MICROBIAL TESTING IN SUPPORT OF ASEPTIC PROCESSING

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Abstract

Sterile products produced in staffed cleanrooms are subject to microbial contamination from the environment in which the process is carried out. The role of microbial testing to ensure the sterility of aseptically filled sterile products is explained, from the product development phase to in-process monitoring to finish product testing. Microbial testing is conducted in the sterile pharmaceutical industry in support of sterile product development; for in-process monitoring during aseptic processing and filling operations; and for testing finished products. In this study will be discussed the role that microbial testing plays in promoting sterility and assurance of aseptically filled sterile products.

Key words: microbial testing, sterile product.

Product development

The objective of the product development process is to take successfully progressing discovery leads from preclinical trials through development with the purpose of defining the formulation, delivery system, manufacturing process, and product specifications. The following microbial tests can be used during sterile product development and scale-up:

- microbial limits and bioburden testing
- bacterial endotoxin testing
- antimicrobial effectiveness testing
- container and closure integrity testing
- bacterial challenge testing for sterilizing filters
- aseptic processing validation using media fills

Pharmaceutical ingredient and packaging component evaluation.

Microbial considerations play a key role in the successful development of new sterile drug products. During formulation development, the potential microbial and endotoxin content of the active pharmaceutical ingredients and excipients should be considered. The testing used to evaluate the ingredients should comply with *USP* General Tests <61> "Microbial Limit Tests" and <82> "Bacterial Endotoxins Tests" [1,2]. Typically, USP- or NF-grade raw materials are selected for use in the formulation and the possible contibution each ingredient would make the product bioburden are evaluated. Recently, United States Pharmacopeia (USP) has begun adding bacterial endotoxin requirements on the basis of maximum human dosage for monograph ingredients that may be used in sterile products. In some cases, the blanket compendial Microbial Limits for the Total Aerobic Microbial Count not more than 1000 cfu/g or mL, and Total Combined Yeast and Mold Count not not more than 100 cfu/g or mL found in the draft *USP* General Chapter <1111> may be too loose for some sterile products [3]. The contribution that each individual ingredient may make to the presterile filtration bioburden, in terms of its concentration in the formulation, must be evaluated to minimize the bacterial challenge to the sterilizing filter and the endotoxin content of the product. Information about the properties and specifications of pharmaceutical ingredients is available in the *Handbook of Pharmaceutical Excipients* [4].

The bioburden of packaging components must be evaluated with respect to the sterilization process that will be used in the manufacture of sterile products. The bacterial endotoxin levels and the potential populations of gram-positive, spore-forming bacteria associated with stoppers and vials are a consideration. Vials must be inspected and packaged for shipment to the customer in a controlled environment. Individual vials must be separated with non-shredding dividers and shrink-wrapped to prevent glass-to-glass contact and particulate contamination. Vials are washed to remove particulates, depyrogenated to remove bacterial endotoxins, and sterilized before aseptic filling. The maximum temperature and belt speed are established for a depyrogenating tunnel that adequately depyrogenates and concurrently sterilizes the vials as they move through the tunnel into the aseptic filling area. Stopper preparation methods should physically remove bacterial endotoxins and nonviable particulates before siliconization. The cleaning and siliconization process should not contribute to the bioburden. The sterilization cycle ensures that the stoppers are sterile and dry so they can be stored before the aseptic filling operation. It should be noted that steam sterilization is not a depyrogenation step. Typically, stoppers are steam sterilized in heat-sealed, nonwoven, high-density polyethylene bags

that enable steam penetration and moisture removal during the sterilization, exhaust, and drying processes within a pass-through autoclave. Cleanroom personnel usually size the bags to replenish the stopper hopper with a single load to reduce the potential for microbial contamination during multiple handling. Alternately, semi-automated stopper preparation equipment can be used to prepare sterile siliconized stoppers. Stoppers also can be purchased clean, siliconized, and sterile. Initial bioburden and endotoxin monitoring of incoming packaging components should be conducted to establish whether the challenge levels for the cleaning, depyrogenation, and sterilization processes are adequate [5].

