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# The Activity of Alkaline Glutaraldehyde Against Bacterial Endospores and Select Non-Enveloped Viruses

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The Activity of Alkaline Glutaraldehyde Against Bacterial Endospores and  
Select Non-Enveloped Viruses

Justen Thalmus Despain

A thesis submitted to the faculty of  
Brigham Young University  
in partial fulfillment of the requirements for the degree of  
Master of Science

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## ABSTRACT

### The Activity of Alkaline Glutaraldehyde Against Bacterial Endospores and Select Non-Enveloped Viruses

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Alkaline glutaraldehyde (GTA) has been used as a high level chemical disinfectant and sterilant for many years and is known to kill a broad spectrum of organisms ranging from vegetative eukaryotes to bacterial endospores. Although the mechanism of sporicidal action has been studied on numerous occasions, GTA's exact mechanism(s) of action are still debated.

In addition to the uncertainty of GTA's mechanism(s) of action, GTA has also shown significant variability in the time required to kill endospores and naked viruses. A better knowledge of the lethal mechanism(s) of GTA is needed to understand this discrepancy in kill times for GTA against spores of different species. Similar trends have been observed in GTA's activity against non-enveloped viruses.

Based on previous work, one proposed major mechanism of GTA's sporicidal activity is related to the number of available primary amines located on the surface of microbes. In this study, we have compared the efficacy of GTA on spores from 5 *Bacillus* species. We have also developed a method for staining these spores with amine reactive dyes to create fluorescent profiles correlating to the abundance of free amino groups on each spore type. We also describe a method for staining non-enveloped viruses to identify exposed primary amino groups on capsid proteins that may act as targets for GTA, using amine reactive Gold nanoparticles.

We found that GTA 6-Log<sub>10</sub> reduction times for various spore types varied at both the batch and species level. Spore coat thickness and fluorescence were useful tools in predicting the susceptibility of spores to GTA. Amine reactive gold particles (AuNPs) also proved useful in identifying virus susceptibility to GTA. Ultimately, more reliable disinfection testing methods are needed, and caution should be used when trying to extrapolate data generated from surrogate organisms to other species.

Keywords: endospore, glutaraldehyde, Alexa Fluor, poliovirus, human papilloma virus

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## Introduction

Disinfection of surfaces and materials is a subject that has been studied extensively in an effort to provide safe and effective inactivation of infectious organisms on everything from children's toys to precision surgical instruments. Yet, regardless of test standardization efforts and optimal selection of surrogate test organisms, there is still a lot of variability seen in both the time required to kill organisms, and the spectrum of organisms that are susceptible to a given disinfectant. Bacterial endospores and non-enveloped (naked) viruses have received particular attention, as their destruction is often the most difficult. Therefore, these agents are used in testing disinfectants purported to be used as sterilants for sensitive medical devices. These two classes of microbes are also responsible for a number of clinically relevant diseases. This study aims to bring better understanding to some of the factors involved in glutaraldehyde-based disinfection testing. To begin, we will review what is known about bacterial endospores and naked viruses and their mechanisms for resistance to chemical disinfectants. This will be followed by an overview of some of the current methods and regulations for testing candidate disinfectants and a review of one of the more well studied disinfectants, glutaraldehyde.

## Bacterial Endospores

### *General Characteristics and Significance*

Bacterial endospores (spores) are formed primarily by a small number of gram-positive bacteria that are members of the *Bacillus* and *Clostridium* genera. Unlike spores from other organisms such as fungi and protozoans, bacterial spores are not part of the replication cycle, rather they are a defensive mechanism designed to allow bacteria to survive extreme conditions that would otherwise destroy vegetative organisms. Spores are resistant to the lethal effects of several classes of biocides, and can persist in extreme heat and dehydration conditions for

extended periods of time. Spores are virtually ametabolic, but can convert back into a vegetative form upon sensing favorable environmental conditions (1-3). In the food and health industries, spores are considered the most resistant type of microorganisms, and are used to validate sterilization claims.

### *Sporulation and Development of Resistance*

Sporulation is a process that is typically initiated as a result of nutrient starvation, though other factors are also involved in starting this process (4). Some Clostridia species are known for their ability to create organic solvents such as acetone during fermentation. This process is known as solventogenesis and it has been linked to induction of sporulation in clostridia spores (5). Divalent cations are generally added to media to produce high yields of resistant spores, as they are required for normal sporogenesis (6). The primary molecular signals of sporogenesis are still being defined. What is known, is that upregulation of histidine sensor kinase A moves phosphate through a relay system that phosphorylates and activates SpoA, the master regulator for initiating sporulation genes (1, 3, 7). Sporulation is a highly regulated process that once begun, cannot be stopped (8, 9). Once properly formed, bacterial endospores become highly resistant to heat, radiation, chemical disinfection, desiccation and mechanical destruction (10, 11)

At the onset of nutrient deprivation, the bacterial cell is split asymmetrically creating two compartments designated the mother cell or sporangium and the forespore, both of which contain a copy of the genome. The forespore is then engulfed by the mother cell creating a double lipid membrane around the spore. At this point, the proteins required for the spore coat and cortex peptidoglycan are formed in the mother cell and attached to the spore while the core begins to replace water with calcium and dipicolinic acid. Next, the cell's metabolism begins to shut down.

Once the forespore has completed the coat and peptidoglycan layers, the mother cell will lyse and release the fully formed spore (1, 4, 12, 13). Sporulation in *Bacillus subtilis* has been thoroughly studied and used as a model for defining sporulation events in other spore-forming bacteria (10, 14).

### *Spore Coat*

There are three basic structures to bacterial endospores; the coat, cortex and core. Figure 1 shows an electron micrograph of an endospore with its various parts labeled. The coat is a thick layer of protein responsible for environmental fitness and in many cases, resistance to external stresses such as disinfection or extremes in temperature or salinity (15). Its thickness and protein composition can be modified by



Figure 1: A bacterial endospore. Exo – Exosporium; Co – coat; Cx – Cortex

changing sporulation media (4). Removal of spore coats renders spores susceptible to treatment with lysozyme, disinfectants and organic solvents (11, 16, 17). The spore coat has other functions in addition to those listed above. For example, the coat acts like a molecular sieve that can exclude large toxic molecules such as lysozyme but allow low molecular weight germinants such as D-alanine, access to receptors at the cortex (4, 15, 17). Some spore species, such as *Bacillus anthracis*, also have a loose fitting outer layer known as an exosporium in addition to their coat, although its function is not fully understood (17). The coat of *B. subtilis* is made up of

at least 70 different proteins (1) and makes up 78% of the protein weight of spores (18). Of these proteins, there are some that regulate germination such as an alanine racemase enzyme, which converts D-alanine to L-alanine. *B. subtilis* mutants for this protein have been observed to germinate early while still encapsulated in the mother cell (19). CotA is a copper-dependent laccase found in the coat of *B. subtilis*, that contributes to UV and peroxide protection by creating a pigment similar to melanin (20). The coat can also provide protection from phagocytosis by immune cells as well as protection against predation by nematodes and other predators (21, 22). Endospores generally have coats with two separate layers referred to as an inner and an outer coat (15). Coat compositions can be quite diverse between species (1). Between the coat and cortex is the outer membrane, the function of which is not well understood, though it is implicated in aiding spore formation. Its removal has little effect on the resistance or survival of the spore (23, 24).

### *Cortex*

The cortex is composed of two distinct layers of peptidoglycan. The inner most layer is called the germ cell wall and is composed of peptidoglycan similar to that of vegetative organisms (25). The outer and thicker layer has the same general structure as normal peptidoglycan with alternating N-acetyl-glucosamine (NAG) and N-acetyl-muramic acid (NAM) and peptide side chains (26, 27), however, the outer layer contains muramic- $\delta$ -lactam and significantly fewer peptides attached to NAM units. As a result, outer spore peptidoglycan is also significantly less cross-linked than the germ cell peptidoglycan (26, 28). Between the cortex and the core is a highly impermeable inner lipid membrane that repels even small nonpolar molecules (29). The lipids found in the inner membrane have extremely low mobility, but they share

structure similar to that of the vegetative organism's membranes. What exactly causes this low mobility is not known, though it likely contributes to spore resistance (24, 30).

### *Core*

The core is severely dehydrated and is made up of ribosomes, DNA, dipicolinic acid (DPA) and small acid-soluble proteins (SAPS). DPA makes up 5-15% of the spores dry weight and is involved in resistance to UV radiation and in the desiccation process of the core during sporulation (31). The replacement of water by DPA in the core is known to promote resistance to wet heat, while SASP are thought to contribute to spore resistance to dry heat, further desiccation and other forms of DNA damage (32, 33). SAPS make up 3-6 % of dry spore weight and saturate dormant spore DNA. They also have been shown to alter the conformation of DNA from the B to the A form, which contributes to UV resistance (33, 34).

### *Resistance to Biocides*

In general, compounds that are bacteriostatic will also be sporostatic at similar concentrations. However, sporicidal and bactericidal concentrations usually differ significantly (35). Spores are resistant to several bactericidal agents, and therefore are often used as the standard for testing label claims of high level disinfection and sterilization processes (36). Spore are generally resistant to alcohols, quaternary ammonium compounds, many aldehydes, phenolic compounds, and organic solvents (2, 37-39).

### *Bacillus anthracis*

*Bacillus* are a genus of Gram positive spore-forming rods. *B. anthracis* is one of the better known species in this genus. It has a number of unique biochemical properties that distinguish it from the other *Bacillus* species (40). *B. anthracis* spores are best known for their potential use as biological weapons due to their ability to cause pulmonary anthrax, a rapidly

progressing disease with a high mortality rate (41). These spores were mass produced as bioweapons during the Cold War and the organism is currently on the Centers for Disease Control's list of Tier 1 select agents (42). As such, *B. anthracis* has received a significant amount of interest and has been well studied. In addition to a protein coat, *B. anthracis* spores also have a loose fitting exosporium composed of protein, lipid, and carbohydrates. However, this exosporium has not been shown to contribute to either the spore's protection or pathogenesis (43, 44). The spore coat of *B. anthracis* is thinner than that of *B. subtilis*, though in overall size, *B. anthracis* is a mid-sized spore measuring about 1.5  $\mu\text{m}$  in length. *B. subtilis* measures about 1.07  $\mu\text{m}$  in length (10, 45).

#### *Bacillus subtilis*

*B. subtilis* is currently a model organism used to study general characteristics for most Gram positive rods and forms one of the smallest of the bacillus spores (45). The molecular mechanisms of sporulation have been heavily studied in *B. subtilis* such that most of what is currently known about endospores in relation to sporulation, dormancy, resistance and germination, has come as a result of studying *B. subtilis* (9, 14, 46). Its natural competency has made it popular for use in molecular biology (47). It is also used industrially to produce some enzymes and solvents (48). Compared to the other *Bacillus* species spores, *B. subtilis* has a very thick coat (10, 49, 50). It does not have an exosporium, though some studies have reported a top layer of the spore coat that is easily removed without removing the outer coat (4, 21, 51). *B. subtilis* has also been used as a surrogate for *B. anthracis* in testing biocides (52, 53).



### *Bacillus pumilus*

*B. pumilus* spores are currently used in testing for the microbiocidal activity of UV light and other radiation based sterilization methods (36, 54). One study performed by NASA included a strain of *B. pumilus* that was more resistant to UV than any other *Bacillus* species ever reported (55). It has also been employed as an agricultural fungicide (56). *B. pumilus* has a thinner coat than *B. subtilis* which also is comprised of relatively few coat proteins, compared to other *Bacillus* species spores (50, 57). It is not known to cause disease in humans and is primarily used as a bioindicator for radiation sterilization.

### *Bacillus atrophaeus*

*B. atrophaeus*, formerly *B. globigii*, is a Gram positive black or orange pigmented bacilli that is closely related to *B. subtilis*. The pigmentation is retained during sporulation and is thought to aid in resistance to UV radiation (58). It has been employed as a surrogate for *B. anthracis* in testing biocides and decontamination procedures of spores (59). It is currently used as a bio indicator to test dry heat and ethylene oxide sterilization methods (36). *B. atrophaeus* spores are similar in size to *B. anthracis* spores (45). It also is not known to cause disease in humans.

### *Geobacillus stearothermophilus*

Formerly *Bacillus stearothermophilus*, *G. stearothermophilus* is a thermophilic bacterium first isolated in 1920 from canned corn (60). *G. stearothermophilus* can be found in hot springs as well as in soil (61). It has been shown to grow between 45 and 75 °C, though poorly below 50 °C, and has not been observed to cause disease in humans (60). Its spores are commonly employed as biological indicators in steam sterilization processes (36). Comparatively little has been studied on these spores. The outer coat is reported to be thinner than other *Bacillus* species,

yet interestingly, *G. stearothermophilus* makes one of the largest of the *Bacillus*-related spores (45, 50, 62).

## Naked Viruses

### *General Structure and Characteristics*

Viruses can be broadly classified into two categories: naked (hydrophilic) and enveloped (lipophilic). Naked viruses include those from the following families:

*Adenoviridae*, *Papillomaviridae*,  
*Polyomaviridae*, *Reoviridae*, *Caliciviridae*,  
*Picornaviridae*, *Astroviridae*, *Hepeviridae*,  
*Parvoviridae*, and *Anelloviridae*. Naked viruses have a very simple structure consisting of a protein capsid containing a

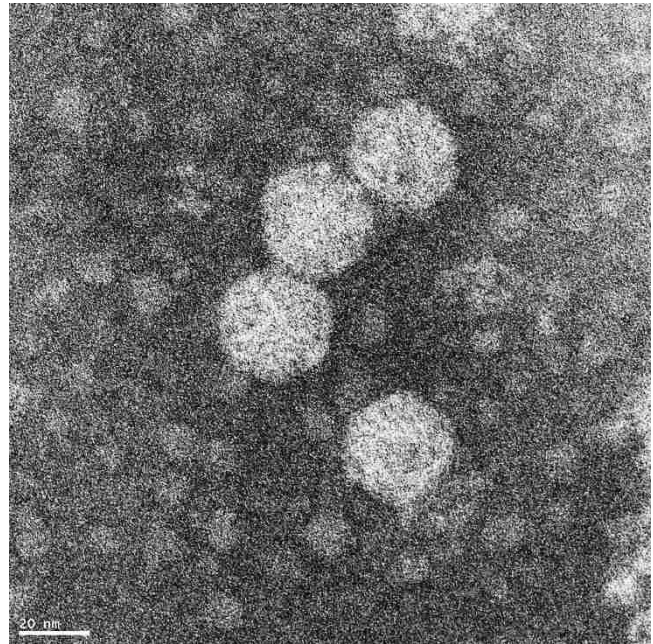


Figure 2: Poliovirus, a non-enveloped virus

DNA or RNA genome. Viruses are obligate intracellular parasites and as such, require the use of cell culture for scientific study. Many of them enter cells using receptor mediated endocytosis, then release their genome into the host cells cytoplasm for replication or transport to the nucleus. After several replication cycles, the virus progeny will typically lyse the host cell to reenter the surrounding environment, although this strategy is not the case for all of the above listed virus families. Their infection and replication strategies can vary significantly.

### *Mechanisms of Resistance*

Disinfection of viruses is somewhat different than that of vegetative organisms or even spores. The general mechanism for killing vegetative organisms with chemical biocides is by

destroying structures related to metabolism such as denaturation of protein or fixation of structures to disable function. Since viruses are ametabolic, the action of many chemical disinfectants is to inactivate them by blocking viral entry into cells or destroying the genome (63). As such, there are much fewer targets, especially on naked viruses, on which disinfectants can act. In addition, naked viruses can aggregate into bodies while replicating within cells that can act as barriers to disinfection for those virions buried deep within the aggregate (63, 64). Naked viruses are considered to be more resistant to chemical disinfectants and have greater environmental stability than enveloped viruses (65, 66). Two examples of naked viruses will be discussed below.

