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BIOLOGICAL INDICATORS

Understanding Biological Indicator Grow-Out Times

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In the mid 1980s, the US Food and Drug Administration's Center for Devices and Radiological Health (CDRH) published a protocol for establishing a reduced incubation time (RIT) less than seven days that focused on the last surviving spore. This protocol has not been widely accepted outside the United States. The data collected in this study are being submitted to facilitate ISO development of a globally accepted RIT protocol. This study used BIs containing 10^5 *Geobacillus stearothermophilus* spores and a new technology that continuously monitors incubated BIs and records nonsterile (growth-positive) results on a minute-by-minute basis, which allowed for a deeper understanding of spore germination and generation capabilities. Analysis of nearly 4000 data points indicated that there is an inverse relationship between the number of surviving spores on a BI and the overall incubation time needed for a nonsterile result to be detected.

Nonsterile results in BIs with several hundred surviving spores appear normally distributed. As surviving spores per BI decrease, variability and grow out time increase, and BIs that contained only one viable spore exhibit the longest grow-out times.

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Biological indicators (BIs) are used globally to monitor the efficacy of sterilization processes for medical products (1,2,3,4,5). BIs contain high numbers—generally 10^4 to 10^6—of bacterial endospores that are highly resistant to the sterilization process for which they are designed. The spores are placed on or in a carrier material such as paper and packaged to protect the BI before as well as after sterilization and before culturing. BIs are useful for validation and routine monitoring of ethylene oxide, steam, dry heat, low temperature steam formaldehyde, vapor hydrogen peroxide, and related plasma sterilization systems.

In the United States, BIs sold for use in healthcare facilities are considered medical devices and are regulated by the US Food and Drug Administration good manufacturing practices (GMPs) as promulgated in the Quality System Regulation of 1996 (6). BIs sold to manufacturers of sterile medical products are considered manufacturing components. Regardless of the intended use, BIs must be manufactured under carefully controlled conditions to assure quality, reproducibility, and predictable performance (7).

Reducing biological indicator incubation time and the existing FDA protocol

How much incubation time is required to provide sufficient information to allow the user to make a correct decision regarding the acceptability of the sterilization process? In the mid-1980's, the FDA Center for Device and Radiological Health (CDRH) issued a protocol (hereafter termed the RIT [reduced incubation time] protocol) for BI manufacturers and users to follow to reduce the incubation time for BIs from the normal seven days to a shorter time based on the results of testing defined in the protocol (8). The RIT protocol requires manufacturers to expose 100 BIs from each of three lots to separate sterilization processes; the results of the exposure to the sterilization processes must result in 30 to 80 nonsterile BIs from each lot after incubation at the appropriate conditions for seven days. Achieving this outcome requires carefully-controlled reduced lethality (fractional) sterilization runs

for which highly specialized sterilization equipment is often used.

To determine a reduced incubation time for a BI/sterilization process according to the RIT protocol, the number of nonsterile BIs for each of the three lots is recorded after periodic reviews up to seven days of incubation; the number of nonsterile BIs at seven days is considered "100%." The number of nonsterile BIs at incubation times less than seven days is compared with the respective seven-day value, and a percentage is calculated; the qualified reduced incubation time is the longest time, for one or more of the three lots, to first exhibit a number of nonsterile BIs that is ≥ 97.0% of that observed after seven days of incubation. A typical example appears in Table I with the BIs being scored daily.

As required, for each of the three lots tested, the number of nonsterile BIs after seven days of incubation was greater or equal to 30 and less than or equal to 80. For lots 2 and 3, the 97.0% criterion was met after one day of incubation; for lot 1, however, the 97.0% criterion was met after two days of incubation. Based upon these results, a reduction of incubation time from seven to two days is acceptable.

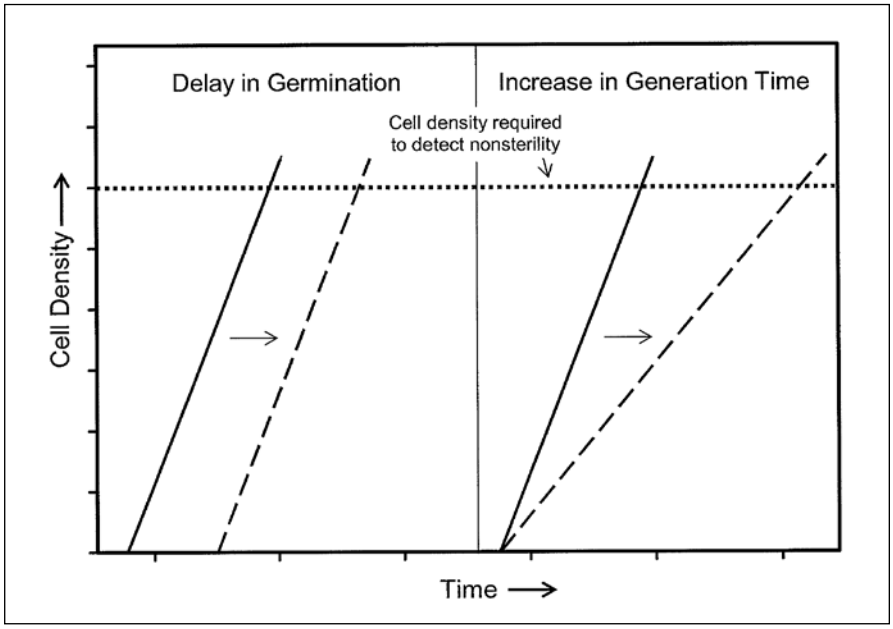

Figure 1: Effects of delayed germination and increased generation time on the detection of a positive test.

