



The Test for Sterility of Medicinal Products

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INTRODUCTION

This paper examines the one of the most important tests required for products purportedly to be sterile: sterility test.

Sterility is an absolute term: either something is sterile or it is not. The definition of sterility for pharmaceutical products is not a strict biological definition that would be based on 'the total absence of viable life'. The sterility test only examines for those bacteria and fungi which can grow under the particular cultural conditions of the test. Even with this more limited 'pharmaceutical definition' of sterility, the term sterility cannot be proved, and it can only be quoted in terms of probability. One of the ways that the probability of sterility is given greater assurance is through the sterility test (Gilbert and Allison, 1996).

Although today a significant number of experts question the need for sterility testing of a given batch that has terminally received validated amount of heat or irradiation sterilization conditions, most do agree that aseptically processed batches as well as gas sterilized batches (since the process is considered difficult to control) require proper testing and release.

Several types of product are required to be sterile. These include parenterals, ophthalmics and inhalations. By their very nature, a significant number of these types of products cannot be terminally sterilised. Biological vaccines may be destroyed by the sterilization process, for example, the influenza vaccine. Additionally, some products are prescribed to immunocompromised patients and therefore must be sterile to avoid causing harm (e.g. antibiotics delivered by inhalation for Cystic Fibrosis patients).

Short History of the Sterility Test

The first sterility testing methodology, which involved use of direct inoculation with five days incubation period, appeared in 1932 in the British Pharmacopoeia. Through the decades of years several changes in the British Pharmacopoeia (and, by association, the European Pharmacopoeia), as well as United States Pharmacopoeia and Japanese Pharmacopoeia were implemented and today a harmonized monograph exists. These harmonized monograph has two basic procedures of which the most common -membrane filtration method- is used for most pharmaceuticals while direct inoculation method is usually used for medical devices.

Can The Sterility Test Really Confirm Product Sterility?

However, this applied statistical probability results in the sterility test being one of the most meaningless microbiological tests performed. This is because very little of the batch is tested (the EP and USP allow a maximum of 20 units to be tested from a batch size of 500 or more). For batches of a size in the several 1000s the chance of detecting a contaminated unit are very slim. For example, if 5% of a batch was contaminated and only 10 samples are tested for sterility then 84 / 100 sterility tests would pass each time (refer to TGA Guidelines, 2002). Therefore, the sterility test can only detect gross contamination. There is no value in increasing the number of samples presented for sterility testing by doubling, as some companies have done, in response to something like a series of poor environmental monitoring results. Statistically there is no greater chance of detecting a failure (coupled with the lack of any conclusive link between the amount of contamination detected through environmental monitoring and sterility test failures) (Sandle, 2013).

Even under conditions of heavy contamination the sterility test will only show the presence of those micro-organisms that will grow under the test conditions. These conditions include the particular culture media and incubation conditions; recovery methods and diluents; and assumption that micro-organisms will not pass through a filter of 0.45µm porosity when the membrane filtration method is employed. Therefore, the test can only detect gross contamination of those micro-organisms which will produce visible turbidity in the culture media under the conditions applied.

Data that is far more meaningful to give a probability of sterility is derived from the quality systems; sterility assurance and the microbiological and physical environmental monitoring employed during the production process (Sandle, 2000). Furthermore, more accurate results can be derived from new technology like real time epi-fluorescence counting can theoretically detect any microbial genetic material in a product. These methods, however, are far away from regulatory acceptance.

Not only are there limitations with what the result of the sterility test is telling us but there are limitations with performing the test itself. The risk of a obtaining a false positive result during testing must be managed as stringently as we manage the aseptic processing of the batch itself. There are a number of potential risk areas that may lead to a false positive result:

- A compromise of the sterility test zone by the technician, possibly due to repetitive actions and poor ergonomics.
- Poor establishment of the aseptic environment prior to performing the test, typically due to the lack of the bio contamination process.
- Over reliance on barrier technology to exclude microbial contamination can mean that good aseptic technique is not employed.

- Environmental monitoring interventions can compromise the aseptic testing zone if not part of the system design.

