Sterility Testing

Introduction

This chapter examines some practical approaches to sterility test method validation, focused on the culture based method. Sterility test method validation, for routine products that filter readily, is relatively straightforward and the method for doing so is laid out in the pharmacopoeias (such as USP <71> and Ph. Eur. 2.6.1). However, for a variety of other products the prescriptive instructions in the pharmacopoeias are not readily adaptable and therefore some modification of the standard method is required.

This chapter discusses practical approaches that can be taken for products where pharmacopoeial methods cannot be readily applied. Examples of potentially difficult products are:

- Mercurial compounds
- Antibiotics
- Turbid samples
- Medical devices
- Oily samples
- Catgut
- Radiopharmaceuticals
- Cell lines

Given that the sterility test has the inherent limitation that the test will only show the presence of those microorganisms that will grow under the test conditions, care must be taken with the establishment of any method for difficult products and the success or otherwise of any manipulation must be validated.

For testing products using the culture based method, the pharmacopeia offers two choices:

a) Membrane filtration
b) Direct inoculation

The method of choice for the culture based sterility test method, as this book has emphasises, is the membrane filtration method. The same rank order applies when difficult products are considered for an attempt should be made to test the product using the membrane filtration method first and then only resorting to the direct inoculation method when the product can be shown not to filter. Membrane filtration is the preferred method 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 large sample size is tested than is for the direct inoculation method, where, with the inoculation method, the amount of product is only between 1ml to half the container contents. It also stands that the membrane filtration method is easier to validate. When validating a product for the first time, the selection of the direct inoculation method over the membrane filtration method should be justified in the validation report.

Validation

For products which can be tested satisfactorily and for those which cannot be tested in a straightforward manner, the pharmacopoeia requires that the selected method is validated. Unlike conventional assays, where the 'assay' is validated, sterility test validation is the validation of each product with the method; that is the validation of culture media in the presence of product

The validation approach is relatively straightforward, for standard products. However many pharmaceuticals are not readily testable using the method as described in the pharmacopoeias without some form of test modification or product neutralisation.

Practical approaches for 'difficult' products

Any method designed for testing 'difficult' products with the sterility test must be validatable and, consequently, validated. This should be central to the design of any method.

Some products (or the preservative that is added to them) possess an anti-microbial activity. These products will not pass the sterility test validation without some form of manipulation. It may be that only some microorganisms will be inhibited and not others (for example, it is often suggested that *Aspergillus niger* is the most resistant of the standard set of validation organisms to anti-microbial substances and a hostile cultural environment). However, a method needs to be established whereby each of the required test panel of microorganisms exhibit growth.

Some general variations to technique that can be adopted for dealing with products that will not pass the sterility test validation are listed below. The list is not necessarily in order of priority and the laboratory will need to consider the cost and time implications of each. It may also be that some manipulations are easier to perform in a conventional cleanroom than they are inside an isolator, which may have a bearing on the method selection.

<u>Membrane Filtration</u>

a) Type of membrane filter

There are different types of membrane filter that can be used for the sterility test. Principally these are divided by properties: hydrophobic or hydrophilic; and by the primary material of manufacture: nylon, cellulose acetate, cellulose nitrate or polycarbonate.

All standard filters have a porosity of 0.45 μm with low product binding characteristics. For a 'standard' aqueous based product, hydrophilic filters are the most commonly used (such as those manufactured from mixed esters of cellulose). Hydrophilic filters are easily wet with water. Hydrophilic filters can be wetted with virtually any liquid, allowing the liquid to pass through the filter effectively.

Hydrophobic edged filters are widely used for the membrane filtration of antibiotics (such as those manufactured from mixed esters of cellulose, polyvinylidene difluoride

or polycarbonate). The use of a conventional hydrophilic filter with antibiotics can lead to the antibiotic remaining at the periphery of the membrane (which would affect bacterial growth). A hydrophobic filter overcomes this affect by minimising the anti-microbial residues at the filter edge (which are difficult to rinse out). If antimicrobial residues remain then this can lead to a false negative result (although, before this stage, the product under test would not pass the validation test in terms of inhibiting the growth promotion of the challenge microorganisms).

