Antibiotics and preservatives

14

14.1 Introduction

An antimicrobial agent is a chemical that either kills or inhibits the growth of microorganisms. Each class of antimicrobial agents has a unique mode of action. Antimicrobial agents that inhibit microbes, such as tetracycline, have the suffix "static" added to their root (e.g., "bacteriostatic"); whereas agents that kill microbes, such as fluoroquinolones, have the suffix "cidal" added to their root (e.g., "bactericidal"). Antimicrobial agents include antibacterial, antiviral, antifungal, and antiparasitic agents.

The first antibiotic was serendipitously "discovered" in 1928 by Alexander Fleming when *Penicillium novatum* spores were observed to inhibit the growth of *Staphylococcus* on agar. The first wave of antibiotics was derived from microorganisms; latterly, following advances in medicinal chemistry, most modern antibacterials are semisynthetic modifications of various natural compounds [1]. These often differ from their parent compound in their antimicrobial activity or their pharmacological properties.

Antibiotic sensitivity is the susceptibility of bacteria to antibiotics. Antibiotic susceptibility testing (AST) is undertaken to determine which antibiotic will be most successful in treating a bacterial infection *in vivo*. Although it is more commonly an aspect of clinical microbiology, the performance of antimicrobial susceptibility testing of significant bacterial isolates plays an important part of the pharmaceutical development of new antimicrobials. The objective of this testing is to detect possible drug resistance in common pathogens and to assure susceptibility to drugs of choice for particular infections. The most widely used testing methods include broth microdilution or alternative rapid automated instrument methods that use commercially marketed materials and devices. Manual methods include the disc diffusion and gradient diffusion methods. Each method has strengths and weaknesses, although, in general, testing methods provide accurate detection of common antimicrobial resistance mechanisms. That said, newer or emerging mechanisms of resistance require constant vigilance regarding the ability of each test method to detect resistance accurately.

A preservative is a natural or synthetic chemical that is added to products such as foods, cosmetics, or pharmaceuticals to prevent spoilage [2]. A preservative is added to pharmaceuticals to prevent decomposition by microbial growth or by undesirable chemical changes. Antimicrobial preservatives are substances added to products to protect them from microbiological growth or from microorganisms that are introduced inadvertently during or subsequent to the manufacturing process.

Upon determination that a product has been properly neutralized and has very low levels of contamination (<10 colony-forming units (CFU)/g), microbiologists can conduct a preservative efficacy test (PET) (alternatively termed an antimicrobial efficacy test). With this test, the product is challenged within individual containers, separately, using one of the five required microorganisms. These organisms, as dictated by

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compendia, are: *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans,* and *Aspergillus brasiliensis*. The PET assay measures the reduction of a high inoculum (10^6–10^7 CFU/g) in the presence of a product containing preservative over 28 days. The method tests the ability of the preservative to prevent a re-bound of an organism (re-growth).

14.2 Antibiotic susceptibility testing

14.2.1 Antimicrobials

Resistance to antibiotics can either be naturally occurring for a particular organism and drug combination or acquired resistance can occur. This is where over-use (misuse) of antimicrobials results in a population being exposed to an environment in which organisms that have genes conferring resistance (either spontaneously mutated or through deoxyribonucleic acid (DNA) transfer from other resistant cells) have been able to flourish and spread.

Antimicrobial agents are classified by their specific modes of action against bacterial cells. These agents may interfere with cell wall synthesis, inhibit protein synthesis, interfere with nucleic acid synthesis, or inhibit a metabolic pathway. Mechanisms include:

- interference with cell wall synthesis (such as activity of beta-lactams);
- interference with the cytoplasmic membrane;
- interference with protein synthesis by binding to the 30S ribosomal subunit;
- inhibition of protein synthesis by binding to the 50S ribosomal subunit;
- inhibition of protein synthesis by inhibition of the 70S initiation complex;
- interference with nucleic acid synthesis;
- inhibition of the metabolic pathway for folic acid synthesis.