Container–closure integrity. The container–closure integrity of the packaging components also is addressed during product development using a sensitive and adequately validated test. Recommendations for various container–closure combinations from packaging suppliers are usually helpful. A physical container–closure integrity test may be selected and validated using a bacterial liquid immersion or aerosolization test. In general, physical tests are more sensitive than bacterial challenge tests. Therefore, the leakage observed during a physical test may not be indicative of sterility assurance loss. A comprehensive discussion about leak testing of pharmaceutical packaging systems has been published [6]. When selecting a test method, the container–closure type should be considered. Although stoppered vials are subjected to a bacterial immersion test, prefilled syringes are subjected to a bacterial aerosolization test because the latter has a more torturous path for container–closure integrity. Physical test methods described in the literature include the bubble method, helium mass spectrometry, liquid tracer (dye), headspace analysis, vacuum and pressure decay, weight loss or gain, and highvoltage leak detection. There are two phases to the container–closure integrity assessment: the initial evaluation and selection of the container–closure system and integrity testing within the premarketed stability program. Suitable testing intervals are 0,3,6,9,12,18, and 24 months during the premarketed stability program and annually during the postmarketed stability program. The number of samples tested at each time interval reflects the sampling requirements found in *USP* General Chapter <71>, "Sterility Test" [7]. Whenever possible, physical container–closure integrity tests for product monitoring should be substituted for sterility testing.

Sterility test development. The development of a sterile product requires initial and ongoing consultation with an experienced pharmaceutical microbiologist. The application of the sterility test is one indicator of the presence or absence of contaminating microorganisms in a sterile batch, but the sterility assurance is established by process design and validation, not simply finished product testing. Validation of the sterility test includes bacteriostasis and fungistasis testing and follows a procedure that is defined in *USP* General Chapter <71>, "Sterility Tests" Biological products marketed in the United States that must meet the 21 *CFR* 610.12 sterility testing requirements and products that differ in the subculturing requirements are an exception. The development microbiologist uses results from sterilization process validation, aseptic process simulation using media fills, and congruent environmental and personnel monitoring to generate an assurance level that is satisfactory for sterile product production. The use of biological indicators for sterilization process cycle development is another way for microbiologists to help build robustness into the production process. In cases in which sterile filtration is part of aseptic processing, the microbiologist should be aware of the filtration process capabilities by reviewing microbial challenge data generated during the validation of the filtration, filter integrity testing results, filtration operating parameters, and prefiltration bioburden levels. Because the sterility assurance level is higher with terminally sterilized products than with aseptically filled products, aseptic filling is used only when justified by the heat instability of the product or when the packaging systems cannot be subject to terminal sterilization. An example of the latter are prefilled syringes used for emergency drug administration or home care. Emphasis should be given to the establishment and monitoring of critical operating parameters used in the sterilization process with the aim of using parametric release for terminally sterilized products. Guidance is provided in *USP* General Information Chapter <1222>, "Terminally Sterilized Pharmaceutical Products: Parametric Release" [8].

Manufacturing process development. The individual manufacturing process steps for a new sterile drug product must be reviewed to determine their potential for sterility assurance loss. On the basis of risk assessment, critical control points can be established and, if necessary, monitored to minimize the risk of microbial contamination. The risk assessment includes the appropriateness of the aseptic manufacturing environment; aseptic

techniques; quality of the water systems; the sanitary design of processing equipment; equipment cleaning; sterilization and storage procedures; the establishment of immediate holding times for sterilized aseptic processing equipment and bulk solutions; the level of exposure of product to manufacturing personnel; aseptic sampling methods for product testing; and the establishment and monitoring of critical aseptic operating parameters.

In-process monitoring

The following microbial tests may be used during in-process monitoring:
- microbial limits and bacterial endotoxin monitoring of incoming pharmaceutical ingredients and packaging components
- presterile filtration bioburden monitoring
- bacterial endotoxin monitoring
- air, surface, and personnel monitoring in aseptic processing areas
- disinfectant effectiveness testing.