### *Poliovirus*

Human poliovirus is a small (~30 nm), non-enveloped, icosahedral, (+) ssRNA virus from the *Picornaviridae* family and is known for its ability to cause poliomyelitis. As an enterovirus, polio spreads via fecal-oral transmission and replicates in the gastrointestinal tract using CD155 for cell entry. Poliovirus replicates in the cytoplasm and its genome is translated into one long peptide chain that self cleaves to yield the capsid proteins comprised of VP1, VP2 and VP3, as well as various replication components including an RNA-dependent RNA polymerase and an RNA helicase. How poliovirus lyses and exits cells is not fully understood (67). There are three serotypes of poliovirus designated type 1 (Mahoney), type 2 (Lansing), and type 3 (Leon), with type one being the most prevalent and most often used in virucidal testing. In a report by the CDC, the last reported case of type 2 poliovirus was in India in 1999, while no incidences of type 3 poliovirus have been reported since November of 2012. Type 1 has still been reported in Europe and the West Pacific (68). Poliovirus displays significant resistance to biocides and is often used in virucidal disinfectant testing, since it is relatively easy to culture

(69-71). Disinfectants effective against poliovirus include hypochlorite, aldehydes, and peroxide (63). Ethyl alcohol is effective against poliovirus producing a 4 log kill in less than 3 minutes, while isopropyl alcohol seems to have little to no effect on the virus at all (72, 73). GTA shows good virucidal activity ( $> 3 \log_{10}$  reduction) after 3 minutes against poliovirus (71, 74, 75). From 1991 to 2004, a series of papers were published on GTA's effect in low concentrations on poliovirus and its capsid proteins. They concluded that location of lysine residues on VP1's surface were related to level of glutaraldehyde inactivation of poliovirus (75-77).

### *Human Papillomavirus*

Human papillomavirus (HPV) is another small (~50 nm) non-enveloped circular dsDNA virus. HPV infects keratinocytes at the base of the epidermis and as a result of infection, can create warts, and in some cases, cancer. HPV-16 and 18 are best known for their role in causing genitorectal cancers in men and women (78, 79). HPV is non-cytolytic and does not induce visible cytopathic effects in traditional cell cultures. It is difficult to culture *in vitro* since its replication cycle is tied to the differentiation state of the epidermal tissues it infects. Organotypic raft cultures were developed in the 1990's to allow HPV to be grown *in vitro* (80). Because of its difficulty to culture and lack of an infectious virus assay, there was very little known about the susceptibility of HPV to chemical disinfection until recently. Saitanu suggested in 1975 that viruses from the then *Papovaviridae* family may be more resistant, given their greater environmental stability (81). Recent developments have allowed HPV disinfections studies to be done. Viable HPV can be quantified by PCR amplification of the spliced E1<sup>^</sup>E4 mRNA produced by HPV upon successful infection (82). In 2014, our lab published the first known disinfectant susceptibility data for HPV-16. We showed that HPV-16 displays resistance to low level, broad spectrum disinfectants such as alcohols and phenols, similar to enteroviruses and

other naked viruses. HPV-16 however was found to be completely resistant to commercial GTA, even at extended contact times (82).

## Disinfection

### *Types of Disinfection and the Spaulding Classification*

There are two categories of pathogen-destroying agents: those that are used on animate surfaces (antiseptics) and those used on inanimate surfaces (disinfectants). The goal of disinfection is to remove or kill infectious pathogens from objects that could possibly transmit these organisms. Sterilization is defined as the complete absence of life. To further classify disinfectants, Earl Spaulding published a paper in 1939 on the disinfection of medical instruments in relation to how and where the instruments were used (83). He divided medical instruments into three categories; Critical, Semi-critical and Non-critical, which called for treatment by sterilants, high level-disinfectants, and low-level disinfectants, respectively.

Critical instruments are those that if used when contaminated would pose a high risk for infection. These instruments are typically used in sterile body sites such as the cardiovascular system and are therefore required to be sterilized using an autoclave at 121 °C for a recommended time. For instruments that are water or heat sensitive, liquid chemical sterilants, such as GTA were recommended as a substitute (83, 84).

Semi-critical instruments are those that contact mucus membranes or non-intact skin. This includes endoscopes, anesthesia equipment, etc., and as such, must be processed minimally using high-level disinfectants, such as hydrogen peroxide, glutaraldehyde, ortho-phthaldehyde or peracetic acid. The FDA defines a high-level disinfectant as a “sterilant used for a shorter contact time to achieve a 6-log<sub>10</sub> kill of an appropriate *Mycobacterium* species” (72).

Non-critical instruments would be things that contact intact skin, such as stethoscopes, or contact thermometers. These can be disinfected with alcohols, quaternary ammonium compounds, or halide-based disinfectants. Intermediate disinfectants have also been added as a category for the disinfection of semi-critical instruments that have contact with areas of the body such as non-intact skin or mucosal tissues (72, 83). Disinfectants can further be classified functionally as bactericidal, tuberculocidal, fungicidal, virucidal and sporicidal, to more clearly indicate the types of organisms they can kill. Organized in Table 1 are the various classifications for instruments and the levels of disinfection required for each class, as described by Spaulding and updated by the CDC.

Table 1: A simple representation of the Spaulding Classification. Information taken from: [http://www3.gehealthcare.com/en/Products/Categories/Ultrasound/~-/media/Images/Product/Product-Categories/Ultrasound/probe\\_care\\_chart.gif](http://www3.gehealthcare.com/en/Products/Categories/Ultrasound/~-/media/Images/Product/Product-Categories/Ultrasound/probe_care_chart.gif)

Classification	Definition	Level of Disinfection Required	Test Organism
Critical	Device enters otherwise sterile tissue	Sterilization	Endospores
Semi-Critical	Device contacts mucous membranes or may come in contact with unhealthy or non-intact tissue	High	Mycobacteria
Non-critical	Device contacts intact skin	Intermediate	Small Non-enveloped viruses
Non-critical	Device contacts intact skin	Low	fungi, yeasts, enveloped viruses, vegetative bacteria

### *Disinfectant Testing*

In 1881, Robert Koch published a forty-eight page study entitled “On Disinfection” which was one of the first real comprehensive studies on the ability of chemical disinfectants to destroy bacterial endospores (85, 86). A few years later in 1897, Kronig and Paul published a study in which they observed that bacteria are not all disinfected similarly, and kill rates depended heavily on factors such as contact time, concentration of the disinfectant, and

temperature of the reaction. They were also the first to use a survivors per plate count method to determine a disinfectant's biocidal activity (87). In 1903 Rideal and Walker introduced phenol as a standard for comparison of disinfectants, and standardized methods such as growth conditions, media and specific bacteria for use in disinfectant testing. This would lay the foundation for the standardized methods for testing chemical disinfectants used today (86, 88). Virucidal testing would come much later with the development of cell culture methods.

Currently there are several standardized methods available to test the efficacy of disinfectants in different scenarios and against different types of organisms (86). AOAC International and the American Society for Testing and Materials (ASTM) have published many of these methods, several of which are employed by government agencies for validating the label claims of commercial disinfectants.

There are three general kinds of disinfection tests: suspension, carrier and in-use or field tests. Suspension tests are useful for determining the potency of a disinfectant against the organism of interest while suspended in a solution such as physiological saline. At predetermined contact times, aliquots of a disinfect/organism suspension are removed and sub-cultured for survivor counts. The conditions of the test can be manipulated in many ways for a variety of possible scenarios, such as the addition of protein to simulate a bioburden. These tests, however, are limited in their ability to accurately predict kill times in more realistic situations and as such, represent an ideal scenario for the disinfectant. They are often used as preliminary tests (63). The ASTM method E231-03 is an example of this type of test (86).

Carrier tests employ the use of a small smooth surfaced object that is inoculated with the test organism and dried, in an attempt to better simulate disinfection of a contaminated hard surface. The carrier is then treated with disinfectant for predetermined contact times. The

disinfectant on the carrier is then neutralized either by dilution or by a neutralizing solution, after which it is placed in recovery medium and incubated for several days. Positive cultures indicate the failure of the disinfectant to kill all of the intended organisms. Although they better simulate disinfection in real-life scenarios, carrier tests are prone to inconsistency, due to a number of factors including carrier condition, organism preparation, neutralizing method, etc. (86, 89). This topic will be discussed further later.

In-use tests are usually an extension of, or are used to validate the results of the two previous types of tests. These types of tests are usually the application of the disinfectant being tested in a real-life setting. In-use tests involve disinfecting a test instrument using the protocol designed for it, then washing or swabbing for viable survivors. Generally, these tests aim for complete kill after the required contact time (86).

#### *Approval of Chemicals used in the Reprocessing of Medical Instruments*

The United States Food and Drug Administration (FDA) is the regulating body for claims involving liquid chemical sterilants used on reusable medical instruments (84). The regimen of tests required to qualify an agent as a liquid sterilant is quite rigorous and expensive. The manufacturer must provide documentation of testing for potency of the proposed sterilant using AOAC Official Test Methods 966.04 (sporicidal carrier test), 965.12 (tuberculocidal carrier test), 6.2.01:1995 (Salmonellicidal, Staphylocococidal and Pseudomonacidal), 964.02, and the EPA approved virucidal test DIS/TSS-7. In addition to these tests, the manufacturer must provide evidence of a simulated-use test proving that a Sterility Assurance Level (SAL) of 6 can be achieved by the disinfectant. The SAL refers to the time require to kill a certain quantity of organisms, in this case 6 logarithms or  $10^6$  spores. The FDA requires the use of *Bacillus subtilis*



spores for this test. Finally the manufacture must provide confirmatory results to the simulated-use test with an in-use test (84).

#### *AOAC Sporocidal Test Method*

For liquid chemical sterilization and sporicidal disinfection in clinical settings not associated with the use of medical instruments, the United States Environmental Protection Agency (EPA) is the regulating body. They recommend the use of AOAC method 966.04 for testing disinfectant efficacy and require the use of two kinds of carriers with spore concentrations of  $\sim 10^6$  CFU per carrier.

AOAC Method 966.04 is a carrier style test intended for liquid and gaseous chemical disinfectants. It employs the use of spores from *Bacillus subtilis* and *Clostridium sporogenes*, which are dried onto either porcelain penicylinders or silk suture loop carriers. These are then submersed in the test disinfectant for the recommended contact time. The carrier is then removed from the disinfectant and added to nutritive broth which is incubated for 21 days. If no growth is observed, the tubes are heat shocked and incubated for an additional 72 hours. If no growth is observed after these incubations, the culture is reported as negative. In order to be considered sporicidal, a disinfectant must effect at least 59 of 60 replicates as negative. In order to be considered a sterilant, no cultures can test positive after the given incubation period (90). For disinfection claims against *Clostridium difficile* spores, manufactures must test *C. difficile* spores on carriers as well (84).

#### *ASTM Virucidal Test Method*

In general, virucial testing is used to indicate intermediate level disinfection, although this is not a hard and fast rule. For example, poliovirus and some other small naked viruses are not readily inactivated by isopropyl alcohol, which is often considered an intermediate level

disinfectant and easily kills most vegetative bacteria (73). Virucidal testing requires  $>10^4$  viable virus particles per carrier with an effective exposure time less than or equal to 10 minutes. The EPA recommends ASTM 1053-11 for validating virucidal efficacy claims for disinfectants, as well as testing with the intended target virus to be listed on the label of the disinfectant (63, 91, 92).

ASTM Method E1053-11 is also a carrier style test intended for testing liquid or aerosolized disinfectants. This method involves drying virus as a film onto a carrier, then incubating with the test disinfectant. At the appropriate contact time the virus is diluted in buffer or neutralizing buffer and then assayed for infectivity in appropriate cell cultures. This method recommends that at least one non-enveloped virus in addition to the virus of interest is tested to demonstrate broad virucidal activity. Non-enveloped viruses are typically used for this method since they exhibit greater resistance to destruction than enveloped viruses. This test is also more difficult to perform than most sporicidal or bactericidal tests since the use of cell culture is required to count surviving viruses.

#### *Test Method Associated Discrepancies in Disinfection Data*

As described above, the FDA specifies a number of AOAC and ASTM methods for the validation of disinfectant claims. In the last 40 years, there have been numerous reports of inconsistencies in the accuracy and reproducibility of these test methods, especially amongst carrier tests (93-99). Many of the discrepancies studied have been found to be a result of the factors associated with the test method; i.e. carrier washing methods, recovery media, neutralization methods, and so forth. In 1990, Cole *et al.* showed that the neutralizing solution in the approved AOAC method for testing tuberculoicidal activity was bacteriostatic, and therefore made results from that test unreliable (93). The AOAC sporicidal test used to validate liquid

sterilant and high level disinfectant claims has also been criticized as being inaccurate and having inconsistent results, due to test method factors (100-105). In 1995, Miner showed that homogenization and filtration of the spore suspension by various methods not specified in the test, affected the ability of glutaraldehyde to effectively disinfect spores, resulting in false positives. They also evaluated the effect of carrier history on the generation of randomly positive carriers, and showed that newer carriers tended to have more false positives as a result of cracks and pits that could protect spores from GTA disinfection (89, 105). Loyd-Evans showed in 1986 that recovery of viruses from carriers depended on the carrier type and porosity, which lead to variable results in the determination of viral infectivity after disinfectants were tested (106).

#### *Organism Associated Discrepancies*

Even when tests methods are refined and strictly controlled, there can still be a significant difference in disinfection results from lab to lab. Poliovirus is known to be susceptible to glutaraldehyde. However, strain related differences in disinfection susceptibility have been observed between reference strains and wild strains of poliovirus, with some of the clinical isolates proving to be more resistant than the type strains. The authors suggested that the differences in kill times for these viruses was likely due to slightly different capsid amino acid sequences. (107). Previously our lab tested the efficacy of chlorine, a quaternary ammonium compound and glutaraldehyde against 5 *Mycobacterium bovis* isolates provided by certified disinfection testing laboratories, using the EPA approved tuberculocidal method, and found there were significantly different kill times as well as phenol resistances between the isolates (108). It is reasonable to conclude as well that high titer bacterial and even viral suspensions contain enough organisms that subpopulations begin to arise that may change the kinetics of how the suspension will disinfect.

Generally the use of surrogates for target organisms is accepted if the organism of interest is difficult to culture or dangerous to work with. There are numerous criterion that may be used to select surrogate test organisms (86, 109, 110). Table 2 show a small list of target organisms and their surrogates, as well as some of the reasons for choice of the surrogate.

Table 2: Examples of Surrogates from: Criteria for Selection of Surrogates Used To Study the Fate and Control of Pathogens in the Environment (110)

Case	Target	Practical	Biological	Surrogate
Fomite survival of anthrax	<i>B. anthracis</i>	safety, cost	morphology, genetic relationship	<i>B. thuringensis</i> , <i>B. subtilis</i> , <i>B. atrophaeus</i>
Water purification	coliforms	common in environment	similarity	<i>Kelbsiella terrigena</i> , <i>E. coli</i>
	viruses	common in environment	Morphological similarity, resistance	Poliovirus, Rotavirus
	cyst forms	common in environment	resistance, functional morphology	<i>Cryptosporidium parvum</i>
	Disinfection-medical	<i>M. tuberculosis</i> viruses	safety, cost, cultivation	morphology, genetic relationship cultivation
Sterilization-food	spoilage organisms	safety, cost	resistance, cultivation	<i>Geobacillus stearothermophilus</i>
Sterilization-medical	<i>Clostridia</i> spp.	safety, cost	Genetic relationship, morphology, resistance	<i>C. sporogenes</i> , <i>B. subtilis</i>

The use of surrogate organisms to predict resistance of other organisms is another source of concern associated with these tests (82, 104, 111-113). For over 50 years, *B. atrophaeus* and *B. subtilis* have been used as surrogates in testing decontamination procedures for *B. anthracis* (86, 113). However, our lab previously showed that *B. subtilis* is almost 50 times more resistant to disinfection by CIDEX™ (2.3% alkaline glutaraldehyde) than *B. anthracis* (104). This and other studies have led to the presumption that *B. subtilis* is the most resistant of the spore forming organisms to disinfectants. However, in this same study, we also showed that when tested against SteriPlex™ (0.2% peracetic acid), *B. anthracis* was 4 times more resistant than *B.*

*subtilis* (104). Recently our lab has shown that HPV-16, a naked virus that causes genitoretal cancers, is completely resistant to glutaraldehyde and other common clinical disinfectants.