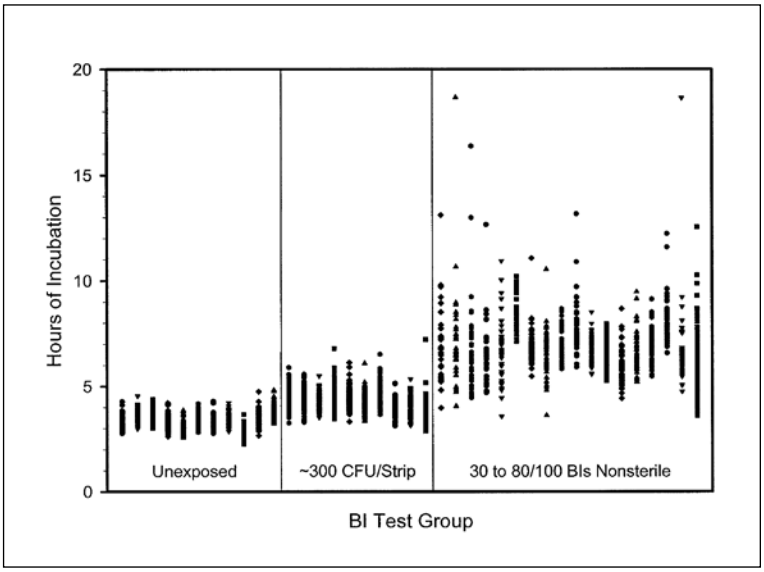

Figure 2: Vertical scatter plots of incubation time of biological indicators (BIs) tested.

Application of the RIT protocol

Both users and manufacturers of BIs have voiced concerns about several aspects of this approach to qualifying a reduction of incubation time for BIs:

- The 30 to 80 "window" for the number of nonsterile BIs is too narrow; identifying sterilization conditions that yield such results is difficult. Often, numerous sterilization runs must be performed on a given lot to provide results that fall within this window.
- The 97.0% criterion is arbitrary and does not take into consideration the distribution of grow-out times observed for a given BI lot and the sterilization conditions used in the protocol-specified testing.
- Industry users of BIs have repeatedly challenged the necessity for protracted incubation times. Longer incubation times for BIs do not, de facto, make medical products more safe and clearly result in higher costs for users of BIs.

This RIT protocol has not been widely accepted outside the United States. The International Standards Organization (ISO) BI working group of TC198 (WG04) has been directed to develop a new RIT protocol that will be acceptable to the global community (9).

Data presented in this paper are based on *Geobacillus stearothermophilus* self-contained BIs that have been exposed to fractional moist heat sterilization processes. We present these data for the development of a new approach to qualify a reduced incubation time based upon a detailed analysis of grow-out times for numerous lots of BIs exposed to fractional moist heat sterilization processes.

The wording of the current RIT protocol suggests that it only applies to BI manufacturers unless the user desires to apply the product in a manner other than that stated by the manufacturer. This protocol has been applied to users by some regulatory auditors and notified bodies. In such cases, users are required to expose BIs in specific product loads in production scale sterilizers. Successful execution of the RIT protocol in a production sterilizer is extremely difficult since the outcome of the sterilization exposure must result in at least 30 and no more than 80 BIs having surviving spores; i.e. nonsterile. It is important that the exposure of BIs for an RIT study be performed in a manner that ensures that they are statistical replicates. In a small research vessel, each of the 100 BIs is exposed to nearly identical sterilization conditions; in a production-scale sterilizer, there can be significant variability in exposure conditions. The less uniform conditions in the production-scale sterilizer, particularly when performing a reduced lethality exposure, can result in larger variability in the number of surviving colony-forming units (CFU) among the nonsterile BIs.

The application of the RIT protocol in this manner suggests that the sterilizer and the associated load of product in some way influence the grow-out time of spores exposed to a fractional sterilization process. There are no published data to support the notion that the molecular-level action of the sterilizing agent on the spore is influenced by the size

of the sterilizer, the presence of product, or process variations that affect the rate of spore inactivation.

We should emphasize that inactivation of microorganisms by sterilizing agents generally follows first-order kinetics (10, 11). In the case of BIs, spore death is the result of the destruction of a critical molecule or chemical reaction such that the spore can not develop into a replicating vegetative form (12, 13, 14). The longer a spore is exposed to a sterilization process without a lethal event, the more collateral (nonlethal) damage can occur. Collateral damage could affect the time required to complete germination and/or the time required for the vegetative cells to divide (generation time). Such germination- and generation-time effects could influence the time required to have a visible/chemical indication that a given BI had one or more surviving spores after exposure to a sterilizing agent.

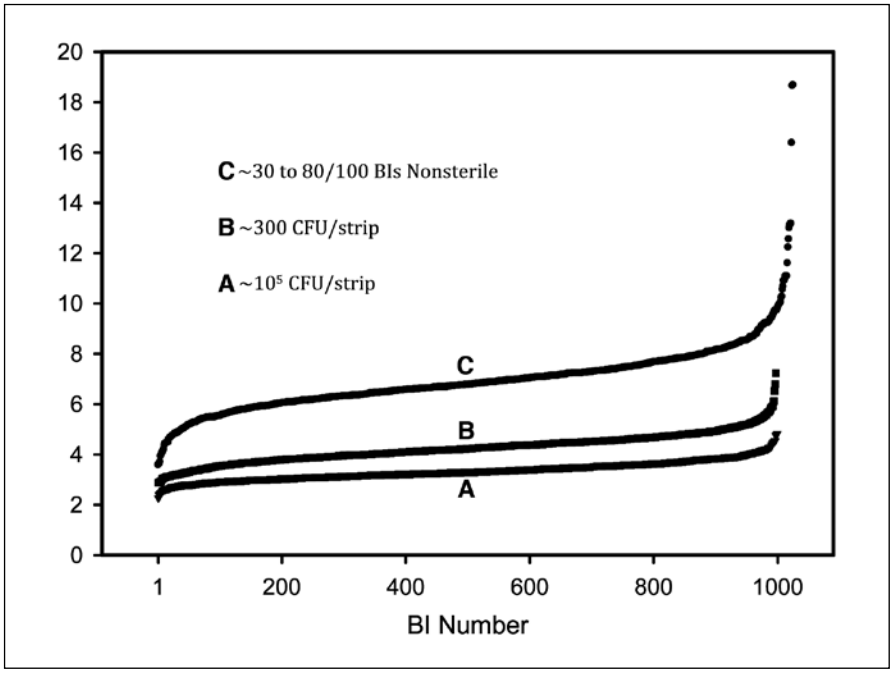

Figure 3: Horizontal scatter plots of incubation time for the three ranges of surviving biological indicators.

