTS

***C. difficile* biofilm.** *C. difficile* vegetative cells and spores were grown from all ribotypes regardless of the type of biofilm growth (single versus mixed species or anaerobic versus aerobic) or biofilm growth time. Biofilms were visualized starting on day 2 of growth attached to the base of 24-well plates. No statistically significant difference was observed among the three modes of biofilm formation ( $P = 0.243$ ). Overall, all ribotypes were able to form biofilms although amounts of biomass differed among them (Fig. 1). The mean log CFU counts of vegetative cells and spores for 72-h biofilm between ribotypes were  $2.77 \pm 0.07$  and  $1.77 \pm 0.06$  CFU/ml, respectively. Average log CFU counts of vegetative cells and spores were  $3.11 \pm 1.62$  CFU/ml and  $1.99 \pm 1.46$  CFU/ml, respectively, for *C. difficile* grown in 72-h biofilms,  $2.84 \pm 1.43$  CFU/ml and  $1.78 \pm 1.14$  CFU/ml, respectively, for *C. difficile* grown in monospecies anaerobic biofilms, and  $2.35 \pm 1.36$  and  $1.54 \pm 0.97$  CFU/ml, respectively, for multispecies anaerobic biofilms. Average log CFU counts of vegetative cells and spore counts for 72-h biofilm by precleaning status were  $3.06 \pm 1.40$  CFU/ml and  $2.04 \pm 1.22$  CFU/ml, respectively, for washed biofilm and  $2.47 \pm 1.54$  CFU/ml and  $1.51 \pm 1.17$  CFU/ml, respectively, for unwashed biofilms. Similar results were seen in 120-h biofilms with the exception that the vegetative cell counts were lower than those observed in biofilms grown for 72 h ( $P < 0.01$ ).

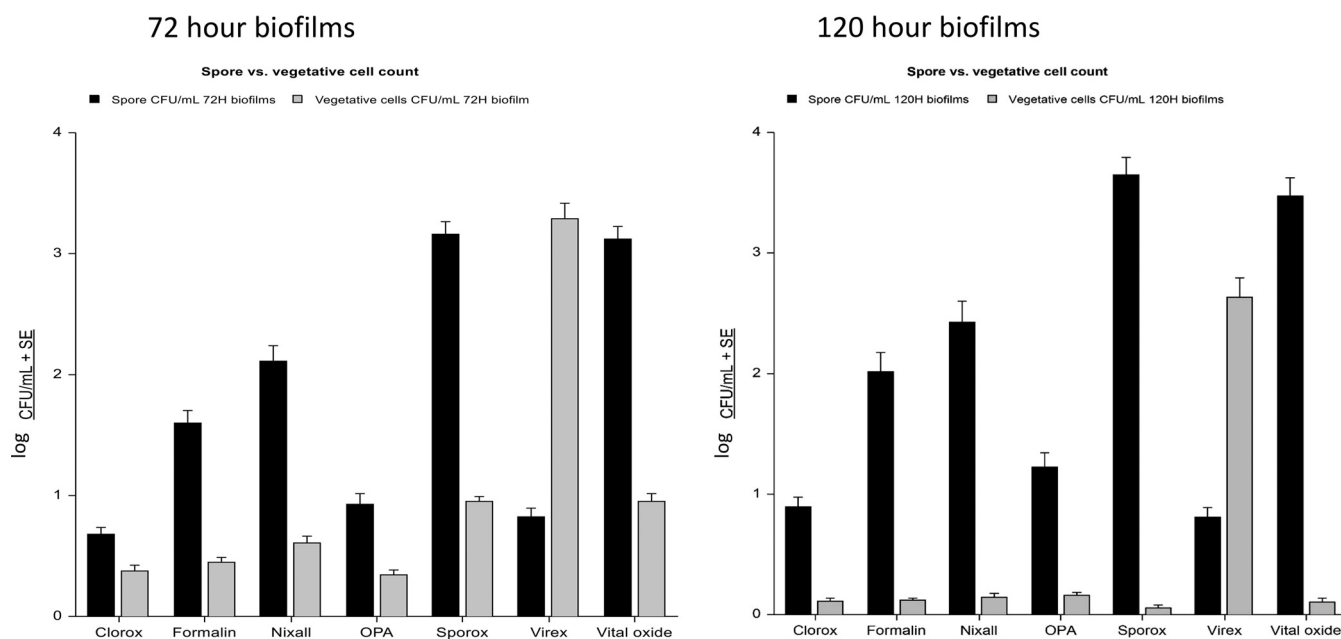
***C. difficile* spore and vegetative cells embedded within biofilms after disinfectant exposure.** The efficacy of seven hospital disinfectants to kill *C. difficile* embedded within biofilms grown for 72 h and 120 h is shown in Fig. 2. No disinfectant was able to completely eliminate *C. difficile* from the biofilms. Overall, Clorox, *ortho*-phthalaldehyde (OPA), and Virex were most effective at killing *C. difficile* spores regardless of biofilm age, ribotype, or wash conditions (i.e., washed or unwashed biofilm) ( $P = 0.001$ , each). Clorox and OPA were also effective at killing vegetative cell growth ( $P = 0.001$ , each) but Virex was found to be ineffective against the vegetative cell growth ( $P = 0.77$ ). This