Glutaraldehyde is a common liquid sterilant used to sterilize flexible endoscopes. This is the first report of a naked virus being completely resistant to glutaraldehyde (82). From these evidences, it is plain that the use of surrogates to produce reliable generalized disinfection data, though convenient and in some cases effective, should be done with caution and the data acquired should be used to create clinical disinfection protocols with a reasonable degree of skepticism.

### Glutaraldehyde

#### *Structures in Solution*

Glutaraldehyde (GTA) is a 5 carbon dialdehyde (1,5-pentanedial) commonly used as a cross-linking agent. There have been several studies published on the predominant structure of GTA in commercial and reagent grade solutions. GTA solutions typically have varying amounts of intermediates and polymer sizes depending on pH, temperature, and concentration which can affect its reactivity as a biocide and cross-linker (114-117). GTA can be found as a monomer, dimer, trimer, polymer, cyclic hemiacetal, acetal-like polymer, and as a mono or dehydrate (116, 118). In acidic solutions, GTA is known to be in equilibrium between cyclic hemiacetals and polymerized hemiacetals (119, 120). As the pH and temperature are increased GTA will begin to undergo an aldol reaction and subsequent dehydration resulting in an  $\alpha$ - $\beta$  unsaturated polymer that will continue to grow until the length of the polymer causes it to precipitate (120, 121). Because of these myriad structures, the reactivity of GTA with other molecules can be difficult to predict. It is also relatively difficult to manufacture GTA solutions containing high amounts of free aldehyde. Figure 3 shows a number of the GTA structures that can be found in solution.

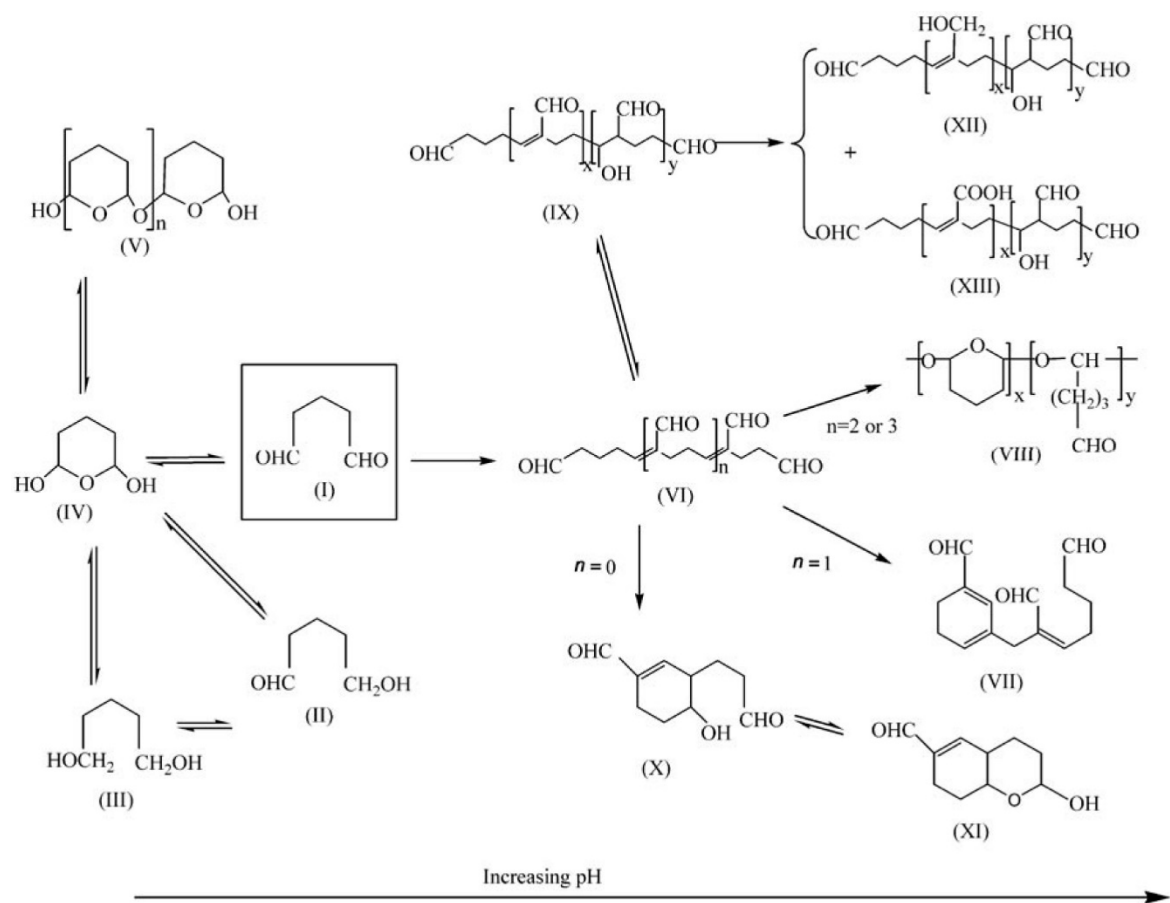


Figure 3: A representation of the many forms of aqueous glutaraldehyde and changes induced by increasing pH (116).

### *Chemistry and Reaction with Various Biomolecules*

GTA exhibits aldehyde chemistry at both ends with a variety of nucleophiles such as deprotonated amines. In this particular reaction, a primary amine will attack the carbonyl carbon of the aldehyde group in nucleophilic addition to form a carbinolamine. This will dehydrate to form an imine, the final product of the reaction (122). Figure 4 shows the general reaction of an aldehyde and an amine and the end product.



Figure 4: Reaction of glutaraldehyde with a primary amine

GTA was first employed as a tissue fixative for electron microscopy in 1963 and has since found numerous uses including chemical disinfection and leather tanning (123). GTA's reactivity with side chains of amino acids and proteins under various conditions has been studied in depth. The reactivity of GTA with glycine, serine and proline peaks at around pH 6-7 while its reactivity with lysine and arginine keeps increasing with pH (122, 124-126). Generally, aldehydes, including GTA, react with amines to produce imines via nucleophilic substitution. In addition to primary amines, GTA has also been shown to have limited ability to interact with several other functional groups on proteins, such as secondary amines, thiol, phenol, imidazole, guanidyl and hydroxyl groups (116, 127, 128). Of all these groups, previous studies suggest that the  $\epsilon$ -amine group on lysine is most likely the main functional group involved in protein crosslinking (116, 129). Woodroof proposed that GTA protein crosslinking is carried out through the reaction of free aldehyde with lysine residues on the same protein or between proteins to form a pyridinium salt similar to desmosine (130). Another study showed that GTA reacts with the  $\epsilon$ -amino groups on diaminopimelic acid, a carboxylated derivative of lysine, in *B. subtilis* peptidoglycan (131). The interaction of GTA with other vital biomolecules such as carbohydrates, lipids and nucleic acids, has not received much attention (132, 133). Studies in cell models showed that GTA would preferentially bind to nuclear protein and histones before DNA. Nucleic acid crosslinking was also shown to be temperature dependent (134). Limited RNA fixation has also been documented in poliovirus, though it is still not thought to be as likely

a candidate for GTA reactivity (75). There are lipids that contain primary amine groups that could react with GTA such as phosphatidylserine and phosphatidylethanolamine, though again, there is little data to support that it plays any major role in GTA's ability to kill microbes (116, 127).

### *Sporicidal Activity*

GTA's sporicidal activity is likely one of its most important and useful properties. It is the most effective sporicidal aldehyde known, killing spores effectively at a ~2% concentration (114, 135, 136). Studies have shown GTA to be able to kill  $10^6$  spores from both *Bacillus* and *Clostridium* species in under 3 hours, with *B. subtilis* spores proving to be one of most resistant. Spores from *B. anthracis* and *C. difficile* are typically killed in under 10 minutes (115, 137, 138). Although there are more powerful sporicidal compounds than GTA, it has some significant advantages to other chemical disinfectants. GTA is non-corrosive and has low toxicity, and retains its sporicidal activity in the presence of considerable organic matter (127, 139). Under acidic conditions, GTA can be stored almost indefinitely, until an alkalinizing agent is added for better sporicidal action (121, 140). Currently it is thought that GTA kills bacterial endospores by crosslinking proteins important in germination of the spore, though the exact location GTA acts is not well known. Crosslinking of vital proteins could inactivate spores by making them unable to transmit environmental signals favorable to spore germination. Studies have also documented that GTA does not kill by fixing DNA (23, 132, 141).

### *Virucidal Activity*

GTA is an effective virucide. In the original article describing GTA's biocidal activity, GTA was tested against an array of viruses including Poliovirus-1 and 2, Coxsackie virus, Influenza A-2 and Herpes Simplex, all of which were inactivated in less than 10 minutes (140).



Later studies showed enveloped viruses to be more susceptible to GTA than non-enveloped viruses (69). As was noted earlier, HPV-16 is the only known organism that is completely resistant to GTA disinfection.

#### *Effect of Concentration*

GTA has been tested at various concentrations in an effort to determine the optimal concentration for efficient sporicidal action. At concentrations of 1 and 2%, GTA has been shown to be more effective than a 4% formaldehyde solution. Lower concentrations (0.1%) are shown to be sporostatic. Currently commercial GTA disinfectants are sold as a 2.3% solution in alkaline or acid (115, 138, 140, 142).

#### *Effect of Temperature*

As a crosslinker, GTAs reactivity increases and decrease with temperature (114, 119, 143). Studies have also shown that at room temperature, GTA acts effectively as a sporicide and high level disinfectant, and its sporicidal activity increases with temperature (120, 144).

#### *Effect of pH*

After concentration, GTA solution pH seems to be the most significant factor in the sporicidal activity of GTA. Studies have repeatedly documented the effect of pH on the biocidal activity of GTA (137, 145, 146). Although acid GTA has been shown to be much more stable than alkaline GTA, it also exhibits slower sporicidal activity than basic solutions (142). As a result, many commercial GTA solutions use 0.3% sodium bicarbonate as an activator and buffer. As was described earlier, the addition of alkalizing agents allows GTA to self-polymerize more rapidly, leading to a loss of free aldehyde necessary for crosslinking (147). Ultimately, while GTA kills spores faster at alkaline pH, it also losses its sporicidal activity with time on the order of days and weeks. One suggested reason for acid GTA's weaker sporicidal action is the loss of

deprotonated primary amines as a result of the acidic environment. Acid GTA is also thought to not be able to penetrate as deep into spores as alkaline GTA (137).

### *Effect of Sodium Bicarbonate*

Some studies have suggested that sodium bicarbonate, in addition to being an alkalinizing agent, also acts on endospore coats by some not well understood mechanism(s) to make them more penetrable to GTA. The pH of the solution alone does not fully explain alkaline GTA's increased sporicidal activity. Power showed that the addition of NaOH as an alkalization agent does not improve GTA's activity to the same degree as bicarbonate (114, 137). Sodium bicarbonate is hypothesized to disrupt spore coats in such a way as to make them more permeable to GTA. The ability of GTA to penetrate deeper into endospores allows it to act on targets that may be more sensitive than the spore coat, such as the cortex or possibly even the core where proteins vital to germination are located. Gorman *et al.* showed using electron microscopy that when adding bicarbonate to spore suspensions, the spore coats from *B. subtilis* are less tightly associated with the spore, and show areas of decreased density when compared to non-treated spores (137). Cheung suggested that sodium bicarbonate may interact with the carbonyl groups on acidic amino acid residues in protein. This was thought to alter spore coat structure negatively, and may possibly explain the observations of Gorman. The same study showed that 0.3% sodium bicarbonate was able to inhibit spore germination (137, 148).

### *Clinical Use*

GTA is still one of the most widely used liquid sterilants for reusable medical instruments such as endoscopes, bronchoscopes, etc (149). It is recommended that endoscopy clinics soak instruments in GTA for at least 10 minutes for normal patients with an increase to 20 minutes for patients with known HIV and pulmonary TB infections (118). It has also been recommended for

use in disinfecting dental equipment, cystoscopes, food containers, hemostats and anesthetic equipment (150-152). CIDEX™ is a commonly used commercially available alkaline GTA solution. The manufacturer recommends a 45-minute soak time for high level disinfection and a 10-hour soak time for sterilization, for the processing of semi-critical and critical medical instruments, respectively. CIDEX™ is recommended for use up to 14 days after activation.

### Amine Reactive Probes

#### *Structure and Properties*

Molecular Probes™ has created a wide variety of amine reactive probes for use in flow cytometry and other fluorescent applications. These probes can be separated into three separate categories: activated esters, isothiocyanate esters, and sulfonyl chlorides. This section will focus mostly on the activated esters which includes N-hydroxysuccinimide esters and tetrafluorophenyl esters. These esters generally react with primary amines to form a stable carboxyamine in a pH-dependent reaction. Generally a pH of around 8.3 is required for good bioconjugation. Although bioconjugation with these functional groups can increase with pH, these esters also can hydrolyze in aqueous solution to form non-reactive carboxylic acids in a reaction that increases with pH. These probes show little reactivity with other functional groups associated with proteins, even with other amino acids containing amines on their side chains. They have been shown to be almost unreactive with other amine containing amino acids such as glutamine, asparagine, arginine, and histidine, as well as with amines on adenosine and guanosine bases (153).

### *N-hydroxysuccinimide and Tetrafluorophenyl Esters*

N-hydroxysuccinimide (NHS) esters are used commonly in bioconjugation of molecular probes to proteins via acylation of  $\epsilon$ -amino group on lysine residues as stated above. In addition to the reactivity listed above, NHS –esters have also been shown to have very low reactivity with hydroxyl functional groups found on amino acid side chains, and this only occurs when the probe is added in significant excess and with much longer reaction times than those typically used for bioconjugation. NHS-esters are also susceptible to hydrolysis in aqueous solution, though this side reaction is observed to be slow at a pH less than 9. The 2,4,5,6-Tetrafluorophenyl (TFP) esters have similar properties and reactivates to the NHS ester probes, but are less resistant to spontaneous hydrolysis than the NHS ester group (153-155).

### *Alexa Fluor<sup>TM</sup> Amine Reactive Dyes*

Since the discovery of green fluorescent protein in the 1960's, the use of fluorophores in science, especially in biology, has become extremely popular. Fluorophores are particularly useful in biology due their ability to be selectively excited and give good contrast in samples being studied. The Alexa Fluor<sup>TM</sup> dyes are a series of fluorophores developed by Molecular Probes Inc. They were designed to have high fluorescent yields and high photostability. They are currently sold as a wide variety of bioconjugates and commonly employed in immunology to stain cells using antibody conjugates of the dye, but they can be attached to other biomolecules as well (156-158). The Alexa Fluor 488 TFP-ester is a fluorescein derivative with an excitation maximum at 488 nm and an emission maximum at 525 nm (153). The Alexa Fluor NHS/TFP dyes have been used to label surface protein on various organisms. (159-161). In 2008, Boyana *et al.* labeled *Bacillus anthracis* spores using the Alexa Fluor 488 NHS-ester dye to visualize spores that had been phagocytosed by macrophages (161). In 2010, Zhang *et al.* described a

method for labeling live Dengue virus using a similar fluorescent dye for use in infectivity assays (159, 160). Figure 5 shows the structures of the Alexa Fluor dyes used in this study.

