Microbiology laboratory techniques

6

6.1 Introduction

In the manufacture of all types of pharmaceuticals, quality assurance represents a major consideration. It is important that products are not contaminated with microorganisms that might affect their safety, efficacy, or acceptability to the patient.

During pharmaceutical product manufacture, microbiological contamination is controlled by the application of good manufacturing practice (GMP). Nonetheless, contamination risks remain an ever-present threat. In practice, the presence of microorganisms in pharmaceutical products constitutes two main hazards.

(1) It could result in spoilage of the product; the metabolic versatility of microorganisms is such that any formulation ingredient may undergo degradation in the presence of a given microorganism.

(2) It may provide an infection hazard to the patient. Although the degree of hazard will be dependent on the product's intended use and route of administration (i.e., oral, topical, parenteral, application to the eye, and so on), With nonsterile products, certain pathogens present a hazard; with sterile products, any contamination presents a potential risk.

For these reasons, the microbiological contamination control of pharmaceuticals is evaluated during various phases of product development and during routine commercial manufacture [1]. This requires a range of microbiological tests to be conducted. Such tests focus on the number and type of microorganisms (and any potential microbial impurities such as bacterial endotoxins). These tests are applied to raw material ingredients (including pharmaceutical waters), in-process product checks (including bioburden levels) during manufacture, environmental controls and ultimately finished product microbiological tests. Many of these tests are described in the various national and international pharmacopeia. To add to these, there are techniques to assess cleanrooms through the environmental monitoring program.

Microbiology plays a critical role in pharmaceutical quality control, specifically evaluating raw materials, process controls, product release tests, and product stability tests. The quality and interpretation of the data from these tests critically impacts product safety. It is the quality control function that assures that data from these tests are meaningful (reliable and precise) and have a minimum of error [2]. Microbiologists must evaluate the suitability for the use of microbiology tests, the limitations of their applicability and measurements, and whether acceptance criteria were met. Furthermore, microbiologists must understand both the nature of the tests and the data derived from them.

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This chapter describes some of the more commonly performed tests and focuses on those tests that are not described elsewhere in dedicated chapters.

6.2 Good laboratory practice and laboratory safety

The use of good laboratory practice is an important factor in safeguarding the health and safety of laboratory personnel. It should be remembered that many of the bacteria that are cultured in microbiological laboratories are capable of producing disease in humans. This, coupled with the fact that, potentially more virulent, pure strains of such bacteria are often being produced, means that there is considerable risk to the health of microbiology laboratory workers if adequate precautions are not taken.

The basis of good practice in a microbiological laboratory can be summed up by the following:

- ensure all necessary equipment and media are sterilized prior to use;
- ensure that all sterilized equipment and media is not re-contaminated after sterilization by allowing it to touch, or rest on, any unsterilized surface;
- frequently disinfect hands and working surfaces;
- as far as possible, eliminate flies and other insects that can contaminate surfaces, equipment, media, and also pass organisms to laboratory personnel;
- never pipette by mouth samples that are suspected to have high bacterial concentrations;
- wear appropriate protective clothing: laboratory coat, safety glasses, and gloves;
- do not eat, drink, or smoke in the laboratory;
- sterilize contaminated waste materials prior to disposal;
- take care to avoid operations that result in bacterial aerosols being formed.

Each laboratory should have a risk assessment system in place. This is based on possible hazards and the risks associated with them. Taking microorganisms as an example, here a hazard is the danger or harm that a microorganism may cause to a person. A risk is the probability or likelihood that a person will be harmed by the microorganism. Safety issues, including protective clothing, are considered in Chapter 4.

6.3 Aseptic technique

Due to the fact that microorganisms can be present virtually anywhere, it is important to take measures to avoid contamination of microbiological experiments with extraneous bacteria. The measures used to prevent this cross-contamination in microbiological laboratories are collectively known as aseptic techniques.

Aseptic techniques usually involve disinfection of working areas, minimizing possible access by bacteria from the air to exposed media and use of flames to kill bacteria that might enter vessels as they are opened.

Asepsis can be achieved by laboratory staff washing their hands and disinfecting the bench area. Work should be conducted in a dust-free and draught-free area, using a unidirectional airflow cabinet for critical activities. In terms of the application of techniques, staff should not touch any part of the container, pipette, and so, that will come in contact with the sample or culture.

6.4 Cultures and identifications

An important aspect of microbiology is with cultural techniques and in obtaining a pure culture. Microbiological culture describes a method of multiplying microbial organisms by allowing them to reproduce in predetermined culture media under controlled laboratory conditions (time, temperature, and atmospheric conditions). Microbial cultures are used to determine the type of organism, its abundance in the sample being tested, or both.

Furthermore, microbial cultures are foundational, and they are required for basic diagnostic methods. For these, it is necessary to isolate a pure culture of microorganisms. A pure (or axenic) culture is a population of cells or multicellular organisms growing in the absence of other species or types. A pure culture may originate from a single cell or single organism, in which case the cells are genetic clones of one another.

