Vapor-Phase Hydrogen Peroxide Resistance of Environmental Isolates

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The authors examine the vapor-phase hydrogen peroxide resistance of microbial isolates recovered from the controlled environments of pharmaceutical and/or medical device companies and compared them with commercially available biological indicators under various test conditions.

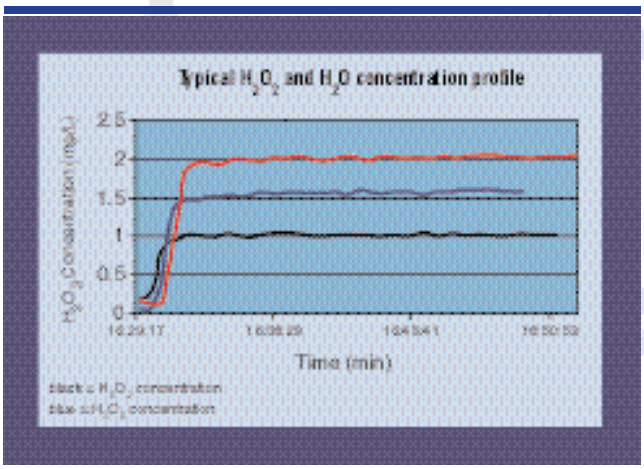

Figure 1: Typical vapor-phase hypdrogen peroxide concentration profiles.

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Barrier technology has become the de facto standard in aseptic processing and sterility testing, with more than 200 units in use worldwide. Isolators in aseptic processing applications were featured prominently in the US Food and Administration good manufacturing practice (GMP) guidance about sterile drug products produced by aseptic processing (1) as well as in several presentations delivered by FDA's team leader for guidance and policy, Richard Friedman (2, 3). The Pharmaceutical Inspection Convention recommendation document, "Isolators Used for Aseptic Processing and Sterility Testing," (4) and the PDA technical report, "Design and Validation of Isolator Systems for the Manufacturing and Testing of Health Care Products" also discuss using isolators for these applications (5).

Although using isolators in the pharmaceutical and medical device industries continues to increase—as does the generation of technical reports, guidelines, and publications for their design and validation—the industries and regulatory agencies do not have consensus on several issues. Two issues are germane to the present study: (a) the air classification for the background environment where an isolator is located, and (b) the level at which microbial reduction is appropriate to demonstrate a validated isolator decontamination process.

The isolator's interior should meet Class 100 (ISO 5, European Union [EU] Class A) standards as a minimum, but the classification of the environment surrounding an isolator is not as clear cut. FDA has recently stated that a Class 100,000 (ISO 8) background environment "can be appropriate, depending on isolator design and manufacturing situations. An aseptic processing isolator should not be located in an unclassified area" (1). The EU guidance on GMPs states in Annex 1 that the background environment for an isolator "should be controlled and for aseptic processing it should be at least Grade D" (6). PDA has suggested that the background isolator environment be controlled but not be classified (5).

The air classification of the isolator background environment will influence the microbial challenge to the isolator system. Indigenous flora, either airborne or operator- or personnel-related vectors, is a major potential source of contamination to the interior isolator environment. This contamination could occur through integrity breaches in the gloves, rapid transfer

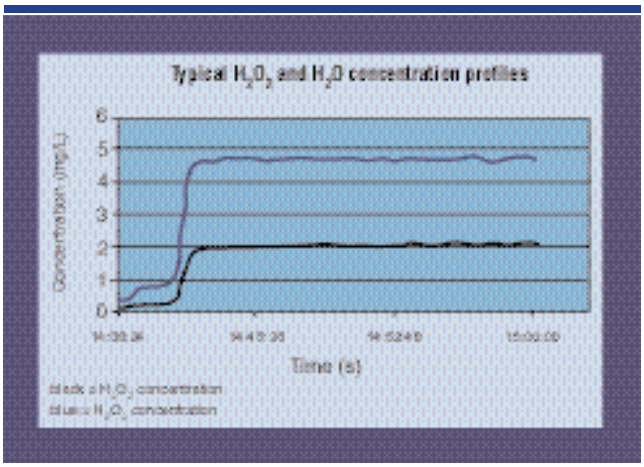

Figure 2: Typical vapor-phase hypdrogen peroxide and water concentration profiles.

ports, or isolator structure or through mouse holes in open isolators. Therefore, indigenous organisms challenge isolator decontamination procedures. Decontamination cycles must allow an appropriate safety margin to provide confidence in the cycle's robustness.

