Potential Surrogates for Evaluation of Decontamination Methods Under Field Study Conditions or BSL-2 Biosecurity Lab Conditions: A Review

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Abstract: Surrogate species are commonly used to evaluate the ability of decontamination, sterilization, and/or disinfectant methods to sanitize bio-contaminated surfaces, equipment, facilities, soil, or water. As new decontamination technologies become commercialized there is an ongoing need to evaluate them using field studies, or on-site for large, stationary systems, to determine if they are more environmentally friendly, less expensive, or more effective than the current sanitation practices. This surrogate review compares potential surrogate species such as MS2 bacteriophage, *Clostridium difficile Bacillus subtilis,* and *Cytisus scoparius* for their ability to accurately estimate the efficacy of decontamination, sterilization methods or commercial systems when evaluated under field conditions. Evaluation of decontamination systems, using field or on-site studies conducted under real-world conditions provides realistic estimates of sanitation and insights into potential risks to health or the environment. Multi-stage decontamination systems, or semi-sterilization methods, such as concentrated, or high-level, disinfectants, pressure washing equipment with steam, or extended ultra-violet (UV-C) radiation, require hard-to-kill surrogates, such as *B. subtilis*, to determine effective treatments. Use of multiple surrogates for decontamination or sterilization research alleviates several concerns about selecting a single surrogate species that may only perform well only under specific treatments or environmental conditions.

Keywords: Surrogate, Decontamination, Disinfectant, Sterilization, Efficacy testing.

1. INTRODUCTION

Surrogates are used to evaluate decontamination, sterilization, and disposal methods or commercial products or systems for their effectiveness for sanitizing surfaces, equipment, or facilities from bio-contaminants [1, 2]. Surrogates serve as substitutes for target pests or pathogens due to their non-pathogenic properties, low biosecurity risk, ease of culturing and assaying, and relatively low cost. Also, surrogates allow disinfection efficacy studies to be conducted under field or real-world conditions and evaluate new decontamination and sterilization methods, technologies or commercial systems that are impractical to test under highly controlled or laboratory conditions [1,3]. Efficacy trials are the gold standard for evaluating new decontamination and sterilization methods or commercial systems. These new methods and systems may have the potential to improve sanitation practices, lower health and safety risks, promote environmentally friendly methods, or introduce less expensive methods [4-6].

Surrogates are often selected based on their similarity to the target pest or pathogen [1]. They are also selected for hardiness and ability for surviving normal storage and handling protocols. Surrogates are also selected for availability, ease of culturing, and high density counts after sample inoculation, which translates into improved statistical accuracy and precision [1, 2]. Also, surrogates may be chosen for their higher resistance ranking, i.e. they are “harder-to-kill” and, therefore, all other pathogens with a lesser resistance should be easier to inactivate. The ideal surrogate should be easy to identify, culture, handle, and store, while easily sampled quantitatively, have even distributions within a wide range of ecological requirements, and possess low genetic variability [2, 7].

Spaulding’s hierarchy of disease was introduced in 1957 but is still relevant today with the time-tested ranking of microbial organisms by their chemical resistance [8]. Selecting potential surrogates, based on this hierarchy, assumes that any decontamination treatments that inactivate the hardest-to-kill class of microbes will also inactivate any other class of pathogens that have lesser resistance. The use of multiple surrogates or several bacterial life stages to broaden and strengthen the efficacy testing would greatly improve the evaluation process. Surrogate samples that include both spore and vegetative cells of spore-forming bacteria would provide efficacy information that covers both easy and hard-to-kill target pests and pathogens.

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E-ISSN: 2409-9813/20

Based on Spaulding's hierarchy of disease classification, bacterial spores have the highest resistance, after prions, to decontamination treatments. Vegetative bacteria are typically selected as surrogates for disinfectant studies due to their association with public health and the spread of diseases [8]. However, vegetative bacterial cells do not survive long without a sustaining media or liquid and they generally have weak resistance to most disinfectants [8]. Highly controlled laboratory tests that use vegetative cells may result in inflated efficacy results, which can not be reproduced under harsher, real-world conditions.