Developing pure culture techniques is crucial to the observation of the specimen in question. The most common method to isolate individual cells and produce a pure culture is to prepare a streak plate. This method is a means to separate the microbial population physically and is performed by spreading and then inoculating back and forth with an inoculating loop over the solid agar plate. Upon incubation, colonies will arise, and single cells will have been isolated from the biomass.

In essence, the steps required are, for transfer onto solid agar:

- Sterilize a wire loop (or use a sterile plastic disposable loop) by heating it until red hot in a flame; allow it to cool for several seconds. Test for coolness by touching the agar at the edge of the plate;
- Pick up a loop full of liquid inoculum or bacterial growth from the surface of an agar plate and, starting about 2.5 cm in from the edge of the plate, streak lightly back and forth with the loop flat, making close, parallel streaks back to the edge of the plate;
- Sterilize the loop and cool again, then with the edge of the loop, lightly make another set of nearly parallel streaks about 0.32 cm apart, in one direction only, from the inoculated area to one side of the uninoculated area, so that about one-half of the plate is now covered;
- Flame and cool the loop again, and make another set of streaks in one direction, perpendicular to and crossing the second set of streaks, but avoiding the first set.

The pure culture is a foundation method for conducting microbial identifications, as described in Chapter 9.

6.5 Microscopy

The light microscope is an important tool in the study of microorganisms, particularly for identification purposes. The compound light microscope uses visible light to directly illuminate specimens in a two-lens system, resulting in the illuminated specimen appearing dark against a bright background. The two lenses present in a compound microscope are the ocular lens in the eyepiece and the objective lens located in the revolving nosepiece. Compound light microscopes typically have the following components (as outlined below and set out in Figure 6.1):

- Illuminator: the light source in the base of the microscope;
- Abbe Condensor: a two lens system that collects and concentrates light from the illuminator and directs it to the iris diaphragm;
- Iris diaphragm: regulates the amount of light entering the lens system;
- Mechanical stage: a platform used to place the slide on which has a hole in the center to let light from the illuminator pass through. Often contains stage clips to hold the slide in place;
- Body tube: houses the lens system that magnifies the specimens;
- Upper end of body tube—oculars/eye pieces: what you view through;
- Lower end of body tube—nose-piece: revolves and contains the objectives.

Essentially, a light microscope magnifies small objects and makes them visible. The science of microscopy is based on the following concepts and principles:

- Magnification is simply the enlargement of the specimen. In a compound lens system, each lens sequentially enlarges or magnifies the specimen;
- The objective lens magnifies the specimen, producing a real image that is then magnified by the ocular lens resulting in the final image;
- The total magnification can be calculated by multiplying the objective lens value by the ocular lens value.

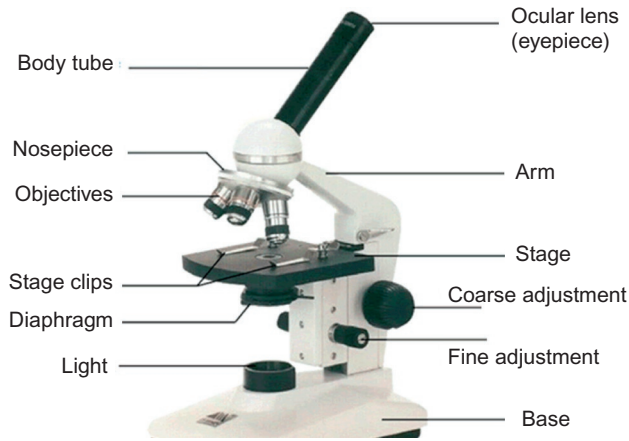

Figure 6.1 Microscope design.

6.6 Pharmacopeia and microbiological tests

The majority of tests to assure the microbiological quality assurance of pharmaceutical products are described in the major pharmacopoeias (such as the British Pharmacopoeia (BP), European Pharmacopoeia (Ph. Eur.), US Pharmacopoeia (USP), Japanese Pharmacopoeia (JP), and the World Health Organization International Pharmacopeia). Of these, the Ph. Eur, USP, and JP constitute the primary texts. The tests described constitute the set of basic microbiological laboratory techniques in relation to pharmaceuticals and healthcare [3]. Alternative tests to the pharmacopeia described methods can be validated and employed [4], but the pharmacopoeial method remains the referee test in the event of any dispute over product quality (this is the case, for example, with many of the types of rapid methods described in Chapter 17).

The basic methods are shown in Table 6.1 with some additional supporting documents that provide guidance on the microbiological quality expectations of pharmaceutical preparations and good microbiological laboratory practice.

