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Selecting A Biological Indicator for Sterilization Processes

Joseph Dunn

Performance Packaging, 811 Fentress Court, Daytona Beach, FL 32117

*Corresponding author: Joseph Dunn, Performance Packaging, 811 Fentress Court, Daytona Beach, Florida 32117, Tel: 815-979-9749; E-mail: jdunn@pplv.co

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Abstract

The selection of a biological indicator organism for use with a new or existing sterilization method is an important endeavor because it will serve as a benchmark for validation, safety, and quality assessments across a range of current and future sterilization systems and machines. By convention, the process for bioindicator selection is to choose an organism demonstrating high relative resistance for the sterilization agent and method under examination and, therefore, includes the evaluation and comparison of the resistance of multiple candidate organisms and spores. This report reviews the basic tenants and principles of modern sterilization science as they relate to the determination and comparison of microbial resistance, uses the pulsed light treatment of Bacillus pumilus spore preparations, and spore stocks prepared via different methods to illustrate how different preparation procedures can affect the results seen with resistance testing, particularly survival/end point testing, and provides insights and experiences into microbial spore resistance testing and examines relevant cases in point.

Introduction

The U.S., British, Japanese and the Pharmacopeia of the World Health Organization recommend bioindicators for use with a range of traditional sterilization technologies. For example, the spores of *Bacillus atropheous* ATCC 9372 [1] (formerly *Bacillus subtilus* strain niger variety globigii, or "Big" A in European repositories and laboratories) has for many decades been the recommended bioindicator for sterilization methods and systems using dry heat or a broad range of chemical sterilants such as hydrogen peroxide, ethylene oxide, or chlorine dioxide.

Occasionally reports appear proposing a recommended bioindicator for a new sterilization method, or challenging an earlier recommended bioindicator for an established technology and proposing that an alternative spore is more resistant (and by inference therefore, a more appropriate indicator organism). An example for consideration, Blakistone et al. [2] presents results of experiments using the peroxide based sterilization agent Oxonia Active (Ecolab) to inactivate a range of bacterial spores and suggests that *Bacillus cereus* spores represent a more appropriate relatively resistant bioindicator organism than *Bacillus atropheus*, i.e., *Bacillus subtilis* A spores, for use with peroxide based agents in quality control audits and challenge tests. It is important to evaluate the methods and procedures in this report before recommending wide acceptance of its conclusions.

The present review provides a general overview of appropriate methods for selecting a bioindicator organism of relatively high resistance for use with sterilization safety, quality assurance audits, and challenge testing.

Materials and Methods

Spore Preparations used in Pulsed Light Inactivation Tests

Two *Bacillus pumilus* ATCC 27142 spore preparations were used in the pulsed light results reported herein:

Preparation 1: Used for pulsed light treatment in these studies was obtained from Air Dispersions, Ltd. (Manchester, England and included passage through a Collison nebulizer as part of the preparation procedure;

Preparation 2: Employed for pulsed light tests this in report was prepared using conventional methods that included three cycles of water washes comprised of centrifugation and resuspension in sterile, distilled water.

Pulsed Light Treatment Equipment and Procedures

The pulsed light system used was fabricated by Pure Pulse Technologies (formerly of San Diego, CA) and included two lamps enclosed in reflector units with quartz windows. The pulsed light referred to is emitted by a tubular xenon-filled fused sapphire flash lamp (sapphire is a more robust envelope with a greater Young's modulus than quartz). The lamp is caused to flash by using pulsed power engineering technologies to convert alternating current line-voltage to a high voltage, high power direct current pulse. In this process, electrical energy is stored and concentrated in a capacitor over a relatively long time, i.e., seconds or tenths of a second, and then conducted through cabling and released across the electrodes of the flash lamp as a high power electrical pulse (of approximately 10,000 volts) a few hundred millionths of a second in duration (in this instance 125 μs). The electrical pulse ionizes the gas within the lamp to create a plasma that expands to fill the lamp. The wall of the lamp eventually confines the plasma and it becomes superheated by further electrical energization. During thermal ionization of the gas, outer shell electrons are stripped away and an intense flash of broad spectrum radiation is emitted. The spectrum of the emitted flash is equivalent to blackbody radiation source at a temperature of about ten thousands of degrees Kelvin, with superimposed xenon line-structure and extends from the ultraviolet to the infrared (the wavelengths emitted are largely controlled by the transmission properties of the lamp envelop). The process is very efficient in converting electrical to light energy with approximately 75% of the electrical energy into the lamp converted into light emission. The pulsed light employed here is a rich source of ultraviolet light with about 25% of the light in the ultraviolet and approximately 20% of the light residing in the 200-300 nm wavelength range. However, unlike most conventional ultraviolet sources, the emitted pulsed light is a high power flash (approximately 30,000 times brighter than sunlight at sea level) with a spectrum that is a continuum from the far ultraviolet, through the visible, and into the infrared.