There are a number of ways by which microorganisms are resistant to antimicrobial agents. These include:

- the bacteria produce enzymes that either destroy the antimicrobial agent before it reaches its target or modify the drug so that it no longer is recognized by the target;
- the cell wall becomes impermeable to the antimicrobial agent;
- the target site is altered by mutation so that it no longer binds the antimicrobial agent;
- the bacteria possess an efflux pump that expels the antimicrobial agent from the cell before it can reach its target;
- specific metabolic pathways in the bacteria are genetically altered so that the antimicrobial agent cannot exert an effect.

With the above, in some species antimicrobial resistance is an intrinsic or innate property using one of the aforementioned mechanisms. Bacteria also can acquire resistance to antimicrobial agents by genetic events such as mutation, conjugation, transformation, transduction, and transposition.

These variable aspects of resistance make antimicrobial susceptibility testing of importance.

14.2.2 Antimicrobial susceptibility test concepts

Identification of a microorganism in the clinical setting normally goes hand in hand with the AST test. This is because knowing what microorganism has isolated together with knowledge of the isolation site, will give an indication of what type of antibiotics should be considered. The sensitivity of an isolate to a particular antibiotic is measured by establishing the minimum inhibitory concentration (MIC) or breakpoint. MIC is the lowest concentration of antibiotic at which an isolate cannot produce visible growth after overnight incubation.

MICs can be determined by agar or broth dilution techniques by following the reference standards established by various authorities. These include the Clinical and Laboratory Standards Institute (CLSI, USA), British Society for Antimicrobial Chemotherapy (BSAC, UK), AFFSAPS (France), Deutsches Institut für Normung e.V. (DIN, Germany), and ISC/World Health Organization (WHO).

With each method, one of the most important steps in the testing process is preparing the inoculum of the test microorganism. This involves selecting appropriate colonies for testing, suspending them in broth, and standardizing the suspension.

14.2.3 Broth dilution method

The broth dilution method depends upon microbial inoculation at a specific inoculum density of broth media (in tubes or microtiter plates) containing antibiotics at varying levels—usually doubling dilutions are used (e.g., 1, 2, 4, 8, and 16 µg/mL). The standardized bacterial suspension is typically $1–5 \times 10^5$ CFU/mL. Following incubation at 35 °C, turbidity is recorded either visually or with an automated reader, and the breakpoint concentration established. The lowest concentration of antibiotic that prevented growth represents the MIC. The precision of this method is to be ±1 twofold concentration [3].

Microtiter plates or ready-to-use strips are commercially available with antibiotics ready prepared in the wells. Standard trays contain 96 wells, each containing a volume of 0.1 mL that allows approximately 12 antibiotics to be tested in a range of eight × twofold dilutions in a single tray [4].

A variation on this approach is the agar dilution method where a small volume of suspension is inoculated onto agar containing a particular concentration of antibiotic, when the inoculum has dried the plate is incubated and again examined for zones of growth. With this microdilution testing, the method uses about 0.05–0.1 mL total broth volume and can be conveniently performed in a microtiter format.

14.2.4 Disc diffusion method

Disc diffusion or the Kirby–Bauer test is one of the classic microbiology techniques, and it is still very commonly used. Because of convenience, efficiency, and cost, the disc diffusion method is probably the most widely used method for determining antimicrobial resistance around the world.

A suspension of the isolate (of approximately $1–2 \times 10^8$ CFU/mL) is prepared to a particular McFarland standard, then spread evenly onto an appropriate agar (such as Müller-Hinton agar) in a Petri dish. The agar typically contains (weight/volume) [5]:

- 30.0% beef infusion;
- 1.75% casein hydrolysate;
- 0.15% starch;
- 1.7% agar;
- pH adjusted to neutral at 25 °C.

With the test, the discs are impregnated with various defined concentrations of different antibiotics are placed onto the surface of the agar. A multichannel disc dispenser can speed up placement of the discs. After incubation (16–24 h at 35 °C) zones of growth inhibition around each of the antibiotic discs are measured to the nearest millimeter (as shown in Figure 14.1). A clear circular zone of no growth in the immediate vicinity of a disc indicates susceptibility to that antimicrobial [6]. Using reference tables, the size of zone can be related to the MIC and results recorded as whether the organism is susceptible (S), intermediately susceptible (I), or resistant (R) to that antibiotic [7].