Monitoring incoming pharmaceutical ingredients, intermediates, and packaging components.
Incoming shipments of pharmaceutical ingredients used in the sterile product are routinely tested for bioburden and bacterial endotoxin levels. The concept of objectionable microorganisms is not useful for pharmaceutical ingredients used in sterile products; it is best reserved for the evaluation of the bioburden of nonsterile pharmaceutical products as used in the control of microbial contamination, per 21 *CFR* 211.113. It is not recommend screening pharmaceutical ingredients used in sterile products for USP specified microorganisms. Controlling bioburden to limit the microbial challenge to the sterilizing filter will readily control microbial toxins within sterile products because of the physical presence of microorganisms. The sterility assurance requirements will result in the numbers of organisms at least three magnitudes lower that those needed to control microbial toxins.

Sterile products are manufactured using water for injection (WFI) as ingredient water. Water must be manufactured by distillation. The storage tanks, loops, and points of use in a WFI system are routinely monitored for microbial content and bacterial endotoxins. The USP-recommended specifications are as follows: Total Aerobic Microbial Count not more than 10 cfu/100 mL and Bacterial Endotoxin Levels not more 0.05 endotoxin units/ mL [9]. When a validated, well-designed water system is used, each loop must be monitored weekly and each point of use must be monitored on a weekly or biweekly rotation. Although the use of a low

nutrient microbiological medium such as R2A agar incubated at 20–25ÚC for at least 7 days may yield the highest count, the USP-recommended method of using plate count agar incubated at 30–35ÚC for 48–72 h may be more suitable for the routine monitoring of a fully validated water system when the objective is to detect adverse trends in the water system in a timely manner and not to generate the highest possible count. Two additional advantages of using the plate count agar are greater growth promotion capacity for fungi and the inability to consistently subculture bacterial isolates from nutrient-poor R2A agar. In July 2002, the European Pharmacopeia Commission (EP) adopted the use of an R2A medium incubated at 30–35ÚC for at least 5 days as its official test method for water for pharmaceutical use. This medium is usually incubated at 20–25ÚC [10]. For US-based companies that make sterile products for a global market, the routine monitoring with the USP recommended method and periodic monitoring (e.g., each loop monthly monitoring of all loops using the EP-recommended method) is a practical monitoring program. Opportunities exist for the application of rapid microbial methods to water monitoring to generate the result earlier than 48 h. Packaging components such as molded or tubular glass vials or vulcanized rubber stoppers are manufactured with high temperatures and pressures and are unlikely to have an inherent bioburden or be contaminated with bacterial endotoxins. Thus, reduced testing of these packaging components is justified following a supplier's qualification through a site audit and incoming testing. The presterilized filtration bioburden is a critical parameter for the maintenance of a high level of sterility assurance of aseptically filled pharmaceutical products. Presterilized filtration bioburden monitoring of bulk solutions is a common practice in the manufacture of sterile pharmaceutical products. Singer and Cundell indicate that real-time measurement of the number and size of bacteria in the bulk solution could control the bacterial challenge to the sterilizing filter [11]. However, an appropriate bioburden level for a specified bulk solution volume and sterilizing filter surface area first must be determined. It is assumed that the alert and action levels are related to the recommended microbial count for bulk WFI (i.e., not more than 10 cfu/100 mL) and the sterilizing filter rating of the retention of 10^7 colonyforming units (cfus) of the challenge organism *Brevundimonas diminuta*/cm^2 of filter surface [12]. To exceed this filter capacity, a 50-L bulk solution passed though a 5-ft^2 sterilizing cartridge filter (filter surface area 4645 cm^2) must

contain more than 9 x 10^5 gram-negative bacteria or 18cfu/mL. Typically, bulk solution limits are set in the pharmaceutical industry at 1 or 10 cfu/100 mL, which is 180–800 times lower than the sterilizing filter rating. Furthermore, this limit is 3–4 magnitudes lower than the 10^4–10^5 gram-negative bacteria/mL that represents sufficient bacterial endotoxin to elucidate a pyrogenic response.

