CHAPTER FOUR

Antimicrobial testing

Contents

4.1 Introduction

Antimicrobial agents have been used extensively across the world to inhibit microbial growth in a range of situations including medical, agricultural and environmental purposes. An understanding of how antibiotics target different sites within bacteria, such as cell wall components and protein or RNA synthesis among other targets has allowed different classes of antibiotic to be used for varying functions. Some are used for their broad range of activity, whereas others are employed for their narrow range of activity and ability to inhibit or kill particular types of bacteria.

The heavy use of antibiotics over many years has led to a rise in antibiotic-resistant bacteria and a corresponding decrease in the efficacy of many antibiotics. Therefore, the ability to determine the sensitivity of bacteria to antibacterial compounds is an essential technique that can be undertaken in a variety of ways.

4.1.1 Antibiotics and targets

The activity antibiotics have falls into two broad categories: bactericidal (they kill the bacteria) or bacteriostatic (they inhibit bacterial growth). The effect they have depends on the part of the bacteria they target. A wide range of antibiotic classes have been developed targeting varying parts of the bacterial cell. Examples of some antibiotic classes and their bacterial targets are given in Table 4.1.

Ideally, an antibiotic will display selective toxicity and target a component that is unique to the bacteria and will not cause damage to human

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Table 4.1 Examples of some antibiotic classes with their corresponding target sites and known resistance mechanisms.

Antibiotic class	Example antibiotic	Target site	Common resistance mechanisms
B-Lactams	Penicillin	Cell wall	• Enzymatic degradation • Altered penicillin binding protein
Tetracyclines	Tetracycline	Protein synthesis (30s)	• Efflux pumps • Altered target site
Aminoglycosides	Gentamicin	Protein synthesis (30s)	• Enzymatic modification • Altered target site • Decreased uptake
Lincosamides	Clindamycin	Protein synthesis (50s)	• Altered target site
Glycopeptides	Vancomycin	Cell wall	• Altered target site
Polymyxins	Colistin	Cytoplasmic membrane	• Altered target site
Macrolides	Erythromycin	Protein synthesis (50s)	• Efflux pumps • Altered target site
Quinolones	Ciprofloxacin	DNA gyrase	• Efflux pumps • Altered target site
Rifampicins	Rifampicin	RNA synthesis	• Altered target site

cells. In practice, many antibiotics will have some level of host toxicity in addition to their antibacterial effect. There are several ways in which you can test the efficacy of antibiotics against the bacterial isolates in which you are interested. Once familiar with standard testing methods, those methods can also be adapted to test new compounds or materials that you may have developed to inhibit or kill bacteria. Commonly, these methods include testing the activity of antibiotics against bacteria on agar plates or in broth. A range of methods that can be used are outlined in this chapter.

4.1.2 Disc diffusion

One of the easiest and quickest methods that can be used to test the antibiotic sensitivity of a bacterial isolate is disc diffusion. When testing a conventional antibiotic, this involves creating a lawn of bacteria on an agar plate and then placing an antibiotic disc or discs onto the lawn before incubation it overnight. This method works because the antibiotic in the disc diffuses out into the agar, creating a concentration gradient. The concentration of antibiotic near the disc is high and gradually decreases the farther from the disc it diffuses.

The zone of inhibition caused by the antibiotic is measured the next day, giving an idea of how effective the antibiotic is against a particular bacterium. Standardised methods are available that provide detailed instructions on how to carry this process out in reproducibly: for example, from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org/) or the Clinical and Laboratory Standards Institute (CLSI). These organisations provide detailed methods describing how to perform standardised testing, including information about which concentration antibiotic discs to use and what the breakpoints are for a variety of bacteria. When using the disc diffusion method, the breakpoint is the size of the zone of inhibition below which a bacterium is resistant. The organisations also provide the size of zone above which the bacteria are considered to be sensitive. If you are working with antibiotics, it is worth following these guidelines because they ensure that you are creating reproducible tests that can be compared with antibiotic sensitivity tests carried out in other laboratories. They provide details about how to prepare the media and inoculum, as well as information about how to calibrate and validate your work. These guidelines and breakpoints are updated regularly, so you should always check for the latest updates before performing this type of experiment.

