Minireview

Validation of Sterilization Procedures and Usage of Biological Indicators in the Manufacture of Healthcare Products

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Received 20 April, 2011/Accepted 21 May, 2011

Healthcare product manufacturers strive to provide safe, sterile products by validating and controlling manufacturing procedures. Validation study is the result of a multidisciplinary team effort. This successful effort relies on each discipline understanding the fundamentals of each technical disciplines and applying those fundamentals in terms of their own technical background. This paper is to introduce the basic concepts of sterilization methodology which are involved in validating various technical methods of sterilization and biological indicators (Bls). These will mainly address sterilization procedures in general terms and highlight on their microbiological aspects. The proper starting point is defining what sterility is and how it can be achieved through the validation studies by using BI.

Key words: Validation / Sterilization / Biological indicator / Healthcare Products / Sterility.

INTRODUCTION

One of the most important points in the manufacture of healthcare products is the production of a sterile product. Healthcare product manufacturers strive to provide safe, sterile products by validating and controlling manufacturing procedures. Validation is the result of a multidisciplinary team effort. Typically, personnel with backgrounds in engineering, physics, pharmacology, chemistry, biochemistry, and microbiology work together to demonstrate that a process does what it is designed to do. The success of this effort relies on each discipline understanding the fundamentals of other technical disciplines and applying those fundamentals in terms of their own technical background. In this way, there will be synergy in problem solving and a successful end result.

The scope of this paper is to introduce the basic concepts of sterilization methodology to those who are involved in validating various methods of sterilization (e.g., steam, dry heat, radiation, lethal gas, and filtration), and biological indicators. This review article will mainly address sterilization procedures in general terms and highlight their microbiological aspects. The proper starting point for this paper is defining what sterility is and how it can be achieved. This article is prepared based on the description of six books and several ISO documents. These editors/authors of the books are Seymour S. Block, Adam P. Fraise et al, Gerald E. McDonnell, Robert A. Nash and Alfreed H. Wachter, and Anurag S. Rathore and Gail Sofer, and Akikazu Sakudo and Hideharu Shintani.

STERILITY AND STERILIZATION

The definition of sterility is the complete absence of microorganisms. It is an absolute term; a product may be almost sterile when no growth of a bioburden is observed. The conventional practice is to define sterility as the absence of microorganisms as demonstrated by growth and reproduction by the sterility test, which is scientifically old fashioned and furthermore no sterility assurance level (SAL) from sterility test can be confirmed. Thus, the most modern way of demonstrating sterility is by using a biological indicator (BI) and attaining an appropriate SAL such as
$10^{-6}$. A material is sterile when BI tests show that nothing grows in or on it. The absence of BI growth means there is no living material and, therefore, signifies sterility even though no definite SAL is clarified. The above description seems reasonable until one realizes the range of environmental conditions that can support growth and the variety of living things that can make something nonsterile.

There are a variety of methods for obtaining a sterile condition. In fact, the control of microorganisms has been central to the field of microbiology. Microbial growth, although primarily beneficial, is responsible for economic losses and diseases that profoundly affect people. It is not surprising, therefore, that the various means of preventing growth and destroying microorganisms have been studied extensively.

There are many approaches to the inhibition, destruction, or removal of microbial growth, it is convenient to categorize these approaches as either physical or chemical. Physical methods include moist or dry heat and filtration. Examples of agents used in chemical methods are ethylene oxide gas (EOG), disinfectants, vapor phase of liquid hydrogen peroxide (VHP), peracetic acid, formaldehyde, chlorine dioxide and so on. The sterilization mechanism for ionizing radiation is a combination of physical and chemical effects. These broad-based examples are not intended to be all-inclusive, but rather, to illustrate the variety of methods available for controlling the microbial content of a substance.

The extent to which microbial control is applied, from preventing the growth of microorganisms to achieving sterility, also varies widely. In many instances, the mere inhibition of microbial growth is sufficient. An example of this approach is found in food preservation. Other situations (particularly those associated with formulation) require the selective destruction or removal of offensive microorganisms while leaving other microorganisms unaffected. Sanitizers, such as phenolics, are designed to kill pathogens, whereas they often allow other microorganisms to survive. It is clear, however, that the complete destruction or removal of microorganisms, commonly termed sterilization, represents the most effective control method available.