A hydrophobic filter can additionally help to separate out microbial cells from the product. The rinse solution is then employed to rinse away product residues, leaving only microbial cells trapped in the filter matrix.

The two main filter types are cellulose nitrate and cellulose acetate. Sometimes the key to a successful validation is as straightforward as selecting the correct filter type. Cellulose nitrate filters are used for testing aqueous, oily and weakly alcoholic solutions, whereas cellulose acetate filters are used for the testing of strongly alcoholic solutions.

b) Pump speed

When undertaking the membrane filtration test, controlling the pump speed can assist with the filtration of certain products, such as reducing the amount of foaming or reducing the tendency for the filter to block. It is unlikely that variations to the pump speed alone will make a significant difference to whether a material can be successfully tested or not. It is important, incidentally, that the pump speed is recorded when undertaking test validation, as this can avoid problems when the method is transferred for routine testing.

c) Type and number of rinse solutions

If inhibition cannot be overcome by the act of membrane filtration alone then the rinsing of the filter can often over come anti-microbial effects. The common rinse solutions used in the sterility test are saline; phosphate buffered saline or Ringer's Solution. Proud and Sutton found that a 'Universal Diluting Fluid' (UDF), based on Dey-Engley neutralising broth, was the optimal solution for neutralising anti-microbial activity . The UDF was effective against such compounds as thiomersal, benzalkonium chloride, biguandies and so on.

Anti-microbial effects can further be overcome by varying the number of rinse solutions. The pharmacopeia places a limit on the number of rinse solutions that can be performed (this is 5 x 500 ml after a recommended 3 x 100 ml has been attempted). The pharmacopeia did once dispensation for the product to be released without validation if this is acceptable with the Regulatory Authority; however, the text in the harmonised pharmacopeia states: "Modify the conditions in order to eliminate the antimicrobial activity and repeat the method suitability test."

Variations can be made to the rinse solution through the addition of neutralisers. Common general additives include polysorbate-80 or the surfactant Triton X-100. Polysorbate 80 is a nonionic surfactant and emulsifier derived from polyethoxylated sorbitan and oleic acid. Triton X-100 ($C_{14}H_{22}O(C_2H_4O)_n$) is a nonionic surfactant

which has a hydrophilic polyethylene oxide chain and an aromatic hydrocarbon lipophilic or hydrophobic group. It has detergent properties. There is some uncertainty as to whether this substance is toxic to some micro-organisms and would thereby create a false negative.

For antibiotics, the main neutraliser is penicillinase. Penicillinase is a specific type of β-lactamase, showing specificity for penicillins. Beta-lactamases are enzymes produced by some bacteria and are responsible for their resistance to beta-lactam antibiotics like penicillins, cephamycins, and carbapenems (ertapenem).

Even by varying the amount of rinse solutions or the formulation of the rinse solution, some products remain very difficult to counteract through rinsing because of their tenacious attachment to the filter. An example is erythromycin lactiobionate.

Direct Inoculation

a) Type of neutralising agent

For the direct inoculation test, there are different neutralising agents that can be added to the culture medium (or even directly into the product) and therefore be used to inactivate different anti-microbial compounds. Some products require very specific neutralisation agents, for others multipurpose agents like lecithin or polysorbate-80 ('Tween') can be used. The table below shows **some** examples of neutralising agents appropriate for different anti-microbial agents:

Anti-microbial agent / product	Neutralising agent
Benzalkonium chloride 0.01%	0.5% lecithin and 3% polysorbate-80
Chlorohexine	Lecithin and polysorbate-80
Parabenz	5% polysorbate-80 or 0.07% lecithin and 0.5% polysorbate-80
Mercurial compounds	Thioglycollate / sodium thiosulphate / thioglycollate with cysteine
Azide	Azolectin
Sorbic acid	Dilution and polysorbate-80
Collageb implant	3% polysorbate-80
Organic acids	Polysorbate-80
Penicllin / cephalosporins	Penicillinase (β-lactamase – volume determined from antibiotic assay). Considered less effective for cephalosporins – membrane filtration recommended
Chloraphenicol	Chloramphenicol acetyltransferase
Sulphonamide	P-aminobenzoic acid