The lamp/reflector units were located in a test stand in a horizontal position facing one another (Figures 1 and 2). This arrangement has advantages over the use of a single lamp reflector because it creates a reflective cavity that increases the incident energy and uniformity of the flash. The position of the lower lamp/reflector was fixed, while the upper unit could be raised or lowered by a screw arrangement. The relative distance between the two was variable over a range of 1-60 cm. A sample stage comprised of a quartz plate and holder was located between the two lamp/reflector units (one above and one below the sample stage). The position of the sample stage could also be adjusted to vary its distance relative to the pulsed light sources. A test sample could be treated from one side using only one lamp (by shielding the lower lamp/reflector unit with opaque material) or from both sides by simultaneously flashing both the upper and lower lamps. Digital linear scales were associated with the upper lamp/reflector unit and the sample stage to facilitate accurate determination of lamp and sample positions. The walls of the cabinet in which the test stand was located were flat black in order to reduce extraneous reflection.

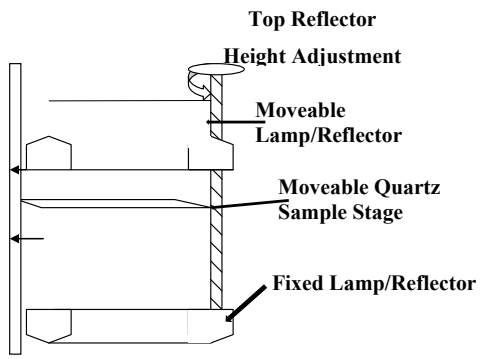

Figure 1. Schematic illustration of the pulsed light test stand.

Figure 2. The pulsed light test stand.

Pulsed light dose response studies were performed using a sample holder/treatment jig in which an aqueous microbial suspension was treated as a 1-mm deep fluid layer in a quartz sample holder (Figure 3). The quartz sample holder was designed to treat aqueous or saline microbial suspensions as a 1-mm thick layer and allow a direct comparison of the response of microbial suspensions with minimal interference from absorption or refraction artifacts inherent in many other treatment arrangements. Some small reflective losses of pulsed light energy are expected as the light transverses the interfaces between media (from air to quartz to water) and for this reason we have termed the effective fluence reaching the sample as nominal incident dose. The apparatus employed two 3-inch diameter flat quartz disks held 1-mm apart by spacers and a holder. The microbiological sample, generally 0.6-0.7 mL in volume, was injected and withdrawn using a positive displacement pipette. The approximate diameter of the treated droplet was less than about 4 cm. The droplet was centered in the apparatus, treated, and recovered for quantitative microbiological assays (plate counting). When performing tests on a particular microorganism preparation, a highest to lowest energy sequence of treatments was employed. Between samples, the quartz disks of the sample holder were rinsed with distilled water, hand-dried using nonabrasive laboratory wipes, and rinsed with alcohol and dried by evaporation.

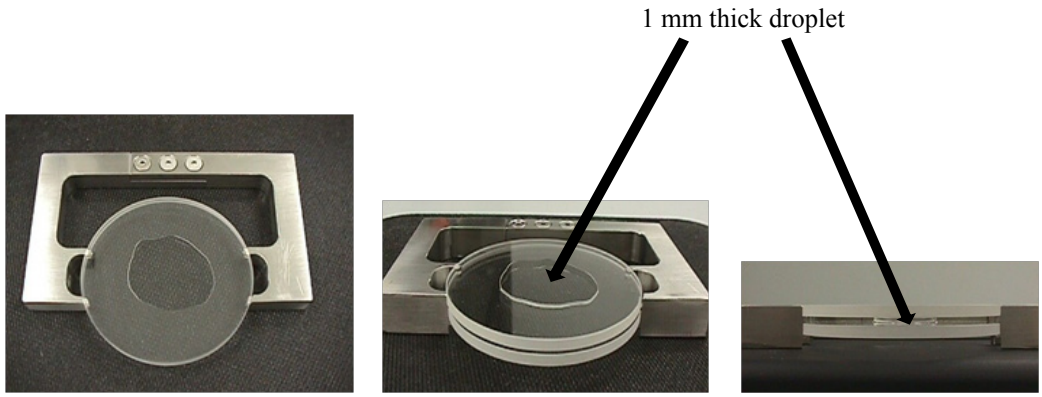

Figure 3. Pulsed light treatment test jig designed to treat aqueous suspensions of microorganisms as a 1 mm thick droplet held between two quartz disks.

