A REVIEW OF STERILITY TESTING IN PHARMACEUTICALS AND BIOTECHNOLOGY

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Abstract

Sterility testing is a critical quality control procedure used to ensure that pharmaceutical products, medical devices, and biological preparations are free from viable contaminating microorganisms such as bacteria, fungi, and yeast. The test is particularly important for products that are required to be sterile, including injectable medications, intravenous fluids, ophthalmic preparations, and implantable devices. The main objective of sterility testing is to confirm the absence of microbial contamination in the final product, which could lead to serious infections or adverse reactions in patients.

The testing process is typically performed using two main methods: membrane filtration and direct inoculation. In membrane filtration, the product is passed through a sterile filter that traps any microorganisms, which are then incubated in a growth medium. In direct inoculation, the product itself is introduced directly into a growth medium and monitored for microbial growth over a specified period, usually 14 days. Both methods require stringent aseptic techniques, appropriate environmental conditions, and validated procedures to prevent false positives or negatives.

Advances in rapid microbiological methods are being explored to reduce the time required for sterility testing, which traditionally takes weeks. However, conventional methods remain the gold standard in many regulatory environments. Ensuring the accuracy of sterility testing is essential for patient safety and regulatory compliance in pharmaceutical manufacturing.

Keyword-*Aseptic technique, Sterilization, Contamination prevention, Microbial control, Germ free environment*

Introduction.

It is easy to become complacent with our habits, whether those habits are good or bad. Bad medical habits, however, can lead to detrimental health conditions and untimely deaths. In 1847, when Vienna, Austria, was considered the world's leading medical center, physician Ignaz Semmelweis discovered by simple observation the cause of puerperal fever, then known as childbed fever. Semmelweis implemented a solution, and, in 3 months, the death rate in the maternity ward fell from 18% to 1%. Semmelweis' solution was simple—he ordered doctors and medical students to wash their hands. He determined that a doctor going from patient to patient without handwashing could carry and spread puerperal fever, which today is known to be caused by *Streptococcus pyogenes*. Although his findings were published and even duplicated, nobody listened. It would be many years and thousands more deaths before handwashing became accepted clinical practice. Those involved in quality control are also subject to complacency.

Quality-control methods often are accepted because "that's the way it has always been done." This is an especially dangerous attitude if the test result is biased or based on the conditions set forth, or if a better method is overlooked. A quality-control laboratory has a great responsibility to not only adhere to the guidelines, but to continually examine its employees and methods, and should refer to the literature critical to the field involved; microbiological testing is no exception. Microbial testing in pharmaceutical compounding should be examined in two parts: (1) process validation and (2) end-preparation sterility testing. United States Pharmacopeia (USP) Chapter <797> outlines process validation in the sections titled "Personnel Training and Evaluation in Aseptic Manipulation Skills" and "Environmental Quality and Control." End-preparation sterility testing falls under the "Finished Preparation Release Checks and Tests" section. Each section is important, but the focus here is only on sterility testing.

The pharmacopeial standard applying to sterile products is that sterile products must be capable of passing a test for sterility. The sterility test is an important, microbiological test in terms of regula-tory expectation rather than probability of detecting gross contamination. It allows for the examination of products purportedly to be sterile. This paper fo-cuses on the conventional sterility test method as set Out in the harmonized pharmacopoeias (i.e., Euro-pean, Japanese, and United States). The sterility test is used as a product release test, where the sterility of A product is defined by the absence of viable and ac-tively multiplying microorganisms when the product Or pharmaceutical preparation is tested in specified culture media. Detectable microbial growth is con-firmation of non-sterility, whereas sterility can be as-Summed from the absence of growth. In other words, the test assumes sterility unless non-sterility can be demonstrated. A failure with product sterility could lead to an adulterated product should the product be released onto the market (1). The method for conducting the sterility test is clearly documented in the European, Japanese, and United States pharmacopeias. Oc-casionally, the sterility test will produce a positive result. This demands both an examination of the Laboratory test and an examination of the production process to determine why the sterility test failure occurred. The conclusion of such an investigation Will be either that the sterility test was invalid due to some type of "laboratory error" (a position for which a great deal of caution is required given that regula-tory agencies require a robust rationale) or that the product was contaminated due to some event or inci-dent in the manufacturing, sterilization, or filling of the product or with the sterilization of components. This paper examines some of the areas to consider When looking at sterility test failures and in doing so draws upon the guidance in the FDA Guideline on Sterile Drug Products Produced by Aseptic Processing and from the author's experiences of failure investi Gations (2). The emphasis is on the established con-ventional method. Although it is acknowledged that some

companies have adopted rapid methods and in This context some of the guidance in this paper is not applicable, the conventional sterility test remains the most widely practiced and one of the most important Release tests for sterile products.