Definitions

D-value. Time or dose required to achieve inactivation of 90% of a population of the test microorganism under stated dose conditions (15).

Biological indicator (BI). A test system containing viable microorganisms providing a defined resistance to a specified sterilization process (15).

Most probable number (MPN). The estimated population of maximum likelihood responsible for producing the observed combination of positive and negative outcomes (16).

Sterility assurance level (SAL). the probability of a single viable microorganism occurring on an item after sterilization. Note that the term SAL takes a quantitative value, generally 10^{-6} or 10^{-3}. When applying this quantitative value to assurance of sterility, a SAL of 10^{-6} has a lower value but provides a greater assurance of sterility than a SAL of 10^{-3} (15, 17).

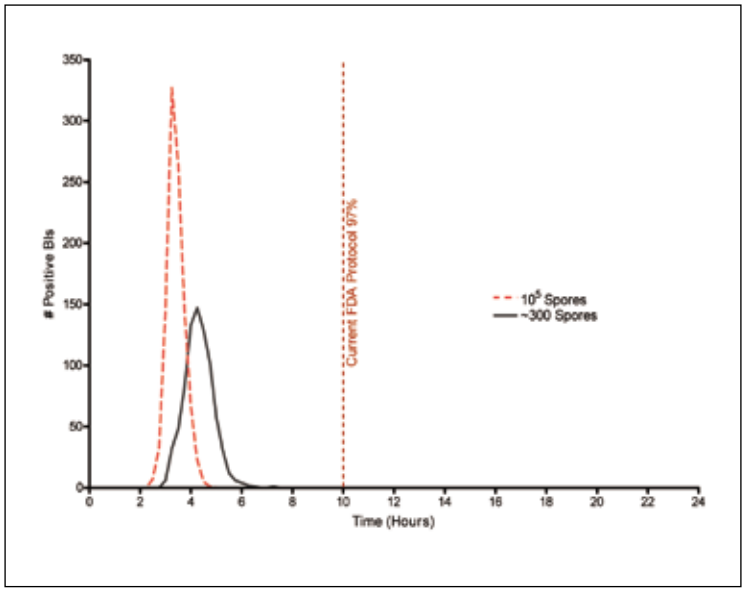

Figure 4: Incubation times for biological indicators with 10^5 and 300 spores.

MPN considerations

The science of sterilization and disinfection has received considerable attention in the last 100 years (10, 11, 13, 18). Its importance was initially linked to an understanding of prevention and transmission of disease. Later, the consistent production of quality products such as wine, milk, and milk products became the emphasis. Significant strides were made in the last century in defining and predicting rates of microbial inactivation using the concept of decimal reduction values or D-values (18–20). As the science of sterilization was refined, it became necessary to be able to predict the surviving numbers of microbes as their population approached zero. Thus the probabilistic nature of microbial lethality and first order kinetics has become a well recognized applied science. One method of predicting low numbers of surviving microbes is the MPN approach of Halvorson and Ziegler (16). MPN gives an estimate of the average number of surviving spores per BI.

MPN is calculated using the formula:

$$\text{MPN} = \ln (n \div r)$$

where n is the total number of units tested, and r is the number of sterile units.

The MPN approach can be applied to all fraction negative-type data (number tested/number nonsterile) as long as at least one of the replicate BIs is nonsterile. The MPN approach is generally applied over the range of 1% to 99% sterile BIs. The experimental design must ensure that all these BIs are statistical replicates.

The validity of the MPN approach for determination of inactivation rates and D-values has been affirmed by comparing results obtained by the survivor curve method (13, 19). D-values determined by the latter method generally agree well with those determined by the MPN approach.

The MPN approach is valuable because it allows prediction of the distribution of surviving spores over a wide range of fraction negative-type data. Table II lists the average number of surviving spores over the range of 99% to 1% nonsterile determined by the MPN approach.

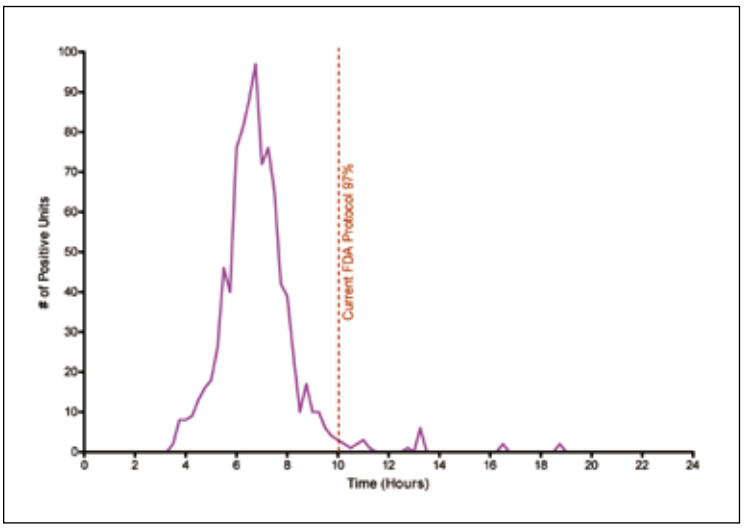

Figure 5: Summary of all biological indicator incubation times yielding 30–80 positives/100 units tested.