Sterilization is achieved by any one of several methods. These include thermal, lethal gas, radiation, and filtration processes. The most widely used and most extensively studied methods are based on heat. Thermal sterilization methods are well-characterized approaches to complete microbial removal and are often considered the method of choice. Characterization of a sterilization method includes the following major elements. First, a BI of performance is chosen that is resistant to the desired sterilization method. *Geobacillus stearothermophilus* ATCC 7953 spores fill this role for steam sterilization (ISO 11138-1,3, ISO 11134). A second consideration is the influence of other variables on the destruction or removal of the indicator. Examples of such variables in heat studies include spore propagation, interactions between BIs and the medium being sterilized, and the physical characteristics of the sterilized medium in response to heat. Third, a quantification of microorganism removal efficiency, under defined conditions, is established for the sterilization method. For example, the exposure time at a given temperature during thermal sterilization predicts the rate of spores killed. Finally, the concept of assigning the probability of sterility to materials undergoing sterilization represents a natural extension of the orderly and progressive destruction of BI under defined conditions. More succinctly put, characterization of sterilization implies a collection of studies that prove the reliability and predictability of the chosen method. A more detailed discussion of sterilization and BI will follow hereafter.

**STERILIZATION**

There are five principal sterilization methods from which manufacturers of sterile products may choose. These include steam, dry heat, gas, ionizing radiation, and filtration. Each method differs in the mechanisms of microbial removal, parameters of operation, and applicability to any given product. However, all of the methods provide sterility, and they all require validation and monitoring to demonstrate their effectiveness. This paper will discuss sterilization in terms of how to select a method, how the methods remove or destroy microorganisms, and how to prove microbiologically that the methods remove microorganisms.

**Method Selection**

Selection of an appropriate sterilization method is based on such items as the effect of the method on product quality or esthetics, regulatory requirements, industry practice, the economics of the sterilization process, and the logistics of sterilization compared with those of the overall manufacturing process. Safe and effective should be the guiding phrase in the regulated manufacture of healthcare products. This forms the basis of a complex network of statutes and regulations that dictate many of the practices and procedures employed in the healthcare production industry. Any sterilization method that compromises the safety or the effectiveness of the product is precluded from use.
There are situations, however, when a property of the product unrelated to either safety or effectiveness may be altered by a given sterilization method. To illustrate this, consider plastic polymers, which are commonly used in the production of medical devices. The sterilization methods typically applied to these devices are either EOG or ionizing radiation. Some plastic polymers, when exposed to sterilization doses of ionizing radiation such as 15 or 25 kGy, will discolor and decompose to produce toxic compounds such as 4,4’-dimethylaniline from polyurethane or bisphenol A from polycarbonate or polysulfone. This may affect the performance of a device and it can result in a product that is unacceptable to health facilities and users. In this circumstance, EOG may become the alternative sterilization method even though the residual toxicity is still being discussed at ISO TC 194 (ISO 10993-7).

Clearly, an important consideration in manufacturing processes is the economic effect of each process step on the cost of the final product. Thus, process development personnel strive to choose steps, or unit operations that are cost-effective. When the option of more than one sterilization method is open to the process engineer, he or she will usually select the least costly method in terms of price, time, or test requirements. Moreover, the economics of sterilization method can be optimized by proper design of the sterilization process.

Mechanisms of Destruction and Removal

In the broadest sense, all sterilization methods perform identically; they all render products sterile. Close examination of the individual methods demonstrates that each operates in a different manner. This section describes the biological basis of microbial removal or destruction in each of the five major methods of sterilization: moist heat, dry heat, sterilant gas or vapor, ionizing radiation, and filtration.

Moist Heat

Moist heat, or steam under pressure, is the most widely studied method of sterilization. As with all sterilization methods, the cellular function that is of primary interest is reproduction. No reproduction means no growth. The reproductive process of microorganisms is directed by nucleic acids (DNA and RNA) and mediated by enzymes-protein biocatalysts-which, among other things, direct nucleic acid synthesis and the construction of cellular components. Generally speaking, the three-dimensional (tertiary) structure of proteins, especially enzymes, determines their function. This structure is the result of the primary linear arrangement of amino acids, each of which has different chemical properties. When proteins are formed, the sequence of amino acids dictates the shape of the protein. This is because the individual amino acids interact with each other and their aqueous environment to yield the most stable shape. If the shape of the protein is changed after its formation, e.g., through protein denaturation, then its function changes. Often this change is irreversible and results in nonfunctionality.