Although the definition of the "appropriate" level is open to discussion, FDA's latest draft aseptic-processing guideline says, "Normally a four- to six-log reduction can be justified, depending on the application" (1). Yet PDA contends that a three-log reduction is sufficient (5). The FDA guideline further advises that the specific biological indicator (BI) spore titer should be known and justified. Neither the FDA nor PDA document addresses the resistance of the BI, which is an equally important performance parameter to total count. The total resistance of the BI is a function of its population and organism resistance (D-value). This principle has been stressed by Pflug (7).

Vapor-phase hydrogen peroxide (VHP) is currently the most widely used isolator decontamination agent. Many BIs for monitoring the effectiveness of the VHP decontamination cycle are commercially available. Unfortunately, no VHP BI monograph or performance standard specifies the minimum acceptable resistance values, standard test conditions, or the testing equipment. These standards are available for commercial BIs designed to monitor other antimicrobial processes—such as saturated steam, ethylene oxide, or dry heat. As a result, BI manufacturers certify their monitors at various conditions using different test apparatuses. In fact, some manufacturers only certify the population of their BIs and do not address VHP resistance at all. This situation makes it difficult for users to select an appropriate BI for their particular application and to generate meaningful performance comparison data. Ultimately, one validates the isolator decontamination process to demonstrate microbial control of the interior environment in support of aseptic manufacturing and/or sterility testing activity. The correlation of the BI resistance to the potential indigenous microbial contamination must be understood to make the BI an effective tool.

Khorzad reported on the design, performance, and validation of a VHP biological indicator evaluator resistometer (BIER) unit (8). This unit precisely and accurately controls the exposure conditions of temperature, relative humidity (RH), and VHP concentration, and delivers them to the test BIs in a square wave fashion. This allows us to determine VHP D-values and standardize BI performance testing. The equipment's performance is analogous to ANSI/AAMI BIER unit standards for steam and ethylene oxide (9). Using this equipment, Caputo examined the resistances of commercially available VHP BIs (10). He reported a wide range of VHP resistance values among commercial BIs, large disparities among the manufacturers, resistance claims, and discrepancies with the actual experimental resistance values.

Materials and methods

Environmental isolates. Microbial isolates were obtained from the controlled environments of various US pharmaceutical and medical device facilities from the contract PSI environmental monitoring and microbial taxonomy program. Identical microorganisms, isolated from various locations, were selected for evaluation to ascertain whether there were any differences in resistance. Bacterial isolates were identified using the RiboPrinter technology (DuPont Qualicon, Wilmington, DE), which is based on the DNA sequence of the 16S RNA gene (11). Molds were identified using traditional methods. Representative colonies were selected for identification and study.

A total of 26 isolates—19 bacterial and 7 fungal species—were examined and are listed below. The number in parentheses represents the number of isolates of that species evaluated.

Bacterial species:
- Bacillus subtilis (2)
- Bacillus cereus (2)
- Bacillus licheniformis (2)
- Staphyloccus aureus (5)
- Staphyloccus warneri (3)
- Micrococcus luteus (2)
- Staphyloccus epidermitis (3).

Fungal species:
- *Candida albicans* (1)
- *Penicillium* species (3)
- *Aspergillus* species (2)
- *Aspergillus niger* (1).

