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ROLE OF DISINFECTANTS IN THE ERADICATION AND PROPAGATION OF CLOSTRIDIODES DIFFICILE INFECTION

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**ROLE OF DISINFECTANTS IN THE ERADICATION AND
PROPAGATION OF CLOSTRIDIODES DIFFICILE INFECTION**

By

TASNUVA RASHID, MD, MPH

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by

Tasnuva Rashid, MD, MPH, PhD

2019

DEDICATION

To all six of my parents; my husband and my baby brother

**ROLE OF DISINFECTANTS IN THE ERADICATION AND PROPAGATION OF
CLOSTRIDIoidES DIFFICILE INFECTION**

by

TASNUVA RASHID, MD, MPH

Presented to the Faculty of The University of Texas

School of Public Health

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

THE UNIVERSITY OF TEXAS

SCHOOL OF PUBLIC HEALTH

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PREFACE

Now that I am sitting back thinking about what inspired me to take this long path to a culminating degree. The answer that echoes in my mind is that I wanted to embark on a journey of a lifetime and wanted to explore the various aspects of research and how it relates to human health. I still remember the days when I was looking for a project idea for my dissertation. I always knew Infectious disease is the call.

However, what topic. Like everyone, I was also initially drawn to the most obvious ones like surveillance, antibiotic resistance, pharmaceuticals and so on. However, something in me always attracted me to primary prevention aspects and hence disinfectants and human health. The more I delved in the field of disinfectants, the more I realized that the world of disinfectants and disinfection is still in the very primitive stage with no clear cut and consistent, evidence-based guideline for proper use. Even less is known about disinfectants and *Clostridioides difficile* and hence the interest. All my hard works will pay off if any part of my research can play even a tiny role in updating the infection control guideline in the US and globally.

ACKNOWLEDGMENT

First of all, I will like to express my gratitude to Dr. Sharma who is not only my advisor but also my inspiration and role model. Thank you for being there throughout the years and for leading by example. I will also like to thank Dr. Garey without whom this research would not have been possible. Thank you for guiding me through the projects and bearing with me. I learned a lot working with you. I am also grateful to my committee members for their time and input on my dissertation manuscripts. A special thanks to Dr. Alam and Khurshida for teaching me various laboratory, microbiological and genetics techniques; thanks to Eugenie for teaching me various lab techniques and having confidence in me. I truly appreciated that. I will also like to thank my lab members as without their support none of this would have been possible. I am truly grateful to my family whose love and guidance are with me in whatever I pursue. Last but not least I will like to thank my loving husband for his continued support and encouragement. Could not have done this without you.

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The University of Texas

School of Public Health, 2019

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Abstract

Introduction:

Clostridioides difficile is a motile, gram-negative, toxin-producing bacteria responsible for the majority of the nosocomial as well as community-acquired infection in the US and worldwide. A high rate of morbidity, mortality, recurrences, healthcare cost coupled with frequent epidemic outbreaks with drug-resistant strains has classified *C. difficile* as a pathogen with an urgent threat to human health. Current guidelines regarding disinfectant use are either limited or absent or are not effective in reducing the environmental burden of *C. difficile*. The aims for this study was first to determine the in vitro efficacy of select hospital disinfectants in

eradicating the spores of *C. difficile* both in planktonic form as well as in biofilm and secondly, to determine the effect of community disinfectants on the eradication and propagation of *C. difficile*.

Methods

Following standardized protocol, low, medium and high category spores were produced and exposed to the original concentration of disinfectants at both label determined and standardized time in presence or absence of organic matter. Minimum disinfectant concentration and minimum organic matter concentration for with bactericidal effect were also determined. The efficacy of hospital disinfectants on the strictly anaerobic mono organism, mixed multi organism and strictly aerobic multi organism 72 hours and 120 hours *C. difficile* biofilm was quantified using both microbiological techniques as well as microscopically. The effect of community household disinfectants on *C. difficile* spores was determined using time-kill experiments as well as time-kill curves. Germination assay, Ca-DPA assay, spore cortex fragmentation assay, the effect on the dormant vs. activated spore, qPCR and microscopy was done to characterize the role of community disinfectants on *C. difficile* propagation.

Results

The effect of 7 hospital disinfectant and 7 community disinfectant were tested against 16 *C. difficile* spores of 6 different ribotypes. All tested hospital disinfectants

were effective in eradicating almost 100% *C. difficile* spores at manufacturer suggested contact time in absence of organic matter and at all spore concentration. Using the general linear model, sporicidal activity of the disinfectants was affected by a shorter contact time (0.52 ± 0.10 log decrease; $p < 0.0001$), and presence of organic matter (low organic substance: 0.77 ± 0.11 log decrease; $p < 0.0001$; high organic substance: 2.02 ± 0.12 log decrease; $p < 0.0001$). *C. difficile* vegetative cells and spores were recovered from biofilms regardless of type or duration of biofilm formation. No disinfectant was able to completely eliminate *C. difficile* from the biofilms. Overall, Clorox, OPA, and Virex were most effective at killing *C. difficile* spores regardless of biofilm age, ribotype, or wash conditions ($p = 0.001$, 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 the total vegetative cell growth ($p = 0.77$). Clorox and Virex were most effective in reducing biomass followed by Nixall, OPA, and Vital oxide. None of the community disinfectants except Clorox was found to be effective against *C. difficile* spores. Increased spore germination was observed for Lysol hydrogen peroxide and Lysol. Effect on active vs dormant spores, germination assay, Ca-DPA assay, spore cortex fragmentation assay, qPCR and microscopy ass indicate the potential for Lysol hydrogen peroxide to act as a germinant for *C. difficile* spores.

Conclusion:

None of the disinfectants could consistently reduce *C. difficile* spore count under all conditions. Furthermore, this is the first study to look at the efficacy of hospital disinfectants on *C. difficile* spores encased in a biofilm. This study reported for the very first time about the germinant potential of certain household disinfectants. Thus, the findings for this study may help to inform the infection control guidelines and aid to fulfill the CDCs goal of reducing *C. difficile* infection by 30% by 2020.

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INTRODUCTION/BACKGROUND

Overview of Clostridioides difficile organism

Clostridioides difficile is a motile, gram-positive, toxin-producing anaerobic bacillus that causes 20 to 25% of antibiotic-associated diarrhea (AAD), 50 to 75% of antibiotic-associated colitis, and 90% of pseudomembranous colitis. ⁽¹⁾ ⁽¹⁾ ⁽¹⁾ This bacteria was discovered in 1935 from newborn infant feces. ⁽²⁾ The name *C. difficile* was coined by Hall and O'Tode based on the relative difficulty in culturing and isolating the organism due to slow growth compared to other infectious agents. ^(3, 4) *C. difficile* is a unique component of the gut microflora persisting and thriving in the distinct ecosystem of the hospital environment and following antibiotic treatment. ⁽⁴⁾ About 2 to 3 % of healthy adults and 15 to 70% of healthy neonates have positive *C. difficile* stool cultures. ^(5, 6) *C. difficile* can stay in free form (planktonic) or in a monocellular or poly cellular biofilm. ^(7, 8) Biofilm production is often linked with survival, persistence, colonization, resistance to anti-microbial agents and disease causation. ⁽⁸⁾

Like other spore-forming bacteria, *C. difficile* also has two forms—the vegetative cell and the spore form (**figure 1**). During the logarithmic phase, the vegetative cells predominate, which are very aero-intolerant and susceptible to various antibiotics, environmental conditions, and disinfectants. ⁽²⁾

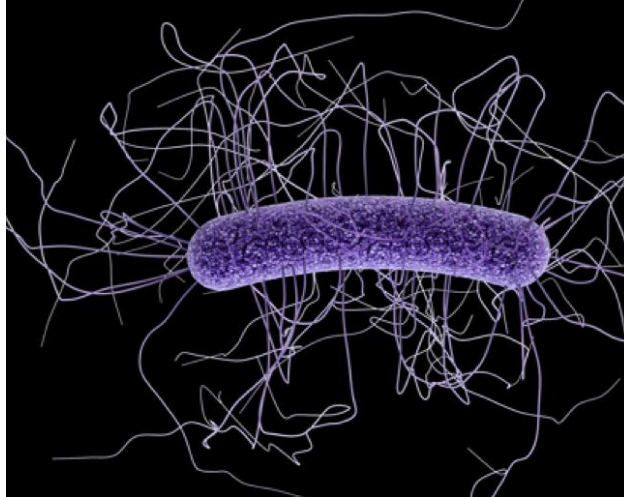


Figure 1: Vegetative cell

The vegetative cell consists of an outer wall with two S-layer proteins, which are highly conserved across strains and shows sequence diversity coupled with an inner circular genome. ⁽⁹⁾ The genome of CD630, a reference strain of toxigenic *C. difficile* has been completely sequenced providing information regarding genes related to the organism survival, virulence, toxin production, pathogenesis, and resistance ⁽²⁾ Fifty (50%) of the coding sequences of the genes are unique to *C. difficile* with 11% mobile genetic element (conjugate transferase) capable of being excised and integrated to various hosts. ⁽¹⁰⁾ The pathogenicity loci of the genome are responsible for the synthesis and regulation of toxin A (tcd A) and toxin B (tcd B), which are the most important virulence factors for *C. difficile*. ⁽¹⁰⁾ Toxin A is found in 70% of the strains and toxin B is found in all strains. ⁽¹¹⁾ Another toxin, the binary toxin (CDTab) is produced by only certain strains. ⁽¹²⁾ Other virulence factors include adhesins, extracellular enzymes, fimbriae, flagella, Para crystalline S layer, and capsule⁽¹³⁻¹⁷⁾ The expression of toxin A and B is repressed during the exponential phase of the disease but is expressed during the stationary phase. ⁽¹⁸⁾ This prioritization of growth and toxin release is thought to contribute to

proper colonization and disease production.⁽¹⁸⁾ *C. difficile* strains can be characterized by more than 150 ribotypes and 24 toxin types.⁽⁶⁾ In the European Union (EU) the 12 most common ribotypes that account for 65.5% of strains include 001, 002, 012, 014, 017, 020, 027, 048, 077, 078, 126, and 168.⁽¹⁹⁾ The most common ribotypes in the US are 002, 106, 017, 027, and 078.⁽²⁰⁾

During extreme environmental conditions including low pH of the gastric environment, antibiotic use, high heat, various stresses (osmotic distress, acid and heat shock) *C. difficile* forms spores (**figure 2**).^(21, 22) *C. difficile* spores are metabolically dormant, resistant to various harsh physical and chemical exposures, and have prolonged survival.⁽²⁾ Spores represent the transmissible form of *C. difficile* and contribute to its survival and persistence in aerobic environments outside the host as well as inside the host during microbial therapy and recurrence.⁽²⁾ *C. difficile* spore has a unique architectural structure (**figure 3**). At the very core, there is a partially dehydrated spore core containing 1M Calcium -dipicolinic acid (Ca-DPA) which is a major contributor for spore dormancy and resistance.⁽²³⁾ The spore core is in turn surrounded by permeability barrier inner membrane and germ cell wall. A thick modified peptidoglycan layer called the spore cortex to surround the germ cell wall. The cortex wall is in turn surrounded by spore coat containing enzymes for cortex hydrolysis. Exosporium is the outermost layer which plays a vital role in host-pathogen interaction and persistence of spore in recurrent infection.⁽²⁴⁾

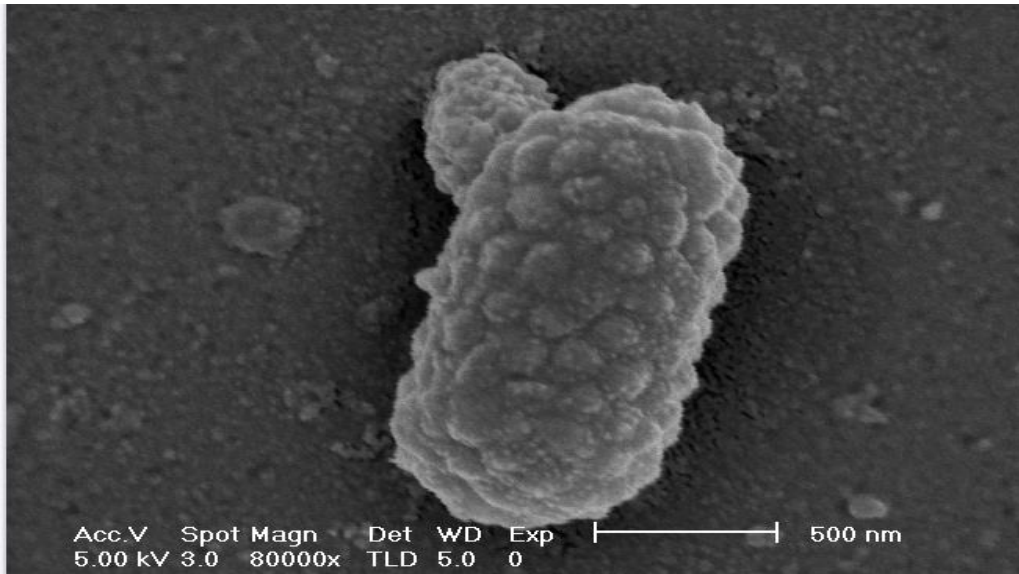


Figure 2: *C. difficile* spores

In the dormant state, the outer surface is smooth with numerous filamentous projections that present during germination.⁽²⁵⁾ These are the various proteins on the spore wall including proteases, stress response proteins, metabolic proteins and surface exposed proteins, which are responsible for spore germination, metabolism, and protection from oxidative stress.⁽²⁶⁾ When conditions are optimal, spores germinate to vegetative cells. The regulation of sporulation of *C. difficile* is maintained by a signal transduction pathway called the sporulation phosphorelay, which is intimately associated with toxin regulation.^(26, 27)

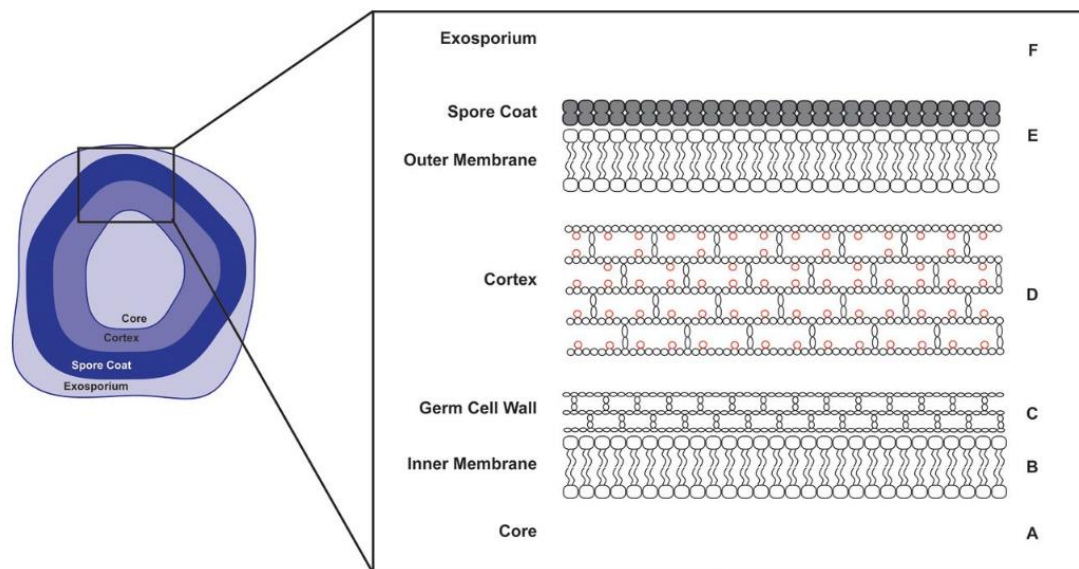


Figure 3: Layers of *C. difficile* spore wall ⁽²³⁾

***C. difficile* Infection (CDI):**

Any healthcare-associated infections occur primarily in hospitals, nursing homes, long term care facilities, outpatient clinics, and daycare facilities. ⁽²⁸⁾Community and healthcare-acquired CDI are defined in **Table 1**.

Table 1: <i>C. difficile</i> Infections (CDI)
Community-acquired CDI
<p>A CDI which is diagnosed when</p> <ul style="list-style-type: none"> • A patient has diarrhea with positive <i>C. difficile</i> laboratory assay within 48 to 72 hours of hospitalization with no previous history of a recent stay at any healthcare facility. ^(28, 29)

Hospital-acquired CDI

A CDI which is diagnosed when

- A patient has diarrhea and a positive *C. difficile* stool assay within 48-72 hours of admission or
- If a patient has a positive stool *C. difficile* assay within 72 hours of admission in phase of a recent hospital stay in the last 3 months.

(28, 30, 31)

The *C. difficile* organism can be acquired directly from hospital environment or it could be a part of the patient's gut microbiota flora getting upper hand due to antibiotic use. (25, 26)

The prevalence of community-acquired CDI accounted for 5% of admitted cases in the late nineties to around 41% of the cases in 2012. (28, 32) One study found that 97 per 100,000 of CDI cases in Sweden were hospital-acquired compared to 25 per 100,000 of cases which were community acquired. (33) Compared to hospital-acquired CDI, patients with community-acquired CDI are more likely to be female, younger, have fewer comorbidities, less chance to be exposed to antibiotics, and present with less severe disease. (32)

The sources and reservoirs of community-acquired *C. difficile* involve exposure to spores present in soil, animals, household surfaces, shoe bottoms, contaminated foods and contact with family members with diarrhea. (28, 34) A report of 2,580 environmental

sources found *C. difficile* isolates in 47% of river water, 21% of soil samples, 15% of retail meats, and 2% in home environments. ⁽²⁾ Another study found 32.3% household contamination with *C. difficile*. ⁽³⁴⁾ In the hospital setting, the major reservoirs for *C. difficile* include CDI patients, asymptomatic carriers, direct inoculation of spores through contact with contaminated surfaces including floors, bedrails, windowsills, toilets, bed sheets, wall bottoms, scales, blood pressure cuffs, electric thermometers, feeding tubes, ICU instruments, pulse-oximeter, procedural instruments and flow control devices with IV catheter. ⁽³⁵⁻³⁷⁾ *C. difficile* spores are resistant to desiccants and can persist for up to 5 months with around 49% of sites in rooms of CDI patients and 29% of rooms of asymptomatic patients being contaminated. ^(36, 38)

Transmission:

C. difficile is mainly transmitted in the form of spores as a result of oral-fecal contamination. The infected patients or attending healthcare workers can act as reservoirs that facilitate transmission. A study found that approximately 59% of healthcare workers caring for the *C. difficile* positive patients had positive *C. difficile* hand cultures. ⁽³⁰⁾ Transmission can also occur through contact with contaminated surfaces or via direct inoculation into the bowel. ⁽³⁹⁾ There is also evidence for airborne transmission of *C. difficile* spores during periods of activity and with bed making ^(40, 41). Foodborne transmission has also been documented. Factors influencing the transmission of spores include the resistance of spores to disinfectants, antibiotic presence in hospitalized patients, and promiscuity of patients. ⁽⁴²⁾

Risk factors:

There are various traditional and emerging risk factors associated with *C. difficile* infections (**Table 2**).

Table 2: Risk factors for <i>C. difficile</i> infection ^(28, 43-48)			
Risk factors	Hospital-acquired		Community-acquired
Traditional risk factors	Agent factor	Strain types	Strain type
	Host factor	The severity of underlying diseases	Use of proton pump inhibitors (PPI)
		Transplantation	Young Children
		Anti-ulcer medication	Chronic intestinal conditions like polyp, IBD, diverticulitis
		Nasogastric tube	
		Comorbidities	
		Impaired immune system	
		Low antitoxin A and B	
		Nonsurgical GI procedure	
	Environmental factors	Duration of hospital stay	Pets
		Prolong antibiotic use	Ineffective disinfectants

		Use of multiple antibiotics	Infected family members
		Infected roommates	Consumption of contaminated meals and feed
		Hand transfer by healthcare professional	
Emerging risk factors	<ul style="list-style-type: none">- Young children- Young peripartum women- Patients without prior exposure to antibiotics- Patients with IBD		

Pathogenesis:

Pathogenesis of CDI results from alterations to the gut microbiota, predisposing risk factors, virulence of *C. difficile* strains, colonization resistance and host factors and is modified by a variety of factors. The major factors associated with the severity and disease diversity with *C. difficile* include- a) toxigenicity and virulence of the colonizing strain; b) infective dose of *C. difficile* c) host susceptibility; d) adhesion to the colonic epithelium and e) the presence of organisms affecting toxin expression and activity. ⁽²⁾ Following exposure, ingested *C. difficile* spores initially germinate in the terminal ileum and later multiply in the colonic lumen along with another resident *C. difficile* resulting in a downstream cascade of events leading to CDI. In the presence of various secreted factors e.g., adhesins, *C. difficile* adhere to the gut mucosa⁽⁴⁹⁾ resulting in the release various hydrolytic enzymes and toxins as well as in the recruitment of polymorph

nuclear cells (PMNs).^(50, 51) Production of toxin A and B results in various cytopathic and enterotoxic effect on the gut mucosa characterized by disruption of gut cell-cell tight junctions resulting in gut tissue damage accentuated by enzymatic connective tissue degradation.⁽⁵²⁻⁵⁵⁾ Recruited PMNs and gut environment promote successive cycles of *C. difficile* pathogenesis ultimately leading to local tissue necrosis, apoptosis, and origination of CDIs (**figure 4**).

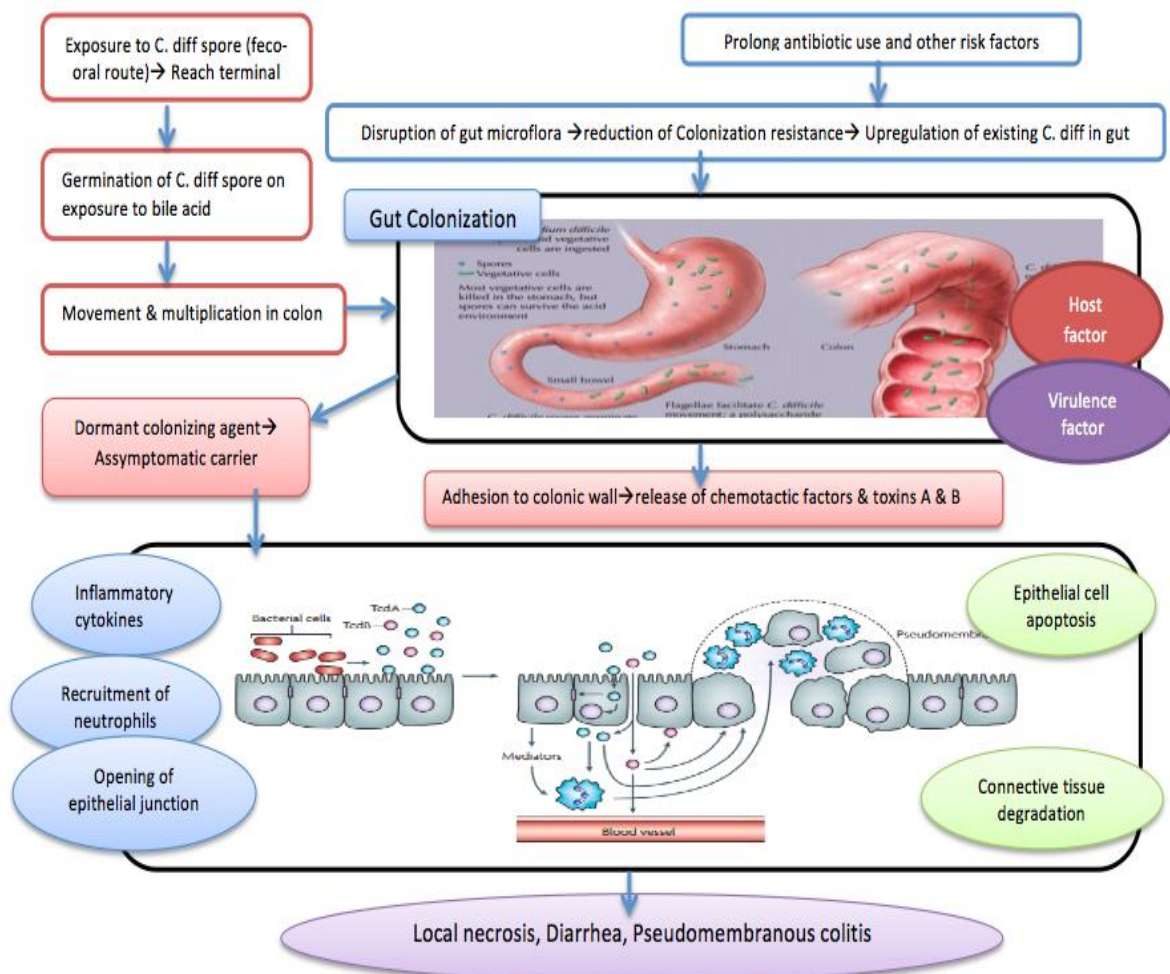


Figure 4: Pathogenesis of CDI (48, 56)

Spectrum of CDI:

The spectrum of diseases associated with *C. difficile* encompasses asymptomatic carrier states to severe fulminant disease including the toxic mega colon.^(6, 57) About 20% of hospitalized adults and up to 50% of long-term care facility patients are *C. difficile* carriers and shed *C. difficile* spores in their stool.^(30, 58) Mild CDI is characterized by diarrhea with colitis. *C. difficile* toxin-induced cytoskeleton disruption characterized by shallow intestinal ulcers is manifested as pseudo membranous colitis.⁽⁵⁵⁾ The more severe form of CDI consists of fulminant colitis, toxic mega colon, bowel perforation, and death.^(59, 60) Other unusual manifestations include protein-losing enteropathy, IBD, and other extra colonic involvements. The recurrence rate for *C. difficile* ranges between 5-47% and poses a great challenge to CDI management.^(61, 62)

C. difficile in a biofilm

In addition to the planktonic form, *C. difficile* can also exist in the environment encased in a biofilm. Biofilm formation constitutes a unique and dynamic ecosystem of microbial communities encased in an extracellular matrix for bacterial survival and persistence in various diverse environmental niches. Cells in a biofilm can be differentiated from their planktonic counterpart in terms of secretion of the extracellular matrix, growth rates, up or down regulation of various genes as well as communication through quorum sensing.⁽⁶³⁻⁶⁸⁾ The formation and attachment of biofilm to various biotic and abiotic surfaces is a function of a property of surface (texture, hydrophobicity, etc.), the property of overlying medium as well as the property of microorganism (motility, extracellular matrix, hydrophobicity).⁽⁶³⁻⁶⁸⁾ Biofilms commonly form on various biotic surfaces like living

tissues, gut, teeth and abiotic surfaces including indwelling medical devices, water system, piping, medical devices, industrial aquatic surfaces as well as regular flooring surfaces. ⁽⁶³⁾ Biofilm formation is a complex cycle of initial attachment and microcolony formation followed by maturation into the differentiated biofilm and finally detachment and dispersal of planktonic cells from biofilm(**figure 5**). ⁽⁶⁹⁾ Various environmental signals including change in nutrition, pH, temperature, O₂, osmolality induces the initial reversible cellular attachment. ⁽⁷⁰⁾ At this phase, all cells are in the logarithmic growth phase. During the second phase there is irreversible binding and intercommunication of bacteria, the release of chemical signals, decrease in motility and progressive aggregation of cells in various layers. ⁽⁷⁰⁾ When chemical signals reach threshold various transcription factors and genes are activated making the bacterial colonies resistant to phagocytes, antibodies, and antibiotics. ⁽⁷¹⁾ In this phase, microcolonies differentiate to mature biofilm through various pathways with resultant biofilm thickness >100µm. ^(72, 73) The dispersal pathway may either include a programmed set of chemical pathways leading to hydrolysis of the extracellular matrix, reactivation of dormant cells or physical detachment through physical sheer forces. ^(71, 74-76) This phase occurs several days after biofilm maturation.