There are a number of critical steps in this approach, such as which medium is used; depth and moisture content of the agar in the plate; incubation conditions; accurate inoculum density; discs must be firmly placed in contact with the agar surface otherwise the diffusion rate will not be correct. The advantages of the disc method are the test simplicity that does not require any special equipment.

Preprepared antibiotic discs with full quality control documentation provided by the manufacturer maintain reproducibility and considerably increases assay reliability. Discs should always be manufactured to an acceptable specification, for example, from the US Food and Drug Administration (FDA), WHO, and DIN. The DIN standard has the tightest range with antibiotic concentrations within 90–125% of that stated.

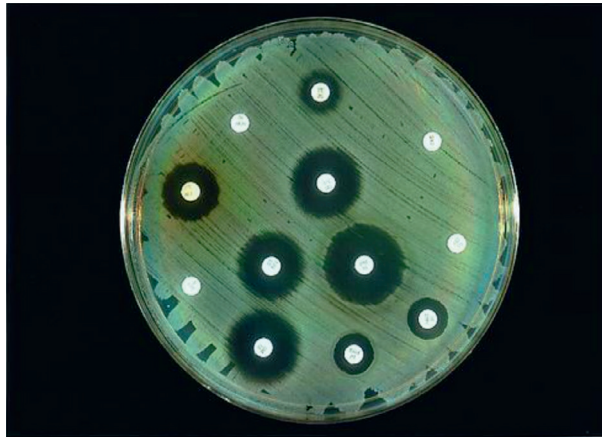

Figure 14.1 Classic disc diffusion assay.
Photograph courtesy of Pharmig.

A variation on this approach is to use a strip impregnated along its length, with a gradient of different concentrations of antimicrobial. This method employs thin plastic test strips that are impregnated on the underside with a dried antibiotic concentration gradient and are marked on the upper surface with a concentration scale. Following incubation this creates an ellipse-shaped zone of no growth, where the ellipse meets the strip, the MIC can be read from the concentration markings on the strip. These are easy to read, no tables need to be referenced to get an MIC value, and the test requires fewer manipulations, as one strip will cover the whole concentration range. These again can be manually or instrument read [8].

14.2.5 Test variability

There are several factors can affect the accuracy of the AST results. These conditions are pH, moisture, effects of medium components, and microbial inoculum.

14.2.5.1 pH

pH of the medium is an important factor that influences the accuracy of the AST. If the pH of the medium is too low than the desired pH, certain drugs, such as aminoglycosides, quinolones, and macrolides, lose their potency. In contrast, antibiotic classes, such as tetracyclines, appear to have excess activity a lower pH, and the vice versa happens in the case of the higher pH.

14.2.5.2 Moisture

The presence of moisture content on the medium can counter act with accuracy of the susceptibility testing. It is important to remove the excess moisture present in the agar surface, by keeping it in the laminar flow hood for few minutes.

14.2.5.3 Effects of medium components

If the media selected for the antibiotic susceptibility contains excessive amounts of thymine or thymidine compounds, they will reversibly inhibit the action of certain antimicrobial agents such as trimethoprim groups. This reversible inhibition yields smaller or less distinct or even no zones and will be misinterpreted as resistant antibiotics. Mueller-Hinton agar is low in thymine and thymidine contents, and it can be used successfully to study the susceptibility of antibiotics. Also, the medium containing excessive cation reduces the zone size, while low cation content results in unacceptably large inhibition zones.

14.2.5.4 Microbial inoculum

The amount of the organism used for the susceptibility testing is standardized using a turbidity standard. This is obtained by a visual approximation using McFarland standard of 0.5, or else it can be determined by using a spectrophotometer with optical density of 1 at 600 nm wavelength.

14.2.6 Automated methods

Disc diffusion and broth dilution techniques can be semiautomated by using image analysers to read zones or turbidity readers for the broths, these give a more objective result and can come with software for automatically interpreting results. Automated systems that are widely used, these normally combine identification with sensitivity testing. The use of instrumentation can standardize the reading of end points and often produce susceptibility test results in a shorter period than manual readings because sensitive optical detection systems allow detection of subtle changes in bacterial growth.