However, these limitations accepted, the sterility test is a regulatory requirement for the release of products that cannot be terminally sterilised (such as aseptically filled or heat-labile products). A sterility 'pass' remains a criterion for product release using the relatively unchanged pharmacopoeial methods. Parametric release remains some way off in the future. The FDA 'Sterile Products Produced By Aseptic Processing' (issued in 2004) only cites the compendial sterility test.

Sterility Test Methods

There are two principle methods of sterility testing as defined in the pharmacopoeias:

- a) Membrane filtration
- b) Direct inoculation

Of these methods, membrane filtration is the method of choice because all of the contents of a small volume product are filtered or at least half the contents of a large volume product through a membrane filter. Therefore, a much larger sample size is tested than is for the direct inoculation method, where the amount of product can vary from 1- 2 ml to half the container contents. Furthermore, any micro-organisms present are far more likely to be separated from potentially inhibitory substances in the product through the act of filtration or can be eliminated by rinsing the filter. It is also common for membrane filtration systems to be enclosed, such as the Steritest system (introduced in 1975), which minimises risk of contamination by reducing transfer steps (Van Doorne et al, 1998).

Membrane filtration is the appropriate method for all aqueous, alcoholic, oily and solvent products that can pass through a sterile filter with a porosity of 0.45µm. The standard filter is manufactured from cellulose esters or other similar plastics. The filter acts to separate the product from any micro-organisms so that the product passes through the filter and any micro-organisms present in the product are trapped within the filter matrix. A rinse solution (such as phosphate buffered saline, saline or Ringers solution) is used to remove any product residues. This washing process is normally performed three or four times and filter should remain wet throughout.



Figure 1: Dr. Tim Sandle inspecting a membrane filtration test chamber (image: courtesy of Tim Sandle)

The filter is divided into two portions (or more than one filter is used as in the widely used Steritest polycarbonate filtration system). To each filter, culture media is added, so that any micro-organisms trapped in the filter membrane, following incubation at a suitable temperature, will replicate. Two culture media are used. The pharmacopoeias recommend fluid thioglycollate medium, incubated at 30-35°C to isolate bacteria (aerobic and anaerobic); and soya bean casein digest medium (tryptone soya broth), incubated at 20-25°C to isolate aerobic bacteria and fungi.

However many products will not readily filter (such as protein based products which will block the filter pores) or are so inherently anti-microbial that the direct inoculation method is used. Direct inoculation may also be preferred over membrane filtration if the membrane filtration method simply cannot be validated. When the direct inoculation method is selected, the laboratory should be able to justify why it has selected this method over the membrane filtration technique. The direct inoculation technique involves the addition of a portion of the product to two different culture media (FTB and TSB, as per the membrane filtration technique). This is half the contents of the product vial to each culture medium - for product between 50 mg and 300 mg - or the entire contents for products less than 50 mg. For very large volumes of product a concentrate of the culture medium is sometimes added to the product.

For the direct inoculation technique, products which have anti-microbial activity must be neutralised before a portion of the product is added to the culture medium. This is performed either by the addition of a neutraliser or by dilution of the product.

According to pharmacopoeial procedures, sterility testing of a given batch requires 4-20 containers (depending on the product type and batch size) randomly collected samples.

The incubation time for both test methods is fourteen days. The previous incubation, which stood for fifty years, was seven days. This was increased to fourteen, in the EP, in 1997, following the adoption by Australia, and later matched by the USP and JP. This was because it was estimated that 30% of sterility test failures occurred between seven and fourteen days due to the time taken for sublethally damaged or stressed micro-organisms to grow. Micro-organisms isolated from a sterility test are more likely to be stressed due to the transfer from their environment into a more hostile environment (the product) and then into a completely different nutrient rich environment (the culture media). These micro-organisms are also likely to be low in number (as little as one cell). These factors contribute to a relatively long lag phase at the start of the microbial cell growth cycle in the culture medium.



Figure 2: A technician preparing direct inoculation test bottles (image courtesy of Tim Sandle)

For products which produce suspension, flocculation, and turbidity or deposit so that the presence or absence of microbial growth cannot be readily seen, a subculture step is required. To subculture a suitable portion of the culture media is transferred to a container of the same media type and incubated for a further time period (as discussed below).

