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The Effects of Goldshield Antimicrobial on Bacillus anthracis Sterne and Clostridium difficile

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Goldshield’s active reagents were tested against the isolated spores of *C. difficile* and *B. anthracis* Sterne, by the method of environmental sampling. Its durability on a surface (fabric and plastic), was tested through different stressors, such as heat and washes. The spores were applied to surfaces treated with varying amounts of Goldshield.

Results indicate that G.S. 48 inhibited the growth of both organisms under normal conditions and heat. When the surface was washed and the spores reapplied, growth was present in all tested application amounts. Applications of G.S. 48 below 50µl were proportional to the inhibition of growth.

Goldshield proved to be an efficient product in single applications against both organisms, in all tested conditions. Although, its usefulness as a coating system was not satisfactory, given it was easily removed from the surfaces, indicating poor durability. Goldshield could be considered a disinfectant alternative in healthcare settings given its non-toxic and water based composition, but its use as a coating layer is not recommended since it would not bring sustainable results.

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**Department**
Health Sciences
The Effects of Goldshield® Antimicrobial on
*Bacillus anthracis* Sterne and
*Clostridium difficile*

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Abstract

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durability. Goldshield could be considered a disinfectant alternative in healthcare settings given its non-toxic and water based composition, but its use as a coating layer is not recommended since it would not bring sustainable results.
Introduction

Microbiology is the field of study concerned with microorganisms, which are defined by being unicellular or cell-cluster microscopic organisms (Martinko, 2006). Living organisms include eukaryotes such as protists and fungi, and prokaryotes, which are bacteria and archaea. Although viruses are not strictly qualified as living organisms, they are also studied in microbiology (Rice, 2007).

Microorganisms’ omnipresence affects the balance of virtually all environments. Practically any area of the earth is inhabited by a form of micro ecosystem of life that may be uniquely adapted to that environment (Larkin, 2009). Even though many organisms are essential to maintain harmony in many forms of life, studies tend to focus on those bacteria that could be potentially harmful and pathogenic. The goal of most studies is to find treatments against diseases caused by certain organisms or toxins, or in the case of this study, to prevent its propagation by disinfecting environments.

This research focus is on the activity of a new product known as Goldshield Antimicrobial, a patented nano-molecular-assembly technology developed at Emory University (Viking, 2007). There is currently a need for antimicrobials that are effective against important pathologic microorganisms, without being harmful to sensitive medical equipment. Given that Goldshield is a water-based, non-toxic, non-leaching, environmentally-benign antimicrobial solution, it could potentially be a good substitute over more toxic counterparts such as bleach (Viking, 2007).
Goldshield covalently bonds to surfaces, including textile and plastic, forming a shield against bacterial and microbial contamination. The solution has been proven to be effective against mold, fungi, algae, yeast, and some bacteria. Recent studies completed by Dr. Baxa, Ph.D., who is Director of Infectious Disease Research at Henry Ford Hospital in Detroit, have shown the product’s efficacy against MRSA (methicillin-resistant Staphylococcus aureus) and VRE (vancomycin-resistant Enterococci) (Czech, 2007). Despite being efficient against very relevant organisms, Goldshield’s activity against spore formers had yet to be tested.

Sporulation only occurs in bacteria pertaining to the genera Bacilli and Clostridia. In this process, successive changes in cell structure occur. These changes in bacteria produce dormant structures. This progression by which a vegetative cell becomes converted into a dormant structure is called 'sporogenesis' while the reverse process to the vegetative stage is known as 'spore germination,' followed by outgrowth.

The ability of forming spores is very important for the survival of organisms; it gives spore-forming bacteria an ecological advantage, since it enables it to survive under several adverse conditions. Sporulation will normally occur under conditions in which nutrients are depleted. Factors such as pH, media composition, ionic strength, aeration, temperature and salinity are known to affect sporulation (Sporulation of
bacteria, 2009).

The process of sporulation can be divided in a few main stages. On stage I, the chromatin is visible as a single axial rod. The second stage is marked by an invagination of cytoplasmic membrane, completing the spore septum. Following this step (stage III), a double-cell organism, the sporangium, is created. The membrane develops to the point that the spore is carved out of the mother cell. The next couple stages are characterized by the formation of new envelopes around the spore, which develops the spore’s thermo-resistance. In the final stage, the mother cell is lysed, setting the spore free (Schaeffer, P. 1969). The total time for sporulation may vary between different bacterial strains and species.