With automated methods, the whole test is set up and read automatically not only is the workload reduced but also the result is less subjective, more reproducible. Results are usually faster, with same day results possible as the instruments monitor growth by taking continuous readings and base results on growth kinetics. While automated systems have many advantages, they can be less flexible in terms of the choice of antibiotics available, consumable costs are usually higher, and equipment costs need to be met whether by outright purchase, leasing or reagent rental deals, together with service and maintenance charges.

For both the semiautomated zone readers and the fully automated ID and susceptibility systems, the data collected can be assessed by expert or smart software systems for interpretation, highlighting unusual anomalous results, suggesting other possible antibiotics to try and can be exported to other laboratory information management systems for further reporting.

The use of genotypic approaches for detection of antimicrobial resistance genes has been promoted as a way to increase the rapidity and accuracy of susceptibility testing. Methods that employ the use of comparative genomics, genetic probes, microarrays, nucleic acid amplification techniques (such as polymerase chain reaction (PCR)), and DNA sequencing are capable of increased sensitivity, specificity, and speed in the detection of specific known resistance genes [9].

14.2.7 Results interpretation

Whichever method is used, the result provides a key cut-off point that equates to the MIC of antibiotic for that test isolate, and methods initially require a pure culture to be prepared, which may take 1–2 days.

With antimicrobial efficacy testing, different products will have different pass criteria. This will be based on the route of administration [10]. However, the general interpretation criteria will be [11]:

- a “susceptible” result indicates that the patient’s organism should respond to therapy with that antibiotic using the dosage recommended normally for that type of infection and species;
- an organism with an MIC or zone size interpreted as “resistant” should not be inhibited by the concentrations of the antibiotic achieved with the dosages normally used with that drug;
- an “intermediate” result indicates that a microorganism falls into a range of susceptibility in which the MIC approaches or exceeds the level of antibiotic that can ordinarily be achieved and for which clinical response is likely to be less than with a susceptible strain.

As a note of caution, sometimes the "intermediate" result can also mean that certain variables in the susceptibility test may not have been properly controlled, and that the values have fallen into a "buffer zone" separating susceptible from resistant strains.

While the AST, in its various forms, more often than not produces accurate results, it should be borne in mind that the effectiveness of individual antibiotics varies with the location of the infection, the ability of the antibiotic to reach the site of infection, as well as with the ability of the bacteria to resist or inactivate the antibiotic.

14.3 Antimicrobial efficacy testing (preservative efficacy testing)

The antimicrobial effectiveness test (AET) is used to assess preservative efficacy of products in multi-dose containers. The AET is sometimes referred to also as the preservative effectiveness test. The preservative efficacy of products can either be due to added preservatives, or the inherent properties of the product without the addition of preservatives.

More often, the preservative is added to the product at some point during formulation. If a multiuse pharmaceutical preparation does not itself have adequate antimicrobial activity, antimicrobial preservatives may be added, particularly to aqueous preparations, to prevent proliferation, or to limit microbial contamination which, during normal conditions of storage and use, particularly for multidose containers, could occur in a product and present a hazard to the patient from infection and spoilage of the preparation.

The AET consists of challenging the preparation, wherever possible in its final container, with a prescribed inoculum of suitable microorganisms storing the inoculated preparation at a prescribed temperature, withdrawing samples from the container at specified intervals of time and counting the organisms in the sample removed.

Importantly, the test is not intended to challenge the ability of product in multidose containers to withstand in-use contamination. Instead, the test is designed to show that the product will be stable over a prolonged period of time in relation to microbial contamination. The purpose of the test is, therefore, designed to focus on activity of the preservative systems as a protection against inadvertent contamination during storage and use of the product. The test is used during product development to determine the effectiveness of the product and during stability to demonstrate the preservative system is stable over time. Certain microorganisms can adversely impact (reduce or inactivate) the activity of certain products. It is important to understand the microbial load of the nonsterile finished products in order to determine if the product will react the way it was intended. Likewise, it is important to understand the effectiveness of the preservative system to ensure the product will remain active as intended overtime without garnering microorganisms that can cause human infection [12].