Environmental monitoring. Several key documents describe environmental monitoring programs for manufacturing sterile pharmaceutical products [13,14,15,16]. These documents, although not fully consistent in their approaches, are the basis of the environmental monitoring programs implemented by sterile product manufacturers in the United States. An unfortunate trend in the pharmaceutical industry is the compliance-inspired drive to take recommended target microbial levels for cleanrooms and other controlled areas and use them for the evaluation of microbial monitoring to detect adverse trends and make product release specifications. The methods used in microbial monitoring using contact plates and swabs for surface and personnel monitoring and using airsettling plates and active air samplers for air monitoring—have poor recoveries, low precision, and microbial levels that are typically at or close to the limit of detection of the methods. For example, the judgement that the isolation of 3 cfus/contact plate surface in a cleanroom is unsatisfactory although 1 cfu/contact plate is satisfactory is difficult to defend given the analytical capabilities of the microbial methods and the heterogeneity of the distribution of microorganisms within a cleanroom. Several air samplers are available for microbial monitoring. An ideal air sampler would use standard petri plates or surface contact plates; be battery-operated and portable; have high flow rates; be equipped with a delay function to enable the operator to position the air sampler and exit the area; have interchangeable heads for flexibility; and be self-calibrating or easy to calibrate.

A major function of microbial monitoring is to identify outof- trend conditions that can indicate a loss of environmental control or a breakdown in aseptic practices [16]. Typically, action levels are established on the basis of regulatory, compendial, or standard documents. Alert levels are established on the basis of statistical analysis of historic microbial monitoring data. Because environmental monitoring data typically are not distributed (i.e., the data exhibit high levels of skew toward zero counts), a nonparametric-tolerance-limits approach to setting

alert and action levels is recommended. These limits enable a confidence level of e" 95% (K = 0.95) that 100(P) or 99% of a population lies below the value for the respective data, and is depicted by the stated action limits. For distribution-free tolerance limits, the minimum sample sizes are $N = 60$ for 95/95 (alert limit) and $N = 300$ for 95/99 (action limits). Out-of-trend situations can be detected by flagging consequential alert level events that are statistically unlikely by monitoring the frequency of alert and action level events within a set period of time or by monitoring the average time lapse between alert or action events. For the latter case, if the time between events increases, the environmental control is improving; if the time remains constant, a level of environmental control is being maintained; or if the time lessens, the environmental control is deteriorating. The most frequently isolated microorganisms in controlled areas used for aseptic processing are bacteria from the human skin (e.g., the gram-positive cocci *Staphylococcus epidermidis, Staphylococcus hominis, Staphylococcus simulans, Micrococcus luteus,* and *Micrococcus varians*), skin diphtheroids (e.g., *Corynebacteria spp.*), and airborne bacterial spores, (e.g., *Bacillus sphaericus, Bacillus cereus, Bacillus thuringiensis,* and *Bacillus subtilis*); occasionally, airborne fungal spores (e.g., *Aspergillus niger, Penicillium spp.,* etc.); and most infrequently, gramnegative bacteria (e.g., *Enterobacter cloacae, Burkholderia cepcia,* etc.) are used. Given this pattern of isolation, the use of a general microbiological culture medium such as soybeancasein digest agar incubated at 30–35ÚC for 48–72 h is supported. Gram-positive cocci are found in high numbers on human skin and are readily shed. Controlled areas are protected from the cocci with the use of suitable gowns, hoods, facemasks, gloves, proper gowning techniques, and good aseptic practices. Bacterial spores that are formed during adverse conditions as a survival mechanism can be found in dust, cellulosic material, or foot traffic. However, fungal spores reproduce asexually and are shed from actively growing fungal colonies within damp building materials such as cardboard packaging material or vegetation surrounding the facility.