During moist heat sterilization, it is the irreversible denaturation of vital enzymes that results in cell death. Both water vapor and elevated temperature are required to effectively denature proteins and kill cells. If water vapor is present, much lower temperatures are required in heat sterilization. Generally, moist-heat sterilization is performed with water-saturated steam under pressure at 121.1°C. The water vapor contributes to the available heat at any temperature (e.g., saturated steam at 121.1°C provides at least seven times as much available heat as air at the same temperature). This alone, however, does not explain the efficiency of killing cells by moist heat. The water vapor also interacts directly with the protein at the elevated temperature to denature proteins and enzymes. The exact protein or proteins that are rendered nonfunctional by moist-heat sterilization are widely known and this phenomenon is academically and economically unimportant because denaturation by heating is nonselective with regard to proteins and enzymes. The process of cell destruction is predictable and reproducible under defined conditions of operation.

Dry Heat

Dry heat is used to sterilize materials, such as glassware, metal parts, dry powders, and other substances, that can withstand heat. The lack of moisture in this sterilization process, compared with moist-heat sterilization, necessitates higher temperatures, on the order of 160 °C-170 °C, and longer time periods. Sterilization by dry heat actually represents an incineration process during which the cells are destroyed. This is generally considered an oxidative process during which the cellular components are destroyed at elevated temperatures. Other factors that may play a role include water content, water location within the spore or cell, and possible effects on DNA. The exact site of action has still not been clarified, but the process is predictable and reliable. To illustrate, dry heating is so often applied to the inactivation of endotoxins from gram negative bacteria at 250 °C for 30-60 mins to result in a three log reduction of endotoxins, which reduction FDA demands.
Sterilant Gas or Vapor

Sterilant gases and vapors, such as EOG, formaldehyde, VHP, peracetic acid, and chlorine dioxide are used for a variety of applications in aseptic processing. Gaseous and vaporous sterilants are most often used for heat-labile or radiation-incompatible plastic containers, closures, and drug delivery systems, as well as for sterility test isolators, manufacturing isolator systems, lyophilizers, and medical devices. Because EOG and VHP are the most commonly encountered gaseous or vaporous sterilants in aseptic processing, their microbiocidal effects are described in this paper.

EOG is the most commonly used gaseous sterilant. It works through a chemical reaction with cellular components, such as nucleic acids and macromolecules such as proteins and enzymes. Its chemical activity is as an alkylating agent, and it is through this mechanism, acting on nucleic acids and proteins, that it is thought to kill cells. EOG replaces labile hydrogen atoms with hydroxy ethyl (-CH₂CH₂OH) groups. Macromolecules, such as proteins, contain functional groups such as carboxyl (COOH), hydroxyl (OH), sulfhydryl (SH), amino (-NH₂), and imino (-NH), the hydrogens of which are labile to alkylolation. Because many of these groups play an important role in protein structure and function, their modification by EOG will disrupt or destroy the protein's activity. If the protein is vital to cell replication, then death occurs after its activity is lost. EOG is well known to effectively cause spore death. Therefore, the spore of Bacillus atrophaeus ATCC 9372 is selected as the BI for EOG (ISO 1138-1, 2, ISO 11135).

The VHP is widely used for the surface sterilization of isolators and, when optimized with pulsed vacuum cycles, can be used to sterileize certain types of packaging components. VHP cannot be used as a sterilant for liquids owing to condensation. The bactericidal, virucidal, and fungicidal mechanism of VHP is attributed to the oxidation of sulfhydryl groups (-SH) and double bonds in proteins, lipids, and surface membranes. Hydroxyl radicals are produced by the following reaction:

\[ \text{O}_2^- + \text{H}_2\text{O} \rightarrow \text{OH}^- + \text{OH}^- + \text{O}_2 \]

The OH radical plays the major role for VHP sterilization.

Compared with hydrogen peroxide in its liquid form, VHP has been reported to be highly sporicidal at low concentrations. The action of VHP on spores appears to be the removal of proteins from the spore coat. The use of G. stearothermophilus ATCC 7953 for the validation of VHP sterilization is at present widely known and utilized for the validation of the sterilization of isolators and cleanrooms.