Table 6.1 Primary pharmacopoeial microbiology tests

Pharmacopoeial chapter/section	Application
Microbiological examination of nonsterile products: total viable aerobic count (Ph. Eur. 2.6.12, USP <61>) USP <2021> Microbial enumeration tests-nutritional and dietary supplements USP <2023> Microbiological attributes of nonsterile nutritional and dietary supplements	Number of organism in raw materials, water, product in-process controls (bioburden), finished products
Microbiological examination of nonsterile products: tests for specified organisms (Ph. Eur. 2.6.13, USP <62>) USP <2022> Microbiological procedures for absence of specified microorganisms-nutritional and dietary supplements	Type of organisms present in raw materials, water, product IPC (bioburden), finished products
Ph. Eur. 5.1.4 Microbiological quality of pharmaceutical preparations/USP <1111> Microbiological attributes of nonsterile pharmaceutical products	Setting of limits and control factors
Sterility (Ph. Eur. 2.6.1, USP <71>)	Sterility test for finished products
Pyrogens/endotoxin Rabbit Pyrogen Test (Ph. Eur. 2.6.8, USP<151>) *Limulus* amoebocyte lysate (LAL) bacterial endotoxin test (Ph. Eur. 2.6.14, USP<85>)	Pyrogen/endotoxin test for raw materials, pharmaceuticals waters, product IPC, finished products

Continued

Table 6.1 Continued

Pharmacopoeial chapter/section	Application
Antimicrobial preservative efficacy testing (Ph. Eur. 5.1.3, USP <51>)	Product formulation challenge to microbiological contamination
Microbiological assay of antibiotics (E.P 2.7.2., USP<81>)	Potency assays for antibiotic pharmaceutical preparations
USP <1112> Application of water activity determination to nonsterile pharmaceutical products	Assessment of water activity (can affect microbial growth and survival)
USP <1211> Sterilization and sterility assurance of compendial articles. There are a series of subchapters that describe specific sterilization methods	Sterilization and microbial kill
USP <55> Biological indicators	Biological indicators for assessing microbial kill
USP <1113> Microbial characterization, identification, and strain typing	Microbial identification methods
USP <1117> Microbiological best laboratory practice	General laboratory methods and management
USP <1116> Microbiological control and monitoring of aseptic processing environments	Environmental monitoring and cleanroom design for aseptic environments
Ph. Eur. 5.1.6 Alternative methods for control of microbiological quality/ USP <1223> Validation of alternative microbiological methods	Rapid microbiological methods
USP <1115> Bioburden control of nonsterile drug products	Bioburden control
USP <1227> Validation of microbial recovery from pharmacopeial articles	Microbial method validation

In addition to the above, the chapters on water testing in the pharmacopeia include information relating to microbiological testing.

The microbiological test method and guideline general chapters (USP <61>, <62>, <71>, and <1111>, Ph. Eur. 2.6.1, 2.6.12, 2.6.13, and 5.1.4) are harmonized. That means that the basic text is the same for Ph. Eur, JP, and USP, and that tests conducted under one pharmacopeia are accepted by another.

Many of the above tests are culture based. Here microorganisms are grown in the laboratory by providing them with an environment suitable for their growth. The growth medium should contain all the correct nutrients and energy source and should be maintained at an appropriate pH, salinity, and oxygen tension and be free of antibacterial substances. Control of culture media, as detailed in Chapter 5, is of great importance.

Some of the different tests are discussed in more detail in the proceeding sections.

6.7 Microbiological examination of nonsterile products

6.7.1 Total viable aerobic count (Ph. Eur. 2.6.12, USP <61>)

These established tests are described in Chapter 8, for this reason they will not be outlined in detail here. In summary, the test is designed to count the number of microorganisms (as colony forming units, CFUs) in a nonsterile product or raw material. There are two parts: total aerobic microbial count (TAMC) and total yeast and mould counts (TYMCs).

With the method, a test sample is taken and processed (e.g., diluted and neutralized) and then:

- filtered and the filter placed on defined media (membrane filtration technique), or
- a sample aliquot is taken and placed in a Petri dish and specified media poured onto the sample (pour plate technique), or
- a sample aliquot is placed on the surface of defined media and smeared evenly over the surface (spread plate technique), or
- for mainly insoluble materials, sample dilutions are placed into a series of replicate tubes and the number of tubes showing growth give a statistical evaluation of the number of microorganisms in the sample (most probable number, MPN technique).

With these methods, it is necessary to demonstrate that the sample material, test reagents or any aspect of the test procedure, adversely affects the outcome of the test.

Before embarking on the test, it is important to compile information about the material to be tested such as: physical and chemical attributes, base formulation, and the estimated bioburden. This information helps considerably with experimental design, and test method selection and validation.

6.7.1.1 Bioburden determination

Tests for bioburden determination used, for example, for the examination of in-process material, are broadly similar to the TAMC method. The optimal counting range for colonies, on a 9-cm agar plate, is 20–250 CFU [5]. Consideration must also be given to incubation times and temperatures [6]. Further information on bioburden testing is outlined in Chapter 8.