Typical categories of products tested include:

- parenteral and ophthalmic preparations;
- topical preparations;
- oral preparations.

The first contamination-related consideration is with the selection of the preservative and its efficacy. An ideal preservative is a rapidly effective and topically nonirritating. It may be a single antimicrobial agent or a mixture of such agents. Preservatives are designed to prevent the growth or to destroy microorganisms accidentally introduced into the product when the container is opened during use. Preservatives are a necessary additive.

There are several critical considerations in selecting a preservative for inclusion in the dosage form. These include preservative stability, chemical compatibility with the other components of the formulation, compatibility with packaging materials, and concentration. Preservative agents must to be effective throughout the entire shelf life of the product. A preservative will only provide protection from microbial growth for a short time period. For this reason, 28 days is typically stated as the maximum shelf-life after the preservative-containing product is opened [13].

14.3.1 Antimicrobial efficacy test

The compendial AET is essentially a suspension test designed to demonstrate the extent of microbial kill. The AET test comprises a controlled inoculum of the challenge organisms is placed in suspension with the preservative sample to be tested, and the number of survivors determined at different time points. Key aspects of the test are with developing a method for neutralization of the preservative. Residual preservative in the recovery agar could artificially depress the recovery of viable cells. It is, thus, important to neutralize this residual activity to get accurate counts of survivors [14].

The test is not designed to be a quality control release test. To verify effectiveness of the preservative, the AET is assessed during product validation. Obtaining successful validation of the assay can be complex depending on the product. Key factors in developing a proper method include some experimentation as well as knowledge of the test material properties.

The test is not (yet) subject to international harmonization. It is important to note that while the US Pharmacopeia (USP), Japanese Pharmacopoeia (JP), and European Pharmacopoeia (Ph. Eur.) all describe the assay in a similar fashion, but the acceptance criterion varies amongst the different compendial chapters.

The references are:

- USP <51> Antimicrobial Effectiveness Test (AET);
- Ph. Eur. 5.1.3 Efficacy of Antimicrobial Preservation;
- JP 19. Preservatives-Effectiveness Tests (PET);
- ICH Guidance Q6A Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances.

With the pharmacopeia chapters, there are some differences with the microorganisms to be used in the tests [15]. These are shown in Table 14.1.

Antimicrobial preservatives are substances that are added to products to limit microbial contamination during normal use or storage. Antimicrobial agents may be harmful to patients. If possible, it is important to demonstrate that the agents are effective in the final package and that they are safe for human use. Products that have

Table 14.1 Comparison of test microorganisms across the main pharmacopeia

Pharmacopeia	Microorganisms
USP <51>	*Candida albicans* ATCC 10231, *Aspergillus brasiliensis* ATCC 16404, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538
JP 19	*Candida albicans* ATCC 10231, *Aspergillus brasiliensis* ATCC 16404, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538
Ph. Eur. 5.1.3	*Candida albicans* ATCC 10231, *Aspergillus brasiliensis* ATCC 16404, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538 In addition, environmental isolates can be used to substitute any of the above if they are considered to be more appropriate

inherent antimicrobial properties must also be analyzed for antimicrobial effectiveness. Antimicrobial effectiveness must be demonstrated for all products multidose containers.

The AET is used to evaluate the effectiveness of preservative systems in multidose dosage forms. Products in multidose forms that do not contain added preservatives must still comply with the test to demonstrate that the inherent antimicrobial properties of the product are effective.

The test involves inoculating a measured amount of product with known amounts of microorganisms. Whenever possible, the original containers are utilized for the assay. The containers are protected from light and incubated at ambient temperature for 28 days. The death rate is measured over a 28-day period and compared with the acceptance criteria outlined in the compendial guidance documents.

While the method across the three pharmacopeia varies, they are broadly similar. The section below describes the basis of the Ph. Eur. method. Readers who have an interest in other compendial methods are advised to refer to these.