After each organism was identified, it was grown on the appropriate culture medium, harvested, washed in a physiological buffer, and prepared in a test-suspension. For resistance determination, approximately 10^3–10^6 cells were inoculated onto a 316 stainless steel substrate. Conidia spores were used to challenge the resistance of the mould cultures and endospores were used for the challenge of bacteria of the genera *Bacillus* and *Geobacillus*.

The resistance testing of the isolates was performed in a VHP BIER unit (VHyPer, PSI) using the following exposure conditions, unless otherwise noted:
- VHP: 1.25 mg/L
- RH: 25%
- temperature: 30 °C
- time: 0.5–2.5 min, in 0.5-min increments
- pre- and post-temperatures: 30 °C
- packaging: no packaging.

Table I: Comparative resistance of multiple lots of BIs (packaged).

Manufacturer	D-Value (min) 15% RH
AS	1.2
AS	1.3
SB	2.8
AB	0.5
AB	0.5
AB	0.5
AB	0.8

Table II: Comparative resistance of multiple lots of BIs (unpackaged).

Manufacturer	D-value (min) 1.0 mg/L	1.3 mg/L	1.5 mg/L	1.7 mg/L
BR	—	—	1.3	—
BR	—	—	1.3	—
BR	—	—	2.1	—
AB	—	—	—	0.1
SB	19.6	1.9	—	—
SB	10.1	3.1	—	—
OB	5.8	2.8	—	5.3
OB	7.3	2.2	—	1.7
OB	9.8	3.0	—	3.6

The exposure sequence was as follows:

- pretreatment for 5 min at 30 °C
- exposure to square wave conditions, which is defined as moving from zero-VHP concentration to the desired VHP concentration and conditions in ~3 s. Flow velocity of the defined gaseous exposure mixture to the test sample was ~30 ft/min.
- post-treatment for 5 min at 30 °C to aerate samples and ensure that residual VHP does not kill additional microorganisms and skew the killing time results.

We assessed resistance by one of two methods: (a) a fraction/negative (F/N) screening method to indicate whether a particular isolate's D-value was smaller than that of a conventional commercial BI, or (b) a five-point survivor curve method. F/N D-values were calculated using the Stumbo, Murphy, and Cochran or Spearman–Karber (12) methods. A total of 10 samples were used for each F/N screening time. For the survivor curves, quadruplicate samples were used for each time cut. Each dilution was plated in duplicate. A least-squares analysis was used to determine the line of best fit. Samples were incubated for a minimum of 72 h at a temperature appropriate for the individual isolate.

Commercial BIs. A total of five commercial *G. stearothermophilus* BIs were evaluated. For the purpose of this study, the manufacturers were designated by the codes AB, AS, BR, OB, and SB. Resistance was evaluated at various exposure conditions designed to mimic actual isolator decontamination conditions. Resistance testing was conducted in the VHP BIER unit using the following conditions:

- VHP: 0.9–1.8 mg/L
- RH: 10–30%
- temperature: 30–35 °C
- time: 0–5.5 min, in 0.5-min increments
- pre- and post-temperatures: 30–35 °C
- packaging: nonwoven high-density polyethylene (1073B Tyvek, DuPont, Wilmington, DE) or unpackaged
- substrate: 316 stainless steel or glass cover slips.

The exact testing conditions will be indicated in the results section for each data set. The VHP BIER unit exposure sequence was similar to that described for the environmental isolates.

As described for the environmental isolates, we conducted resistance by either the F/N approach or the survivor curve method. Incubation of the *G. stearothermophilus* BIs was conducted at 55–60 °C and plate counts were performed after 48–72 h.

Results

This section summarizes the results of BIER performance, commercial BIs, and environmental isolates. The resistance values are presented as relative values for the purpose of illustrative experimental analysis rather than values to be taken literally.

BIER performance. Figure 1 shows typical VHP concentration profiles for BIER unit exposures at three VHP concentrations. Figure 2 demonstrates typical VHP and water concentration profiles for a BIER unit exposure.