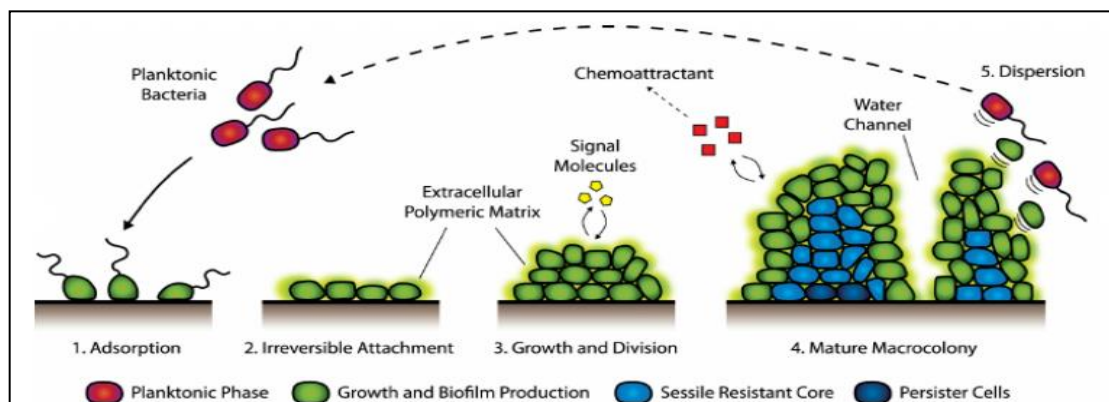


Figure 5: Phases of biofilm formation⁽⁷⁷⁾

Although there have been various studies looking at the biofilm architecture in several dimensions, the basic structure of mature biofilm consists of three layers. It is comprised of mushroom-shaped microcolonies suspended in an extracellular matrix containing extracellular polymeric substances (EPS), DNA and proteins.⁽⁷⁰⁾ The mushroom and stalk together form an architecture with water channel between bacterial colonies providing them with nutrition and protecting against the buildup of toxic materials.⁽⁷⁰⁾ The biofilm structure and formation play a vital role in infectious disease transmission and persistence processes through producing emboli of detached cells, exchange of resistance plasmid between cells in biofilm, development of virulent phenotypes, reduced antimicrobial susceptibility and evasion of human innate and acquired immune response.^(63, 78) Biofilms on various biotic and abiotic surfaces account for 65% of microbial infections and 80% of chronic infections in the body.⁽⁷⁶⁾ Worldwide 65% of all nosocomial infections and 2 to 14% of all surgical wound infection are associated with biofilms.⁽⁷⁹⁾ The economic implication of biofilms also costs nations billions of dollars. Some of the common organisms in abiotic biofilm which are associated with health care associated infection (HAI) include *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, gram-negative bacteria (*E. coli*, *Enterococcus faecalis*, *Klebsiella pneumoniae* etc.), *Listeria monocytogenes* and *Clostridioides difficile* (*C. difficile*).^(79, 80)

Biofilm formation by *C. difficile* strains was first reported in 2012.^(81, 82) Till then various studies have indicated that biofilm production might be one of the crucial mechanisms

for persistence and survival of *C. difficile* in the abiotic environment. ⁽⁸³⁾ Studies were done on a few strains of *C. difficile* name R220291 and CD630 demonstrated that this bacterium forms a complex multilayered biofilm in a matrix of protein, DNA and polysaccharide on abiotic surfaces as well as biotic surfaces in the gut. ⁽⁸³⁾ EPS matrix provide scaffold for vegetative cells and spores and provide protection against oxygen stress. ⁽⁸¹⁾ Various factors including virulence-associated protein (cwp84, flagella, transcription factors, SpoOA, and quorum sensing regulator LuxS regulates biofilm formation by *C. difficile*.⁽⁸³⁾ The robustness and integrity of the biofilms of *C. difficile* vary among the different clinical strains. ⁽⁸²⁾ It has been found that *C. difficile* forms both mono and multicellular biofilm through coaggregation phenomenon. One study found that either due to coexistence with strong biofilm producer (*F.magna*) or due to increase in biofilm production in *C. difficile* in response to biofilm promoting substance released by a co-organism, *C. difficile* multicellular biofilm may have different characteristics as compared to a monocellular biofilm. ⁽⁸⁴⁾ The presence of various proteins in the *C. difficile* matrix and the lysis rate indicates a high level of metabolism and cannibalistic phenomenon in the biofilm. ⁽⁸⁵⁾ Biofilms not only protect *C. difficile* from environmental toxins like antibiotics and oxygen stress but also promoted increased resistance to various antibiotics like metronidazole. ⁽⁸⁵⁾ Although the level of sporulation of CD in biofilm varies by strain type the level of germination is usually lower. ^(85, 86) All in all, *C. difficile* biofilm play a vital role in environmental adaptation, drug resistance, virulence, and transmission dynamics.

Formation of *C. difficile* biofilm in various environmental surfaces results in not only various drug-resistant HAIs but also are linked to several disease outbreaks related to

contamination of drinking water in water networks, food industries, and medical facilities. ⁽⁸⁷⁾ Biofilms also pose a great impact in various industrial sectors resulting in failure of production. Thus, there is a need for adequate and coordinated steps to prevent biofilm formation on abiotic surfaces either through modification of environmental surfaces to prevent bacterial adhesion and biofilm formation or through eradication of formed biofilms using various chemical agents including disinfectants.

***C. difficile* spore germination**

In response to various environmental stimuli including chemical, nutrient, metabolic the *C. difficile* spores are irreversibly activated into metabolically active vegetative cells **(figure 6)**. *C. difficile* spore germination is initiated by various germination pathways including amino acid-dependent pathways like a bile-salt amino acid pathway, alanine racemase-dependent d-amino acid pathway and amino acid-independent pathways like bile salt-divalent cation pathway. ⁽⁸⁸⁾ Bile salt amino acid pathway is the most common pathway where bile salt in the presence of a co germinant binds with cspC receptor. Various environmental sensing pathways initiate a signaling cascade with ultimate activation of Slec. Slec activation, in turn, leads to degradation of the cortex, rehydration of core and release of Ca-DPA from core and spore germination. ⁽²³⁾

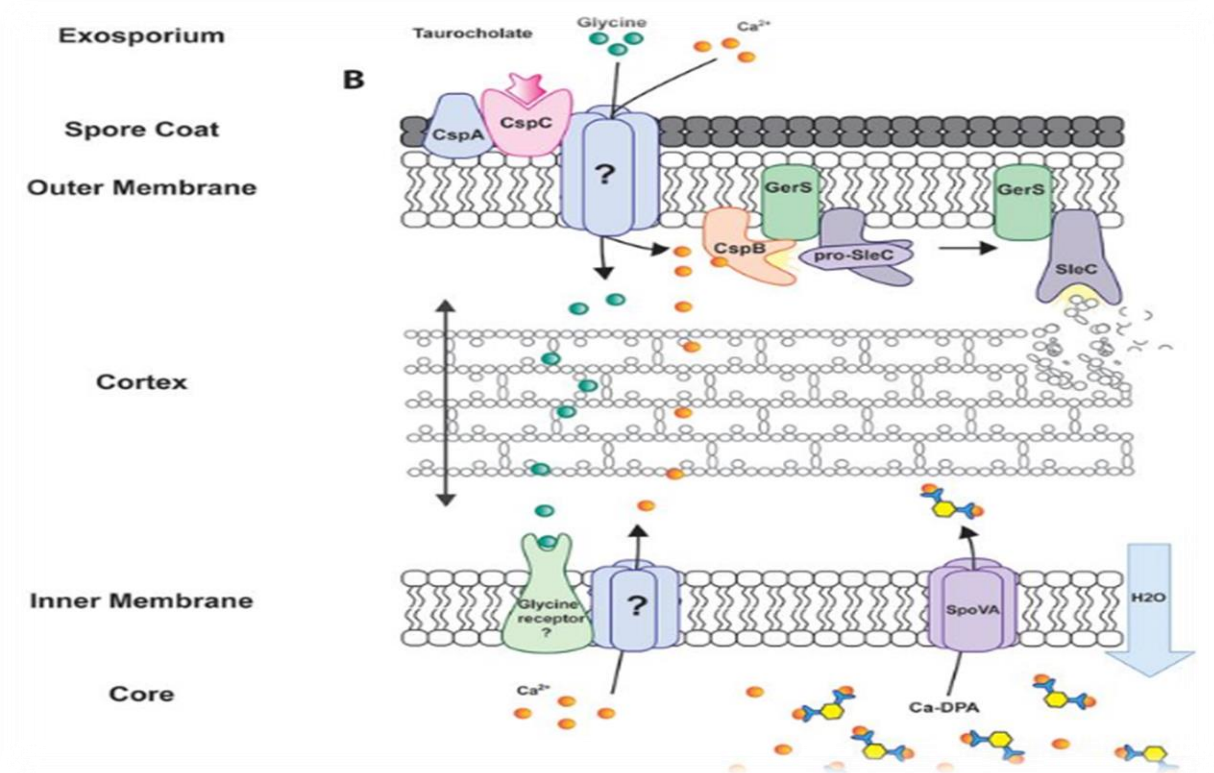


Figure 6: Model for *C. difficile* spore germination⁽²³⁾

PUBLIC HEALTH IMPORTANCE

C. difficile epidemiology

C. difficile is a major nosocomial enteropathogen and a leading cause of hospital-acquired infection in the US and worldwide replacing MRSA. ⁽⁸⁹⁾ There has been a dramatic increase in the incidence, severity, strain variance, recurrence, and associated

morbidity and mortality particularly in people 65 years or older. ⁽⁹⁰⁾ In 2011, it was estimated that *C. difficile* caused around half a million illnesses and 29,000 deaths per year. ⁽⁹¹⁾ The recurrence rate of CDI is around 16.6 to 27.4%. ⁽⁹²⁾ Around 333,000 primary and 145,000 recurrent healthcare-associated CDI cases occur annually in the US with more than half originating from a long term care facility. ⁽⁹³⁾ In Canada, the incidence of nosocomial CDI ranged between 38-95 per 100,000 and accounted for 3.4 to 8.4 per 1000 admissions mirroring that of the US. ⁽⁵⁶⁾ The incidence is higher in females compared to males [RR 1.26 (1.25-1.27)], whites compared to non-whites [1.72 (1.56-2.00)] and those ≥ 65 years of age compared to those <65 years [8.65 (8.6-9.31)]. ⁽⁹⁴⁾ It is the 18th leading cause of death in those aged ≥ 65 years. ⁽⁹⁴⁾ Among the *C. difficile* cases, around 159,700 cases were community acquired, 293,000 healthcare associated, and 107,600 were hospital-acquired. ⁽⁹⁴⁾ It was found that new exposure and colonization with *C. difficile* more frequently lead to CDI compared to existing colonization. ⁽⁹⁵⁾ The incidence of *C. difficile* colitis increased from 0.68 to 1.2% and that of fulminant colitis increased from 1.6 to 32%. ⁽⁹⁶⁾ Around 20% to 27% of all CDI are community associated with an average incidence rate of 20-30 per 100,000. ⁽⁹⁷⁻⁹⁹⁾ in urban and semi-rural areas of the US. ⁽⁴⁸⁾ The community associated CDI cases were more severe and occurred mostly in younger individuals and pregnant women. ⁽⁴⁸⁾ According to one study, around 1/3 of community-associated CDI did not have any hospital contact and another 1/3 contracted the disease from the family member. ⁽⁴⁴⁾

C. difficile hospitalization and healthcare costs

In 2009 nearly 1% of all hospitalizations (336,600) were complicated by a CDI. ⁽¹⁰⁰⁾ Between 1996 and 2010 there has been a four-fold increase in hospital-associated *C. difficile* in the US and Canada. ⁽¹⁰¹⁾ One study found a 23% increase in annual hospitalization and a 3-fold increase in hospitalization stays due to CDI over a 6 year period (2000 to 2005). ⁽⁹⁴⁾ Another study in Canada found that CDI was associated with an increase in the length of hospital stays by 21.3 days on average ⁽¹⁰²⁾ Among the hospitalized asymptomatic patients, around 15% were colonized with a toxigenic strain of *C. difficile* compared to 6% infected with a non-toxigenic strain. ⁽¹⁰³⁾ The rates of hospital stay varied by region, age group, and gender. The rate was highest in the North West (138 per 100000) and lowest in the West (95 per 100000). ^(29, 100) The rate of hospital stays was higher for female CDI patients compared to male CDI patients⁽²⁹⁾. A study found that CDI hospital stays patients were more severely ill than the general hospitalized patients with a predicted mortality rate of 9.1% compared to <2% in general inpatients. ^(100, 104) The rate of hospital discharge listed with CDI as a diagnosis in the US increased from 3.82 per 100000 in 2000 to 8.75 per 100000 in 2008. ^(100, 104) CDI is also associated with patient isolation, ward closure, and hospital closures in the UK and is a notifiable disease in the UK.⁽⁹⁴⁾ Vital records in the US indicate that the number of death certificates with primary CDI was 7483 in 2008 compared to 793 in 1999. ⁽⁹⁴⁾ It is estimated that *C. difficile* complicated cases account for about 54% higher hospital costs over general cases and average about \$3,669 in the US and £4000 per case in the UK^(47, 102) for a total of \$ 1.1 billion healthcare cost per year. ⁽⁴⁷⁾ Another study estimated a cost ranging between \$2871 to \$4846 per case for primary CDI and

\$13,655 to \$18,067 per case in recurrent CDI cases in the US. ⁽⁴⁷⁾ In Canada and the UK, the cost per case for primary and recurrent CDI was \$5243 to \$8570 and \$13655 per case respectively. ⁽¹⁰⁵⁾

C. difficile associated mortality

There has been a significant increase in the mortality rates associated with CDI from 5.7 deaths per million to 23.7 deaths per million in the US approximately 35% adjusted per annum increase during a period between 2000 and 2005. ^(106, 107) About 1 in 11 people aged 65 years or older die within a month of CDI diagnosis. ^(1, 45) According to one study, the increase in mortality due to CDI mirrors the increase in virulence of the organism. ^(107, 108) The in-hospital mortality rates for patients with secondary CDI diagnosis (11.7%) is threefold compared to patients with primary CDI (3.7%). The overall death rate for *C. difficile* colitis was 57% in patients undergoing colectomy. ⁽⁹⁶⁾

Emerging epidemiologic transition for C. difficile

There is an ongoing epidemiological transition in *C. difficile* infection risk stratification and virulence factors. *C. difficile* is now increasingly occurring in previously low-risk group individuals including children and peripartum women. ⁽⁹⁴⁾ The estimated incidence of CDI in children in 2011 was 24.2 per 100,000 with more than 2/3 occurring in the community. ⁽⁹⁴⁾ There was a 57% (12.5 fold) increase in *C. difficile* related hospitalization in children from 1997 to 2009. ⁽¹⁰⁹⁻¹¹¹⁾ There is an increasing trend in pediatric CDI especially in those 1 to 5 years of age. Though neonates have high levels

of toxigenic *C. difficile* they rarely develop symptomatic disease.^(42, 112, 113) Since 2005, there have been reports of severe CDI among peripartum women.⁽¹¹⁴⁾ The incidence increased from 0.04 to 0.07 per 1000 discharges in 2006, which might be a surrogate for antimicrobial use in this group.⁽¹¹⁴⁾ In addition to prevalent endemic infections, there has been frequent epidemic outbreaks in the US and worldwide. The beginning of the epidemic was characterized by a continuous rise of CDI in the US, Canada, and Europe that started in the US in 2002.⁽⁴⁸⁾ By 2006 there was a doubling in the incidence of CDI and associated deaths in the US.⁽⁴⁸⁾ In Europe, all countries except the UK experienced a significant increase in CDI from around 1.7 to 3.8 per 100,000 to 14.8 per 100,000.⁽⁴⁸⁾ The reduction in CDI cases in the UK paralleled the national efforts to reduce *C. difficile* infections by 30% by 2011.⁽⁴⁸⁾ The outbreak of severe CDI was associated with decreased treatment effectiveness and increased mortality.⁽³⁰⁾ The strains responsible for the epidemic included O17 and O78 possibly due to increased survival, transmissibility and virulence of the clonal lineage.⁽⁹⁴⁾ Hypervirulent and hyper toxin producer BI/NAP1/O27 was isolated from 50% of the patients during various epidemic outbreaks.^(115, 116) The epidemic was characterized by an increase in community-acquired CDI as well as affecting previously low-risk segments of the population, emphasizing the need for early interventions. According to a study two distinct epidemic lineages, FQR1 and FQR2 emerged in North America within a relatively short period of time.⁽¹¹⁷⁾ These two lineages were Fluoroquinolone resistant and had a distinct pattern of global spread resulting in outbreaks in the UK, Europe, and Australia. In other parts of the world, there have been reports of isolated outbreaks with O27 and O17, but no epidemic has been reported. However, in the event of an epidemic, developing

countries with poor overall hygienic practices and sanitation may make this global challenge even worse.

With the rapid increases in hospitalization and mortality rates due to *C. difficile* and the associated increase in virulence of the strains and diminished anti-microbial sensitivity, *C. difficile* may prove to be a challenge to the healthcare system globally. This highlights the need to focus on setting up a surveillance system to survey trends in outbreaks and develop better awareness and prevention techniques with the goal of reducing newly diagnosed *C. difficile* cases by 2020. Currently, there are two surveillance systems including the National Nosocomial Infections Surveillance system (NHSN) and CDI surveillance system of the Emerging Infections Program (EIP) to monitor *C. difficile* rates across the US. ⁽⁹⁴⁾ A joined effort of raising awareness regarding implementation and adherence to infection control practices and prevention strategies are the key to reducing CDI. According to the Society for Healthcare Epidemiology of America, infection control measures include patient isolation, contact precaution, environmental sanitation, avoidance of rectal thermometer use, and washing hand with soap and water. ⁽¹¹⁸⁾ In this proposal, we will look at the effect of disinfectants on *C. difficile* and its transmission.

INTRODUCTION TO CURRENT PROPOSAL

Disinfectants and C. difficile:

Disinfectants are antimicrobial chemical compounds used alone or in combination in healthcare or community setting to prevent or control the spread of common and emerging pathogens⁽¹¹⁹⁾. Based on the site and nature of use there are various formulations and modes of use for different disinfectants. The choice of disinfectant depends on the type pathogen, site of use, toxicity of the disinfectant, environmental and occupational concerns associated with the use of disinfectants, and the type of healthcare equipment or complex medical instruments needing disinfection.

Environmental disinfection, personal hygiene practices, and barrier methods are the three key elements of in-hospital *C. difficile* infection control. *C. difficile* spores shed through feces can contaminate and persists on the hard surfaces for a long time. ^(37, 120)

According to Rachel et al., appropriate use of disinfectants could result in a 59% decrease in CDI cases. This can be translated to the prevention of 59,000 CDI cases and 82,000 CDI deaths over a period of 5 years resulting in a saving of \$2.5 billion. ⁽¹²¹⁾

Despite various guidelines describing the judicious and proper selection of disinfectants, *C. difficile* infection control presents a significant challenge both at the hospital and community levels. ⁽³⁶⁾

Evidence suggesting current disinfectant practices are sub-optimal

Disinfectant use is a widely practiced infection control technique at the level of hospitals. However, there are very few measures in place to monitor the efficacy and effectiveness of the disinfectants. A study done at a University-affiliated community teaching hospital found that high touch areas in the hospital were often not cleaned and even after disinfectant use 16 to 24 % of the surfaces were still contaminated indicating suboptimal use of disinfectants⁽¹²²⁾ Another study found that only 47% of the high touch areas were cleaned after 2 to 3 terminal cleanings. ⁽¹²³⁾ Absence of very few recognized techniques in place to measure disinfectant efficacy, wide variation in disinfectant use, differential exposure times to disinfectants, and lack of adequate knowledge and research regarding proper disinfectant use are some of the most important steps leading to suboptimal disinfectant use. ⁽¹²⁴⁾

Evidence that currently used disinfectants may not kill C. difficile spores

a. Hospital-based disinfectants

Up to 49% of samples obtained from CDI patient rooms and 29% of samples obtained from an asymptomatic *C. difficile* carrier patient rooms are contaminated with *C. difficile* spores. ^(30, 38) Various commonly used hospital disinfectants include alcohol-based disinfectants, chlorine-based disinfectants, iodine-based disinfectants, phenol, quaternary ammonium compounds, and hydrogen peroxide based disinfectants. ⁽¹²⁵⁾ However, with the exception of chlorine and iodine-based disinfectants, none of the other

disinfectants display consistent sporicidal effects. ⁽¹²⁵⁾ Also, chlorine- and iodine-based disinfectants have only fair sporicidal effects. ⁽¹²⁵⁾

Furthermore, the evidence is lacking regarding the disinfection of hospital environments based on the level of spore contamination. ⁽¹²⁶⁾ There is evidence that disinfectants might not be enough to decrease the CDI incidence with the need for more effective and less toxic sporicidal agents. ^(127, 128) Some studies found no significant role of disinfectant used in reducing nosocomial with *C. difficile* infection rates. ⁽¹²⁹⁻¹³¹⁾

Other studies found a formulation dependent effectiveness of hospital-based disinfectant in reducing *C. difficile* incidence and transmission rates. ⁽¹³²⁾ A hypochlorite containing disinfectant was found to reduce *C. difficile* incidence (8.6% compound to 3.3% after disinfection use), whereas a shift to quaternary ammonium compounds increased *C. difficile* incidence from 3.3 to 8.1 patient days. ^(126, 132) Wilcox *et al.* found that the same disinfectants may have varied effects based on the intensity of environmental contamination, hand hygiene practice of the hospital personnel, and various confounding variables including changes and levels of antibiotic use, prescription, patient type, and cleaning efficiency. ^(30, 126) Another group found the working strength concentration of different disinfectants used (except chlorine-based compounds) only had an impact on vegetative cells but not spores. ⁽¹³³⁾ A study looking at the effect of different environmentally safe hospital

disinfectants found that glutaraldehyde and para acetyl ions were highly sporicidal independent of organic load conditions. ⁽¹³⁴⁾ However, another study found that diluting glutaraldehyde <2% resulted in the inability of the disinfectants to kill spores at commonly employed contact time. ⁽¹³⁵⁾

Disinfectants are currently the main modality for control of biofilms on abiotic surfaces. ⁽¹¹⁵⁾ Various chemical-based commercial disinfectants with a different mechanism of action are used in various concentration to disrupt biofilm on the surfaces. Chemical disinfectants currently used against biofilms include acidic compounds, chlorine-based compounds, hydrogen peroxide, para acetic acid, phenolics, surfactants, iodine and so on. ^(136, 137) However, the efficacy of most of the currently used disinfectants is based on the planktonic form of the microorganism. ⁽¹³⁸⁾ Microorganism in biofilm may be up to 1000-fold more resistant to disinfectants in comparison to their planktonic counterpart. ⁽¹³⁹⁻¹⁴²⁾ Currently, there is no study which looked at the efficacy of various hospital-based disinfectants against *C. difficile* in a biofilm.

b. Household disinfectants

Although there are numerous studies looking at the effect of hospital-grade disinfectants with varied findings very few studies to date have looked at the sporicidal effect of household and eco-friendly disinfectants. ^(36, 126, 128) A study by Vohra et al. found that only chlorine-based disinfectant was able to

eliminate *C. difficile* spore from the community environment and prevent lab to community transmission of Bacillus spores. ⁽¹⁴³⁾

Thus, in spite of increasing incidence and severity of *C. difficile* and growing demand for adequate environmental disinfection, there is no consensus on the appropriate use of chemical disinfectants for environmental disinfection of *C. difficile* spores. This is further coupled by issues of safety, access, sporulation capacity and various disinfectant related confounding factors and a variety of effect based on *C. difficile* strains. **This dissertation aims to look at the effect of hospital and community household disinfectants on multiple clinical and community-acquired environmental *C. difficile* strains both in planktonic and in biofilm and to further explore various mechanistic theories underlying their effect on *C. difficile* eradication and propagation.**

Gap in current literature and Public Health Significance of this study

As previously stated, *C. difficile* is a major nosocomial infection and is responsible for one-third of all nosocomial infections in the US. With the increasing prevalence, incidence, mortality, hospitalization and health care cost due to *C. difficile* infection coupled with prediction of frequent epidemic and pandemic there is a need to focus on the *C. difficile* infections worldwide. Keeping in pace with the situation the available antibiotics are becoming resistance. This window of coexistence of clinical and environmental *C. difficile* with newly developing antibiotic resistance has validated the need to focus on the primary prevention of *C. difficile* infection. One of the primary prevention strategies is the use of disinfectants. There is almost no study which looked

at the effect of disinfectants on the environmental *C. difficile* spores. There are some articles which looked at the effect of hospital disinfectants on *C. difficile* spores. However, there is lack of consistency between the results of those studies in determining the exact role of disinfectants on *C. difficile* spores, the effective concentration, optimal contact time, effect of organic matter on the efficacy of disinfectants and the effective formulation. Currently, there is no study which looked at the effect of hospital disinfectants on *C. difficile* spore encased in a biofilm. Again, there is little or no study which looked at the effect of household disinfectants on *C. difficile* spores or on their virulence. Our study will look at these gaps in the literature. Also, based on our findings we might do explorative research to determine the mechanism of action of disinfectants on *C. difficile* spores. This study is novel as we will try to characterize the actual effect of disinfectants on the spores and whether the disinfectants enhance the growth of spores. This study has enormous public health significance. Firstly, we can determine the disinfectants which are actually working in the hospital environment both in planktonic form as well as in biofilm and advocate for their use in the hospitals so, as to be able to reduce the *C. difficile* spore load in the hospitals. Secondly, though the experiments will be done in vivo, care will be taken to make the environment of the experiments as close to the hospital environment. Thirdly, we can characterize which disinfectants are working in the household environment. We will try to see whether any disinfectant increases the growth of spores. If any such disinfectants are found then that might change the protocol for household disinfection use worldwide. This may play a vital role in the environmental control of *C. difficile*. Finally, we will also try to look at the microscopic effect of disinfectants activity on the

spores both in planktonic form and in biofilm and if possible, explore the mechanism of action of these disinfectants. Thus, we will apply a holistic approach to correctly classify the sporicidal efficacy of disinfectant and their overall effect which might, in turn, change the protocol of disinfectant use worldwide and provide a check to the impending epidemic and pandemic.

AIMS AND OBJECTIVES

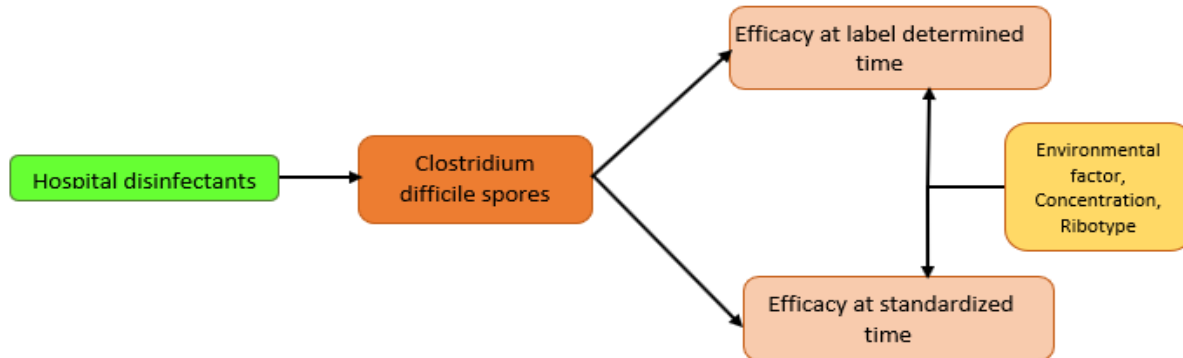
The overall objective of this proposed study was to determine the effect of hospital and community household disinfectants on multiple clinical and community-acquired environmental *C. difficile* strains both in planktonic and in biofilm and to further explore various mechanistic theories underlying their effect on *C. difficile* eradication and propagation. To achieve these objectives, we pursued the following aims:

Specific Aim 1: To determine the *in vitro* sporicidal efficacy of commercially available hospital disinfectants against clinical and environmental *C. difficile* spores.

- **Sub-aim 1:** To determine the sporicidal efficacy of hospital disinfectants against clinical and environmental *C. difficile* isolates at high and low spore concentrations and in the presence and absence of organic substrates at label determined time.
- **Sub-aim 2:** To determine the sporicidal efficacy of hospital disinfectants against clinical and environmental *C. difficile* isolates at high and low spore concentrations and in the presence and absence of organic substrates at label determined time.
- **Sub-aim 3:** To determine the effect of various environmental factors, the concentration of disinfectants and ribotype on disinfectant efficacy.

Summary: Time-kill microbiology techniques were used to assess the killing of *C. difficile* spores by disinfectants at various organic matter concentrations based on label determined and standardized contact time.

Conceptual model:



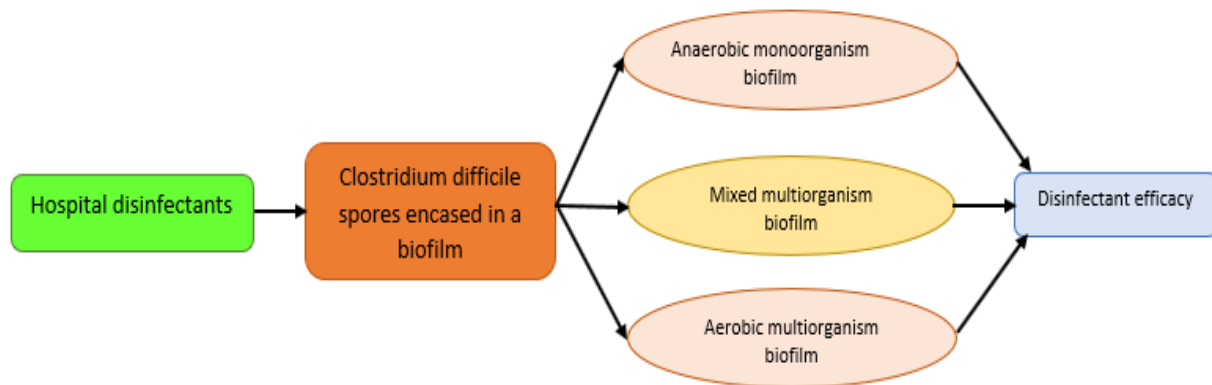
Specific Aim 2: To determine the efficacy of Hospital disinfectants on *C. difficile* spores and vegetative cells encased in a biofilm.

- **Sub-aim 1:** To determine the efficacy of hospital disinfectants on *C. difficile* spores and vegetative cells encased in an anaerobic mono organism *C. difficile* biofilm
- **Sub-aim 2:** To determine the efficacy of hospital disinfectants on a laboratory-derived *C. difficile* biofilm colony encased in a multi-organism biofilm and grown aerobically
- **Sub-aim 3:** To determine the efficacy of hospital disinfectants on *C. difficile* spores and vegetative cells encased in an aerobic multiorganism *C. difficile* biofilm.

Summary: *C. difficile* biofilms were grown over three conditions – anaerobic mono organism, mixed multi-organism, and aerobic multi-organism over 72 and 120 hours.

The biofilms were exposed to disinfectants and efficacy tested against vegetative cells, spores, and biomass.

Conceptual model:

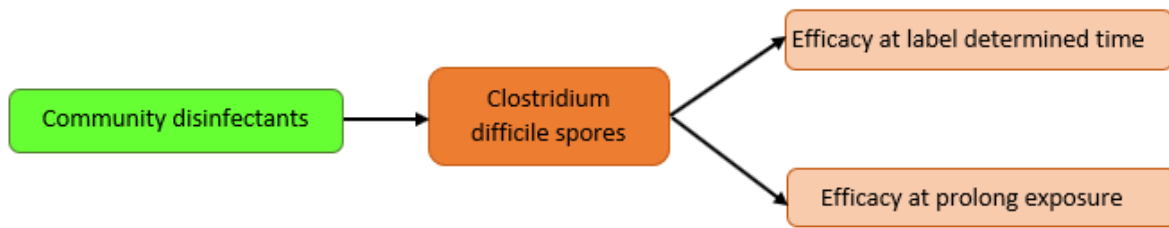


Specific Aim 3:

Specific Aim 3A: To assess the effect of community disinfectants on clinical and environmental *C. difficile* spores.