Varied requirements are needed to initiate sporulation, such as the decrease of the rate of the TCA cycle. In addition, highly phosphorylated nucleotides (HPN) such as tetra and pentaguanosine phosphates have been found necessary to initiate sporulation. Given that sporulation is defined as a differentiation process, different proteins are synthesized depending on which stage of the sporulation cycle the organism is found. In order to achieve this new protein formation, enzymes that alter RNA polymerase trigger new m-RNA that is not normally produced in the vegetative state (Schaeffer, P. 1969).

As previously mentioned, the two genera of bacteria that are known for producing spores are *Bacillus* and *Clostridium*. The spores of bacilli and clostridia contain DNA that is very similar in chemical composition to the vegetative DNA. In some instances the spores have been found to contain a higher amount of DNA. The main difference lies in the fact that spores contain a protein-synthesizing scheme
that is partially inert (Spore chemistry, 2009).

The efficacy of Goldshield antimicrobial was tested on both *Bacillus anthracis* Sterne and *Clostridium difficile*, in order to prove its effects on both aerobics and anaerobics. These two species were chosen due to the fact that they are currently very relevant.

*B. anthracis* is mostly known for being used as a bioweapon in terrorist attacks. This organism is the causative agent of the often-fatal bacterial infection known as anthrax. Human infections normally occur from contact with contaminated animals or animals’ products. To this date there are no known human-to-human infections. The infection is initiated by the endospores of the soil organism *B. anthracis*.

Anthrax endospores are often worrisome because despite not having the ability to divide, they have no measurable metabolism, and are resistant to drying, heat, ultraviolet light, gamma radiation, and many disinfectants (Watson and Keir, 1994). Due to this hardiness and dormancy, the spores allowed nations to use the organism as biologic weapon. The spores allow the transmission of the organism while the anthrax virulence genes become expressed once the spores reach the vegetative form of *B. anthracis*, which results from the germination of spores within the body. Figure 2 depicts the infection process of *B. anthracis*.

Endospores may contaminate a human host by three main routes: abrasions in the skin (cutaneous infection), inhalation (pulmonary infection) or ingestion (gastrointestinal infection) (Hanna P., 1998). The course of infection is shown in figure 1 (Hanna, P. et al, 1999).
Endospores introduced into the body by inhalation, abrasion or ingestion are phagocytosed by macrophages and carried to lymph nodes. The endospores germinate while still inside the macrophages becoming vegetative bacteria (Ross, J. 1957). The vegetative bacteria are released from the macrophages into the lymphatic system, where they are allowed to multiply and enter the bloodstream. As the number of organisms increase, the host eventually reaches massive septicemia. There is no evidence that an immune response is initiated against vegetative bacilli.
once they are released from macrophages (Hanna, P. 1998). The virulence factors from Anthrax bacilli include both toxin and capsule. The resulting toxemia has systemic effects that lead to the death of the host (Hanna, P. 1998).

As mentioned, due to the ability of producing spores and its relevancy in the current health care and political settings, *B. anthracis* was one of the organisms considered important to be tested in this experiment.

As an anaerobic counterpart, *Clostridium difficile* was the second organism of choice in this experiment, given that it qualifies in the first basic criteria: spore former. In addition, similarly to *B. anthracis*, *C. difficile* is also a very important organism to be combated in health care (although for fairly different reasons).

*Clostridium difficile* is an organism of great concern, since it is responsible for a great parcel of nosocomial infections. Every year, anywhere from 300,000 to 3,000,000 cases of diarrhea and colitis are caused by *C. difficile* in the United States.

Infections with *C. difficile* are often associated with certain antibiotics such as amoxicillin and cephalosporin. The first step of the development of colonization occurs when the flora of the colon is disrupted (usually by medications). Colonization occurs by the fecal-oral route, when the patient ingests the spores. The spores survive the gastric acids and finally germinate once they reach the colon. The clinical presentation can vary from asymptomatic colonization to mild diarrhea to severe debilitating disease, which is characterized by high fever, severe abdominal pain, paralytic ileus, megacolon, or even perforation (McFarland, M. 1989).