If you are using the disc diffusion method with the intention of checking results against the breakpoint tables, it is important to check that the antibiotic concentration in the disc is correct, because some antibiotics have more than one concentration available. It is also useful to familiarise yourself with the antibiotic abbreviations, because not all of them are immediately obvious as short versions of the original antibiotic name (Fig. 4.1).

A general overview of the method is given here, but you should check the protocols provided by the organisations discussed, to ensure that the specific details are correct for the bacteria and antibiotic being tested.

The antibiotic disc susceptibility testing method is briefly:

1. Aseptically pick a few bacterial colonies from an overnight agar plate culture and suspend these in sterile saline to give a density equivalent to a 0.5 McFarland standard. You can do this using a spectrophotometer or by comparing the density with a standard if you have one in the laboratory.
2. When the suspension is at the correct turbidity, take a sterile cotton swab and dip it in the suspension. Press it against the inside of the suspension tube to remove excess liquid. Then, apply it to an agar plate. The swab should be swabbed across the plate, moving from side to side

Figure 4.1 Range of antibiotic discs available for sensitivity testing. Letters on the discs give the identity and concentration in micrograms of the antibiotic in the disc. *ATM*, aztreonam; *C*, chloramphenicol; *CIP*, ciprofloxacin; *CN*, gentamicin; *CT*, colistin; *DA*, clindamycin; *E*, erythromycin; *FOX*, cefoxitin; *LZD*, linezolid; *MEM*, meropenem; *TE*, tetracycline; *TOB*, tobramycin; *VA*, vancomycin.

all the way from the top to the bottom of the plate before rotating the plate approximately 60 degrees. Before swabbing again, rotate the plate once more and swab again. The plate should then have been swabbed in three directions across the plate. The swab should be dipped into the inoculum only before the swabbing starts, not between each rotation.

3. Once the lawn of bacteria has been created, the antibiotic discs can be applied to the plate. These should be removed from the fridge or freezer before use and allowed to come to room temperature before use. The discs can be applied using an antibiotic disc dispensing machine if you have one in your laboratory; use sterile forceps if you do not have a dispenser (Fig. 4.2). If using forceps, take care not to rip or damage the disc when handling it.
4. Ensure that you press the disc down onto the agar so that it is secure and does not fall off. Then, invert the plate for incubation. Incubate the plates for 16–20 h at 35°C.

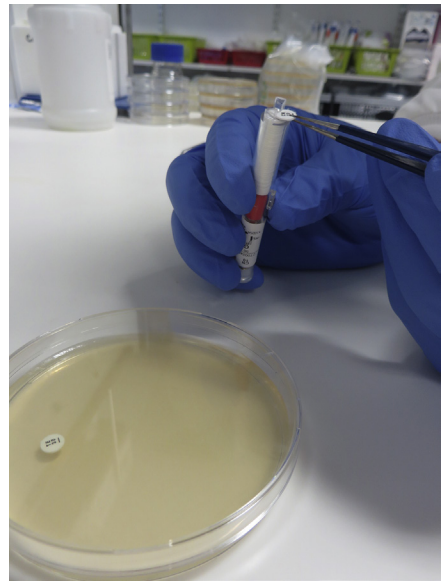

Figure 4.2 Forceps used to remove an antibiotic disc from its tube. The discs will come out of the tube only in one direction. If a disc is damaged because it has been forced out of the tube in the wrong direction, dispose of it and remove one in the correct direction.

If using forceps, these can sterilised between uses by dipping the tip of the forceps into ethanol and passing through a blue flame. Hold the forceps away from the flame and allow the flame to go out. Wait a few seconds for the forceps to cool before using it. Do not dip the forceps into the ethanol when it is hot, or the ethanol could catch fire. If in doubt, wait slightly longer before dipping the forceps. It is a good idea to have something nonflammable, such as a metal lid, that can be put across the top of the container, which holds the ethanol. If the ethanol catches fire, the lid can be placed across the top to remove the oxygen supply to the fire.

When undertaking disc diffusion testing, it is important to get the antibiotic discs onto the plates within 15 min of creating the bacterial lawn. After applying the antibiotic discs, you should aim to get the plates into the incubator within 15 min. So, think carefully when setting up your experiments, because you might need to prepare the lawn plates and apply the antibiotic discs in batches so that you do not stray outside those time limits.