Table 1: Neutralisation agents

b) Dilution

The dilution of some products prior to direct inoculation can overcome anti-microbial properties as can varying the volume of the culture media used. For culture media volumes, the USP, until version 27, allowed up to 2000 ml of culture media to be used if other attempts at neutralisation are not successful (although it is important to continue to allow sufficient air space in the tryptone soya broth). If 2000 ml was not successful, the USP then allowed the sterility test to proceed using a media volume of 2000 ml (provided the laboratory can be granted acceptance by the Regulator). It may be possible to argue for a volume greater than 2000 ml if this is required for the immersion of a medical device. The problem with increasing volumes is that there is a danger of this resulting in a low recovery of any low level contamination or a possible of increasing the likelihood of there being a false positive through increased manipulations. In contrast the Ph. Eur., and the 2004 version of the USP (#27) onwards, contains no set dilution limit provided that "volume of the product is not more than 10 per cent of the volume of the medium."

Instead of increasing the volume of the culture media an inhibitory effect can be overcome through dilution of the product. An example of varying the product dilution is the dilution of benzyl alcohol or phenol, which requires a 1:50 dilution with sterile water. Furthermore, the type of diluent used can have an impact upon antimicrobial activity. The use of some solvents or solutions with neutralisers can be more effective than simple dilution or reconstitution with sterile water.

c) Turbid samples

Turbid samples present a problem, especially when the direct inoculation method is used. For normal sterility testing turbid samples require subculturing. When this occurs is a matter of debate. The pre-2012 version of the FDA CFR 610 described this as taking place at 3 – 7 days after the initial test and then re-incubating the subcultured product for an additional seven days alongside the original sterility test (therefore total test time becomes 14 + 7 days). Whereas the PHR. EUR., and the USP from #27 onwards, described this taking place after the end of the standard sterility test incubation (i.e. after 14 days have elapsed). With no direct reference in the current CFR to such aspects of the cultural method, the subculture step is undertaken after the initial read.

The incubation time for the 'second' test is not clearly specified, the text: "transfer portions (each not less than 1 mL) of the medium to fresh vessels of the same medium and then incubate the original and transfer vessels for not less than 4 days." The laboratory will need to determine an appropriate incubation time. To validate this, either a similar subculture is required to demonstrate if there is microbial growth (because such growth cannot be seen in a broth that is rendered turbid by the product itself) or plating out.

<u>For both techniques</u>

a) Pre-treatment of the product

Some products, particularly solids or articles, require manipulation prior to filtration or direct inoculation. Typically, this involves either dissolving the product in water (if it is water soluble or has been freeze-dried) or dissolving with a solvent (such as creams

or water insoluble substances). For direct inoculation, either dissolving or adding the solid (or disassembling the article) directly into the culture media. The addition of a heating step can either facilitate or speed up the dissolution. Variation to these approaches can influence the success or otherwise of the validation. However, such approaches can often be variable and it is important to cover all possible differences in product volumes and consistency in the validation exercise.

b) Use of non-standard culture media

Some products are not testable using the culture media described in the pharmacopoeias; therefore, alternative or modified media may be used. Some companies select Fluid Sabouraud Media instead of, or along with, tryptone soya broth if the potential for there being fungal contamination is high. For the testing of water assumed sterile, some data indicates that the use of R2A is preferable to tryptone soya broth. This is due to the mediums ability yield higher numbers of bacteria from water (the bacteria being in low nutrient environments and in a stressed or damaged state and therefore they may not be able to multiply in the nutrient rich tryptone soya medium). To go down this path would be a variation from the pharmacopeia and this would need justifying and validating.

An example of modified media is for the testing of penicillins where the addition of penicillinase to media is required (β-lactamases are discussed above). Furthermore, the testing of medical devices, according to the USP, is performed using alternative thioglycollate medium (this is not listed in the Ph. Eur. because the pharmacopoeia does not cover medical devices).