6.7.1.2 Method validation

Bioburden tests, and the TAMC/TYMC tests, need to be qualified to show the appropriateness of the method to the material under test. This is particularly with the areas of sample preparation and the ability of the media to recover microorganisms in the presence of the test sample. The following four "validation" areas need to be considered:

(1) media growth promotion;
(2) sample preparation;
(3) test method;
(4) sample neutralization.

6.7.1.3 Media growth promotion

Media growth promotion is required to demonstrate that it supports growth and has the ability to detect organisms in the presence of the test sample [7]. This is achieved by using not more than 100 CFU of the following specified organisms.

(1) For tryptone soy agar (TSA) and tryptone soy broth (TSB):
  a. *Staphylococcus aureus* ATCC 6538 (NCIMB 9518, CIP 4.83, NBRC 13276);
  b. *Pseudomonas aeruginosa* ATCC 9027 (NCIMB 8626, CIP 82.118, NBRC 13275);
  c. *Bacillus subtilis* ATCC 6633 (NCIMB 8054, CIP 5262, NBRC 3134).
(2) For sabouraud dextrose agar (SDA):
  a. *Candida albicans* ATCC 10231 (NCPF 3179, IP 48.72, NBRC 1594);
  b. *Aspergillus niger* ATCC 16404 (IMI 149007, IP 1431.83, NBRC 9455).

Various equivalent strains can be used as obtained from approved culture collections. These are: American Type Culture Collection (ATCC), National Collection of Industrial and Marine Bacteria (NCIMB), Collection of Institute Pasteur (CIP), Imperial Mycological Institute (IMI), National Collection of Pathogenic Fungi (NCPF), and National Biologicals Resources Centre (NBRC).

For the media control, a comparison is made between the recovery of organisms from the specific media against the calculated inoculum. For the test method validation, a comparison is made between the recovery of organisms from media with test material with that of a diluent control. Results in both cases should not differ by more than 50%.

6.7.1.4 Sample preparation

This area for consideration and should prompt the questions:

(1) Where will the sample be prepared, such as on a laboratory bench, within unidirectional airflow cabinet or an isolator. They may also be safety concerns with require the use of a Microbiological Safety Cabinet (MSC). The primary purpose of an MSC is to protect the laboratory worker and the surrounding environment from pathogens. All exhaust air is high efficiency particulate air (HEPA) filtered as it exits the biosafety cabinet, removing harmful bacteria and viruses. This device is different from a unidirectional airflow cabinet, which blows unfiltered exhaust air toward the user and is not safe for work with pathogenic agents.
(2) In what will the sample be prepared? For example, a sterile plastic container.
(3) Agitation—does the sample need vortexing or sonication?
(4) Dilution—does the sample need diluting because of a high natural bioburden/because it is highly antimicrobial?

Some products are more complex to test and require pre-treatment. Examples of best practice for different products include:

Water-soluble products

- Dissolve or dilute (usually a 1 in 10 dilution is prepared) the product to be examined in phosphate buffer solution pH 7.2, If necessary, adjust to a pH of 6–8.
- Further dilutions, where necessary, are prepared with the same diluent to yield not more than 250 CFU/plate in case of TAMC, 50 CFU/plate in case of TYMC.

- For products or raw materials that do not dissolve completely, grind them in a sterile mortar and pestle, in an aseptic environment, to a fine powder.

Nonfatty products insoluble in water

- Suspend the product to be examined (usually a 1 in 10 dilution is prepared) in phosphate buffer solution pH 7.2.
- A surface-active agent such as 1 g/L of polysorbate 80 may be added to assist the suspension of poorly wettable substances.
- If necessary, adjust to a pH of 6–8. Further dilutions, where necessary, are prepared with the same diluent to yield not more than 250 CFU/plate in case of TAMC, 50 CFU/plate in case of TYMC.

Fatty products

- Dissolve in isopropyl myristate sterilized by filtration, or mix the product to be examined with the minimum necessary quantity of sterile polysorbate 80. Heated, if necessary, to not more than 40 °C or, in exceptional cases, to not more than 45 °C. Mix carefully and if necessary maintain the temperature in a water bath.
- Add a sufficient quantity of the pre-warmed chosen diluent to make a 1 in 10 dilution of the original product.
- Mix carefully, while maintaining the temperature for the shortest time necessary for the formation of an emulsion. Further serial tenfold dilutions may be prepared using the chosen diluent containing a suitable concentration of sterile polysorbate 80 or another noninhibitory sterile surface-active reagent.

6.7.1.5 Test method

Which test method is selected is based upon sample characteristics and the required microbial limits. The choice is between membrane filtration, pour plate, spread plate, and the MPN methods.