*Clostridium difficile* forms spores, which due to its hardy nature are able to
persist in the environment for years. The spores may be spread through the hands of health care professionals or just by attaching to environmental surfaces (e.g. floor, counters, gowns). Contamination by \textit{C. difficile} is common in hospitals and long term care facilities, especially in rooms occupied by an infected individual. Patients with diarrhea, especially if accompanied by incontinence, may spread the spores in the environment, unintentionally spread the infection to other patients and health care workers (C. diff support, 2004). The organism can also be easily cultured from environmental surfaces from the rooms of infected patients.

Figure 3 describes the course of infection of \textit{Clostridium difficile}.
Both *C. difficile* and *B. anthracis* have great virulence and persistence in the environment due its spores. It is a major interest in health care and public safety that preventions against both organisms are developed. Goldshield is being studied as a possible disinfectant, emulsifier and coating system (due to its durability in a surface). If the product is able to destroy spores, then it could be considered a good alternative over bleach and other harsher chemicals. Advantages such as non-
toxicity and water based would also be ideal for health care workers, since the
Goldshield does not stain and is not harmful to the skin.

The goals of this project involved measuring the effectiveness of varying
application amounts of G.S. 48 necessary to inhibit growth of the organisms and also
its endurance in a surface. Tests were done in fabric surfaces (100% cotton) and
plastic.
Materials and Methods

The protocol used in this experiment was a modification of ATCC carrier methods testing.

Environmental sampling was the method of choice to test the efficacy of Goldshield. In order to execute this method, the isolation of Clostridium difficile and Bacillus anthracis Sterne was necessary. The spores of C. difficile were attained according to the protocol kindly provided by Abraham L. Sonenshein of Tufts University. The steps from the protocol for isolating C. difficile spores and further on germinating it are shown below:

1) Measure the optical density of a overnight culture and determine the amount of overnight culture to add to fresh BHIS to obtain an OD\textsubscript{600} = 0.2

*Remove the aliquot from the anaerobic chamber to measure the density of the culture*

2) Mix the solution from Step 2 thoroughly

3) Add 150 µL of this culture to each well of a 6-well tissue culture dish containing 5-ml of BHIS agar

*To ensure that the culture is evenly spread over the entire surface of each well, tilt the plate on each axis. This will allow the liquid on top of the agar to spread over the surface*

4) Incubate the plate in the anaerobic chamber for 4-7 days
C. difficile will not sporulate in liquid culture. It is necessary to induce sporulation on solid medium. We have found that using a 6-well tissue culture dish with 5ml of BHIS agar rather than a standard petri plate increases the yield of C. difficile spores.

5) After 4-7 days, using a sterile, disposable inoculating loop gently swipe the top of the agar to collect a small amount of cells

6) Resuspend these cells in 50 µL sterile water and remove from the anaerobic chamber

7) Add 4 µL of the culture to a microscope slide and cover with a coverslip

8) Inspect the progress of sporulation using a phase-contrast microscope

The C. difficile culture should contain approximately 1x10^7 spores/well (there may be variation between strains in the total number of spores). Spores should be readily visible after 7 days growth on the agar medium. Spores appear as bright and ovular when using a phase-contrast microscope. Most of the spores may not have been released from the mother-cell. If there are few spores present, continue to incubate on the agar medium and follow the progress until spores are visible.

Vegetative bacteria will likely always be present.

9) Purify the spores by flooding each well of the 6-well tissue culture dish with 1 ml of ice-cold water.

Note: This and all subsequent steps may be done outside the anaerobic chamber

10) Pellet the suspension of vegetative C. difficile and spores at 14,000 x g for 1 min
11) Wash the pellet with 1 ml ice-cold water

12) Repeat steps 10 & 11 5 times to ensure spores are released from the mother cells

13) Suspend each washed pellet with an aliquot of 20% w/v HistoDenz (Sigma-Aldrich)

14) Gently layer the 5 ml of 20% HistoDenz onto the 10 ml of 50% HistoDenz and centrifuge 15 min at 15,000 x g

*The HistoDenz gradient will separate free-spores from vegetative bacteria and cell debris. Spores will pellet to the bottom of the tube and bacteria and debris will remain at the interface of the gradient*

15) Gently remove all liquid from the tube taking care to remove the debris at the interface first to minimize the amount that would contaminate the purified spores

16) Resuspend the spore pellet in 1 ml ice-cold water

17) Wash the resuspended pellet twice in 1 ml ice-cold water to remove trace amounts of HistoDenz

18) Resuspend the spore pellet in 200 μL water

*Inspect the purity of the spores by phase contrast microscopy. Spores should be >99% pure*

19) To recover *C. difficile* spores as vegetative bacteria, remove a sample to the anaerobic chamber and streak on a pre-reduced BHIS agar plate containing 0.1% taurocholate
C. difficile spores will not form colonies in the absence of taurocholate.