During installation, the BIER unit was challenged 30 times at each of the two operating conditions to establish interrun and intrarun variations. These data demonstrated the precise and accurate delivery of challenge parameters (8).

Commercial BIs. Table I lists the comparative resistance of multiple lots of BIs from three manufacturers, packaged as they were received and challenged under identical test conditions: 1.7 mg/L H_2O_2, packaged, 35 °C, and D-value in minutes.

Table II lists the comparative resistance of multiple lots of BIs, challenged without primary packaging, under identical test conditions: 10–15% RH, 35 °C, and D-value in minutes. Data again show a great variation in resistance among various manufacturers.

Table III demonstrates the effect of testing the same lot of BIs within their primary packaging (as received from the manufacturer) at the following test conditions: 1.5 mg/L H_2O_2, 15% RH, 35 °C, and D-value in minutes.

Table IV demonstrates the effect of testing the same lot of BIs removed from the packaging at the following test conditions: 1.7 mg/L H_2O_2, 15% RH, 35 °C, and D-value in minutes.

Table V shows RH's affect on the resistance of the same lot of BIs, challenged under identical conditions except for the % RH: 1.0 mg/L H_2O_2, 35 °C, and D-value in minutes.

Table VI demonstrates the effect of inoculating the same suspension of *G. stearothermophilus* spores onto various carrier substrates on the resistance of the suspension. The SB lot was run under the following conditions: 0.9 mg/L H_2O_2, packaged, 10% RH, 30 °C. The BR lot was run under the following conditions: 1.5 mg/L H_2O_2, packaged, 15% RH, 35 °C. The humidity difference between the two test conditions may have contributed to both the high resistance values in Table VI and the resistance disparity between lots SB and BR.

Environmental isolates. Table VII tabulates the VHP resistance of the bacterial isolates under the following conditions: 1.25 mg/L H_2O_2, 28% RH, and 30 °C. The resistance (D-value) of these isolates varied by as much as one log and all the *S. aureus* strains were catalase positive. On the basis of these results, the production of catalase did not afford these organisms any unique VHP resistance. Finally, the *S. warneri* strains, which were catalase negative, exhibited higher resistance than the *Bacillus* spores. This result can probably be attributed to clumping when drying on

Table III: Same-lot testing (packaged and unpackaged).

Manufacturer	D-value (min) Packaged	Unpackaged
BR	3.9	1.3
BR	—	2.1
BR	—	1.3

Table IV: Same-lot testing (packaged and unpackaged).

Manufacturer	D-value (min) Packaged	Unpackaged
AB	0.5	0.1
AB	0.5	—
AB	0.5	—
AB	0.8	—

Table V: RH effect.

Manufacturer	D-value (min) 10% RH	35% RH
OB	5.8	2.8
OB	7.3	3.7
OB	9.8	3.4
SB	19.6	2.8
SB	10.1	2.4

Table VI: Inoculation effect.

Manufacturer	D-value (min) Steel	Glass
SB	28.1	57.8
BR	3.9	3.1

Table VII: Resistance summary of bacterial isolates.

Organism	Estimated D-value (min)
B. subtilis (1)	0.7
B. subtilis (2)	0.8
B. cereus (1)	<0.1
B. cereus (2)	<0.1
B. licheniformis (1)	0.1
B. licheniformis (2)	<0.1
S. aureus (1)	0.2
S. aureus (2)	0.5
S. aureus (3)	0.5
S. aureus (4)	0.1
S. aureus (5)	<0.1
S. warneri (1)	1
S. warneri (2)	0.8
S. warneri (3)	<0.2
S. epidermidis (1)	0.3
S. epidermidis (2)	0.5
S. epidermidis (3)	>0.13
M. luteus (1)	0.2
M. luteus (2)	0.2

the carrier, which tends to result in artificially high resistance.

Table VIII tabulates the VHP resistance of the fungal isolates under the following conditions: 1.25 mg/L H_2O_2, 28% RH, and 30 °C. As with the bacterial isolates, the resistance (D-value) varied by as much as 1 log between the fungal isolates.