**FIG 1** Biofilm mass for different *C. difficile* ribotypes. B1, *C. difficile* monospecies biofilm; B2, mixed-species biofilm grown anaerobically; B3, mixed-species biofilm grown aerobically.

same effect was noted in biofilms grown for 72 and 120 h. Formalin and Nixall were not as effective as Clorox, OPA, or Virex but were more effective than Sporox, which did not have a sporicidal effect. Similar results were observed regardless of biofilm preparation method (*C. difficile* single species, anaerobic mixed species, or aerobic mixed species) (Fig. 3).

**Efficacy of disinfectants against *C. difficile* in a biofilm.** Figure 4 shows the log reduction in CFU count for vegetative cells and spores in 72- and 120-h biofilms. The mean log reduction of CFU count for all disinfectants was  $1.74 \pm 0.07$  and  $1.83 \pm 0.06$  CFU/ml, respectively, for vegetative cell counts and spores in a 72-h biofilm. Similar results were observed for 120-h biofilm. Most of the disinfectants were ineffective in reducing the vegetative cell and spore counts by more than 2 logs. A regression analysis was done to look at the effect of disinfectants on total and spore counts of 3-



**FIG 2** Biofilm embedded *C. difficile* spore and vegetative cell counts (log CFU counts/milliliter) after exposure to disinfectants grown in biofilms for 72 or 120 h, as indicated.