Summary: Time-kill microbiology technique and time kill curves will be used to assess the effect of community disinfectants on *C. difficile* spores.

Conceptual model:



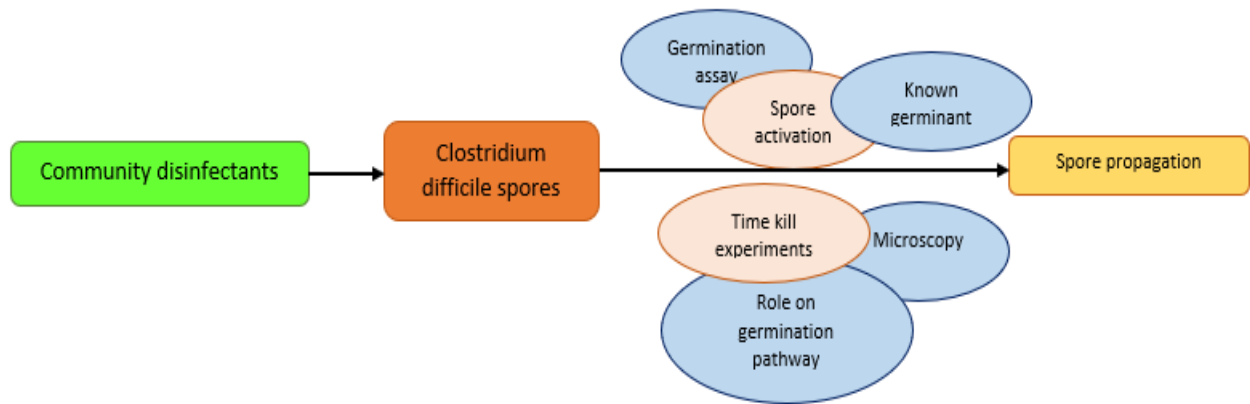
Specific Aim 3B: To investigate the role of Community disinfectants in the propagation of *C. difficile* spores.

- **Sub-aim 1:** To compare and quantify the effect of community disinfectants with known germinant for *C. difficile* spores.
- **Sub-aim 2:** To determine if any active ingredient in community disinfectants predict *C. difficile* growth
- **Sub-aim 3:** To determine if community disinfectants are activating *C. difficile* spores
- **Sub-aim 4:** To determine the role of community disinfectants on *C. difficile* spore germination pathway
- **Sub-aim 5:** To investigate the effect of community disinfectants on *C. difficile* spores microscopically

Summary: Time-kill curves, the effect on the active vs. dormant spore, germination assay, Ca-DPA assay, spore cortex fragmentation assay, qPCR and

confocal microscopy techniques were used to assess the role of disinfectants on *C. difficile* spore propagation.

Conceptual model:



METHODS

Study Design:

A lab-based study was undertaken to look at the effect of various disinfectants on *C. difficile* spores. All experiments were carried out in a controlled environment, following strict protocols and various biosafety rules and regulations. For the purpose of this research, we worked with 7 household disinfectants, 7 hospital-grade disinfectants and 16 *C. difficile* isolates of 6 different ribotypes. A list of *C. difficile* isolates, as well as disinfectants used, is shown in **Table 3 and 4** below.

Study Setting:

This study was done at a lab at the University of Houston College of Pharmacy. This study was conducted between May 2016 and Dec 2018. The stool samples for this study were deidentified and mostly collected before or during the study as a part of another IRB approved the project.

Study Sample:

The study sample included the following:

❖ ***Clostridioides difficile* sample processing:**

A. Clinical *C. difficile* isolates-

Stool sample from hospitalized patients with CDI that were deidentified, stocked and ribotyped was used. Stool processing included the following⁽³⁴⁾-

- Enrichment of sample in brain heart infusion (BHI) broth with 0.05% sodium taurocholate (Sigma chemicals).
- Anaerobic culture of enrichment broth at 37°C in an anaerobic chamber for 3 days.
- Centrifugation of 1 ml from enrichment broth, resuspension of pellet in 100µL DW and plating on CCFA (cycloserine cefoxitin fructose) agar and anaerobic incubation for 48 hours.
- Suspected colonies tested using latex agglutination reagent (*C. difficile* test kit) stocked and frozen at -80°C.
- Presence of toxin genes including toxin (A), toxin (B) and binary toxin (CDT A and CDT B) identified through a multiplex polymerase chain reaction.
- Ribotyping was done based on Walk *et al.* protocol⁽⁵⁹⁾.

B. Environmental *C. difficile* isolates:

Trained research staff collected specimens from household items or environmental dust from houses in Houston, Texas. The samples were collected in 50 ml size pre-sterilized tube and transported to the lab within 12 hours of collection. Following similar protocol as for clinical samples,

suspected *C. difficile* colonies were stocked, ribotyped and assessed for toxin types.

For the purpose of this study, deidentified, toxin positive and ribotyped samples from these stock vials were randomly selected for further processing and isolation in pure culture. The clinical and environmental *C. difficile* isolates which were randomly chosen are given in the table below-

Table 3: <i>Clostridioides difficile</i> strains		
Type of Isolate	<i>Clostridioides difficile</i> ribotypes	Number of strains
Clinical Isolates	O14-20	2
	O27	3
	O78-126	2
	UM 11	2
	O12	1
Environmental Isolates	O14-20	2
	O78-126	2
	UM 11	2

❖ ***Clostridioides difficile* spore preparation:**

C. difficile sample stock from samples described above was used for spore preparation. Initially, samples from *C. difficile* stock were plated at least two times

on blood agar until a pure culture of *C. difficile* isolates was obtained. Once a pure culture was obtained and confirmed by latex agglutination and PCR, spore preparation was done. A spread plating of *C. difficile* isolates was done on a blood agar plate and incubated for at least 72 to 96 hours for spore preparation.

For the preparation of *C. difficile* spore stock, two loops of spore from the plate was suspended in 1000 µL DW, mixed uniformly and centrifuged at 10000 G for two minutes. The pellet obtained was treated with 1000 µL of 70% isopropanol to kill vegetative cells. After re-centrifugation of the mixture, 1000 µL of DW was added to complete the spore preparation. The spore stock was serially diluted and plated on blood agar for spore count. Ideal countable plate count was between 30 to 300 CFU/ plate. The ideal spore count expected for low concentration spore will be 10^5 . The spore count will be calculated using the formula:

Spore count = CFU count on blood agar plate * 10 * dilution factor

Ideal low, medium, and high spore count were $<10^3$, 10^3 - 10^5 and $>10^5$ CFU/ml respectively.

Spore preparation for specific aim 2 was done as previously described. ⁽¹⁴⁴⁾ Briefly, *C. difficile* strains were streaked onto brain heart infusion-supplemented (BHIS) agar and incubated anaerobically for 4 days. Surface microbial growth was scraped and mixed with 1 ml sterile ice cool water in an Eppendorf and incubated overnight at 4°C. The

suspension was then washed five times in ice-cold sterile deionized water (DW). The washed pellet was suspended in 3ml ice cold deionized water (DW). The suspension was then layered on top of a 10 ml bed of 50% sucrose in water and centrifuged at 3,200g for 20 minutes. The spores formed a pellet at the bottom of the tube. The spore pellets were washed 5 times to remove sucrose and was then re-suspended in DW and stored at 4°C.

❖ Disinfectant selection

A. Community disinfectants

Community disinfectants were defined as chemical anti-microbial component commercially available for common household or institutional use against potential human pathogens.⁽¹³⁵⁾ For the purpose of this research, we visited a commercial supermarket and visually monitored the disinfectant choice of the first 50 shoppers and made a list using a form shown in Appendix A. Based on study aims and feasibility of testing, only disinfectant sprays and refills were considered. No cleaners or degreaser, disinfectant swabs or deodorizer were considered. The top 5 disinfectants from the list in the form were considered as the 5 household disinfectants for this study. In addition, two ecological community disinfectants were chosen based on availability and feasibility for the study purpose as shown in **table 4**

Table 4: Community disinfectants		
Name of Disinfectant	Active ingredient	Label determined contact time
Spic and span	Octyl decyl dimethyl ammonium chloride Dioctyl dimethyl ammonium chloride Didecyl dimethyl ammonium chloride Alkyl (50%C14, 40%C12, 10%C16) dimethyl benzyl ammonium chloride	10 minutes
Windex	Lactic acid	10 minutes
Clorox	Sodium hypochlorite	1.5 minutes
Lysol with H2O2	H2O2	10 minutes
Lysol	Citric acid	10 minutes
Seventh Generation	Thymol, Sodium Lauryl sulfate, copper sulfate, pentahydrate, sodium citrate, essential oil	10 minutes
Pure Green 24	SDC (Silver ions and citric acid)	10 minutes

B. Hospital-grade disinfectants

Hospital grade disinfectants were defined as substances which were suitable for general purpose disinfection of hospital buildings, patient rooms or wards as per protocol and did not include instrument grade disinfectants, anti-bacterial cloths preparations, sanitary fluid, sanitary powder or sanitizer. ⁽¹⁴⁵⁾ According to CDC, hospital grade disinfectants are those disinfectants which are registered with EPA for use in hospitals, clinics, dental offices or any other medical related facility with efficacy tested against *Salmonella choleraesuis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. ^(146, 147) Commonly 1,200 hospital grade disinfectants are registered with EPA. Some of the EPA registered disinfectants are labeled to kill *C. difficile*. There are various disinfectants based on the mechanism of use as chemical disinfectant, thermal disinfectant, UV rays, vapors, steam sterilization, gas sterilization, and ionizing radiation, etc. For the purpose of this study, we only considered chemical hospital grade disinfectants. The chemical hospital grade disinfectants used were chosen from the list of top 10 hospital disinfectants based on the availability, exposure hazards, and cost. ⁽¹⁴⁷⁾ Two additional commonly used eco-friendly hospital grade disinfectants were also selected for this study based on feasibility and availability. The disinfectants chosen were given in **table 5** below-

Table 5: Hospital-grade disinfectants			
Name of Disinfectant	Active ingredient	Label determined contact time	Sporicidal claim
Formalin	Formaldehyde (We used 4% formaldehyde based on CDC recommendation)(146)	2 hours	Yes
McKesson OPA/28	Ortho- phthaldehyde	10 minutes	Yes
Sporox II	7.5% H ₂ O ₂	30 minutes	Yes
Clorox	10% Sodium hypochlorite	2 minutes	Yes
Virex	Quaternary ammonium compound (5-10%)	10 minutes	Yes
Vital Oxide	Stabilized ClO ₂	10 minutes	No
Nixall	Hypochlorous acid	10 minutes	Yes

These are the basic study samples required for this lab-based research. A quick summary of the study done is given in **Table 6**.

Table 6: Summary Table	
AIMS:	
AIM 1: Hospital Disinfectant study	
AIM 2: Biofilm study	
AIM 3: Community disinfectant study	
DISINFECTANTS:	Clostridioides difficile strains
7 Community disinfectants	10 Clinical isolates
7 Hospital grade disinfectants	6 Environmental isolates
GENERAL:	
Replicates – 2 independent replicates	
Time- Label determined contact time and standardized time of 10 minutes	
Plating- Blood Agar plate	
Anaerobic chamber	

Data Collection Procedure:

All the experimental procedures for this study are mentioned in the 3 manuscripts attached. Briefly, the experimental procedures are given as follows-

- **Specific Aim 1:** Manuscript 1 summarized the data for specific aim 1 which looked at the efficacy of hospital disinfectants against clinical and environmental *C. difficile* spores. Time-kill studies were done by exposing the *C. difficile* spores of different concentrations to the original concentration of different hospital disinfectants at label determined time and the spore count measured post exposure and compared with unexposed controls. These experiments were repeated at different disinfectant concentration, presence or absence of organic matter and standardized time. Minimum organic matter concentration for disinfectants affected by organic matter was also determined.

- **Specific Aim 2:** The data for specific aim 2 is summarized in manuscript 2. Manuscript 2 looked at the efficacy of hospital disinfectants on *C. difficile* spores encased in a biofilm. Three different biofilms were produced in this study. Strictly anaerobic mono-organism *C. difficile* biofilm was produced by adding 72 hour culture to 24 well plates and incubating anaerobically for 72 and 120 hours respectively. For mixed multi-organism biofilm, a mixture of overnight *Enterococcus faecium* and *Staphylococcus aureus* culture was added to an anaerobically grown 48 hour *C. difficile* biofilm and the mixture incubated aerobically for 24 and 72 hours. Aerobic multi-organism biofilm was produced by

adding 72 hours *C. difficile* culture was added to an overnight culture of *Enterococcus faecium* and *Staphylococcus aureus* in a 24 well plate and the mixture incubated for 72 and 120 hours respectively. Following exposure, the biomass was measured using crystal violet assay and the biofilms were exposed to hospital disinfectants. Total count, vegetative cell count, and spore count were measured post exposure and compared with unexposed biofilms. The biofilms were visualized using inverted light microscopy to indicate the surface biofilm cleaning post-exposure to disinfectants. Confocal microscopy was done using Live/dead biofilm viability staining to assess the viability of cells in a biofilm and effect of disinfectants on biomass.

- **Specific Aim 3:** Manuscript 3 summarized the data for specific aim 3. For this aim, time-kill study and time-kill curve were done by exposing the clinical and environmental *C. difficile* strains to different concentrations of community disinfectants. To further characterize the effect of disinfectants on *C. difficile* spores, experiments were done to determine the effect of the active ingredient of disinfectants and effect on active spore vs. dormant spore. Effect of disinfectant on *C. difficile* spore germination was determined through germination assay, Ca-DPA assay, and spore cortex fragmentation assay. qPCR was done to determine whether disinfectants down or up-regulated any gene in the germination pathway. The effect of disinfectants was further visualized microscopically through the confocal microscope.

Data Analysis:

Data were collected, summarized, tabulated and analyzed using Excel and STATA12.1 (STATA Corp LLC, College Station, TX). GraphPad Prism 8.02 was used to analyze the results of optical density and creating the data graphs and standard curves for various assays. The results are presented through bar charts, box plots, tables, regression analysis tables, growth curves, kinetics curves, curves for Ca-DPA assay and spore cortex fragmentation assays, microscopic pictures and so on. Efficacy for the disinfectants will be expressed as log reduction of percent reduction. In percent scale, 1 log reduction means 90% reduction, 2 log reduction means 99% reduction, 3 log reduction means 99.9% reduction, 4 log reduction means 99.99% reduction and so on.

JOURNAL ARTICLE: A

Title of Journal article:

Organic material, spore concentrations, and contact time reduces the effectiveness of hospital disinfectants against *C. difficile* spores regardless of PCR-ribotype

Name of Journal Proposed for Article Submission: Infection Control and Hospital Epidemiology or Diagnostic Microbiology and Infectious Diseases

Organic material, spore concentrations, and contact time reduces the effectiveness of hospital disinfectants against *C. difficile* spores regardless of PCR-ribotype

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Running title: C diff spores and hospital disinfectants

Keywords: in vitro study; anaerobic infections; environmental decontamination; ribotype 027;

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Abstract

Background: Chemical disinfection is the most common strategy employed to eradicate *C. difficile* spores from the environment. Causes for the poor sporicidal effect include the degree of spore contamination, the presence of organic substances, and contact time. Thus, the aim of this study was to determine the effect of these variables on the *in vitro* sporicidal efficacy of hospital disinfectants against a variety of *C. difficile* ribotypes.

Methods: Six sporicidal hospital disinfectants were tested against *C. difficile* spores of known ribotypes. Mean \log_{10} colony forming reduction \pm SE was calculated for each disinfectant by ribotype, contact time, organic substrate, and initial spore concentration. To assess the independent effect of the experimental conditions, a general linear model was built testing the effect of \log_{10} colony forming reduction by each disinfectant along with ribotype, initial spore concentration, contact time, and organic substrate.

Results: All tested disinfectants were effective against *C. difficile* spores using the manufacturer suggested contact time at all spore concentrations. However, the killing effect decreased with increasing amounts of organic substances and using a standardized contact time. Using the general linear model, sporicidal activity of the disinfectants was affected by a shorter contact time (0.52 ± 0.10 log decrease; $p < 0.0001$), and presence of an organic substance (low organic substance: 0.77 ± 0.11 log decrease; $p < 0.0001$; high organic substance: 2.02 ± 0.12 log decrease; $p < 0.0001$).

Conclusion: Using multiple *C. difficile* ribotypes, we demonstrated the independent effect of organic substrates, contact time, and spore concentrations to affect the

sporicidal activity of hospital disinfectants. Although differences were noted amongst disinfectants, no ideal disinfectant was identified.

Introduction

Clostridioides difficile, a motile, gram-positive spore-forming anaerobic bacillus is the most common cause of healthcare-associated infections in the USA.^(1, 2) *Clostridium difficile* infection is a major challenge for the health care industry both in terms of high morbidity, mortality, hospital stay, recurrence rate as well as frequent outbreaks of highly virulent antibiotic resistance strains.⁽³⁾ *C. difficile* exists in two forms, a strict anaerobic vegetative cell, and a highly resistant, metabolically dormant spore.⁽⁴⁻⁶⁾ Transmission of spores can occur through direct inoculation or contact with contaminated surfaces.⁽⁷⁾ Hospital surfaces including walls, floors, bedpans, stethoscope, blood pressure cuffs, and hands, and shoes of healthcare workers have all shown high rates of *C. difficile* contamination.^(8, 9) Chemical disinfection is the most common strategy employed to eradicate *C. difficile* spores from the environment. Although widely employed, the efficacy rates of chemical disinfection of *C. difficile* spores vary.⁽¹⁰⁻¹²⁾ Causes of poor sporicidal effect include the degree of spore contamination, presence of organic substances, the concentration of disinfectant and contact time of the disinfectant with the environment.^(11, 13-15) In addition, the sporicidal effect among different ribotypes is unclear and has not been adequately studied.⁽¹⁶⁾ Studies have documented the concept of disinfectant resistance in various hypervirulent *C. difficile* strain.⁽¹⁶⁾ However, there are few studies that investigated the effect of commercially available hospital disinfectants on *C. difficile* spores. Thus, the aim of this study was to determine the factors affecting the *in vitro* sporicidal efficacy of commercially available hospital disinfectants against a variety of *C. difficile* spores.

Methods:

Bacterial Isolates

Clinical and environmental *C. difficile* spores of known ribotype were obtained from an ongoing prospective cohort study.⁽¹⁷⁾ For this study, the following ribotypes (number of isolates) were used, ribotypes O14-20 (n=4), O27 (n=3), 078-126 (n=4), 106 (n=3), and laboratory strains R20291 (ribotype 027), strain 630 (ribotype 012), and a non-toxigenic *C. difficile* strain (ATCC700057).

Spore preparation

C. difficile spores were prepared from the stocked *C. difficile* strains by incubating pure isolates anaerobically at 37°C on blood agar for 72 to 96 hours.⁽¹⁸⁾ A 10 µL loop was used to lift colonies of spores which were then suspended in 1000 µl deionized water (DW), centrifuged at 10000 g for 2 min, and then treated with 1,000 µl of 70% isopropanol to kill the vegetative cells. The mixture was then centrifuged, re-suspended in 1000 µl DW and stored aerobically at 4°C. Spore counts were determined on plates after 72 hours using serial dilution and plating technique, and calculations were performed to give CFU/mL. This modified spore preparation protocol was validated and standardized. Spore count from the stock was done before every experiment to ensure standardization.

Spores were prepared for low (<10³ CFU/mL), medium (10³-10⁵ CFU/mL) and high (>10⁵ CFU/mL) spore experiments.

Disinfectants tested

Seven hospital disinfectants were used in this study (**Table A.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 preparation, sanitary fluid or hand sanitizers. Disinfectants were chosen from a list of top 10 hospital disinfectants. ⁽¹⁹⁾ Among the seven disinfectants chosen for this study all except Vital oxide were documented to have sporicidal activity against spores. ⁽²⁰⁾ Vital oxide, which is not marketed as an anti-sporicidal agent, was used as an active control in all experiments. The common mechanism for the disinfectants used in this study is given in **supplementary figure A.1**. For each disinfectant, contact time experiments were conducted based on manufacturer determined contact times, or study standardized contact time of 10 minutes.

Sporicidal assay

The disinfectants were tested for their sporicidal activity in suspension test at the recommended concentration as well as 1/2, 1/5 and 1/10 of the same. ⁽¹⁸⁾ For each test, around 100 µl spore suspension was added to 900 µl disinfectants of different dilutions, mixed thoroughly and incubated based on label determined and standardized time. Following incubation, 100µl from spore disinfectant mixture was added BHIS and bile salt and incubated anaerobically at 37°C for 5 days. There was positive and negative control for each disinfectant tested. To identify bactericidal activity, the mixture was also plated on blood agar and growth observed after 3 days.

Experimental procedures

The protocol for determining the sporicidal activity was developed and standardized in-house and adapted from various published methods. ⁽²¹⁾ Spore stock (20 µl) was added to 500 µl of disinfectants and incubated aerobically at ambient room temperature (20-25°C) for appropriate contact time (label determined and standardized). After incubation, 100 µl of heat-killed *Candida* in DW was added to concentrate spores. ⁽²²⁾ Carrier *Candida* significantly improved the recovery of spores during centrifugation. The spore, disinfectant and candida mixture was centrifuged at 15000 g for 2 minutes and spore pellet washed and resuspended in 200 µl DW. 100 µl of the suspension was plated on blood agar and incubated anaerobically at 37°C for 72 hours. Each experiment was done in duplicate and repeated on different days. For each time point and each strain, there was a positive control without disinfectant maintained simultaneously and in duplicate and a negative control plate to check the purity and contamination of DW. After 72 hours, viable spores were counted and compared to positive control plates. The limit of detection of the assay is 2 CFU/plate. Results were reported as logarithmic reductions compared to positive controls; errors bars are a standard error of the mean (SEM). These experiments were repeated at low, medium and high concentration of spores and at two previously specified contact time. The percent eradication of spores by the disinfectants was also determined.

In separate experiments, the effect of organic substrates was tested using a complex organic matter called BHI (Brain Heart Infusion) broth. The effect of organic substrate on disinfectant efficacy at high and low spore concentration was tested by adding 40 µl

of organic matter (protease peptone 10gm/L) from low (30 mg/mL) or high (300 mg/mL) concentration into the experiment. The final concentration of organic substrate in the test mixture was 2.4mg/ml for low concentration and 24mg/ml for high concentration. All experiments were done in duplicate on different days. Positive and negative controls were included in each experiment.

Analysis

The efficacy of the disinfectants was expressed as a \log_{10} reduction in CFU count. Mean \log_{10} colony forming reduction \pm SE was calculated for each disinfectant by ribotype, contact time, organic substrate, and initial spore concentration. To assess the efficacy of the tested disinfectants controlling for experimental conditions, a general linear model was built using the \log_{10} colony forming reduction as the dependent variable and each disinfectant along with ribotype, initial spore concentration (low, medium, high), contact time (standardized vs. manufacturer recommended), organic substrate (none, low, high) as covariates. To determine the effect of these covariates on each disinfectant, separate general linear models were built for each disinfectant. SAS version 9.3 (SAS Institute, Cary NC) or STATA/IC 12.1 (STATACorp LLC, College Station, TX) was used for all analyses. A p value of <0.05 was considered significant.

Results

Sporicidal assay

All experiments were done on 7 hospital disinfectants and 17 *Clostridioides difficile* spores of various ribotypes to determine disinfectant efficacy. The sporicidal

concentration for the disinfectants tested was ascertained at label determined time and study standardized contact time for exposure to disinfectants (**Table A.2**). Although there was no significant difference among the ribotypes for the sporicidal concentration of disinfectants, it was observed that a slightly higher concentration of disinfectants was required for killing the spores of O27 strains. For Nixall any concentration below original concentration was not sporicidal. Some of the disinfectants were sporicidal even at one-fifth of the recommended concentration.

Spore eradication

The percent eradication of spores post-exposure to disinfectants was calculated both, at label-determined time of exposure, as well as standardized exposure in the presence or absence of organic matter (**Figure A.1**). At the label-determined time, there was almost 100% spore eradication post-exposure to Clorox, Sporox, OPA, Nixall, Formalin, and Virex. However, with an increase in organic matter concentration, there was a gradual decrease in spore eradication reaching up to 10% for some of the disinfectants. Clorox, OPA and Nixall were comparatively more affected by the presence of organic substrate compared to other disinfectants tested. However, at a study standardized contact time of 10 minutes, the sporicidal efficacy of Clorox increased and was found to be effective (>90% spore eradication) even at high organic substrate concentration. The efficacy of Sporox and Formalin whose label determined contact time was higher than standardized time, reduced drastically and were ineffective even in the absence of organic matter.

Efficacy of disinfectants and the effect of organic matter on the efficacy

Mean \log_{10} colony forming reduction \pm SE reduction was calculated for each disinfectant by contact time, initial spore concentration, and organic substances (**Figure A.2**). All tested disinfectants were effective against *C. difficile* spores using the manufacturer suggested contact time at low, medium, and high spore concentrations in the absence of organic substrate. However, mean \log_{10} colony forming reduction was decreased with increasing amounts of organic substances and using a standardized contact time. Using the general linear model, \log_{10} CFU reduction by the disinfectants was affected by increasing spore concentration (medium spore: 0.76 ± 0.13 log increase; $p < 0.0001$; high spore: 1.72 ± 0.12 log increase; $p < 0.0001$), a shorter contact time (0.52 ± 0.10 log decrease; $p < 0.0001$), and presence of organic substance (low organic substance: 0.77 ± 0.11 log decrease; $p < 0.0001$; high organic substance: 2.02 ± 0.12 log decrease; $p < 0.0001$). For disinfectants mostly affected by organic substrate, a 10-fold reduction in organic substrate concentration significantly improved the disinfectant efficacy. A linear trend in increasing disinfectant efficacy with a decrease in organic substrate concentration was seen for Nixall disinfectant (**supplementary figure A.2**). The sporicidal activity of the disinfectants did not differ between the various ribotypes or between environmental vs. clinical strains or between the toxigenic and non-toxigenic strains ($p > 0.05$, each). Controlling for significant variables, sporicidal activity was highest for Virex (3.00 ± 0.16 log decrease; $p < 0.0001$) followed by Clorox (2.90 ± 0.16 log decrease; $p < 0.0001$), OPA (2.12 ± 0.16 log decrease; $p < 0.0001$), Sporox (1.76 ± 0.16 log

decrease; $p < 0.0001$), Formalin (1.69 ± 0.16 log decrease; $p < 0.0001$), and Nixall (1.36 ± 0.16 log decrease; $p < 0.0001$).

Individual general linear models were built for each disinfectant to assess the effect on spore concentration, organic substance, contact time, and ribotype on sporicidal activity (**Table A.3**). All disinfectants had reduced sporicidal activity at high organic substance levels as well as with increased spore concentrations.

Disinfectant efficacy by ribotype

None of the ribotypes had any inherent resistance to the disinfectants tested. Although there was no significant difference in disinfectant efficacy by ribotype, there was variation in the magnitude of log reduction. Interestingly, the \log_{10} reduction was more affected in the presence of organic matter for two of the epidemic strains O27 and O14-20 (**Figure A.3**).

Efficacy of Chlorine-based disinfectants against spores of *Clostridium difficile*

A closer look at the three Chlorine-based disinfectants Clorox (Sodium hypochlorite), Vital oxide (Chlorine dioxide) and Nixall (Hypochloric acid) indicate that presence of chlorine ion in a disinfectant did not guarantee effective killing. Of the three disinfectants, Vital oxide was ineffective irrespective of contact time, spore concentration or organic substrate concentration. The killing effect of Nixall was almost 100% in the absence of organic matter concentration but even any presence of organic matter rendered it inefficient. Although Clorox was the most sporicidal chlorine-based

disinfectant, the effect of Clorox varied greatly between duration of exposure and concentration of organic matter (**supplementary figure A.3**).

Discussion

Chemical disinfection is the most common infection control strategy to decrease the environmental bioburden of *C. difficile* spores in the healthcare environment. ⁽²³⁾

Despite multiple disinfectants with *in vitro* potency against *C. difficile* spores, clinical studies have demonstrated continued environmental contamination after disinfectant use. ^(24, 25) A number of studies have demonstrated that certain variables affect the sporicidal activity of disinfectants including the presence of organic substrates, contact time, spore concentrations, and ribotype. ^(16, 26, 27) In this study, we performed a large-scale, *in vitro* experiment to test the effect of these variables on a wide variety of hospital disinfectants using *C. difficile* strains of different ribotypes. Some of the disinfectants tested in our study had sporicidal property even in 1/5th dilution from the recommended concentration. Findings from our study demonstrate that spore concentrations, presence of organic substances, and contact time significantly change the sporicidal activity of disinfectants. Differences were noted among disinfectants although all were affected by spore concentrations and presence of organic substrates. No single disinfectant was identified in this study which worked consistently against *Clostridioides difficile* spores at different organic matter and spore concentration. Among the disinfectants tested, Virex was the most potent disinfectant followed by Clorox, OPA, Sporox, Formalin, and Nixall. The eradication of spores post-exposure to disinfectants in presence of organic matter may decrease up to 10% for some potent disinfectants. Although no significant difference was observed in disinfectant efficacy by

ribotype, there was a reduction in disinfectant efficacy in the presence of organic substrate for epidemic ribotypes. Only chlorine-based disinfectant, Clorox was found to be effective in different exposure conditions as compared to another chlorine-based disinfectant.