Radiation

Ionizing radiation can be delivered to a product to be sterilized either by gamma rays (cobalt-60 or cesium-137, mainly Co60 is used) or by accelerated electrons (electron beam, E-beam). Although the method of depositing the radiation energy is different, the lethal effects are believed to be the same; gamma photons or E-beam electrons strike free electrons within the product which, in turn, strike other electrons resulting in ionization and the formation of free radicals. Large organic molecules, such as DNA, appear to be particularly susceptible to ionizing radiation. Because DNA is the essential template of living systems, any major changes in the template result in an improperly built structure and, in the cell's case, nonviability of the microorganism. Direct effects of ionizing radiation on organic molecules appear to be bond scission and free radical formation. Indirect effects are attributed to reaction products produced by ionization and water (e.g., hydroxyl radicals and peroxides). These highly reactive compounds can interact with macromolecules and disrupt their activity. It appears, however, that the direct interactions with DNA are more important. The sensitivity of a microorganism to radiation is a function of the volume of DNA within the microorganism.

Filtration

Removal of microorganisms (this is incorrectly called sterilization because microorganisms are not destroyed by filtration) by filtration represents a special case relative to the mechanism of action. In contrast with the other methods described thus far, filtration relies on the physical removal of microorganisms, rather than their destruction. In that sense, this procedure is not correctly classified as sterilization because no SAL is attained from this procedure. Filtration, limited to fluids, restricts the passage of microorganisms as the fluid passes through a filter. The mechanism of removal is a function of the filter type. Depth filters, composed of fibers randomly pressed together, remove microorganisms by a combination of adsorption and entrapment in their internal structure. Membrane filters, formed by a controlled polymer precipitation process, can retain microorganisms by sieving, entrapment, or adsorption, or a combination thereof. Size exclusion, a combination of sieving and entrapment mechanisms, is one of the most reliable removal mechanisms operating for all fluids. It is critical that the appropriate BI and processing conditions are used to validate the sterilizing filter. For
example, if adsorption is the predominant mechanism of removal in a membrane filter, changes in fluid physicochemical properties could serve to desorb BIb and allow them to pass into the product.

**VALIDATION OF STERILIZATION: BIOLOGICAL INDICATORS**

All sterilization methods are routinely validated through a common approach. First, a BI of performance is chosen that is resistant to the sterilization method. A second consideration is the influence of different variables affecting the resistance of the BI to the sterilization cycle, such as indicator propagation and interactions between the indicator and the target product being sterilized. Third, the quantitative nature of indicator destruction by the sterilization process is established. Finally, the concept of assigning sterility probabilities to materials undergoing sterilization is determined for a specific process. More succinctly, the validation of sterilization methods implies a collection of studies that prove the reliability and predictability of the method.

**CHARACTERISTICS OF INDICATORS**

The principal function of any sterilization method is to remove or destroy microorganisms. This function is usually validated by studies that use a particular microorganism as the BI of performance. Although each sterilization method uses a specific BI microorganism, all indicators share certain common characteristics, including the following.

**An Inherent Resistance to the Sterilization Method**

An inherent resistance to the sterilization method is clearly the primary characteristic to consider when choosing an indicator. It makes little sense to characterize a sterilization method if the indicator is easily removed or destroyed by the method when compared with other microorganisms.

**A Stable and Reproducible Resistance to the Sterilization Method under Defined Conditions Usage**

The effectiveness of a sterilization method is expressed in terms of its ability to remove a BI located in the worst place (cold spot in case of moist heating). If a BI shows wide variability in resistance, then it is of limited usefulness for either characterizing new-sterilization processes or monitoring existing ones; therefore it must demonstrate consistent resistance over time. This is defined by ISO 11138 1-5. Thus, the results obtained during validation will accurately predict the effectiveness of the sterilization method in routine operation.

**Efficient Recovery After Exposure to the Sterilization Method**

Typically, sterilization studies expose an indicator to a variety of sterilizing conditions, and the effectiveness of these conditions is expressed in terms of indicator survival. If a test microorganism is not capable of reproducing, for reasons other than the effect of sterilization conditions, then the effect of these conditions will be overestimated. In other words, any indicator that survives sterilization should be amenable to cultivation after exposure.