The limitations with these methods, and other culture-based assessments of bioburden, should be understood. The "colony count" is an indirect count with variable insensitivity, and it is very imprecise when very few colonies are counted. Furthermore, with the CFU, which is an artefact-based count relying on cellular replication to produce a visible speck of cells (the "colony") on the growth medium, if the medium or physical conditions are not adequate, then no colony appears. Another weakness is that if a clump of many cells lands in one place and only a single colony forms, then the count of "one" underestimates the total. Therefore, plate counts are not always precise or accurate [8]. The MPN, which is not a direct cell count, is also dependent on a cell's ability to multiply in growth medium under the physical incubation conditions.

To add to these issues, if the sample possesses antimicrobial activity that requires neutralization then there are principally three approaches to consider:

(1) chemical neutralization;
(2) enzymatic neutralization;
(3) dilution.

The appropriate method selected needs to be qualified.

Once the sample preparation has been developed and validated, the media performance qualified, and the test method selected then the routine test can be implemented.

6.7.2 Tests for specified organisms (Ph. Eur. 2.6.13, USP <62>)

The test for specific organisms is to determine the absence, or limited occurrence of specified microorganisms in a given pharmaceutical sample, that may be detected under the test conditions. These tests are described in Chapter 8, and they focus on screening for one or more of the following organisms:

(1) *Escherichia coli*: natural inhabitant of gut flora. Some species are pathogenic and cause diarrhoea. If recovered, the organism indicates fecal contamination;
(2) Salmonellae: common inhabitant of gut flora. If recovered, the organism indicates fecal contamination and of high pathogenicity;
(3) *S. aureus*: common inhabitant of human skin and nose, detectable in feces. If recovered it indicates high pathogenicity potential. There may also be a risk to product quality due to resistance to preservatives. The bacterium has a low nutrient demand can grow to high numbers in certain materials;
(4) *P. aeruginosa*: a common water inhabitant, especially of stored water. If recovered it indicates high pathogenicity potential;
(5) Clostridia: potential pathogens relating to specific situations, especially where anaerobic conditions are prevalent (e.g., with talc or bentonite);
(6) *C. albicans*: potential pathogens relating to specific situations, such as vaginal preparations.

Special, selective or differential agars are required for the examination of the above indicator microorganisms.

In addition to these described species, there may be a requirement to identify and test for specific "objectionable" microorganisms. These are undesirable organisms from a product quality/efficacy point of view or from a patient risk situation. Such organisms require the adoption of appropriate selective agars.

6.7.3 Specification limits (harmonized method)

The limits for total viable aerobic count and the tests for specified microorganisms are displayed in Tables 6.2 and 6.3.

It should be noted that the pharmacopeia allows variability in test results equal to a factor of two if the specified microbial limit is 10, the maximum accepted microbial count is 20 CFU and still meets the product specification, if the specified microbial limit is $100(10^2)$, the maximum accepted microbial count is 200 CFU and still meets the product specification and so on.

- 10^1 microorganisms: maximum acceptable count = 20.
- 10^2 microorganisms: maximum acceptable count = 200.
- 10^3 microorganisms: maximum acceptable count = 2000, and so forth.

Table 6.2 Acceptance criteria for microbiological quality of nonsterile dosage forms and raw materials

Route of administration	TAMC	TYMC	Specified microorganisms
Nonaqueous preparations for oral use	10^3	10^2	*Escherichia coli* absent in 1 g or 1 mL
Aqueous preparations for oral use	10^2	10^1	*Escherichia coli* absent in 1 g or 1 mL
Rectal use	10^3	10^2	*If required*
Oromucosal use Gingival use Cutaneous use Nasal use Auricular use	10^2	10^1	*Staphylococcus aureus* absent in 1 g or 1 mL *Pseudomonas aeruginosa* absent in 1 g or 1 mL
Vaginal use	10^2	10^1	*Staphylococcus aureus* absent in 1 g or 1 mL *Pseudomonas aeruginosa* absent in 1 g or 1 mL *Candida albicans* absent in 1 g or 1 mL
Inhalation use (special requirements apply to liquid preparations for nebulization)	10^2	10^1	*Staphylococcus aureus* absent in 1 g or 1 mL *Pseudomonas aeruginosa* absent in 1 g or 1 mL Bile-tolerant gram-negative bacteria absent in 1 g or 1 mL
Transdermal patches (limits for one patch including adhesive layers and backing)	10^2	10^1	*Staphylococcus aureus a*bsent in 1 g or 1 mL *Pseudomonas aeruginosa* absent in 1 g or 1 mL

Table 6.3 Acceptance criteria for microbiological quality of raw materials for nonsterile manufacturing

Material	TAMC	TYMC	Specified microorganisms
Substances for pharmaceutical use	10^3	10^2	*If required*

6.8 Measurement of cell concentration in suspension by optical density

A common issue for the microbiology laboratory is the determination of starting inoculum concentration. If the inoculum concentration is determined by plating, the inoculum is several days old before use, and if the inoculum is out-of-range, the resultant test will be invalid. A means to avoid this is to estimate the population through an assessment of cellular optical density.