The items incubated must be clearly labelled with the identity of the product; the medium used; the temperature of incubation and the date of testing. Throughout the incubation of the sterility test the articles must be examined regularly for growth (this is often every day or every other day). When inspecting the items on test care must be taken to prevent undue agitation, especially of the thioglycollate medium. If anaerobic conditions are not maintained, this will be indicated by the resazurin indicator. At the end of the incubation period the articles must be inspected, by gentle swirling, for visible turbidity against an artificial light source.

If turbidity is seen an investigation must be performed. This is examined later in this paper. For the test to be valid, certain conditions must be met:

- The culture media used is sterile (often shown by incubating articles of culture media alongside the sterility test)
- The culture media can support microbial growth (from growth promotion testing)
- The product does not have a microstatic or microbicidal effect (or can be eliminated) – as indicated by sterility test validation
- Contamination is not introduced into the test by external sources

Test environment

The sterility test must be undertaken in an environment which does not introduce contamination and lead to the potential of a false positive occurring. A suitable environment is either a unidirectional airflow device held within a cleanroom or an isolator. Environmental monitoring should be undertaken during each test session in order to demonstrate that the environment is within control.

Sterility test media

All media used for the sterility test must have passed a growth promotion test for nutritive properties and must not have exceeded its nutritive properties expiry time. The Ph. Eur. requires the following micro-organisms to be used:

- a) For FTB, between 30°C and 35°C, inoculate with *Clostridium sporogenes* (ATCC 11437) and *Staphylococcus aureus subsp. aureus* (ATCC 6538) and one of the following aerobic bacteria:
 - i) *Bacillus subtilis subsp. spizizenii* (ATCC 6633)
 - ii) *Pseudomonas aeruginosa* (ATCC 9027)
- b) For TSB, at between 20°C and 25°C, inoculate with a minimum of one of the following fungi:
 - i) *Candida albicans* (ATCC 10231)
 - ii) *Aspergillus brasiliensis* (ATCC 16404) [Formerly *Aspergillus niger*, taxonomic change by ATCC August 2008]

and with a minimum of one of the following aerobic bacteria:

- i) *Staphylococcus aureus subsp. aureus* (ATCC 6538)
- ii) *Bacillus subtilis subsp. spizizenii* (ATCC 6633)
- iii) *Pseudomonas aeruginosa* (ATCC 9027)

USP chapter <71> Sterility Tests requires the following regime:

- a) For FTB:
 - a) Anaerobic micro-organism: *Clostridium sporogenes* (ATCC 11437 or ATCC 19404) or *Bacteroides vulgatus* (ATCC 8482)*
 - b) One of either *Staphylococcus aureus subsp. aureus* (ATCC 6538) or *Bacillus subtilis subsp. spizizenii* (ATCC 6633)
 - c) One of either *Pseudomonas aeruginosa* (ATCC 9027) or *Micrococcus luteus* (*Kocuria rhizophila*) (ATCC 9431)
- b) For SCDM:
 - i) *Candida albicans* (ATCC 10231)
 - ii) *Aspergillus brasiliensis*¹ (ATCC 16404)

* Only where a non-spore forming micro-organism is required. This is not the case at BPL.

Sterility test method validation

Each product type must be validated to show that the product in the presence of the culture media does not possess any anti-microbial activity (this is sometimes referred to as bacterostasis and fungistasis). Validation is also performed if there is a substantial change to a previously validated product or to the culture media.

The sterility test validation involves, for each type of micro-organism listed below:

- a) Membrane filtration method: after transferring the contents of each final product vial/bottle through the membrane, less than 100 cfu is added to the final portion of the saline rinse.
- b) Direct inoculation method: after transferring the contents of each final product vial/bottle into the culture medium, between 10 and 100cfu is added to the test culture media bottle.

<u>Microorganism</u>	<u>Media</u>
<i>Staphylococcus aureus subsp. aureus</i> (ATCC 6538)	FTB, TSB
<i>Bacillus subtilis subsp. spizizenii</i> (ATCC 6633)	FTB, TSB
<i>Pseudomonas aeruginosa</i> (ATCC 9027)	FTB, TSB
<i>Clostridium sporogenes</i> (ATCC 19404)	FTB
<i>Candida albicans</i> (ATCC 10231)	TSB
<i>Aspergillus brasiliensis</i> ² (ATCC 16404)	TSB

The above list is based on Ph. Eur. recommendations. It is also in keeping with USP. The USP requirements are the same as those for the validation of culture media, which are described above.