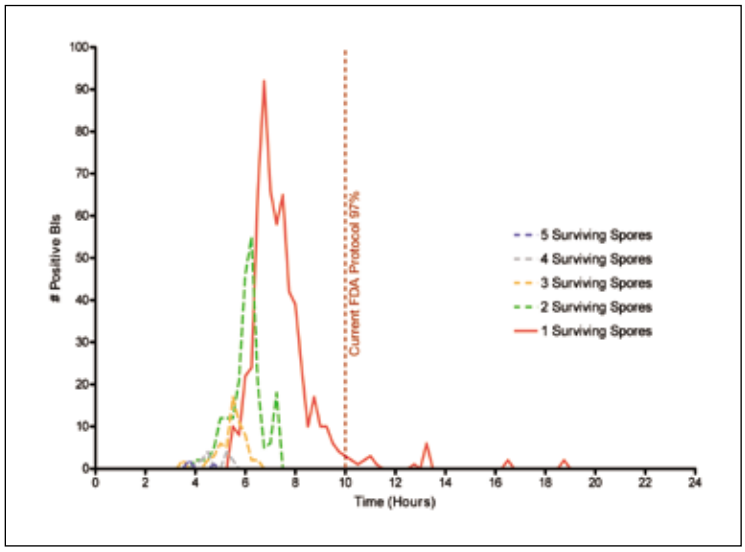

Figure 6: Biological indicator (BI) incubation times with approximately 1, 2, 3, 4, and 5 spores per BI.

Also listed, for each average number of surviving spores, is the number of nonsterile BIs with 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10, surviving spores determined by a Poisson distribution calculation based upon the given average number.

For a set of 100 BIs exposed to a sterilization process where only one BI is found to be sterile, the average number of surviving spores is 4.605 as determined by the MPN calculation. In the cases of 80 and 30 nonsterile BIs out of 100 tested, the average number of survivors, as would be expected, is lower at 1.609 and 0.357, respectively. The cases of 80 and 30 nonsterile BIs per 100 tested are particularly relevant to a discussion of the RIT protocol.

If a set of 100 BIs is exposed to a sterilization process and 80 of the BIs are nonsterile, it is clear that 20 of the BIs have "0" surviving spores. The MPN calculation gives an average number of surviving spores of 1.609 or a total of ~161 spores distributed across the 80 nonsterile BIs. The Poisson distribution analysis shows that ~32 of the BIs would be expected to have only one surviving spore and ~2 BIs would be expected to have five surviving spores. In the case where only 30 nonsterile BIs were found after an exposure to a sterilization process, 70 would have 0 spores and ~25 would be expected to have one surviving spore. In this case, a BI with five surviving spores would be very unlikely.

Germination and growth considerations

Out growth of a nonsterile BI is a function of:

- Initiation of spore germination
- Conversion to the cell form
- Cellular metabolism
- Cell division and multiplication.

Cell division and multiplication are required for identification of a nonsterile BI whether the determination of nonsterility is based upon the visual detection of turbidity and/or a metabolic reaction that may result in, for example, a change in pH. Visual detection of turbidity requires cellular metabolism and abundant growth of the microorganisms in the medium. For this growth to occur, initiation of germination, conversion to the cell form, cellular metabolism, and cell division must first occur. As illustrated in Figure 1, the time required to first discern that a BI is nonsterile, by turbidity or pH change, is a function of both the time it takes for a spore to germinate and become a cell form and the cell division or generation time. As can be seen (left panel), if the time for a spore to germinate and turn into a cell form is delayed by 1 hour but the generation time is unaffected, the time delay for detection of nonsterility would also be delayed by 1 hour. If the generation time of the cell form is affected (right panel), the delay in the time to detect nonsterility is related to the magnitude of the increase in the generation time.

Visual detection of turbidity due to microbial growth requires on average a cell density greater than 10^6 cells/mL with smaller cells requiring a somewhat higher number. Table III gives the number of generations required to attain greater than 10^6 cells/mL for starting numbers of cells ranging from 1 to 10^5; bacterial growth is by binary fission, therefore, each generation is a doubling of the population. Approximately 20 generations (doublings) are required for one cell to provide detectable turbidity; i.e. greater than 10^6 cells/mL. The time for one cell to produce detectable turbidity would, therefore, be 20 times the generation time. BIs that have been exposed to an ineffective sterilization process, with most or all BIs having a large number of surviving spores, will yield visible turbidity in a sterility test sooner than those exposed to a process that yields a low percentage of nonsterile BIs with most having 0 or one surviving spore.

Considerable research has been conducted with different sterilization methods that demonstrate that the processes can adversely impact spore germination (21). One could predict that for stressed populations of spores, delayed outgrowth would generally be the result of longer germination times rather than longer generation times. To result in the latter, a process must inflict an inherited mutation producing a rate-limiting effect on reproduction without it being a lethal mutation. However, many adverse effects on the spore coat, spore cortex, or ionic constitution, for example, have been shown to delay germination without resulting in longer generation times of the offspring (22). Therefore, merely on the basis of probability, one may conclude that most outgrowth delays result from delayed germination time.

Targeting an average number of one surviving spore per BI appears to be a crucial aspect of the RIT protocol; the 30 to 80 nonsterile BI

"window" provides a MPN average of 0.357 to 1.609 spores per BI. Therefore, the protocol is suited to accomplish its intended purpose. When exposure to a sterilization process yields a BI with a single viable but possibly damaged spore, variations in grow out time can be ascribed to the metabolic/genetic constitution of that spore. However, if multiple spores are present on an exposed BI, the resulting nonsterile outcome will be the result of the most rapid out-grower in the group. This is true whether the spore driving the nonsterile outcome does so by faster germination and conversion to a cell form, shorter generation time, or a combination of both. The fact that the outgrowth characteristics of individual spores can be masked when multiple spores survive on a given BI is important for the subsequent discussion here; BIs that exhibit prolonged grow-out times likely have one surviving but damaged spore.