Several spore-forming bacteria are extremely resistant to chemical and heat treatments while posing few safety concerns. Three genera of spore forming bacteria, *Bacillus*, *Geobacillus,* or *Clostridium,* are widely used as surrogates, and all of these are gram positive with rod shaped cells. Several *Bacillus* and *Geobacillus* species are non-pathogenic and can be readily cultured. A potential disadvantage of using bacterial spores is that they should be refrigerated if stored over long periods before starting an efficacy study to reduce spore germination rates to vegetative cells [9]. Spores in general are much more resistant to heat and chemicals in comparison to their vegetative counterparts, therefore any germination of spores due to activation at room temperature has the potential to increase treatment efficacy and introduce bias into the treatment effectiveness.

2. POTENTIAL BIOLOGICAL SURROGATES

2.1. MS2 Bacteriophage

The bacteriophage MS2 is a non-enveloped, positive-sense, single-stranded RNA, or (+) ssRNA virus that infects the *Escherichia coli* and other members of the *Enterobacteriaceae*. The host for MS2 is an enteric bacterium (*E. coli*), thus this bacteriophage is commonly found in sewage and animal feces. MS2 is a biosafety level 1 microorganism and is non-pathogenic to humans.

The use of MS2 bacteriophage as a surrogate for viral pathogens in food safety and disinfectant studies has gained recent acceptance. Shin and Sobsey [10] used the MS2 phage as a viral surrogate in an ozone disinfectant study. Also, Hosseini *et al*. [11] used MS2 in a food safety study as a surrogate for enteric viruses for thermal inactivation in milk. Dawson *et al*. [12] found that MS2 had a prolonged survival rate when inoculated onto fresh produce. A laboratory study evaluated the survival rate for MS2, which resulted in a 2.1 log-10 reduction after 48 hr on a clean, coupon surface (unpublished data). The low survival rate for the MS2 phage, when tested under ambient environmental conditions, requires that treated samples have a rapid, one-day assay turnaround time to ensure adequate phage survival rates. Also, it is essential that phage survival rates for transport and control samples be used to differentiate between inherent phage mortality over time and phage inactivation rates due to decontamination treatments.

Coronaviruses are also positive-sense single-stranded RNA, or (+) ssRNA viruses. Due to their taxonomic similarity, the MS2 virus would make an excellent surrogate for any coronavirus. Research involving decontamination of surfaces, air and food for coronaviruses is in high demand and a low health risk surrogate such as the MS2 virus should be fully exploited in these studies. Previous research has shown that the MS2 phage is more resistant to disinfectants and heat treatments than other non-enveloped RNA viruses. Also, MS2 is a proven, low-level health risk for animals and humans. All these factors make the MS2 phage a good surrogate for the evaluation of disinfectants or decontamination methods, if the study designs can compensate for their low survival rates.

2.2. Clostridium Difficile

Clostridium difficile is a gram-positive, rod-shaped bacteria that produces endospores and is anaerobic [20]. It is commonly found in soil, water, feces, and in the gastrointestinal tract of both humans and animals [21-23]. It has been detected on farms, in public lawns [24], in meat products and fresh vegetables [25 - 26], and in hospitals [27]. The survival stage of *C. difficile* is a dormant endospore that is extremely resistant to antibiotics and resistant strains can grow in the presence of antibiotics. The vegetative form of *C. difficile* produces toxins but is susceptible to antibiotics [27].

There are no documented cases of *C. difficile* uptake in vegetable or plant roots, however spores have been found on the surface of raw and ready-to-eat foods such as deli meats and minimally processed fruits and vegetables [26]. *C. difficile* spores can germinate into vegetative, disease-causing cells when it reaches the intestinal tract in humans and animals, which contain glycine and cholate derivatives needed for germination [28]. *C. difficile* has been detected in

the common house fly [29], which could readily contaminate raw produce or any uncovered food. Contaminated food products can vector *C. difficile* transmission resulting in disease outbreaks in humans [30].