To determine whether the bacteria are sensitive to the antibiotic discs you have placed on the agar, you will need to measure the zones of inhibition around the discs from the back of the plate. You can measure the zones using digital or manual callipers or a ruler. The measurement should be

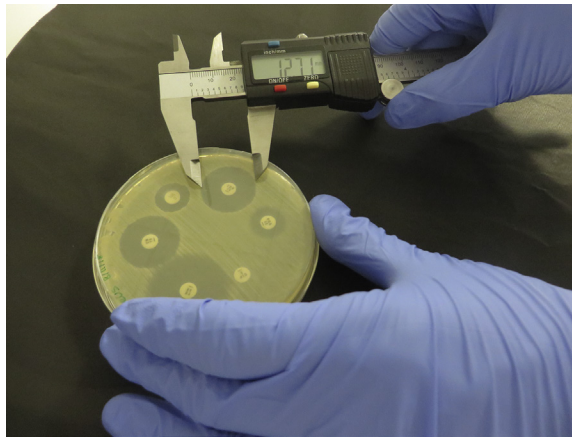

Figure 4.3 Digital callipers used to measure the zone of inhibition in millimetres around each antibiotic disc on the agar plate.

Table 4.2 Sample layout for a table to collect zone inhibition measurements.

	Antibiotic discs (µg/mL)					
Bacteria	**CIP (5)**	**CN (10)**	**TOB (10)**	**ERY (15)**	**C (2)**	**TET (30)**
MRSA1						
MRSA2						
MRSA3						
MRSA1						
MRSA2						
MRSA3						
MRSA1						
MRSA2						
MRSA3						

This table allows triplicate data for each organism to be collected. Some of the antibiotic disc abbreviations are easy to remember (e.g., TET for tetracycline) but others are less straightforward (e.g., CN for gentamicin). *C*, chloramphenicol; *CIP*, ciprofloxacin; *ERY, Erythromycin; MRSA*, Methicillin-resistant *Staphylococcus aureus*; *TOB*, tobramycin.

the diameter of the zone of inhibition (where no bacteria have grown) to the nearest millimetre (Fig. 4.3). The zones should be read against a dark background using reflected light, or from the front using reflected light. Whichever is used depends on the agar employed, so check the published guidelines to determine which is needed.

It helps to have a pre-prepared table in your lab book in which to record your results, especially if you have multiple isolates and antibiotics. A pre-prepared table ensures that the data are recorded accurately. Table 4.2 shows a sample data collection table that could be used to record zone of inhibition measurements from triplicate isolates.

Once the zone of inhibition is established, the sensitivity or resistance status of the bacteria can be determined by comparing that value with published breakpoint tables. It is good practice to run a control bacterium alongside the test bacteria. Testing a bacterial isolate with a known antibiotic susceptibility profile alongside the test bacteria allows you to check that the method and antibiotics are working as they should be and that the results obtained are reliable.

When inspecting the zones of inhibition, if they do not appear to be straightforward (e.g., double zones, fuzzy-edged zones or colonies growing within the inhibition zone), often detailed standardised protocols will offer advice on how these should be interpreted. It is worth checking the detailed protocols if there are unusual results, because advice on how to interpret the varying zones can differ among species.

Analysis: Once the data have been collected, they can be processed and presented as a bar graph to make it easy for others to see the results quickly. If the tests have been carried out in triplicate, there will be multiple values for the zone of inhibition for each antibiotic. If tested in triplicate, the mean value should be calculated. This can be done by adding together the zone of inhibition measurements for each antibiotic and dividing by the number of replicates; this will generate one mean value for each antibiotic, which can be plotted onto a graph. The standard deviation should also be calculated for each antibiotic to ensure that replicate data are similar. The standard deviation can then be added to the graph as error bars (Fig. 4.4). The standard deviation can easily be calculated and often is available as a function in a programmes such as Excel. To calculate standard deviation in a programme such as Excel, select an empty cell, press the Equals key, and then select standard deviation from the Functions menu bar (often the top left in Excel and displayed as STDEV or a variation of this). Select all replicates for the antibiotic of interest and then press Return. This should generate a value for the standard deviation. This process can be repeated for each antibiotic, and once generated, can be plotted onto the graph. The error bars should provide an easy way to see whether the zone sizes were similar between replicates or whether there is a lot of variation, which could indicate that the experiment needs to be repeated.