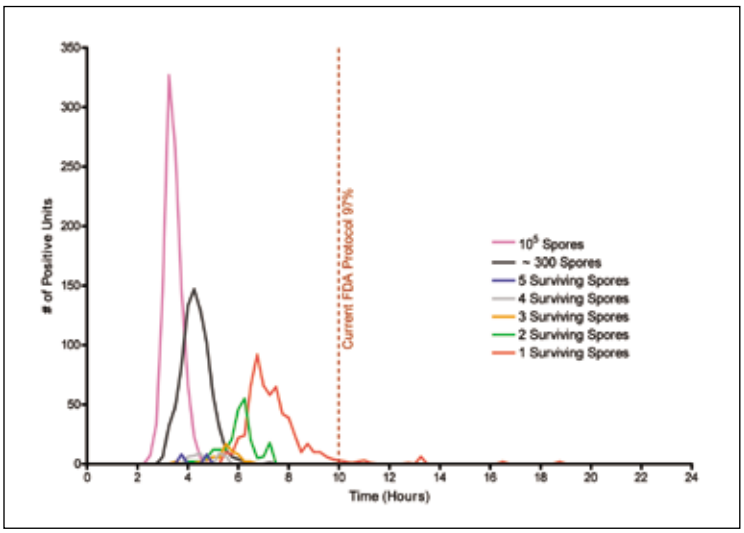

Figure 7: Summary of biologicial indicator incubation for units from 10^5 to 1 spore.

Analysis of biological indicator incubation grow-out times

The following data were generated to form a basis for the analysis of BI grow-out time following exposure to a moist heat sterilization process; the analysis presented here is based on the results from testing approximately 4,000 BIs. Self-contained BIs (EZTest, SGM Biotech) inoculated with approximately 10^5 *Geobacillus stearothermophilus* spores were used.

Groups of 100 replicate BIs were exposed to a moist heat sterilization process designed to result in sterilization of only a fraction of the BIs with a target of 30 to 80 of the BIs per group to be nonsterile. For each group of 100 BIs, the average number of surviving spores was calculated by the MPN approach, and the number of BIs with 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 spores was estimated by a Poisson distribution analysis.

Table II gives the average number of surviving spores per BI and the Poisson distribution analysis for all possible experimental outcomes from 1 to 99 nonsterile BIs.

Table I : Example of data necessary to meet the FDA CDRH criteria of determining a reduced incubation time of less than 7 days.

Lot Number	Number of BIs Nonsterile (% of Day 7 Value)						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
1	60 (95.2%)	62 (98.4%)	63 (100%)	63 (100%)	63 (100%)	63 (100%)	63 (100%)
2	42 (100%)	42 (100%)	42 (100%)	42 (100%)	42 (100%)	42 (100%)	42 (100%)
3	73 (98.6%)	74 (100%)	74 (100%)	74 (100%)	74 (100%)	74 (100%)	74 (100%)

Materials and methods

A series of experiments were performed using an ISO 18472-compliant resistometer (23). Self-contained BIs produced from four separate *Geobacillus stearothermophilus* spore crops were tested. The four spore crops, ATCC #7953, were produced over a span of seven years. Fourteen distinct manufacturing lots of BIs were produced from these spore crops; these lots were produced over a span of approximately 18 months. All BI lots and resistance testing results met the requirements of ISO 11138-1:2006, 11138-3:2006, and ANSI/AAMI ST79:2006 (4, 7, 24). For the sterilization exposures, BIs were selected randomly from the 14 production lots.

Groups of BIs were tested in the following manner:

1. Nine groups of 100 and two groups of 50 BIs were tested for sterility with no exposure to the moist heat sterilization process.

2. Ten groups of 100 BIs were exposed to a moist heat sterilization process targeted to result in approximately 300 surviving spores per BI. This number of surviving spores was chosen because it approximates the outcome of a "survival time" exposure as defined in USP 32 (5).

3. Eighteen groups of 100 BIs were exposed to moist heat sterilization processes targeting results of 30 to 80 nonsterile BIs per 100, the "window" in the RIT protocol.

SGM's Smart-Well system (60 + 2° C) was used to incubate the BIs and detect color change in the BI growth medium. When growth was detected, the system recorded the incubation time as hours:minutes; e.g. 2:53. All BIs in these experiments were incubated for a total of seven days.

Results

Grow-out time. The data presented in the left panel of Figure 2 give the results for the 11 sets of BIs that were not exposed to moist heat sterilization prior to incubation; this set had a population of approximately 10^5 CFU/BI at the start of incubation. The first nonsterile BI was recorded at 2:16; the last nonsterile BI was recorded at 4:33.

The data presented in the center panel of Figure 2 give the results for the 10 sets of 100 BIs exposed to moist heat sterilization processes that resulted in approximately 300 surviving CFU/BI at the start of incubation. The first nonsterile BI was recorded at 2:53; the last nonsterile BI was recorded at 7:14 (see Figure 4).

The data presented in the right panel of Figure 2 give the results for all 18 sets of 100 BIs exposed to moist heat sterilization processes, where 30 to 80 nonsterile BIs were observed per 100 tested. At the start of incubation, the number of surviving CFU ranged from 0 to ~6/BI. The first nonsterile BI was recorded at 3:35; the last nonsterile BI was recorded at 18:41. These data are arranged in increasing order with respect to the absolute number of nonsterile BIs per test group. The left-most data set in the panel reflects 33 nonsterile BIs; the right-most data set had 80 nonsterile outcomes.

Scatter plots of all of the grow-out times for each of the three sets of BIs are shown in Figure 3.

Table II: Most probable number of surviving spores per biological indicator.