The pulsed light energy measurements in this report are cited as total nominal full spectral fluence, or nominal incident radiant energy per square centimeter of surface area in J/cm^2, at the sample treatment plane. Fluence was measured using a thermopile calorimeter (9OSC-2, Ophir Optronics Ltd., Jerusalem, Israel)) to measure the incident light energy. The calorimeter was comprised of a detector head and a signal processor with digital display. The head assembly contains a flat carbon disk, one square centimeter in area, which acts as an efficient broad-spectrum light absorber of relatively flat spectral response. A temperature measurement device in intimate contact with the back surface of the carbon disk generates an electrical signal proportional to the amount of light energy absorbed by the carbon disk during each pulsed light flash. The response of the calorimeter assembly is calibrated using a Joule-source traceable to national standards.

The dose values used in the quartz sample holder studies represent the light energy at the sample position in the absence of the quartz sample holder and do not take into account light energy losses due to reflection or absorption within the sample holder itself. Such losses undoubtedly occur. When a disk from the quartz sample test jig holder is superimposed over the calorimeter, a mean 4.1% loss in energy measurements is observed. This loss is primarily reflective in nature, therefore, microorganism inactivation kinetics presented in this report are given as nominal incident light, in J/cm^2.

A Further Note on Pulsed Light Systems and Test Results

Pulsed light food processing, and surface disinfection and sterilization is studied in many laboratories on an international basis, and the U.S. FDA have approved the use of pulsed light for the treatment of foodstuffs [3]. Commercial systems are available, however, these systems vary widely in design, capabilities, and efficacy. Some of the pulsed light systems available for purchase and represented by reports in the literature, bear little and only superficial resemblance tothat reported on in this review, in Dunn [4,5,8], and Dunn et al. [6,7]. Many, but not all, of the pulsed light systems commercially available and reported in the literature more closely resemble strobe lights (such as Dr. Edgerton's strobesor larger lamp versions of the flash lamps used on the wingtips of aircraft) in energy and spectral output and qualify as only pulsed light due to the presence of a switching mechanism that enables the lamp to be turned on and off. It is to be emphasized that the Pure Pulse puled light systems used for the tests in this report was in several ways unique as compared to pulsed light systems used for dermatological applications (dermatology systems generally use broad spectrum white light with little to no content at wavelengths less than about 400 nm) and to some other systems sold for disinfection and the treatment of foods and the author is hopeful that some of the pulsed light testing reported here will be repeated by investigators using other systems for the purposes of comparison in order to better understand pulsed light system design and functionality.

The deficiencies inherent in some pulsed light commercial systems and literature reports relate to historic flash lamp industry convention and dogma. The shot-life of conventional flash lamps is proportional to how energetically they are driven (power into the lamp or Joules of energy into the lamp per flash duration); flash lamp life formulae exist and are commonly used by manufacturers in designing flash lamp systems, however, it is important to understand that such formulae are by definition for conventional flash lamps and that modifications and improvements in flash lamp design and construction can allow flash lamps to be operated at significantly higher powers and with significantly longer life than predicted by convention and these formulae.

Pure Pulse Technologies (no longer in business) designed and fabricated the pulsed light system used for some of the tests in this report. Pure Pulse carried out an extensive program to develop more robust, longer life flash lamps. This program successfully developed an advanced flash lamp and operating system that extended the lamp life and reliability of a system that conventional flash lamp dogma predicted a maximum life of about a few tens of thousands of flashes under the operating conditions employed to over one million flashes with three-sigma reliability and confidence. Some of the implemented lamp and system improvements included: 1) the use of lamp simmer circuitry, 2) lamps with fused sapphire envelops rather than quartz, and 3) specially designed electrodes (electrode sputtering plays a major role in limiting flash lamp life).