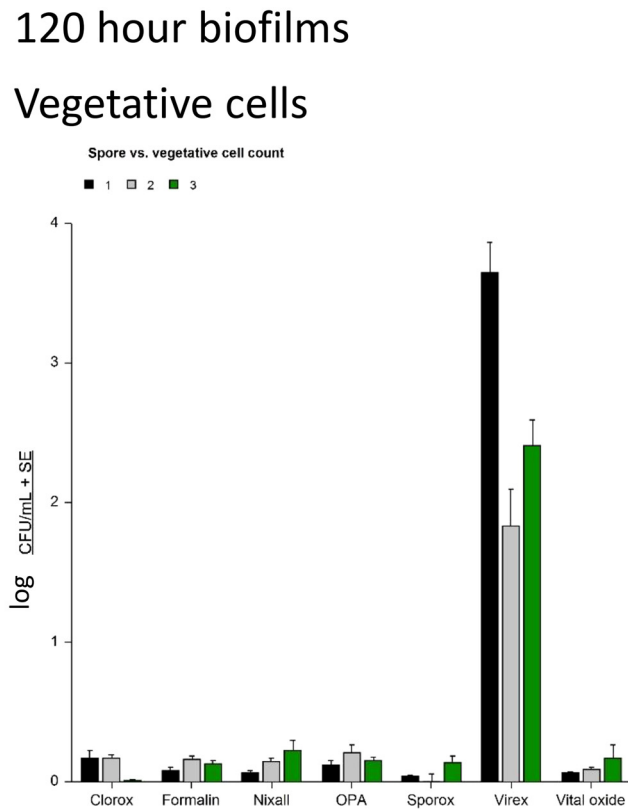
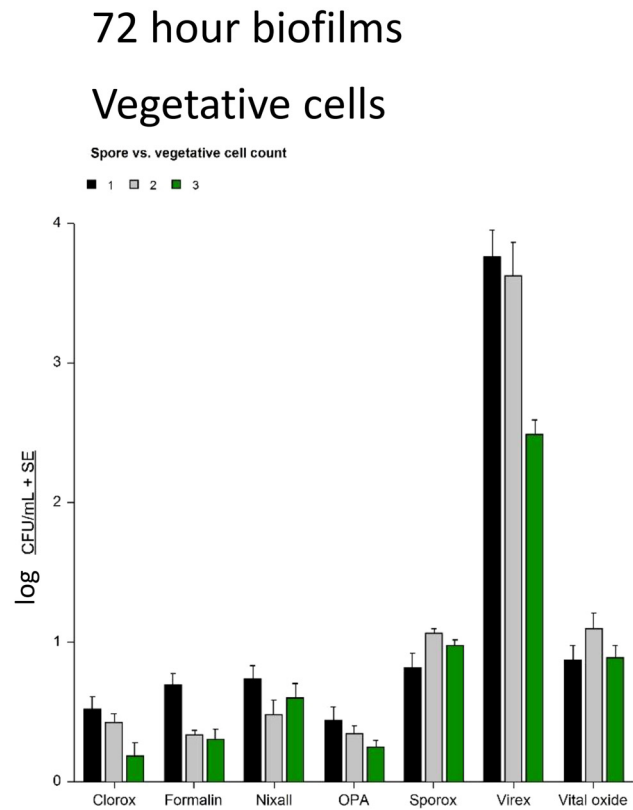
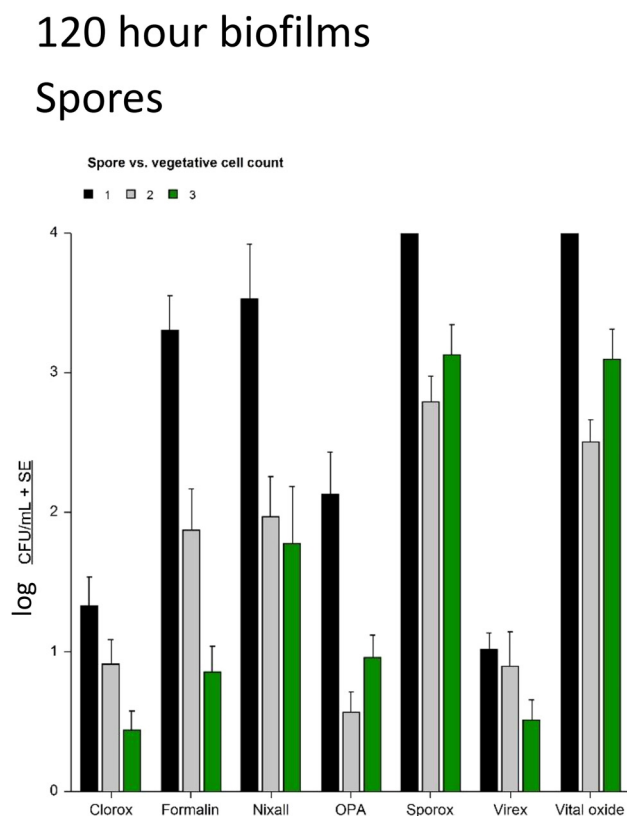
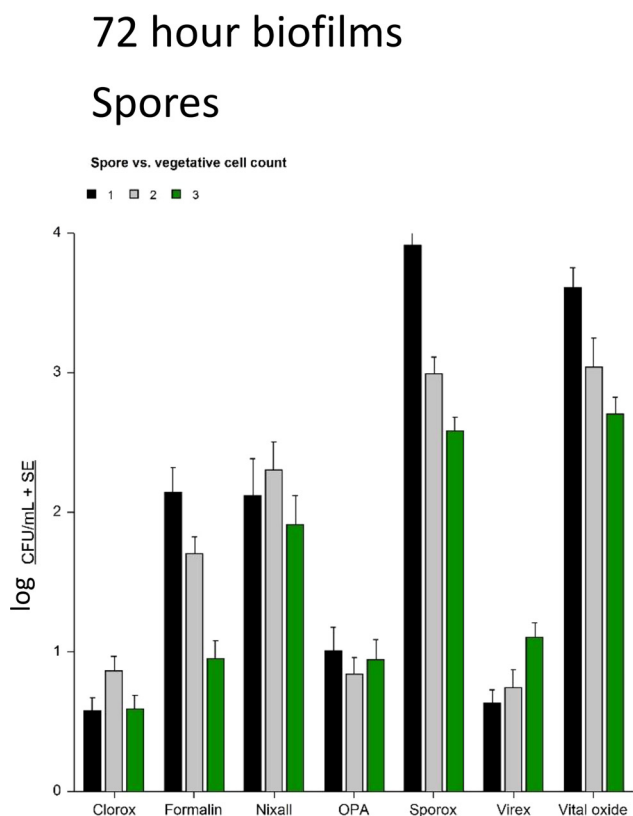


FIG 3 Killing effect of disinfectant based on biofilm type.

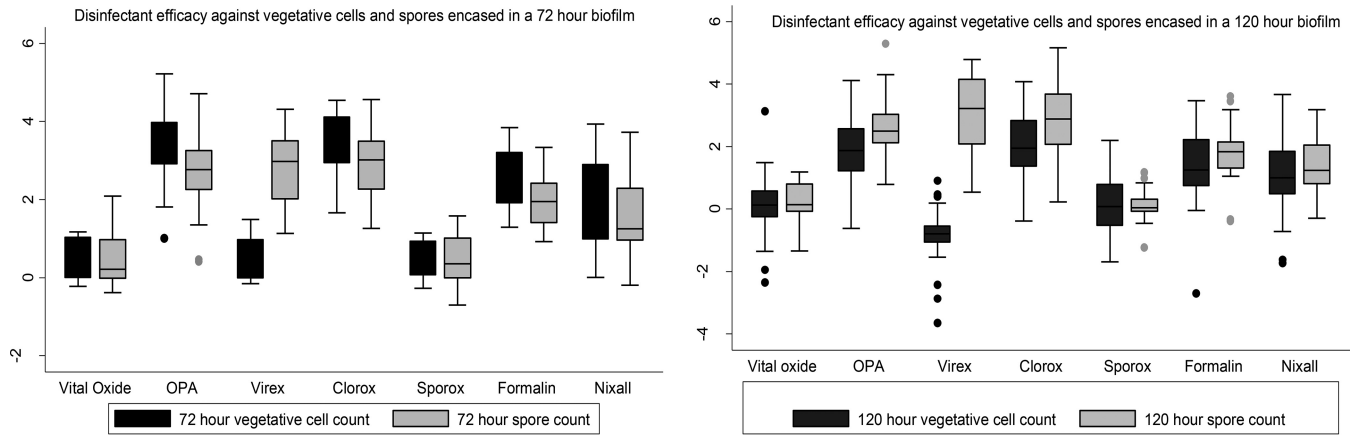


FIG 4 C. difficile vegetative and spore cell counts based on disinfectant and biofilm growth time.