# Nonsterile	#Sterile	#Tested	MPN Avg	Number of Surviving Microorganisms											Sum
				0	1	2	3	4	5	6	7	8	9	10	
99	1	100	4.605	1.0	4.6	10.6	16.3	18.7	17.3	13.2	8.7	5.0	2.6	1.2	99.2
98	2	100	3.912	2.0	7.8	15.3	20.0	19.5	15.3	10.0	5.6	2.7	1.2	0.5	99.8
97	3	100	3.507	3.0	10.5	18.4	21.6	18.9	13.3	7.7	3.9	1.7	0.7	0.2	99.9
96	4	100	3.219	4.0	12.9	20.7	22.2	17.9	11.5	6.2	2.8	1.1	0.4	0.1	99.9
95	5	100	2.996	5.0	15.0	22.4	22.4	16.8	10.1	5.0	2.1	0.8	0.3	0.1	100.0
94	6	100	2.813	6.0	16.9	23.7	22.3	15.7	8.8	4.1	1.7	0.6	0.2	0.1	100.0
93	7	100	2.659	7.0	18.6	24.8	21.9	14.6	7.8	3.4	1.3	0.4	0.1	0.0	100.0
92	8	100	2.526	8.0	20.2	25.5	21.5	13.6	6.9	2.9	1.0	0.3	0.1	0.0	100.0
91	9	100	2.408	9.0	21.7	26.1	20.9	12.6	6.1	2.4	0.8	0.3	0.1	0.0	100.0
90	10	100	2.303	10.0	23.0	26.5	20.3	11.7	5.4	2.1	0.7	0.2	0.1	0.0	100.0
89	11	100	2.207	11.0	24.3	26.8	19.7	10.9	4.8	1.8	0.6	0.2	0.0	0.0	100.0
88	12	100	2.120	12.0	25.4	27.0	19.1	10.1	4.3	1.5	0.5	0.1	0.0	0.0	100.0
87	13	100	2.040	13.0	26.5	27.1	18.4	9.4	3.8	1.3	0.4	0.1	0.0	0.0	100.0
86	14	100	1.966	14.0	27.5	27.1	17.7	8.7	3.4	1.1	0.3	0.1	0.0	0.0	100.0
85	15	100	1.897	15.0	28.5	27.0	17.1	8.1	3.1	1.0	0.3	0.1	0.0	0.0	100.0
84	16	100	1.833	16.0	29.3	26.9	16.4	7.5	2.8	0.8	0.2	0.1	0.0	0.0	100.0
83	17	100	1.772	17.0	30.1	26.7	15.8	7.0	2.5	0.7	0.2	0.0	0.0	0.0	100.0
82	18	100	1.715	18.0	30.9	26.5	15.1	6.5	2.2	0.6	0.2	0.0	0.0	0.0	100.0
81	19	100	1.661	19.0	31.6	26.2	14.5	6.0	2.0	0.6	0.1	0.0	0.0	0.0	100.0
80	20	100	1.609	20.0	32.2	25.9	13.9	5.6	1.8	0.5	0.1	0.0	0.0	0.0	100.0
79	21	100	1.561	21.0	32.8	25.6	13.3	5.2	1.6	0.4	0.1	0.0	0.0	0.0	100.0
78	22	100	1.514	22.0	33.3	25.2	12.7	4.8	1.5	0.4	0.1	0.0	0.0	0.0	100.0
77	23	100	1.470	23.0	33.8	24.8	12.2	4.5	1.3	0.3	0.1	0.0	0.0	0.0	100.0
76	24	100	1.427	24.0	34.3	24.4	11.6	4.1	1.2	0.3	0.1	0.0	0.0	0.0	100.0
75	25	100	1.386	25.0	34.7	24.0	11.1	3.8	1.1	0.2	0.0	0.0	0.0	0.0	100.0
74	26	100	1.347	26.0	35.0	23.6	10.6	3.6	1.0	0.2	0.0	0.0	0.0	0.0	100.0
73	27	100	1.309	27.0	34.4	23.1	10.1	3.3	0.9	0.2	0.0	0.0	0.0	0.0	100.0
72	28	100	1.273	28.0	35.6	22.7	9.6	3.1	0.8	0.2	0.0	0.0	0.0	0.0	100.0
71	29	100	1.238	29.0	35.9	22.2	9.2	2.8	0.7	0.1	0.0	0.0	0.0	0.0	100.0
70	30	100	1.204	30.0	36.1	21.7	8.7	2.6	0.6	0.1	0.0	0.0	0.0	0.0	100.0
69	31	100	1.171	31.0	36.3	21.3	8.3	2.4	0.6	0.1	0.0	0.0	0.0	0.0	100.0
68	32	100	1.139	32.0	36.5	20.8	7.9	2.2	0.5	0.1	0.0	0.0	0.0	0.0	100.0
67	33	100	1.109	33.0	36.6	20.3	7.5	2.1	0.5	0.1	0.0	0.0	0.0	0.0	100.0
66	34	100	1.079	34.0	36.7	19.8	7.1	1.9	0.4	0.1	0.0	0.0	0.0	0.0	100.0
65	35	100	1.050	35.0	36.7	19.3	6.7	1.8	0.4	0.1	0.0	0.0	0.0	0.0	100.0
64	36	100	1.022	36.0	36.8	18.8	6.4	1.6	0.3	0.1	0.0	0.0	0.0	0.0	100.0
63	37	100	0.994	37.0	36.8	18.3	6.1	1.5	0.3	0.0	0.0	0.0	0.0	0.0	100.0
62	38	100	0.968	38.0	36.8	17.8	5.7	1.4	0.3	0.0	0.0	0.0	0.0	0.0	100.0
61	39	100	0.942	39.0	36.7	17.3	5.4	1.3	0.2	0.0	0.0	0.0	0.0	0.0	100.0
60	40	100	0.916	40.0	36.7	16.8	5.1	1.2	0.2	0.0	0.0	0.0	0.0	0.0	100.0
59	41	100	0.892	41.0	36.6	16.3	4.8	1.1	0.2	0.0	0.0	0.0	0.0	0.0	100.0
58	42	100	0.868	42.0	36.4	15.8	4.6	1.0	0.2	0.0	0.0	0.0	0.0	0.0	100.0
57	43	100	0.844	43.0	36.3	15.3	4.3	0.9	0.2	0.0	0.0	0.0	0.0	0.0	100.0
56	44	100	0.821	44.0	36.1	14.8	4.1	0.8	0.1	0.0	0.0	0.0	0.0	0.0	100.0
55	45	100	0.799	45.0	35.9	14.3	3.8	0.8	0.1	0.0	0.0	0.0	0.0	0.0	100.0
54	46	100	0.777	46.0	35.7	13.9	3.6	0.7	0.1	0.0	0.0	0.0	0.0	0.0	100.0
53	47	100	0.755	47.0	35.5	13.4	3.4	0.6	0.1	0.0	0.0	0.0	0.0	0.0	100.0
52	48	100	0.734	48.0	35.2	12.9	3.2	0.6	0.1	0.0	0.0	0.0	0.0	0.0	100.0
51	49	100	0.713	49.0	35.0	12.5	3.0	0.5	0.1	0.0	0.0	0.0	0.0	0.0	100.0
50	50	100	0.693	50.0	34.7	12.0	2.8	0.5	0.1	0.0	0.0	0.0	0.0	0.0	100.0
49	51	100	0.673	51.0	34.3	11.6	2.6	0.4	0.1	0.0	0.0	0.0	0.0	0.0	100.0
48	52	100	0.654	52.0	34.0	11.1	2.4	0.4	0.1	0.0	0.0	0.0	0.0	0.0	100.0