To assess optical density, the most common method to use is spectrophotometry [9]. A spectrophotometer measures turbidity directly. The theory is that light passing through a suspension of microorganisms is scattered, and the amount of scatter is an indication of the biomass present in the suspension. Each spectrophotometer used must be independently calibrated for use in estimating microbial concentrations. The absorption of light is affected by the width of the instrument's slit, the condition of the filter, the size and condition of the detector, and the total output of the lamp [10].

With the use of optical density, the correlation of absorption to dry weight is very good for dilute suspensions of bacteria, and this relationship seems to hold regardless of cell size. In developing a method to estimate CFUs, a calibration curve is constructed. The calibration study must demonstrate the linear range of the absorbance against CFU values and the relevant values. It is important to note that, in more concentrated suspensions, this correlation (absorption to dry weight) does not correlate well.

6.9 Sterility testing

The sterility test applies primarily to finished products that are required to be sterile such as eye drops and intravenous products. The sterility test is a referee test; however, it is not intended as a sole release test. To verify sterility, other GMP expectations should be in place, controlled, and monitored such as environmental control, environmental monitoring, validated aseptic processing, validated sterilization processes, and so forth. The sterility test thus represents one set of data that contributes to the decision of whether or not the product lot meets the stated claims to be sterile. The test was first introduced in 1932 in the British Pharmacopeia as a direct inoculation test. This was followed by the USP in 1935. A membrane filtration version was introduced in 1957.

The test has several flaws. Most evidently, from Bryce's critique of the methodology. This highlighted the fact that "the sample size is so restricted that it provides only a gross estimate of the state of 'sterility' of the product lot" and that it "can only recognize organisms able to grow under the conditions of the test" [11]. Statistical evaluation of the sterility test indicates that it is limited use in assuring product sterility, only capable of detecting gross contamination. Moreover, the test will only detect those microorganisms that are capable of reproducing within the prescribed culture media and at the preselected temperature and for the described time period. Nevertheless, it is the recognized method, and it remains a component of any release strategy for a sterile product filled by aseptic processing (with terminally sterilized products, a case can be made for parametric release).

The sterility test is a qualitative, presence/absence test based upon growth for bacteria and fungi in two types of media. There are principally two methodologies applied which ask slightly different questions [12]:

1. Membrane filtration (funnel open method and, more commonly, a closed system method—the open funnel method poses a greater contamination risk).

With this method, the sample is filtered, then a rinse is undertaken to remove or to neutralize any product residues. Then, either two filters are used or a filter is cut filter

in half. The two portions are placed into specified media and incubate at a defined temperature for 14 days.

The membrane filter requirements are:

(1) cominal pore size 0.45 µm;
(2) diameter about 50 mm;
(3) cellulose nitrate filters (e.g., for aqueous, oily, and weakly alcoholic solution);
(4) cellulose acetate filters (e.g., for strongly alcoholic solutions);
(5) other filter types may apply (e.g., antibiotics).

The question asked is: are there viable cells on or in the filter?

2. Direct inoculation

With this method, a sample is placed directly into specified media, containing neutralizers if required, and incubated at a defined temperature for 14 days.

The question asked is: are there viable cells in the sample?

The pharmacopeias indicate that, wherever possible, the membrane filtration technique is utilized due to the greater likelihood of recovery of contamination by virtue of the greater sample size. However, certain test articles, such as viscous oils, creams ointments, and medical devices, may not be filterable therefore direct inoculation will be the method of choice.

The following media is for sterility testing:

(1) Fluid thioglycollate medium: primarily for anaerobic bacteria, but will also isolate aerobic bacteria;
(2) Soya-bean casein digest medium (TSB): for the isolation of fungi and aerobic bacteria.

Other media can be used so long as they meet the requirements for growth promotion. Where neutralization is required, the neutralizer can be added to the media; for example, ß-lactamase (penase) for the testing of penicillins and cephalosporins. In the case of neutralizers, the type and volume required, and the efficacy/toxicity must be part of the sterility test validation. With penase, a specific validation using *S. aureus* is described.

All batches of media must be shown to promote growth. Specific organisms are described to demonstrate growth promotion:

a. Fluid thioglycollate medium:
  i. *Clostridium sporogenes*, *P. aeruginosa*, *S. aureus*;
b. Soya-bean casein digest medium:
  i. *A. niger*, *B. subtilis*, *C. albicans.*

Each medium is inoculated with not more than 100 CFU and incubated for 3 days (bacteria) or 5 days (fungi). Clearly visible growth must be observed.

Incubation conditions and times are as follows:

(1) fluid thioglycollate medium at 30–35 °C;
(2) soya-bean casein digest medium at 20–25 °C;
(3) for a total incubation period of 14 days with visual examination for turbidity.