**Characteristic of the Microorganisms Commonly Occurring in the Product to be Sterilized**

Although the bioburden of products to be sterilized varies widely, there are some commonly occurring microbial species that require destruction or removal. The type of BI used in sterilization validation should reflect and be more resistant than those that must be removed during the actual operation of the sterilization process. Historically, each sterilization method has been validated employing a bacterium that possessed the foregoing characteristics. With the exception of a few sterilization methods (BIs of filtration are exceptional), BIs conventionally used have been a spore that is dormant form of some gram-positive bacteria such as Bacillus sp. and Geobacillus sp. (ISO 11138 1-5). This stands to reason, because the spore confers survival ability (high resistance) to the microorganism under a variety of adverse environments. The spore state is essentially a form of suspended animation.

**RECOMMENDED BIOLOGICAL INDICATORS**

Sterilization by moist heat (steam), dry heat, and sterilant gas, are validated with spores of gram-positive bacteria. Although B. pumilus spores have been used in the past to validate sterilization of ionizing radiation (gamma or E-beam), validation by a naturally occurring bioburden is currently preferred. Furthermore, BIs used to validate the effectiveness of filtration are for the most part small gram-negative rods. Spores of BI are generally the genus of Bacillus sp. and Geobacillus sp. Bacillus sp. and Geobacillus sp. are commonly found in the soil and, because their spores are resistant to desiccation, are often airborne contaminants. These BIs are either mesophilic, such as B. atrophaeus, or thermotolerant, such as G. stearothermophilus. The particular species or subspecies is selected, based on the resistance of that species to the sterilization method to which it has been assigned. Thus, it may be inappropriate to
substitute one species with another when characterizing a particular sterilization method unless information about its resistance and recovery is known.

The method for validating sterilization by ionizing radiation is based on the varying resistance of microorganisms to the radiation process. In fact, some microorganisms such as *B. megaterium* or *Deinococcus radiodurans* have a higher resistance to radiation sterilization than the previously recommended BI, *B. pumilus*. It is important to evaluate the naturally occurring bioburden of the product to be sterilized and to monitor both the bioburden and the sterilization dose on a regular basis, because a change in the bioburden character could decrease SAL (ISO 11137).

The indicator used for 0.22 μm filtration, *Brevundimonas diminuta*, is different from other BIs. This BI is not a spore type, but a vegetative cell type. The mechanism of filtration sterilization is that filters eliminate microorganisms by physically excluding them. Thus, the size of the indicator microorganism becomes the critical resistance factor. *B. diminuta* is a small rod type bacterium that, in addition to meeting the resistance criteria (size exclusion), also satisfies the other BI criteria and is a mesophilic, neutrophilic, heterotrophic aerobe. When *B. diminuta* is put in the water in the oligotrophic status, the size of the rod becomes much smaller and has the possibility of passing through 0.22 μm-sized pores. *Acholeplasma laidlawii* serves the same purpose for 0.1 μm filters and meets the same criteria.

**VALIDATION OF STERILIZATION: QUANTITATION OF INDICATOR REMOVAL**

The underlying rationale for sterilization validation by BI is that removal or destruction of high numbers of the indicator microorganisms will confirm the removal or destruction of the microorganisms that make up the product bioburden. Typically, large numbers of BIs are exposed to a set of sterilization conditions, and the effectiveness of the sterilization method is expressed in terms of the removal or destruction of the BI. Because sterilization methods are broken down into two basic categories, those that destroy microorganisms (heat, gas, and radiation) and those that remove them (filtration), the discussion of indicator removal quantitation will be presented in two parts.

**INDICATOR DESTRUCTION**

The basic tool in sterilization studies involving the destruction of BIs is the survival curve, an exponential relation derived by plotting the number of surviving BIs as a function of some parameter of the sterilization method. For sterilization by heat and EOG, survivor numbers are plotted as a function of exposure time under fixed conditions of sterilization (i.e., temperature for heat and the combination of gas concentration, temperature, and relative humidity for EOG). Ionizing radiation survival curves are plotted as a function of total radiation dose expressed in kGy (10 kGy=1 Mrad). Generally speaking, such a curve, when plotted on a semilogarithmic scale (with number of survivors on the logarithmic y axis, and time in minutes on the x axis), results in a negative slope. These curves may be linear or nonlinear. For simplicity this discussion will focus on linear survival curves.

It is from such survival curves that D values, a measure of sterilization resistance, are calculated. The D value (decimal reduction value) is defined as the amount of time, at a given temperature (dry or moist heat) or dose (radiation), that is required to reduce the microbial population by one order of magnitude (1 log). Put in terms of the survival curve, the D value is the slope of the line. Typical microbiological determinations of the D value start with BI at an initial known concentration and expose that population to an abbreviated sterilization process.

