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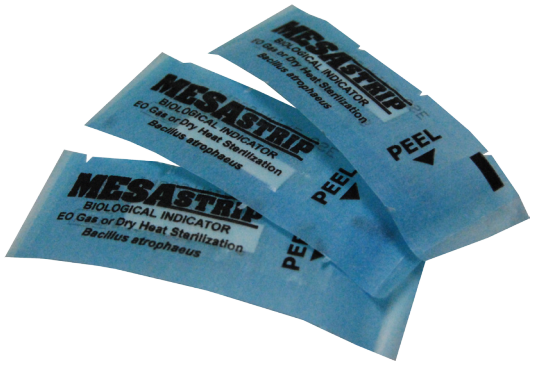

Resistance Analysis of Mesophilic Spores to Ethylene Oxide Sterilization

by Beth Ridgeway

Occasionally a customer will contact us in a case where they have identified an organism in their biological indicator (BI) that is not the organism labeled on the Certificate of Analysis (CofA). Some of these situations arise when a customer streaks for isolation a cultured positive control, an unexpected positive that shows in their cycle, or during initial species identification for their in-house qualification testing. Most of the time the BIs in question are those containing mesophilic spores of *Bacillus atrophaeus* used in ethylene oxide (EtO) and dry heat sterilization processes. These BIs in particular will be the main focus of this Spore News. So, where did this contamination come from? Possible sources include, but are not limited to:

- Improper aseptic technique when streaking or culturing the BI can lead to post-exposure contamination, i.e. the microbe came from the technician.
- Environment; airborne microbes can contaminate the BI or the culture medium during the culturing process.
- Non-sterile culture medium.
- Contamination during the manufacturing process of the BIs.

Because the number of contaminating microbes is typically very low {under 10 colony forming units (CFUs)}, it is hard to ascertain an accurate count through plating techniques because of the presence of 10^6 spores of *Bacillus atrophaeus* on the BI. *Bacillus atrophaeus* does have an orange colony morphology which helps to differentiate this species from others that may be present. Again, the level is so low that in order to enumerate the contaminant, it is usually necessary to plate directly from a 10^{-1} dilution which means careful evaluation of the plates as this dilution factor will produce a lawn of *Bacillus atrophaeus* growth. Some users have tried enumerating or determining a percent contamination level by culturing a positive control (PC) and letting the PC proliferate. This is a very inaccurate assessment mainly because each organism has a different growth and death rate. This test is good for establishing whether another organism is present assuming strict aseptic technique was employed but should not be used to determine percent contamination. With all this being said, is contamination really an issue to the extent that it would negatively impact the intended performance of the BI? Let's take a look at what the standards say regarding purity of the BI.

USP 37, General Chapters: <1035> BIOLOGICAL INDICATORS FOR STERILIZATION

"Carriers and primary packaging shall not contain any contamination (physical, chemical, or microbial) that would adversely affect the performance or the stability characteristics of the biological indicator."

USP 37, Monographs: Biological Indicators for Moist Heat, Dry Heat, and Gaseous Modes of Sterilization, Nonpaper Carriers
'Purity - There is no evidence of contamination with other microorganisms following examination of spores recovered from the carriers using a suitable plate culture medium."

USP 37, Monographs: Biological Indicators for Ethylene Oxide Sterilization, Paper Carrier
"Purity - By examination of the spores on a suitable plate culture medium, there is no evidence of contamination with other microorganisms."

ANSI/AAMI/ISO 11138-1:2006, Sterilization of Health Care Products-Biological Indicators-Part 1: General Requirements
4.1.1 Quality systems
"The manufacturer shall establish, document and maintain a formal quality system (e.g. ISO 13485, GMPs, or other national or regional requirements) to cover all operations required by this part of ISO 11138. In particular, the manufacturer shall take precautions at all stages of production to minimize contamination that would adversely affect the performance of the biological indicator."

5.2 Carrier, primary and secondary packaging
5.2.1 "The materials of the carrier and the primary and secondary packaging shall not contain any contamination (physical, chemical or microbial) that would adversely affect the performance of the biological indicator."

5.3 Inoculated carrier
5.3.2 "Only one strain of test organism shall be used in a batch of inoculated carriers, unless the manufacturer has demonstrated that the use of multiple strains does not significantly affect test organism performance in the specified sterilization process."

