The Use of Biological Indicators for Steam Sterilization

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Introduction

Steam sterilization is widely used as a terminal sterilization process for pharmaceutical drug products in glass ampoules, vials, syringes, and plastic containers. It is also used for sterilizing closures, filters, manufacturing equipment, cleaning equipment, and so forth. Steam sterilization in autoclaves has a strong scientific basis. The essence of validation of steam sterilization processes is to demonstrate that temperature and time conditions are being achieved uniformly through every item included in the autoclave load, and that the lethality being achieved in practical situations corresponds to that which would be expected from sterilization theory.

Thus, compliance with limits for temperature uniformity throughout empty autoclaves (heat distribution studies) is an index of the way in which the autoclaves are engineered. Compliance, or biovalidation, of steam sterilization is demonstrated through the use of thermocouples and biological indicators. This paper addresses the use of biological indicators. The use of biological indicators is a requirement of the FDA Code of Federal Regulations (21 CFR 880 2800).

Biological indicators are preparations of a specific microorganism, with high resistance towards particular sterilization methods. Since sterilization is only a probability of there being an absence of microorganisms (where true sterility can only be demonstrated through infinite exposure), then greater assurance for sterilization can be measured through physical (thermometric) data and from biological indicators to provide confidence that a sterilization process has been successful. There is a recurrent debate as to whether physical data or biological data is the most important when examining a sterilization processes, as well as considering if both measures are needed simultaneously. This paper does not set out to discuss the respective merits of these two approaches, although the importance of biological indicators is well established (and this author considers that biological monitoring is the most effective method).

Biological indicators are "standardized" preparations of certain microorganisms with known characteristics (a defined population, purity and resistance)[1]. The microorganisms used to prepare biological indicators are those capable of forming endospores and the microorganism is used in the "spore state." A biological indicator is prepared by depositing bacterial spores, from a spore crop, onto a carrier, such as filter paper. The carrier may be wrapped in a suitable primary package. In preparing a biological indicator, the object is to use a microorganism of a known population, purity and resistance characteristic.

Different biological indicators are used for different sterilization processes. Biological indicators are designed for use with:

- ethylene oxide gas
- hydrogen peroxide vapour
- dry heat
- steam
- radiation

With each of these:

- Ethylene oxide gas is used to kill bacteria, mold and fungi in medical supplies such as bandages.
- Dry-heat sterilization uses an oven to raise the temperature of items that are wrapped in foil or fabric.
- Steam sterilization uses an autoclave, a self-locking machine that sterilizes its contents with steam under pressure.
- Irradiation is used to sterilize materials that may be damaged by moist heat, such as plastics.

The microorganism used will vary depending upon the means of sterilization which requires testing. Microorganisms are selected depending upon how resistant they are to the chosen method of sterilization. Different microorganisms are more resistant than others to different types of sterilization. With steam sterilization, for example, spore-bearing microorganisms are more resistant than non-spore bearing microorganisms. A microorganism like *Staphylococcus* (commonly carried on human skin) would have a typical D-value at 121^{o}C for a 15-minute autoclave cycle of only 15 seconds, whereas an endospore forming thermophilc *Bacillus* would have one of at least 1.5 minutes. For steam sterilization, *Geobacillus stearothermophilus* (formerly described as *Bacillus stearothermophilus*) is most commonly used (as required by the pharmacopoeias). This microorganism is used due to its theoretical resistance to particular types of sterilization, including heat.

[1] Outside of pharmaceutical microbiology the term "biological indicator" has a more generalized term in biology defined as a species which is used to monitor the health of an environment or ecosystem.