Evaluation of survivors can be accomplished by two different methods. The first, known as the survivor curve method, is a quantitative method. The number of surviving viable cells after a defined exposure is quantitated by standard microbiological techniques (plate counts) at various time intervals during the sterilization process. The resulting data are plotted as a survival curve and the D value obtained from the curve (ISO 14161). A second method of D value determination, known as the fraction-negative method, is a semiquantitative method because this has a premise that the survivor curve was straight from the initial population to the fraction negative range of SAL between 5 to 10^-2_. The fraction-negative methods are the Sperman-Karber Method and the Stumbo-Murphy-Cochran Method (ISO 14161). The number of surviving viable cells is evaluated by most probable number (MPN) techniques. Inoculated samples are incubated in a nutritive medium, after a defined exposure, and examined for the presence or absence of growth. The pattern of positive versus negative results are scored and used to calculate the D value for each time interval.

For applying the D value, if one wanted to reduce a population of cells by six orders of magnitude and the population had a D value of 2 min (for heat), then the total exposure time required to kill the population would be 12 min. Usually, sterilization methods are validated by an overkill approach. By this, it is meant that a sterilization procedure is extended beyond a 6-
log kill to, for example, a $10^{-6}$ probability of a survivor (12-log reduction or a 24-min exposure time).

A second quantitative expression used in thermal sterilization studies is the $Z$ value. The $Z$ value of a particular microorganism is the number of degrees of temperature necessary to change the $D$ value by a factor of 10. The $Z$ value reflects that microbial resistance to heat can change as the temperature changes. For example, if the sterilization temperature in a moist-heat process was increased from 121.1 $^\circ$C to 131.1 $^\circ$C for a microorganism with a $Z$ value of 10 $^\circ$C and a $D$ value of 5 ($D_{121.1^\circC} = 5$), the resistance to heat would decrease and the $D$ value would decrease by a factor of 10 ($D_{131.1^\circC} = 0.5$). $T$ is in degrees celsius and $D$ is the $D$ value corresponding to the specified temperature in the numerator. $Z$ values are obtained experimentally by plotting the logarithm of the $D$ values as a function of the temperature at which they are derived. Such a relation is known as a thermal death curve. The $Z$ value is the negative reciprocal of the slope of the thermal death and is calculated in the ranges of temperature between 110 $^\circ$C to 130 $^\circ$C (ISO 11138-3).

The third term commonly applied in thermal sterilization validation is the $F$ value (thermal death time). The $F$ value is the equivalent time, in minutes, at a specific temperature delivered to a product to produce a given sterilization effect at a reference temperature and specific $Z$ value. In reality, thermal sterilization processes do not reach a fixed sterilization temperature, for example 121.1 $^\circ$C, instantaneously. More likely, a product undergoing thermal sterilization will gradually heat up to and cool down from the sterilization temperature. In addition, the heat destruction of a microbial population is not a discrete phenomenon during which all the cells die at, and only at, the sterilization temperature. Rather, microbial destruction is a continuous process during the product heat-up and cool-down for which the rate of kill (the $D$ value), at any one temperature, changes as the temperature changes (reflected in the $Z$ value). Thus, it is possible to integrate the individual kills for each temperature during product heat-up and cool-down by knowing the thermal profile of the product (temperature change as a function of time) and the $Z$ values [Eq. (1)]. In other words, $F$ values account for the real world of the process heat-up and cool-down during which microbial destruction is occurring throughout the process cycle. In the equation below,

$$F = \int 10^{\frac{T_t - T_{ref}}{Z} / \alpha} \, dt \quad (1)$$

$T_{ref}$ = the exposure temperature within the product being treated, $T_t$ = the process reference temperature, and $Z$ = the $Z$ value

As mentioned earlier, the mechanism of microbial destruction is different for moist heat and dry heat. Dry heat inhibits the transfer of the moisture necessary for protein denaturation. Thus, microbial destruction in a dry-heat process will require longer processing times at higher temperatures than in a moist-heat process. This is accounted for in separate $F$ equations for moist heat ($F_m$) and dry heat ($F_d$). Note that the $Z$ value is assumed to be 10 $^\circ$C in Eq. (2) for $F_m$ and 20 $^\circ$C in Eq. 3 for $F_d$.

$$F_m = \int \frac{10^{\frac{T_{ref} - 121.1^\circC}{10}}}{10} \, dt \quad (2)$$

$$F_d = \int \frac{10^{\frac{T_{ref} - 170^\circC}{20}}}{10} \, dt \quad (3)$$

The $F$ value is dependent on the $D$ and the $Z$ values which, in turn, are dependent on the microorganisms tested, the microbial inoculum concentration, inoculum carrier, and the properties of the test product. Determination of resistance should be performed with calibrated BIs and, if necessary, on resistant product isolates as determined from bioburden testing.