When assessing the contamination issue, Mesa Labs believes that the contamination is typically not going to have a negative affect on the resistance performance of the BI. This is especially true if the contamination was picked up during the BI manufacturing process. If the contaminant was picked up through improper aseptic technique or airborne contamination when culturing, this poses a different kind of problem altogether, but does not necessarily negatively impact BI performance. When you look at it from a resistance performance standpoint, D-value is defined as, "the time or dose required to achieve inactivation of 90% of a population of the test microorganism under stated dose conditions". Thus, consider a typical EtO BI that contains 1.0 x 10^6 spores of *Bacillus atrophaeus* and has a D-value of perhaps 3.5 minutes. Even if it were contaminated with 100 CFUs per BI and those contaminating microbes has a resistance that matched that of the test microorganism, the contaminant would be killed in the early stages of exposure with virtually zero potential to "out-survive" the test species.

The questions still arise though as to "what is the resistance of some of these organisms?" and "how do we know for sure that there is no impact on the resistance performance?".

To assess this aspect, four different organisms were tested for ethylene oxide resistance. Two of the organisms, *Bacillus cereus* and *Bacillus thuringiensis*, were picked because they are commonly isolated spore forming contaminants. The two other organisms, *Bacillus subtilis* "5230" and *Bacillus pumilus,* were randomly chosen for no specific reason other than being two other mesophilic spore formers. For the testing, 4 batches of Strip BIs were manufactured with each of the four organisms using a 10^6 population. A population assay was performed on each batch and then fraction negative analysis using an ethylene oxide biological indicator evaluator resistometer (BIER) was performed using 10 units/exposure for each batch. The ten units from each batch were exposed in the same cycle. Units were then allowed to properly aerate and cultured into Tryptic Soy Broth (TSB) and incubated at 30°C - 35°C for 7 days. During the 7 days, data was recorded and post incubation D-values were calculated using the Stumbo Murphy Cochran method. These results are listed in Table 1.

Table 1: Ethylene Oxide Resistance of Mesophilic Spore

Organism	Population	D-value (in minutes)
Bacillus atrophaeus	2.7 x 10^6	3.8
Bacillus subtilis 5230	2.4 x 10^6	2.9
Bacillus cereus	3.5 x 10^6	2.0
Bacillus pumilus	3.0 x 10^6	3.7
Bacillus thuringiensis	3.1 x 10^6	1.6

Another test was performed to determine whether the presence of spores on the BI does have an effect on the resistance performance labeled on the CofA. Two organisms were chosen for this study, *Bacillus cereus* and *Bacillus subtilis 5230*. *Bacillus atrophaeus* MesaStrips were removed from the glassine and each strip was inoculated with 10^6 spores from each organism. Strips were allowed to dry overnight and then repackaged into glassine envelopes. Fraction negative analysis using an ethylene oxide (BIER) was performed using 10 units/exposure for each batch including MesaStrips only containing *Bacillus atrophaeus*. All batches were exposed in the same cycle. Units were then allowed to properly aerate and cultured into TSB and incubated at 30°C - 35°C for 7 days. During the 7 days, data was recorded and post incubation D-values were calculated using the Stumbo Murphy Cochran method. At the end of the 7 days, positive tubes from the longest cycle giving fractional data were streaked for isolation to confirm presence of only *Bacillus atrophaeus*, all plates indicated only *Bacillus atrophaeus* growth. Resistance performance is listed in Table 2.

Table 2: Resistance of *Bacillus atrophaeus* Strips Inoculated with Another Organism

BI configuration	D-value (in minutes)
Bacillus atrophaeus only	3.8
Bacillus atrophaeus inoculated with 10^6 *Bacillus cereus* spores	3.9
Bacillus atrophaeus inoculated with 10^6 *Bacillus subtilis 5230* spores	3.9

The data illustrated above reaffirms the position of Mesa Labs regarding purity and contamination. Of the organisms tested, all showed resistance below the resistance of *Bacillus atrophaeus* (data from Table 1). Even if the BIs were grossly contaminated with another organism, data from this study indicates there is negligible change to the overall resistance measurement as the data in Table 2 shows only a 0.1-minute increase to the measured D-value. It is worth noting that the purity acceptance criteria of Mesa Labs requires a level of ≤0.0001%. This equates to not more than one contaminating microbe for every one million test spores on the BI. From the data presented above, it is evident that this is a very reasonable and overly conservative acceptance level. Process controls are put in place during every step in the manufacture of the BI to assure that we are able to achieve this level of purity.

To summarize, if you find yourself asking any of the following questions, you should feel confident with the answers presented below.

"Is my BI OK to use with these low levels of contamination?" **Answer: Yes**
"Will it affect my process?" **Answer: No**
"Am I in compliance with the guidelines set forth in the USP and ISO?" **Answer: Yes**

Beth graduated from University of Montana with a B.S. in Microbiology and Medical Technology. She has worked for Mesa Labs for nine years holding positions in the BI production laboratory, Research and Development, and Contract Studies. Beth's previous position was Spore Lab Supervisor and currently she is Director of Laboratory Production.