and 5-day-old biofilms adjusting for ribotypes, duration of exposure, type of biofilm, and presence of organic matter. As Vital Oxide was found to be ineffective against planktonic *C. difficile* and all three stages of *C. difficile* biofilm, the efficacy of other disinfectants was compared to that of Vital Oxide. There was mostly a statistically significant ( $P < 0.001$ , all) reduction in total and spore counts for both 3- and 5-day-old biofilms except for Sporox and Virex. Sporox increased the total and spore counts in all cases, and Virex increased the total count but reduced the spore count.

**Biomass of biofilms after exposure to disinfectants.** The effect of disinfectants on biomass of biofilms is shown in Fig. 5. Overall, Clorox and Virex were most effective in reducing biomass, followed by Nixall, OPA and Vital Oxide. There was no reduction of biomass postexposure to Sporox and formalin. The same effect was noted in biofilms grown for 72 h and 120 h. An *F* test revealed a strong association between disinfectant efficacy in reducing vegetative cell count and biofilm mass ( $P = 0.0002$ ), which remained significant even after adjusting for the type of disinfectant, ribotype, and

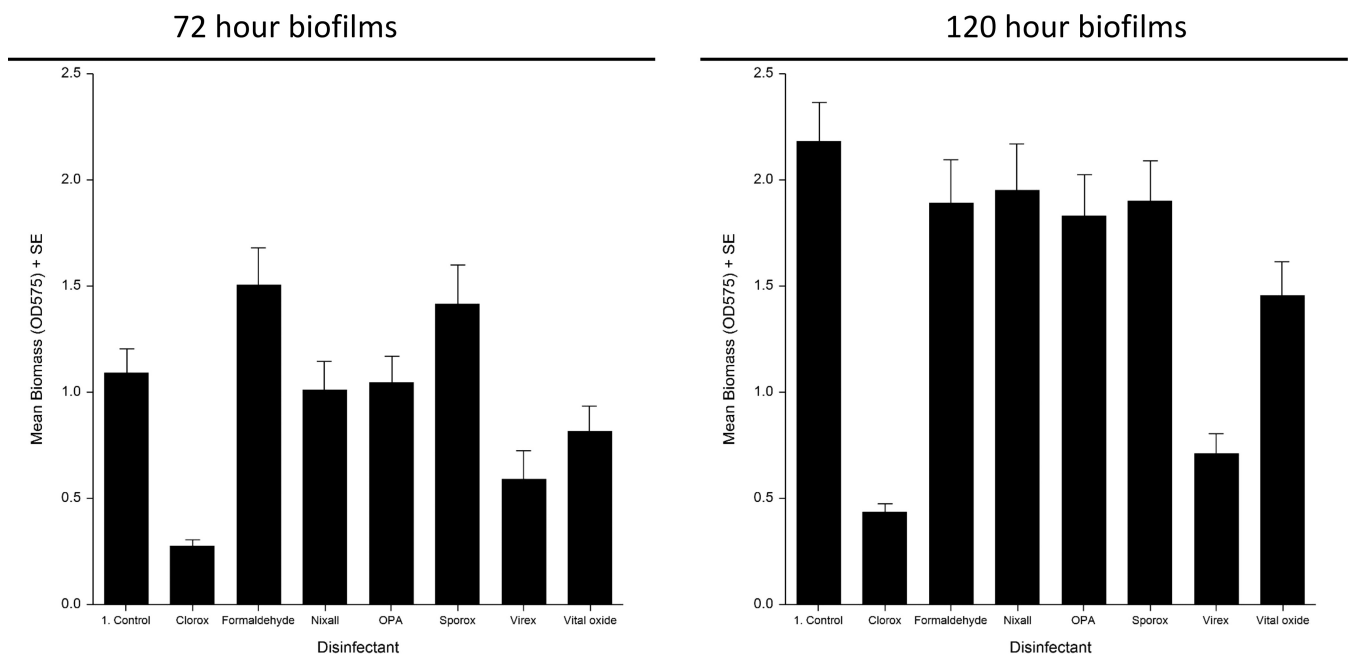
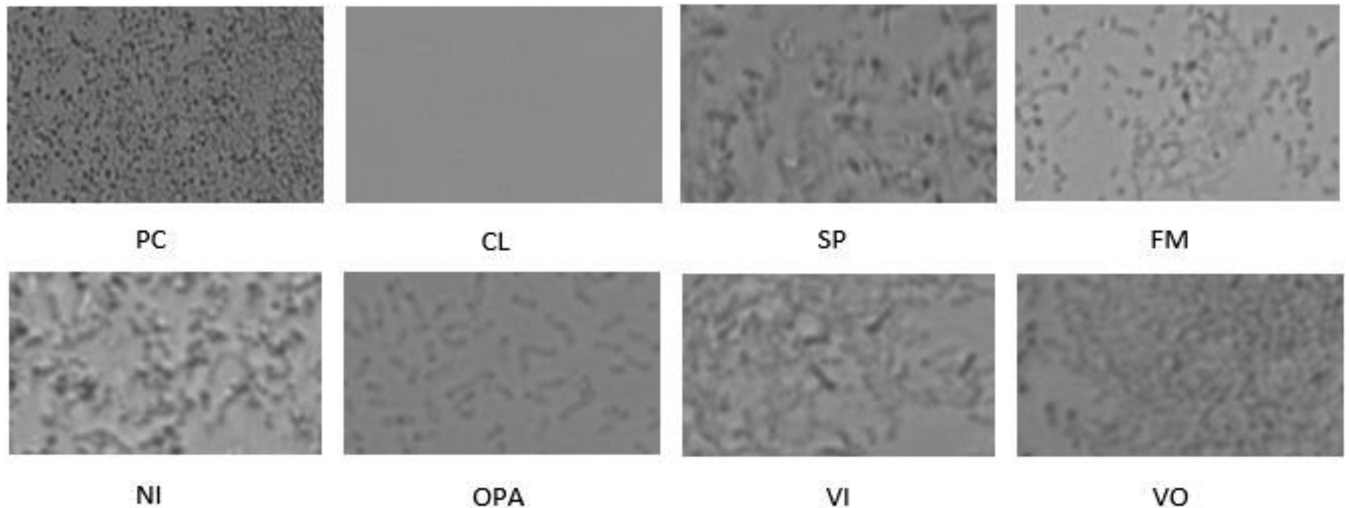