Different studies looking at the sporicidal concentration for the disinfectants showed varied results.^(18, 28) Some studies found that sporicidal property was observed at the recommended concentration of the disinfectants and others found efficacy even at 1/5th dilution. In our study, we found sporicidal efficacy for some disinfectants even at 1/5th original concentration and 1/10th for Clorox at an exposure time of 10 minutes. Current guidelines and publications advocate for use of Chlorine-based disinfectants for hospital environmental cleaning but in our study, only one of the three chlorine-based disinfectants were found to be effective against *C. difficile* spores consistently.⁽²⁹⁾ In our experiment we observed that the sporicidal effect of most of the disinfectants reduced considerably in presence of increasing concentration of organic substrate as well as increase in spore concentration as shown in a study by Vohra et al.⁽¹⁸⁾ We found quaternary ammonium disinfectants to be the most effective sporicidal followed by hypochlorite and hydrogen peroxide disinfectants. Other studies found Clorox to be a better disinfectant compared to Virex and Sporox.⁽³⁰⁾

Although we did not show any disinfectant resistance for epidemic *C. difficile* strains, we did observe a reduction in log CFU count for ribotypes O27 and O14-20 especially in presence of organic matter. Previous studies have shown that the ribotype 027 strain

demonstrated less sporicidal effects against disinfectants.⁽¹⁶⁾ In our experiments, we standardized spore concentrations in order to be able to test this effect as an independent variable. Other studies have demonstrated increased sporulation ability of ribotype 027 and other ribotypes and it is possible that spore concentrations in the healthcare environment are variable by ribotype.⁽²⁵⁾ Methods to assess sporicidal abilities of disinfectants are not standardized across laboratories, we used contemporary clinical strains and types of disinfectants not studied in previous publications.

These results have a strong implication for environmental cleaning services in a healthcare institution and can be used to strengthen the rationale for stringent measures to assure appropriate pre-cleaning and contact time are adhered to by hospital cleaning staff. It is estimated that up to 50% of cleaning is not done appropriately during terminal cleaning of a patients room.⁽³¹⁾ It is also important to note that environmental disinfection will not be effective unless a strong infection control plan including appropriate hand washing, patient accommodations, and contact precautions are maintained.⁽³²⁾ We have also recently shown that *C. difficile* spores are highly present in the shoe bottoms in community homes and elsewhere in the community, healthcare environment.⁽¹⁷⁾ Thus, environmental disinfection will always require a sustained effort as new spores are introduced into the healthcare environment from symptomatic patients or elsewhere. Decontamination of shoe bottoms entering the healthcare system is also a possibility to prevent the re-introduction of spores into the healthcare environment from the community.^(9, 33)

Some of the strengths of this study include testing the efficacy of commercially available hospital disinfectants against a large number of strains of *Clostridium difficile* including highly virulent strains. There were at least two to three replicates for each experiment. Most of the studies usually look at a few disinfectants or many disinfectants with a similar active ingredient. In this study, we looked at 7 hospital disinfectant with seven different active ingredients. This study has limitations. This study tested a limited number of disinfectants, results will need to be replicated and expanded. We used a number of clinical and environmental isolates for a variety of ribotypes. However, these isolates are unique to our biobank. Ideally, an international standard needs to be developed to standardize disinfectant research among laboratories.⁽³⁴⁾ We performed enough replicates in this experimental study to be able to test the independent effect of sporicidal activity on a number of variables against a variety of disinfectants. These results will need to be confirmed in real-world settings. Last, no ideal disinfectant was identified that was not affected by the variables we tested. Novel disinfectants, disinfectant techniques, or combination disinfectant use is required to identify effective, cost-effective, and practical disinfectant strategies.

Conclusion

In conclusion, using multiple *C. difficile* ribotypes, we demonstrated the independent effect of organic substrates, contact time, and spore concentrations to affect the sporicidal activity of hospital disinfectants. Although differences were noted amongst disinfectants, no ideal disinfectant was identified.

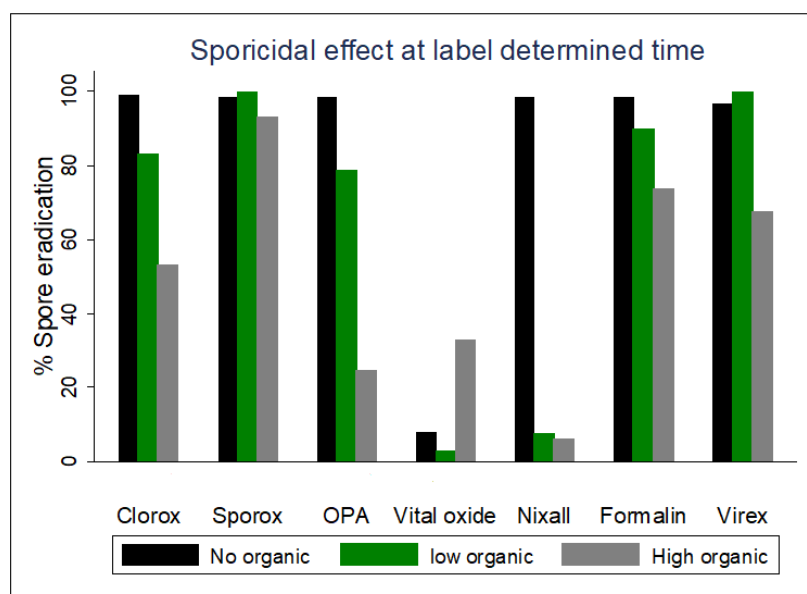
Table A. 1: Commercial Hospital Disinfectants

BEST	Disinfectant name	Biocide type	Active ingredient(s)	Manufacturer minimum contact time (min)	Standardized contact time	Effective vs. C. difficile spores	Manufacturer	Sporicidal claim
1	Clorox (CL)	CRA	Sodium hypochlorite (10%)	1.5	10	Yes	Clorox Company	Yes
4	Formalin (FM)	FORM	Formaldehyde (4%)	60	10	Yes	Pure Health	Yes
5	Nixall (NI)	CRA	Hypochlorous acid (0.046%)	10	10	Yes	Nixall Company	Yes
3	Cidex OPA (OPA)	OPA	Ortho-phthaldehyde solution (0.575% w/v)	10	10	Yes	McKesson	Yes
5	Sporox (SP)	H2O2	Hydrogen peroxide solution (7.5%)	30	10	Yes	Sultan Healthcare	Yes
2	Virex (VI)	QAC	Quaternary ammonium compound (5-10%)	10	10	Yes	Diversey	Yes
5	Vital Oxide (VA)	CRA + QAC	Chlorine dioxide (0.2%) & Quaternary ammonium compounds (0.125% + 0.125%)	10	10	No	Vital Oxide Company	No
Chlorine releasing agents (CRAs), quaternary ammonium compounds (QACs), formaldehyde (FORM),								

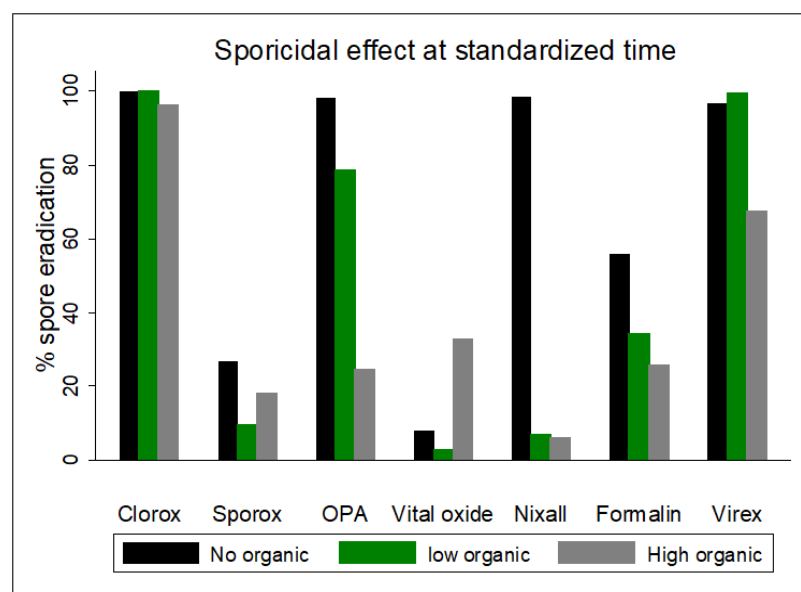
Table A. 2: Minimum Sporicidal concentration

Disinfectants	Time of exposure (min)	Minimal sporicidal concentration					
		Ribotype	Ribotype	Ribotype	Ribotype	Ribotype	NT
		O27	O12	O14-20	O78-126	106	
Clorox	Label	1/5	1/2	1/5	1/2	1/5	1/5
	Standard	<1/10	1/10	<1/10	<1/10	1/10	<1/10
OPA	Label	OR	1/2	1/2	OR	1/2	1/2
	Standard	OR	1/2	1/2	OR	1/2	1/2
Nixall	Label	OR	OR	OR	OR	OR	OR
	Standard	OR	OR	OR	OR	OR	OR
Vital oxide	Label	OR	OR	OR	OR	OR	OR
	Standard	OR	OR	OR	OR	OR	OR
Virex	Label	1/2	1/5	1/5	1/2	1/2	1/2
	Standard	1/2	1/5	1/5	1/2	1/2	1/2
Sporox	Label	1/2	1/5	1/5	1/5	1/5	1/5
	Standard	OR	1/2	1/2	OR	1/2	1/2
4% Formalin	Label	1/2	1/5	1/2	1/5	1/5	1/5
	Standard	OR	OR	OR	OR	OR	OR

Figure: A. 1a & b: Effect of disinfectant on spore eradication



1A: Sporicidal effect at label determined time



1B: Sporicidal effect at standardized time

Figure: A. 2: Efficacy of hospital disinfectants on *C. difficile* spores under various environmental factors

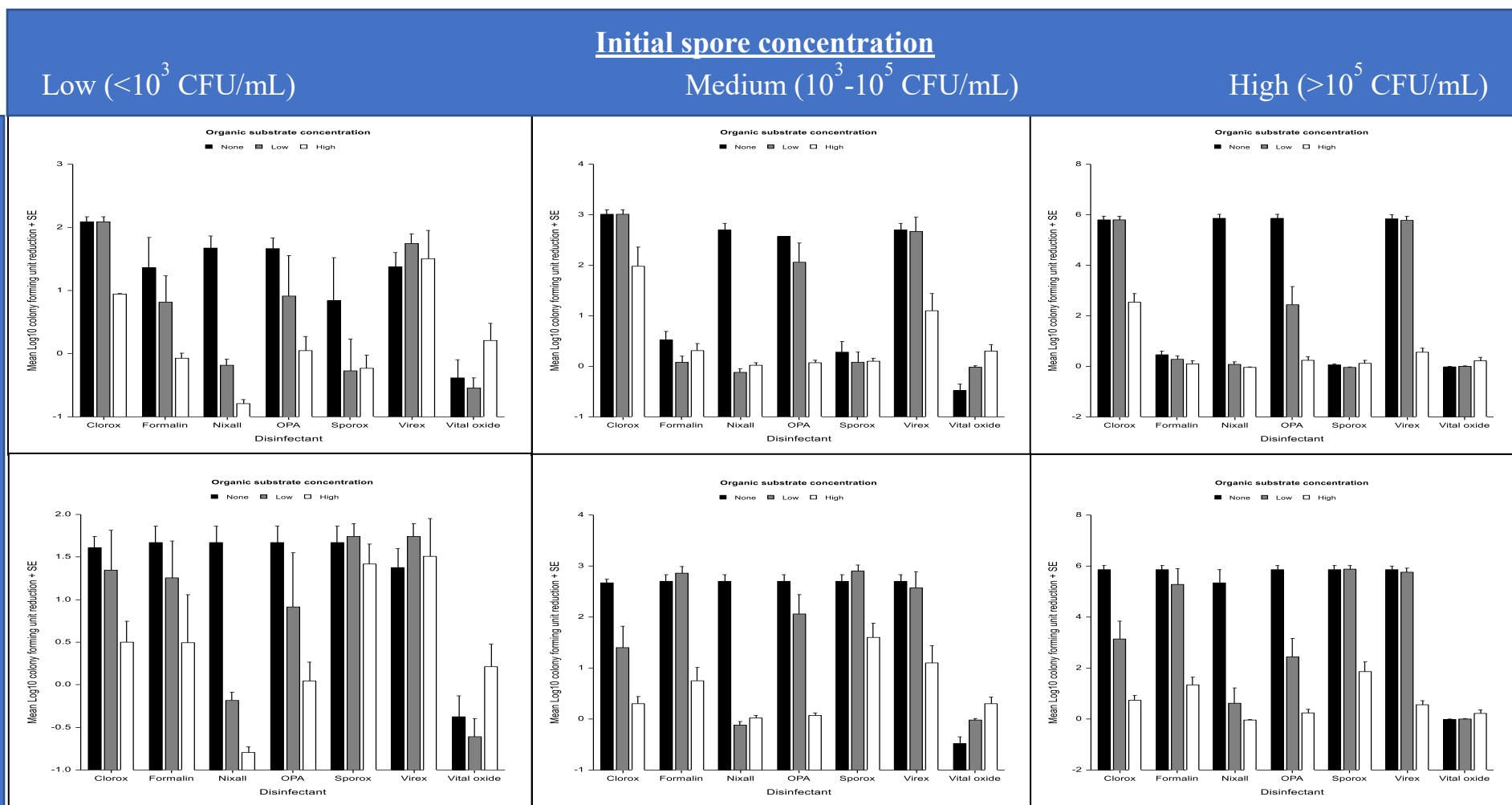


Table A. 3: General linear model to assess the effect of various factors on disinfectant efficacy

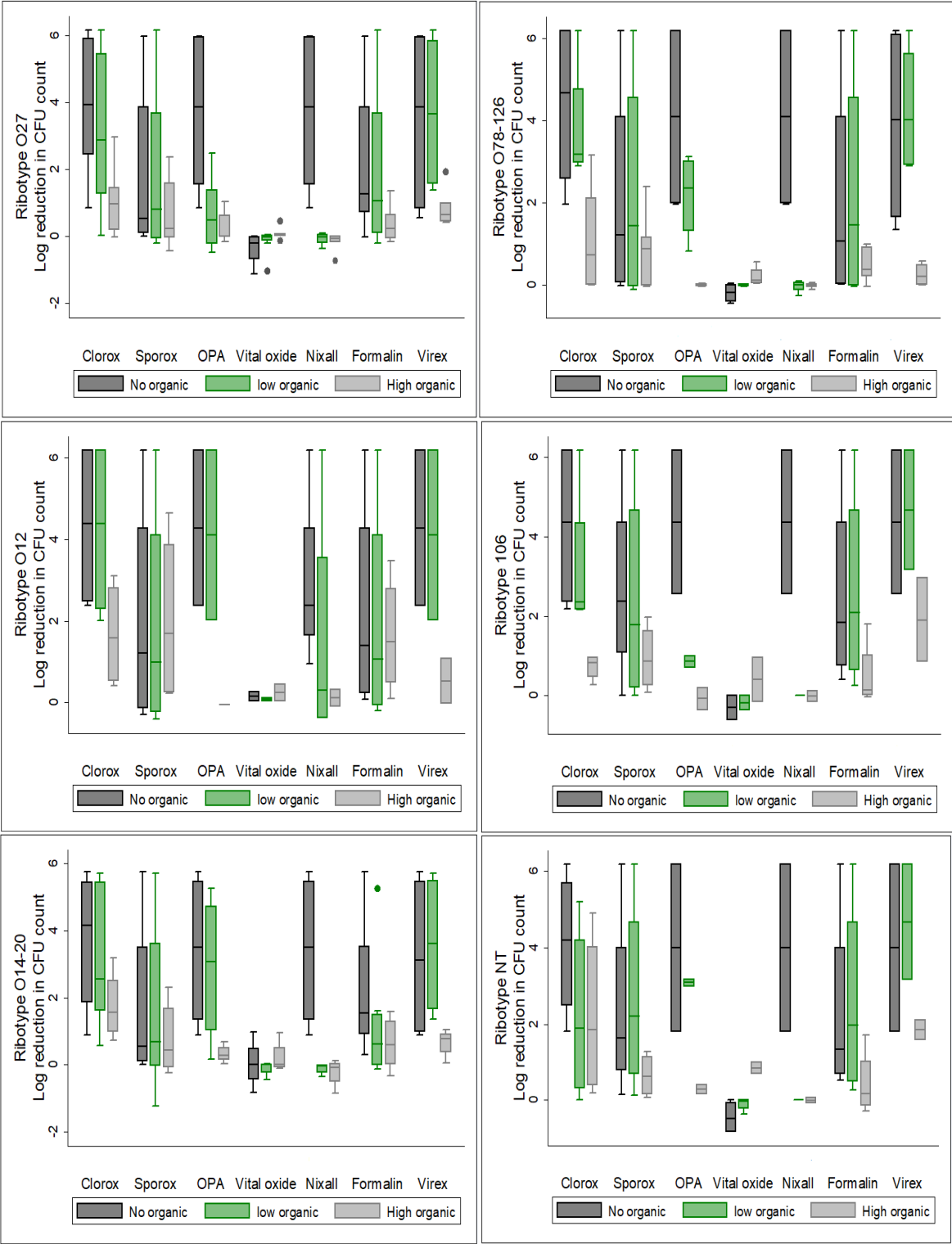
Disinfectant	Organic substrate low	Organic substrate high	Spore concentration	Contact time	Ribotype
Clorox	-0.91	-2.94	1.45	1.22	0.04
Formalin	-0.31	-1.80	0.86	-2.61	-0.01
Nixall	-3.85	-4.15	1.05	0.00	0.03
OPA	-2.08	-4.09	1.23	-0.01	0.14
Sporox	-0.10	-1.33	0.91	-3.23	0.01
Virex	0.02	-3.33	1.60	-0.01	0.01

Multivariable regression analysis of the effects of confounding variables on disinfectants. Values represent log reduction attributed to each variable stratified by disinfectant.

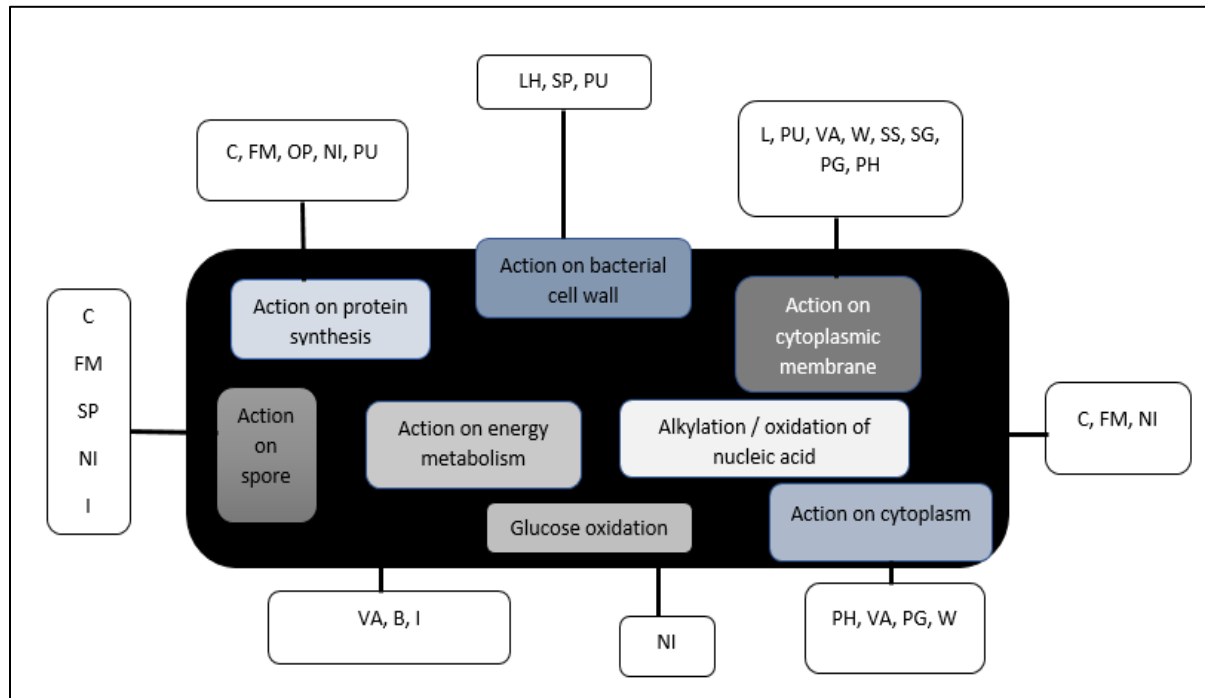
Bold: $p < 0.001$

***Vital oxide was not effective at reducing spore concentrations**

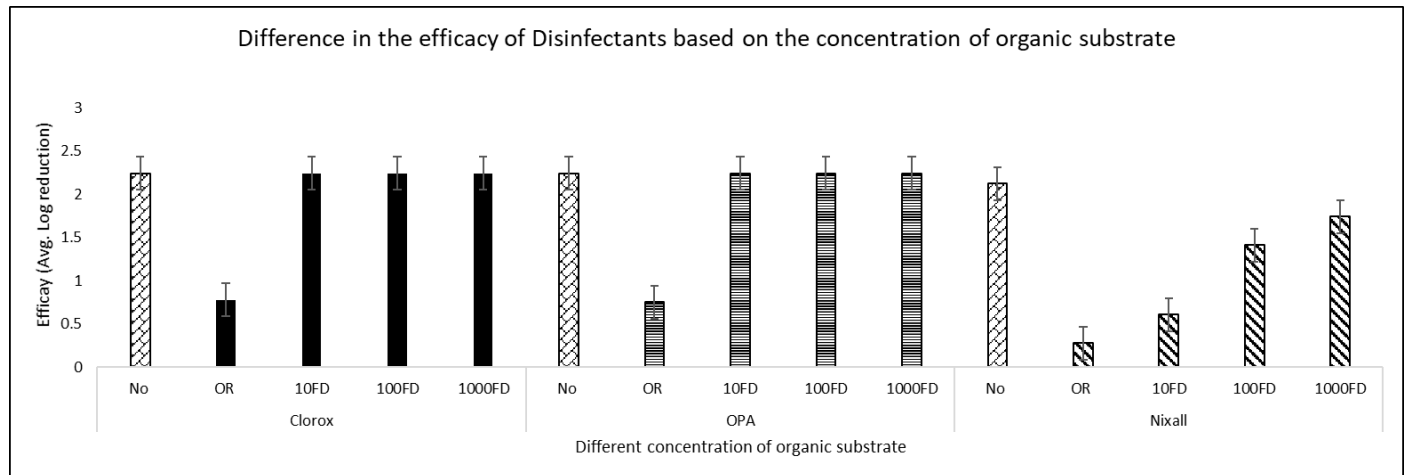
Figure: A. 3: Efficacy of disinfectants against different *C. difficile* ribotypes



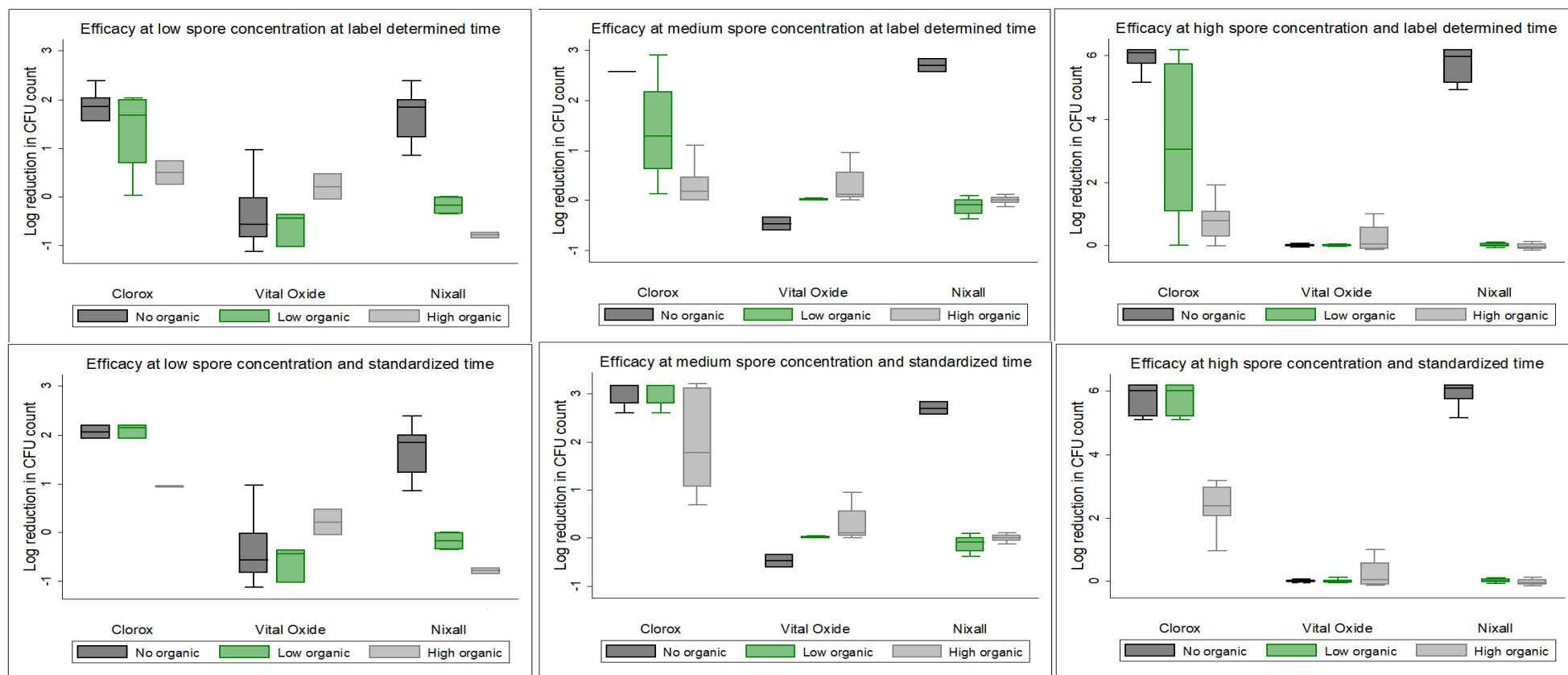
Supplemental figure A. 1: Mechanism of action of disinfectants tested in the study



Supplemental figure A. 2: Titration of organic matter concentration for disinfectant efficacy



Supplemental figure A.3: Efficacy of chlorine-based disinfectants



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JOURNAL ARTICLE: B

Title of Journal article:

Efficacy of Hospital Disinfectants against Vegetative Cells and Spores of *C. difficile* encased in Biofilms

Name of Journal Proposed for Article Submission: Antimicrobial Agents and
Chemotherapy

Activity of Hospital Disinfectants against Vegetative Cells and Spores of *C. difficile* in Biofilms

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Running title: *C. diff* spores and hospital disinfectants

Keywords: in vitro study; anaerobic infections; biofilm; environmental decontamination; ribotype 027;

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Abstract:

Objective: *Clostridioides difficile* spores survive in the environment either in mono- or mixed-species biofilm. Most sporicidal disinfectants lose effectiveness against biofilm-embedded spores. However, no previous studies have investigated the 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.

Methods: Five *C. difficile* strains (ribotypes 027, 012, O14-20, O78-126 and 106) embedded in three different types of biofilms grown for 72 or 120 hours were exposed to seven different hospital disinfectants. *C. difficile* log CFU/mL was calculated after manufacturer determined contact times along with biofilm biomass and microscopy. The primary analysis was to compare the log reduction of *C. difficile* vegetative colonies and spore counts and biomass after exposure to disinfectants.

Results: *C. difficile* vegetative cells and spores were recovered from biofilms regardless of the type of biofilm growth (single vs. mixed species or anaerobic vs. aerobic) or biofilm growth time. No disinfectant was able to completely eliminate *C. difficile* from the biofilms. Overall, Clorox, OPA, and Virex were most effective at killing *C. difficile* spores regardless of biofilm age, ribotype, or wash conditions ($p=0.001$, 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 the total vegetative cell growth ($p=0.77$). Clorox and Virex were most effective in reducing biomass followed by Nixall, OPA, and Vital oxide.

Conclusion: 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 persister reservoir.

Introduction

Clostridioides difficile is a Gram-positive, obligate anaerobic, spore-forming bacterium and the most common health-care acquired infection in the United States (U.S.)⁽¹⁾ Spores can be transmitted via symptomatic and asymptomatic carriers to the environment or via healthcare personnel.^(2, 3) To break the transmission cycle, hospitals and healthcare institutions commonly use chemical sporicidal agents for environmental surface cleaning.⁽⁴⁾ Studies have found that sporicidal agents could reduce the *C. difficile* spore count by around 4 logs.⁽⁵⁾ Current guidelines of the United Kingdom Department of Health recommend the use of chlorine-based disinfectants to reduce *C. difficile* spores in clinical settings.^(5, 6) As per Infectious Disease Society of U.S. (IDSA), daily cleaning with a sporicidal agent in conjunction with other prevention modalities is recommended in case of an outbreak, hyperendemic settings, or high rate of repeated infection.⁽⁷⁾ However, clinical studies investigating chlorine-based disinfectants found that none of the disinfectants achieved adequate disinfection within labeled determined contact time in either clean or dirty environment.⁽⁶⁾ The reasons for this lack of efficacy are unclear but may relate to the presence of biofilms which are groups of microorganisms embedded in an extracellular matrix.

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* survive in the abiotic environment either in 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, DNA and polysaccharide biofilm in the gut as well as abiotic surfaces. ^(9, 12, 13) The extra polysaccharide (EPS) matrix provide an anaerobic scaffold that contained both vegetative cells and spores. ⁽¹⁰⁾ Multiple variables influenced biofilm formation including virulence-associated proteins (cwp84, flagella, transcription factors, SpoA) and quorum sensing regulator, LuxS. ^(9, 11) Using mixed-species biofilms, a co-infection model of *Finnegoldia magna* and *C. difficile* enhanced biofilm formation of both bacteria⁽¹¹⁾ and another polymicrobial biofilm demonstrated *C. difficile* spores embedded in the biofilm. ⁽¹⁴⁾

Most sporicidal disinfectants are not as effective against biofilm embedded spores as compared to planktonic spores. ⁽¹⁵⁻¹⁸⁾ Microorganism in biofilm may be up to 1000-fold more resistant to disinfectants in comparison to their planktonic counterpart. For example, spores of *Bacillus cereus* embedded in a biofilm are highly resistant to cleaning procedures. ⁽¹⁹⁾ After six days of exposure to chemical disinfection, *Bacillus* spores could still be observed within a biofilm using micro slicing 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 affected the lack of efficacy of chemical disinfectants included the age of the biofilm and cell density. ^(22, 23) Despite this known association on spores and biofilms, no 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.