The plastic tests were done in two twenty four (4x6) wells plates. A separate plate was used to test each microorganism. To observe in which amounts Goldshield is effective, the rows were coated with different concentrations of the substance. Row one was coated with 100 µl of Goldshield®, row two with 50 µl, row three with 25 µl and row four (control) had no Goldshield applied. The wells were allowed to dry overnight.

Once the wells were coated and dry, they were coated with 75 µl of a \(10^{-1}\) serial dilution of the isolated spores of C. difficile. Spores were applied in the second plate, except that B. anthracis Sterne was used. Once again, this was repeated and the plates were allowed to dry overnight.

After a period of approximately 24 hours, when the plates were completely dry, all wells were flooded with 1 ml of double distilled water. The content of each well were subjected to three different treatments prior to plating: heat, wash and room temperature. The wash step used 300 µl dispensed into an epi-tube. Another 300 µl was heated in a water bath at 64 degrees for 30 minutes. The remaining 400 µl was incubated for 15 minutes at room temperature before being plated. After being treated, the samples recovered by environmental sampling of C. difficile were plated on appropriate anaerobic media (with taurocholate). The plates were incubated in an anaerobic environment at 37°C (jar) for 48 hours. Colony growth before 48 hours was not adequate in order to measure inhibition appropriately. The colonies growths were counted after the incubation period.
The same treatments done with *C. difficile* were done with *B. anthracis* Sterne: room temperature, heat and wash. The contents recovered from the *B. anthracis* Sterne twenty-four well plates by environmental sampling were plated in regular BHI media and incubated at room temperature in an aerobic 37°C environment. After 24 hours, the numbers of colonies grown were counted.

Once the numbers of colonies seen were recorded, the two twenty-four wells plates were re-incubated with 75 µl of a $10^5$ serial dilution of the isolated spores of *C. difficile* and *B. anthracis* Sterne for the endurance test (Goldshield was not reapplied, so its endurance capacity on a surface could be tested).

In addition to the hard surface testing, Goldshield was also tested on fabric. In order to do this test, fabric was soaked on a Goldshield solution and allowed to dry for 48 hours. *C. difficile* was incubated on the fabric for 24 hours, before being recovered by environmental sampling and plated on anaerobic media. A single piece of fabric used as the control was not treated with Goldshield. The plates were incubated at 37°C in an anaerobic environment. After 48 hours the colony growth was quantified and recorded.
Results

The isolated spores for both *C. difficile* and *B. anthracis* Sterne were plated in the appropriate media and environmental conditions once they were recovered from the surfaces treated with Goldshield antimicrobial. All the experiments were repeated at least three times in order to obtain a trustable average of results.

Initial studies included factoring proper dilutions needed for the assays. After numerous trials the ideal dilution for *C. difficile* was found to be $10^{-1}$, of an original titer of $14 \times 10^4$ organisms. The dilution for *B. anthracis* Sterne was also $10^{-1}$, from an original titer amount of $24 \times 10^5$ organisms.

Three main treatments were developed to study varied features and interactions between organisms and G.S. 48. Room temperature indicates spores that were simply recovered through environmental sampling without any stressor. Heat indicates when the recovered solution of spores was heated at 65°C in a water bath for 20 minutes. Lastly, the wash procedure compiles of spinning the recovered solution and removing the supernatant, and finally re-suspending the spores pellet with double distilled water.

The growth of *C. difficile* observed with the different treatments and Goldshield concentrations are represented in the graph below (Figure 3).

Fig. 4 – *C. difficile*
The first application of the microorganisms on the coated surfaces displayed that the colony expansion was successfully inhibited. When the concentration of Goldshield was equal or higher than 50µl, the growth of *C. difficile* was inhibited under room temperature and heat. Although, the results show that some growth occurred when the sample recovered by environmental sampling was washed.