FIG 5 Effect of disinfectants on biomass of biofilms grown for 72 and 120 h.



**FIG 6** Visualization of killing effect of disinfectants on monocellular *C. difficile* biofilm on a surface using light microscopy. PC, positive control; CL, Clorox; SP, Sporox; FM, formalin; NI, Nixall; OPA, *ortho*-phthalaldehyde; VI, Virex; VO, Vital Oxide.

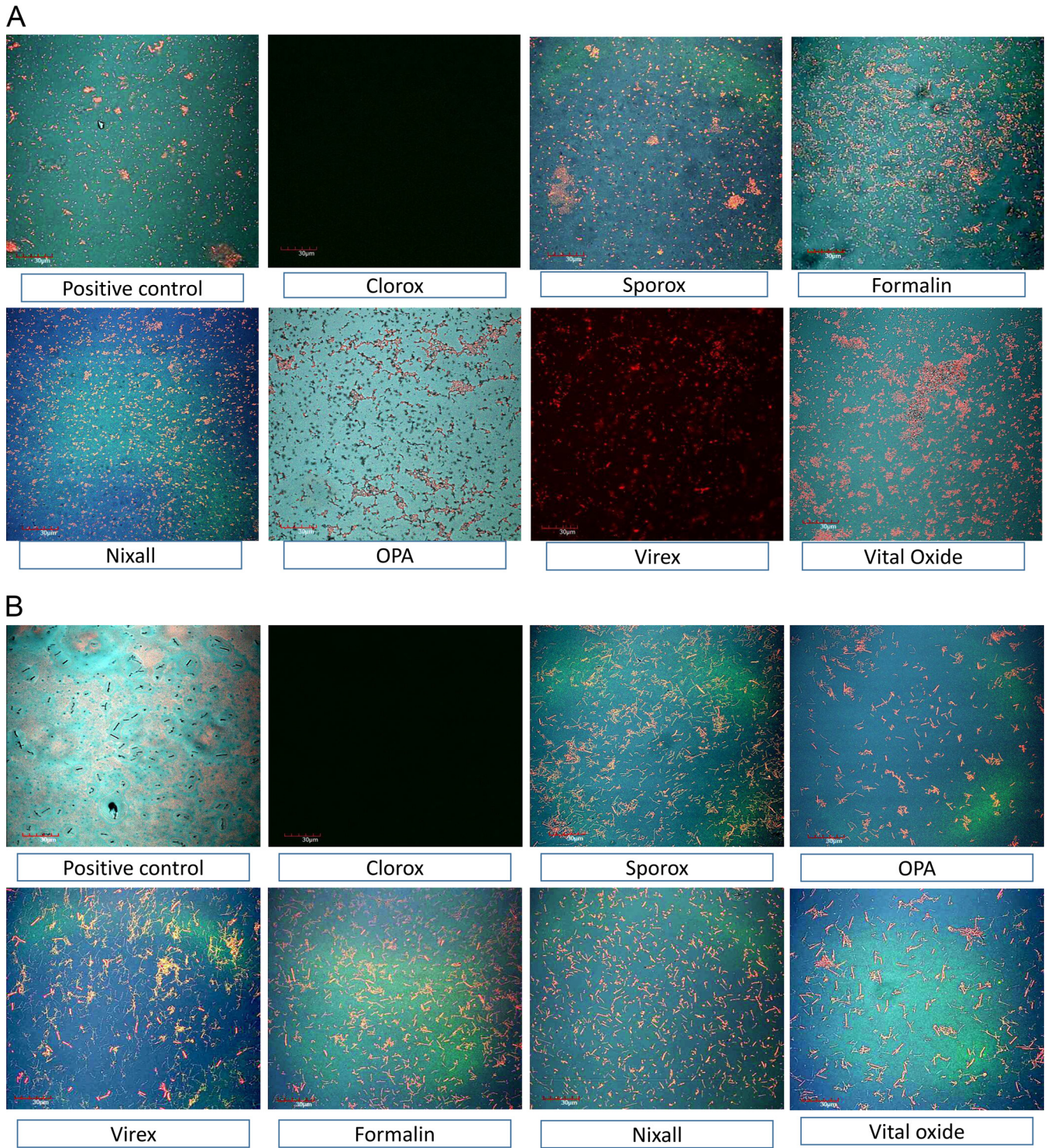
stage of biofilm growth ( $P < 0.01$ ). Similar associations were observed for disinfectant efficacy in reducing spore count.