Depending on what is being tested, there are two ways in which the data could be presented. If you are testing multiple isolates of the same species and the same range of antibiotics on each isolate, a graph that contains all the isolates and only one antibiotic could be created. This type of graph provides an opportunity for the breakpoint line plotted onto the graph so

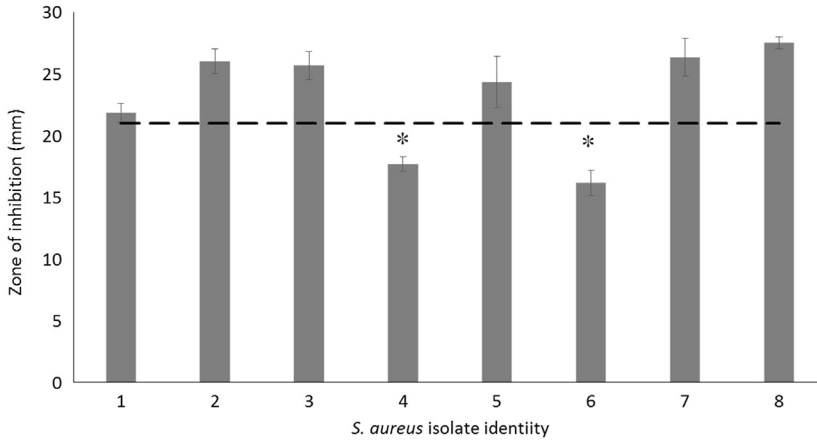

Figure 4.4 Zones of inhibition (in millimetres) generated by for ciprofloxacin against a range of *Staphylococcus aureus* isolates. The *dashed black horizontal line* represents the breakpoint beneath which isolates are resistant (marked with *asterisks*). Error bars represent standard deviations of the mean.

that anyone looking at the graph has an easy way to see which isolates are sensitive and which are resistant (Fig. 4.4).

If you are working with different species of bacteria and a range of antibiotics, it might be better to plot one bacterial species per graph with the whole range of antibiotics shown. Breakpoint lines can still be plotted onto this type of graph, but they will vary depending on which antibiotic and bacteria are being presented (Fig. 4.5).

It is important to follow one of the standardised methodologies that are available. Without a standardised protocol, you risk making incorrect judgements about whether a bacterial isolate is sensitive or resistant and limit the comparisons you would be able to make within your own experiments.

Common problems with technique include not swabbing the whole of the plate with the inoculum and therefore not getting an even lawn of bacteria. To avoid this, make sure the plate is swabbed in three orientations to create a confluent lawn.

Moreover, if the initial inoculum is too light or heavy, it will not create a suitable lawn, giving misleading zones. To avoid this, ensure the inoculum is the correct density before starting work.

Wet plates can cause the bacterial inoculum to smudge or run across the plate, making it difficult to measure the zone sizes accurately. Ensure your plates are properly dried before using them for this technique. Antibiotic discs can fall off the plate upon inversion, leading to a missing data point for

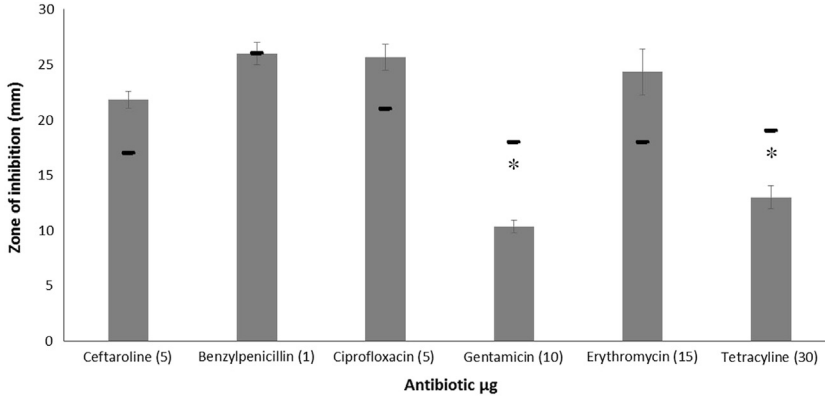

Figure 4.5 Zones of inhibition (in millimetres) generated by a range of antibiotics against one strain of *Staphylococcus aureus*. The *black horizontal lines* represent the breakpoint beneath which isolates are resistant (marked with *asterisks*). Error bars represent standard deviations of the mean.

that antibiotic. To avoid this, check that antibiotic discs are firmly in place before inverting the agar plates for incubation.