Discussion

The data indicate that the VHP BIER unit provides precise and accurate delivery of standardized challenge conditions, thereby providing a standardized method of evaluation of BI resistance. The data further suggest that a wide variation in the resistance of VHP commercially available BIs exists. Depending on the methodology used for resistance determination, these values can vary by as much as one log. This finding is consistent with data from studies that indicate a wide variation in reported BI VHP resistance values (e.g., D-value of 2.0 min at 1.5 mg/L VHP concentration for *B.* [now *G.*] *stearothermophilus* [13] and D-values in the 5–10 second range for the same organism [14]). This widely reported resistance variation is probably at least partially caused by the testing methodology. If these values are used to develop decontamination cycles or to calculate or support decontamination safety factors, greatly divergent cycles could result.

Both the testing of RH as well as the BI packaging also have been shown to greatly influence the D-value. The RH situation appears very analogous to that of ethylene oxide, in which the resistance of organisms is increased at RH levels below ~30% (15–17). In addition, the nonwoven, high-density, polyethylene pouch typically used to package BIs increases the resistance of the BI unit. Unpackaged BIs demonstrate a considerably lower resistance. Isolator-validation practitioners must appreciate and understand this fact. BIs are sometimes removed from their package for easy suspension in the isolator or to facilitate post-exposure sterility testing. These procedures should not be performed without realizing the potential ramifications.

FDA has suggested, in its draft aseptic processing guideline, that when evaluating the efficacy of an isolator decontamination procedure, "an appropriate, quantified BI challenge should be placed on various materials ..." (1). The present data indicate that the supporting substrate can influence the BI resistance. This phenomenon has been observed with gaseous ethylene oxide processes (18). Although many factors, including the texture of the surface, the porosity of the surface, the surface tension of the substrate—which could lead to clumping of the inoculum—and the cleanliness of the carrier material, can influence BI resistance on a particular substrate, the observed substrate effect appears to be real.

The VHP resistance of environmental isolates varies greatly. The data showed differences among organism types as well as the same organism isolated for various areas of the country. Most isolates had a lower VHP resistance than that reported for most BIs. Some isolates, however, had a greater VHP resistance than that reported for some commercially available VHP BIs. This fact emphasizes the need to know and then calibrate the resistance of the BI selected to conduct isolator cycle development and validations. This fact further highlights the need for BI manufacturers and the pharmaceutical industry to work together to establish consensus on appropriate D-value ranges for commercially available BIs used with VHP. In addition, the typical indigenous flora of the isolator environment and its correlation to BI resistance must be well understood. Finally, proper calibration of the BIs would also help to eliminate some of the observed variability. It is therefore recommended that VHP resistance be considered as part of the initial characterization of the indigenous flora of the environment enclosed by the isolator.

A reduction of six logs of a certified BI is achievable when the conditions such as VHP concentration, RH, load configuration, or substrates are controlled. If any of these variables are uncontrolled or misunderstood and a BI positive is generated, then too often the cause is immediately attributed to "bad" BIs. Microbiologists and engineers experienced in sterilization science understand the concept of approximate first-order kinetics: the more robust the decontamination/sterilization cycle, the straighter the line. If this logic

Table VIII: Resistance summary of fungal isolates.

Organism	Estimated D-value (min)
C. albicans	0.7
Penicillium species (1)	<0.1
Penicillium species (2)	<0.1
Penicillium species (3)	0.9
Aspergillus species (1)	0.2
Aspergillus species (2)	0.1
Aspergillus niger	1.3

of linear extrapolation is accepted, then the first solution to a BI positive should not be to decrease the acceptance criteria for a successful cycle from a six-log to a three-log reduction. If the decontamination/sterilization conditions are less than robust, the answer should not be to reduce the challenge but to fix the exposure parameters. The microbial load of the product and environment will continue to be unknown and must be managed and addressed through a deep understanding of the cycle parameters and their interrelationships.

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