**Biofilm visualization and viability testing.** Using inverted microscopy, biofilms were not evident after treatment with Clorox and were markedly reduced after treatment with OPA or formalin (Fig. 6). Visualization and viability of cells were also assessed using live/dead biofilm viability staining by confocal microscopy (Fig. 7a and b) for both monospecies anaerobic and multispecies aerobic biofilms. Visually, no difference was found in the viability of cells between the two types of biofilm. More live cells were observed in 3-day-old biofilm than in 5-day-old biofilm for both ribotypes.

## DISCUSSION

The purpose of this study was to assess the *in vitro* effectiveness of hospital-based disinfectants against *C. difficile* spores within a mono- or mixed-species biofilm at various stages of biofilm development. Three disinfectants (Clorox, OPA, and Virex) were most effective at killing *C. difficile* spores regardless of experimental conditions. Clorox and OPA were also effective at killing vegetative *C. difficile* within biofilms although Virex was found to be ineffective against vegetative cell growth. Biomass studies were consistent with time-kill results, and light and confocal microscopy demonstrated a significant reduction in biofilm grown on slides after exposure to Clorox. Multivariate analysis demonstrated differences in disinfectant killing effects based on experimental conditions of the studies (ribotype, biofilm age, biofilm type, and washed versus unwashed). To the best of our knowledge, this is the first comprehensive study to evaluate the effect of hospital disinfectants on *C. difficile* embedded in biofilms. Strengths of the study include a large number of experiments under different growth conditions using a large number of clinically significant *C. difficile* ribotypes.

No previous study has investigated the killing effects of disinfectants on *C. difficile* embedded in a biofilm. However, previous studies have investigated the impact of disinfectants against other organisms embedded in biofilms (24). For example, 11% of multidrug-resistant *Staphylococcus aureus* (MRSA) and 80% of *Pseudomonas* cells survived in a biofilm after disinfectant exposure (25). Not only was *Escherichia coli* embedded in a biofilm resistant to sodium hypochlorite disinfectant but also the biofilm was able to reform on disinfectant-treated surfaces after initial disruption (26). Efficacy of sodium hypochlorite disinfectant was also dependent on the composition of the biofilm, with disinfectants having decreased killing effect in multispecies biofilms, results that are consistent with our study (27). Hypochlorite and quaternary ammonium



**FIG 7** (A) Visualization of killing effect of disinfectants on a 72-h anaerobic *C. difficile* biofilm on a surface using confocal microscopy. (B) Visualization of killing effect of disinfectants on a 72-h aerobic multispecies *C. difficile* biofilm on a surface using confocal microscopy.

disinfectants were ineffective against *B. cereus* embedded in biofilms even at high concentrations (24, 28). Studies using OPA have demonstrated decreased killing effects for *Pseudomonas fluorescens* (13) but not for *Klebsiella pneumoniae* (29) when the organism was embedded in a biofilm. Studies using H<sub>2</sub>O<sub>2</sub>-based disinfectants were effective against *P. aeruginosa* and *S. aureus* embedded in biofilms, which differs from

**TABLE 1** Commercial hospital disinfectants used in this study

Active ingredient(s) (concn) <sup>a</sup>	Disinfectant name	Biocide type <sup>b</sup>	Manufacturer	Minimum contact time (min) <sup>c</sup>	Sporicidal
Sodium hypochlorite (10%)	Clorox	CRA	Clorox Company	1.5	Yes
Formaldehyde (4%)	Formalin	Formaldehyde	Pure Health	60	Yes
Hypochlorous acid (0.046%)	Nixall	CRA	Nixall Company	10	Yes
OPA solution (0.575%, wt/vol)	Cidex OPA	OPA	McKesson	10	Yes
Hydrogen peroxide solution (7.5%)	Sporox	H <sub>2</sub> O <sub>2</sub>	Sultan Healthcare	30	Yes
QAC (5–10%)	Virex	QAC	Diversey	10	Yes
Chlorine dioxide (0.2%) + QAC (0.125% + 0.125%)	Vital Oxide	CRA + QAC	Vital Oxide Company	10	No

<sup>a</sup>OPA, *ortho*-phthalaldehyde; QAC, quaternary ammonium compounds.