Methods:

Bacterial strains and spore preparation

Two laboratory strains of *C. difficile* (R20291; ribotype 027: CD630: ribotype 012) and three clinical strains of different ribotypes (ribotypes O14-20, O78-126 and 106) were used for all experiments.

Chemical disinfectants

Seven hospital disinfectants were used in this study, six with sporicidal properties and one non-sporicidal as an active control (**Table B.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 preparation, sanitary fluid or hand sanitizers. For each disinfectant, contact time experiments were conducted based on manufacturer determined contact times or 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 in 3 different conditions namely strictly anaerobic, a mix of aerobic and anaerobic and strictly aerobic. To form a *C. difficile* single species biofilm grown anaerobically (mono-species anaerobic biofilm), overnight cultures of *C. difficile* were diluted 1:100 in fresh brain heart infusion salt (BHIS) broth containing 0.1M 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 multi-species biofilm grown

anaerobically (*multi-species anaerobic biofilm*), 1 mL of overnight cultures of *Enterococcus faecium* (*E. faecium*) and *Staphylococcus aureus* (*S. 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 hours to maintain a consistent biofilm age of 3 or 5 days. Finally, to grow a multi-species biofilm in an aerobic environment (*multi-species aerobic biofilm*), an overnight culture of *C. difficile* grown anaerobically along with *E. faecium* and *S. aureus* culture were 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 five-day biofilms were washed twice with sterile phosphate buffer saline and allowed to air dry for 10 minutes. Following drying, 1 ml of filter sterilized 0.2% crystal violet was added to each well and incubated for 30-minutes. 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 buffer saline. Crystal violet dye was re-eluted from the biofilm by adding 1 ml of methanol and incubating at room temperature for 30 minutes. Methanol extracted dye was then diluted 1:1 and 1:10 and optical density (OD) measured on a plate reader at wavelength A570. ⁽⁹⁾

Biofilm imaging and viability assay

Biofilm was imaged using both an inverted microscope (Evos) and confocal laser scanning microscope. Light microscopy was done to visualize structure and formation differences of a 3- and 5-day old biofilm pre and post-exposure to disinfectants. Cells in the biofilms were also visualized using a confocal laser-scanning microscope following Joseph et al protocol.⁽²⁴⁾ Briefly, bacterial biofilm from all stages was formed on a 4 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, 24) A live/dead viability kit contained Syto9 and propidium iodide stains were added to visualize *in vitro* killing effects. Slides were fixed, washed with PBS, and plastic wells were removed from the slide. Saline was added to the plastic well and covered with a coverslip. The edges of the coverslip were sealed with mounting media and air dried for 1 hour before microscopy. Samples were imaged under oil immersion using a laser confocal microscope.⁽²⁴⁾ The excitation/emission for Syto9 was 488nm/505-550 nm and 543/>650 nm for propidium iodide.⁽²⁴⁾ The assays were performed in duplicate and images analyzed.

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 unwashed. This was done to assess the effect of organic substrate on disinfectant efficacy. For each experiment, 500 µL of disinfectant at original concentration was added in duplicate to both, the washed and unwashed biofilms, based on label-determined contact time (**Table B.1**). 500 µL of PBS

was added to the wells for 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 42Hz for 10 minutes followed by manual scrapping for exactly 1 minute and pipetted into an Eppendorf tube. Removal of the biofilms was confirmed by light microscopy (Evos digital inverted microscope (AMG)). For total count, the cells in the Eppendorf were diluted and 100 μ L plated on blood agar plates and incubated anaerobically at 37°C for 48 hours. For spore count, the detached cells were heated at 65°C for 30 minutes to kill the vegetative cells. Vegetative cell and spore *C. difficile* colony forming units were measured using the dilution and plating method.⁽²⁵⁾ In the case of multispecies biofilm, plates were read carefully to count only the *C. difficile* colonies. The morphology of the colonies for the three types of the organism used was 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 the CFU count of *C. difficile* vegetative cell and spore count and biomass measured by the crystal violet assay pre and post-exposure to disinfectants. Secondary aims were to compare log reduction of *C. difficile* vegetative cell and spore count based on the type of biofilm (mono, mixed, or mixed aerobic), duration of biofilm formation (3 or 5 days), ribotype, and presence of organic matter (washed vs. unwashed biofilm). The third aim was to compare the overall efficacy of disinfectants against the 3 different types of biofilm for different ages of biofilm using

confocal microscope. Vegetative cell and spore mean \log_{10} colony forming reduction \pm standard error (SE) were calculated for each disinfectant by biofilm type and duration, ribotype, and experimental procedure. Linear regression analysis was done comparing the efficacy of sporicidal disinfectants against the efficacy of non-sporicidal disinfectant Vital oxide (control) for biofilms of different ages. SAS version 9.3 (SAS Institute, Cary NC) or STATA/IC version 12.1 (STATACorp LLC, College Station, TX) was used for all analyses. A $p < 0.05$ was considered significant.

Results:

***C. difficile* biofilm**

C. difficile vegetative cells and spore were grown from all ribotypes regardless of the type of biofilm growth (single vs. mixed species or anaerobic vs. aerobic) or biofilm growth time (**Figure B.1 and B.2**). Biofilms were visualized starting on day two 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 biomass differed among different ribotypes (**Supplemental Figure B.1**). The mean log CFU of vegetative cell and spore count for 72-hour biofilm between ribotypes was 2.77 ± 0.07 and 1.77 ± 0.06 respectively. Average log CFU of vegetative cell and spore count for *C. difficile* grown in 72-hour biofilms was 3.11 ± 1.62 CFU/mL and 1.99 ± 1.46 CFU/mL for *C. difficile* grown in mono-species anaerobic biofilms, 2.84 ± 1.43 CFU/mL and 1.78 ± 1.14 CFU/mL, respectively for

multi-species anaerobic biofilms and 2.35 ± 1.36 and 1.54 ± 0.97 , respectively for multi-species aerobic biofilms. Average log CFU of vegetative cell and spore count for 72-hour biofilm by pre-cleaning status was 3.06 ± 1.40 and 2.04 ± 1.22 , respectively for washed biofilm and 2.47 ± 1.54 and 1.51 ± 1.17 , respectively for unwashed biofilms. Similar results were seen in 120-hour biofilms with the exception of that the vegetative cell counts were lower than that observed in biofilms grown for 72 hours ($p < 0.01$).

***C. difficile* spore and vegetative cells embedded within biofilms after disinfectant exposure**

The overall efficacy of seven hospital disinfectants to kill *C. difficile* embedded within biofilms grown for 72 hours and 120 hours is shown in **Figure B.3**. No disinfectant was able to completely eliminate *C. difficile* from the biofilms. Overall, Clorox, OPA, and Virex were most effective at killing *C. difficile* (less than 1 log CFU/ml) spores regardless of biofilm age, ribotype, or wash conditions ($p = 0.001$, each). Clorox and OPA were also effective at killing vegetative cell growth (less than 0.5 log CFU/ml with $p = 0.001$ each) but Virex was found to be ineffective against the vegetative cell growth (more than 3 log CFU/ml with $p = 0.77$). This same effect was noted in biofilms grown for 72 and 120 hours. 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; **Figure B.4**).

Efficacy of disinfectants against *C. difficile* in a biofilm

Supplemental Figure B.2 shows the log reduction in CFU count for vegetative cells and spores in a 72 and 120-hour biofilm. The mean log reduction of CFU count for all disinfectants was 1.74 ± 0.07 and 1.83 ± 0.06 respectively for vegetative cell count and spores in a 72-hour biofilm. Similar results were observed for 120 hours biofilm. Most of the disinfectants were ineffective to reduce the vegetative cell count and spore count by more than 2 logs. A regression analysis was done to look at the effect of disinfectants on Log CFU of vegetative cell count and spore count of 3- and 5-day old biofilms adjusting for ribotypes, duration of exposure, type of biofilm and presence of organic matter (**supplementary table B.1**). As Vital Oxide was found to be ineffective against planktonic and all three stages of *C. difficile* biofilm so, the efficacy of other disinfectants were compared against the efficacy of Vital oxide. There was a statistically significant reduction in Log CFU/ml of vegetative cell and spore count for both 3- and 5-day old biofilm except for Sporox and Virex. Sporox increased the vegetative and spore count in all cases and Virex increased the vegetative count but reduced the spore count.

Biomass of biofilms after exposure to disinfectants

Visually no significant difference was observed for wells treated with Nixall, Sporox, Virex, and Vital Oxide. The effect of disinfectants on the biomass of biofilms is shown in **Figure B.5**. Overall Clorox and Virex were most effective in reducing biomass followed by Nixall, OPA, and Vital oxide. There was no reduction in biomass post-exposure to Sporox and Formalin. The same effect was noted in biofilms grown for 72 hours and 120 hours. 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 type of disinfectant, ribotype, and stage of biofilm ($P<0.01$).

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 (**Figure B.6**). Visualization and viability of cells were also assessed using Live/dead biofilm viability staining under a confocal microscope (**Figure B.7a and b**) for both mono species 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 compared to 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. Although hypothetical this efficacy may be related to the chemical composition of the disinfectants as well as the degree of penetration into the biofilm. Clorox and OPA were also effective at killing vegetative *C. difficile* within biofilms although Virex was found to be ineffective against

the vegetative cell growth. Biomass studies were consistent with time kill results. 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 wash vs. 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, other studies have investigated the impact of disinfectants against other organisms embedded in biofilms. ⁽²⁶⁾ For example, 11% of multidrug-resistant *Staphylococcus aureus* (MRSA) and 80% of *Pseudomonas* cells survived in a biofilm after disinfectant exposure. ⁽²⁷⁾ *E. coli* embedded in a biofilm was not only resistant to sodium hypochlorite disinfectant, but the biofilm was able to reform on disinfectant treated surfaces after initial disruption. ⁽²⁸⁾ Efficacy of sodium hypochlorite disinfectant was also dependent on the composition of biofilm with disinfectants having decreased killing effect in multi-species biofilms; results that are consistent with our study. ⁽²⁹⁾ Hypochlorite and quaternary ammonium disinfectants were ineffective against *B. cereus* embedded in biofilms even at higher concentration. ^(26, 30) Studies using OPA have demonstrated decreased killing effect when the organism was embedded in a biofilm for *Pseudomonas fluorescence*⁽¹³⁾ but not for *Klebsiella pneumoniae*. ⁽³¹⁾ Studies using H₂O₂ based disinfectants were effective against *P. aeruginosa* and *S. aureus* embedded in biofilms which differs from our study in which H₂O₂

was ineffective against *C. difficile* embedded in a biofilm.⁽¹²⁾ Sporox (hydrogen peroxide) was displayed reduced killing effect against *Pseudomonas aeruginosa* embedded in a biofilm which was postulated to be due to genetic products released by cells in a biofilm that may reduce their susceptibility to oxidative disinfectants like H₂O₂.⁽³²⁾ Hospital and nursing home environments are known to harbor *C. difficile* spores and may be important sources for healthcare-associated infection. Biofilm removal methods may be required to properly clean these environments.⁽³³⁾ Although we show efficacy for Clorox, OPA and Virex as per recent EPA guideline, to be effective against biofilms, disinfectants need to show at least log reduction in viable cell count in biofilm.⁽³⁴⁾

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.⁽³⁵⁾ Sodium hypochlorite-based disinfectants denatured proteins in a biofilm and inhibited the major enzymatic function of bacterial cells.⁽³⁵⁾

There are certain limitations to this study. This study used in vitro techniques to compare the killing effect of hospital-based disinfectants against *C. difficile* embedded in biofilms. Future studies are required to confirm and replicate the finding from this study in a hospital setting using various surfaces. Accurate quantification of the organism within biofilms by sonication or scraping is sometimes limited by the ability to remove all the organism from the biofilm.⁽²⁷⁾ To overcome this issue, we used manual scrapping in addition to sonication.

Although our study looked at disinfectant efficacy at label determined contact time and concentration, it did not investigate any off-label use or effect of other environmental factors. This study was done on polystyrene surface and hence may not necessarily represent how cells grow on other surfaces. ⁽²⁷⁾ For example, the rate of biofilm formation was enhanced on stainless steel surfaces as compared to other metals or plastic surfaces possibly due to the hydrophilic nature of the material or surface irregularities leading to the increased surface area. ⁽²⁷⁾ The viable cells post disinfectant exposure may have the ability to resurrect the biofilm, act as a reservoir for spread and preservation of recalcitrant infection or simply a source of environmental contaminant. ⁽³⁶⁾ 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 an in vitro study to assess the effect of hospital-based disinfectants 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 the persister reservoir of pathogens in biofilms.

Table B. 1: Disinfectants used in this study

Biocide type	Active ingredient(s)	Manufacturer minimum contact time (min)	Disinfectant name	Manufacturer
CRA	Sodium hypochlorite 10%	1.5	Clorox	Clorox Company
FORM	Formaldehyde 4%	60	Formalin	Pure Health
OPA	Ortho-phthaldehyde solution 0.575% w/v	10	Cidex OPA	McKesson
H202	Hydrogen peroxide solution 7.5%	30	Sporox II	Sultan Healthcare
QAC	Quaternary ammonium compound (5-10%)	10	Virex	Diversey
CRA	Hypochlorous acid 0.046%	10	Nixall	Nixall Company
CRA + QAC	Chlorine dioxide (0.2%) & Quaternary ammonium compounds (0.125% + 0.125%)	10	Vital Oxide	Vital Oxide Company
Chlorine-releasing agents (CRAs), quaternary ammonium compounds (QACs), formaldehyde (FORM),				

Figure B. 1: *C. difficile* spores and vegetative cells in biofilms by ribotype and growth conditions

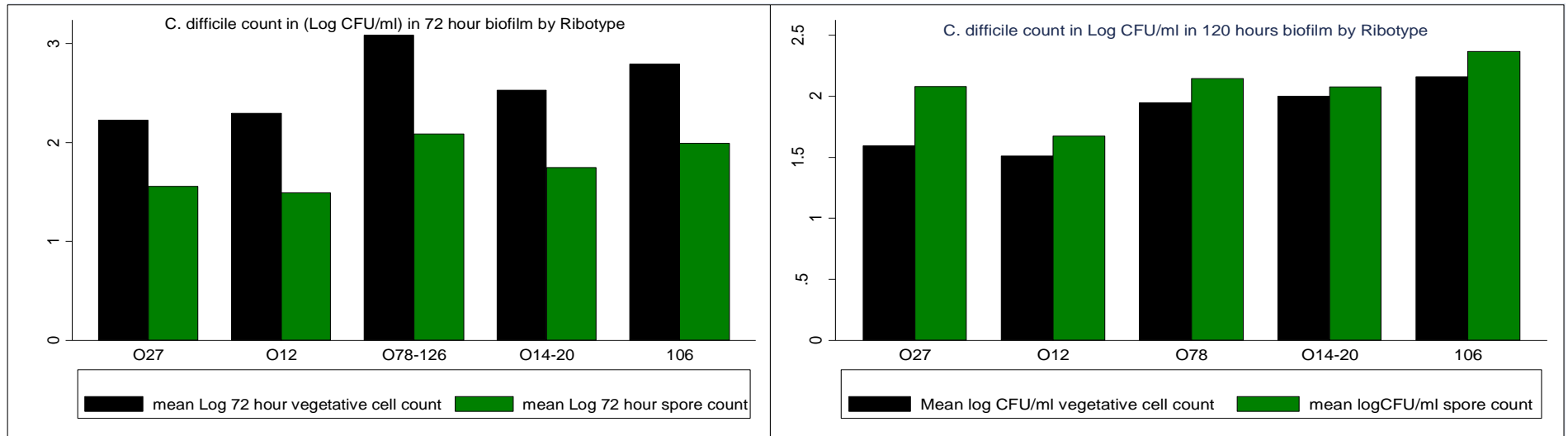


Figure B. 2: C. difficile spores and vegetative cells in biofilms by ribotype and growth conditions

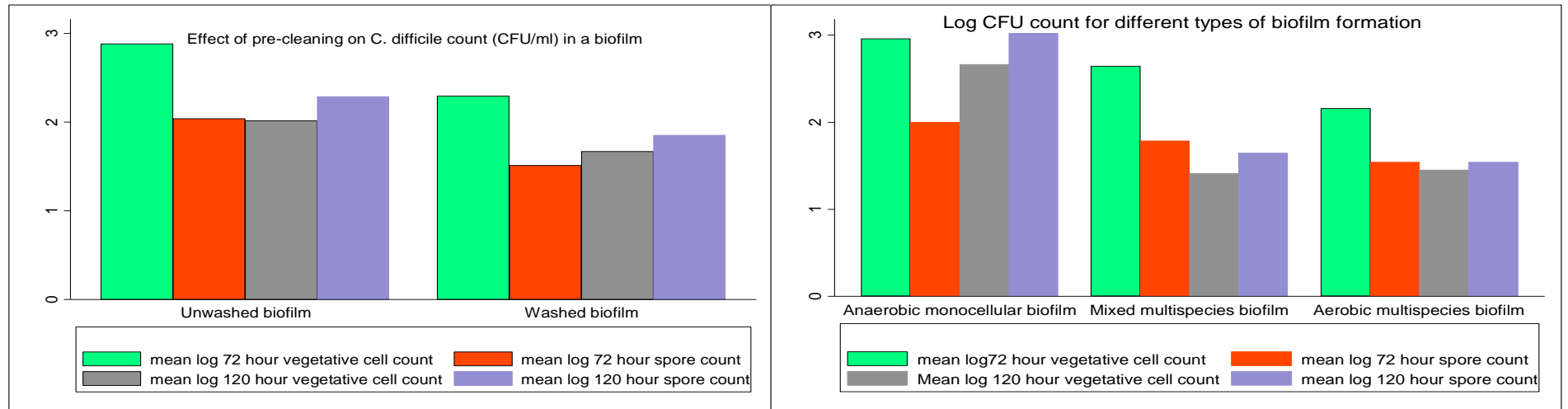


Figure B. 3: Biofilm embedded *C. difficile* spore and vegetative cell counts (log CFU/mL) after exposure to disinfectants grown in biofilms for 72 or 120 hrs.

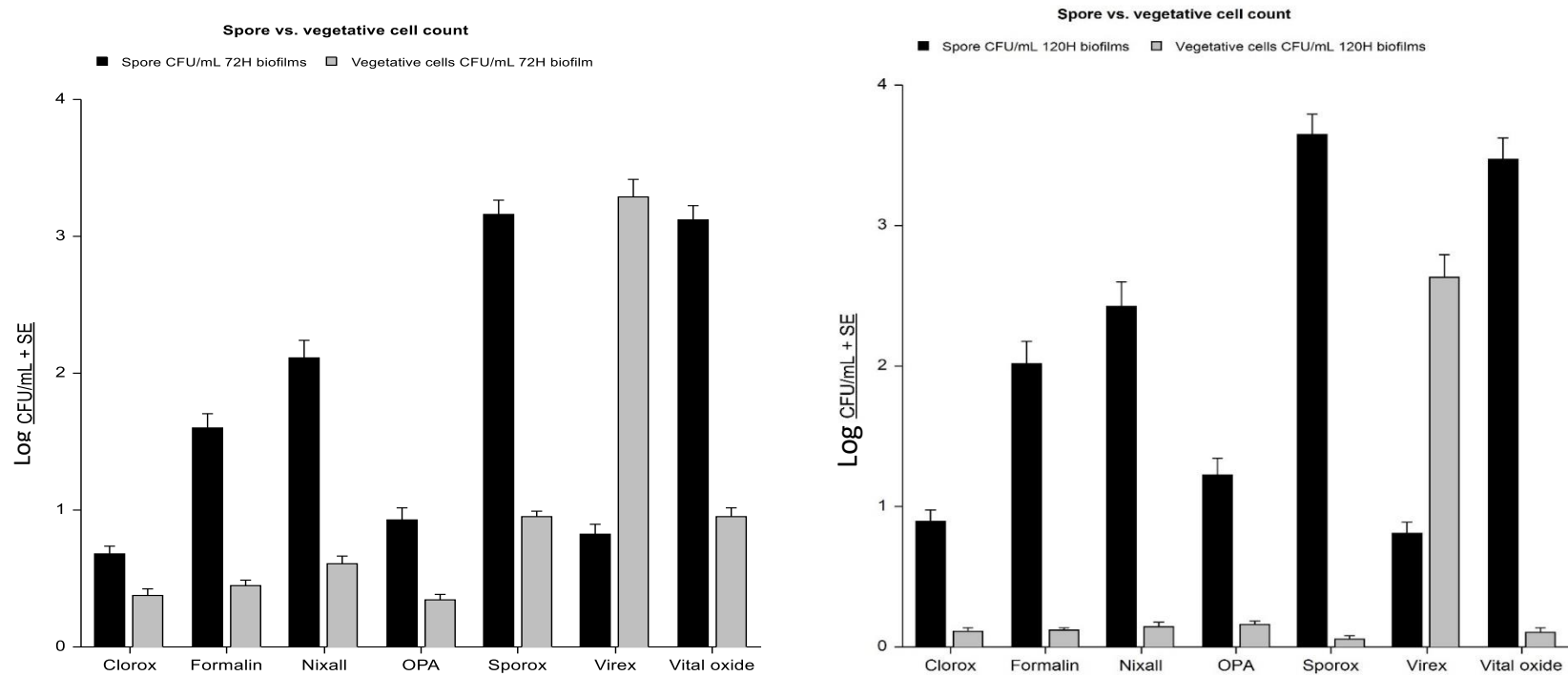


Figure B. 4: Killing effect of disinfectants based on biofilm type

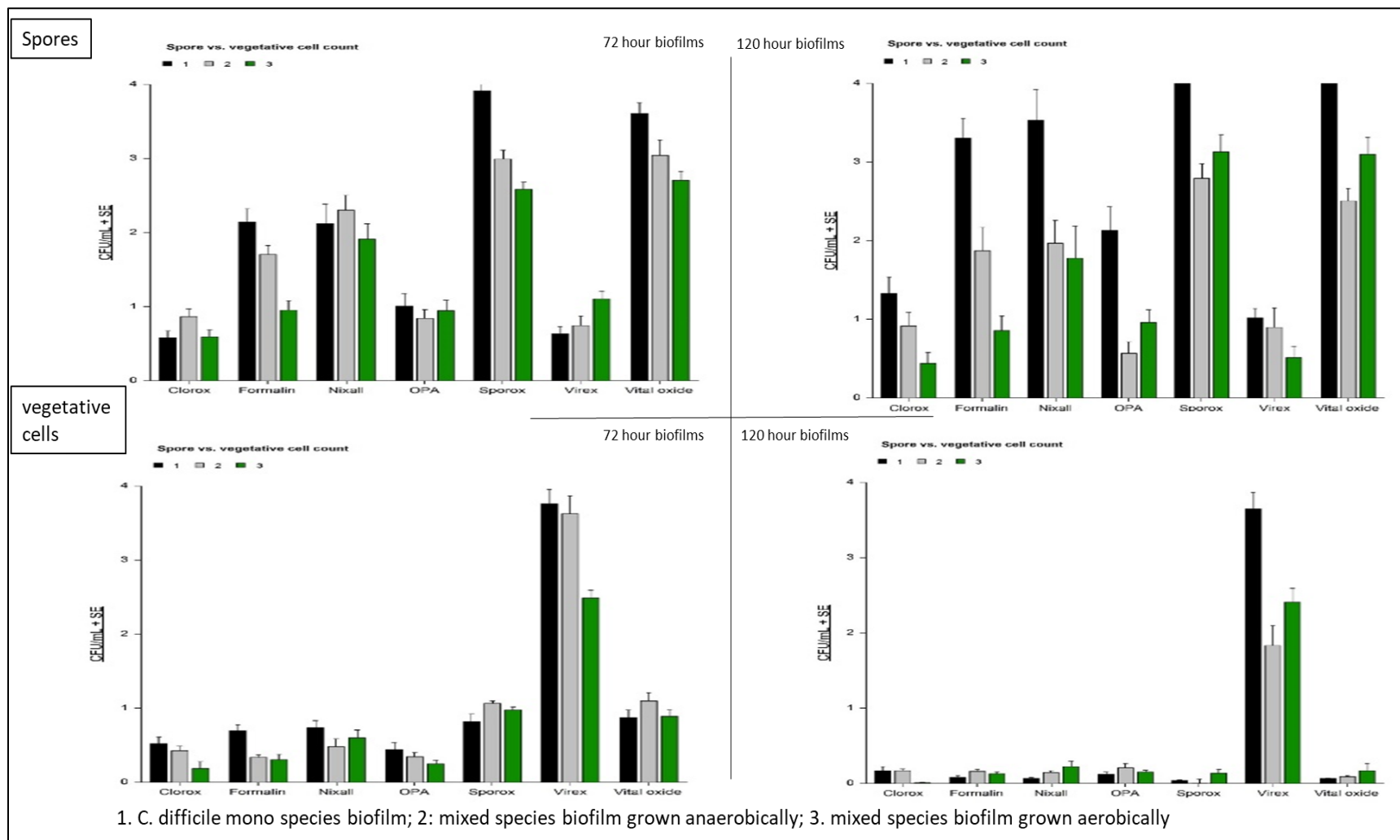


Figure B. 5: Effect of disinfectants on the biomass of biofilms grown for 72 and 120 hours

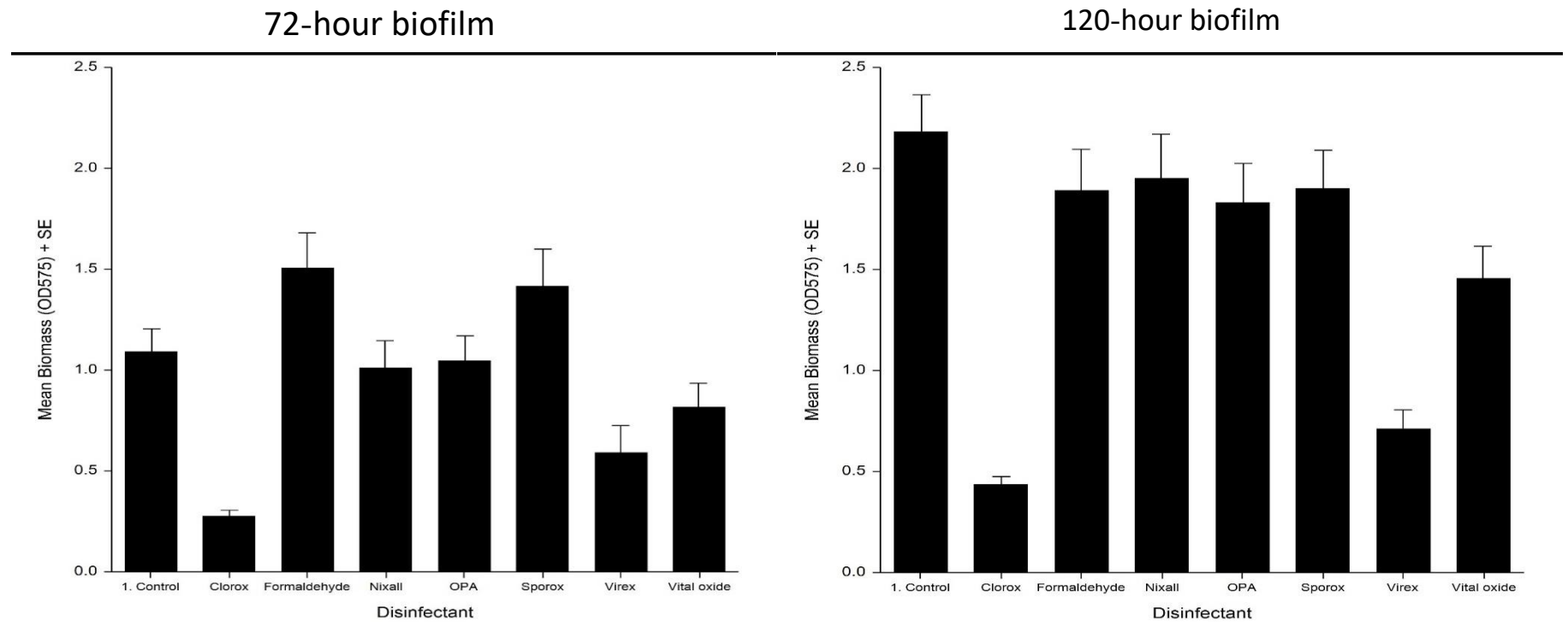


Figure B. 6: Visualization of killing effect of disinfectants on a 72-hour monocellular *C. difficile* biofilm on a surface using Light microscopy