47	53	100	0.635	53.0	33.6	10.7	2.3	0.4	0.0	0.0	0.0	0.0	0.0	0.0	100.0
46	54	100	0.616	54.0	33.3	10.3	2.1	0.3	0.0	0.0	0.0	0.0	0.0	0.0	100.0
45	55	100	0.598	55.0	32.9	9.8	2.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	100.0
44	56	100	0.580	56.0	32.5	9.4	1.8	0.3	0.0	0.0	0.0	0.0	0.0	0.0	100.0
43	57	100	0.562	57.0	32.0	9.0	1.7	0.2	0.0	0.0	0.0	0.0	0.0	0.0	100.0
42	58	100	0.545	58.0	31.6	8.6	1.6	0.2	0.0	0.0	0.0	0.0	0.0	0.0	100.0
41	59	100	0.528	59.0	31.1	8.2	1.4	0.2	0.0	0.0	0.0	0.0	0.0	0.0	100.0
40	60	100	0.511	60.0	30.6	7.8	1.3	0.2	0.0	0.0	0.0	0.0	0.0	0.0	100.0
39	61	100	0.494	61.0	30.2	7.5	1.2	0.2	0.0	0.0	0.0	0.0	0.0	0.0	100.0
38	62	100	0.478	62.0	29.6	7.1	1.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	100.0
37	63	100	0.462	63.0	29.1	6.7	1.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	100.0
36	64	100	0.446	64.0	28.6	6.4	0.9	0.1	0.0	0.0	0.0	0.0	0.0	0.0	100.0
35	65	100	0.431	65.0	28.0	6.0	0.9	0.1	0.0	0.0	0.0	0.0	0.0	0.0	100.0
34	66	100	0.416	66.0	27.4	5.7	0.8	0.1	0.0	0.0	0.0	0.0	0.0	0.0	100.0
33	67	100	0.400	67.0	26.8	5.4	0.7	0.1	0.0	0.0	0.0	0.0	0.0	0.0	100.0
32	68	100	0.386	68.0	26.2	5.1	0.7	0.1	0.0	0.0	0.0	0.0	0.0	0.0	100.0
31	69	100	0.371	69.0	25.6	4.8	0.6	0.1	0.0	0.0	0.0	0.0	0.0	0.0	100.0
30	70	100	0.357	70.0	25.0	4.5	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
29	71	100	0.342	71.0	24.3	4.2	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
28	72	100	0.329	72.0	23.7	3.9	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
27	73	100	0.315	73.0	23.0	3.6	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
26	74	100	0.301	74.0	22.3	3.4	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
25	75	100	0.288	75.0	21.6	3.1	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
24	76	100	0.274	76.0	20.9	2.9	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
23	77	100	0.261	77.0	20.1	2.6	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
22	78	100	0.248	78.0	19.4	2.4	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
21	79	100	0.236	79.0	18.6	2.2	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
20	80	100	0.223	80.0	17.9	2.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
19	81	100	0.211	81.0	17.1	1.8	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
18	82	100	0.198	82.0	16.3	1.6	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
17	83	100	0.186	83.0	15.5	1.4	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
16	84	100	0.174	84.0	14.6	1.3	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
15	85	100	0.163	85.0	13.8	1.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
14	86	100	0.151	86.0	13.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
13	87	100	0.139	87.0	12.1	0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
12	88	100	0.128	88.0	11.2	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
11	89	100	0.117	89.0	10.4	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
10	90	100	0.105	90.0	9.5	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
9	91	100	0.094	91.0	8.6	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
8	92	100	0.083	92.0	7.7	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
7	93	100	0.073	93.0	6.7	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
6	94	100	0.062	94.0	5.8	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
5	95	100	0.051	95.0	4.9	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
4	96	100	0.041	96.0	3.9	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
3	97	100	0.030	97.0	3.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
2	98	100	0.020	98.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
1	99	100	0.010	99.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0