STERILITY TESTING

The conventional sterility test is one of the oldest and Most established pharmaceutical microbiology tests (3). It was first described, as a direct transfer meth-Od into broth media, in the British Pharmacopeia in 1932 The conventional sterility test is an established cultural test method, both as a membrane filtration method, which is the method of choice for any filterable product, and as a direct inoculation (direct transfer) method for products that cannot be readily filtered or for medical devices. The three pharmacopoeias (i.e., European Pharmacopoeia [EP 2.6.1], the United States Pharmacopeia [USP <71>], and the Japanese Pharmacopoeia [JP 4.06]) harmonized the sterility test in 2009. In addition, reference to the sterility test is made in the FDA Code of Federal Regulations. With the federal regulations, a change in direction away from cultural-based methods and towards consideration of rapid microbiological methods was proposed in a 2011 edition of the Federal Register. Rapid methods include Adenosine Triphosphate (ATP) bioluminescence, chemiluminescence, and carbon dioxide headspace measurement. These are methods that have been approved in the US. There are, nonetheless, other types of rapid methods.

Sterility – Process of removing all viable forms of microorganisms.

Sterility test – A test that critically assesses whether a sterilized pharmaceutical product is free from contaminating microorganisms.

(or)

Acc to IP – The sterility tests are intended for testing the absence of viable forms of microorganisms in or on the pharmacopeial preparations.

With membrane filtration, the concept is that the microorganisms will collect on the surface of a 0.45-micron pore size filter after the product has been passed through the filter membrane. This filter is segmented, or two separate filters are used, and transferred to appropriate media. The typical test media used are fluid thioglycollate medium (FTM) and soybean casein digest medium (SCDM), which is commercially known as tryptone soya broth (TSB). Alternative culture media may be suitable for particular applications, such as testing products with preservatives, and can be used provided that these are validated. FTM is selected based upon its ability to support the growth of anaerobic and aerobic microorganisms. TSB is selected based upon its ability to support a wide range of aerobic bacteria and fungi. The incubation time is 14 days at 30-35°C for the FTM media and 20-25°C for the TSB media. The outcome of the sterility test is assessed as a presence or absence test (i.e., microbial growth either occurs or it does not). Growth, or absence of growth, is determined by

examining the media, which are generally clear and transparent. For this, some elect to inspect the media against an artificial light source. Turbid (cloudy) areas in or on the media are indicative of microbial growth.

To be used as a product release test, the sterility test requires validating. This consists of validating the media before use (i.e., growth promotion) and in the presence of the product. This is done to determine if the test sample will inhibit the growth of microorganisms in the test media, as the tests for bacteriostasis and fungistasis. For the growth promotion testing of the cultural method, the microorganisms should include those stated in the pharmacopoeias (i.e., Gram-positive coccus, a Gram-positive rod, Gram-negative rod, and fungus). In addition, the FDA guidance document on sterile drug products recommends that if a sterility test failure has previously occurred, the isolate should be additionally used to challenge the culture media. Each different type of product requires validating using the selected test method. Often the use of multiple rinses (e.g., the membrane filtration test), the use of different types of filters (e.g., cellulose acetate or cellulose nitrate), or the addition of neutralizers to media is required in order for many products to pass the test.

The sterility test is conducted in a controlled environment, or in an isolator, to a comparable standard to the environment used for aseptic filling operations. The test environment is described in USP General Informational Chapter <1211>, the FDA guidance document for sterile drug products, and in the PIC/S Annex to the Guide to Good Manufacturing Practice for Medicinal Products—Manufacture of Sterile Medicinal Products. It is commonplace to conduct the sterility test in an isolator. The isolator does not need to be located within a controlled environment, although many organizations choose to place the isolator within an ISO Class 8 area. The use of isolators is recommended by the FDA guidance document for sterile drug products. It is recognized that some facilities conduct the test within an EU GMP Grade A/ISO 14644 Class 5 unidirectional airflow cabinet located within an EU GMP Grade B/ISO 14644 Class 7 cleanroom. The use of a cleanroom with a laminar flow hood is less desirable because a sterility test failure could be the result of a less controlled laboratory environment.