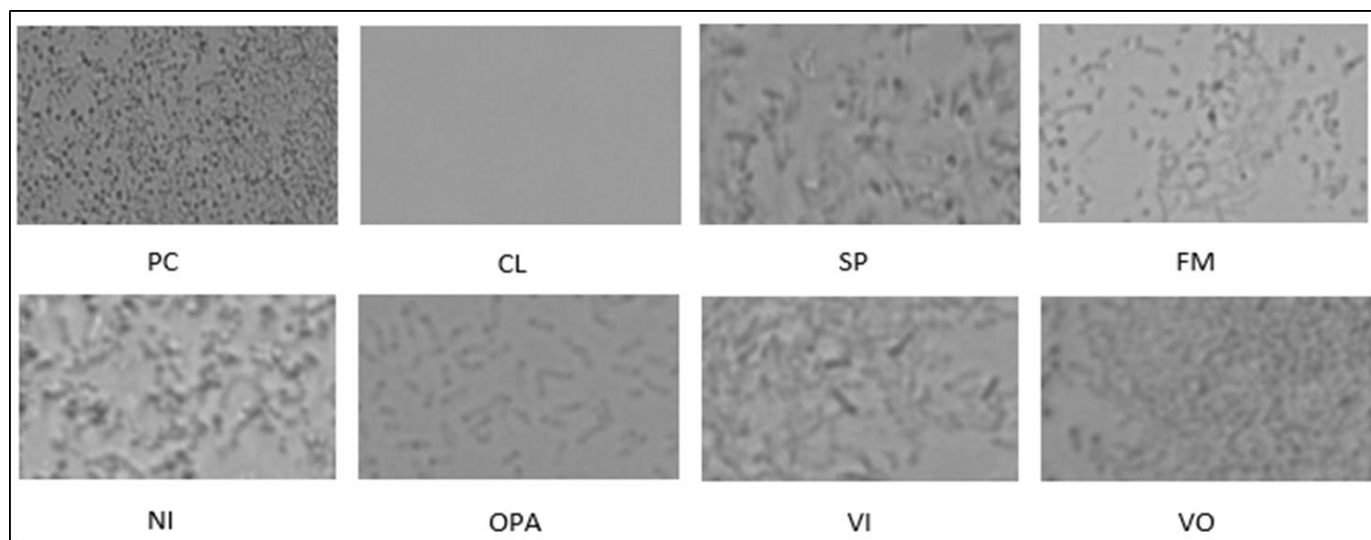
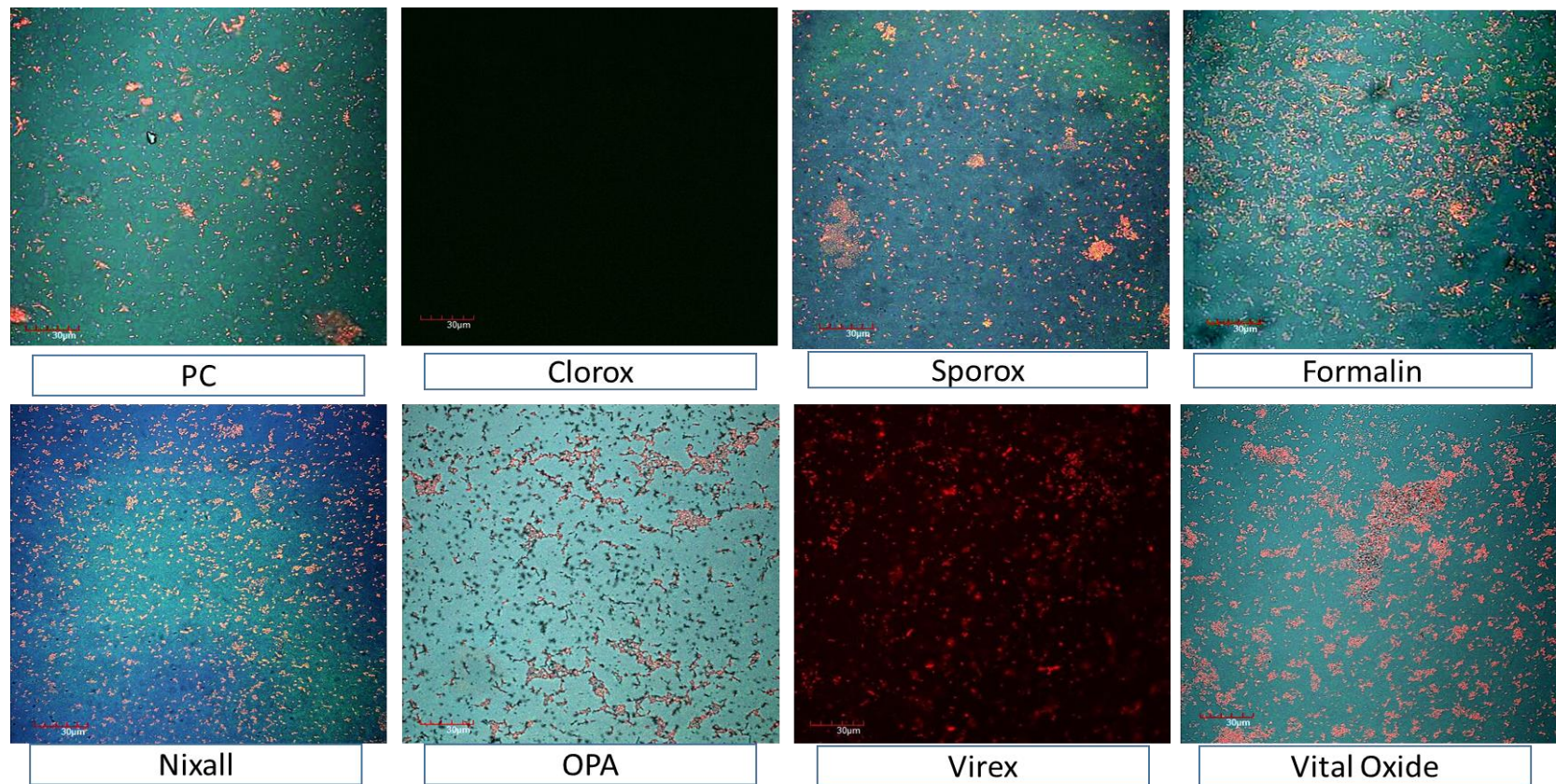
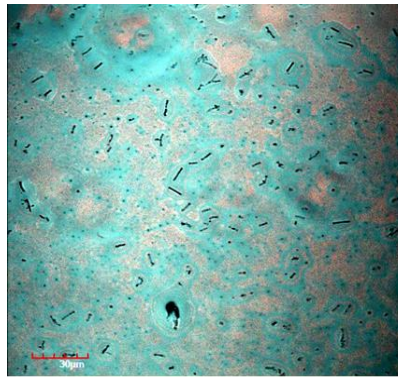


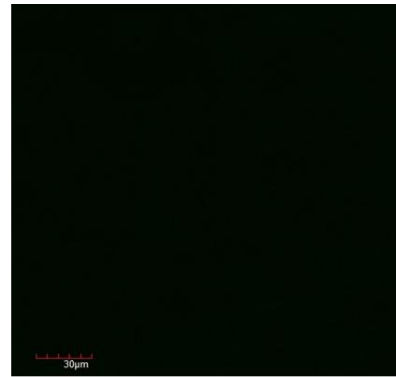
Figure B. 7. a & b: Confocal microscopy of Biofilms



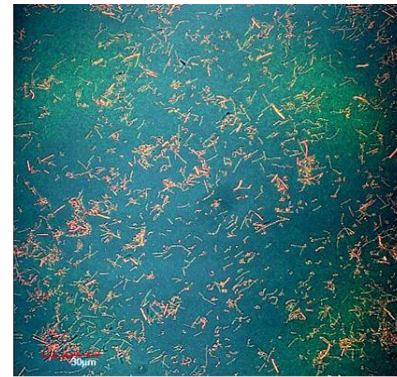
7. a: Visualization of killing effect of disinfectants on 72-hour anaerobic *C. difficile* biofilm on a surface



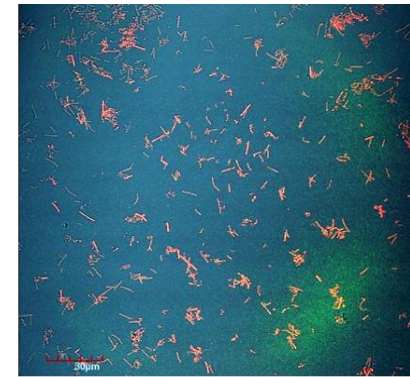
PC



Clorox



Sporox



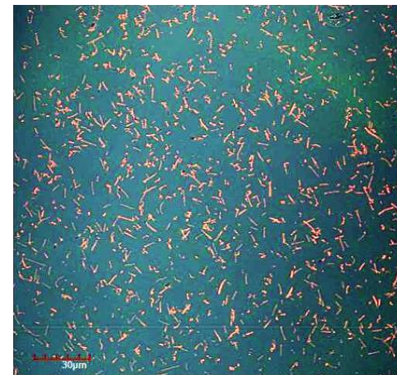
OPA



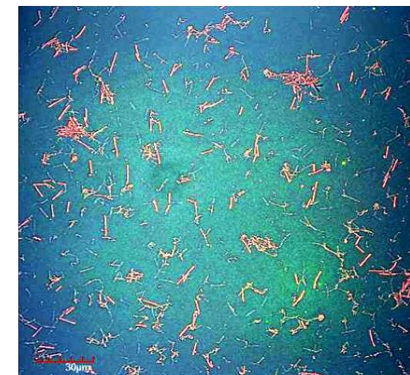
Virex



Formalin



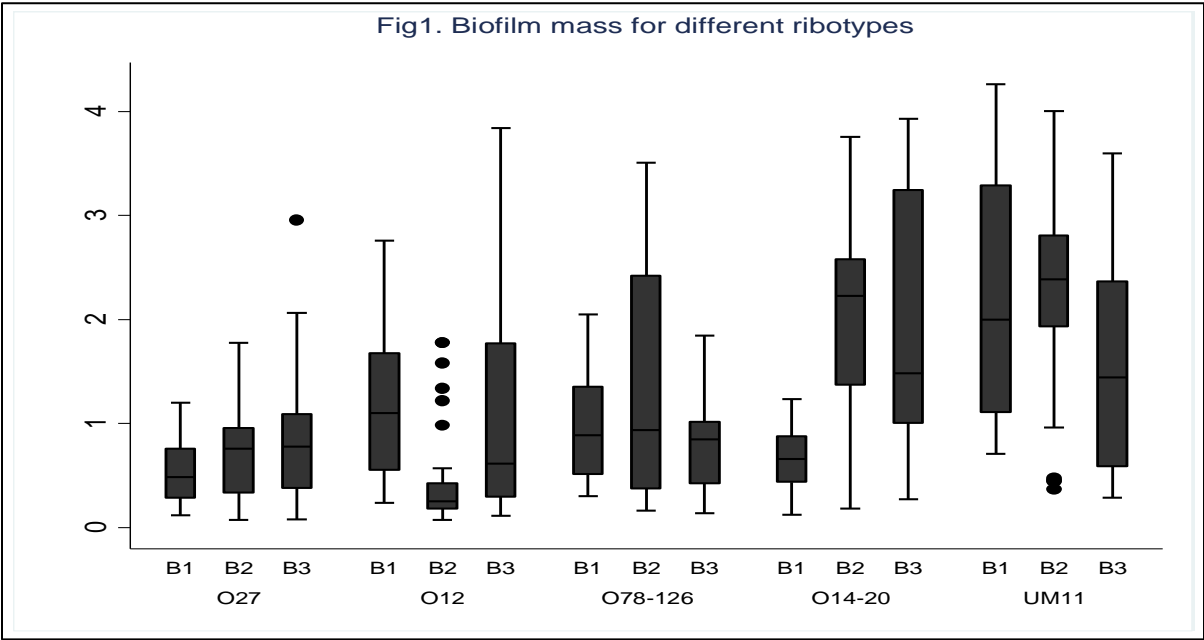
Nixall



Vital oxide

7. b: Visualization of killing effect of disinfectants on 72-hour aerobic multispecies *C. difficile* biofilm on a surface using Confocal microscopy

Supplemental figure B. 1: Biofilm mass for different *C. difficile* ribotypes



Supplemental figure B. 2 a & b: Efficacy of disinfectants against Biofilm embedded *C. difficile* vegetative cells and spore counts

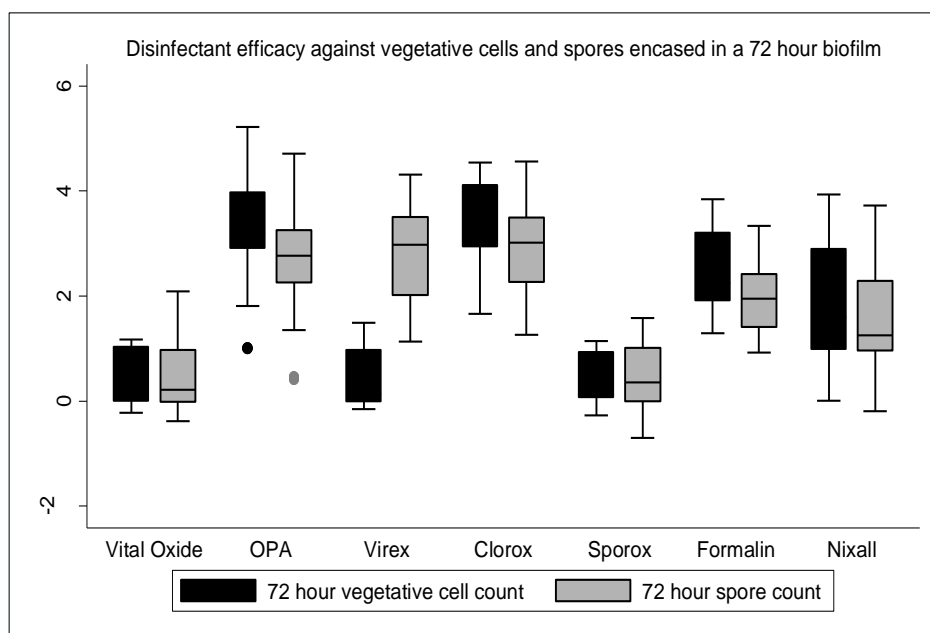


Fig 2a

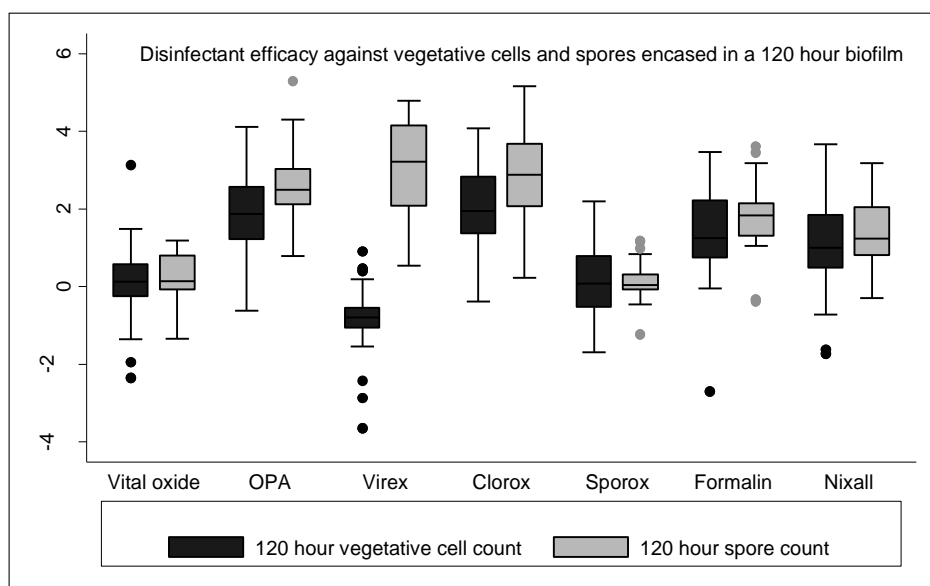


Fig 2b

Supplemental table B. 1: Regression analysis of disinfectant efficacy against vegetative cells and spores in a biofilm

Table 2: Regression analysis of disinfectant efficacy (Log CFU/ml) against vegetative cells and spores encased in a biofilm				
Category	Count type	Disinfectants tested	Regression analysis	
			Coefficient* (log CFU)	P value
3-day old biofilm	Vegetative cell count	Vital Oxide (Control)	0	Reference
		OPA	-2.9	0.000
		Virex	0.19	0.17
		Clorox	-3.12	0.00
		Sporox	0.05	0.75
		Formalin	-2.22	0.00
		Nixall	-1.49	0.001
3-day old biofilm	Spore count	Vital Oxide (Control)	0	Reference
		OPA	-2.19	0.00
		Virex	-2.29	0.00
		Clorox	-2.45	0.00
		Sporox	0.057	0.69
		Formalin	-1.48	0.00
		Nixall	-1.00	0.00
5-day old biofilm	Vegetative cell count	Vital Oxide (Control)	0	Reference
		OPA	-1.86	0.00
		Virex	0.73	0.00
		Clorox	-2.17	0.00
		Sporox	-0.02	0.92
		Formalin	-1.27	0.00
		Nixall	-0.99	0.00

5-day old biofilm	Spore count	Vital Oxide	0	Reference
		OPA	-2.25	0.001
		Virex	-2.67	0.001
		Clorox	-2.59	0.000
		Sporox	0.20	0.27
		Formalin	-1.39	0.00
		Nixall	-1.05	0.00
		• Adjusted for ribotypes, type of biofilm, duration of exposure, presence of organic substrate		

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JOURNAL ARTICLE: C

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Common household disinfectants act as germinating factors for *Clostridioides difficile* spores

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Common household disinfectants act as germinating factors for *Clostridioides difficile* spores

[Authors]

Running title: Household disinfectants and *C. difficile*

University of Texas School of Public Health and the University of Houston College of Pharmacy, Houston, TX USA

Keywords: C diff spores, community disinfectant, bile salt, germinant

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Abstract

Introduction: With the increased incidence of community-associated *Clostridioides difficile* infection (CA-CDI), there is a need for effective infection control and preventive measures for preventing CDI in the community setting. Thus, the purpose of this study was to assess the in vitro killing effect of common household disinfectants against *C. difficile* spores.

Methods: Sixteen clinical and environmental toxigenic *C. difficile* strains were exposed to seven commonly used community disinfectants. Spore counts were assessed after exposure to disinfectants or controls. Sub-studies investigated certain disinfectants as possible germinant for *C. difficile* spores.

Results: Among the disinfectants tested, only household Clorox was able to reduce the spore count by more than 99%. All other disinfectants were either ineffective (n=4) in reducing *C. difficile* spore count or increased *C. difficile* counts higher than positive controls (n=2) in a concentration-dependent manner. Sub-studies demonstrated the effect was primarily due to increased dormant spore germination through upregulation of two genes, SleC, and cspC involved in the *C. difficile* germination pathway.

Conclusion: This study identified Clorox as a potent disinfectant against *C. difficile* spores in the community setting. This study also identified that certain disinfectants can act as potential spore germinant in the community environment. These results can help form a vital role in informing current infection control and prevention guideline for community household environments against *C. difficile* spores.

Introduction

Clostridioides difficile is a gram-positive spore-forming anaerobic bacillus and a leading cause of hospital-acquired infection in the US and worldwide. ⁽¹⁾ *C. difficile* infection (CDI) occurs in approximately 453,000 newly diagnosed cases per year with associated 29,000 deaths. ⁽²⁾ Classically considered a nosocomial infection, community-acquired CDI has emerged in low-risk individuals including younger females with fewer comorbidities and lacking traditional CDI risk factors. ^(3, 4) Of all new CDI cases, approximately 10 to 37% are community-acquired with the incidence of 20-30 per 100,000 population in the USA and Europe. ^(5, 6)

The microbiological features of *C. difficile* spores including prolonged environmental survival, low inoculating dose, frequent environment contamination and relative resistance to various disinfectants contribute to the pathophysiology of the disease. ^(7, 8) Although hospital environmental contamination of *C. difficile* spores has been well studied, fewer studies have investigated community environmental contamination. *C. difficile* spores were identified in various household environment sites in homes of persons with recurrent CDI including vacuum cleaner bags, bathroom sinks, toilets, and kitchen surfaces. ^(7, 9, 10) A Canadian study looking at the household of patients without *C. difficile* infection found 31% of the household to have *C. difficile* spores which were predominantly O27 ribotype. ⁽⁹⁾ *C. difficile* spores have also been found in soil, waterways, water treatment plants, animals, and food. ⁽¹¹⁻¹³⁾ The similarities in the prevalence of common ribotypes and toxigenic strains between environmental, animal and food sources with human indicates a transmission chain between them. ⁽¹⁴⁾ Thus,

the transmission model for community CDI can be categorized broadly into consumption (ingestion of *C. difficile* spore in contaminated food), person to person contact transmission from one infected person to another) and environment to human contact (ingestion/contact with contaminated spores from environmental sources). ⁽¹⁵⁾

With the epidemiologic shift and emerging concern for community-associated CDI (CA-CDI) as a public health threat, there is a need for effective infection control and preventive measures for preventing CDI in the community setting. Although several guidelines are available for preventing CDI in a health care setting, environmental control of *C. difficile* in the household setting is poorly studied. Specifically, there are no studies looking at the effect of commonly used household disinfectants against *C. difficile* spores. Disinfectants are effective against non-spore forming organisms such as *E. coli*, *S. aureus*, *Salmonella*, coliforms, and common viruses. ⁽¹⁶⁻²¹⁾ However, spores can be resistant to many disinfectants and require independent study. Thus, the purpose of this study was to assess the in vitro killing effects of common household disinfectants against *C. difficile* spores.

Methods

Nine clinical and six environmental toxigenic *C. difficile* strains of different ribotypes (O27, O14-20, O78-126, O12, 106) and one non-toxigenic strain was used in this study along with two reference strains (R20291; a ribotype 027 strain and a nontoxigenic *C. difficile* (ATCC 700057)). Clinical *C. difficile* strains were isolated from stool samples of de-identified patients and the environmental strains were collected by trained research

staff from household items or environmental dust from houses in Houston.⁽¹⁰⁾ *C. difficile* strains were isolated, purified, stocked and ribotyped as previously described.⁽²²⁾ Presence of toxigenic genes (toxin A, toxin B, and binary toxin) was identified through multiplex PCR. Seven commonly used household disinfectant sprays and refills were used for this study (Table 1). Household disinfectants were defined as any chemical antimicrobial component that was commercially available for common household use against potential human pathogens. To identify commonly used disinfectants, one investigator (TR) visually observed 50 shoppers select a household disinfectant from the disinfectant aisle of a local supermarket. The top 5 disinfectants from the list were selected for this study. Another two disinfectants were chosen from a list of eco-friendly disinfectants based on feasibility and availability.

***C. difficile* spore preparation**

C. difficile spore preparation was done as previously described.⁽²³⁾ Briefly, *C. difficile* strains were streaked onto brain Heart Infusion-Supplemented (BHIS) agar and incubated anaerobically for 4 days. Surface microbial growth was scraped and mixed with 1 ml sterile ice cool water in an Eppendorf and incubated overnight at 4°C. The suspension was then washed five times in ice-cold sterile deionized water (DW). The washed pellet was suspended in 3ml ice cold DW. The suspension was then layered on top of a 10 ml bed of 50% sucrose in water and centrifuged at 3,200g for 20 minutes. The spores formed a pellet at the bottom of the tube. The spore pellets were washed 5 times to remove sucrose and was then re-suspended in DW and stored at 4°C.

Spore counts

For spore counts, 100 μ L of killed *Candida* DW was added to work as a carrier to concentrate spores after incubation.⁽²⁴⁾ The mixture was centrifuged at 15,000 g for 2 minutes, washed, diluted in DW, plated on blood agar plates and incubated anaerobically for 72 hours. After 72 hours, the viable spores were counted for disinfectant-treated and positive control plates.

Germination kinetics assay

The initiation of spore germination was monitored aerobically at 600 nm using Cytation Gen 3 Cell imaging multi-mode reader as previously described.⁽²⁵⁾ To initiate spore germination, purified spores were suspended in germination buffer containing 10mM Tris (pH 7.5), 150 mM sodium chloride, 100 mM glycine with or without 10mM bile salt.

Ca-DPA assay

Ca-DPA assay for both dormant and activated spores was monitored real-time using terbium fluorescence (TbCl₃).^(25, 26) Both dormant and heat activated spores were centrifuged at 15,000 x g for 2 minutes and re-suspended in an equal volume of DW to remove any Ca-DPA release from auto germinating spores. Fluorescence due to Ca-DPA release was monitored using Cytation Gen 3 at excitation 270 nm, emission 545 nm, and cutoff 420 nm over a period of 1 hour.

Spore cortex fragmentation assay

Spore cortex fragmentation assay based on colorimetric detection of reducing sugars released during spore germination was done on activated spores. ⁽²⁷⁾ Samples were collected at 0, 5, 10, 15, and 30 minutes, centrifuged and supernatant frozen at -80°C for lyophilization. As a positive control for reducing sugar detection and quantification, 0, 12.5, 25, 50, 100, 250, 500 of N-acetyl glucosamine was prepared and standard curve created. Cortex fragments were measured using the Cytation Gen 3 plate reader at 585 nm.

Confocal microscopy

Microscopy samples (pellets), either immediately post-exposure to disinfectants for 10 minutes or post-exposure to disinfectant followed by incubation at room temperature for 30 minutes in germination buffer without bile salt were resuspended in 200µL of 4% paraformaldehyde (PFA) and incubated at room temperature for 1 hour. The samples were centrifuged for 2 minutes at 15,000 g, re-suspended in 60 µL DW, stained for 1 hour with 500 µg/ml FM4-64 (Life Technologies) in DMSO. Cells were then washed twice, resuspended in DW and stored at 4°C prior to microscopy experiment. For microscopy, cells were mounted on slides and transferred to a laser-scanning confocal microscope (Olympus FU 1000). Images were taken using 515 nm and 640 nm lasers and an X60 objective lens. ⁽²⁸⁾

qRT- PCR

Quantitative real-time PCR (qRT-PCR) was performed to look at gene expression during spore germination for both treated and untreated spores of standard strain R20291. Both Lysol hydrogen peroxide-treated and untreated spores were washed and resuspended in PBS alone, germination buffer with a bile salt, germination buffer without bile salt and in BHIS and bile salt for 30 minutes. Following exposure for 30 minutes, RNA was extracted using a modified protocol using Zymo QuickRNA kit with mechanical lysis.⁽²⁹⁾ Briefly, an equal volume of methanol was added to the spore sample to fix followed by centrifugation and resuspension in STE buffer and mechanical lysis using glass bead in fast prep tubes. The samples were then lysed with RNA lysis buffer. RNA was extracted using the protocol mentioned in the kit. In column DNase treatment was done and DNA contamination checked with agarose gel run. The RNA concentration and ratio were measured using QuickDrop spectrophotometer.

Complementary DNA (cDNA) was synthesized from RNA using Invitrogen Superscript IV reverse transcriptase.⁽³⁰⁾ Briefly, 50µM Oligo d(T) primer is annealed with template RNA from our samples in the presence of dNTP at 65°C for 5 minutes. Reverse transcriptase reaction mix (consisting of 5xSSIV, DTT, RNase inhibitor and superscript IV RT) is added with annealed RNA and incubated at 55°C for 10 minutes and reaction is inactivated by heating at 80°C for 10 minutes. RNA is removed from the samples by adding 1µL of RNase and incubation at 37°C for 20 minutes.

The expression of two genes commonly involved in the germination pathway, SleC and cspC was determined from complementary DNA using qRT-PCR as described by Pishdadian et al.^(31, 32) Primers for SleC and cspC was designed as previously

described.⁽³³⁾ Briefly, qPCR was performed using SYBR Green qPCR master mix, 250 nM of gene-specific primers and ABI (Applied Biosystem) 7500 Real-time PCR system. Transcript levels for *SleC* and *cspC* were measured from cDNA and normalized against referenced housekeeping gene *rpoB* using the $\Delta\Delta CT$ method. There were 3 replicates for each experiment. The level of transcripts was calculated for Lysol hydrogen peroxide (Lysol H₂O₂) treated sample relative to untreated samples.

Experimental procedures

The killing effect of 7 household disinfectants was tested against 10 clinical and 6 environmental *C. difficile* spores. Ten μ L from dormant spore stock was added to 500 μ L of disinfectants and incubated based on the label determined contact time for each disinfectant. Spore counts were calculated for disinfectant treated plates and positive controls. Changes in spore counts were also assessed at different contact times (10 minutes, 6 hours and 24 hours) and with or without the presence of organic matter (40 μ L protease peptone 10gm/L). Finally, the *in vitro* killing of *C. difficile* spores was assessed at three concentrations (original, 50% dilution and 25% dilution).

Effect of disinfectant on dormant vs activated spore propagation

Results of the *in vitro* studies demonstrated an unexpected finding of increased CFU spore count after exposure to certain disinfectants. To investigate whether disinfectants might activate or initiate spore germination, a variety of experiments were undertaken. All experiments were done on 10 clinical *C. difficile* strains of six ribotypes. Separate experiments were performed on active and dormant spores. Spores were heat activated

at 65°C for 30 minutes and placed on ice. In a separate set of experiments, *C. difficile* spore CFU count following exposure to disinfectants was quantified and compared with known germinating factors; potassium chloride (KCL), aspartate (Asp) and bile salt (sodium taurocholate) and disinfectant active ingredients.

To assess germination kinetics, 100 microL of dormant and activated spores were added to 500 µL of Lysol hydrogen peroxide disinfectant or DW (untreated sample), exposed for contact time, washed and re-suspended in 5 µL of buffer. Disinfectant treated and untreated samples were then added to 995 µL germination buffer with and without bile salt and the change in optical density at 600 nm was measured over a period of 30 minutes as described above. To assess calcium DPA release, 100 µL of spores were added to 500 µL of two disinfectants (Lysol and Lysol hydrogen peroxide) and 500 µL of DW (untreated sample) and exposed for 10 minutes and centrifuged. The pellets were re-suspended in 5 µL of germination buffer and then added to 125 µL of germination buffer supplemented with 800 µM TbCl₃ in 96 well opaque plates. Finally, to assess spore cortex fragmentation, heat activated spores were exposed to one disinfectant (Lysol hydrogen peroxide) or an equal volume of DW and added at an OD of approximately three to germination buffer with or without bile salt. One ml of germination solution without spores served as a negative control for these experiments.