Because of the unique features of the pulser and lamps used in this report, the author is desirous to have other researchers attempt to duplicate the results shown herein with their pulsed light equipment. Any typical quartz lamp system generating a lamp plasma temperature of about ten thousand

degrees Kelvin should provide similar results treating test suspension as a 1mm thick layer at equivalent fluence. Though we used a two lamp system, we saw no significant change in the results using a single lamp treating from one side only at equivalent sample fluence.

Plating of Spore Preparations

Control and pulsed light treated spore preparations were serially ten-fold diluted in sterile saline and spread plated onto tryptic soy agar (TSA) followed by incubation for 48 hours at 35C.

Results and Discussion

Sterility Assurance

Early in the evolution of the canning industry, it was concluded that in order to assure commercial processes provided a high level of safety to the consuming public, a sterility assurance level (SAL) of 10^{-6} was necessary [8]. This involved assuring processing methods provided a statistical probability that not more than one viable microorganism would survive in one million processed cans.

In order to facilitate testing the efficacy of processing procedures, relatively resistant bioindicator microorganisms were selected and employed for challenge tests and auditing procedures. In such challenge tests and audits the inactivation of populations of the relatively resistant bioindicator microorganism was used to assure the inactivation of less resistant organisms and pathogens.

A corollary to these methods was that the chosen bioindicator was of relatively high resistance to potential pathogens in the processed product. For the canning industry the most resistant pathogen of concern were the spores of the bacterial pathogen *Clostridium botulinum* and thus, it was reasoned that the selected bioindicator should be of known high resistance to the applied process relative to the resistance displayed by *Clostridium botulinum* spores.

Dose Setting for Sterilization Technologies

Procedures were developed and are available for defining an appropriate process cycle for a new sterilization method. These procedures use both quantitative assays, i.e., dose response count reduction assays to define the kinetics of inactivation across a range of greater than or about 10^6 colony forming units (CFU) to about 30 CFU (below which plate count methods lose statistical validity); and qualitative assays, i.e., end point or fraction negative quantal determinations, treating a number of replicate samples at a particular dose or a range of doses and subsequently determining the number of samples capable of survival and growth (Figure 4). For statistical relevance in end point and fraction negative quantal assays the meaningful results are seen in fractionally negative sample sets (some units positive and some units negative) occurring between sets treated at higher dose levels and demonstrating no survival (all units negative) and sample sets treated at a lower dose and demonstrating 100% survival (all units positive).

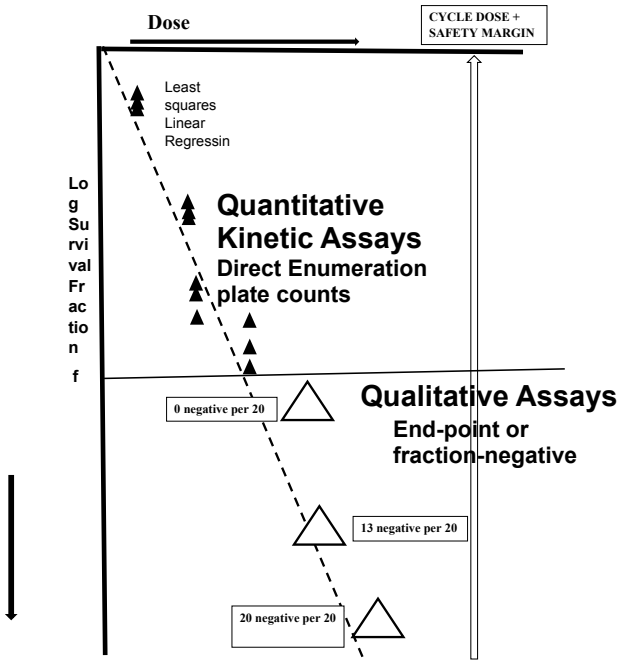

Figure 4. Schematic illustration of how quantitative count reduction assays and quantal fraction negative assays are used to determine a sterilization cycle dose (plus some safety factor) for a new sterilization process.

Tailing during Disinfection and Sterilization

It is generally expected that quantal assays should and will follow and continue the dose response kinetics observed in quantitative assays. However, quite often end point or fraction negative quantal assays exhibit a significant deviation in dose response as compared to the results predicted by quantitate kinetic assays extended. This phenomenon is commonly termed "tailing" in the literature [10,11]; tailing is a widely observed phenomenon and commonly attributed to clumping in a preparation. Gross clumping can be observed using microscopic examinations, however, it is often difficult to quantitate lesser degrees of clumping that can significantly impact end point survival testing but may represent a feature present in only 1 out of 104-107 total particles.