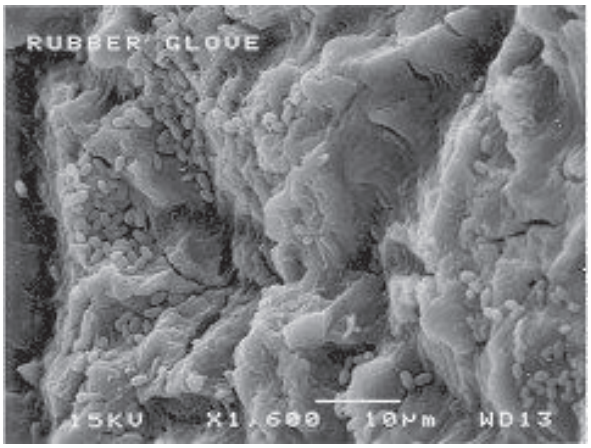
The principle is that if these spores are destroyed, it can be assumed that any contaminating microorganisms in the sterilization load would also have been killed, as these microorganisms will have lower resistance than any spores that might be present (and such environmental microorganisms will have been present in far lower numbers).

The target population for biological indicators is $>10^6$. The reason this population is used is because it is generally accepted that "devices purporting to be sterile," such as an autoclave, are designed to achieve a 10^{-6} microbial survival probability (that is, there is less than one chance in a million that a microorganism would survive the sterilization process).

Biological indicators are available in many different forms. Examples include strips (the classic "spore strip"), discs, suspensions, test tubes and ampoules. With these:

- Spore strips are biological indicators that are packaged in a pouch made of glassine, a paper that is resistant to moisture and air at ambient temperatures and pressures.
- Spore discs are usually made of borosilicate paper or stainless steel. Spore suspensions are diluted aliquots that are derived from a primary batch of spores.
- Other spore suspensions which are inoculated directly onto surfaces, such as rubber closures.
- Test tubes that are available in a variety of sizes and are usually made of expansion-resistant glass. Ampoules are small, self-contained vials, which are hermetically sealed with a flame. They have a score mark around the neck so that the sealed top can be snapped off by hand. Typically, ampoules are used to contain hypodermic injection solutions.

Objective



The objective of any sterilization process is to kill the microorganisms naturally present in the product (the product bioburden). Although sterilization can be measured thermometrically using thermocouples, it is increasingly common for the validation and routine requalification to use biological indicators. Theoretically the reason for this is dry heat in comparison with moist heat (saturated steam). A thermocouple might be reading the correct temperature, however the local environment may consist of a "dry pocket." A good practice is a combination of a BI and a thermocouple to ensure saturated steam exists in the region where the thermocouple was placed. Both the European Pharmacopoeia (section 5) and the United States Pharmacopoeia (in <1211> and <1035>) require the use of biological indicators in order to validate sterilization processes.

There are some recommended parameters in the pharmacopoeias for the use of biological indicators. These are for the European Pharmacopoeia:

- Population of more than 1x 10^5
- D-value$_{121\,^{o}C}$ of more than 1.5 minutes

For the United States Pharmacopoeia:

- Population of between 1x 10^5 and 5 x 10^6
- D-value$_{121\,^{o}C}$ of between 1.5 and 3.0 minutes

These parameters are discussed later.