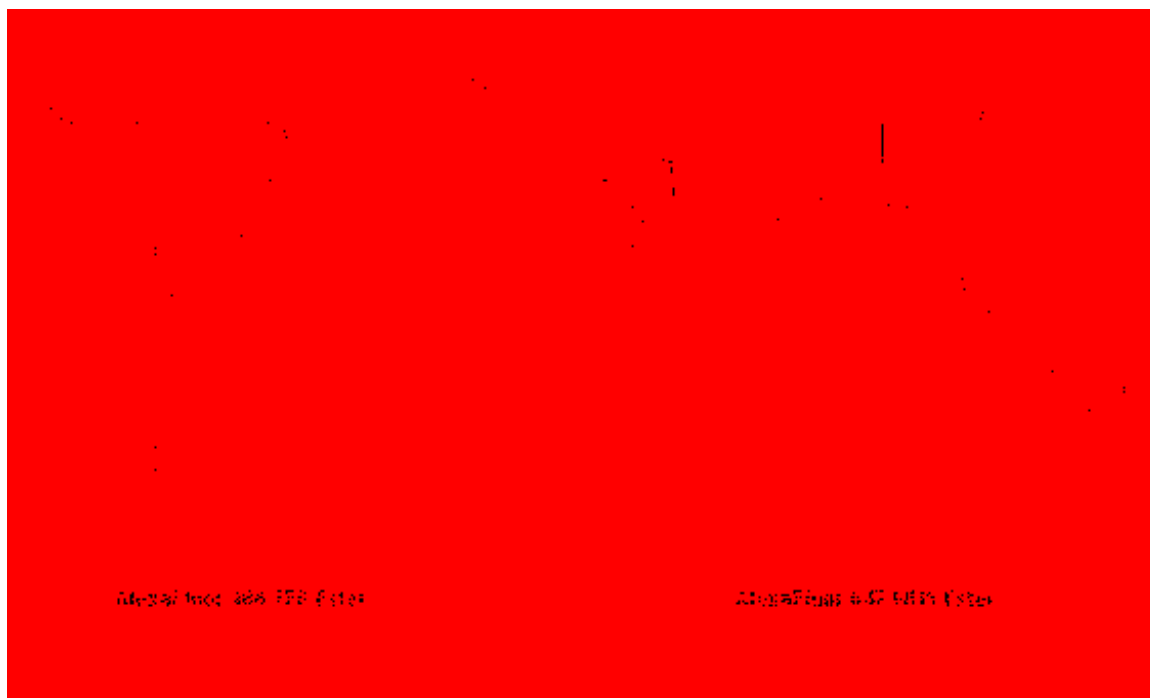


Figure 5: Structure of the Alexa Fluor dyes used in this study.

Taken from:

<http://www.thermofisher.com/content/dam/LifeTech/Documents/chemstructures/images/36236.jpg>, <http://www.lumiprobe.com/img/p/structure/sulfo-cy5-nhs-ester.gif>

### *NHS Activated Gold Nanoparticles*

Gold has had useful scientific, as well as obvious practical value since the early years of the scientific method. Gold is a Nobel Metal and as such, has several desirable properties such as being inert under standard conditions, resistant to oxidation and is non-toxic. Typically, gold colloids are produced synthetically by growing crystals from gold ions, although there has been a fair amount of research into the use of microorganisms to produce gold nanoparticles (162). The use of gold nanoparticles (AuNPs) has been gaining traction in the field of biology, as a result of the above characteristics, as well as the improved ability to functionalize AuNPs for bioconjugation, for example with NHS groups. AuNPs have also been used for years in electron

microscopy as size indicators, as well as catalysts. Current research is being done to improve the use of AuNPs in immuno-sensing using AuNP-conjugated antibodies (163, 164). Bioconjugation of proteins with AuNPs for visualization in electron microscopy is one of the most common uses of gold colloids in biology. Although there have been a number of studies published on the immune-sensing and protein conjugation properties of AuNP conjugates, not much has been done in the way of labeling organisms using the non-specific binding of NHS-activated AuNPs. One study did investigate the use of AuNP-conjugated adenovirus to carry AuNPs to tumor cells for photothermal cancer therapy (165). Figure 6 shows a representation of the linkage between a gold colloid (yellow-orange) and its NHS functional group via a polyethyleneglycol (PEG) linker arm.



Figure 6: Representation of NHS Gold nanoparticle with PEG linker.

Taken from: <http://www.cytodiagnosics.com/store/pc/catalog/NHS-Activated-Gold-Nanoparticles-Category.jpg>

## Current Study

It is apparent that the current methods used for testing disinfectants for use in clinical settings are not entirely reliable for determining an organism's resistance to a given disinfectant in real life scenarios. This comes in part as a result of a lack of reliable testing methods, and only a simple understanding of the damaging mechanism(s) of many chemical disinfectants. This, combined with the physiological variability seen among various organisms and their approved surrogates, makes predicting and extrapolating disinfection trends a difficult task. In addition, it is known that the testing and approval methods for registering disinfectants with their various label claims, are time consuming, laborious and expensive. The ability to quickly and efficiently predict kill times for various organism/disinfectant combinations would be invaluable.

Our recently published HPV-16 and HPV-18 disinfection data also demonstrates the need for alternative methods to predict disinfection susceptibility, especially for those organisms that are difficult to culture or are untestable due to logistical constraints.

The goal of this study is to investigate the relationship between endospore and non-enveloped virus resistance to GTA and the prevalence of available primary amines on the surface of these organisms. Since GTA has been shown to preferentially react with lysine residues on exposed proteins, it may be possible to quantify the relative amount of amines available to GTA using amine-reactive dyes and labels. These could be quantified on bacterial endospores, using mean fluorescence generated by the amine-reactive Alexa Fluor 488 TFP ester dye, and on viruses by quantifying binding of small, activated NHS-Gold nanoparticles.

To do this, we first studied the labeling profiles of various bacterial spores, and compared them to their corresponding 6-log reduction times when treated with GTA.

Similar experimentation was performed with non-enveloped viruses. Two small, non-enveloped viruses were used in these studies: poliovirus, known to be inactivated by GTA, and HPV, known to be resistant to GTA. Viruses were stained with 5 nm AuNPs and visualized with electron microscopy. The number of AuNPs attached to capsid proteins was evaluated for each virus. This was then compared to the known GTA susceptibilities of these viruses.

In addition to fluorescent labeling, electron microscopy was also performed on endospores to investigate the possible correlation between spore coat thickness and other factors with GTA kill times.



## Materials and Methods

### *Bacterial Strains and Spores Production*

*Bacillus anthracis* Stern 1043, *Bacillus subtilis* ATCC 19659, *Bacillus atrophaeus* ATCC 51189, and *Bacillus pumilus* ATCC 7061 were maintained on Columbia (BD Diagnostic Systems) medium and grown at 37 °C. Spore suspensions were prepared using the method of Leighton and Doi (18). Spore stock solutions were quantified by serial dilutions and viable plating. Stocks typically yielded between  $1 \times 10^8$  -  $1 \times 10^9$  CFU/ml. Suspensions were stored at 4 °C until use. *Geobacillus stearothermophilus* ATCC 12980 was maintained on Columbia medium at 55 °C. Spore suspensions were created by growing lawns of *G. stearothermophilus* on nutrient agar supplemented with  $Mg^{+2}$ ,  $Mn^{+2}$ ,  $Ca^{+2}$ ,  $K^+$  and  $Fe^{+2}$  ions at 55 °C for 8 - 10 days, until spores made up greater than 90% of the culture (19). Spores were then harvested by adding cold PSS + 0.01% Tween 80 to plates, scraping off the spores, and centrifuging at 4000 x g for 15 minutes. Washing by centrifugation was repeated 5 times and suspensions were checked for purity before storage at 4 °C until use.

### *Viruses and Viral Growth*

Poliovirus-1 Mahoney strain (PV-1) kindly provided by Dr. Dale Barnard, Utah State University, was grown, purified, and titered as previously described (166). Human Papilloma Virus 18 (HPV-18) was kindly provided by Dr. Craig Meyers at Pennsylvania State University and was stored at -80 °C until use in labeling studies.

### *Spore Labeling and Flow Cytometry*

Spores were labeled according to a modified protocol from Boyana *et al.* Suspensions were adjusted to  $1 \times 10^7$  CFU/ml, then diluted 1:10 in PSS + 0.01% Tween 80 and 0.1 M sodium bicarbonate, pH 8.3 (wash buffer) to give approximately  $10^6$  CFU/ml. Desiccated Alexa Fluor™

488 TFP-ester (AF488) dye was re-suspended in sterile DMSO to give a final concentration of 1 µg/µl. Fifteen µl of dye was added to each of the spore suspensions, which were incubated in the dark for at least 2 hours at room temperature. Unbound dye was removed by centrifuging at 14,000 x g for 30 minutes, and washing the suspensions three times in 1 ml of wash buffer. Prepared samples were kept at room temperature in the dark until use in a Blue/Red Applied Biosystems Attune Flow Cytometer (14). Since spore preparations were all greater than 90%, bright phase spore gates were made around populations that showed relatively high side scatter and low forward scatter, suggestive of spore complexity and size respectively, to reduce noise from other particulates and contaminants. Unstained spores were used as standards for gating stained spore populations. Runs were done in triplicate, and each experiment was performed six times. Raw data was processed using FlowJo™ where fluorescent means were obtained by gating on spores, then sub-gating peaks to exclude extreme outliers and unstained spores.

### *Suspension Tests*

All spore suspensions were tested against GTA using the ASTM Standard Guide for Assessment of Antimicrobial Activity using a Time-Kill Procedure, designated E2315-03. One-hundred µl of a 10<sup>9</sup> CFU/ml spore suspension was added to 9.9 ml of GTA and incubated at 20 °C for predetermined contact times. At the given contact time, 1 ml of this GTA-spore suspension was added to 9 mL of 1% glycine for at least 5 minutes, before serially diluting the suspension further to predetermined dilutions. One ml from these dilutions were then plated in triplicate onto Columbia agar using membrane filtration, and incubated at 37 °C (55 °C for *Geobacillus* spores). Colonies on each membrane were counted at 24 and 48 hours. The average number of survivors per plate were used to determine the Log<sub>10</sub> reduction in spores for each contact time. Tests were plated in triplicate and replicated three times using four contact times

per test. Time-Kill regression curves and 6-Log<sub>10</sub> reduction estimates were generated using Minitab™ from contact times and their associated Log<sub>10</sub> reductions.

#### *Glutaraldehyde*

CIDEX™ (2.3 % alkaline glutaraldehyde) was purchased from Advanced Sterilization Products™ and was activated prior to use in disinfection tests.

#### *Fluorescent Quenching Assay*

To test the ability of GTA to inhibit binding of AF488 to bacterial spores, stock spore suspensions were diluted to approximately 10<sup>7</sup> CFU/ml and 100 µl of this stock was added to 900 µl of GTA. Spores of *B. anthracis* were incubated for 5, 10, 15, and 30 minutes before 1ml of 6% glycine was added to neutralize the reaction. *B. subtilis* spores were incubated for 45, 90, 130 and 150 minute contact times. The spores were then washed 3 times at 14,000 rpm for 30 minutes and re-suspended in wash buffer to remove the glycine-GTA solution. Ten µg of AF488 was added to each tube and these suspensions were incubated at room temperature in the dark for at least 2 hours. Once staining was complete, the suspensions were washed 3 times as described above and analyzed by flow cytometry. Generated peaks were analyzed and mean fluorescence was recorded in triplicate. Each spore species was tested three times.

#### *Protection Assay*

To test the ability of AF488 to block GTA sporicidal activity, 10<sup>7</sup> spores were stained with 100 µg of Alexa Fluor 647 NHS-ester (AF647) for 2 hours. AF647 was used in the place of AF488 (TFP-ester) for this experiment because of the presence of a primary amine on AF488 that could possibly react with GTA. The Alexa Fluor NHS-esters exhibited the exact same reactivity as the TFP-esters (167). After incubation at room temperature, the stained suspensions were washed three times as described above and re-suspended in 1 ml of wash buffer. These

suspensions were then used in a modified version of the ASTM Time-Kill Suspension test. Instead of 100  $\mu$ l of this suspension, 1ml of stained spores were added to 9 ml of GTA.

#### *Spore Coat Removal*

Spore coats were removed as described earlier (137, 168). About  $10^8$  spores were suspended in de-coating solution (containing 8 M urea and 10% 1-mercaptoethanol) and incubated at 60 °C for 60 minutes. Spores were then washed three times at 10,000 x g with cold PSS and re-suspend in cold PSS with 0.01% Tween 80.

#### *Viral Labeling*

Five nanometer NHS activated Gold Nanoparticles (AuNPs) were purchased from Cytodiagnosics and used to stain  $10^7$ - $10^8$  PFU of either PV-1 or HPV-18, according to the manufacturer's instructions. Briefly, each virus was purified through a sucrose gradient and re-suspended in protein resuspension buffer (Cytodiagnosics). Sixty  $\mu$ l of virus in buffer was then added to 48  $\mu$ l of reaction buffer (Cytodiagnosics) and the mixture was added to a vial containing 0.1 mg of lyophilized AuNPs. This mixture was then shaken to homogenize and incubated at 22 °C for 2 hours. Ten  $\mu$ l of quencher solution (Cytodiagnosics) was added, then the reaction mixture was diluted in 3.9 ml of PBS (pH 7.3). The mixture was then placed in an Amicon® Ultra-4 MWCO 100K ultrafiltration membrane tube and spun at  $\sim 3000$  x g for 3 minutes, or until only 250  $\mu$ l of solution remained on top of the filter. About 3.75 ml of PBS was added to each tube and the flow through solution was discarded. This was repeated 3 times to also exchange buffers. The last centrifugation was used to concentrate the virus with AuNPs to a total volume of 250  $\mu$ l. These were then stored at 4 °C until preparation for electron microscopy.

### *Electron Microcopy*

Endospores were prepared for electron microscopy by fixing with 2% glutaraldehyde overnight. Spores were washed and then fixed with OsO<sub>4</sub> for 3 hours. After fixation, spores were embedded in 2% LMP agar and dehydrated using an acetone series. Spore-agar pellets were then soaked in 50% acetone/50% resin of Spurr for 4 hours, two times, and then 100% resin overnight. Spore pellet-resin solutions were then cured overnight at 70 °C. Thin sections were cut to a gold color and stained with 2% uranyl acetate and lead citrate. Thin sections were then attached to copper grids with formvar-carbon supports and imaged in a FEI Helios Nanolab 600 by STEM.

After viruses had been labeled with AuNPs, 20 µl of labeled virus and 2% phosphotungstic acid (PTA) were placed on parafilm. A 300 mesh copper grid coated with 3 nm amorphous carbon (TedPella) was then floated on the labeled virus for 1 minute. Excess virus was wicked off the grid with filter paper, and the grid was then placed on the PTA for 1 minute. Excess stain was also wicked off the grid and grids were allowed to dry. Grids were then imaged with a Tenaci F30 or F20 by TEM.

### *Blocking AuNP Binding PV-1 using GTA*

Prior to labeling with AuNPs as described earlier, PV-1 was incubated with 2% glutaraldehyde for 1 hour to test the ability of GTA to block AuNP binding. Preparations were then prepped for and viewed using electron microscopy as described above.

## Results

### *Comparison of Kill Curves and 6-Log Reduction Times for Different Batches of Bacillus Spores*

6-Log<sub>10</sub> reduction times were estimated by generating kill curves of 5 *Bacillus* species against GTA using viable plating by membrane filtration. Each dilution was plated in triplicate and each test was repeated 3 times. Figure 7 shows a wide range of GTA kill times amongst the spores ranging from as short as 6 minutes to up to 2.5 hours. No significant difference was seen between the slopes of spore batches.