With 14 days, Besajew demonstrated that 20% of all contaminants become visible between the 8th and 12th days after a retrospective evaluation of almost 8 years of data. Further, it was found that up to 10% of the time growth did not occur before the 11th and 12th days [13]. There were also issues around suboptimal growth conditions, inherent slow growers and injured cells.

There are two outcomes with the sterility test:

(1) clearly visible growth, which is equivalent in both the test sample and the control tubes indicates a valid sterility test process;

(2) if clearly visible comparable growth is not observed, then the developed sterility test method is not validated, and the test needs to be modified and the validation repeated.

With both the direct inoculation and membrane filtration methods, the sterility test is a demanding procedure where asepsis must be ensured to allow for correct interpretation of the results. Most importantly, the test environment must be adequate (like the production area). This requires the use of an EU GMP Grade A device with a Grade B background or a Grade A isolator operator in a Grade C or D cleanroom. To verify environmental acceptance, environmental monitoring should be undertaken during the monitoring session, and negative controls should be run during the test session.

The test samples should be representative of the batch of material under test, such as being drawn from the beginning, middle, and end of the aseptic fill process. The transfer of samples from the sampling area into the testing area and subsequent handling should be proceduralized. For example, the outside surfaces of vials should sanitized or gassed into the test area. The number of articles taken from a batch and the quantities required to be sterility tested are set out in the pharmacopeia. This relates to the quantity filled per container and to the batch size. For example, with parenteral preparations with batches of not more than 100 containers, the number of containers to be tested is 10% or four containers, whichever is the greater; whereas for more than 100 but not more than 500 containers, the 10 containers are tested; and with more than 500 containers then the number required is 2% or 20 containers, whichever is less *unless*, the product is a large-volume parenteral, in which case the number drawn from the batch is 2% or 10 containers, whichever is less.

6.9.1 Validating the sterility test

The test needs to be validated. For this, the characteristics of the material need to be considered, such as solubility and antimicrobial activity. This information is used to develop a validated sample preparation process. Dispersion of the material in diluents (where required) with or without surface active agents and neutralizers is essential for membrane filtration and direct inoculation to allow for adequate qualification of the sterility test.

Sterility test validation is multifactorial and requires:

(1) defined sample preparation;
(2) appropriately selected test method (i.e., membrane filtration/direct inoculation);
(3) media growth promotion and sterility studies;
(4) environmental control testing;
(5) operator validation;
(6) bacteriostasis/fungistasis effect of the product (now called the validation test in the harmonized methods).

The validation of the sterility test should be performed with the test articles using the developed sample preparation and selected methodology. Three different batches are normally tested. With the challenge microorganisms, these are the same as those

used for media growth promotion. The challenge is fewer than 100 CFU. For membrane filtration, the specified organism is added to the final filter rinse and with direct inoculation, the specified organisms are added to the media. Growth must occur within 3 days for bacteria and within 5 days for fungi.

Rapid microbiological methods have been developed for sterility testing. These have yet to be adopted by the pharmacopeia, although the US Food and Drug Administration (FDA) accepts such methods as alternatives. Chapter 17 contains some information about rapid and alternative methods.

6.10 *In vitro* and *in vivo* testing for pyrogens and endotoxins

Pyrogens and endotoxins are a heterogeneous group of chemical entities that share the characteristic of (when injected) being able to cause fever. Pyrogens can be nonbacterial as well as bacterial in origin [14]. However, the main pyrogen encountered in the pharmaceutical industry is of Gram-negative bacterial origin. That is the lipopolysaccharide (LPS) from the bacterial cell wall. The test for bacterial endotoxins is described in detail in Chapter 11.

In terms of the range of pyrogenic substances, these are displayed in Table 6.4.

Tests for endotoxins are evaluated at various stages during pharmaceutical manufacturing such as water systems, raw materials, in-process steps, and finished product. With finished products, it is more commonplace to test finished products that are to be injected for endotoxin than it is to conduct a test for pyrogens (in the classic form of the rabbit pyrogen test). An alternative method is the monocyte activation test, which uses whole blood and involves the detection of cytokines.

6.10.1 *Rabbit (*in vivo*) pyrogen test*

The basis for the rabbit pyrogen test is that any pyrogen-containing solution injected intravenously will after a short period (circa 15 min) result in fever that peaks after about 90–120 min and then subsides [15]. The body temperature rise is proportional to the level of pyrogen. In reality, a measured dose of sample to be tested is injected into the ear veins of three rabbits. The cumulative rise in body temperature is then

Table 6.4 Sources of pyrogens

Nonbacterial	Bacterial
• Antigens (antibody mediated response) • Poly nucleotides • Steroids • Adjuvants (e.g., muramyl dipeptide) • Viruses • Fungi (yeast, polysaccharide capsules)	• Streptococcal toxins • Staphylococcal enterotoxins • Mycobacterial cell wall components • Bacterial cell wall: lipopolysaccharides (endotoxins)

periodically measured over a 3-h period via thermometers placed into the rectum of each rabbit. The summed temperature changes of the three rabbits is then compared against values representing "Pass," "Fail," and "Retest" acceptance criteria.