**INDICATOR REMOVAL**

Sterilization by filtration is a process of microbial removal rather than destruction. Sterilization terms such as $D$ values and $Z$ values, therefore, do not apply to filtration. Nonetheless, quantitations of sterile filtration performance have been developed and used to validate the filtration processes. The quantitative term widely used to express the efficiency of microbial removal by filters is the log-reduction value (LRV). The LRV is defined as the log$_{10}$ of the ratio of the number of microorganisms challenging a filter to the number of microorganisms passing through the filter. The LRV is determined under “worst-case” conditions of BI numbers ($\geq 10^7$ colony-forming units (CFU)/cm$^2$ of filter surface area) and filtration conditions. Most manufacturers of filters provide sterilizing filters that exhibit no passage of indicator microorganisms (LRVs greater than the total bacterial challenge).

The worst-case indicates a set of conditions encompassing upper and lower processing limits and circumstances including those within standard operating procedures which pose the greatest chance of process or product failure when compared to ideal conditions. The worst-case for a filtration BI can be specific for a given process and may not be described solely by a single parameter such as the bacterial challenge concentration. For example, the
rigors of a high-challenge level may be offset by the microbial-culturing method if BI is not cultured to be of a size and shape most likely to penetrate the test filter.

In considering BIs for filtration, it is also important to understand the characteristic bioburden of a particular process stream before conducting a microbial retention test, because the process stream under investigation may contain a smaller bacterium than the proposed model. Knowledge of the bioburden will enable proper justification of the appropriate BI. For 0.22 μm-sterilizing-grade filters, *B. diminuta* ATCC 19146 is recognized as a BI when cultured according to recommended procedures. Many manufacturers of adjuncts to biotherapeutic products, such as serum and tissue culture fluid, require removal of mycoplasma (which are smaller than *B. diminuta*) from their process streams. In this case, the BI currently used for validation of 0.1 μm membranes is *Acholeplasma laidlawii*. Virus removal can also be validated. Because of the risk associated with mammalian viruses, characterization and modeling can be accomplished first with nontailed bacteriophage particles such as ΦX-174. ΦX-174 is a 30-nm particle, the icosahedral shape of which approximates a sphere and, therefore, is a desirable challenge model.

As implied in the D value concept of indicator microorganism destruction, there is a predictable relation between microbial death and some sterilization parameter (e.g., time and temperature for thermal sterilization). This is important, because the measure of such physical parameters during sterilization helps validate and routinely monitor the effectiveness of the sterilization process. This is especially important for sterile filtration. Worst-case chemical-and physical-processing conditions should be considered, for these can affect not only BI, but also the membrane filter. Physical conditions that should be monitored are flow rate, pressure, time, temperature, and hydraulic shock. Chemical conditions that should be evaluated are pH, viscosity, ionic strength, and osmolality. Routine use of BI during filtration is precluded, as microbial retention testing is a destructive test. The BI of *B. diminuta* is a gram negative bacteria, the primary source of endotoxins. If *B. diminuta* were used during the manufacturing process, it would compromise the nonpyrogenic requirement of healthcare products. Also, such artificial contamination of the product would alter the economics of a filtration process, because the filter would plug prematurely. Thus, there is a requirement for a relation between microbial removal and some measure of filtration performance. Such a relation exists between the LRV of a filter and integrity test values measured during routine filter usage (bubble point and diffusive airflow). Empirically derived under standard conditions and commonly provided by filter manufacturers, these relations provide useful information to users of filters when validating their filtration process.