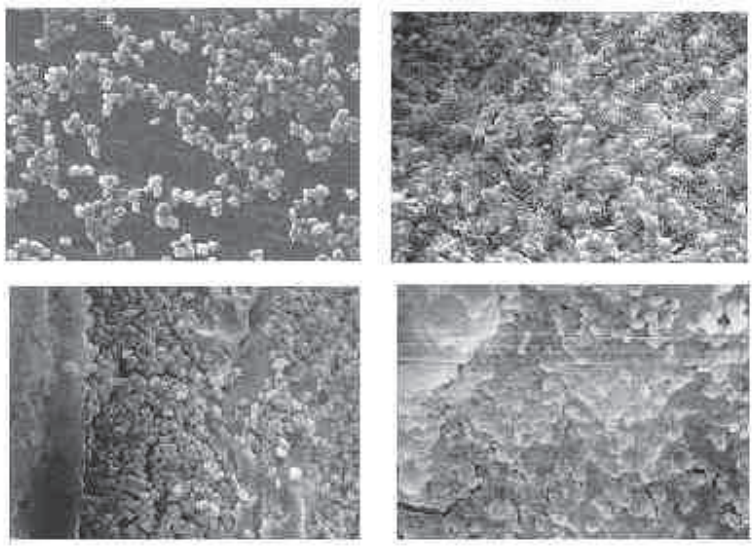
History

The term "biological indicator" has wide usage outside of the pharmaceutical industry. The term "biological indicator" (or "bioindicator") is also applied generally to the application of plants or animals to various conditions where the reaction of the biological material is examination. In one sense, the use of a canary in a cage by a miner to detect pockets of natural gas was arguably one of the first "biological indicators." To assess sterility, in similar presentation and form, biological indicators are commonly used in both the food and pharmaceutical industries.

The original format of biological indicators was inoculated paper strips inside envelopes, which were transferred to sterile culture medium following processing and incubated for seven days. Sterilization failure was measured by turbidity of the growth medium. From this starting point, some organizations elect to incubate biological indicators for up to 14 days, and second-generation biological indicators are commonly used (such as self-contained systems which comprise the micro-organism and growth medium required for recovery in a primary pack ready for use.) Microbial growth is indicated by a change in pH (with a color indicator), which measures the production of acid metabolites in the growth medium by outgrowing spores and replicating microbial cells).

Steam Sterilization

With steam sterilization, *Geobacillus stearothermophilus* is the most commonly used biological indicator. This microorganism is not naturally found in pharmaceutical environments (it is commonly located around hot springs). It has been selected because of its relatively high resistance to heat and because it is nonpathogenic. *Geobacillus stearothermophilus* is a rod-shaped, Gram-positive bacterium and a member of the division Firmicutes. The bacterium is a thermophile and is widely distributed in soil, hot springs, ocean sediment, and is a cause of spoilage in food products.



Photograph showing *Geobacillus stearothermophilus* inoculated onto paper carriers.

The type of *Geobacillus stearothermophilus* used is standardized. The strain used is one traceable to the American Type Culture Collection (ATCC, reference number 7953) and was first referenced in 1952 (Smith *et al*, 1952). This ensures consistency between manufacturers and between successive validation runs.

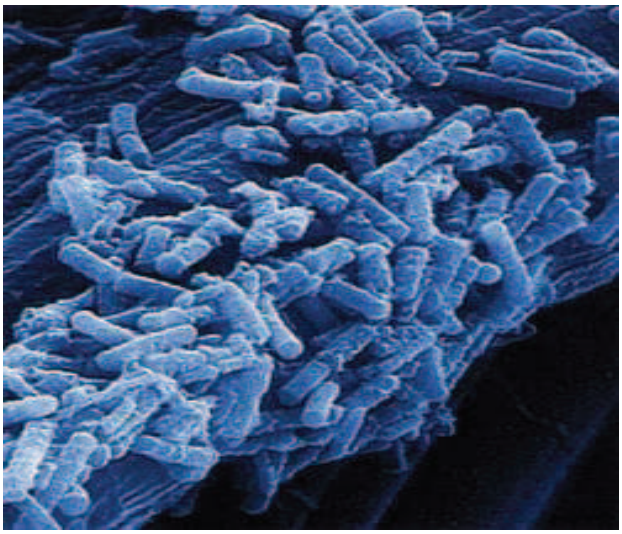
Key Parameters

This section examines some of the key parameters of a biological indicator. The most important characteristic of a biological indicator is that sporulation must readily occur on a defined medium and, if there are any survivors, spore germination will occur. If there are any survivors, then it is important that the survivors form easily countable colonies. Without possessing these characteristics, then the biological indicator is of little value. As this is a critical parameter, it is recommended that a positive control be run alongside each test set of biological indicators.

All biological indicators must come with a certificate of conformity. The certificate should indicate the population, D-value and purity of the microorganism. Due to the variability in the preparation of biological indicators, some users elect to have biological indicators verified (this would be the case with, for example, spores inoculated onto a paper carrier to create a spore strip).