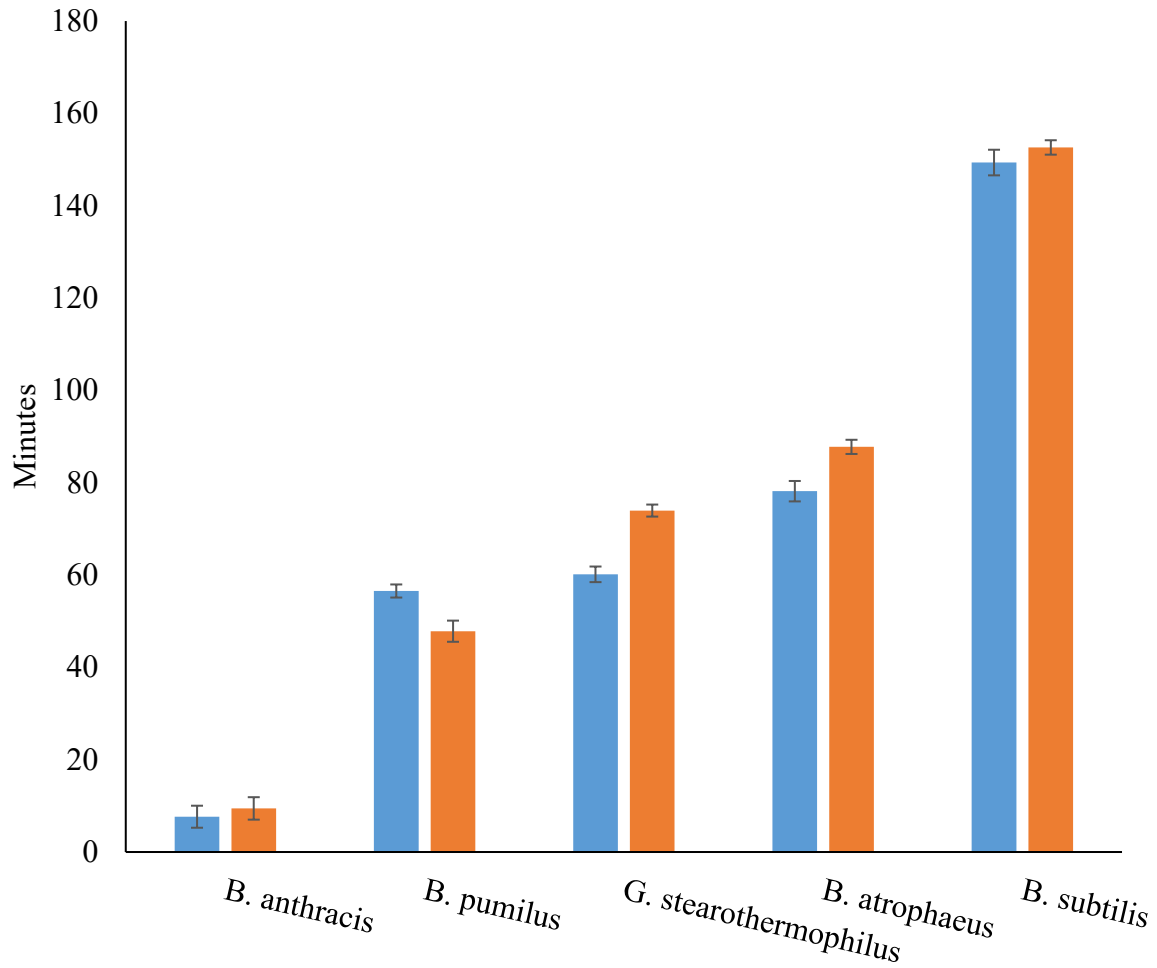


Figure 7: Comparison of Glutaraldehyde 6-Log<sub>10</sub> Reduction Estimates for 2 different batches of spores from 5 different species. Blue columns indicate estimates for spore batch 1, while the orange columns indicate estimates for spore batch 2. Each column represents the mean of 3 replications. Error bars represent  $\pm 1$  standard error.

### *Saturation of Spores with AF488*

To determine the amount of AF488 necessary to saturate all possible binding sites on spores,  $10^6$  CFU of *B. anthracis* and *B. subtilis* spores were incubated with 0.001, 0.01, 0.1, 1, 10, 20 and 50  $\mu$ l of AF488 (1  $\mu$ g / $\mu$ l) for at least 2 hours. ANOVA with Tukey HSD was used to determine statistical difference between fluorescent means. Incubation for longer than 2 hours made no significant difference in mean fluorescence. Figure 8 shows the fluorescent peaks generated from this experiment. Increasing the amount of dye from 20  $\mu$ g to 50  $\mu$ g did not result in a significant increase in mean fluorescence for either spore type. However, increasing from 10  $\mu$ g to 20  $\mu$ g of dye in *B. anthracis* and *B. subtilis* did increase the mean fluorescence significantly. The same difference was not seen in *B. subtilis*. Overall, it was determined that greater than 10  $\mu$ g of AF488 is required for complete saturation of binding sites on *B. anthracis* and *B. subtilis* spores.



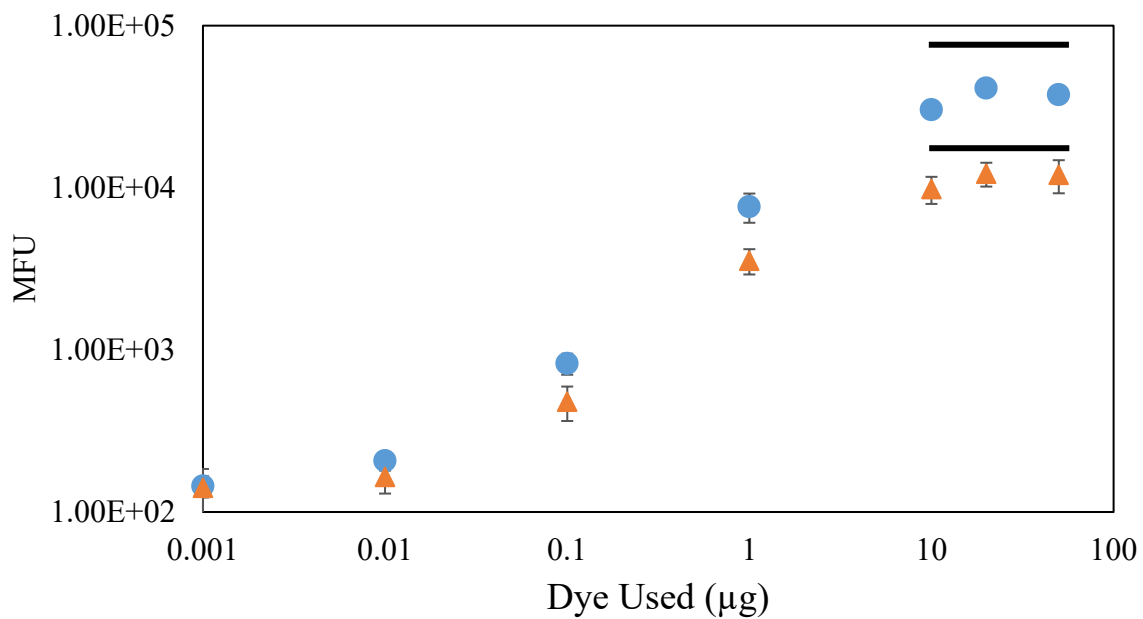


Figure 8: Mean fluorescence (MFU) of *B. anthracis* and *B. subtilis* spores after staining with different concentrations of Alexa Fluor 488. Blue circles indicate MFU for spores from *B. anthracis*, while orange triangles indicate MFU for *B. subtilis*. Each symbol represents the mean of 3 replications. Error bars represent  $\pm 1$  standard error. Horizontal bar indicates means with no statistical difference at a 95% confidence level.

### *Bacillus* Species Fluorescent Profiles

Staining *Bacillus* spores with excess AF488 yielded peak shifts relative to controls. Mean fluorescence for each peak generated by a spore was calculated. Figure 9 shows the mean fluorescence for various spores from *Bacillus* species. *B. anthracis* displayed the most intense fluorescence while *B. subtilis* displayed the least. *G. stearothermophilus* and *B. atrophaeus* displayed fluorescent profiles similar to *B. subtilis*. Figure 9 shows a peak shift for stained spores relative to controls. Statistical analysis showed that the peaks for *B. anthracis* and *B. subtilis* were different from the other spores, but that *G. stearothermophilus* and *B. atrophaeus* had means that were not significantly different from each other.

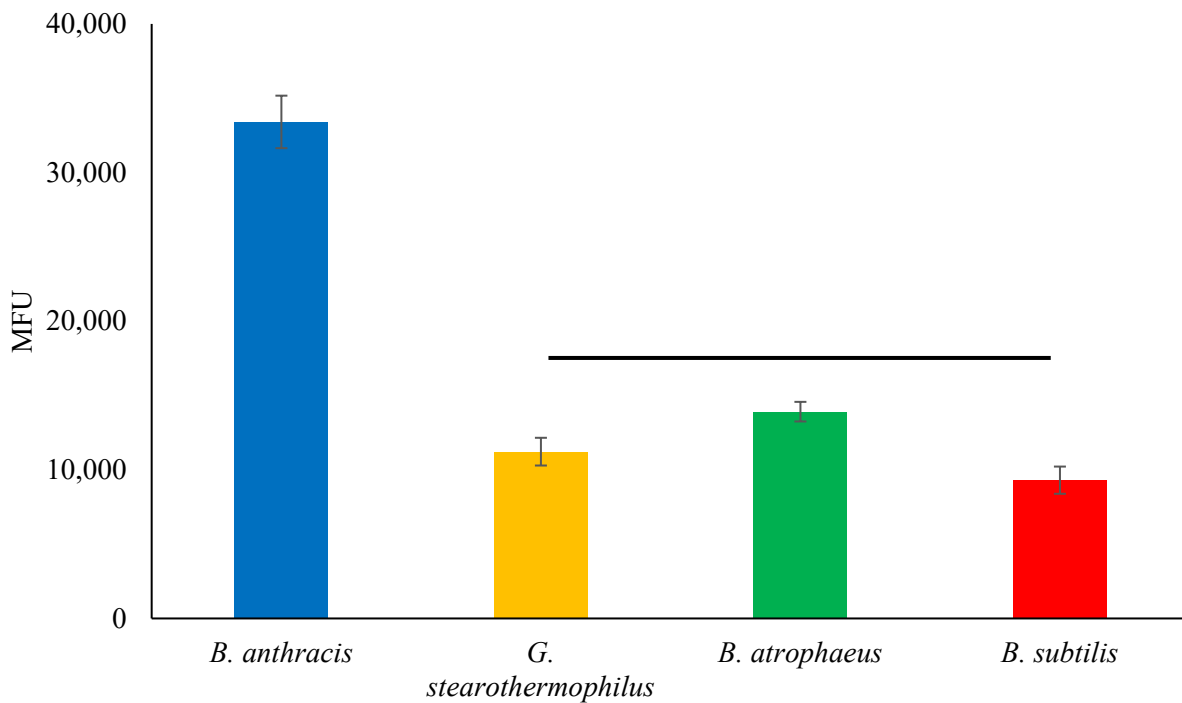


Figure 9: Fluorescent profiles of *Bacillus* species spores. Each column indicates the mean fluorescence (MFU) generated from each spore from batch 1. Each bar represents the mean of 3 replications. Error bars represent  $\pm 1$  standard error. Horizontal bars indicate means that are not statistically different from each other at a 95% confidence level.

### Correlation Between Estimated 6-Log Kill Times and Mean Fluorescent Peaks

Mean fluorescent peaks data was compared with data from Time-Kill assays to investigate a possible correlation between GTA kill times and AF488 binding to reactive groups on spores. From Figure 10, it appears that as fluorescence decreases, kill time increases, although it is not necessarily a linear relationship. ( $R^2 = 0.6187$ )

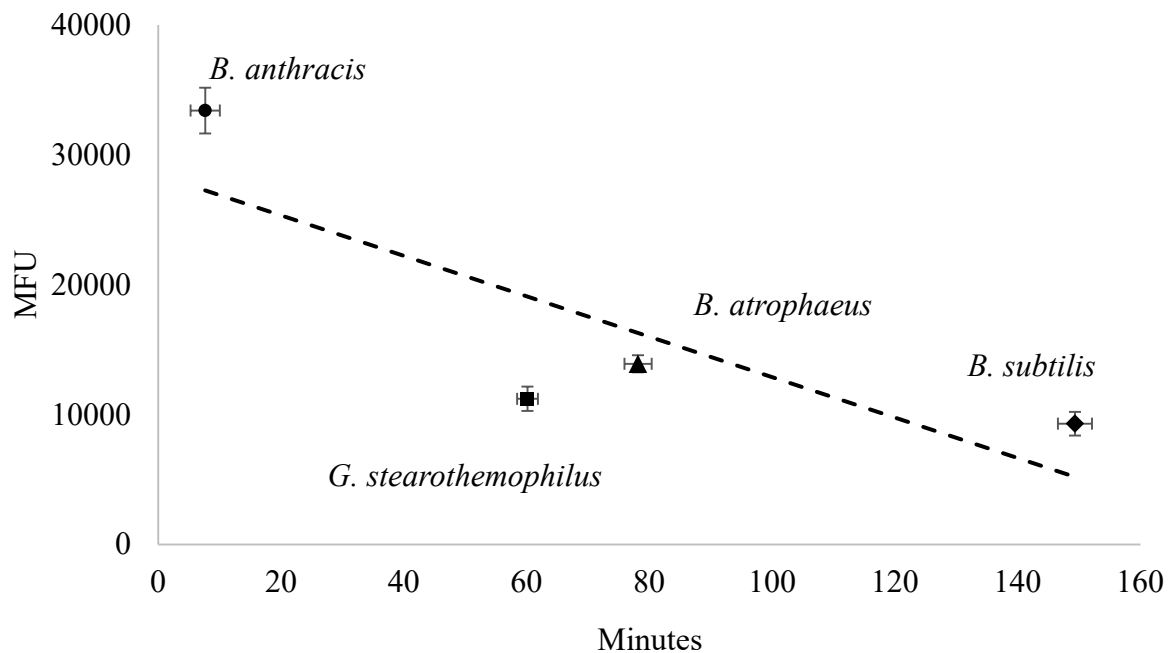


Figure 10: Mean fluorescence vs. 6-Log<sub>10</sub> reduction time estimates. Fluorescent profiles were plotted against 6-Log<sub>10</sub> reduction estimates for *B. anthracis* (circle), *G. stearothermophilus* (square), *B. atrophaeus* (triangle), and *B. subtilis* (diamond). Each symbol represents the mean of 3 replications.  $R^2 = 0.6187$

### *Inhibition of AF488 Binding by Treating Spores with GTA*

The ability of GTA to block AF488 binding to spores was tested to determine the similarity of reactive sites between GTA and AF488. Figure 11 and Figure 12 show the mean fluorescence generated by spores from *B. anthracis* and *B. subtilis* after treatment with GTA for various contact times. Relative to the untreated control, there was a 1.19 log (~94%) drop in fluorescence for *B. anthracis* after a 5 minute treatment with GTA. Fluorescence did not decrease significantly as the final contact time was reached. The same trend was observed with *B. subtilis*. After the initial contact time, mean fluorescence did not significantly decrease beyond the 0.85 log (~86%) loss of fluorescence relative to unstained spore controls.