In the second application of spores to the surface (endurance test) showed that the organism had a significant growth when subjected to all three conditions.

The table below shows the percentage of inhibition of growth of *B. anthracis* Sterne, among all test situations (Negative values indicate when growth surpassed the positive control).
Table 1

<table>
<thead>
<tr>
<th>Room temperature</th>
<th>Wash</th>
<th>Heat</th>
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</thead>
<tbody>
<tr>
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<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>50</td>
<td>99%</td>
<td>98%</td>
</tr>
<tr>
<td>25</td>
<td>99%</td>
<td>96%</td>
</tr>
<tr>
<td>100(Endurance)</td>
<td>9%</td>
<td>62%</td>
</tr>
<tr>
<td>50(Endurance)</td>
<td>-111%</td>
<td>84%</td>
</tr>
<tr>
<td>25(Endurance)</td>
<td>-52%</td>
<td>81%</td>
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</tbody>
</table>

As previously mentioned, the protocol for the testing done with *B. anthracis* Sterne was the same done with *C. difficile*. The bacteria were tested under the same environmental situations: room temperature, heat and wash. The results for *B. anthracis* Sterne were very similar to the results found in *C. difficile*, as shown in the graph below (figure 5).

Fig. 5 – *B. anthracis*
When Goldshield capabilities were tested against an aerobic spore former (*B. anthracis* Sterne), the growth and inhibition patterns were very similar to its anaerobic counter part. In the first application of spores, growth was prevented when the concentration of product coating the surface was equal or higher to 50µl.

In fact, in both room temperature and heat treatments, there was a 99% decrease in colony growth in concentrations of 50µl or higher and a 94% decrease in a concentration of 25µl. The only treatment that showed some significant growth was “wash.” In concentrations of 100µl and 50µl about 90% of the growth was inhibited. In the well with 25µl, 53% of the growth was inhibited. During the endurance part of the test (in which spores were reapplied to the same surface), growth occurred under heat, room temperature and washing. Growth occurred randomly regardless of the concentration of Goldshield that was used originally on the surface. The table below shows the average percentage inhibition in the different volumes of Goldshield applied, in all three treatments.
In addition to testing *C. difficile* on plastic surfaces, pieces of fabric that were soaked in Goldshield were also tested. The results are shown below (figure 6).

Fig. 6 – *C. difficile* test on G.S. 48 treated cloth
Pieces of fabric of two square inches were soaked in Goldshield® and allowed to dry for two days. Once dry, spores were applied to the fabric and allowed to dry overnight. The pieces of fabric were washed with distilled water to recover any remaining spores. A volume of 50ul of the distilled water used to wash the cloth was plated in an anaerobic environment (in Brewer plates) and incubated for 48 hours at 37°C. The graph shows that the growth of *C. difficile* was inhibited in all fabric samples that were treated with Goldshield. The only colony growth observed was in the control sample, which had no Goldshield applied to it.

The spores of *Bacillus anthracis* Sterne were also tested in pieces of cloth that were soaked with Goldshield, yielding results very similar to the ones obtained with *C. difficile*. The results are shown in Figure 7.

Fig. 7 – *B. anthracis* test on cloth treated with G.S. 48
Trials

The results of the cloth test done with *B. anthracis* Sterne were very similar to the results obtained for *C. difficile*. Colony growth was inhibited in all pieces of cloth that received the Goldshield® treatment.

Both organisms, *B. anthracis* Sterne and *C. difficile*, had 99% growth inhibition when they were cultured after the spores were applied to Goldshield treated cloth.
Conclusion

Goldshield® is currently marketed as a long lasting, non-toxic, water based antimicrobial that is effective against several microorganisms that range from MRSA to Staphylococci and fungi. Its uses range from sports hygiene, healthcare, first responders and law enforcement. Since Goldshield does not diffuse or migrate, there is no known or anticipated risk for pathogens to genetically adapt, which is a great advantage over other non-toxic antimicrobials (Goldshield AuProvise, 2009).