Neutralisers can be added to culture media in order to overcome anti-microbial properties. However the growth promotion properties of the modified media must be demonstrated prior to undertaking the main sterility test validation.

This validation approach can be summarised in a flow chart:

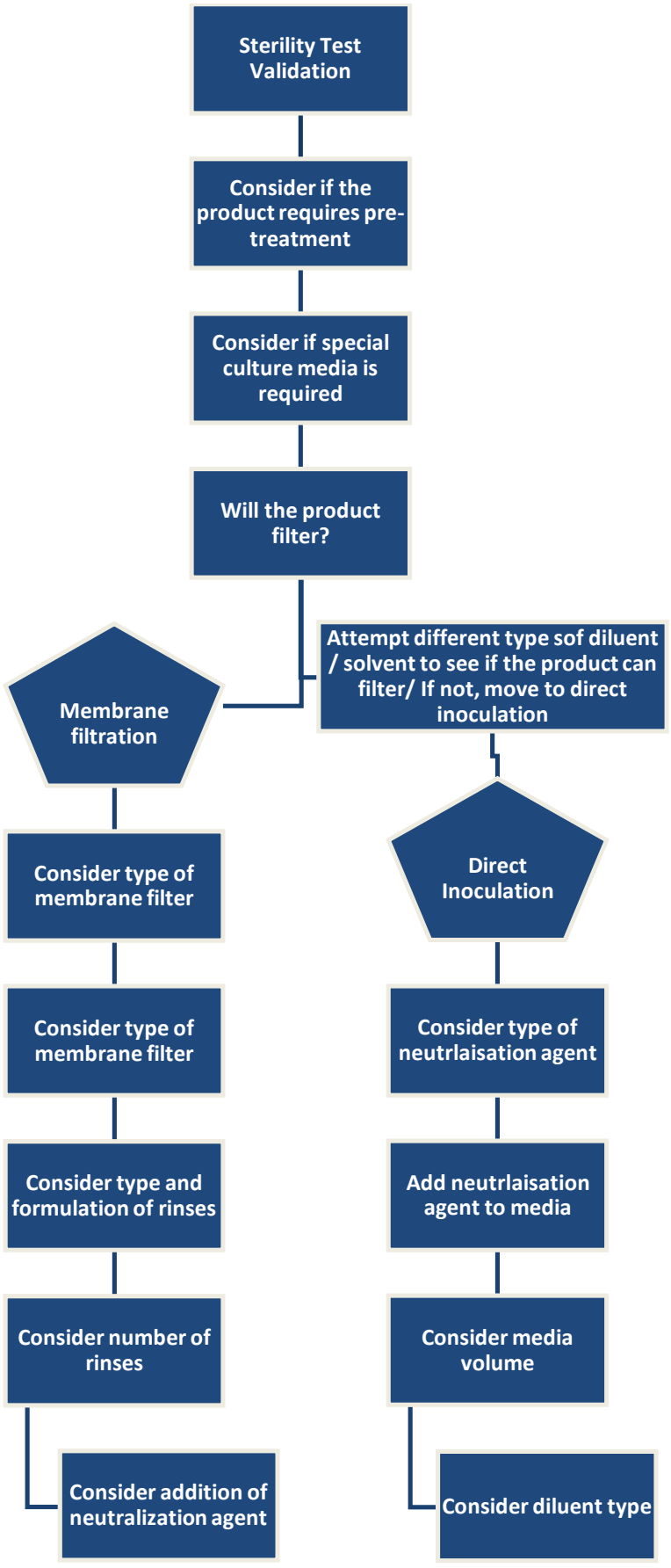

Figure 1: Sterility test validation decision tree

Some Examples of Testing 'special' products

The above section has examined general variations to the sterility test validation method. This part of the chapter looks at some specific product examples.

Antibiotics

When validating antibiotics using the membrane filtration technique it is especially important to pre-wet the filter with some rinse solution (such as saline) and not to let the filter dry-out during testing and it is important to use a hydrophobic edged filter to ensure that no product residues remain. This can potentially cause inhibition. The other important consideration is with the use of neutralisers as outlined above.