¹ Formerly *Aspergillus niger*, taxonomic change by ATCC August 2008.

² Formerly *Aspergillus niger*, taxonomic change by ATCC August 2008.

For the validation, positive controls of media in the absence of product must be used. Bacteria must grow in not more than three days and fungi in not more than five days 'Growth' is defined as 'clearly visible growth' in comparison to the positive controls.

GMP requirements

When conducting sterility tests a number of GMP requirements must be adhered to. Many of these relate to record keeping. During sterility testing, detailed records must be kept. These records should include:

- Description of method
- Details of the method of transfer into clean room or isolator
- Number of product units tested
- Batch / lot number
- Stage of manufacturing (e.g. finished product / intermediate / final bulk).
- Personnel performing the tests.
- Dates of testing.
- Test method.
- Volume tested
- Diluents / solvents used.
- Media batch numbers.
- Temperature and incubation time.
- Date of reading the test and who by.
- The result (pass or fail).
- Environmental monitoring results.
- Negative control results.

Sterility Test Failure Investigation

For every positive sterility test an investigation should be undertaken to consider if the result is valid. That is, does the failed test relate to the sterility of the product or is it due to a laboratory test error (such as environmental contamination during the test session)? (Sandle, 2011).



Figure 3: Investigating a sterility test contaminant (image: courtesy of Tim Sandle)

In the investigation stemming from a sterility test failure consideration should be given to:

- Speciation of the organism
- Record of laboratory results and deviations
- Environmental monitoring of production environment
- Monitoring personnel
- Product pre-sterilization bioburden
- Production record review
- Manufacturing history

Any isolates from sterility testing should be identified to species level. Here:

- The use of genotyping techniques is encouraged.
- Identical methodologies should be employed in species identification in sterility test and environmental monitoring program.

All investigations into sterility test failure should be documented.

Alternative and rapid methods

The fact that the growth based pharmacopoeial procedure requires long incubation period has led to significant research interest which came up with alternative and rapid tests of which some are already commercially available. These methods also termed as Rapid microbiological methods (RMMs) are classified in to three types: Metabolic or growth based, Viability based and those based on technologies that analyze cell component such as nucleic acid (Denoya, 2011). Those which are considered better developed for sterility testing purposes are discussed here.

ATP-bioluminescence

This appears to be by far the most established rapid method which employs enzymes to break down microbial ATP from growing cells and produce visible light which can be measured with ATP-bioluminescence detectors. The method has been described as sensitive and capable of providing results as those of the monograph procedure in less than 7 days Celsis Rapid detection System and Milliflex Rapid microbiology detection and enumeration system are commercial examples. This method however like the monograph procedure is product destructive and growth based (i.e. with specific media and incubation conditions, though growth time is significantly reduced).

Colorimetric growth detection

This method is based on sensitive colour detection systems that can detect carbondioxide produced by growing microbial cells. It is reported that the method can produce results in 3 days BacT/ALERT 3D Dual- T Microbila Detection System from bioMerieux is a commercial example. This method is also product destructive and growth based.

Autofluorescence detection

This method yet to be commercialized for purposes of sterility testing is based on the fact that all living cells produce a small amount of fluorescence which can be amplified from growing cells placed on solid surface (such as agar) and made detectable by naked eye. It is stated that results can be obtained in less than 3 days. Again this method also appears to be product destructive and growth based.

Cytometry systems

Cytometry counting does not rely on growth but uses fluorescent cell labeling techniques. Typically, the microbial cells are labeled using a fluorescent dye or a non-fluorescent substrate which is then converted to fluorescent viable cells. Detection is achieved by laser scanning in either a flow cell or on a solid phase such as membrane filter. Scan RDI is a commercial example which is claimed to detect 1 CFU. This method, though destructive but being non-growth based and rapid, indeed appears to address the two major drawbacks of the monograph method (i.e. spectrum /power of detection and delay).

CONCLUSION

This paper has examined the Sterility Test. In doing so the two primary methods have been outlined, together with some of the practical aspects of these methods. The paper has also discussed the statistical limitations of the test and has emphasised the need for focusing on sterility assurance, including environmental controls during batch manufacture, in order to increase confidence in the probability that the product is sterile.

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