The spores of *C. difficile* are very resilient to drying, heating, and many disinfectants. Perez *et al*. [31] achieved a 6 log-10 reduction of *C. difficile* spores with acidified bleach, bleach and hydrogen peroxide with an exposure time of 10 minutes. Omidbakhsh [32] found that a hydrogen peroxide based gel achieved a 1 and 6 log10 reduction of *C. difficile* spores with an exposure time of 5 and 10 min., respectively. In a large disinfectant study, Speight *et al*. [33] tested the ability of 32 different disinfectants to inactivate *C. difficile* spores in a liquid suspension. He found that 27 of the disinfectants had a greater than 4 log-10 reduction when treated for 60 minutes, based on initial spore counts of 10^6 CFU/ml. There is, however, evidence that *C. difficile* spores may be more sensitive to some disinfectants in comparison to *B. subtilis* spores [34 - 37].

2.3. *Bacillus* and *Geobacillus* Species

Bacillus subtilis is a gram-positive, aerobic, spore-forming bacteria that is commonly found in the soil, air, and plant compost [38, 39]. Other endospore surrogates include *B. atrophaeus*, *B. mycoides*, and *B. thuringiensis*. They are all listed under BSL-1 biosecurity lab restrictions and are considered non-pathogenic surrogates. These endospores are often used as surrogates for *Bacillus anthracis* disinfectant studies [40, 41]. *B. atrophaeus*, *B. subtilis*, and other endospores are commonly tested together in disinfectant studies to compare resistance ranking among the different spore types [42, 43].

Geobacillus stearothermophilus (*G. stearothermophilus*) is a rod-shaped, Gram-positive bacterium [44 - 46]. The endospore was identified in 1920 and named *Bacillus stearothermophilus*, and later it was reclassified in 2001 as a member of the genus *Geobacillus*. The bacterium is a thermophile, is widely distributed in soil, hot springs, ocean sediment, and over 60 *Geobacillus* genomes have been identified from these sites [45]. *G. stearothermophilus* is considered non-pathogenic, but it is a microbial agent that causes food spoilage, especially in milk and dairy products. *G. stearothermophilus* has an optimal growth temperature of 55˚C. The endospores of *G. stearothermophilus* can withstand 121°C for up to 12 min and are able to survive in temperatures as high as 130˚C.

Commercial strips containing *G. stearothermophilus* are widely available as biological indicators for autoclave treatments [47]. Studies involving *G. stearothermophilus* require a BSL-2 site. *G. stearothermophilus* spores are also used to evaluate the steam treatment of waste from contaminated buildings [48]. The spores have also been evaluated in steam, disinfectant, and vaporized disinfectant studies [49-53]. *G. stearothermophilus* spores were also evaluated in an alkaline hydrolysis study, which is an extremely powerful sterilization method that uses chemicals to reduce proteins into simple amino acids, peptides, and salts [54].

The endospore stage of *Bacillus* and *Geobacillus* species produces a durable structure that may remain viable even after 25 to 40 million years. *B. subtilis* has been found encased with a bee, which was preserved in amber [55]. *B. subtilis* spores are extremely resistant to variable temperatures and are non-pathogenic [39]. In response to an environmental challenge (i.e. drought, salinity, extreme pH, radiation, etc.), a bacterial cell produces a spore, which protects the genome until conditions become more favorable to support the germination process [56]. The spores of various *Bacillus* species, including *B. subtilis*, are formed in the process known as sporulation and become metabolically dormant making them resistant to various stress factors within the environment [57, 58]. Sporulation of *B. subtilis* specifically involves the asymmetrical division of the cell followed by the differentiation of the mother cell and the actual endospore [59]. The endospore is composed of a multilayer shell that protects the genome of the bacteria during stressed conditions [56]. Spores are constantly receiving physical and chemical signals from their surrounding environment to determine if favorable conditions have returned so that they may germinate and survive [60]. If conditions are favorable, the endospores rapidly germinate, after which the dormancy and the resistance of the spores are lost. The outer coat of *B. subtilis* spores is made of thick proteins with a dense layer of specialized peptidoglycan called the cortex; if the cortex is formed properly, it helps with heat resistance [60]. The inner membrane of the spore is a permeable barrier that protects the core, the home to the cell's DNA, from any potentially damaging chemicals [61]. *Bacillus* species contain multiple mechanisms to protect the spore against various stresses [58]. Many species of *Bacillus*

contain UV absorbing pigments in their outer layers, which increase the resistance of the spore to ultraviolet (UV-C) radiation [57, 58].