The product tested could have, in fact, been nonsterile. In both the cleanroom and, most importantly, the test environment, environmental monitoring should be undertaken in order to assess the cleanliness of the sterility test environment. For aseptically filled products, the sterility test is a mandatory product release test. For terminally filled products, batch release by parametric release may be undertaken. It is, however, statistically poor at detecting anything other than gross contamination. This limitation has been covered in a number of studies and relates to the few numbers of articles tested. Limitations also arise in relation to the difficulties in selecting a sample representative of all significant events during batch filling. Contamination is unlikely to be uniformly distributed

throughout the batch, and thus, random sampling cannot detect contamination with absolute certainty. Conversely, a pass result does not necessarily mean that the product is sterile. The status of sterility relates to the overall concept of sterility assurance and the methods in place to protect the product during development. Nonetheless, the sterility test is a mandatory test, and over a period of time sterility test failures may occur. When such failures occur, as with any so-termed microbiological data deviation, a documented investigation is required. The object of such investigations is to establish the root cause, to undertake corrective and preventative actions (CAPA), and to demonstrate that the action taken is effective, as well as making a recommendation whether the product is safe to release or if the batch should be rejected.

History/Evolution

Throughout the industry, there is confusion regarding the proper use of sterility testing for monitoring the effectiveness of sterilization, packaging qualification, and shelf life determination. Several decades ago, the pharmacopeial sterility test on fully processed product was acknowledged as the definitive test to determine the sterility of a batch or lot of product. However, as validation methods and principles began to be developed and understood, the pharmacopeial sterility test moved from a batch-by-batch release-type test transitioned to a test associated with process validation. The U.S. Pharmacopeia (USP) acknowledged that the confirmation of sterility should be based on process validation and associated data rather than a single, limited sterility test. This change was largely due to the understanding that the sterility or sterility assurance level (SAL) of a fully processed batch or lot could not be proven using a small number of samples. The term "sterility test" is often used generically to describe various tests used to determine microbial growth. Currently, there are two definitions put forth by ISO/TC 198 (the ISO technical committee for the sterilization of healthcare products) to differentiate the applications of the test. One is the "test of sterility," which is associated with the development, validation, or requalification of a sterilization process. This test is performed on partially processed products during development, validation, or requalification exercises and is intended to provide data for a specific intermediate point in a process, after which further processing can be defined and applied.

The other definition is a "test for sterility," which is the classic pharmacopeial test, typically performed in the validation of an aseptic process or, in some cases, as a referee test when validation is in question or unavailable. This type of sterility test remains in pharmacopeias for certain applications, but for many years it has been specified that the test should not be used alone (in lieu of validation) to confirm product sterility. Throughout this article, the terms "sterility test" and "sterility testing" will be used, except in cases where emphasis is required for a specific application. Current international and national documents related to sterilization specifically acknowledge the limitations of any type of sterility test, especially if used to confirm the sterility or

SAL characteristics of a product outside of process validation.

Many quality-control laboratories often view alternative methods as "taboo." This may be warranted if the methods haven't been validated against the standard of USP Chapter <71>. Furthermore, while the industry may never change the 14-day sterility testing incubation requirement, there are continuous discussions about the potential for improvements in sterility testing methods must still be an effort to improve early detection of microbial contamination.

Material and Methods of Sterility Testing.

Sterility testing is a critical procedure in the pharmaceutical, biotechnology, and medical device industries, where ensuring that a product is free from viable microorganisms is essential for patient safety. This section provides detailed information on the materials and methods commonly used in sterility testing, focusing on the tools, culture media, test methods, and environmental controls required to carry out accurate and reliable testing.

1. Materials for Sterility Testin

The sterility testing process requires specific materials and equipment to ensure that accurate and reliable results are obtained. These materials are designed to maintain aseptic conditions and prevent contamination during the testing process.

1.1. Equipment

- Sterile Isolators or Laminar Flow Hoods: Sterility testing must be performed in a controlled environment to minimize the risk of contamination. A sterile isolator is a closed system that provides a completely sterile environment, while laminar flow hoods maintain an aseptic workspace with a unidirectional flow of filtered air. Isolators are often preferred for their more stringent control over the environment.
- Incubators: Incubators are required to maintain specific temperature conditions for the growth of microorganisms. Two different incubators are often used: one set at 20-25°C for fungi and other microorganisms, and another set at 30-35°C for bacteria.
- Sterile Filtration Units: For membrane filtration methods, filtration units with sterile 0.45-micron pore size filters are used to capture microorganisms from the test sample.
- Sterile Containers and Tubes: These are used to store and transfer test samples, diluents, and culture media under aseptic conditions.
- Pipettes and Pipetting Devices: Used to transfer liquid samples and culture media without introducing contamination. These are typically sterile and disposable.
- Sterile Forceps, Scissors, and Other Instruments: Instruments used to

handle materials, such as membrane filters, must be sterilized before use or should be pre-sterilized and disposable.