When applied to plastic surfaces of dimensions of 128 x 85 x 22 mm (1.91ml), it was observed that a volume of 50μl or higher of Goldshield® was sufficient to inhibit 95% or more of the growth of both C. difficile and B. anthracis Sterne in those wells. The same effectiveness was observed on surfaces of different nature, such as fabric. Goldshield® antimicrobial has surface penetrating characteristics, which disperses over the surfaces more quickly by reducing surface tension. Goldshield forms a cross-linking action with a vast array of organic and inorganic surfaces, and the most complex surface topologies. The multi-dimensional polymeric structure aids on its coverage.

Room temperature was used in order to observe the sole effects of concentration of Goldshield in a surface. The purpose of the heat stressor was to eliminate all vegetative bacteria in order to observe Goldshield effects on spores only, while the washing procedure was done with the intention of analyzing how difficulty was to interrupt Goldshield's action (by removing it from the spores surface). In addition, by washing the recovered samples, the theory that Goldshield
is temporarily inactivating spores is strengthened (instead of completely destroying it), given that random colony growth was observed regardless of the concentration of Goldshield applied to the well under washing condition.

In its first application, given that more than 50μl was applied to the well, Goldshield® effectively inhibited 99% of colony growth in the samples that were subjected to room temperature or heat (65°C), but a slight growth was observed on the samples that were washed (inhibiting 89% of growth), leading to further questions about the product’s durability on a surface.

The heating of the samples was done in order to kill all vegetative form of bacteria reminiscent in the spore isolation. Despite the intentions of eliminating all vegetative form of bacteria from the spores isolate, great difficulty was shown in observing if the samples that were recovered by environmental sampling were pure spore solutions. The results show that the samples that were treated with heat had a greater colony growth, suggesting that perhaps heat could have increased the germination of spores. Although, the number of colonies observed were still inversely proportional to the concentration of Goldshield. The higher the concentration of Goldshield applied, less colony growth occurred.

Goldshield has been proved to be effective against most forms of Gram-positive bacteria, but as mentioned previously, one of the goals of study was to analyze the product’s action on spores. Based on the results obtained, it could be hypothesized that spores were coated with Goldshield, either killing them or keeping them from germinating into their vegetative form. Either way, the result
would be a decrease in colony growth. Additional studies would be necessary to prove if Goldshield is indeed acting on the spores itself, or if germination is occurring and once the bacteria reaches a vegetative stage they start being affected by the product.

Once the bacteria were reapplied, in order to test the endurance of Goldshield on a hard surface, the results proved that the product’s activity was greatly reduced. As an example, inhibition in room temperature went from 100% to 83% in the well that was treated with 50 µl of Goldshield. Considering the number of colonies that grew in the second application, Goldshield should probably not be considered a coating system or even long lasting antimicrobial, given that a single wash of the wells were ample to remove the product from the hard surface. It could possibly be considered that after a first application, the surface has no Goldshield protection.

Reports from AuProvise, the current distributor of Goldshield® antimicrobial, claims that the product is being used in several industry sectors including textiles supplied to the Department of Defense, healthcare apparel, and athletic uniforms. Its characteristics of forming covalent bonds to fibers, not leaching from the materials after application, makes it a great product to be used on textile. AuProvise assures that such properties would provide long-term residual protection after multiple washings. This affirmation agrees with the results obtained for cloth in this research, in which both organisms had a 99% growth inhibition when applied to Goldshield treated cloth. According to AuProvise, technical data
demonstrates antimicrobial inhibition for up to fifty washes conducted under the AATCC 100 protocols.

Providing that Goldshield® has reduced toxicity, the product could be considered a good alternative for disinfectants, replacing harsher chemicals such as bleach and alcohol based products.

In this research, germination of the spores was attempted by treating it with taurocholate and PBS, in order to try to compare the effects of Goldshield on the vegetative form versus the spores. Given that germination was successful, the results obtained could more confidently indicate in which form of the bacteria Goldshield exactly acts upon. Hence, it is still unknown whether the spores were being completely destroyed or if germination was simply inhibited by "paralyzing" the spores, which could be a topic for future research. Either way, the bacteria tested were not successfully proliferating when Goldshield was applied in appropriate concentrations. When applying this fact to practical uses of Goldshield, it could be assumed that with the inhibition of colony growth (regardless if the spores are being allowed to reach a vegetative state) toxins would not be produced. If toxins are not produced, the development of disease in a human host does not occur, which ultimately is the general goal of disinfectants, despite of the method of action.
Bibliography