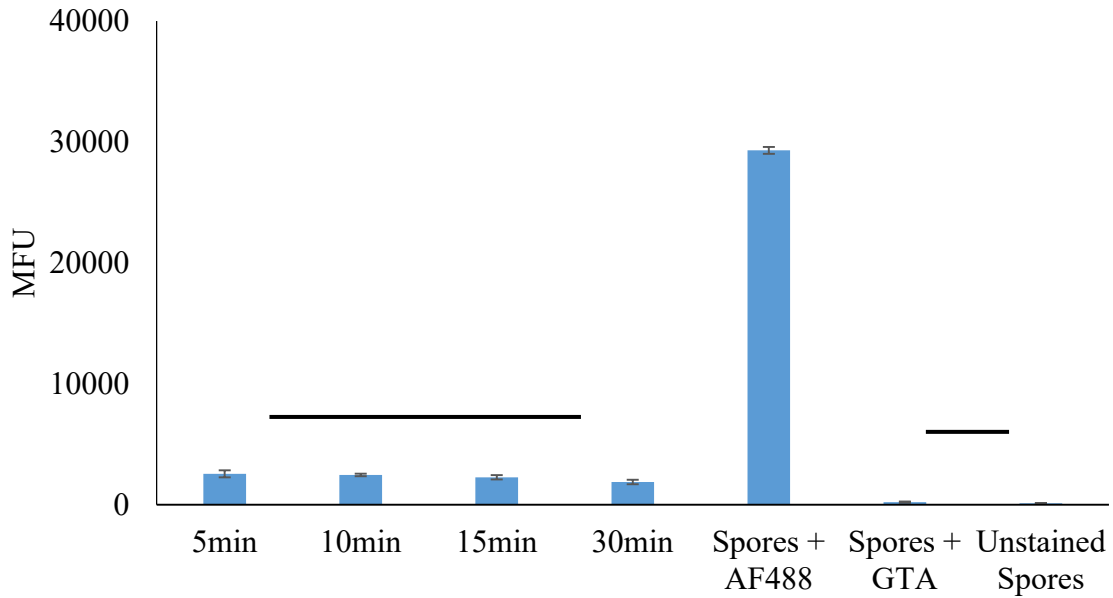


Figure 11: Reduction in fluorescence of *B. anthracis* spores when pretreated with GTA for 5 to 30 minutes prior to staining with AF488. Spores + AF488 were not pretreated with GTA. Spores + GTA were not subsequently stained with AF488. Unstained spores were included as a control. Horizontal bars indicate means that are not statistically different from each other at a 95% confidence level. Each bar represents the mean of 3 replications. Error bars represent  $\pm 1$  standard error.

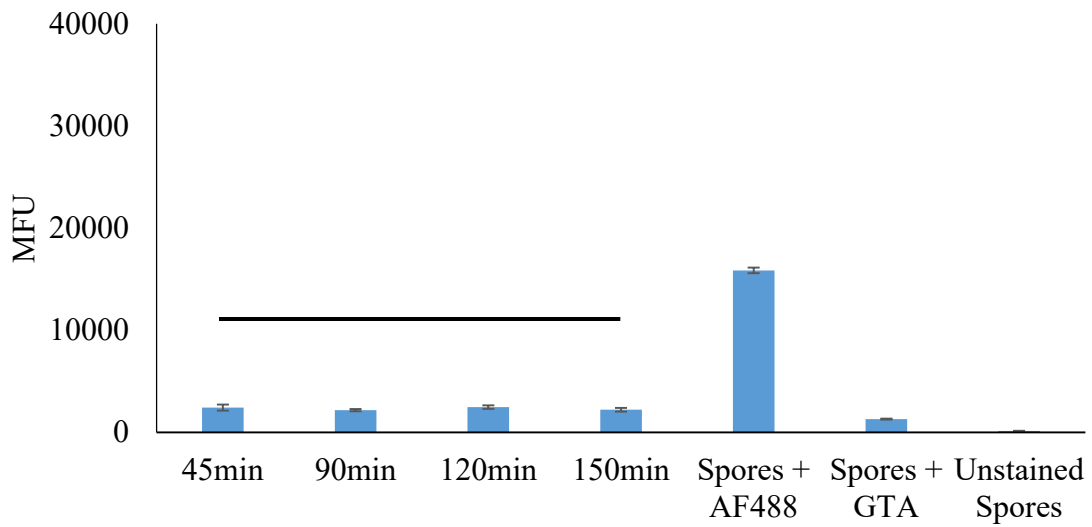


Figure 12: Reduction in fluorescence of *B. subtilis* spores when pretreated with GTA for 45 to 90 minutes prior to staining with AF488. Spores + AF488 were not pretreated with GTA. Spores + GTA were not subsequently stained with AF488. Unstained spores were included as a control. Horizontal bars indicate columns that are not statistically different from each other at a 95% confidence level. Each bar represents the mean of 3 replications. Error bars represent  $\pm 1$  standard error.

### Blocking of GTA Sporocidal Activity Using AF647

The ability of AF647 to block the sporocidal activity of GTA was investigated to further understand the similarity of reactive functional groups used by GTA and AF647. The results are displayed in Figure 13 and Figure 14. No significant difference was observed in the slopes of the kill curves for either *B. anthracis* or *B. subtilis* labeled with any of the above reagents relative to unstained controls.

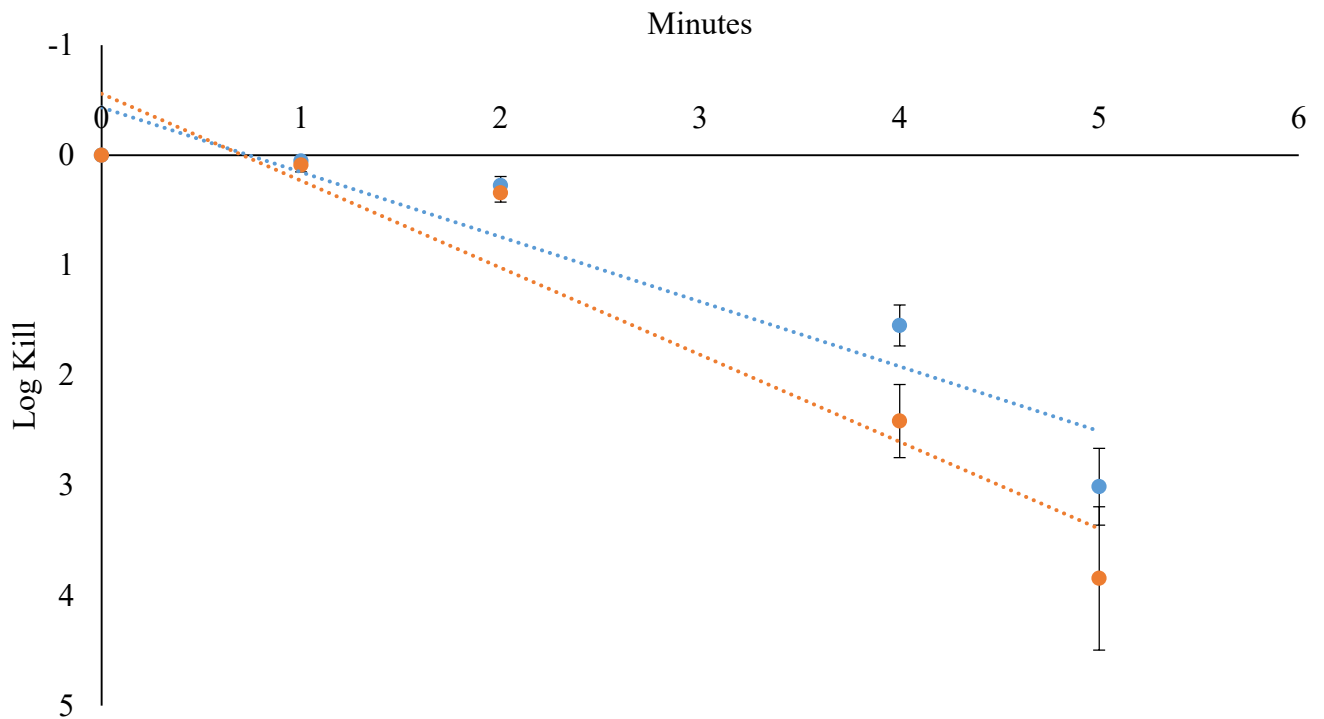


Figure 13: Log<sub>10</sub> reduction plots for *B. anthracis* spores pretreated with AF647. Blue dots indicate spores not treated with AF647 prior to tests with GTA. Orange dots indicate spores treated with AF647 prior to testing. Each symbol represents the mean of 3 replications. Error bars represent ± 1 standard error.

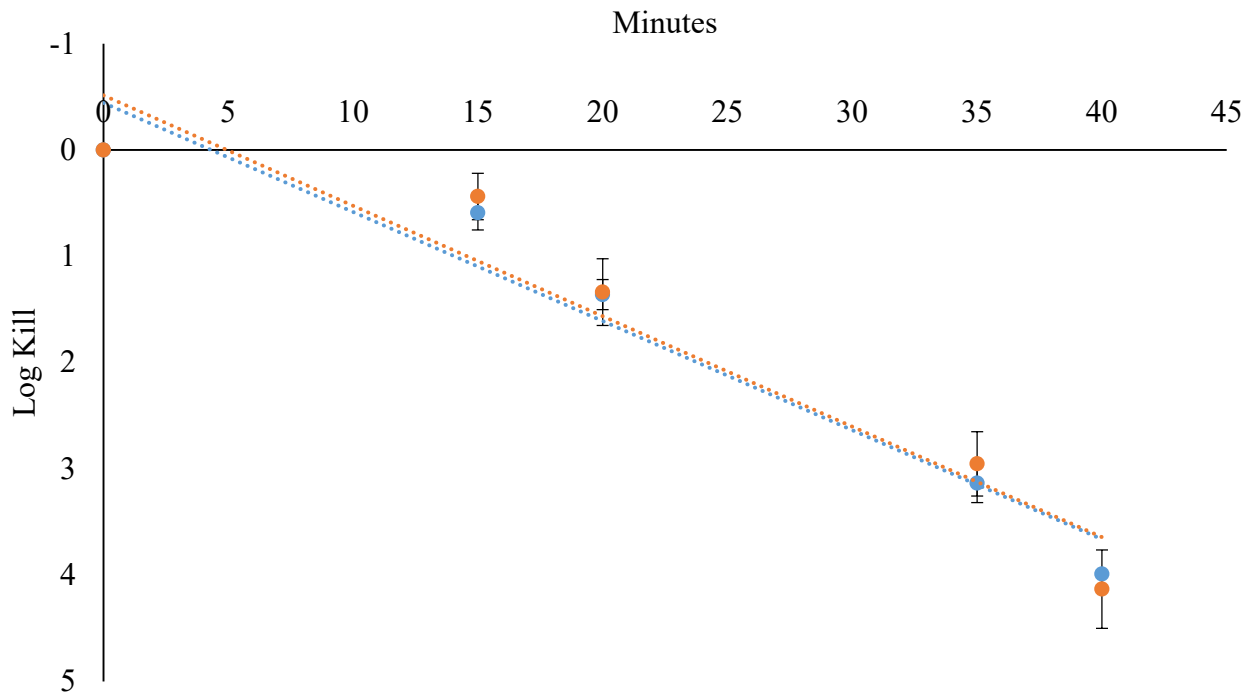


Figure 14: Log<sub>10</sub> reduction plots for *B. subtilis* spores pretreated with AF647. Blue dots indicate spores not treated with AF647 prior to tests with GTA. Orange dots indicate spores treated with AF467 prior to testing. Each symbol represents the mean of 3 replications. Error bars represent  $\pm$  1 standard error.



### *Effect of Removing Spore Coats*

Since protection was not given to spores treated with AF647, we removed the spore coats from *B. subtilis* spores to investigate the ability of AF647 to bind to the spore cortex. The results are shown in Table 3. AF647 gave no protection to spores with their coats removed.

Table 3: Effect of AF647 Staining of De-coated *B. subtilis* spores on Susceptibility to GTA

<b>Spore</b>	<b>Contact Time</b>	<b>Log<sub>10</sub> Reduction</b>
<i>B. subtilis</i>	10 min	>4.5
<i>B. subtilis</i> + AF647	10 min	>4.5
<i>B. anthracis</i>	30 sec	>4.5
<i>B. anthracis</i> + AF647	30 sec	>4.5

### Spore Coat Thickness

Figure 15 shows comparisons of the thickness of spore coats from *Bacillus* spores. Coat thickness was measured at six points on 12 spores from thin section electron micrographs using ImageJ™ imaging software. Mean coat thickness was then compared using ANOVA with Tukey HSD. *B. anthracis* and *B. pumilus* have statistically thinner protein coats ( $p < 0.05$ ) than the other tested spores, while *B. subtilis* has a thicker protein coat. *B. anthracis* also showed a much thinner coat than *B. pumilus* ( $p < 0.001$ ). Statistically, the difference between coats of *B. atropaeus*, *G. stearothermophilus* and *B. subtilis* is insignificant ( $p > 0.05$ ).

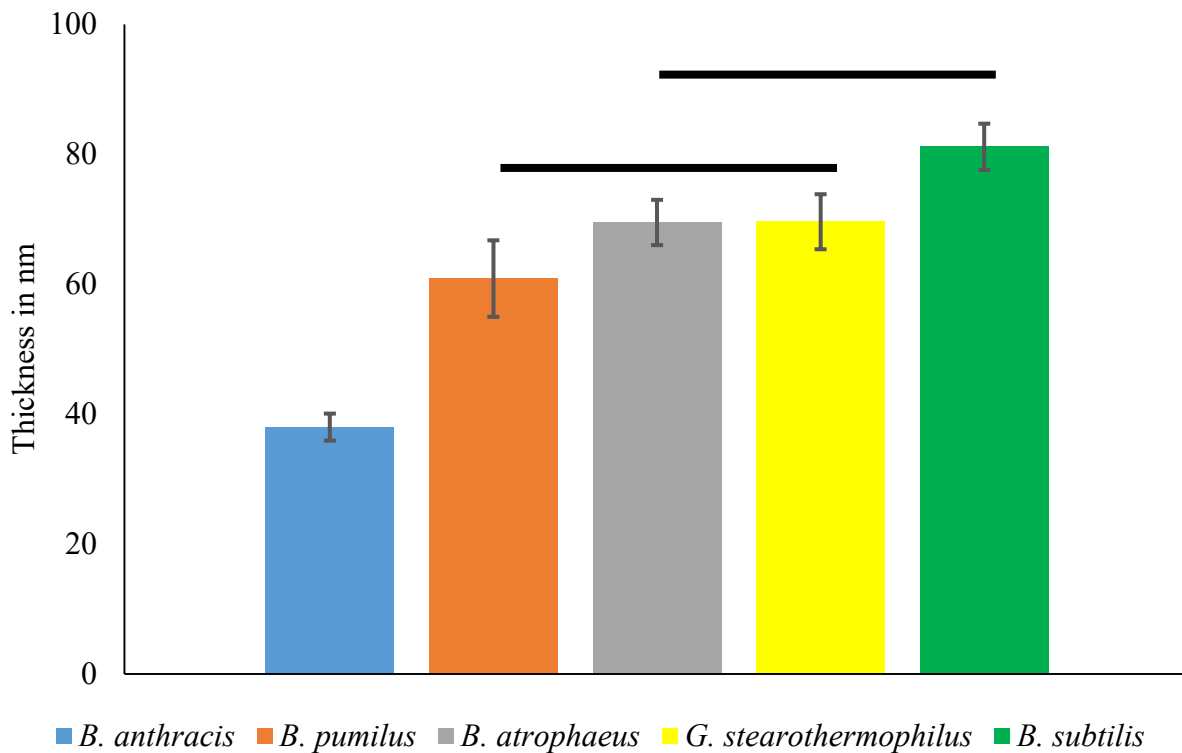


Figure 15: Comparison of protein coat thickness for spores from *B. anthracis* (blue), *B. pumilus*, (orange), *B. atropaeus* (grey), *G. stearothermophilus* (yellow) and *B. subtilis* (green). Horizontal bars indicates means that are not significantly different at a 95% confidence level. Each bar represents the mean of 12 spores. Error bars represent  $\pm 1$  standard error.