If a sample falls into the "Retest" criteria, then a further three rabbits may be tested, and this can be repeated up to four times (i.e., 12 rabbits). The rabbit test is considered to be about 50 times less sensitive that the *limulus* amoebocyte lysate (LAL) test. However, the rabbit sensitivity to pyrogens is similar to humans and, hence, does give an indication of the pyrogenic risk of the material to people. In addition, the rabbit test will react to all potential pyrogens not only LPS endotoxin.

6.10.2 LAL testing for bacterial endotoxin

It has long been known that the blood from the horseshoe crab (*Limulus polyphemus*) when in contact with Gram-negative bacteria becomes coagulated [16]. The mechanism for this coagulation occurs because in the presence of divalent cations (e.g., Ca^{2+}, Mg^{2+}) interaction with a "factor C" in the amoebocyte of the crab activates it. The active factor B induces a pro-clotting enzyme that converts the protein coagulogen into Coagulin resulting in coagulation. This mechanism is the basis of three LAL tests: gelation (or gel-clot) and two photometric methods: turbidimetric and chromogenic. These are outlined in Chapter 11.

6.11 Microbiological assay of antibiotics

The biological determination of antibiotic potency in pharmaceutical preparations is unchanged in principle since the 1950s. Antibiotic substances produced by fermentation are often controlled but representing a collection of closely related substances that individually may exhibit different biological activity [17].

The antibiotic bioassay provides a collective assessment of the potency of the overall biological activity of an antibiotic preparation. This activity (potency) is quoted in terms of international standards, specifically defined and quoted by pharmacopoeias. While many antibiotic assays have given way to chemical analysis such as high-performance liquid chromatography (HPLC), such methods do not reflect the true biological activity. Therefore, antibiotic bioassays still play an essential role in the manufacture and quality control of antibiotic medicines, but the assays still require a considerable amount of expertise and skill to ensure success.

6.12 Environmental monitoring

Microbiological environmental monitoring involves the collection of data relating to the numbers of microorganisms present in a clean room or clean zone. These microorganisms are recovered from surfaces, air, and people. Nonviable particle counting, a physical test, is often included within the program because this function has often

resided with the microbiology department to perform and due to the theoretical relationship between high numbers of nonviable particles and viable counts.

The main aim of microbiological environmental monitoring is to assess the monitoring of trends over time and the detection of an upward or downward movement, within clean areas.

The viable count aspect of environmental monitoring consists enumerating the numbers of microorganisms present in a clean room by collection results by using the following sample types:

(a) passive air-sampling: settle plates;
(b) active air-sampling: volumetric air-sampler;
(c) surface samples: contact (RODAC) plates;
(d) surface samples: swabs;
(e) finger plates;
(f) plates of sleeves/gowns.

These methods, together with the environmental monitoring program, are described in detail in Chapter 16.

6.13 Water analysis

Microbiological water analysis is a method of analyzing water to estimate the numbers of bacteria present and to allow for the recovery of microorganisms in order to identify them.

The method of examination is the plate count. The plate count method relies on bacteria growing a colony on a nutrient medium, so that the colony becomes visible to the naked eye, and the number of colonies on a plate can be counted. Most laboratories use a method, whereby sample volumes of 100 mL (or greater) are vacuum filtered through purpose-made membrane filters, and these filters are themselves laid on nutrient medium within sealed plates [18]. A nonselective medium is used to obtain a total enumeration of the sample (called a heterotrophic plate count). When it is desirable to obtain a specific bacterial species, a selective medium can be used.

Sometimes testing requires an examination of indicator microorganisms. Indicator organisms are bacteria such as nonspecific coliforms, *E. coli* and *P. aeruginosa* that are very commonly found in the human or animal gut and which, if detected, may suggest the presence of sewage. Such organisms are detected using specialist agars or test kits.

Methods for water testing are described in Chapter 10.

6.14 Conclusion

This chapter has provided an overview of some of the common methods found within the microbiology laboratory. For those methods that are not discussed in detail elsewhere, the chapter has provided a general outline together with an indication of any methodological weaknesses. The general weakness pervading over all tests are with

growing microorganisms and then with defining growth, both of which highlight the inherent variability that are commonplace to many microbiological techniques.

Despite the weakness, many of the methods are long established and can trace their methodological basis back to the experiments undertaken by the founding mothers and fathers of microbiology. The extent to which these methods will be replaced by rapid microbiological techniques is likely to be gradual, and, even then, it is unlikely the methods will disappear completely. Many will remain features of the microbiology laboratory for some time to come.

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