Figure 5 shows the results treating with pulsed light a *Bacillus pumilus* ATCC 27142 [12] spore preparation (Preparation 1, prepared using "normal" spore preparation methods followed by passage through a Collison nebulizer) in multiple replicate experiments on different occasions using quantitative plate count methods. The results show 7 logs or greater of *B. pumilus* spore inactivation at nominal incident doses greater than about 4-5 J/cm^2. A regression line and its equation is shown for each occasion and a regression and equation is shown for all the occasions combined (bold line and boxed equation). The large plus signs (+) on the chart indicate samples yielding no survivors and are positioned at the negative value corresponding to the positive value of the logarithm of the inoculation level in the referenced test series; note that this spore preparation (Preparation 1) yielded no survivors on multiple occasions. This spore population was prepared using conventional procedures augmented with passage through a Collison nebulizer in order to provide a spore population relatively free of clumped spores (see Collison Nebulizer below).

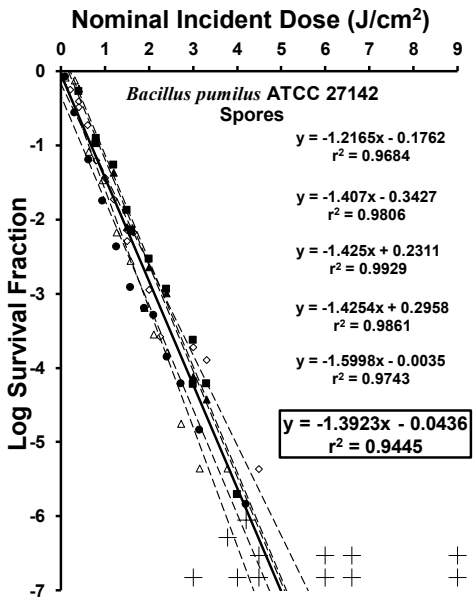

Figure 5. Experimental determination of the dose response of Bacillus pumilus spores (Preparation 1) to pulsed light treatment. The results of quantitative count reduction kinetic assays performed on multiple occasions are shown (the heavier regression line includes all the separate instances treated as a single sample set). The crosses denote samples showing no survival and are plotted as the negative logarithm of the inoculation level.

A second *Bacillus pumilus* spore preparation (Preparation 2) was prepared using only the conventional methods employing multiple water washes encompassing centrifugation and resuspension. This second preparation showed similar quantitative dose response kinetics to the first preparation through about 4-5 logarithm cycles of inactivation, however, it then exhibited tailing with little change in activation level or in in quantal end point fraction negative assays.This preparation did not show 7 logarithm cycles of inactivation until about a nominal accumulated incidence fluence of 30 J/cm^2 was used, and in quantal fraction negative assays using an inoculation level of 106 CFU treatment levels in excess of a nominal accumulated incidence fluence of 45 J/cm^2 or greater were required to yield 6 logs of inactivation (no growth in 20 of 20 replicate samples inoculated at a 10^6 CFU level).

Discussion of Conventional Spore Population Preparation Procedures

The methods employed for conventional spore preparation [13,14] normally involve collecting in aqueous suspension the growth from the surface of media a mature spore crop displaying a large number of phase contrast refractile spores. This suspension is then washed multiple times, often three times, by relatively low speed centrifugation (that will sediment spores and cellular, sporangia, and debris) and resuspension in sterile distilled water. Enzyme treatments with lysozyme or trypsin are sometimes employed to augment autolytic digestion within the sporangium. Different microorganisms vary in the degree to which autolysins are active in the dormant or dying host cell after sporulation and thus, some sporulating organisms are said to be "cleaner" sporulaters than others. The initial centrifugations accompanying the washing of *Bacillus pumilus* spore preparations yield a small white button of spores covered with a thick, viscous, "snotty", over-layer composed largely of cell bodies, cytoplasm, and other cellular debris. Although a large number and percentage of the spore total is trapped in this over-layer, it is this over-layer that must be separated from the spores; my technicians would commonly turn the centrifuge tubes top down on lab paper in order to allow the viscous layer extra time to ooze away before resuspension of the spore pellet for the next wash. I would also note that many technicians inexperienced in the preparation of spore crops will intentionally resuspend the entirety of each centrifugation without attempting to separate the over-layer, thinking that everything that sediments contains valuable spores; such preparations are inherently clumpy and contain much extrasporular debris.