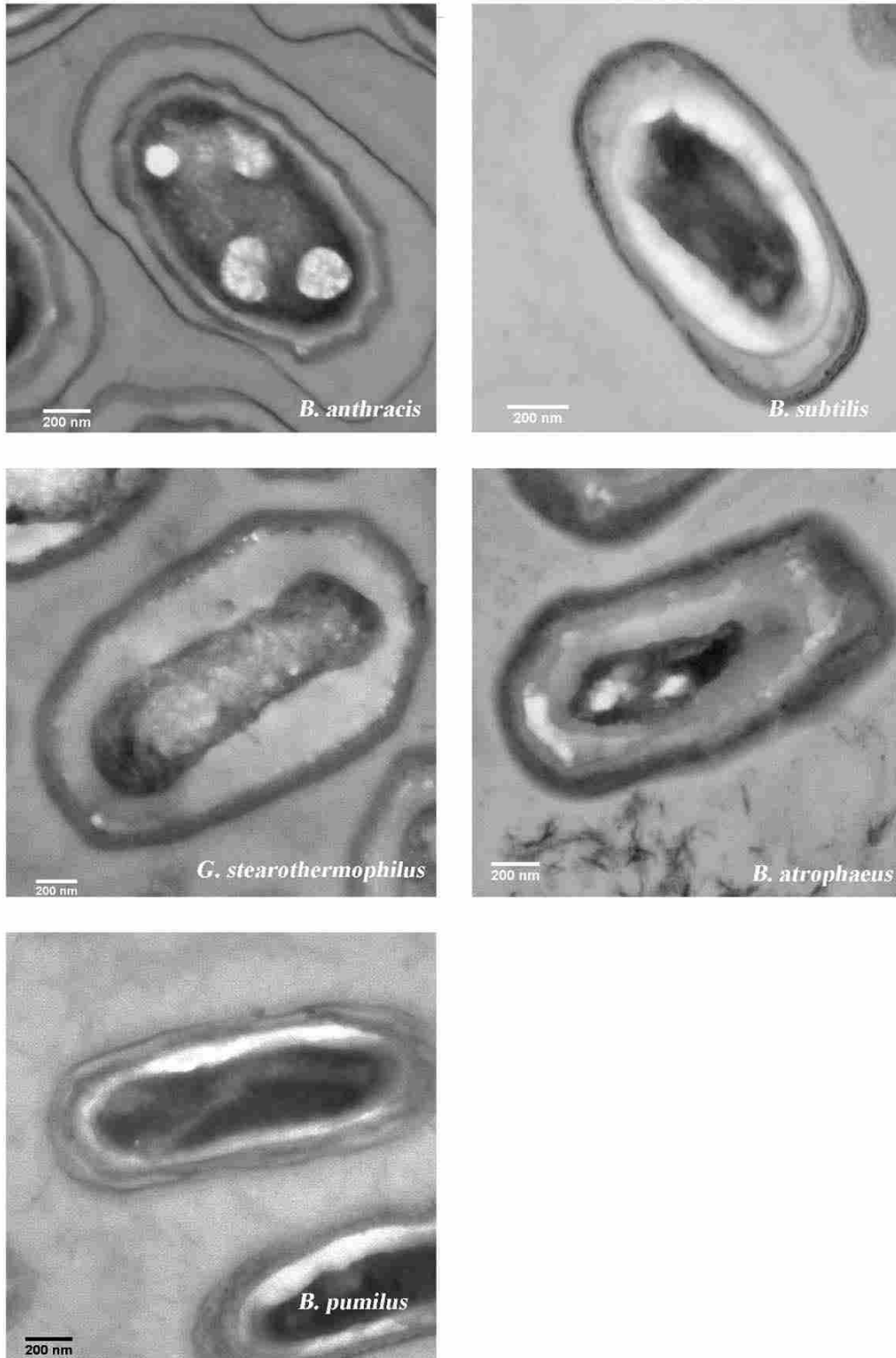


Figure 16: Electron micrographs of the different types of spores used in this study. Refer to Figure 1 for a description of the different parts of a spore. Magnification is 65,000 – 150,000x

*Protein Coat Thickness Compared to 6-Log<sub>10</sub> Reduction Estimates*

When coat thickness was plotted against mean 6-Log<sub>10</sub> reduction kill times for their respective spores, a correlation could be seen. Figure 17 shows the relationship between spore coat thickness and the 6-Log<sub>10</sub> Reduction kill times for each spore type. Generally, as spore coat thickness increased, so did the associated time required to kill the spores, although the correlation between thickness and GTA resistance is only moderate. ( $R^2 = 0.662$ )

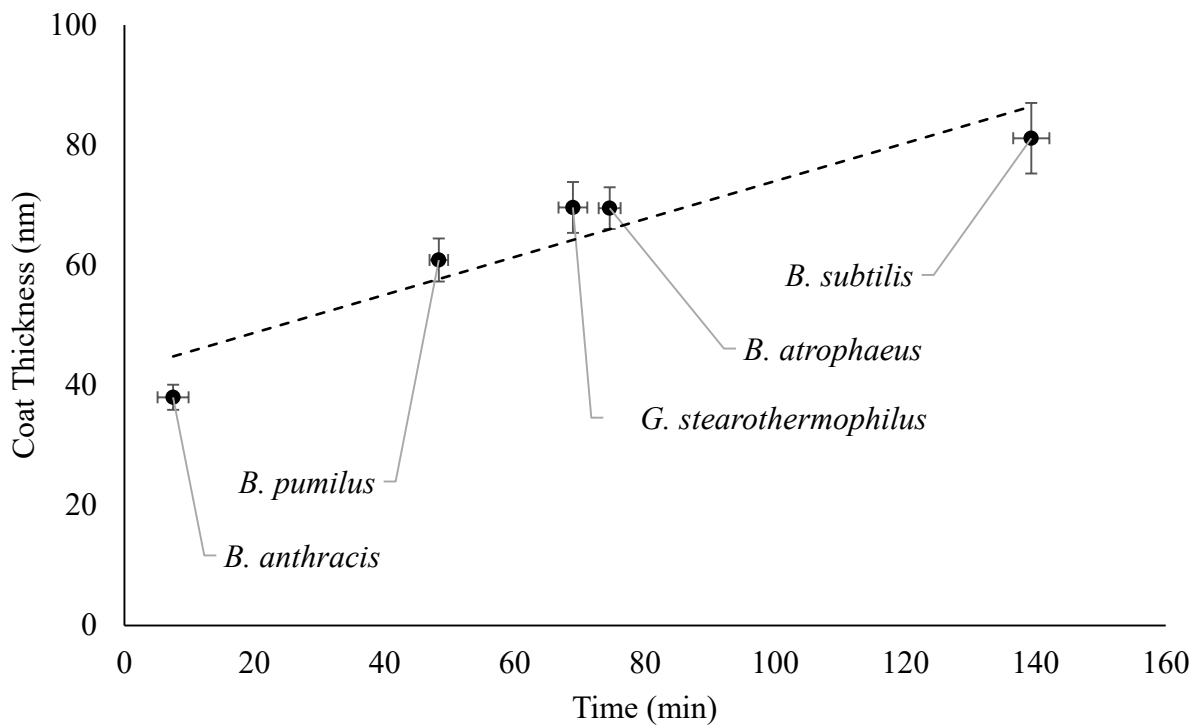


Figure 17: Spore protein coat thickness plotted against GTA 6-Log<sub>10</sub> reduction kill times for various spore species. Points on the plot indicate the average coat thickness and kill time for a given spore. Error bars represent  $\pm 1$  standard error. ( $R^2 = 0.662$ )

### *Viral Staining with Gold Nanoparticles*

Figure 18 shows electron micrographs of latex beads, PV-1, and HPV-18 before and after labeling with gold nanoparticles. Although PV-1 (Figure 18D) is not completely surrounded by AuNPs, it is associated with the AuNPs either in large clusters as seen above, or with a few nanoparticles closely associated with the virus. In the case of HPV-18 (Figure 18 F), no such association is seen. It is important to note that the NHS groups attached to the AuNPs are linked to the particles by a 5kd PEG linker arm. These linkers cause a halo effect around the particles about 5 nm in thickness. Therefore, particles within approximately 5 nm of a virus could still possibly be considered bound to the virus since the NHS functional groups are situated at the end of the PEG linker.

Figure 19 shows plot profiles for latex beads, PV-1, and HPV-18 before and after labeling with gold nanoparticles. The latex particles (B) and PV-1 bound numerous AuNPs, whereas HPV (F) bound none. Profiles were taken using ImageJ™ by selecting between 200-300 pixels in length and 20 pixels in width across a given particle.

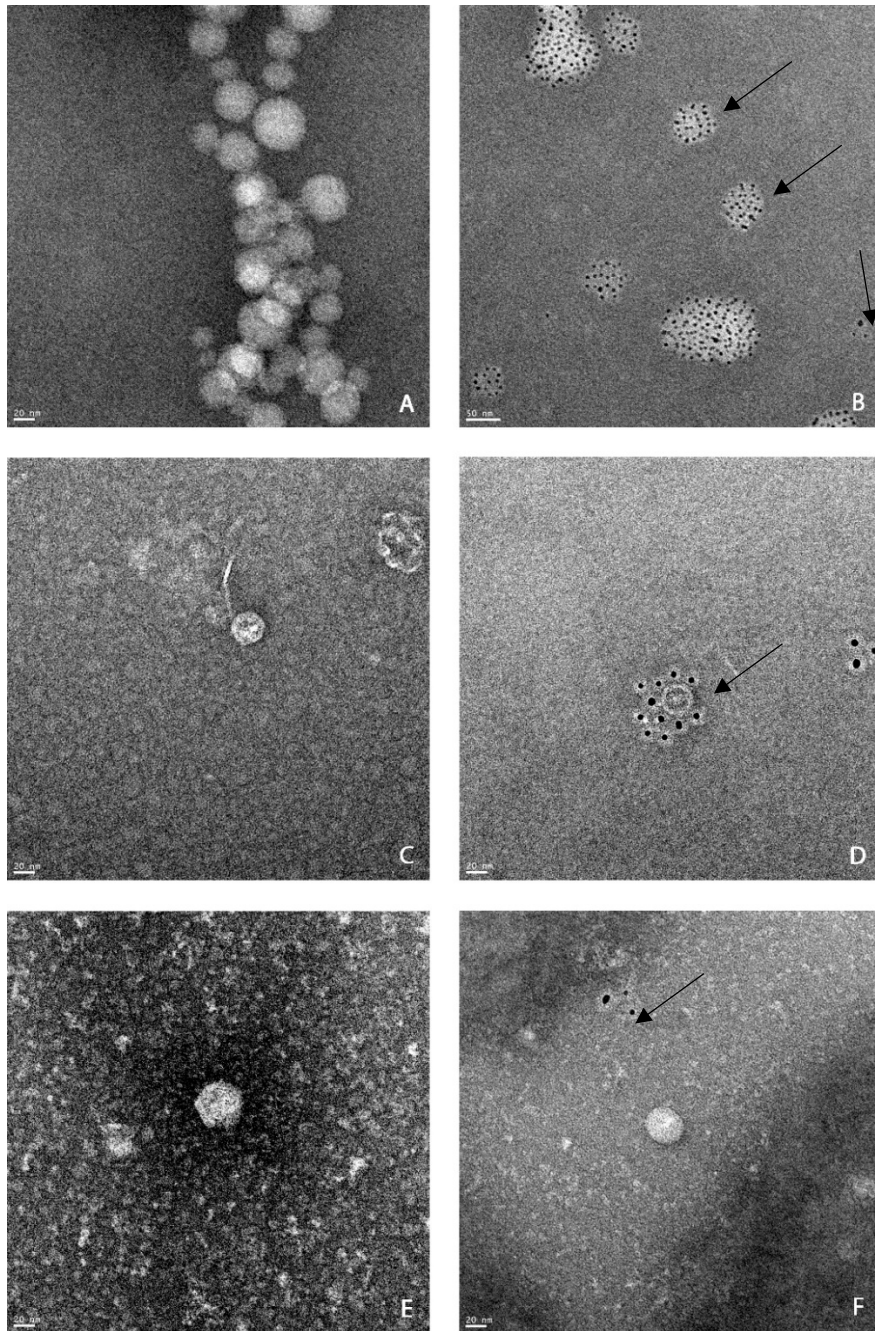


Figure 18: AuNP labeling of latex particles, Polio-1 and HPV-18. Forty nm latex beads (A) labeled with AuNPs (B) were used as controls. C and D show PV-1 before (C) and after (D) labeling with AuNPs. E and F show HPV-18 before (E) and after (F) labeling with AuNPs. Black arrows indicate AuNPs. Magnification is 43,000 – 66,000x

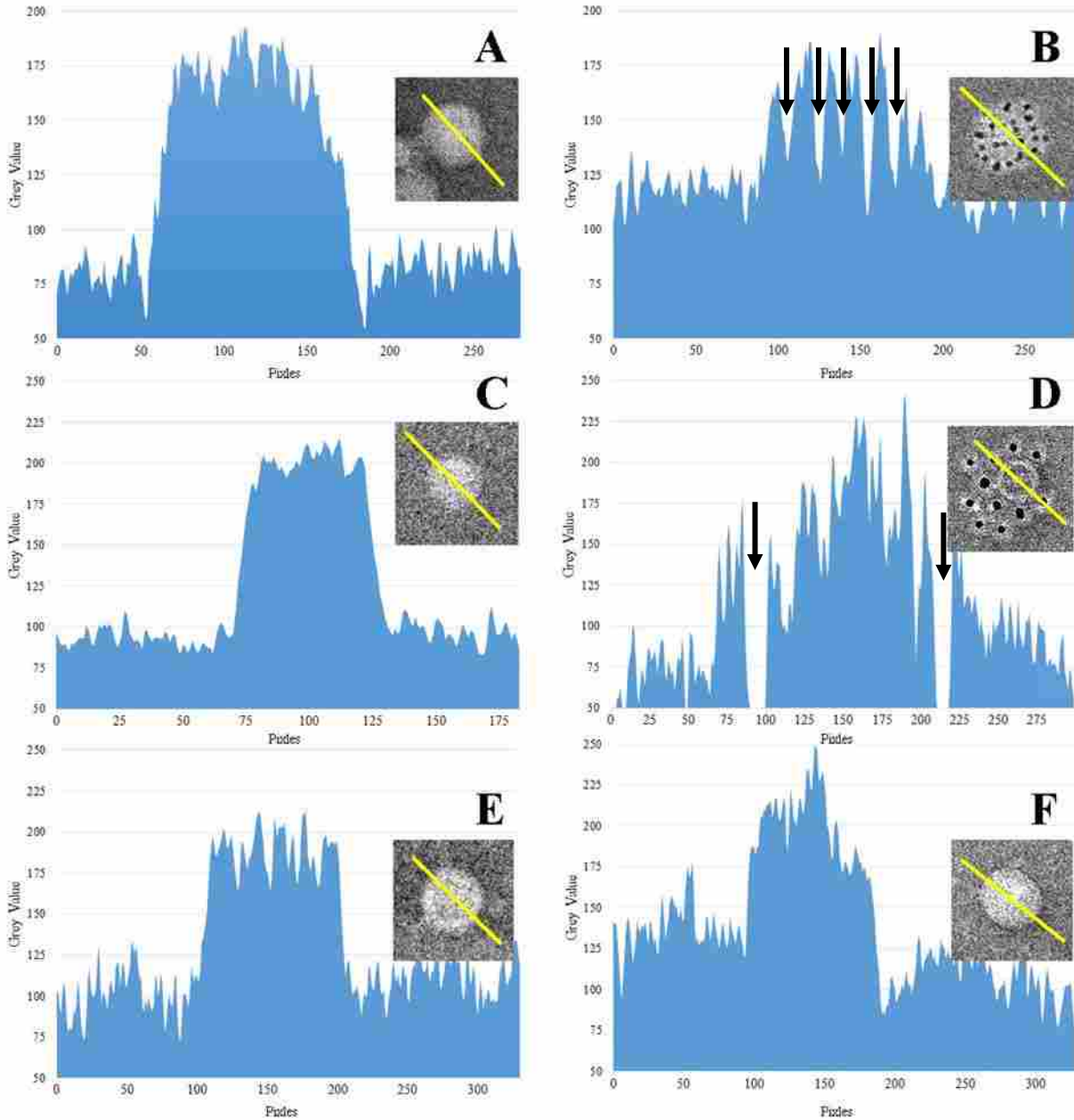


Figure 19: Intensity plot profiles for 40 nm latex particles (A and B), PV-1 (C and D) and HPV-18 (E and F) before (A, C, E) and after (B, D, F) labeling with AuNPs. Grey Value refers to the average intensity of a set of pixels across the analyzed area represented by the yellow bar. The bottom axis is the distance in pixels of the analyzed area. Arrows indicate where AuNPs are located. Insets are the images from where the plot profiles were taken.