- Growth Promotion Test Strains: Sterility testing must include the use of standard microbial strains to ensure that the testing media can support microbial growth. Common strains include *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Clostridium sporogenes*, *Candida albicans*, and *Aspergillus brasiliensis*.

1.2. Culture Media

Two primary types of media are used for sterility testing:

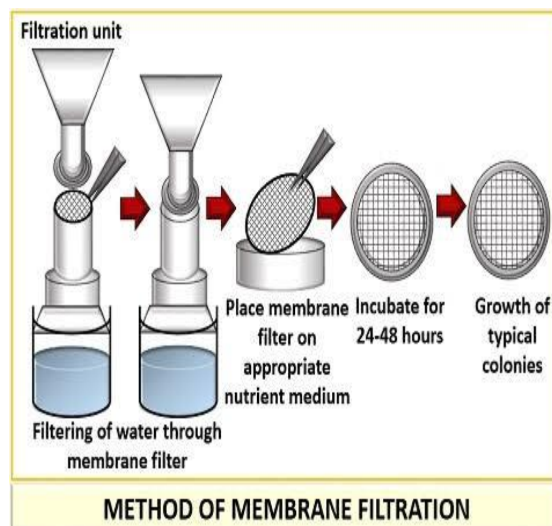
- Fluid Thioglycollate Medium (FTM): This medium is used for the detection of anaerobic and aerobic bacteria. FTM is designed to support the growth of a wide range of microorganisms, including both fastidious and non-fastidious bacteria. The medium contains a reducing agent, such as thioglycollate, which helps maintain anaerobic conditions in the lower part of the medium, while the top portion is exposed to air, allowing aerobic microorganisms to grow.
- Soybean Casein Digest Medium (SCDM): Also known as Tryptone Soya Broth (TSB), this medium is used to detect aerobic bacteria and fungi (molds and yeasts). It supports a broad spectrum of microorganisms, including bacteria, fungi, and yeast, and is widely used in pharmaceutical sterility testing.

Both FTM and SCDM must be prepared according to pharmacopeial standards, sterilized, and validated through growth promotion testing to ensure their ability to support microbial growth

2. Methods for Sterility Testing

Sterility testing is performed using two main methods: membrane filtration and direct inoculation. The choice of method depends on the type of product being tested (e.g., liquids, solids, powders, or medical devices) and its ability to pass through a filter.

2.1. Membrane Filtration Method



METHOD OF MEMBRANE FILTRATION

This method is considered the gold standard for testing filterable products, such as aqueous solutions, oils, and other liquids that can be passed through a filter. Membrane filtration is preferred because it allows for the separation of microorganisms from the

product, reducing the risk of product interference with microbial detection.

Procedure:

- Sample Preparation: The product to be tested is diluted if necessary to avoid clogging the membrane filter. For products with antimicrobial properties, neutralizing agents or diluents (such as polysorbate 80 or lecithin) may be added to neutralize the product's antimicrobial activity.
- Filtration: The sample is passed through a 0.45-micron membrane filter, which traps any microorganisms present in the sample. The filtration process is carried out under vacuum or pressure to ensure efficient passage of the sample.

- Rinsing: After filtration, the filter is rinsed several times with a sterile diluent to remove any residual product that could inhibit microbial growth.

- Incubation: The membrane is then cut into two portions or transferred to two separate culture media: one portion is incubated in FTM for anaerobic and aerobic bacteria, and the other in SCDM for fungi. The incubation period is typically 14 days, with daily monitoring for any signs of microbial growth (e.g., turbidity in the media).
- Observation: After the incubation period, the media are visually inspected for signs of microbial growth. The presence of turbidity, cloudiness, or any sediment indicates microbial contamination. Clear media suggest that the sample is sterile.

2.2. Direct Inoculation Method

This method is used for products that cannot be filtered, such as ointments, creams, or certain medical devices. In this method, the test sample is directly inoculated into the appropriate culture media.