Some antibiotic discs can generate large zones of inhibition that affect the zones of inhibition generated by the disc next to them. If the zones are so large that they merge into one another, there are options for measuring the radius of the zone of inhibition using the opposite side of the zone of inhibition to the part that is affected. Ideally, however, it is best to retest the antibiotics involved on a plate on which they are more spaced out so that no merging of zones occurs.

4.1.3 Antibiotic strip tests

The Etest is another method of determining the antibiotic susceptibility of an organism used in conjunction with the agar plate lawn method. The strips themselves are thin strips impregnated with a predefined gradient of antibiotic. Using an antibiotic strip to test the sensitivity of an organism, it provides the concentration of antibiotic required to inhibit the bacteria being tested. The lowest concentration of antibiotic needed to inhibit an organism is known as the minimum inhibitory concentration (MIC) of the organism. The antibiotic strip method can be used if the MIC is needed and agar lawn plates are already being set up or if facilities to read broth dilution plates are unavailable in the laboratory. The antibiotic strips can be purchased from several manufacturers, including bioMérieux (https://www.biomerieux.co.uk) and Liofilchem (https://www.liofilchem.com/en/).

Briefly, the antibiotic disc susceptibility testing method is thus:

1. Agar lawn plates should be prepared in the same way as described in Section 4.2 for the disc diffusion method.
2. Ensure the antibiotic strips have been allowed to come up to room temperature before using them and always store unused strips according to the manufacturer's instructions. Once the bacteria have been inoculated onto the agar plate to form a lawn, the antibiotic strip should be carefully removed from the packet using sterile forceps. Ensure that the forceps are used to hold the strip only at the top edge of the strip, away from where the antibiotic is impregnated into the strip, because you do not want to damage the antibiotic gradient. Once the strip has been removed from its packaging, it can be placed in the centre of the plate if only one antibiotic is being tested, in pairs on a 9-cm agar plate, or top to tail. Up to six strips can be tested by placing them radiating out in a star shape from the centre of a large (15-cm) agar plate. The highest concentrations of antibiotic should be placed toward the edge of the plate if multiple antibiotic strips are being tested (Fig. 4.6). The strips should be placed so that the scale is facing up so it can be read.

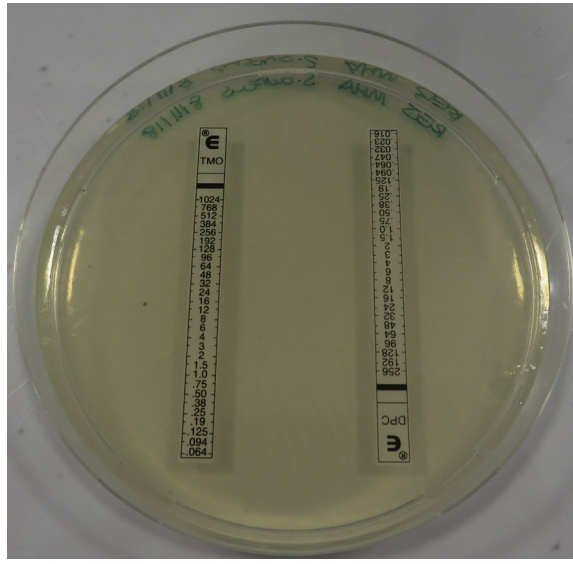

Figure 4.6 Example of how antibiotic strips can be laid out on a 9-cm agar plate to ensure effective use of space on the agar. Note that the strips are laid out top to tail. This ensures that areas where the highest concentrations of antibiotics are and where the largest zones of inhibition will occur are at opposite ends of the agar plate for each strip.

As with the discs, ensure that the whole strip is in contact with the agar. Sometimes air bubbles can become trapped under the strip. If any large bubbles are observed, try to move the bubble up and out from under the strip by gently pushing the bubble up from the low-concentration end of the strip to the high-concentration end. Small bubbles should not affect the zone of inhibition generated, so they can be left under the strip.