Options for testing antibiotics include the following and may be used in combination:

- Adding an antibiotic neutralizing/inactivating agent to the broth/plating media.
- Increasing the concentration of the antibiotic neutralizer/ inactivator in the broth/plating media.
- Physical separation of the organisms from the antibiotic (i.e., membrane filtration). Here it is best to use an alternate membrane filter media (e.g., polyvinylidene difluoride [PVDF] or polyethersulfone [PES]), as cellulose-based membranes tend to bind antibiotics. If the microorganism(s) do not grow on the antibiotic-filtered membrane, this means that there is still activity within the membrane. This may be eliminated by changing the filter material (e.g., PVDF or PES) and/or adding chemical neutralisers to the rinse medium.
- Increase the volume of the rinse

Oily samples

An oily product, such as an eye ointment, can prove difficult to test because any microbial cells present can become embedded in the matrix of the product. In order to remove any micro-organisms that may be present the use of a solvent or an emulsifying agent is required (for example, isopropyl myrisate; polysorbate-80 or light paraffin). The oil should be allowed to penetrate the membrane at its own weight and then be filtered by applying the pressure or suction slowly.

Some general guidance is:

- Oils of a low viscosity: run through a dry membrane.
- Viscous oils*: dilute with a diluent, filter slowly; and then rinse. Sometimes a membrane filter with a larger surface area than the standard 47mm can aid filtration.
- Fatty oils: dilute to 1% in isopropyl myristate at a temperature of 40 – 44^{o}C and filter as rapidly as possible and wash the membrane speedily. Isopropyl myristate is the ester of isopropanol and myristic acid.

Once the test is incubation, cultures essentially comprising of 'oily preparations' should be shaken gently every day. With the fluid thioglycollate medium shaking or mixing must be restricted to a minimum level to maintain anaerobic conditions.

Other ointments and creams

Generally, ointments and creams require dilution at approximately 1 in 10 by emulsifying with a suitable emulsifying agent in a suitable diluent (such as polysorbate 80 or liquid paraffin). This provides an aqueous vehicle capable of dispersing the test material homogeneously throughout the 'fluid mixture'. Furthermore, this will improve contact between the sample and the culture medium. If this is unsuccessful, emulsifying agents can be added to the culture media. Examples include adding 10g/L of polysorbate-80 or 1 g/L of (p-tert-octylphenoxy) polyoxyethanol.

Following the addition of the emulsifying agent, 10 mL of the fluid mixture should be mixed with 80 mL of the medium, and the subsequently tested using the standard method.

Anti-cancer treatments and radiopharmaceuticals

Anti-cancer drugs are very difficult to test using conventional membrane filter techniques because the drugs are readily adsorbed onto the membrane and cannot be easily removed. Many are tested by direct inoculation.

The sterility testing of radiopharmaceuticals poses some problems. First, the total amount of material available for testing is usually limited and may be highly radioactive. Secondly, the shelf life of the material is also limited. Thus, for some radiopharmaceuticals it may not be possible to obtain the results of the sterility test before the product is released. However, cGMP requires that the test is conduced as a monitor for the manufacturing process; or that a system of controls is in place and a justification produced not to undertake the sterility test.

Where the substance is highly radioactive, and where the sterility test is required, a modification to the sterility test is required. A radioactive indicator method for the detection of viable bacteria is based on the monitoring of $^{14}C0_2$ released from growth media containing ^{14}C-labeled substrates.

Implants

Solid substances are difficult to test for sterility. In the case of implants the therapeutic protein / peptide needs to be reverted into a more fluid monomer so that it can be adapted for testing and so that any trapped micro-organisms in the matrix and can eluted. See also solid articles, below.

Sterile Aerosols

The testing of aerosols in cans can prove problematic. Until commercially adaptable membrane filter units became available the main means of testing was to freeze the containers in an alcohol-dry ice mixture for one hour. The container was then aseptically opened (by puncturing) and the contents transferred to a sterile pooling

vessel (by expelling the contents) for testing. The main concerns were to avoid alcohol from entering the vessel; to avoid adventitious contamination and the danger of the can exploding.