Statistical analysis

To assess disinfectant *in vitro* killing effect, CFU spore counts (CFU/ml) was compared between different disinfectants, active ingredients, germinating factors, and positive

controls along with differences based on contact time, presence or absence of organic material, and disinfectant concentration. To assess the killing effect of disinfectants, a linear regression model was built adjusted for ribotypes and strain types (clinical vs. environmental). To assess germination kinetics and Ca-DPA release, the percent change in fluorescence over time for each assay was compared to the baseline (time 0) or untreated controls. For spore cortex fragmentation assay, the amount of cortex was quantified in nmol using the standard curve and plotted against time. STATA/IC version 12.1 (STATACorp LLC, College Station TX) was used for analyses. A P value of < 0.05 was considered significant. GraphPad Prism 8.02 was used to analyze the results for optical density readings.

Results

***In vitro* killing of community disinfectants on *C. difficile* spores**

C. difficile spores were produced from all 16 *C. difficile* strains of 6 ribotypes. The *in vitro* killing effects of the disinfectants was tested on all 16 strains. Germination studies were done on the 10 clinical strains. Initial spore concentrations were approximately 10^3 to 10^4 CFU/ml for all experiments. *C. difficile* spore CFU count post-exposure to disinfectants at label determined contact time is given in **Figure C.1**. The mean \pm SD count for the untreated control samples was 672 ± 809 CFU/mL. Among the disinfectants, only household Clorox was able to reduce the spore count by more than 99%. All other disinfectants were either ineffective in reducing *C. difficile* spore count [Windex(W), Spic & Span (SS), Seventh Generation (SG), Pure Green (PG)] or

increased *C. difficile* count [Lysol, Lysol hydrogen peroxide]. The mean spore count \pm SD for Lysol and Lysol hydrogen peroxide-treated spore was $3,634 \pm 2,359$ and $3,520 \pm 2,043$ CFU/mL, respectively. Both of these concentrations were significantly higher than positive controls ($p < 0.01$). The in vitro killing effects of disinfectants on *C. difficile* spore count was further stratified by different contact time, different concentration of disinfectants and presence or absence of organic matter (**Figure C.2**). For most disinfectants except Lysol hydrogen peroxide, the presence of organic matter further reduced the efficacy at prolong duration of exposure. For Lysol hydrogen peroxide, continued exposure for 24 hours killed *C. difficile* spores irrespective of the presence of organic substrate. A concentration-dependent effect of increased CFU counts was observed for Lysol and Lysol hydrogen peroxide with higher CFU count at original concentration and lowest at 25% of original concentration (**Figure C.3**). Expectantly, a lower concentration of Spic and Span resulted in increased spore count. To characterize the increased spore CFU counts observed with Lysol and Lysol hydrogen peroxide, CFU spore counts were compared to known germinating factors; KCl, aspartate and bile salt (**Figure C.1**). *C. difficile* CFU count for Lysol and Lysol hydrogen peroxide were higher than KCl and aspartate and similar to bile salt. *C. difficile* CFU spore counts were not higher using the active ingredients for Lysol or Lysol hydrogen peroxide (**Figure C.4**). A linear regression analysis was done to assess changes in CFU count by disinfectant type controlling for strain type (clinical vs. environmental) and ribotypes. The only disinfectant that showed effective trend was Clorox (CFU change compared to positive control: -703.9 ± 364.6 ; $p = 0.054$). The disinfectants that increased CFU counts the most was Lysol (CFU change: $2,961 \pm 364.6$; $p < 0.01$) followed by Lysol

hydrogen peroxide (CFU change: $2,847 \pm 364.6$; $p < 0.01$), Seventh generation (CFU change: $1,486 \pm 364.6$ $p = 0.001$); Pure Green (CFU change: $1,175 \pm 364.6$; $p = 0.001$) and Spic & Span (CFU change: 869 ± 364.6 ; $p = 0.02$);

Disinfectants as a germinating/ activating agent

To further investigate Lysol and Lysol hydrogen peroxide as possible germinant, these two disinfectants were tested against 10 clinical *C. difficile* strains. To investigate whether disinfectants activate the spores, both dormant and heat activated spores were exposed to Lysol and Lysol hydrogen peroxide compared with unexposed control (**Figure C.5**). CFU counts were higher (mean CFU count 5.5×10^4 to 7.5×10^4) for dormant spores exposed to disinfectants as compared to unexposed spores (mean spore count 2×10^3). However, for activated spores, the CFU counts were similar between disinfectant exposed spores (mean CFU count 6×10^4 to 7×10^4) and unexposed spores (mean CFU count 4×10^4).

Ca-DPA assay

Calcium DPA release from dormant and activated spores exposed to Lysol, Lysol hydrogen peroxide, or bile salts vs. positive controls is shown in (**Figure C.6**). Using dormant spores, Lysol H202 exposure leads to increased Ca-DPA release (normalized fluorescence of 10) compared to unexposed control without bile salt (normalized fluorescence of 2) and unexposed control with bile salt (normalized fluorescence of 4). Lysol exposure to dormant cells did not increase Ca-DPA release. Using activated

spores, both spores treated with Lysol H2O2 (normalized fluorescence of 3.5) and unexposed spores with bile salts (normalized fluorescence of 3) had a similar level of Ca-DPA release.

Germination and spore cortex fragmentation assay

To further investigate the effect of Lysol H2O2 to trigger *C. difficile* spore germination, the germination efficacy of *C. difficile* spores was determined for Lysol hydrogen peroxide using dormant and active spores in presence or absence of bile salt (**Figure C.7**). Lysol hydrogen peroxide increased germination kinetics for dormant spores compared to positive controls with or without bile salts. Percent of optical density as compared to baseline was around 55% for Lysol hydrogen peroxide with or without bile salt as compared to 95% for control without bile salt and 80% for control with bile salt. For activated spores, germination kinetics were similar for Lysol H2O2 treated spores (55% of original optical density) or positive controls with bile salts (65% of original optical density). Using the cortex fragmentation assay, the amount of cortex hydrolyzed after exposure to Lysol H2O2 was much greater (60 nmol over 30 minutes) as compared to non-Lysol hydrogen peroxide-treated samples regardless of treatment with bile salts (15-25 nmol over 30 minutes) (**Figure C.8**).

Confocal microscopy

Using confocal microscopy for FM4-64 stained spores, the size for 10 minutes post Lysol hydrogen peroxide-treated spores is slightly larger and is between 2 to 5 μm as compared to 2 μm for untreated spores (**Figure C. 9a and C. 9b**). For spore

samples 30 minutes post-suspension in germination buffer, we see significantly more germinating *C. difficile* vegetative cells in the treated sample as compared to the untreated sample. This was true for all focuses of confocal microscopy.

qRT-PCR

The fold increase in the level of expression of *Slec* and *cspC* gene in Lysol hydrogen peroxide-treated spore samples as compared to untreated samples is shown in **Figure C.10**. A 4 to 6.5-fold increase in the level of *cspC* and *SleC* respectively was observed in treated spores compared to untreated spores suspended in PBS alone. As expected, gene expression was also increased if *C. difficile* was grown in germination buffer.

Discussion

Community-associated CDI is an emerging infectious disease affecting persons not commonly thought to be at risk for CDI; namely younger with fewer co-morbid conditions or exposure to antimicrobials. The pathogenesis of CDI involves ingestion of spores found in the environment. Although commonly studied in healthcare systems, disinfection techniques for the community setting are poorly studied. To begin this process, we selected commonly used household disinfectants to test against our biobank of clinical and environmental *C. difficile* strains. Overall, we found that most of the community disinfectants were ineffective against *C. difficile* spores except for one disinfectant (Clorox). We also identified two disinfectants (Lysol and Lysol hydrogen peroxide) that increased *C. difficile* CFU after exposure. This increase in the count was

more at the original concentration of disinfectant and was not related to the active ingredient. The efficacy of disinfectants improved on prolonged exposure at 24 hours with Lysol hydrogen peroxide reaching almost 100% killing at 24 hours. Through a series of experiments, the effect of these two disinfectants was most pronounced on dormant spores in comparison to other known germinating agents. Based on the result from germination kinetics, Ca-DPA release, spore cortex fragmentation assay, confocal microscopy, and qPCR, it was found that Lysol hydrogen peroxide is a potential *C. difficile* spore germinator. This novel finding will require verification but could represent an important new insight into spore propagation in the community setting.

Several studies have investigated the in vitro activity of household disinfectants against other microorganisms.^(17, 34) However, few studies have investigated the in vitro activity of community disinfectants on spore-forming organisms. One previous study has shown in vitro activity of Clorox against *C. difficile* spores.⁽¹⁹⁾ Clorox has also been shown to have in vitro activity against *Bacillus* species, another spore-forming organism.⁽³⁵⁾ As we expand our studies to other community disinfectants, Clorox will be our standard comparator.

The novel finding from this study was the increased spore count observed after a concentration-dependent exposure to Lysol and Lysol hydrogen peroxide. This increased count was more pronounced in dormant vs. active spores and was not due to a single active ingredient of the products. After 24 hours of exposure, Lysol hydrogen peroxide displayed in vitro activity against *C. difficile* possibly due to spore germination leading to susceptibility to disinfectants.⁽³⁶⁾ Increased spore count after exposure to

Lysol and Lysol hydrogen peroxide was due to increased germination of *C. difficile* spores. The architecture of *C. difficile* spores includes a partially dehydrated dormant spore core containing Ca-DPA. The inner layer is a permeability barrier inner membrane followed by germ cell wall surrounded by thick spore cortex which is in turn enclosed in spore coat and external exosporium. ⁽³⁷⁻⁴⁰⁾ In response, external stimuli including bile acids, amino acids, nutrients and electrolytes by CSP proteases various germination pathways are activated including bile acid-amino acid, alanine racemase dependent D- amino acid, or bile salt-divalent pathways. ^(23, 41, 42) Each of these pathways leads to activation of the gene, *Slec* which initiates degradation of cortex resulting in full core rehydration and release of Ca-DPA and initiation of germination. ^(41, 43) Some of the potential ways Lysol hydrogen peroxide can initiate spore germination is given in **(figure 11)**. From our study, we found that the active ingredient of the disinfectants did not cause an increase in spore germination which indicates that non-active additive ingredients in these disinfectants might either act as a nutrient or an ion to stimulate germination. The other potential pathway will probably be through activation of various genes in germination pathway. Through qPCR, we identified a 4 to 6.5 fold increase in two of the genes, *cspC* and *SleC* related to germination pathway. In this study, we demonstrated that Lysol H202 causes lysis in spore cortex resulting in rehydration of spore cortex, the release of calcium DPA and germination as evidenced by our germination assay, Ca-DPA assay, and Spore cortex fragmentation assay as well as confocal microscopy. As we observed an increased effect on dormant spores compared to active spores, the likely pathway for disinfectant action is likely via

activation of spores. However further studies are needed to determine the exact mechanism for disinfectant action.

Strengths of this study include a large number of samples from clinically important ribotypes and a number of commonly used disinfectants. Some of the limitations of this study include the lack of available standardized lab protocol for testing the disinfectants. Being one of the first studies in this field, most of the lab procedures used in this study was developed or modified and validated and optimized in-house. However, further studies are needed to further validate the findings from this study.

Conclusion

In conclusion, this study identified Clorox as a potent disinfectant against *C. difficile* spores. The novel finding from this study is that certain disinfectants can act as potential spore germinant. The results from this study may serve as a reference for future studies on household disinfectants and *C. difficile*. Further studies are needed to investigate which components of disinfectants cause this effect as well as an expansion on other disinfectants. These results can play a vital role in informing current infection control and prevention guideline for community household environments against *C. difficile* spores.

Table C. 1: Disinfectants used in the study

Disinfectant name	Active ingredient	Label determined contact time	Sporicidal agent
Lysol	Citric acid-2.5%	10 min	No
Lysol H2O2	Hydrogen peroxide (H2O2)- 0.88%	10 min	No
Spic and Span	Octyl Decyl Dimethyl Ammonium Chloride- 0.025% Dioctyl Decyl Dimethyl Ammonium Chloride- 0.010% Didecyl Dimethyl Ammonium Chloride- 0.015% Dimethyl Benzyl Ammonium Chloride- 0.034%	10 min	No
Windex multi surface disinfectant	L Lactic acid-1%	10 min	No
Clorox	Sodium hypochlorite- 0.85%	10 min	No
PureGreen	Citric acid- 4.8% Silver ion- 0.003%	10 min	No
Seventh Generation	Thymol- 0.05%	10 min	No

Figure C. 1: *C. difficile* spore CFU count after exposure to disinfectants and germinating factors

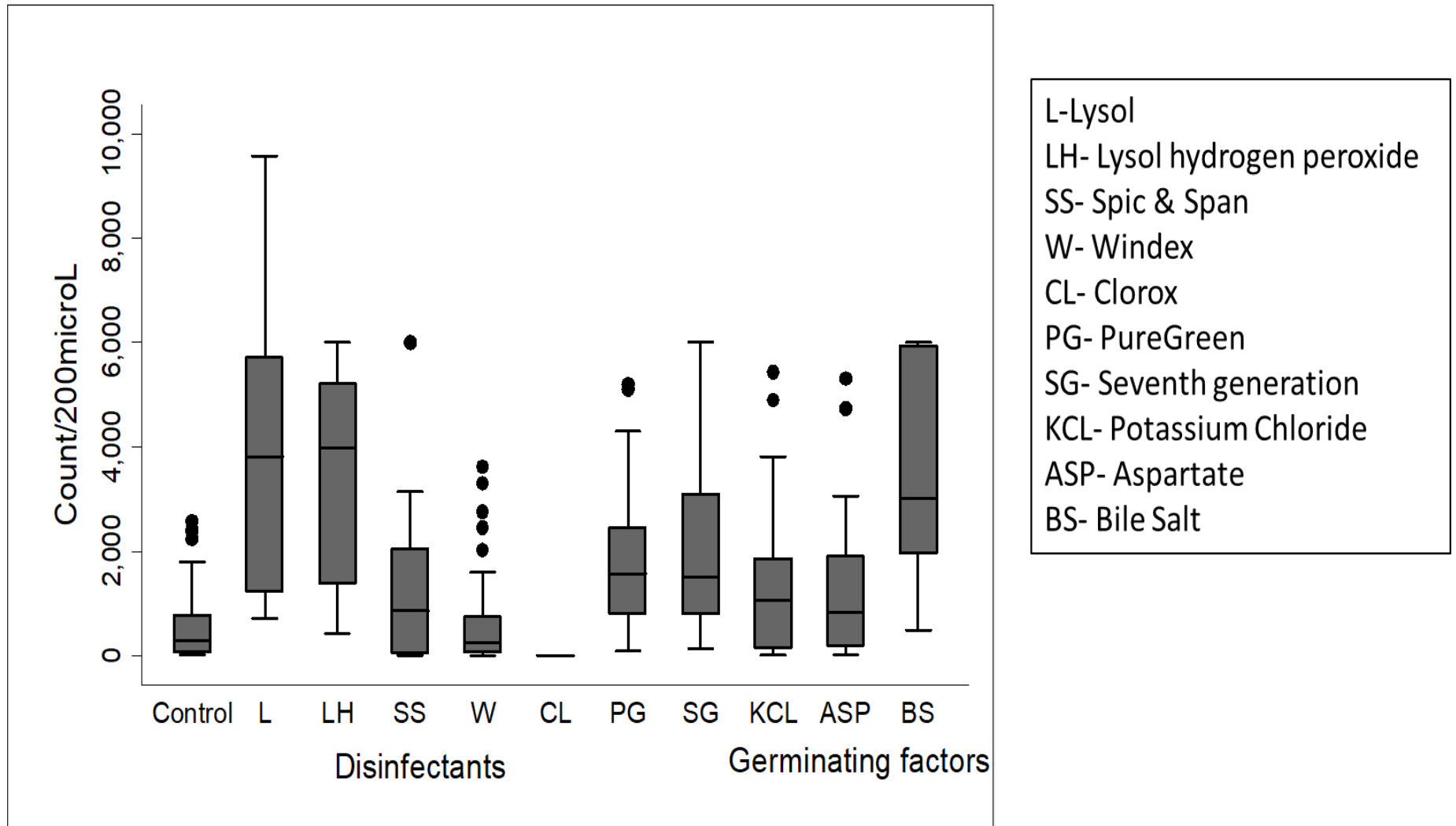


Figure C. 2: *C. difficile* spore CFU count stratified by disinfectant, contact time, and presence or absence of organic matter.

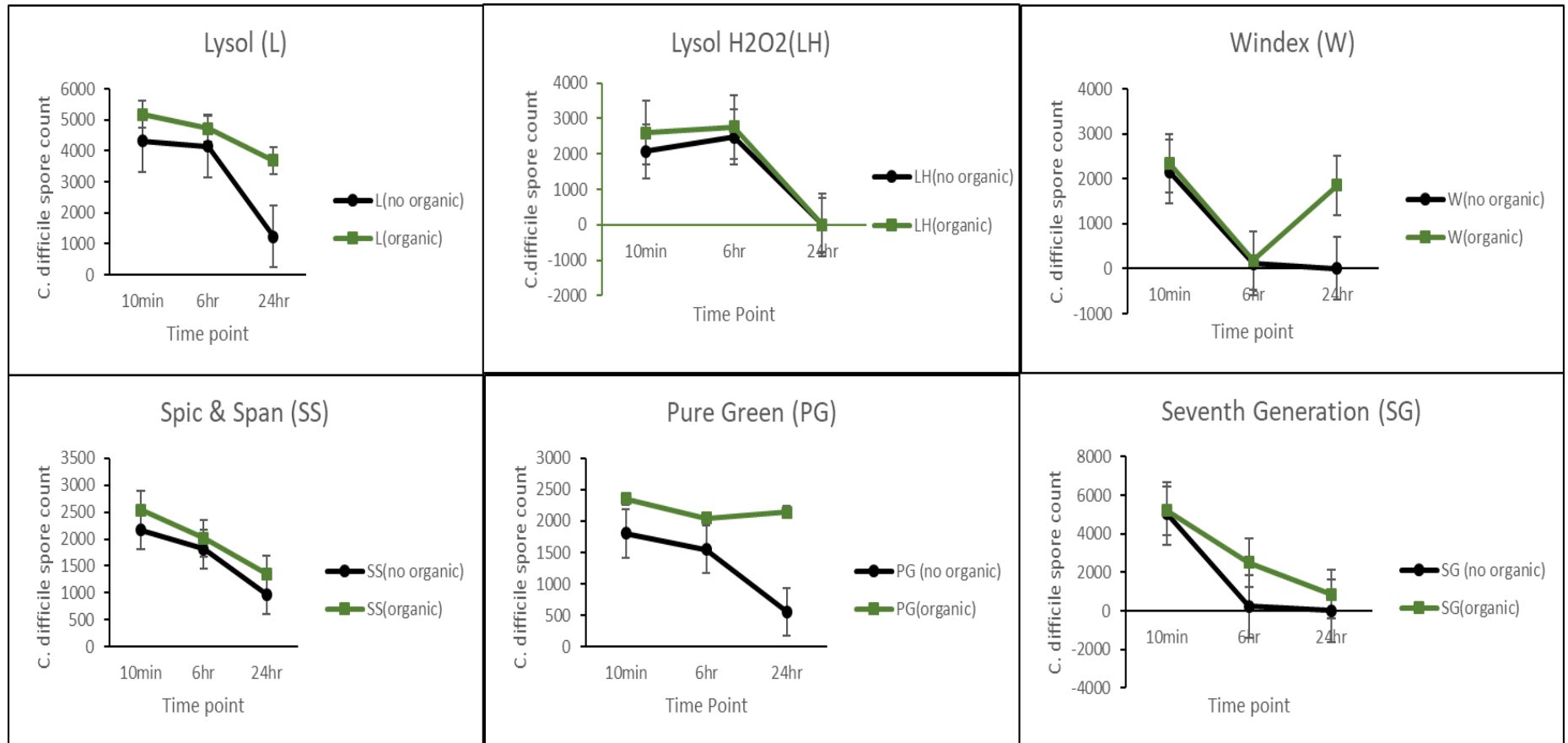


Figure C. 3: Effect of disinfectant concentration on *C. difficile* spore CFU count

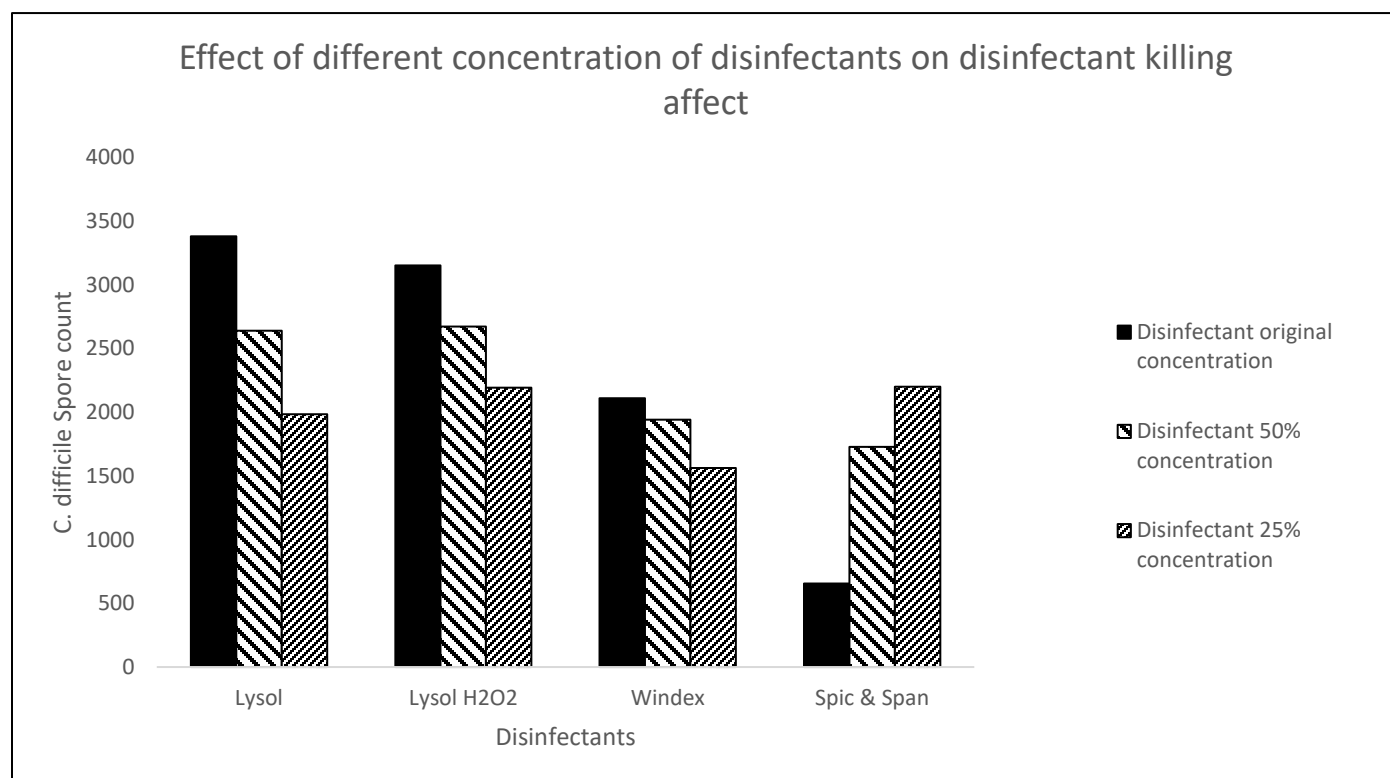


Figure C. 4: *C. difficile* spore count comparing disinfectants with their active ingredients and bile salt

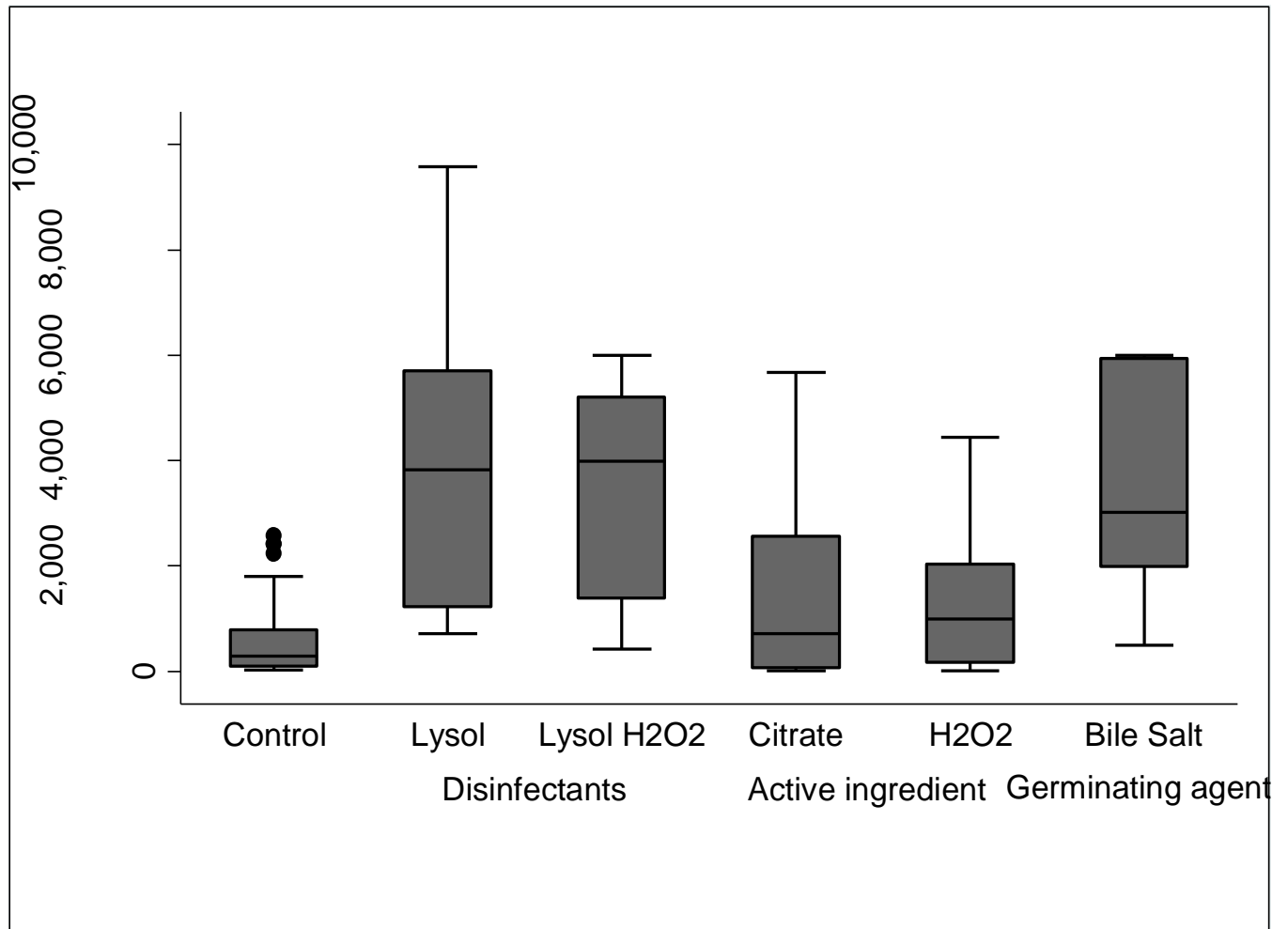


Figure C. 5: *C. difficile* spore count between dormant and activated spores

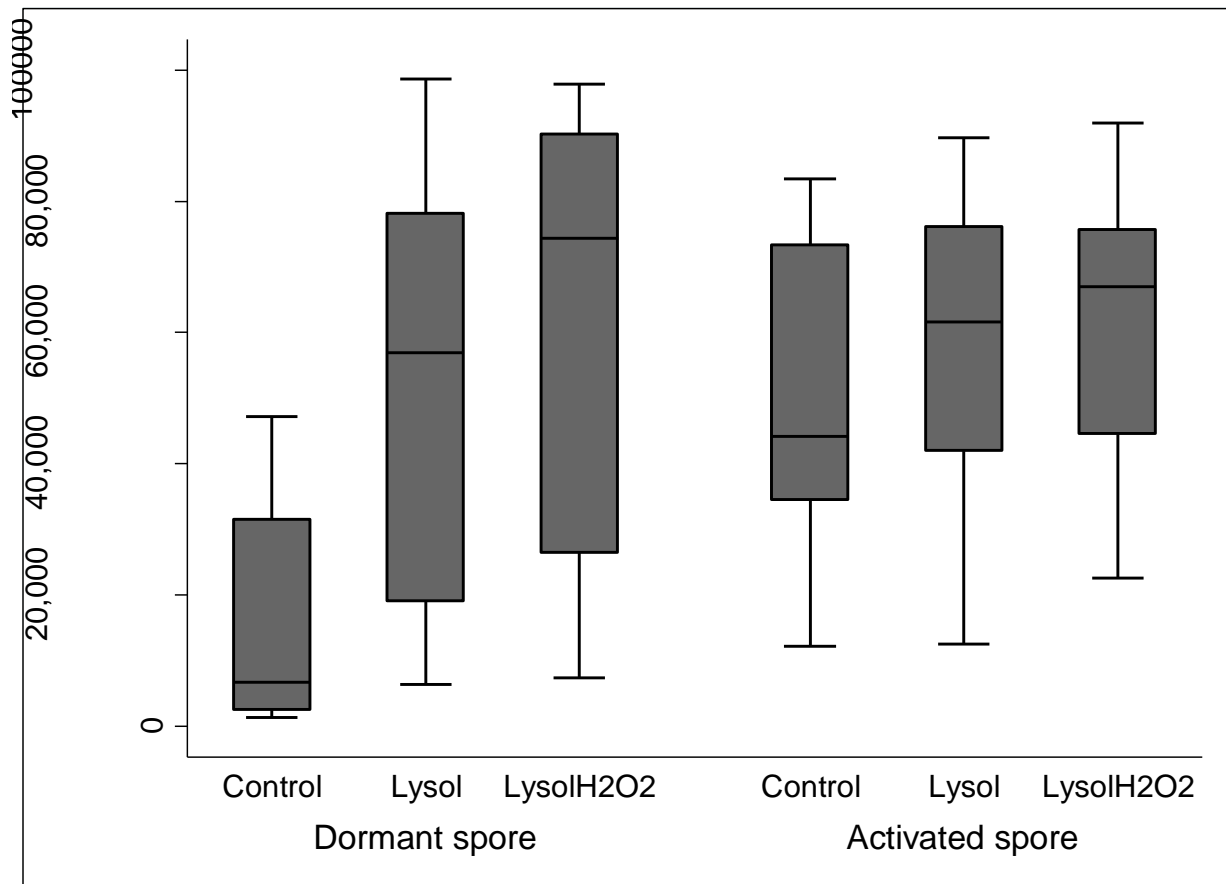
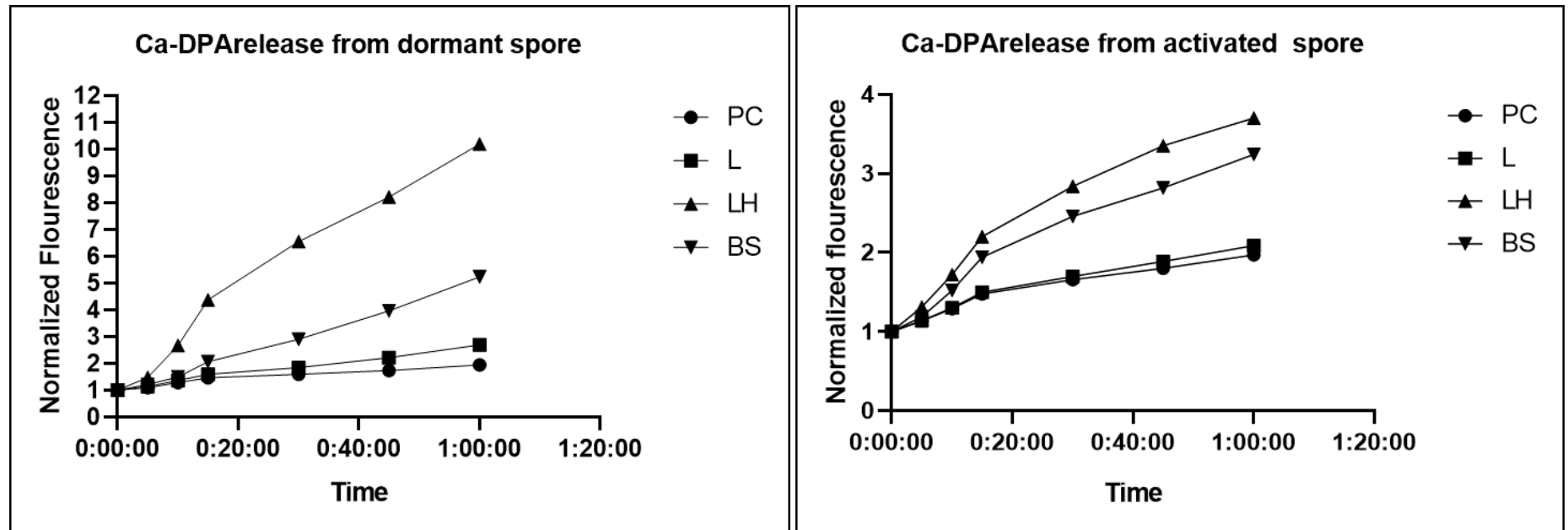