Microbial identification. FDA's "Aseptic Processing Guidance" emphasizes that monitoring should provide meaningful information about the quality of the aseptic processing environment when a batch is manufactured, and should identify adverse trends and potential routes of contamination [17]. The sampling frequency, timing, duration, and size;

equipment and techniques for sampling; alert and action levels; and corrective actions should be defined by standard operating procedures (SOPs). Detection of microbial contamination of a critical site (i.e., product or component contact surfaces) should not necessarily result in batch rejection because the sampling procedures may lead to false positives. FDA has indicated that an increased incidence of contamination over a given period of time is equal to or more important than consecutive isolation. Microorganisms that are isolated during routine environmental monitoring are characterized by their cellular morphology and staining reactions as gram-positive rods, cocci, and rod-shaped spore-formers; gram-negative bacteria; or identified by genus or species depending on the criticality of the sampling location with respect to product exposure. The frequency of isolation and the microbial counts are used to establish trends and to demonstrate a continuous level of environmental control.

In addition, the change in the so-called typical microflora that is found in cleanrooms and other controlled areas may indicate a possible loss of environmental control. Phenotypic microbial identification methods which are determined by morphology, gram reaction, biochemical activity, fatty acid composition, and carbon utilization are the industry practice for the routine identification of microorganisms isolated during the monitoring of pharmaceutical ingredients, water for pharmaceutical use, the manufacturing environment, and the finished product. Microbial identification may be helpful when investigating monitoring results that exceed the alert or action levels or when investigating media fill or sterility test failure to determine the possible source of the microorganisms and corrective actions. Genotypic microbial identification methods (which are determined by nucleic acid analyses) may be less subjective, less dependent on the culture method, and theoretically more reliable than phenotypic methods because nucleic acid are highly conserved by species. These methods include a polymerase chain reaction, 16S and 23S rRNA sequencing, DNA hybridization, and analytical ribotyping. However, these methods are technically more challenging and expensive and frequently are marketed by a single company. Their use should be limited to critical investigations of direct product failure and the identifications should be conducted in a pharmaceutical company's specialized research-orientated laboratory or sent to a contract testing laboratory. The increased accuracy of identification and the ability to determine the strain of microorganism may be considered

molecular epidemiology to more definitively determine the origin of the microbial contamination in media fill and sterility test failures and action level environmental monitoring excursions. Discussions of rapid microbial methods in the pharmaceutical industry have been published [18,19].

Aseptic processing validation. In August 2003, FDA published a draft guidance document to help manufacturers meet the CGMP regulations of 21 *CFR* Parts 210 and 211 when manufacturing sterile drug products and biological products using aseptic processing. This draft guidance document, when finalized, will replace the 1987 "Industry Guideline on Sterile Drug Products Produced by Aseptic Processing" According to the document, media fill studies should simulate aseptic manufacturing operations as closely as possible, incorporating a worst-case approach. At least three consecutive successful media fills should be performed during initial line qualification. It is recommemded that a semiannual requalification be used to evaluate the state of control of each filling line's aseptic process state of control. All personnel who enter the aseptic processing area should participate in a media fill at least once per year. This participation should reflect their routine job responsibilities. The duration of the media fill run should adequately mimic worst-case conditions and include all manipulations without being the same run size as the production fill. Tension exists between the concept of using worst-case conditions in a media fill and not attempting to validate unacceptable aseptic practices. Between 5000 and 10,000 units should be filled during a media fill. For <5000-unit batch sizes, the number of media-filled units should be equal to the batch size. The target should be zero contaminated units. If one out of 5000–10,000 units is a turbid media-filled unit, an investigation must occur and consideration should be given to a repeat media fill. If two units are turbid, the process must be investigated and revalidated. When filling >10,000units, one turbid unit should be investigated and two turbid units should a cause and investigation and revalidation. Media fills should include all vial sizes and fill volumes that are used during production and not simply the worst-case conditions. A general microbiological growth medium such as soybean-casein digest broth (or its growth promotion equivalent) should be used. Growth promotion should be demonstrated using organisms listed in *USP* General Chapter <71> as well as environmental, personnel, and sterility test failure isolates < 100-cfu challenge. The growth promotion testing should reflect the incubation conditions that are used for the media fill. The use