<sup>b</sup>CRA, chlorine-releasing agents.

<sup>c</sup>Per the manufacturer's label.

results of our study in which H<sub>2</sub>O<sub>2</sub> was ineffective against *C. difficile* embedded in a biofilm (12). A hydrogen peroxide disinfectant (Sporox) displayed a reduced killing effect against *P. aeruginosa* embedded in a biofilm. This was postulated to be due to genetic products released by cells in a biofilm that may reduce their susceptibility to oxidative disinfectants such as H<sub>2</sub>O<sub>2</sub> (30). Hospital and nursing home environments are known to harbor *C. difficile* spores and may be important sources for health care-associated infection. Biofilm removal methods may be required to properly clean these environments (31). An interesting observation from our study was that ammonium-based disinfectants (Virex) consistently reduced the spore count in a biofilm but had no effect on vegetative cells. It is possible that quaternary ammonium compounds may interfere with the formation of negatively charged biofilm matrix due to their cationic nature and may also affect spore germination (32). Sodium hypochlorite-based disinfectants denatured proteins in a biofilm and inhibited major enzymatic function of bacterial cells (32).

There are certain limitations to this study. This study used *in vitro* techniques to compare killing effects of hospital-based disinfectants against *C. difficile* embedded in biofilms. Future clinical studies to confirm these results are required. Accurate quantification of the organism within biofilms by sonication or scraping is sometimes limited by the ability to remove all of the organism from the biofilm (25). To overcome this issue, we used manual scraping in addition to sonication. Although our study looked at the killing effects of disinfectant using label-determined contact times and concentrations, it did not investigate any off-label use or effect of other environmental factors. This study was done on a polystyrene surface and, hence, may not necessarily represent how cells grow on other surfaces (25). For example, the rate of biofilm formation was enhanced on stainless steel surfaces compared to that on other metals or plastic surfaces, possibly due to the hydrophilic nature of the material or to surface irregularities leading to increased surface area (25). The viable cells after disinfectant exposure may have the ability to resurrect the biofilm and act as a reservoir for spread and preservation of recalcitrant infection or simply as a source of environmental contaminant (33). Thus, there is a need to combine disinfectant use with other modalities of treatment to eliminate the biofilm structure as well as persister cells.

**Conclusion.** In this *in vitro* study to assess the effect of hospital-based disinfectant to kill *C. difficile* embedded in biofilms, no disinfectant was able to completely eliminate *C. difficile*. Overall, Clorox, OPA, and Virex were most effective at killing *C. difficile* spores regardless of biofilm age, ribotype, or wash conditions. Future research will be required to determine methods to eradicate this persister reservoir.

## MATERIALS AND METHODS

**Bacterial strains.** Two laboratory strains of *C. difficile* (R20291, ribotype 027; CD630, ribotype 012) and three clinical strains of different ribotypes (ribotypes 014-20, 078-126, and 106) were used for all experiments.

**Chemical disinfectants.** Seven hospital disinfectants were used in this study, six with sporicidal properties and one nonsporicidal disinfectant as an active control (Table 1). Hospital disinfectants were defined as chemicals used for general-purpose disinfection of hospital buildings, patient rooms, or wards



and did not include instrument-grade disinfectant, antibacterial clothes preparations, sanitary fluid, or hand sanitizers. For each disinfectant, contact time experiments were conducted based on manufacturer-determined contact times or a standardized contact time.

**Growth of biofilms containing *C. difficile* vegetative and spore cells on 24-well polystyrene plates.** Biofilms containing *C. difficile* vegetative and spore cells were grown under three different conditions, namely, strictly anaerobic, mixed aerobic and anaerobic, and strictly aerobic. To form a *C. difficile* single-species biofilm grown anaerobically (monospecies anaerobic biofilm), overnight cultures of *C. difficile* were diluted 1:100 in fresh brain heart infusion-supplemented (BHIS) broth containing 0.1 M glucose. One milliliter of broth was then pipetted into each well of a 24-well polystyrene plate and incubated anaerobically at 37°C for 3 and 5 days. To form a mixed multispecies biofilm grown anaerobically (multispecies anaerobic biofilm), 1 ml of overnight cultures of *Enterococcus faecium* and *Staphylococcus aureus* diluted 1:100 in BHIS was added to a 2-day-old *C. difficile* biofilm grown anaerobically. The mixture was then incubated aerobically at 37°C for an additional 24 or 72 h to maintain a consistent biofilm at an age of 3 or 5 days. Finally, to grow a multispecies biofilm in an aerobic environment (multispecies aerobic biofilm), an overnight culture of *C. difficile* grown anaerobically along with both *E. faecium* and *S. aureus* cultures was diluted as described above and pipetted simultaneously into a 24-well polystyrene plate. The plates were then incubated aerobically for 3 and 5 days for biofilm formation.