The method involves the following steps:

- prepare suspensions of each microorganism for the study;
- confirm the estimate of the suspensions. Use the plate count or membrane filtration method;
- inoculate a series of containers of the product to be examined, each with a suspension of one of the test microorganisms. The number of containers is left to the user to define; however, this would ordinarily not be fewer than five containers;
- inoculate each container with one inoculum suspension and mix. The volume of inoculum does not exceed 1.0% of the volume of product. Final concentration is between 1×10^5 and 1×10^6 CFU/mL of product;
- incubate the inoculated containers at 22.5 ± 2.5 °C (protected from light) and sample at the appropriate intervals;
- remove a suitable sample from each container, typically 1 mL or 1 g, at 0 h and at appropriate intervals according to the type of product. Ensure antimicrobial properties are removed by dilution, filtration or an inactivator. Determine the number of viable cells by the plate count or membrane filtration method. The procedure needs to be validated:

  - in the membrane filtration method, filtration must be performed with filters that have a pore size not greater than 0.45 µm. The type of filter material is chosen in such a way that the bacteria-retaining efficiency is not affected by the components of the sample. Common filter materials include cellulose, nylon, and polyvinylidene fluoride (PVDF);
  - in terms of culture media, tryptic soy agar (TSA) is used for the culturing of bacteria and Sabouraud dextrose agar (SDA) for the culturing of fungi. For the cultivation of the test organisms, the media should contain a suitable inactivator (neutralizer) for the specific antimicrobial properties in the product to the broth and/or agar media used for the test procedure if required. Media used for testing needs to be tested for growth promotion by inoculating the medium with appropriate microorganisms;
- calculate the log reduction.

In terms of the acceptance criteria, the main pharmacopeia again differ. These differences are shown in Table 14.2.

With the table, the pharmacopoeia divide products into different categories (either by description or using a numbering system).

The Ph. Eur. categories are described (as per Table 14.2), with the USP, the categories are:

- Category 1—injections, other parenteral including emulsions, otic products, sterile nasal products, and ophthalmic products made with aqueous bases or vehicles;
- Category 2—topically used products made with aqueous bases or vehicles, non-sterile nasal products, and emulsions, including those applied to mucous membranes;
- Category 3—oral products other than antacids, made with aqueous basesor vehicles;
- Category 4—antacids made with aqueous bases or vehicles.

Products in different categories require testing at different time intervals. Reference to the compendial text should be sought.

14.3.2 Antimicrobial effectiveness test validation

The suitability of the methods to recover microorganisms if they are present must be established through method verification. Method validation is typically performed on three lots of material to demonstrate the robustness of the method.

With the validation exercise:

- inoculate each container with 0.1 mL (or more as determined by the volume of product) of each of the challenge microorganism inoculums prepared and label the containers accordingly. There should be at least one uninoculated product control container;
- the volume of inoculum and product in the sterile containers may be adjusted as long as the volume of the suspension inoculum used is between 0.5% and 1% of the volume of product;
- mix the solution well to ensure a homogenous distribution of the microorganisms. Determine the number of viable microorganisms in each inoculum suspension by referencing the results from the N0 (or titer);
- then, calculate the initial concentration of microorganisms per milliliter of product under test. The target inoculum should yield a suitable concentration between 1×10^5 and 1×10^6 CFU/mL of product.