of incubation temperatures outside the optimum range for major groups of environmental microorganisms may result in a failure of organisms that grow in the media. Because human-derived *Staphylococci, Micrococci,* and *Corynebacteria* are the most commonly isolated microorganisms in aseptic processing areas, the author favors an incubation temperature of 30–35ÚC for 7 days followed by 7 days at 20–25ÚC. Filled units that are rejected because of the loss of integrity should not be incubated. Written procedures for interventions and line clearance should be assessed during media fills. Media fill inventory documentation should account for and describe units rejected from the run. The media fill should be observed and contaminated units should be traced to the approximate time and to the activity that is being simulated. Contaminated units should be fully investigated. Invalidation of a media fill is a rare occurrence. The differences between FDA's draft guidance and the approach taken by ISO and the European Union is the European assumption that a low, but discernable, contamination rate exists with aseptic processing. FDA has emphasized situational contamination (e.g., failure of aseptic technique during filling station setup), while the Europeans believe that an aseptic process will have a finite contamination rate determined by the environmental control level of the process. This belief is manifested by research relating contamination rate to various air cleanliness standards, and the use of air-setting plates as a tool for continuous monitoring. An acceptance criterion for process simulations of a target with zero contaminated containers, but e" 0.1% contamination rate, has been developed. To make a statistical claim for a 95% confidence level, the number of mediafilled units has been specified (i.e., 4750 filled units), and if one contaminated unit is found, the contamination is < 0.1%. From a statistical point of view, if the true proportion of contaminated media-filled units is $P = 0.001$, then the number of contaminated units will increase as the population size increases. When 3000, 4750, 6300, 7760, 9160, and 10,520 units are filled, the number of turbid units is 0, 1,2,3,4, and 5, respectively, at the 95% confidence level with a 0.1% contamination rate. The ability of inspectors to consistently detect microbial growth in media-filled containers can be questioned. Christensen demonstrated that as the number of units inspected increased and fill volume decreased, the reliability of visual inspection decreased [20]. Vision systems were recommended to screen out presumptive turbid containers that are critically examined by experienced microbiologists, particularly because media fill lot sizes continue to grow.

Finished product testing
The microbial tests used for finished product testing are sterility and bacterial endotoxin tests.
Sterility testing. *USP* General Chapter <71> "Sterility Testing" expresses a preference for the membrane filtration method because it concentrates microbial contaminants within a single container of microbiological broth. However, tripartite bacteriostasis and fungistasis testing does not require that the recovery from the innoculated controls and products samples be comparable throughout the incubation period as is recommended in FDA's "Aseptic Processing Guidance." This requirement is found in the *Australian Therapeutic Goods Authority Regulations* and represents a minority opinion among regulators and pharmaceutical microbiologists [21]. The compendial test does not include information about the selection of the test samples because it is not technically a release test. It is industry practice randomly to select filled containers from the beginning, middle, and end of an aseptic filling operation (i.e., a stratified random sampling plan). The industry does not support the "Aseptic Processing Guide" recommendation that samples be selected from containers filled during processing excursions and/or interventions. Routine interventions are included in the process simulation. Rules for line clearance are developed and included in SOPs and documented in batch records. Some excursions (i.e., environmental monitoring alert levels) would only be determined after the fill is complete, making it impossible to identify the associated filled container. The author believes that this recommendation should be removed from the draft guidance document. It is well known that the compendial sterility test has poor efficacy rates [22]. For a sample size of 20 containers, the test will detect a 1% contamination rate with a > 20% probability. Thus, the test will only consistently detect grossly contaminated product. Furthermore, the probability of passing a repeat sterility test after an initial failure is presumed to be higher because one or more microbial-contaminated containers have been removed from the lot. The poor efficacy of the compendial sterility test in detecting low contamination rates implies that manufacturers must adhere to CGMPs, validation of their aseptic processing methods, good facility design, and training of their employees to achieve high levels of sterility assurance.

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Asbtract

Objectives. The aim was to review our experience with the surgical repair of the anomalous origin of one pulmonary branch from the aorta (AOPA).

Materials and Method. Between January 1995 and March 2013, 10 patients with AOPA underwent surgical correction. Four patients presented isolated AOPA. Six patients presented AORPA and 4 AOLPA.