Rationale for using triplicate biological indicators during VHP decontamination in Isolator

One of the key advantages of using an isolator for an aseptic processing application is that it makes a physical barrier between the operator and the core aseptic processing area. Additional main benefit is that an isolator can be totally sealed before aseptic activity starts, allowing for the exposed interior surfaces within to be bio-decontaminated, typically, via hydrogen peroxide (H_2O_2) vapour or mist.

When isolators were initially introduced in the pharmaceutical industry, the validation of the bio-decontamination technique was mainly patterned after methodology used for steam sterilizer validation. Biological indicators (BIs), typically containing over one million microbes per sample, were used to challenge the bio-decontamination process.

The recommended sampling plan includes:

- ✓ *The distribution of biological indicators on the surfaces and in each corner;*
- ✓ *The positioning of biological indicators at the points judged to be critical, that is:*
  - *Process critical: bowl, needles, filling area, RTP port etc.*
  - *Maximum and minimum temperature locations.*
  - *Worse case locations identified during gas distribution studies using chemical indicators.*
  - *The complexity of the load must be taken into account and the 'Worst Case' approach given preference.*

BIs for use with isolators were mainly prepared with spores of Geobacillus stearothermophilus, because this organism is known to be very resistant to the hydrogen peroxide decontamination process, and the same organism is used for steam sterilization validation. The typical validation approach involved distributing numerous BIs within the interior space of isolator and load items, including in worst case locations where kill of BIs (Geobacillus stearothermophilus) is expected to challenging.

Isolator operators, however, sometimes experienced problems with an occasional positive BI result during the initial qualification or periodic requalification. Following such occurrences, calibration procedure, maintenance procedures such as cleaning the vaporizer or injection needles and transfer tubing, were conducted on the hydrogen peroxide vapour generator. If the next

Physical Parameter impact hydrogen peroxide decontamination process: Pressure, time, surface temperature, airflow, sterilizing agent concentration, air change rate and in some cases ambient temperature and humidity need to be controlled.

decontamination cycle obtained complete kill of the BIs, the maintenance procedure was indicated as the corrective action. Apart from that concentration of H_2O_2 solution, replacement and reconciliation plays an important role.

Rogues One Reason for Positive Results:

Over time, isolator operators began to realize that these occasional BI failure issues were not caused by maintenance. 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.

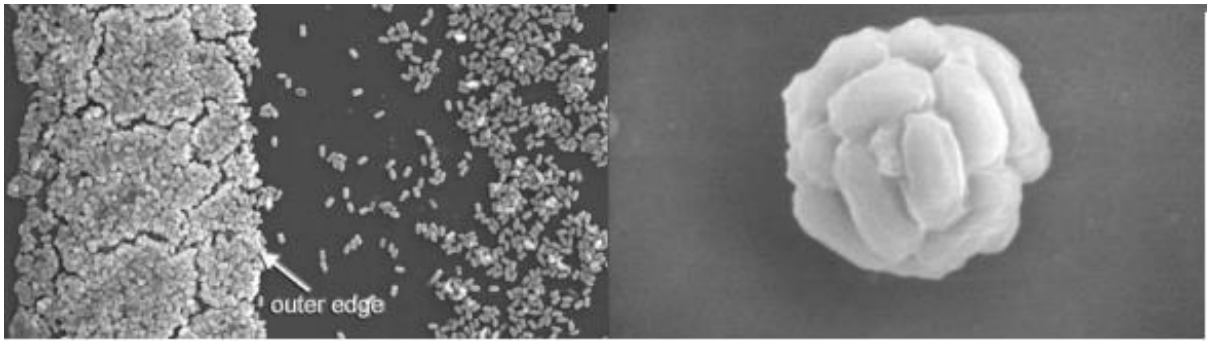

Figure 1: *Rogue's seem to be prevalent where: the spores form clumps or agglomerations; the spores are coated in debris; there are catalytic or protective substances present; the carrier substrate contains fissures into which some spores have become lodged.*

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 H_2O_2 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 prepared 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.

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.

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."*

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 analysed 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.

$MPN = \ln(n/r) = \ln(3/2) = 0.405$

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
N_0 = Initial spore population of the no 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:

$SLR = Log_{10}\ 2 \times 10^6 - Log_{10}\ 0.405$
$SLR = 6.301 - (-0.393)$
$SLR = 6.694$

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 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:

$m = 10^{(\log N0 - t/D)}$

Where:

m = Average number of surviving spores after exposure time
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 $\frac{t}{D} = 6$ 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:

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

Where:

P (a) = The Poisson probability that of a given sample
a = The number of organisms in a specific sample
m = Average number of surviving spores after exposure time

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

$P(0, 2) = \frac{2^0 \times e^{-2}}{0!}$

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:

$P(0) = e^{-2} = 0.135 = 13.5\%$

For this example, if a single BI with 2.0×10^6 spores is exposed to a decontamination process and it is killed, a probability of (100-13.5) = 86.5% for a process lethality of at least a 6 SLR is achieved and 13.5% chance of having a growth negative result.

If three BIs with 2.0 X 10^6 spores are exposed to just a 6 spore log reduction process the probability of killing at least 2 out of 3 can be calculated, according to the binomial distribution:

Probability of 3 negative BIs out of 3
$P_{3n} = (0.135)^3 = 0.0025 = 0.25\%$

Probability of 2 negative BIs out of 3
$P_{2n} = 3 \text{ X } (0.135)^2 \text{ X } (1 - 0.135) = 0.054675 \text{ X } 0.865 = 0.047 = 4.7\%$

Therefore, the probability of killing at least 3 out of 3 and 2 out of 3 of these BIs with just a 6 SLR cycle is:
0.0025 + 0.047 = 0.0495 = 4.95 %. (Approx. 5.0 %)

In other words, there is a certainty of 95.0 % that a SLR of more than 6 orders of magnitude was achieved with the cycle, even if there is one positive BI out of three. Although the occurrence of a sporadic positive BI is very low, it recommended that less than 5 % of the locations used for testing are allowed to have a single positive BI out of three.

Total Result	Possible Outcome	Probability Calculation	Probability	Total Probability	Failure Vs Acceptable
3 Negative	- - -	0.135 x 0.135 x 0.135	0.25 %	0.25%	5 %
1 Positive, 2 Negative	+ - -	0.865 x 0.135 x 0.135	1.58 %	4.74%	
	- + -	0.135 x 0.865 x 0.135	1.58 %		
	- - +	0.135 x 0.135 x 0.865	1.58 %		
2 Positive, 1 Negative	+ + -	0.865 x 0.865 x 0.135	10.12 %	30.36%	95 %
	+ - +	0.865 x 0.135 x 0.865	10.12 %		
	- + +	0.135 x 0.865 x 0.865	10.12 %		
3 Positive	+ + +	0.865x 0.865 x 0.865	64.65 %	64.65%	
				100 %	

Conclusion:

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

1. Coles, T., et al. PDA Technical Report No. 51: Biological Indicators for Gas and Vapor-Phase Decontamination Processes: Specification, Manufacture, Control and Use. Bethesda: PDA, 2010.

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3. Halvorson, H.O. and Zieglar, N.R. “Application of Statistics to Problems in Bacteriology. I. A Means of Determining Bacterial Population by the Dilution Method.” Journal of Bacteriology 25 (1933): 101–121.

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