Biological indicators which the user prepares (such as inoculating a spore suspension onto a rubber closure), must always be verified, as there is no other comparative data available.

Each of the key parameters for biological indicators is examined below.



a) Purity

Although some biological indicators may contain other microorganisms, when subjected to a heat shock challenge, the only thermophilic microorganism detected should be *Geobacillus stearothermophilus.* Biological indicators must be verified for purity by at least a phenotypic identification of the microorganism.

b) Population

Biological indicators must have a minimum population as defined by the pharmacopoeias.

A population verification, per USP <55> total viable spore count, is normally performed. The acceptance criteria state that the results should be no less than 50% or more than 300% of the labelled certified population.

c) D-value

Arguably the most important characteristic of biological indicators is the level of resistance. This is defined by the decimal reduction value (or D-value).

The D-value is the time taken to reduce the population of a known microorganism by 1 log (or 90% of the population). Thus, after an organism is reduced by 1 D, only 10% of the original microbial population remains (that is, the population number has been reduced by one decimal place in the counting scheme). When referring to D values, it is normal to give the temperature as a subscript to the D. For example, a hypothetical organism is reduced by 90% after exposure to temperatures of 121^{o}C for 1.5 minutes, Thus the D-value would be written as D_{121oC} = 1.5 minutes. D-values will vary according to the resistance of the microorganism and the population challenge. Generally, the longer the exposure time and the more resistant the microorganism, the higher the D-value.

Once a D-value has been established, many sterilization cycles have "overkill" built in. This is either simply doubling the cycle time (or sterilization dose), or it is taken from a mathematically calculated Sterility Assurance Level (SAL). Typically the SAL is developed to give a sterilization cycle designed to achieve a 12-log reduction of the challenge population.

The acceptance criteria for the D-value are defined by the USP, which states:

> "The requirements of the test are met if the determined D-value is within 20% of the labelled D-value for the selected sterilizing temperature and if the confidence limits of the estimate are within 10% of the determined D-value."

In order to verify the D-Value, the USP and ISO 11138-14 allows for the use of three methods. These are:

- The Most Probable Number method by direct enumeration;
- A Fraction Negative method (such as Spearman/Karber);
- Assessing the D-Value accuracy by using the USP Survive/Kill calculated cycles.

Regardless of which of the three methods is used, the piece of equipment that will be needed to calculate the D-value is a Resistometer. A Resistometer, also known as a BIER Vessel (Biological Indicator-Evaluator Resistometer), is an item of test equipment that can very quickly and accurately deliver and control very precise sterilization process parameters. The standard ANSI/AAMI ST44:20025 states that with a Steam BIER Vessel, the equipment must be capable of reaching the target temperature set point within 10 seconds or less from the time "steam charge" occurs. Additionally, it must maintain that set temperature to within + or – 0.5^{o}C and then at cycle end, the post-vacuum time to reach atmospheric pressure must be within 10 seconds or less.

The most common method deployed to calculate D-values is a Fractional Negative Method. For this method, multiple groups of biological indicators (typically ten or twenty) are exposed to varying cycle exposure times. The examination is for partial kill (looking for that *fraction* which is *negative*). This is normally running one exposure designed for all test biological indicators to survive; one exposure designed for all test biological indicators to be killed; and several exposures in between, set at equidistant time intervals.

For example, to verify the resistance of a particular biological indicator in a Steam Vessel at 121^{o}C using the Limited Spearman-Karber Fraction Negative Method, 20 biological indicators would be exposed per group to various exposure times at 121^{o}C. After each exposure, each group of biological indicators would be aseptically transferred to a growth medium and incubated at the appropriate temperature.