Procedure:

- Sample Preparation: The sample is prepared according to its physical characteristics. Ointments and creams may be emulsified or diluted with sterile diluents to facilitate mixing with the culture media.
- Inoculation: A predetermined volume of the test sample is aseptically added to two separate containers of culture media. One container contains FTM, and the other contains SCDM. The amount of sample added must not exceed the volume that can be adequately diluted by the media to avoid false-negative results due to the product's antimicrobial activity.
- Incubation: As with the membrane filtration method, the inoculated media are incubated for 14 days, with daily observation for signs of microbial growth.

- Observation: The media are inspected for any signs of microbial growth, such as turbidity or cloudiness. The absence of growth after 14 days indicates that the product is sterile, while the presence of growth suggests contamination.

2.3. Alternative Rapid Microbiological Methods

In recent years, rapid microbiological methods (RMM) have been developed to reduce the time required for sterility testing. These methods include technologies such as ATP bioluminescence, chemiluminescence, and CO_2 headspace measurement. These methods are more sensitive than traditional methods and can provide results in a matter of hours or days instead of weeks.

- ATP Bioluminescence: This method measures the presence of ATP (adenosine triphosphate), a molecule found in all living cells. If microorganisms are present in the sample, the ATP they produce will react with a luminescent substrate, producing light. The amount of light generated is proportional to the number of microorganisms present.
- CO_2 Headspace Measurement: This method detects the production of CO_2 by microorganisms as they metabolize. An increase in CO_2 levels in the headspace of a sealed container indicates microbial growth. This method can be used for both aerobic and anaerobic microorganisms.
- Fluorescent Staining: Microorganisms can be stained with a fluorescent dye, which allows for their detection using fluorescence microscopy or flow cytometry. This method can provide rapid detection of microorganisms, especially in complex samples.

3. Environmental Contro

Sterility testing must be conducted in a controlled environment to prevent contamination from external sources. Environmental control is critical to the reliability of sterility testing results.

3.1. Controlled Environment or Cleanroom

Sterility testing should be carried out in a Grade A or ISO Class 5 environment, which provides a sterile workspace. This environment may be an isolator or a laminar flow hood. In both cases, the workspace must be validated and routinely monitored to ensure that it remains free from contaminants.

3.2. Personnel Aseptic Technique

Personnel performing sterility tests must be trained in aseptic techniques to prevent accidental contamination of the test samples. This includes proper gowning procedures, hand hygiene, and the use of sterile gloves, masks, and gowns. Personnel should avoid any activities that could introduce contaminants into the sterile workspace.

3.3. Environmental Monitoring

Environmental monitoring is critical for maintaining a controlled environment. Monitoring includes regular sampling of air, surfaces, and personnel to detect any potential contaminants. Sampling is usually done using settle plates, contact plates, and active air samplers. Any deviations from acceptable limits must be investigated, and corrective actions should be taken to prevent contamination during sterility testing.

4. Validation and Documentation

Sterility testing requires rigorous validation to ensure that the methods and materials used are reliable and capable of detecting microorganisms. Validation includes:

- Media Growth Promotion Testing: This ensures that the culture media used in sterility testing can support the growth of a wide range of microorganisms. Standard microbial strains are inoculated into the media, and the media are observed for growth.
- Method Suitability Testing: Before sterility testing can be applied to a specific product, method suitability testing must be conducted. This involves spiking the product with known microorganisms to confirm that the testing method can detect contamination, even in the presence of the product.
- Documentation: All sterility testing

Conclusion

Microbiology, like compounding, is a science that must be demonstrated to show that it is reliable, reproducible, and scientifically sound. Aseptic technique must become second nature to the microbiologist and compounder and, as they teach in graduate school, those involved in the sciences must begin to "think like the bugs." Care must be taken during the compounding process to ensure that the preparation being made is of the highest quality, and microbiological testing is no exception. On a daily basis, quality-control laboratories are on the front line of testing newly formulated preparations. With each new drug tested, there is a great responsibility that everything possible is done to ensure that the test result reported is accurate and reliable. While it is recognized that the conventional sterility-testing method has inherent deficiencies, an alternative method cannot be used unless it provides equivalent assurance of detecting microbial contamination.

Too many times, quality-control laboratories get caught up in the old adage "if it ain't broke, don't fix it." Often, it is not just that something is "broken," but that it can be improved. Imagine if Dr. Semmelweis hadn't decided to make a difference; it would have been easier for him to keep going along like everything was fine. Quality-control laboratories should think the same way and want to make a difference.

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