**MICROBIOLOGIC EVALUATION OF CLEAN ROOMS AND OTHER CONTROLLED ENVIRONMENTS**

This section reviews the various microbiological issues involved in aseptic processing, especially the issues relating to clean rooms and other controlled environments for sterile healthcare production. Classification of clean rooms is based on particulate count limits: it is understood by most that the relationship between particulate count and microbiologic counts in clean rooms is not satisfactorily correlated. However, the classification based on particulates gives to the manufacturers of clean rooms specifications of the performance of these rooms that will allow them to design, build, test, and maintain clean rooms in a state of control. The design, development and implementation of a microbiologic evaluation program are an integral part of the assurance that the aseptic processing system is under microbiologic control. The microbiologic evaluation program should include the determination of the number and the type of microorganisms in the environment. It should also include a review of trends over an extended period to evaluate the effectiveness of the microbiologic program. Alert and action levels should be part of the microbiologic program as well as standard operating procedures when these levels are exceeded.

Microbiologic evaluation of clean rooms should include a sampling plan, the frequency of sampling, and the critical sites for sampling. Sampling sites include air, surfaces of equipment, floors, walls and other critical sites. Sites that are in direct contact with product or containers should be tested more frequently than those away from the critical sites. In addition, the need for strict procedures and supervision of personnel involved in aseptic processing is directly related to the sterility assurance of the final product. Attention must also be paid to the media-fill. A microbiologic growth medium instead of a product is used and a simulated aseptic processing is flowed to assess the overall aseptic process in terms of microbiologic contamination. Following aseptic processing, the medium is incubated at 20°C to 35°C with a range of ±2.5°C for 14 days. Visual examination for growth is done, and, if growth is detected, it is identified to the genus level to pinpoint the probable source of contamination. Some of the critical issues
to be considered include the number of units per run, the number of runs, and the interpretation of results. The number of units per run is used in the media-fill in order to assess that not more than one positive unit is encountered in 5000 units. However, the number of units per run seems to go up in order to increase the confidence that the results can be extrapolated for the operational runs with product.

**VALIDATION OF THE ISOLATOR SYSTEM**

Isolators used an aseptic process and isolators used for sterility testing share the characteristic that isolators do not only protect the product from contamination, but also the personnel from contamination by the product. The internal environment of an isolator is sterilized by decontaminating agents such as peracetic acid and/or hydrogen peroxide. At present, the use of hydrogen peroxide is most common. Sterilization of the internal environment is a misnomer, since the internal section is decontaminated. That is, the contaminants inside the isolators are rendered nonviable.

The sterile products and containers are introduced into the internal section of the isolator via transport ports that have been validated to ensure that contamination will not occur during transfer. Operators are not in contact with the product and manipulate the filling and closures of the containers from the outside using gloves or half suits. The air in the isolator is either sealed or supplied through a microbiologically retentive filter (HEPA filter). Isolators need not be placed in a classified environment, thus no microbiologic monitoring of the outside environment is needed. However, the environment outside the isolator should be restricted to only essential personnel.

The decontamination process used must be able to produce a 6-log reduction of an appropriate BI. However, the maintenance of the sterility of the internal portion of the isolator cannot be guaranteed over time. For validation, the fraction negative approach or survivor counts can be used for other methods of sterilization. The maintenance of sterility within the isolator enclosure depends on the operational parameters that control the various barriers to microbial contamination from the outside of the enclosure. The BI commonly used within the isolator is *G. stearothermophilus* ATCC 7953 at 10⁶ CFU/SUS carrier. Careful attention must be paid so that no clumping exists in the BI observed by scanning electron microscopy (SEM, Fig.1), otherwise no correct and reproducible sterility result can be attained.

In general, if contamination in an isolator is detected within the enclosure through a microbiologic monitoring system, it most likely has occurred during the introduction of the sterilized containers, closures and product into the isolator. It is of critical importance to validate the process used to ensure decontamination before the material is entered into the isolator. Other potential sources of contamination are the gloves or half suits that the personnel use when manipulating the products and containers/closures. Small holes in the gloves have been shown to be a source of contamination, and they are difficult to detect.

**CONCLUSION**

This paper serves as a microbiology primer, with particular attention paid to the microbiology of sterilization. Sterilization methods are designed to result in the total absence of living entities in a product, and the success of any method is defined in attaining an
appropriate SAL. The world of microorganisms is highly diverse, with few environments without microbial inhabitants. Nonetheless, there are certain microorganisms typically found in products as bioburden, requiring inactivation. Knowledge of these microorganisms helps define sterilization procedures. Each sterilization method differs in its mechanism of action, but all share the common characteristic of producing sterile products in a predictable and reliable manner.

ACKNOWLEDGEMENT

This paper was prepared through the support of a grant from the Ministry of Economy, Trade and Industry.

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