Heads or Tails

Statistical Methods for Interpreting Multiple Biological Indicator Results

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One of the main advantages of using an isolator for an aseptic processing application is that it creates a physical separation between the operator and the aseptic workspace. Another main advantage is that an isolator can be completely sealed before aseptic work begins, allowing for the exposed interior surfaces within to be biodecontaminated, typically, via hydrogen peroxide vapor or mist.

When isolators were first introduced in the pharmaceutical industry, the validation of the biodecontamination process was largely patterned after methodology used for steam sterilizer validation. Biological indicators (BIs), typically containing over one million microbes per sample, were used to challenge the biodecontamination process. BIs for use with isolators were most often prepared with spores of *Geobacillus stearothermophilus*, because this organism is known to be very resistant to the hydrogen peroxide decontamination process, and by coincidence, the same organism is used for steam sterilization validation. The typical validation approach involved distributing numerous BIs within the interior space and load items of a system, including in worst case locations where kill is expected to challenging. Complete kill of the BIs was usually expected for process qualification to be considered successful.

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Article at a Glance

- ★ Multiple biological indicators are frequently used for isolator validations
- ★ Most Probable Number calculation frequently used for estimating log reduction
- ★ Binomial distribution calculations can also be used to interpret results

tive BI result during the initial qualification or annual requalification. Following such occurrences, maintenance procedures, such as cleaning the vaporizer or injection needles, were conducted on the hydrogen peroxide vapor generator. If the next decontamination cycle obtained complete kill of the BIs, the maintenance procedure was indicated as the corrective action.

Rogues One Reason for Positive Results

Over time, isolator operators began to realize that these occasional BI issues were not caused by maintenance issues. Instead, they started to suspect that some lots contained a very low percentage of "rogue" BI's—individual samples with very high resistance compared to the rest of batch. Rogues can be caused by large clumps of spores, debris, scratches on the surface of the carrier substrate, etc. These samples may be more prone to have some spores survive a hydrogen peroxide decontamination process that relies on direct surface contact than similarly prepared samples exposed to a more penetrating process like steam sterilization.

One must recognize that not all positive BIs that occur during qualifications are rogues. When positive BIs occur, an investigation must be made to make sure the decontamination cycle conformed to the expected cycle parameters, SOPs were followed, etc. If the investigation does not reveal a probable cause, a rationale is required for interpreting the occurrence of an occasional positive BI. PDA Technical Report No. 51 ***(1),*** describes the situation thusly: "Current industrial experience indicates that occasional positive BIs occur even in well-defined cycles. Such rogue results may not be indicative of a failed cycle. Appropriate statistical methods may be used to support the acceptance of such rogue results in both primary validation and revalidation programs." Statistical methods used to interpret BI results require more than one sample per location.

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PIC/S recommendations on isolators ***(2)*** describe the limitations of using single BIs for validation: "If there is only one BI in each position, and only growth/no growth is established, then the number of survivors is unknown and the size of the possible variation in the process cannot be estimated." The document describes approaches using single or duplicate BIs in each location. The surviving number of spores on exposed BIs can be estimated using serial dilutions and counting colonies on media plates, or statistical interpretation of growth/no growth of aliquots of broth. This approach is labor intensive and may not be practical when large numbers of locations are being tested. The PIC/S document goes on to describe the approach that has become common place today: "Another possibility is to place three or more BIs at each position in the isolator and put them individually into broth for incubation. If there are any positive broths, the proportion of positive to negative can be used to estimate the number of survivors and thus the log reduction."

Microbiologists Must Do the Math

The Most Probable Number (MPN) calculation can be used as the basis for estimating the log reduction of spores obtained when using multiple BIs for validation. This method is probably the most familiar one to microbiologists, as the calculation is also used to estimate the initial population of organisms in a sample analyzed by serial dilution techniques. The MPN calculation has different uses in various fields of applied microbiology. This method is alluded to in the previous quotation from the PIC/S guide ***(2).*** The method uses the 1933 Halvorson-Zieglar equation ***(3).*** The equation estimates the most probable number of surviving organisms when multiple BIs are used and a mixture of positive and negative results is obtained.

$$MPN = \ln(^{n}/_{r})$$

Where:

MPN = Most Probable Number of surviving organisms

n = number of replicate BIs at each discrete test location

r = number of growth negative BIs at each test location

The MPN calculation can then be used for estimating Spore Log Reduction (SLR).

$$SLR = \log(N_0) - \log(MPN)$$

Where:

SLR = Spore Log Reduction

$\mathbf{N_0}$ = Initial spore population of the non-exposed BIs

An example follows for the results from triplicate BIs with an initial population of 2×10^6 that have results of one growth positive sample and two growth negative samples:

$$SLR = \log(2 \times 10^6) - \log(\ln 3/2) = 6.69$$

The probability of obtaining cumulative results from series of individual tests with yes/no outcomes is characterized by a binomial distribution. A well-known example of a yes/no outcome is a coin toss which has a 50% chance of yielding a "heads" result and a 50% chance of yielding a "tails" result. The possible outcomes of three coin tosses in a row are: 3 heads, 1 tail and 2 heads, 2 tails and 1 head, or 3 tails. The probabilities of these results occurring are shown in **Table 1.**