B. subtilis is a primary surrogate species because it is not a major agent of food spoilage or diseases [62]. The non-pathogenic spores have been widely tested and reported by a large volume of literature, which serves as reference material for ongoing decontamination studies. Studies involving *B. subtilis* range from sterilization [63], superheating and steam resistance [64-66], ultraviolet radiation resistance [67, 68], and disinfectant testing [68 - 69]. Montville *et al*. [41] found that *B. subtilis* strains were a failsafe non-pathogenic surrogate for other thermal resistance studies. Coleman *et al*. [62] found that heat treatments damaged the spore proteins, which is a major factor for increasing the effective deactivation of spores. Zhou *et al*. [66] measured the differences in length and width of *B. subtilis* spores as heat and pulse times were increased and showed a 46% projected area decrease at the highest temperature of 570°C, which was related to an increased inactivation of spores. Stoeckel *et al*. [70] found that *Bacillus* spores were able to survive treatments up to 140°C for up to 33 seconds. Ghosh *et al*. [71] found that *B. subtilis* spores had a 1 and 0.05% viability for super dormant and normal spores, respectively, when steam treated for 60 seconds at 93°C. Warth [72] found that *B. subtilis* spores had a 1 log-10 reduction, which was equivalent to a 90% inactivation rate when heat treated as a liquid spore suspension at 110°C for 16 minutes. Popham *et al*. [73] found that a cultured strain of *B. subtilis* spores in water were significantly more heat sensitive and hydrogen peroxide sensitive in comparison to a wild strain. Rogers *et al*. [52] used *B. anthracis, B. subtilis,* and *Geobacillus stearothermophilus* spores using vaporous hydrogen peroxide to show significant differences in the decontamination efficacy of the hydrogen peroxide gas on porous and nonporous surfaces. An EPA study [35] found that for fumigation technology, *B. subtilis* spores were as resistant to decontamination as *B. anthracis* spores. *B. subtilis* and other bacteria species are also able to create spores with lower moisture content, which are labeled as super dormant spores. These spores are denser, germinate slower, and are more resistant to heat and chemical treatments [75-77].

An ozonated water study by Ramsey and Newman (unpublished data) evaluated the effects of the disinfectant on normal and super dormant *B. subtilis*. They found that the ozonated water for normal *B. subtilis* spores reduced the colony forming units by 99.65%, while for super dormant *B. subtilis* spores it was reduced to 89.87%. This study validated a study conducted by Ghosh [75], which showed that *B. subtilis* super dormant spores are more difficult to inactivate in comparison to normal spores.

It an attempt to determine why super dormant spores are more difficult to inactivate, samples of both spore types were photographed under a Transmission Electron Microscope (TEM) (Figures **1** and **2**). The images showed that the super dormant spore coats are thinner than the normal *B. subtilis* spore coats. Super dormant spores have a denser outer coat that increases spore resistance to heat and chemicals [75-77]. Super dormant spores germinate more slowly in comparison to normal spores and are more likely to survive environmental changes in which most germinating spores may be rendered inactive.

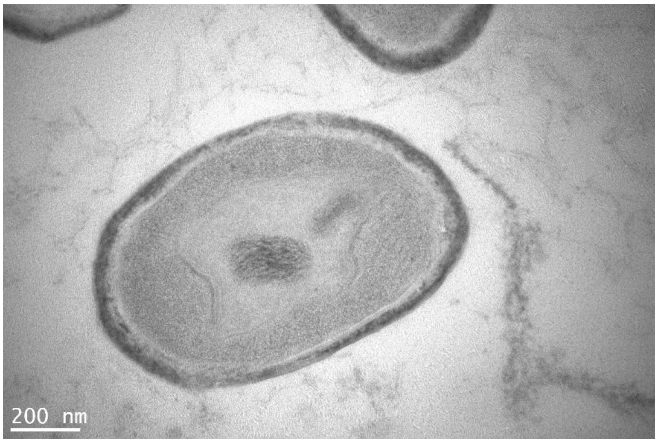

Figure 1: Transmission Electron Microscope (TEM) images of normal *Bacillus subtilis* spores at 200 nm scale.