Table 14.2 Acceptance criteria for the antimicrobial effectives test

Pharmacopeia	Acceptance criteria	Interpretation
USP <51>	Category 1: NLT 1.0 log from initial at 7 days, NLT 3.0 log reduction from the initial count at 14 days, and no increase from the 14 days at 28 days for bacteria. Yeast and moulds—no increase from the initial calculated count at 7, 14, and 28 days Category 2: bacteria—NLT 2.0 log reduction from the initial count at 14 days and no increase from the 14 day counts at 28 days. Yeast and mould—no increase from the initial calculated count at 14 and 28 days Category 3: bacteria—NLT 1.0 log reduction from the initial count at 14 days and no increase from the 14 days count at 28 days. Yeast and moulds—no increase from the initial calculated count at 14 and 28 days Category 4: bacteria, yeast, and moulds—no increase from the initial calculated count at 14 and 28 days	No increase is defined as not more than a 0.5 log unit higher than the previous value measured
JP 19	Category 1A: bacteria: After 14 days 0.1% of inoculum count or less/after 28 days the same level or less after 14 days. Yeasts/moulds: same or less than inoculum count after 14 days/same or less than inoculum count after 28 days Category 1B: bacteria: After 14 days 1% of inoculum count or less/after 28 days the same level or less after 14 days. Yeasts/moulds: same or less than inoculum count after 14 days/same or less than inoculum count after 28 days Category 2: bacteria: same or less than inoculum count at 14 days/same or less than inoculum count at 28 days. Yeast/moulds: same or less than inoculum at 14 days/same or less inoculum count at 28 days Category 1C: bacteria: after 14 days 10% of inoculum count or less/after 28 days the same level or less after 14 days. Yeasts/moulds: same or less than inoculum count after 14 days/same or less than inoculum count after 28 days	When the results are obtained, the product examined is considered to be effectively preserved
Ph. Eur. 5.1.3	Parenteral and ophthalmic preparations: bacteria (A) 6 h = 2 log/24 h = 3 log/28 day = no recover fungi (A) 7 day = 2 log/28 day = no increase/bacteria (B) 24 h = 1 log/7 day = 3 log/28 day = no increase fungi (B) 14 day = 1 log/28 day = no increase Topical preparations: bacteria (A) 2 day = 2 log/7 day = 3 log/28 day = no recover fungi (A) 14 day = 2 log/28 day = no increase/bacteria (B) 14 day = 3 log/28 day = no increase fungi (B) 14 day = 1 log/28 day = no increase Oral preparations: bacteria 14 days = 3 log/28 days = no increase. Fungi: 14 days = 1 log/28 days = no increase	When the results are obtained, the product examined is considered to be effectively preserved. In justified cases where the A criteria cannot be obtained, the B criteria can be used

Note: NLT, not less than.

To assess the log reduction, the following formula should suffice:

$$\begin{aligned}\log\text{reduction} &= \log\text{of initial calculated CFU/mL} \\ &\quad - \log\text{of product challenge results CFU/mL} \\ &= \log\text{of } 1\times10^{5}\ \text{CFU/mL} \\ &\quad - \log\text{of } 1.0\times10^{2}\ \text{CFU/mL}\ \left(\text{or } 100\ \text{CFU/mL}\right) \\ &= 5-2=3\end{aligned}$$

Useful information for the development process includes:

- if the product contains antimicrobial activity, this should be neutralized;
- if inactivators are used, their efficacy and their absence of toxicity for microorganisms must be demonstrated;
- common neutralizing agents and methods include the addition of polysorbate, the addition of lecithin, and/or dilution methods.

During the course of the validation exercise, it is important to note:

- How does the material react to varying scenarios?
- How does the material dissolve?
- How easily/accurately are inoculated microorganisms recovered?
- Does the pH need to be adjusted?
- Which method (membrane filtration or pour plate) is the right method?
- Which neutralizers are needed, if any?

In addition to the above, it should be borne in mind that preservatives are toxic chemicals; therefore, toxicological tests must also be performed.

14.4 Conclusion

This chapter has consider two parts of antimicrobial activity that relate to pharmaceutical products. The first was in relation to antibiotics, used to treat general or specific bacterial infections. Here, although a variety of methods exist, the goal of in vitro antimicrobial = susceptibility testing is the same: to provide a reliable predictor of how a microorganism is likely to respond to antimicrobial therapy in the infected host.

The second area examined in this chapter was with the addition of preservatives to products in order to extent the shelf-life of the product beyond what would be possible from the product ingredients without the addition of the preservative.

Both antibiotics and preservatives are similar in terms of falling under the classification antimicrobials [16]. There are a vast spectra of chemicals that fall within this category demonstrating the effectiveness of these, whether designed to treat, or to preserve, forms an important part of clinical and pharmaceutical microbiology.

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