Common laboratory dogma suggests that clumps are produced during the centrifugations associated with the spore harvesting and washing procedures and that spores clump, stick together, or are multiply trapped in cytoplasmic debris adhering to the surface of the spores or in the pelleting mass.

Besides clumping, a second artifact sometimes found in spore preparations is the presence of a large number of nonviable spores (a poor total particle to viable particle ratio). I once worked with a microbiologist that claimed to be able to grow and prepare spore crops with exceptional high resistance to hydrogen peroxide. It was noted in the laboratory that the inoculation site of a dried 10 μL droplet containing 10^7 CFU of our preparation of the same spore was difficult to detect and required examination at the right angle to the light, whereas this other microbiologist's preparation looked like dried heavy cream under the same conditions. Microscopic examinations showed heavy clumping and comparison of hemocytometer spore counts with spore plate counts showed more than one thousand non-plating spores per CFU.

A Method for Declumping Some Spore Preparations

Both of the Bacillus pumilus spore preparations (Preparation 1 and Preparation 2) treated with pulsed light in the experiments detailed above were prepared using conventional centrifugation and water wash methods, however, after these procedures Preparation 1 was nebulized by passage through a Collison nebulizer and collected by impingement into water and not resubmitted to centrifugation.

The Collison Nebulizer

The Collison nebulizer [15,16] produces an extremely fine mist comprised of droplets with a mean diameter of about one micron. It uses air pressure to force fluid through a series of jet atomizers directed towards the walls of the nebulizer vessel. Larger droplets travel to the vessel walls, coalesce, and run back into the initial fluid pool, however, very small droplets lack the inertial energy to reach the vessel walls and are instead swept out of the nebulizer with the exiting air. The violent shear forces and droplet size selection processes in a Collison nebulizer break up spore clumps and only singlet spores exit the nebulizer and are released [17]. It must be noted that this Collison nebulization procedure should not be expected to solve the problems in grossly problematic and poor spore preparations and such preparations will likely block and clog the atomization jets in the nebulizer.

Quantal assays performed without the benefit of being accompanied by the results of quantitative kinetic assays can lead to misinformation and erroneous conclusions because quantal assays are particularly sensitive to, and influenced by artifacts within a spore preparation, i.e., spore clumping. Spore clumps within a preparation plate as a single CFU, however, inactivating a clump of spores presents an elevated challenge to a sterilization agent in terms of protecting biomass and inhibition of agent penetration. This is apparent in the results treating the two different *Bacillus pumilus* spore preparations: (Preparation 2 produced using centrifugation and water washes only, and Preparation 1 produced using these same methods followed by passing the preparation through a Collison nebulizer to break up and remove spore clumps).

Regarding Blakistone et al. [2] It is important to focus on the methods employed in this earlier research report. It is firstly important to note the differences in the medium used to produce the individual microbial spore crops used in this study differed from strain to strain in a manner that could affect their response to peroxide treatment; of particular note is the difference in the media used for the sporulation of *Bacillus atropheus* (*Bacillus subtilis* A) and that used for the sporulation of *Bacillus cereus*. The medium used in the production of *Bacillus atropheus* spores was supplemented with excess iron (iron sulfate (0.005%). Iron is a transition metal long known to catalyze and accelerate the release of the oxidative activity of peroxides via the Fenton reaction [18] discovered in 1894. It is quite possible, and indeed highly likely, that extra and elevated levels of iron associated with the spore coat and envelope of the *Bacillus atropheus* spore preparation used in this study may have contributed an enhanced sensitivity to peroxide treatment [19,20]. It is for reasons such as this that I prefer not to grow spore crops on special "sporulation" media as I have long had a concern and suspicions that such media could modify the natural susceptibility/resistance of the spores produced. In my experience there seems really no need for exotic media as the strains I have worked with all seem to sporulate quite well on nutrient agar, tryptic soy agar or the like as the culture ages and the medium dehydrates.

It is interesting to note that Blakistone et al. [2], themselves note in the Results section of their report under the heading Morphological Examinations:

> "An inoculating loopful of *B. cereus* from the stock suspension added to a droplet of 2% Oxonia on a glass slide yielded some interesting visual results. Clumping was quite obvious to the unaided eye in the presence of the chemical. Under the light microscope, Oxonia-treated B. cereus spores were seen in pairs and in groups, including at least 1 large clump (Figures 1A and 1B). *B. subtilis* A did not react similarly. Spores remained evenly dispersed."