Figure C. 6: Ca-DPA assay for dormant and activated spores



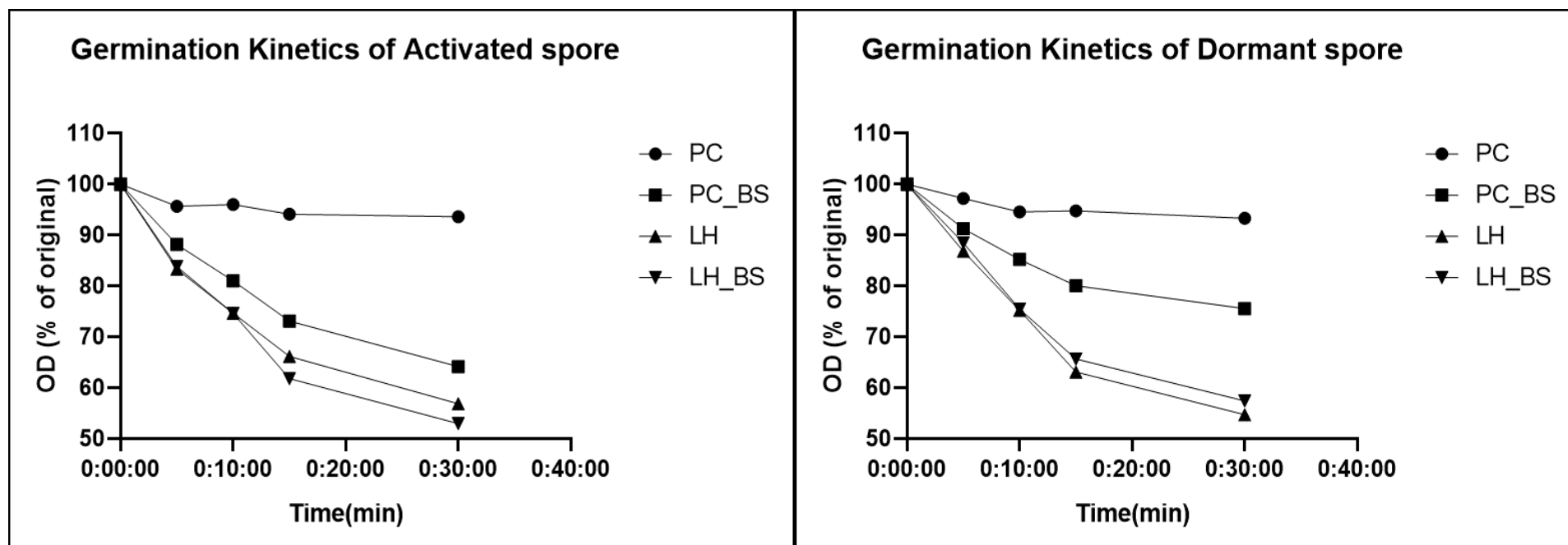
PC: control spores grown in germination medium without bile salt

L: spores pretreated with Lysol H₂O₂ and grown in germination medium without bile salt

LH: spores pretreated with Lysol H₂O₂ and grown in germination medium without bile salt.

BS: control spores grown in germination medium with bile salt

Figure C. 7: Germination kinetics of *C. difficile* spores



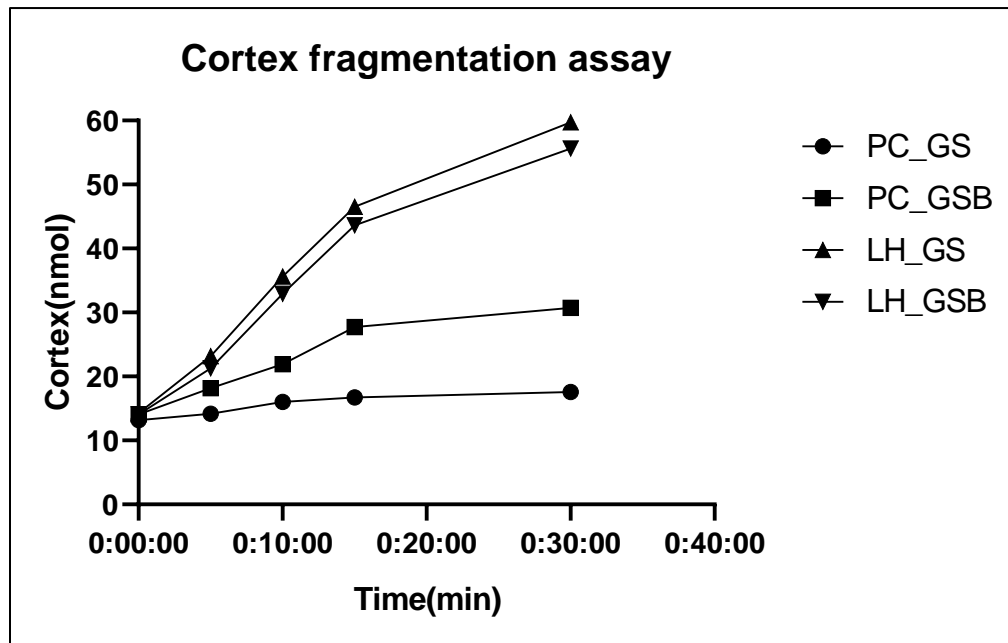
PC: control spores grown in germination medium without bile salt

PC_BS: control spores grown in germination medium with bile salt

LH: spores pretreated with Lysol H₂O₂ and grown in germination medium without bile salt

LH_BS: indicate spores pretreated with Lysol H₂O₂ and grown in germination medium with bile salt.

Figure C. 8: Spore Cortex fragmentation assay



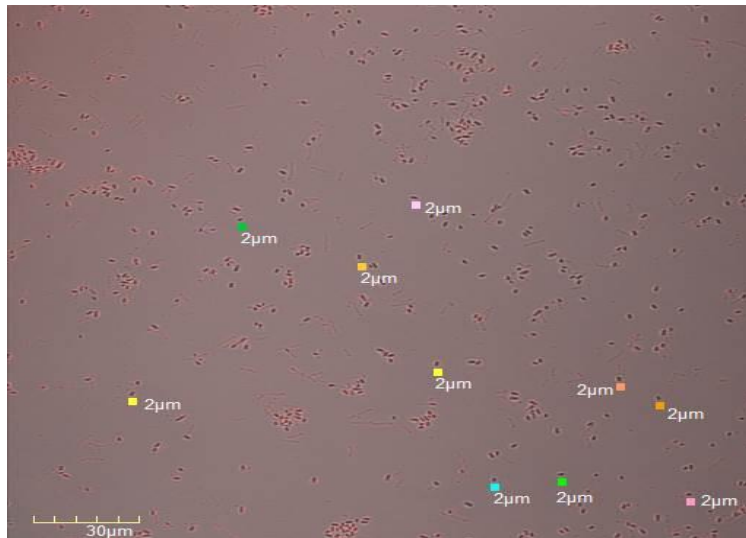
PC_GS: control spores grown in germination medium without bile salt

PC_GSB: control spores grown in germination medium with bile salt

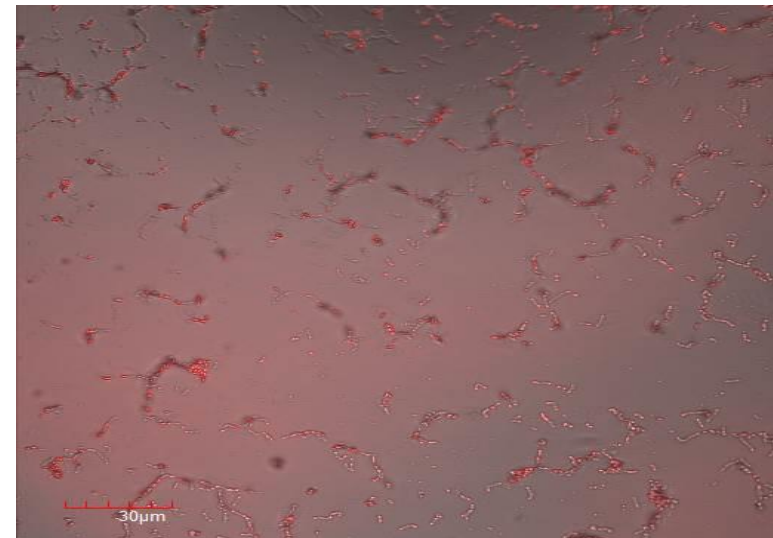
LH_GS: spores pretreated with Lysol H₂O₂ and grown in germination medium without bile salt

LH_GSB: indicate spores pretreated with Lysol H₂O₂ and grown in germination medium with bile salt.

Figure C. 9.a & b: Confocal microscopy of Spores

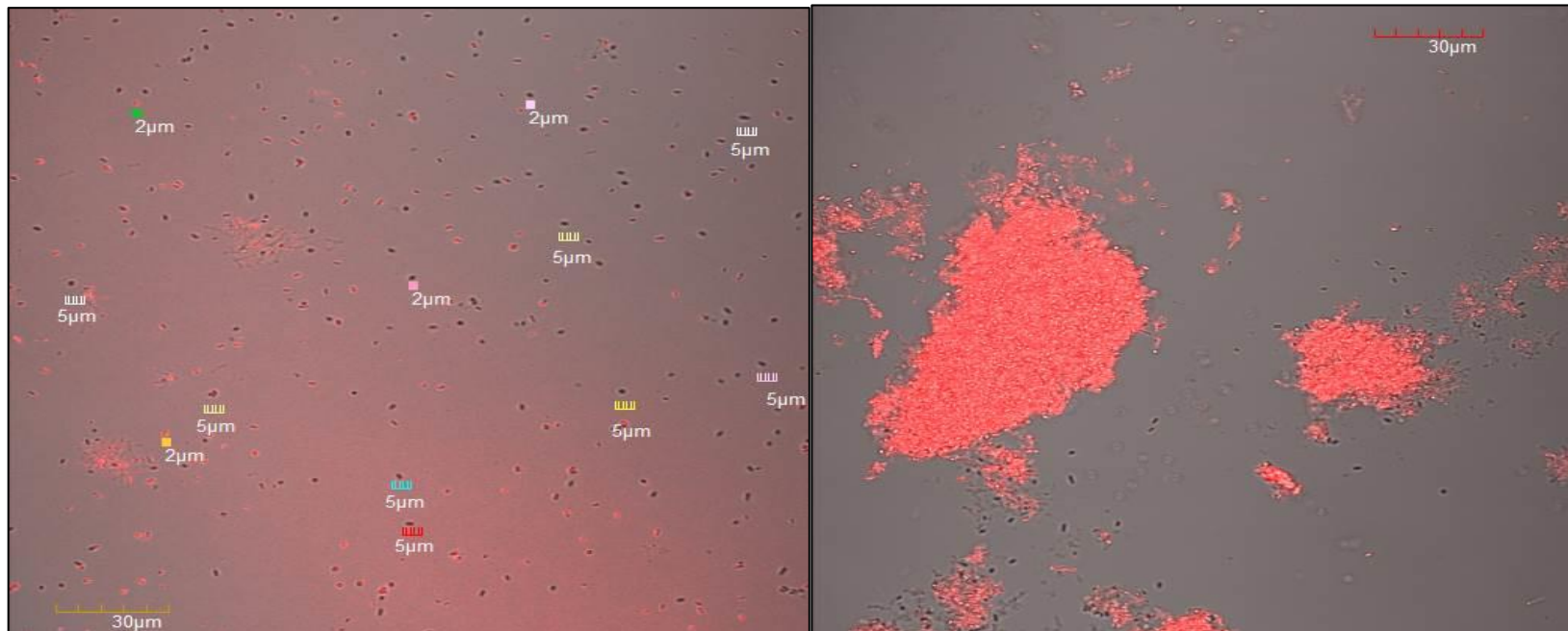


R20291 untreated spore



Germinating R20291 untreated spore (suspended in germination buffer for 30 minutes)

9.a: Spores of R20291 (untreated samples)



R20291 spores treated with LH (10 minutes post exposure)

Germinating R20291 spore treated with LH (suspended in germination buffer for 30 minutes)

9.b: Lysol Hydrogen peroxide treated samples [10 and 30 minutes post exposure]

Figure C. 10: qRT-PCR comparing the relative expression of cspC and SleC gene between Lysol hydrogen peroxide-treated and untreated R20291 spore

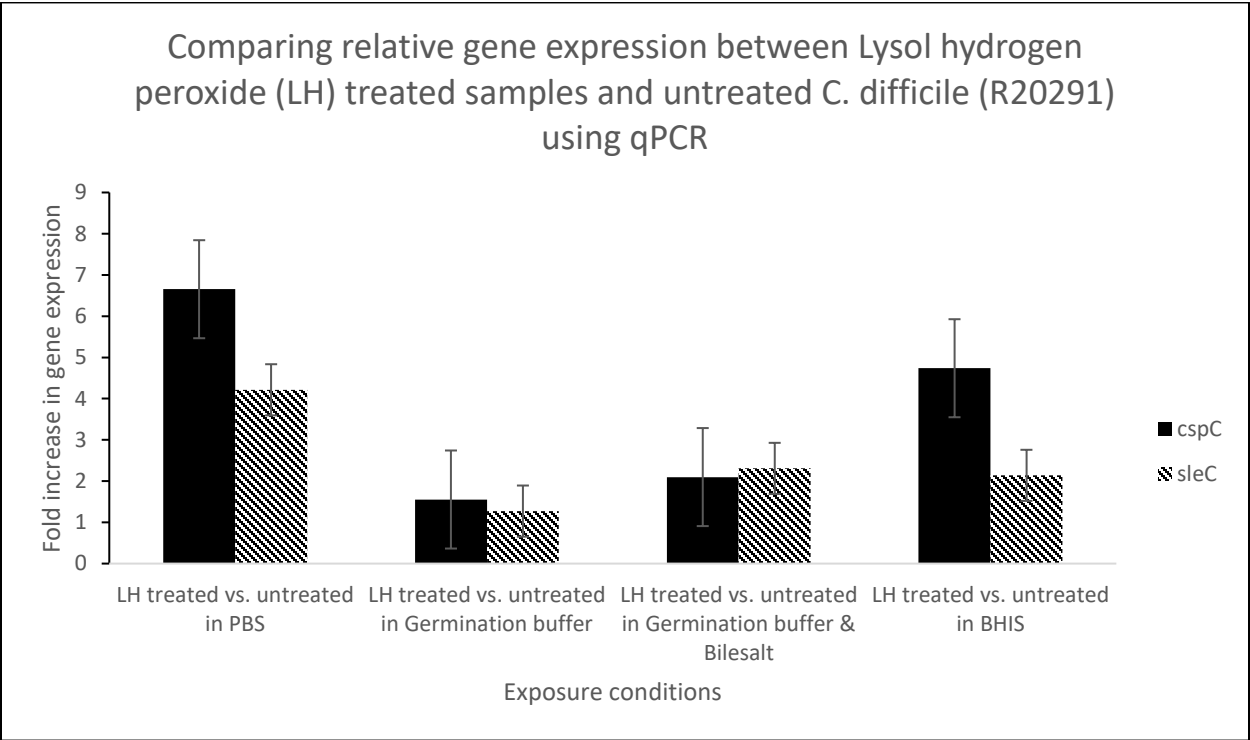
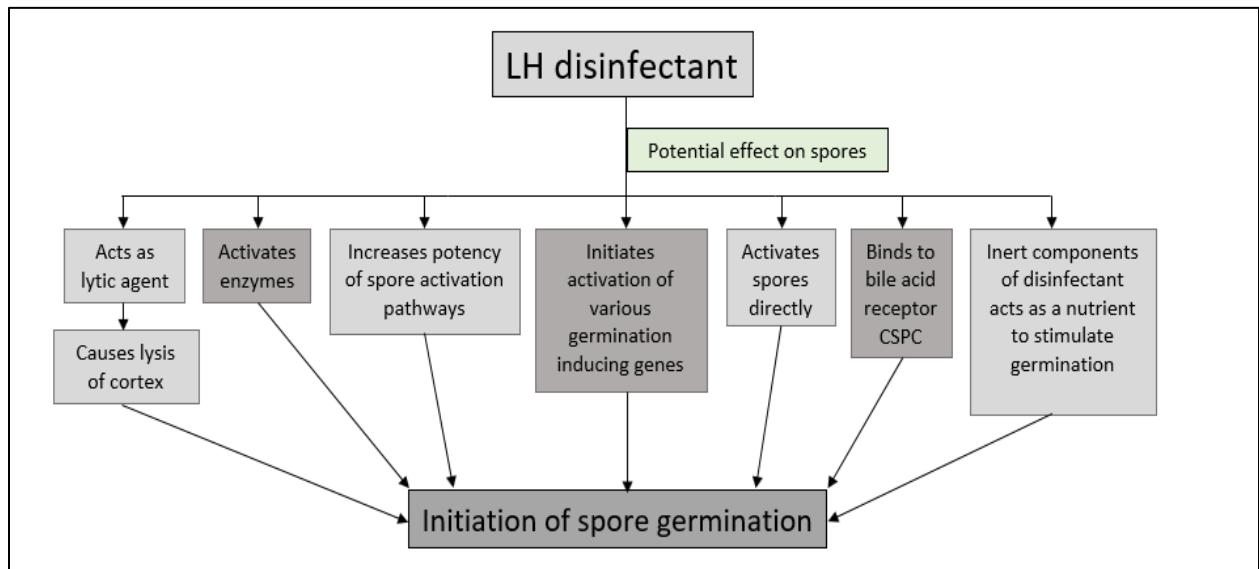
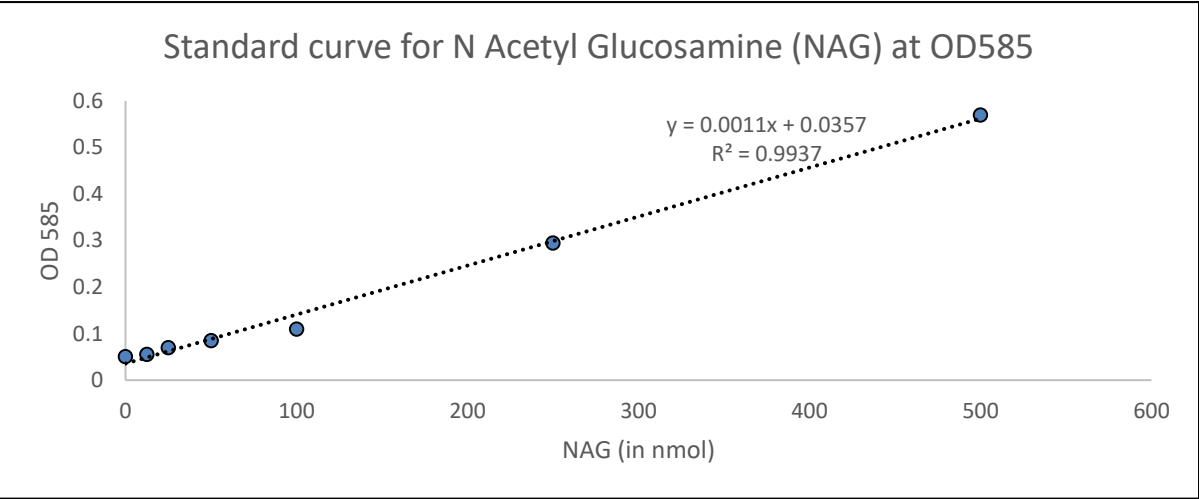


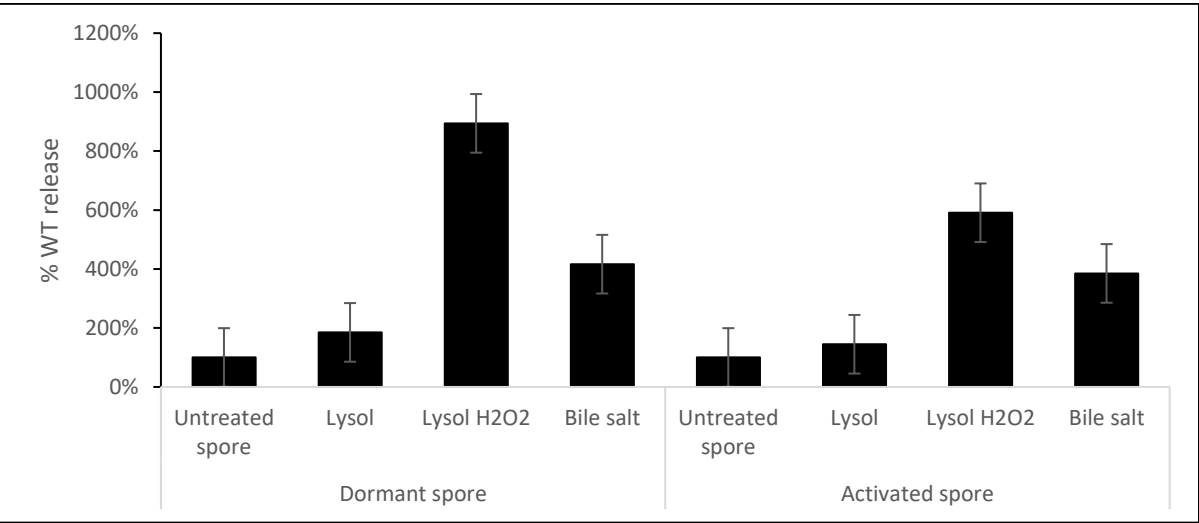
Figure C. 11: Potential effect of Lysol hydrogen peroxide disinfectant on the initiation of *C. difficile* spore germination



Supplemental figure C. 1: Standard curve for Cortex fragmentation assay



Supplemental figure C. 2: Percent total weight release of Ca-DPA compare to untreated spores over the study period



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CONCLUSION

This is one of the most comprehensive studies till date looking at the efficacy of both hospital and community disinfectants on planktonic *C. difficile* spores and *C. difficile* spores encased in a mono or multiorganism biofilm. This study reported the efficacy of hospital disinfectants on *C. difficile* spores and the reduction of efficacy in the presence of an increasing concentration of organic matter and variation in contact time. Although the minimal sporicidal disinfectant concentration was higher for some epidemic ribotypes, overall no significant disinfectant resistance was found among the ribotypes tested. It was also found that not all chlorine-based disinfectants are equally effective against *C. difficile* spores. This study for the first time determined the efficacy of hospital disinfectants against *C. difficile* spore encased in a mono or multi organism biofilm. No disinfectant was able to completely eradicate *C. difficile* from a biofilm. Among the disinfectants tested Clorox, OPA and Virex were most effective against spores in a biofilm. Virex was found to increase the vegetative cell count in a biofilm. Disinfectant efficacy was significantly affected by the presence of organic matter, type of biofilm and ribotype of strains. Community disinfectants except Clorox were mostly found to be ineffective against *C. difficile* spores. This study for the first time reported the potential of Lysol hydrogen peroxide to act as a germinant for *C. difficile* spore.

This study evaluated the efficacy of seven hospitals and seven community disinfectant against 16 clinical and environmental *C. difficile* spores of six different ribotypes. Use of clinical and environmental strains gives a certain generalization to this study. In contrary to other articles, this study looked at disinfectants with a different mode of action based on a list of hospital disinfectants. Most of the disinfectants showing efficacy except Clorox is not listed as a sporicidal agent on the EPA list of hospital disinfectants effective against *C. difficile*. This study may provide evidence for the reclassification of some hospital disinfectants. This study comprehensively looked and quantified various factors affecting disinfectant efficacy. Production of different types of biofilm both aerobic and anaerobic is a unique strength of this study which helped to revalidate the concept of *C. difficile* life cycle in a biofilm and hence the high environmental contamination. This study for the first time looked at the efficacy of community disinfectants on *C. difficile* spores and created a benchmark for the same. Also, no study previously investigated the role of disinfectants as a germinant. Use of various laboratory and microscopic techniques to characterize disinfectant effect is another strength of this study. This study has certain limitations. This study tested a limited number of disinfectants, results will need to be replicated and expanded. We used a number of clinical and environmental isolates for a variety of ribotypes. However, these isolates are unique to our biobank. Ideally, an international standard needs to be developed to standardize disinfectant research among laboratories. This study used in vitro techniques to compare the killing effect of hospital-based disinfectants against *C.*

difficile embedded in biofilms. Accurate quantification of the organism within biofilms by sonication or scraping is sometimes limited by the ability to remove all the organism from the biofilm. Although our study looked at disinfectant efficacy at label determined contact time and concentration, it did not investigate any off-label use or effect of other environmental factors. This study was done on polystyrene surface and hence may not necessarily represent how cells grow on other environmental surfaces. Also, a select number of community disinfectants were chosen which might not be representative of household disinfectant use. Thus, in spite of our uniqueness, further studies are needed to validate the results of this study. There is a need to standardize disinfectant efficacy testing protocols as well as a clear guideline is required for efficacy detection. Future studies need to further elaborate on the concept of the germinant effect of certain disinfectants.

Appendix A

CDID (Clostridium difficile associated diarrhea and Disinfectant) Study

Form 1: List of household disinfectants

Q1. Did the shopper buy disinfectant?

Yes ----- [1]

No ----- [2]

Q2. Number of disinfectants bought -----

Q2. Type of disinfectant bought-

Disinfectant spray -----

Disinfectant refill -----

Disinfectant wipe -----

Disinfectant degreaser -----

Others -----

Specify-----

Q3. If disinfectant sprays or refills were bought, please list the type of disinfectant bought

DISINFECTANTS		
Disinfectant	Yes	No
Lysol		
Lysol and H2O2		
Pine sol cleaner		
Mr. Clean		
Spray nine		
Spic and Span		
Clorox		
Ajax All-purpose disinfectant cleaner		
Windex		
Cavicides		
Evirocid		
Windex		
Other Specify_____		

Appendix B

CPHS Approval letter



Committee for the Protection of Human Subjects

6410 Fannin Street, Suite 1100
Houston, Texas 77030

Tasnuva Rashid
UT-H - SPH - Epidemiology & Disease Control

December 10, 2015

HSC-SPH-15-0959 - Role of disinfectants in the eradication and propagation of *Clostridium difficile*

The above named project is determined to qualify for exempt status according to 45 CFR 46.101(b)

CATEGORY #4 : *Research, involving the collection or study of existing data, documents, records, pathological specimens, or diagnostic specimens, if these sources are publicly available or if the information is recorded by the investigator in such a manner that subjects cannot be identified directly or through identifiers linked to the subjects.*

Health Insurance Portability and Accountability Act:
Exempt from HIPAA

CHANGES: Should you choose to make any changes to the protocol that would involve the inclusion of human subjects or identified data from humans, please submit the change via iRIS to the Committee for the Protection of Human Subjects for review.

STUDY CLOSURES: Upon completion of your project, submission of a study closure report is required. The study closure report should be submitted once all data has been collected and analyzed.

Should you have any questions, please contact the Office of Research Support Committees at 713-500-7943.

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