The application of a binomial distribution to the fractional results of triplicate BIs is only slightly more complicated; the odds of getting positive or negative BI results are not 50–50 because of the natural variability from sample to sample. As the average number of surviving spores approach zero during a lethality ➤

Table 1 Possible Outcomes of Three Coin Tosses

Total Results	Possible Outcome	Probability Calculation	Probability	Total Probability
3 Heads	H H H	0.5 × 0.5 × 0.5	12.5 %	12.5 %
1 Tails, 2 Heads	T H H	0.5 × 0.5 × 0.5	12.5 %	37.5 %
	H T H	0.5 × 0.5 × 0.5	12.5 %	
	H H T	0.5 × 0.5 × 0.5	12.5 %	
2 Tails, 1 Heads	T T H	0.5 × 0.5 × 0.5	12.5 %	37.5 %
	T H T	0.5 × 0.5 × 0.5	12.5 %	
	H T T	0.5 × 0.5 × 0.5	12.5 %	
3 Tails	T T T	0.5 × 0.5 × 0.5	12.5 %	12.5 %
				100 %

Table 2 Possible Outcomes of Three 2×10^6 BIs Exposed to a 6-log Decontamination Process

Total Results	Possible Outcome	Probability Calculation	Probability	Total Probability
3 Negatives	- - -	0.135 × 0.135 × 0.135	0.25 %	0.25 %
1 Positive, 2 Negatives	+ - -	0.865 × 0.135 × 0.135	1.58 %	4.74 %
	- + -	0.135 × 0.865 × 0.135	1.58 %	
	- - +	0.135 × 0.135 × 0.865	1.58 %	
2 Positives, 1 Negative	+ + -	0.865 × 0.865 × 0.135	10.12 %	30.36 %
	+ - +	0.865 × 0.135 × 0.865	10.12 %	
	- + +	0.135 × 0.865 × 0.865	10.12 %	
3 Positives	+ + +	0.865 × 0.865 × 0.865	64.65 %	64.65 %
				100 %

process, the number of spores on individual samples approach a Poisson Distribution *(4),* which is used to describe probability when the average outcome of an event can be calculated and the results of individual events don't influence each other. In this case, the "event" is exposing a BI to a lethal process and the result is the number of viable spores surviving. The average number of surviving viable spores on a BI exposed to a lethal process can be calculated as follows:

$$m = 10^{(\log N_0 - t/D)}$$

Where:

m = Average number of surviving spores after exposure time

$\mathbf{N_0}$ = Initial spore population of the non-exposed BIs

t = exposure time

D = D-value

The average number of surviving spores on a BI with an initial population of 2×10^6 that is exposed to a 6-log decontamination process (t/D=6) is calculated as follows:

$$m = 10^{(\log(2{,}000{,}000)-6)} = 2$$

The probability that various quantities of spores will survive in BI individual samples can be estimated based on the Poisson model when the overall average is known *(4).* The general formula is as follows:

$$P(a) = \frac{m^a}{a!} e^{-m}$$

Where:

P(a) = The Poisson probability that of the quantity of microorganisms exists in a given sample

a = The number of organisms in a specific sample

If complete kill of an individual BI sample is obtained, no spores survived and a=0, in which case the formula simplifies to:

$$P(0) = e^{-m}$$

The probability of obtaining complete kill of a BI that is exposed to a lethality process that yields an average of two surviving spores is calculated as follows:

$$P(0) = e^{-2} = 0.135$$

For this example, a BI with an initial spore population 2×10^6 exposed to a 6-log decontamination process has a 13.5% chance of having a growth negative result and an 86.5% change of having a growth positive result. With these "odds" established, the probability of obtaining the results from triplicate exposed BIs can now be calculated *exactly* in the same way as the coin toss example. A summary is shown in **Table 2.**

The probability that a 6-log decontamination process will produce growth negative results on triplicate 2×10^6 BIs exposed at the same location is only 0.25%. In other words, the probability that the process yielded a greater than 6-log spore reduction is 99.75%. Similarly, if the results produced one growth positive result and 2 growth negative results the probability that the process yielded a greater than 6-log spore reduction is 95.26%. Typically, the acceptance criteria used in validation protocols allow for a small percentage of locations to yield single growth positive results when using triplicate BIs.

Conclusion

Currently, many different strategies are used when implementing BIs to validate the decontamination process. It is advis-

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able that those companies continuing to use single BIs for validation with the expectation of 100% negative growth results allow for a contingent follow-up test using multiple BIs when an occasional growth positive result is noted. More than three BIs per location can be used during a follow-up test, depending upon the physical space available. The MPN calculation and probabilities based on binomial distribution can be used to defend occasional positive BI results when multiple BIs are used. Using BIs with initial spore populations that are slightly greater than the targeted log reduction being validated adds rigor to the statistics involved.

References

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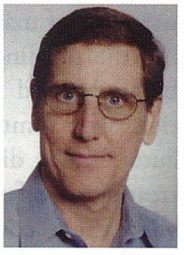
About the Author



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