The authors clearly understand some of the implications of this extensive clumping, seen only with their B. cereus spore preparation, however, it is not mentioned in the Abstract but only late in the Results section and again in the Discussion section regarding their several attempts to minimize clumping, none of which, by their own admission, proved effective; nevertheless, it does not enter into their considerations of relative spore resistance or their reasoning that *B. cereus* is the most resistant of the spores tested. They neglect, however, to discuss the probable effects of this phenomenon on their reported relative resistance of the spore strains included in their report. This is a most important oversight.

The authors of the referenced report do perform some testing they suggest could be effective at minimizing clumps in their B. cereus spore preparation:

> "The effect of Oxonia on this organism did not appear to be related to cell concentration or aided by the addition of Tween. To eliminate the possibility of clumping, microporous filters were used to separate individual spores during challenge tests and 0.1% Tween 80 was added to Oxonia during testing. Unfortunately, none of these approaches proved effective."

It is informative I think to examine and discuss these methods further. The authors report they tried 0.1% Tween 80 to reduce clumping or break up clumps, however the detergent was added to the Oxonia solution prior to the addition of the *B. cereus* spore preparation and it seems they did not try adding the Tween to the spores prior to their addition to the Oxonia.

Another method the authors of the referenced report tried to minimize the effects of clumping was to dilute the spore preparation to 80-200 CFU per mL and inoculate onto 0.45 μm microporous membranes and then test the membranes. This method would only be expected to be effective if clumps only exist within the preparation at higher levels. It also presents the spores on a material surface or within channels with which the peroxide might also react.

The deficiencies in the report cited extend beyond a lack of understanding of the influence of the potential effects of the different media employed but extend to a failure of the experimental methods to integrate both quantitative and quantal assays. The report references the results of end point assays only. This is an unfortunate oversight as the experiments performed at lower doses (time of exposure) surely could have provided insight into the individual quantitative kinetic dose response properties of the spores studied, but were instead were discarded and/or not reported.

As already discussed, the reliance on end point assays only produces the effect of skewing the results and conclusions toward an emphasis on an artifact in the preparation, clumping, that does not correspond to the true relative resistance of spores but relates strongly to the response of each spore population to the spore crop production and concentration methods employed.

Summary

The two methods employed for characterizing the dose response of a spore population to a sterilization agent or for examining the resistance of a particular spore strain or characterizing the resistance of one spore strain to another are reviewed. Quantitative count reduction assays are used to determine the dose-response of the spore preparation down to counts of about 30 survivors and qualitative assays (Quantal assays) are used to determine the response of the spore preparation thereafter. It is important that both assay methods are employed. Reliance on end point or fraction negative quantal assays alone leads to erroneous conclusions due to the inordinate emphasis that such quantal assays place upon an artifact in the spore preparation (clumping) that is very dependent upon the methods employed in production of the spore preparation(s).

An earlier publication in the literature is also reviewed in this report. This often referenced case in point is of interest to review because of the potential for the methods employed to lead to erroneous conclusions. The noted oversights in the methods employed include:

1) Growth of one of the test strains to be used in hydrogen peroxide containing sterilants on ferrous sulfate supplemented media. Iron salts are long well known to catalyze the degradation of peroxides via the Fenton Reaction.

2) The reliance on quantal end point assays alone in this earlier report for determining the relative resistance/sensitivity of a number of spore strains to sterilants.

3) The experiments performed could have been easily used to obtaining quantitative dose response estimates, however, the appropriate assays were either not performed or the results discarded.

4) The authors note extreme clumping in one spore population upon treatment but do not let this observation inhibit their ranking of this spore strain as displaying the highest relative resistance and suggesting that the spores of this strain should replace a long accepted indicator strain. It is of note that the historically accepted bioindicator is the same spore they produced using medium supplemented with iron for testing with peroxide containing sterilants.

5) This extreme clumping of the B. cereus spore preparation when added to the sterilant strongly suggests this preparation is faulty and contains excessive amounts of cytoplasmic and cell debris. This clumping was not noted with the other spore strains tested, only with the spore preparation they rank as the most resistant.

6) They cite multiple attempts they performed using methods they cite as designed to overcome or negate clumping; these methods, by their own observation, were ineffective.

Acknowledgements

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