**Quantification of biofilm biomass.** Three- or 5-day biofilms were washed twice with sterile phosphate-buffered saline (PBS) and allowed to air dry for 10 min. Following drying, 1 ml of filter-sterilized 0.2% crystal violet was added to each well and incubated for 30 min. The strictly anaerobic biofilm was incubated anaerobically, and the other two biofilms were incubated aerobically. After this time, the crystal violet was removed by washing the wells twice with sterile phosphate-buffered saline. Crystal violet dye was reeluted from the biofilm by the addition of 1 ml of methanol and incubation at room temperature for 30 min. Methanol-extracted dye was then diluted 1:1 and 1:10, and the optical density was measured on a plate reader at 570 nm (OD<sub>570</sub>).

**Biofilm imaging and viability assay.** Biofilm was imaged using both an inverted light microscope (Evos Cell Imaging System; Thermo Fisher) and confocal laser scanning microscope. Light microscopy was done to visualize structure and formation differences of 3- and 5-day-old biofilms pre- and postexposure to disinfectants. Cells in the biofilms were also visualized using a confocal laser scanning microscope according to the protocol of Jurcisek et al. (34). Briefly, bacterial biofilms from all stages were formed on a four-well chamber slide. Following incubation, treated or untreated biofilms were washed, stained with BacLight live/dead stain, and fixed with neutral buffered formalin (8, 10, 34). A live/dead viability kit contained Syto9, and propidium iodide stain was added to visualize *in vitro* killing effects. Slides were fixed and washed with PBS, and plastic wells were removed from the slide. Saline was added to plastic wells and covered with coverslips. The edges of the coverslip were sealed with mounting medium and air dried for 1 h before microscopy. Samples were imaged under oil immersion using a laser confocal microscope (34). The excitation and emission wavelengths for Syto9 were 488 nm and 505 to 550 nm, respectively, and 543 and >650 nm, respectively, for propidium iodide (34). All assays were performed in duplicate.

**Experimental procedures.** Immediately prior to experiments, the liquid supernatant of each well was gently removed with a pipette without disturbing the biofilm. Half of the biofilms were gently washed twice with sterile PBS, and the rest were left unwashed. This was done to look at the effect of organic substrate on disinfectant efficacy. For each experiment, 500  $\mu$ l of disinfectant at the original concentration was added in duplicate to both the washed and unwashed biofilms based on label-determined contact times (Table 1). PBS (500  $\mu$ l) was added to the wells as a positive control. Following exposure for the appropriate time, the disinfectants were removed, and wells were washed with 1 ml of sterile PBS. Biofilms were then detached from the bottom of the wells using a sonicator at 42 Hz for 10 min, followed by manual scraping for exactly 1 min, and pipetted into an Eppendorf tube. Removal of the biofilms was confirmed by light microscopy. For total counts of viable cells or spores, the cells in the Eppendorf tube were serially diluted, and 100  $\mu$ l was plated on blood agar plates and incubated anaerobically at 37°C for 48 h. For spore counts, the detached cells were heated at 65°C for 30 min to kill the vegetative cells. Vegetative cell and spore *C. difficile* CFU counts were measured using the dilution and plating method (35). In the case of multispecies biofilms, the morphology of the colonies for the three types of organism used were distinct, allowing for accurate *C. difficile* counts. All experiments were performed at least in duplicate using the appropriate positive and negative controls.

**Statistical analysis.** The primary analysis was to compare *C. difficile* vegetative cell and spore counts as well as biomass measured by a crystal violet assay, pre- and postexposure to disinfectants. Secondary aims were to compare the log reduction of *C. difficile* vegetative cell and spore counts based on the type of biofilm (monospecies, mixed, or mixed aerobic), duration of biofilm formation (3 or 5 days), ribotype, and presence of organic matter (washed versus unwashed biofilm). The third aim was to visually compare the overall efficacies of disinfectants against the three different types of biofilm for different ages of biofilm. The vegetative cell and spore mean log<sub>10</sub> reduction in CFU counts  $\pm$  standard error (SE) was calculated for each disinfectant by biofilm type, age, ribotype, and experimental procedure. Linear regression analysis was done comparing the efficacy of sporicidal disinfectants against the efficacy of the nonsporicidal disinfectant Vital Oxide for biofilms of different ages. SAS, version 9.3 (SAS Institute, Cary NC), or STATA/IC, version 12.1 (STATA Corp LLC, College Station, TX), was used for all analyses. A *P* value of <0.05 was considered significant.

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