D-values vary with different carriers, even where the same spore crop is used. Thus, the same spore crop used to inoculate a paper strip and a rubber closure will give a different D-value (and there is a likely probability that the rubber closure will give a higher D-value). This variation explains why, for instance, the D-value for a self-contained biological indicator in a glass ampoule has a higher D-value than spores inoculated onto a cotton thread.

A similar phenomenon occurs with fluids. Spores suspended in water will have a lower D-value than spores suspended in a saline solution.

d) Z-value

A Z-value is defined as the number of degrees Celsius required to change a D-value by one factor of ten. In the practical sense, it is a measure of how susceptible a spore population is to changes in temperature. For example, if the Z-value of a population is 10 degrees, then increasing the sterilization temperature 10 degrees will result in a log reduction of the D-value.

To work out a Z-value, at least three D-value / temperature pairs are required. Z-values can be estimated graphically (using line of best fit) or calculated mathematically. Z-values are useful for calculating F values (in conjunction with D-values), especially to show the relationship between lethalities.

e) Other factors need to be considered when using biological indicators. These include the shelf life, strip size, and package size of the biological indicator.

Testing Issues

When setting up a biological indicator study, there are a number of issues which need to be considered in advance of undertaking the validation.

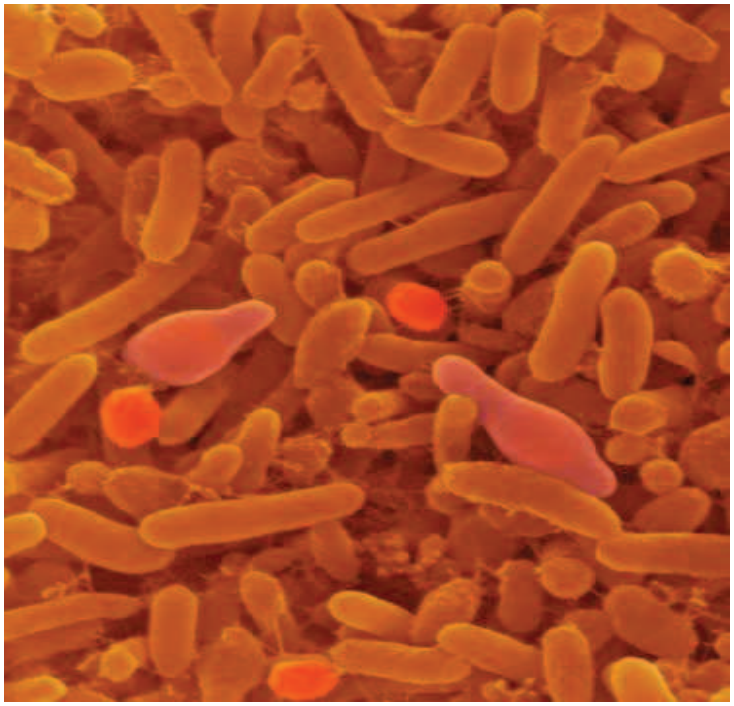
These include:

- The number of biological indicators required should be assessed upfront;
- The locations for the biological indicators should be considered in advance;
- The location where biological indicators are placed in relation to thermocouples should be considered, especially if this might affect air removal, steam penetration, condensate collection or air leakage.

Areas of Concern and Testing Errors

As with any biological test there are aspects of biological indicator testing which can cause testing difficulties. Some of these issues are next examined.

a) The bioburden of the product being sterilized can affect the results of the study, such as leading to an increase in the D-value or promoting survival of spores through a clumping effect by one microorganism covering another. Therefore, the following should be considered:



- Total numbers of organisms present, as the item to be sterilized, just prior to sterilization must be known;
- Types of organisms present;
- Number of resistant spore formers present;
- Resistance of this bioburden;
- Sampling frequency and statistical analysis.

b) Variability Between Different Lots of Biological Indicators

Each lot of biological indicators will vary slightly in its population, resistance, and kill time. This variability can arise from heterogeneity within a spore population, which can be caused by genotypic and phenotypic variations within the spore crop. This is one of the reasons why the USP

recommends that supplier audits take the place of biological indicator manufacturers. In addition, it is good practice to audit any contract test laboratories that may undertake biological indicator testing.