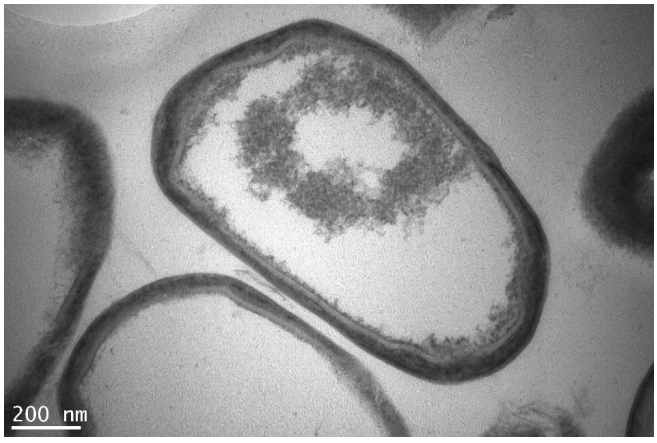

Figure 2: Transmission Electron Microscope (TEM) images of super dormant *Bacillus subtilis* spores at 200 nm scale.

2.4. *Cytisus scoparius*

Cytisus scoparius is a woody shrub, also known as Scotch Broom, and is a member of the

Fabaceaefamily, which includes beans, peas, alfalfa, and clover [13, 14]. It is considered an invasive perennial shrub that is native to Europe and North Africa, which may live for 20 or more years [15 - 16]. It is a fire adapted species and produces seeds with a high heat tolerance. Scotch broom seeds could be used as either dormant and non-dormant seed surrogates in heat treatments or fumigation tests in combination with spore surrogates. Tarrega *et al*. [17] found that Scotch Broom seed germination increased as air temperatures increased above 70 C. Herranz *et al.* [18] found that French Broom (*C. striatus*) seeds heated to 120 C and 150 C for 10 min. had a germination rate of 76% and 2%, respectively. Bossard [19] found that Scotch Broom seeds heated to 100 C and 150 C for 1 min. had a germination rate of 65% and 8%, respectively. These studies indicate that Scotch Broom seeds have a high heat resistance and therefore are potential surrogates for efficacy studies involving heat treatments. The use of multiple surrogates extends and enhances the overall evaluation of decontamination systems and methods.

Table 1: Biosafety Levels for the Potential *Bacillus anthracis* Surrogates. (Biodefense and Emerging Infections Research Resources Repository)

Species	Biosafety Level (BSL lab)
Bacillus anthracis Ames	BSL-3
Bacillus anthracis Sterne	BSL-2
Bacillus cereus	BSL-2
Bacillus megaterium	BSL-2
Geobacillius stearothermophilus	BSL-2
Bacillus atrophaeus	BSL-1
Bacillus thuringiensis	BSL-1
Bacillus subtilis	BSL-1

3. DISCUSSION

The use of *Cytisus scoparius* or Scotch Broom seeds as surrogates in heat treatment decontamination studies is not widely accepted. However, the seeds make exceptional secondary surrogates from fumigation or heat treatment studies. Scotch broom seeds are readily available, inexpensive and do not require any biosecurity level safety precautions. This makes them well suited for field studies that evaluate decontamination systems under real-world condition. Soil fumigation studies have evaluated fumigant treatments using plant seeds as surrogates for target weed species. *C. scoparius* seeds could be used in combination with other microbial surrogates in multi-surrogate studies to obtain advanced information on the effectiveness of a variety of fumigation or heat treatments, especially those in commercial settings.