Although the above method is still used, the advent of commercial membrane filtration units allows the connection of the nozzle directly to the aerosol canister and the transfer of the contents directly to the filtration unit.

Cell lines

A different approach to the testing of cell lines is required. This is primarily in the selection of different culture media and different incubation conditions, depending upon the type of cells that are being tested. The use of an alternative to the thioglycollate medium may be important in some cases due to reports that conventional FTB is toxic to some damaged cells (Baird, 1990: 135). One approach to sterility testing of cell lines is as follows:

a) Thaw the different cell lines to be tested and pool
b) Centrifuge in order to separate the culture from the cells
c) Inoculate 2 – 3 drops (0.5 ml) of the culture into a variety of different media. The type of media used will depend upon the application and cell culture type. Examples include:

Culture Media	**Temperature**	**Incubation: (Min. #Days)**
One Blood Agar plate(aerobic)	37ºC	14
One Blood Agar plate(anaerobic)	37ºC	14
Two Thioglycollate Broth tubes	37ºC and 23ºC	14
Two Tryptone Soya Broth tubes	37ºC and 23ºC	14
Two Sabouraud Broth tubes	37ºC and 23ºC	21

Given the nature of the cell cultures the inoculated media should be inspected after 48 hours incubation. If contamination is observed it may be permissible to have a procedure for a repeat test. Any repeat test should be performed in triplicate.

The above approach will provide a level of assurance that there is no gross contamination from bacteria or fungi. However, another risk to cell cultures is provided from mycoplasmas. Testing for mycoplasmas requires specialist culture media and a nitrogen rich atmosphere (where fungal contamination poses a significant risk). Post-test examination requires the use of a fluorescent microscope. Alternatively, biochemical tests are also available.

Fibrin Sealant

The reconstitution of freeze-dried products can be highly variable and the amount of diluent added and the type of diluent are critical. Other variables include the degree of agitation (or stirring speed) and maintaining the correct temperature. This author has noticed that even +/-1ºC in the reconstitution of a protein based product (in this case, a fibrin sealant) can result in coagulation and an invalid test.

Dressings

For dressings, the entire product does not need to be tested. The recommended amount is normally 100 – 500 mg. His should be the part of the dressing considered to be the most inaccessible to the sterilant. It is also permissible to pool dressing.

Solid articles that cannot be tested using the standard methods

For the testing of sterile devices, where the articles are of an appropriate size and shape, it should be as completely immersed in not more than 1 L of the culture medium. Alternatively, the article, if it can be cut or disassemble, should be divided into smaller proportions to allow for immersion within the culture medium.

For solid articles that cannot be readily cut into pieces or immersed into the largest permitted volume of culture media (2000 ml), the article can be rinsed three times with suitable volumes of medium. In doing this it is important to ensure that all parts of the article come into contact with the medium. The entire washing from the article can then be tested using the membrane filtration method. It must be recognised that this is the least sensitive method available and that some micro-organisms may still adhere to the surface of the solid or that trapped air may prevent the medium from reaching all parts of the article.

Transfusion or Infusion Assemblies

For transfusion or infusion assemblies or where the size of an item almost renders immersion impracticable, flush the lumen of twenty units with a sufficient quantity of fluid thioglycollate medium and the lumen of each of 20 units with a sufficient quantum of soyabeancasein digest medium to give a recovery of not less than 15 mL of each medium. At the end of this process, incubate with not less than 100 mL of each of the two sterility test media.

For some devices, the lumen is so small that fluid thioglycollate medium will not pass through. Here, an alternative thioglycollate medium can be used, provided the alternative medium passes the test for growth promotion.

Summary

This chapter has considered some best practice examples for the sterility testing of ‘difficult’ products. The chapter is has not covered this subject exhaustively, either in terms of the types of products or in the methods that can be applied. The chapter has focused on common product types and widely used techniques. For products not covered in this chapter, the chapter has provided a framework for the appropriate steps in developing an alternative method (such as attempting membrane filtration first, exploring rinsing and neutralisation second, and so forth). The chapter has also emphasised the importance of method validation.