### *Blocking AuNP Binding to PV-1 with GTA*

Figure 20 shows both untreated and GTA-treated PV-1 stained with AuNPs. Treatment of PV-1 with GTA prevented labeling with AuNPs. Plot profiles confirm the absence of AuNPs on GTA-treated PV-1. This suggests that GTA treatment disrupts amine-reactive binding sites on the virus.

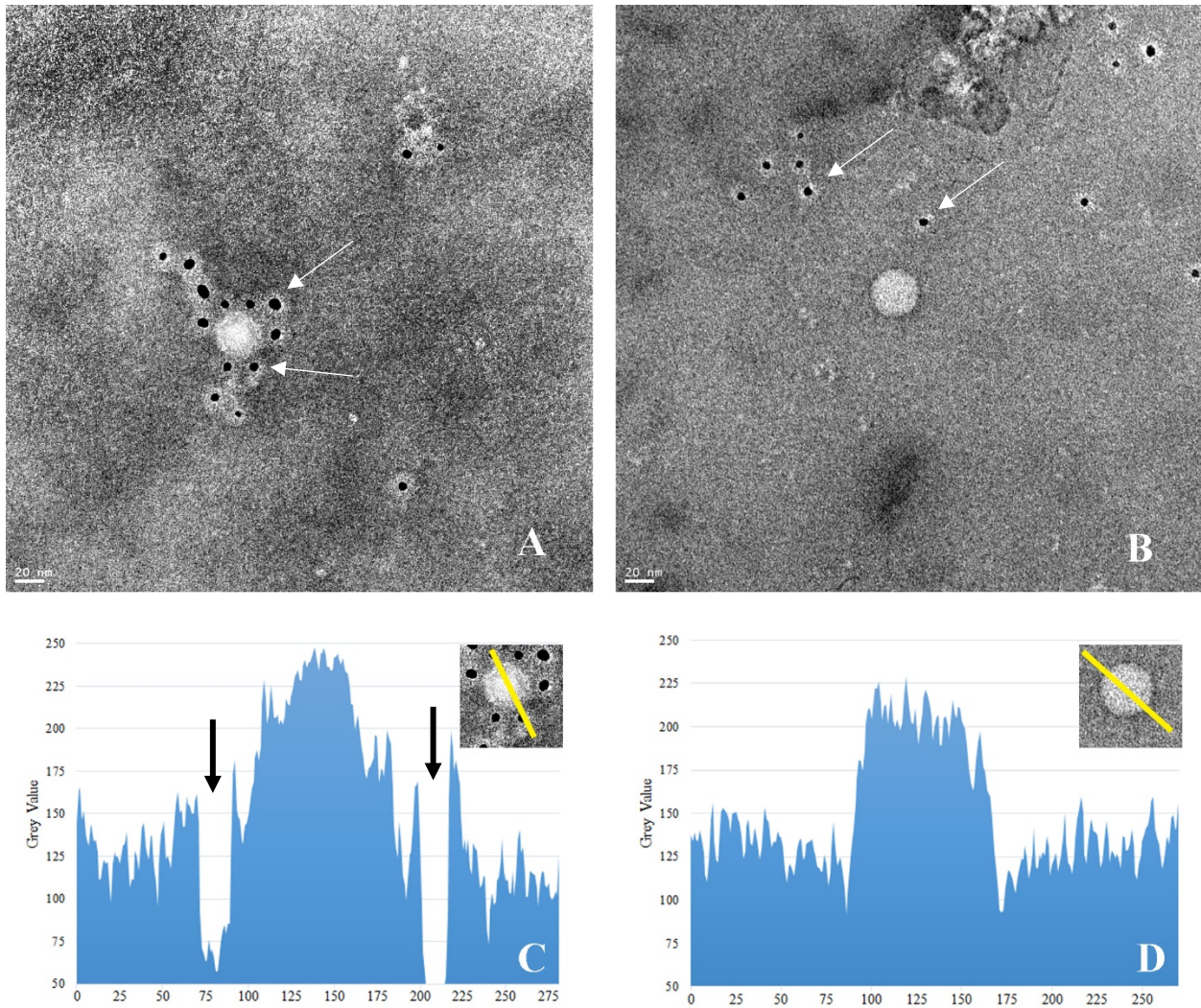


Figure 20: Pretreatment of PV-1 with GTA blocks AuNP binding. A shows untreated PV-1 labeled with AuNPs. B shows PV-1 (center) that was pretreated with GTA for 1 hour prior to AuNP labeling. White arrows indicate AuNPs. C and D show plot profiles of 20 x 270 pixel sections across each inset as in Figure 19.



## Discussion

A comparison of the 6-Log<sub>10</sub> reduction estimates for selected *Bacillus* spores confirms that not all spores share the same sensitivity to GTA (104, 112). A comparison of two batches of each spore also shows that kill times can differ slightly, though not significantly, between batches of spores from the same species created using identical methods, an observation that has also been seen in other types of organisms with GTA (108). In some cases, the type of disinfectant used can reverse trends in susceptibility (104).

In addition to the differences seen in susceptibility, the results in Figure 7 also suggest differentiation of spores into high (*B. anthracis*), intermediate (*B. atrophaeus*, *B. pumilus*, and *G. stearothermophilus*) and low (*B. subtilis*) GTA susceptibility. It has been well-established that there are vast differences in the kill times of *B. anthracis* and *B. subtilis* spores treated with GTA. Several other studies have shown, using various methods, that *G. stearothermophilus*, *B. atrophaeus*, and *B. pumilus* have similar kill times to those presented here, though this is the first time all 5 of these spores have been tested identically and in the same study (86, 115, 137). It should be mentioned that only *B. anthracis* is clinically relevant. Spores of *B. subtilis*, *B. atrophaeus*, *B. pumilus* and *G. stearothermophilus* are all generally used as bio-indicators for sterilization processes (112, 113). Members of the *Clostridium* genus cause a number of diseases including *Clostridium tetani* (tetanus), *C. difficile* (pseudomembranous colitis), *C. botulinum* (botulism poisoning) and *C. perfringens* (gas gangrene), but none were tested in this study. Results of such testing would provide an interesting addition to this data. Previously, GTA has been tested against all of these spores, though in general, there are fewer studies on disinfection of *Clostridium* spores than *Bacillus* spores (5, 138). This is likely because *Clostridium* species are much more difficult to culture, requiring anaerobic environments and producing low spore

yields (138, 169, 170). It would be interesting to see these same experiments performed on the more clinically relevant *Clostridium* spores, in an effort to better determine the effect of species on spore disinfection.

Figure 8 shows a titration of AF488 with *B. anthracis* and *B. subtilis* spores, as well as the difference in mean fluorescence for each spore type. The elevated mean fluorescence generated by *B. anthracis* spores would suggest that they contain more available primary amines, while *B. subtilis* likely has fewer. Since it is known that both GTA and AF488 bind to primary amines, it is likely that increased levels of fluorescence could generally indicate increased susceptibility to GTA (122, 124-126). The fluorescent profiles in Figure 9 support this idea, though only somewhat. Since the mean fluorescence of *B. subtilis*, *B. atropheus* and *G. stearothermophilus* spores were not statistically different from each other, it is likely that up to a certain threshold, it becomes difficult to predict GTA susceptibility for more resistant spores using fluorescent amine reactive probes. When mean fluorescence was plotted against 6-Log<sub>10</sub> reduction times, there did not seem to be a strict linear relationship between fluorescence and kill time, although there was a general trend that as fluorescence decreased, kill time increased. More work needs to be performed on spores with faster kill times (between 4 and 60 minutes) to determine what that threshold might be. Creating fluorescent profiles for spores from *Clostridium* species would also make an interesting addition to these results. Relatively little is known about *Clostridium* spore physiology and development. Given *Clostridium* spores develop under strict anaerobic conditions, it is conceivable that the structure and or physiology of the spore is significantly different from that of *Bacillus* species. This idea is supported by other studies comparing *Clostridium* spores to *B. subtilis* spores (171). In addition, spores from *Bacillus* and *Clostridium* have a number of genetic differences (5, 172).

Evaluating the ability of GTA to block AF488 binding yielded results that are consistent with what is known about AF488 and GTA's binding capabilities (122, 124-126, 153-155). GTA was able to block fluorescence from AF488 relative to a control, which suggests that AF488 binds to similar sites used by GTA. This trend was seen even more dramatically with the poliovirus AuNP labeling experiments discussed later. The residual fluorescence seen in Figure 11 and Figure 12, after different GTA contact times, may indicate fluorophore that was able to bind other functional groups such as secondary amines, thiols, phenols, imidazoles, guanidyl and hydroxyl groups, all of which are likely present on the surface of these spores (167, 173).

Interestingly, we did not see a similar outcome when attempting to block GTA's sporicidal action using Alexa Fluor<sup>TM</sup> 647 NHS-ester (AF647). AF647 was used in these experiments because AF488 contains a primary amine. These experiments, shown in Figure 13 and Figure 14, revealed that GTA could still kill spores pre-stained with fluorophore, with no associated increase in kill time. There are two possible reasons for this. GTA may have the ability to kill spores by unknown mechanisms that are not related to primary amine binding. This is unlikely, given the extensive research that has been done with GTA to determine its preferred binding sites (23, 116, 142, 174). GTA is also not known to bind DNA or lipids to any degree that might harm living organisms, let alone spores (33, 127, 132, 133). A more likely alternative is that GTA has more primary amines available to it than has the fluorophore. Although spore protein coats are dense, they are permeable to certain small molecules, and have been referred to as molecular sieves (15, 17, 29). It is conceivable that the spore coat is able to hinder the movement of the fluorophore (molecular weight = 1250 g/mol) past the outer protein layers, while GTA (molecular weight = 100.1 g/mol) is able to penetrate faster and deeper into the coat.

It is important to also note that Gorman suggested that a possible reason for alkaline GTA's improved sporicidal activity over acid GTA was due to the ability of the alkalinizing agent, sodium bicarbonate, to separate layers of the spore coat from each other, giving GTA more possible targets to bind. It is interesting to note that this separating effect was not observed when other alkalinizing agents were used (137). Penetration of GTA through empty protein coats of *B. subtilis* has also been observed (175).

When the spore protein coat of *B. subtilis* was removed, there was a marked increase in its susceptibility to GTA, though again, there was no protection afforded when coatless spores were stained with AF647 prior to treatment with GTA. This is likely because the peptidoglycan surrounding the spore's core can also act as a barrier to large molecular weight compounds, as it does for vegetative bacteria. Since GTA is known to be able to crosslink peptidoglycan, it is possible that penetration of GTA beyond the limits of the fluorophore would explain why pretreatment with AF647 affords no protection, similar to the observations made above with intact spores (131, 137). Increased susceptibility of de-coated spores to GTA has been observed previously (10, 11, 168). A repetition of this experiment with the removal of the cortex as well as the coat, may provide more information on the role these layers play in GTA susceptibility.

Protein coat thickness of the spores used in this study was measured by electron microscopy. In Figure 16, the differences in spore structure are readily visible. Averages of measurements taken revealed that *B. subtilis* had the thickest coat, while *B. anthracis* and *B. pumilus* had the thinnest. *G. stearothermophilus* and *B. atrophaeus* had coats about the same thickness. Although not statistically different from *B. atrophaeus* and *G. stearothermonphilus*, the small difference in thickness between these and *B. subtilis*, combined with coat composition differences (i.e., density or number of lysine residues), could account for *B. subtilis*'s marked

resistance to GTA. The results shown in Figure 17 suggest a correlation between spore coat thickness and 6-Log<sub>10</sub> reduction estimates for GTA. This agrees with several other studies that noted that *B. subtilis* has a much thicker coat than *B. anthracis*, and suggested that this could be a contributing factor in *B. subtilis*'s marked resistance not only to GTA, but many other disinfectants (10, 16, 18, 50, 176).

Viruses have a much simpler structure than bacterial spores. They are also much smaller and must be visualized using electron microscopy. Gold particles (AuNPs) which covalently bind to primary amines were used to visualize these functional groups on the surface of two viruses; one known to be susceptible to GTA inactivation (poliovirus, or PV-1) and one known to be resistant (human papillomavirus, or HPV). Although PV-1 was not surrounded by AuNPs in a manner similar to the latex bead controls (Figure 18), AuNP binding was still considerable. This was expected since there are likely far fewer primary amine sites on PV-1 than on these latex beads. Similar results were obtained when labeling adenoviruses with 1.4 nm covalently linked AuNPs (165). One study investigated the position of lysine groups on protein capsid secondary structures for various poliovirus-related enteroviruses. They found that the viruses most resistant to GTA had no lysine groups on secondary structures that were accessible on the surface of the virus (77). In addition, PV-1 was generally associated with at least 2-3, and often more AuNPs within 5nm of the virions, which is suggestive that AuNPs are in fact, bound to the viral capsid. Each AuNP has a 5 kd linker that connects each NHS functional group to its AuNP, giving the effect of an electron permissive "halo" sometimes visible around the AuNPs. According to technicians at Cytodiagnosics<sup>TM</sup>, so long as the particle is within 5 nm of the virus, it is generally considered bound (personal communication). Profile plots of PV-1 with associated AuNPs also suggest covalent binding of the particles to the virus (Figure 19). In

addition, Figure 20 revealed that when PV-1 was treated with GTA prior to labeling with AuNPs, no such association was observed, and these treated virions appeared similar to HPV-18. This suggests that GTA is reacting with the binding sites available on the PV-1 capsid surface. PV-1 has been shown to be susceptible to GTA disinfection, and exposed lysine residues have been implicated in its susceptibility, as well as the susceptibility of other non-enveloped viruses (74-76, 107, 135, 140). Since HPV-18 was not observed to be associated with AuNPs, it is reasonable to conclude that little to no AuNP binding occurred. These results are supported by our earlier data showing that HPV is not susceptible to GTA inactivation (82). Interestingly, HPV has been reported to have exposed lysine residues on its capsid surface that are thought to be important in cell entry, though these residues are located in a pocket that may be protected somehow from GTA binding. It is also possible that GTA binding simply has no effect on virus infectivity (177, 178). More research is needed to determine which of these possibilities is correct. Future experiments should endeavor to explore the ability of covalently linked AuNPs to block or reduce GTA inactivation of PV-1. Other clinically relevant non-enveloped viruses such as Hepatitis A, rotaviruses, rhinoviruses, and caliciviruses should be evaluated as well to see if the trend observed here is consistent with other virus types.

## Conclusions

Caution needs to be exercised when attempting to select an appropriate surrogate for disinfection testing and when creating decontamination protocols based on data obtained from surrogate organisms, as kill times can vary greatly between species and even between batches of the same species. The use of *B. subtilis* as a surrogate for *B. anthracis* still seems to be a good choice when using GTA as the disinfectant, in that *B. subtilis* spores are much more resistant. GTA kill for *Bacillus* species spores is likely influenced by a combination of spore protein coat thickness and composition. Amine reactive Alexa Fluor<sup>TM</sup> probes could be used as a screening method when testing novel spores for susceptibility to GTA, although caution should be exercised since there is not a linear relationship between fluorescence and kill times for *Bacillus* spores. The number of available primary amines on viral capsids seems to be related to the susceptibility of a virus to GTA. For non-enveloped viruses, amine reactive AuNPs could prove useful for screening novel or fastidious viruses for susceptibility to GTA. These kinds of rapid preliminary tests could provide valuable susceptibility information that would be useful in avoiding surrogate-subject discrepancies, as was seen in the failure of GTA to disinfect HPV.

## References

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