Clostridium difficle is an endospore bacterium that is resistant to both heat and disinfectants. It can contaminate a wide range of fruits, vegetables, and meat products along with directly infecting humans and animals. Although *C. difficle* is widespread, due to its health risks, *C. difficle* disinfectant studies require a BSL-2 laboratory. The cost of conducting a basic disinfectant test using the Quantitative Disk Carrier Test Method with *C. difficle* samples and testing four disinfectants could reach as high as $12,0000 USD with an additional $3,0000 USD for each additional disinfectant lot tested.

Bacillus subtilis is a widely used non-pathogenic surrogate and is not a major agent of food spoilage [39]. *B. subtilis* spore cultures and strips are inexpensive and readily available with commercial spore strips costing approximately $250 to $270 USD for 100 strips. Working with *B. subtilis* or genetically similar species does not require a special laboratory setting, extra safety precautions, or specially trained staff due to their BSL-1 safety rating. Enveloped spore samples that are air permeable are available from private laboratories for conducting heat, steam, or fumigation efficacy studies. Several private microbiology labs offer services to prepare and assay *B. subtilis* samples to be used in field studies or evaluating on-site decontamination systems or facilities.

A unique feature of using surrogates in fumigation studies is that inoculated samples can be protected inside of gas permeable envelopes that allow fumigants to enter the envelope but also protect samples from any type of contamination [78-80]. Air permeable, Tyvek® envelopes are readily available for preventing cross contamination when testing surrogate samples under heavily soiled conditions, or in soil decontamination studies. Preventing cross contamination of samples sharply increases the accuracy of the assay methods.

Field studies or on-site evaluation of commercial systems require the use of non-pathogenic, surrogate samples that are inoculated with microbes that survive on media coated samples over extended time periods

(weeks to months). Laboratory studies use fresh surrogate samples, thereby minimizing the potentially low microbial recovery rates from long-term storage of field samples. The tradeoff between laboratory and field studies is that the highly controlled efficacy tests in labs often overestimate the effectiveness of decontamination treatments that are less reliable when applied under real world conditions. The most effective decontamination technologies often rely on integrated, multi-stage treatments to sanitize or sterilize surfaces, equipment, or facilities. Single treatment technologies that are highly effective such as vaporized hydrogen peroxide, ultraviolet light (UV-C), or hydroxyl generators, or any multi-stage, integrated system would necessitate surrogate species that are hard-to-kill, or partially resistant to multiple chemical/physical treatments, such as the three *Bacillus/Geobacillus* species.

Well-designed decontamination studies should include multiple surrogates to test a range of species, and/or test a range of microbial resistance to decontamination technologies. Field studies that combine surrogate species, such as *Bacillus* spores with heat resistant plant seeds, for evaluating heat, steam, or fumigation treatments would greatly enhance the study results with minimal added costs. Also, disinfectant efficacy studies could be easily designed with multiple surrogates, such as testing a *Bacillus* species using both vegetative cells and spore inoculated samples. Such multi-surrogate designs would provide a range of disinfectant resistance that would extend the efficacy results across several classes in the Spaulding Hierarchy chart. Finally, efficacy studies using a multi-surrogate design involving different spore species would provide resistance information about the genetic variation among several endospore forming species. Using multiple surrogates alleviates several concerns with choosing a single species that may not perform well under specific treatments or environmental conditions. Multi-surrogate designs generally are not more complicated to conduct or analyze, and they are less expensive than re-running a study that failed to show any valid results or showed no treatment effects.

The goal of choosing effective surrogates for field studies is to develop reliable decontamination methods that work under real world conditions. Practical consideration for selecting a surrogate species include availability, cost effective, ease of use, and correlation with the target pathogen or pest. However, the primary reason for selecting a surrogate for field or on-site studies is that the surrogate is not a health risk for humans, animals, and plants. An example is the use of *Bacillus thuringiensis* as a surrogate for *Bacillus anthracis*. *B. thuringiensis* is not pathogenic, is a biosafety level 1 (BSL1) agent, and is easily obtained.

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Received on 14-09-2020 Accepted on 20-10-2020 Published on 13-11-2020

DOI: https://doi.org/10.15377/2409-9813.2020.07.6