Table III: Generations required to achieve greater than 1 million cells with starting cell numbers 1 to 100,000.

# of generations	Vegetative cells at start								
	1	3	5	7	9	100	1,000	10,000	100,000
1	2	6	10	14	18	200	2,000	20,000	200,000
2	4	12	20	28	36	400	4,000	40,000	400,000
3	8	24	40	56	72	800	8,000	80,000	800,000
4	16	48	80	112	144	1,600	16,000	160,000	1,600,000
5	32	96	160	224	288	3,200	32,000	320,000	
6	64	192	320	448	576	6,400	64,000	640,000	
7	128	384	640	896	1,152	12,800	128,000	1,280,000	
8	256	768	1,280	1,792	2,304	25,600	256,000		
9	512	1,536	2,560	3,584	4,608	51,200	512,000		
10	1,024	3,072	5,120	7,168	9,216	102,400	1,024,000		
11	2,048	6,144	10,240	14,336	18,432	204,800			
12	4,096	12,288	20,480	28,672	36,864	409,600			
13	8,192	24,576	40,960	57,344	73,728	819,200			
14	16,384	49,152	81,920	114,688	147,456	1,638,400			
15	32,768	98,304	163,840	229,376	294,912				
16	65,536	196,608	327,680	458,752	589,824				
17	131,072	393,216	655,360	917,504	1,179,648				
18	262,144	786,432	1,310,720	1,835,008					
19	524,288	1,572,864							
20	1,048,576								

As can be seen in Figures 2 and 3, the BIs that were not exposed to moist heat sterilization (left panel of Figure 2 and bottom plot of Figure 3) exhibited the shortest overall grow-out times (time recorded by the incubator system when growth was detected) with relatively little intra- or inter-lot variability.

When the BIs were exposed to moist heat sterilization in a manner that resulted in ~300 surviving CFU/BI (center panel of Figure 2 and center plot of Figure 3), the overall grow-out times were somewhat increased with an apparent increase in intra-lot variability. The cause of the increase in grow-out time appears to be related to the lower number of surviving spores at the start of incubation. These BIs began incubation with ~300 CFU versus the ~10^5 CFU for the unexposed BIs, a difference of approximately nine generations.

The BIs that were exposed to moist heat sterilization with an outcome of 30 to 80 nonsterile BIs/100 exposed (right panel of Figure 2 and top plot of Figure 3) exhibited grow-out times that again increased with a concomitant large increase in inter- and intra-lot variability. As above, the increase in overall grow-out time is due to the larger number of generations required to produce enough of a pH change to be scored as nonsterile. The large increase in variability is undoubtedly related to the large number of BIs in each test group that would be expected to have only one surviving CFU with a great potential to be impaired with respect to speed of germination and/or generation time (see Figure 5).

MPN and poisson distribution analysis

The results from the moist heat sterilization exposures where 30 to 80 nonsterile BIs were observed per 100 tested were further analyzed using MPN estimate of the average number of surviving CFU and the Poisson distribution prediction shown in Table II. Using the assumption discussed above, for the outcome of each sterilization exposure, a predicted number of surviving CFU was assigned to each observed grow-out time; the highest number of surviving CFU predicted in the Poisson distribution analysis was assigned to the shortest observed grow-out times. The next lowest number of surviving CFU was again assigned to the shortest remaining grow-out times; this process was repeated until all predicted values of surviving CFU > 1 were assigned. The remaining grow-out times were then all assigned a value of one surviving CFU. For example, the lot of BIs that had 80 nonsterile BIs after exposure would be predicted to have approximately two BIs with five CFU, six BIs with four CFU, 14 BIs with three CFU, 26 BIs with two CFU, 32 BIs with only one CFU, and the remaining twenty BIs with no viable CFU. Figure 6 illustrates the BI grow-out times for exposed BIs with five to one CFU predicted. A summary of these data are given in Figure 7 and Table IV.

The first nonsterile outcome recorded from BIs starting incubation with approximately 10^5 spores was 2:16. This was 37 minutes faster than the time for the first nonsterile BI from the group of BIs starting incubation with ~300 spores. This was three hours and three minutes faster than any BI predicted to have one spore. The time between the first positive and the last positive in each group of BI increases as the predicted population decreases.

Discussion

First, it is clear that there is an inverse relationship between the number of surviving CFU on a BI and the overall grow-out time. This was predicted because the time required to attain a detectable cell density and/or cumulative metabolic activity requires less time when the starting level of viable CFU is higher than when it is lower. That is, a cell

Table IV: Grow-out times for biological indicators (BI) with varying numbers of CFU determined by enumeration (10^5) or Prediction Based upon poisson distribution analysis.

CFU/BI	Time					
	First nonsterile BI	50%	68% 1 standard deviation	95% 2 standard deviations	99% 3 standard deviations	100%
~10^5	2:16	3:15	3:25	3:54	4:13	4:33
~300	2:53	4:14	4:29	5:11	5:44	7:14
5	3:37	4:45	5:29	6:35	6:35	6:35
4	3:56	5:05	5:29	6:55	6:55	6:55
3	3:35	5:39	5:56	7:08	7:10	7:14
2	3:59	6:10	6:35	7:32	7:46	7:48
1	5:19	7:13	7:42	9:34	13:00	18:41

density of 10^6 cells/mL can be attained in half the time for a starting population of 10^3 cells/mL compared with a starting population of 1 cell/mL, germination and generation times being equal.

Second, the grow-out times appear to follow a normal distribution for BIs with several hundred surviving CFU; BIs with few surviving CFU have more highly variable grow-out times with a measurable percentage exhibiting relatively long times before being scored as nonsterile. Of the ~1000 nonsterile BIs in the group where 30 to 80 of 100 were nonsterile after exposure, 10 BIs, or ~1%, exhibited a prolonged grow-out time (> 11 hours).

Third, the data support the hypothesis that delayed out-growth of BIs is observed and limited to situations where a significant number or the majority of exposed BIs have only one surviving CFU.

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