c) Shipping Conditions

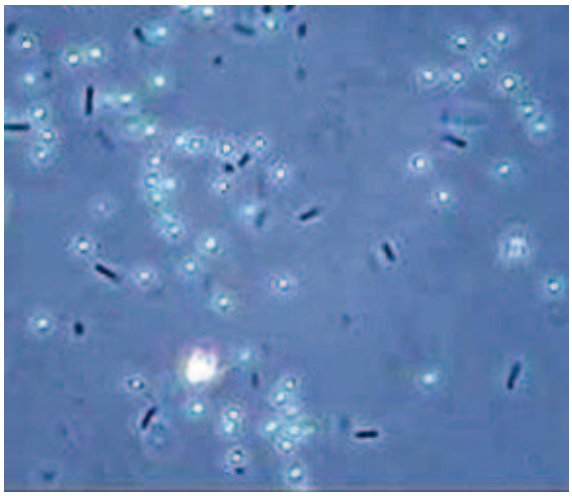
Biological indicators may be affected by the transport from the manufacturer. Any available transport and stability data from manufacturer should be reviewed.

d) Storage Conditions

Most biological indicators will have prescribed storage conditions. These may be strictly defined, or "controlled temperatures" will be referred to. Controlled room temperature is defined in the USP as:

"A temperature maintained thermostatically that encompasses the usual and customary working environment of 20° to 25°C (68° to 77°F); that results in a mean kinetic temperature calculated to be not more than 25°C; and that allows for excursions between 15° and 30°C (59° to 86°F) that are experienced in pharmacies, hospitals and warehouses…"

Humidity, if it is not defined by the manufacturer, is typically 20% to 70% relative humidity.



Storage conditions and times should be assessed by a stability trial. This is of great importance as, theoretically, the D-value of a biological indicator will decrease over time.

e) Delay in Transferring the Biological Indicator to Storage Medium

Theoretically, the ability to recover spores, especially those which are sublethally damaged, may be affected by the time taken to transfer a biological indicator which has undergone steam sterilization to the required culture medium. For this purpose, the USP states in the Guide to General Chapters Microbiological tests<55> Biological Indicators, that:

"…after completion of the sterilizing procedure… and within a noted time not greater than 4 hours, aseptically remove and add each strip to 10 to 30 ml of Soybean Casein Digest medium…"

f) Test Method Used by Contract Test Laboratory to Determine the D-value

Variation can arise when biological indicators are evaluated by contract manufacturers for population and D-value. Variables can include techniques, utensils and equipment. The main source of variation is if the contract test laboratory uses a different technique for D-value determination from the manufacturer. A related variation can arise from the culture medium, and incubation conditions for different brands and different lots of culture media may not have the same degree of "ability to promote growth of injured spores."

g) Preparation of Biological Indicators

Variation can occur with the preparation of biological indicators. This is of particular concern when users prepare their own biological indicators, such as inoculating spores onto stoppers. Areas of concern here include:

- How spores are put onto carriers;
- Places where the inoculation is too thick (and irregular clumps occur);
- How often the spore suspension is resuspended;
- Pipetting technique;
- Drying times;
- The fluid in which the spore suspension is held (typically water or ethanol);
- Problems from media residues;
- Excessive damage to the surface.

Summary

This paper has examined some of the key characteristics of biological indicators. Biological indicators are of great importance in assessing sterilization in the pharmaceutical industry. Thermometric data provides abundant information as to what might theoretically happen, however it is only through biological material that the question "what if my material to be sterilized has a high bioburden?" can be answered.

The emphasis of the paper has been upon some of the factors which might cause variation and testing problems. An element of variation will always be present when biological material is used, however, attempts should be made to reduce this variation to a minimal level.

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United States Pharmacopoeia <1035> Biological Indicators for Sterilization.