The plate is then incubated in an inverted position at 35°C for 18–24 h, but this can change depending on which organisms you are using and which manufacturer's products are being used. Check the manufacturer's guidelines to ensure that the most reliable results are obtained. After the incubation period, remove the plate from the incubator and check that there is a confluent lawn of growth on areas of the plate where the bacteria have grown. If the growth looks wrong (too heavy or too light), disregard the plates and repeat the experiment. If the bacteria are susceptible to any of the antibiotic strips used, a zone of clearing will be seen on the agar. To read the MIC, look for the point at which the zone of inhibition intersects the antibiotic strip. The MIC is the concentration of antibiotic written on the strip at that point (Fig. 4.7). If the

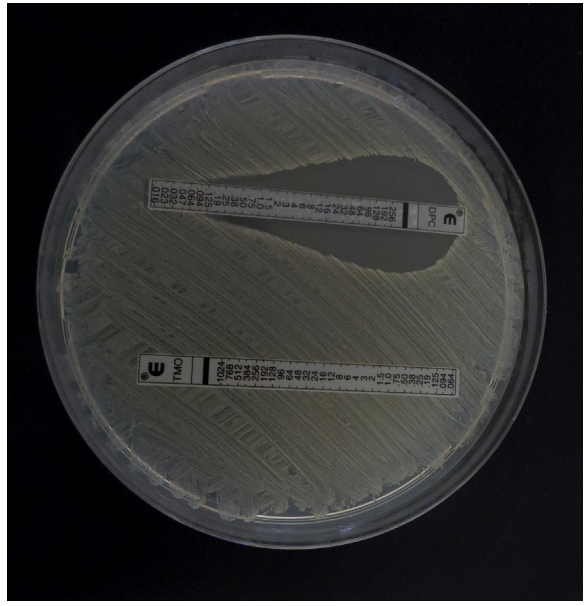

Figure 4.7 Antibiotic strip test using daptomycin (DPC) and temocillin (TMO). There is no minimum inhibitory concentration (MIC) for TMO. The point at which the zone of inhibition intersects the antibiotic strip and for DPC is at 0.125. Therefore, the MIC for this antibiotic is 0.125.

bacterial growth intersects the strip between two values, the higher value will give the more conservative MIC, which means the bacteria is less likely to be accidentally recorded as susceptible when resistant. If results fall between two concentrations, other options are to rerun the susceptibility test or confirm the MIC by another method.

4.1.4 Broth dilution

Another method that can be used to determine the sensitivity of bacteria to antibiotics is the broth dilution method. Like the Etest method, this will give you the MIC of an antibiotic required to inhibit the growth of the bacteria you are testing. In addition, this method provides you with the opportunity to test for the minimum bactericidal concentration (MBC), the lowest concentration of antibiotic needed to kill the bacteria you are testing. This is carried out in an additional step that cannot be performed from the agar plate methods described previously. For this type of test, if you want to ensure reproducible results that can be compared with other published data, or if you want to be able to check whether the bacteria are sensitive or resistant according to published breakpoints, you should follow a standardised method from an organisation such as EUCAST or CLSI. An outline example of this type of method is given here; however, you should check for details at the relevant organisation's website.

Before starting this method, ensure you have all of the equipment you need to avoid having to interrupt the experiment after you have started. You will need a bacterial culture, a Bunsen burner, 96-well microtitre plates (preferably with a lid), inoculating loops, sterile saline and sterile broth (usually Mueller–Hinton Broth), the antimicrobial agent being tested, a low-volume pipette and tips (100–200 μL) and a multichannel pipette if you have one available (Fig. 4.8).

In this method, you will need to prepare a range of antibiotic concentrations before you can start setting up the microtitre plate. To ensure you use a suitable range of antibiotic concentrations so that the test is likely to find an MIC, refer to clinical breakpoint tables to see where the resistance breakpoint is. Using a doubling dilution technique, create a range of antibiotic concentrations. Ideally, as suggested previously, these should go above and below this breakpoint to allow you to determine whether the bacteria are sensitive to the antibiotic you are testing. The number of bacterial strains being tested and the number of replicates that are undertaken will influence how you prepare the doubling dilution antibiotic range.

There are two ways in which you can create the concentration series needed. A stock of each concentration of antibiotic can be created from

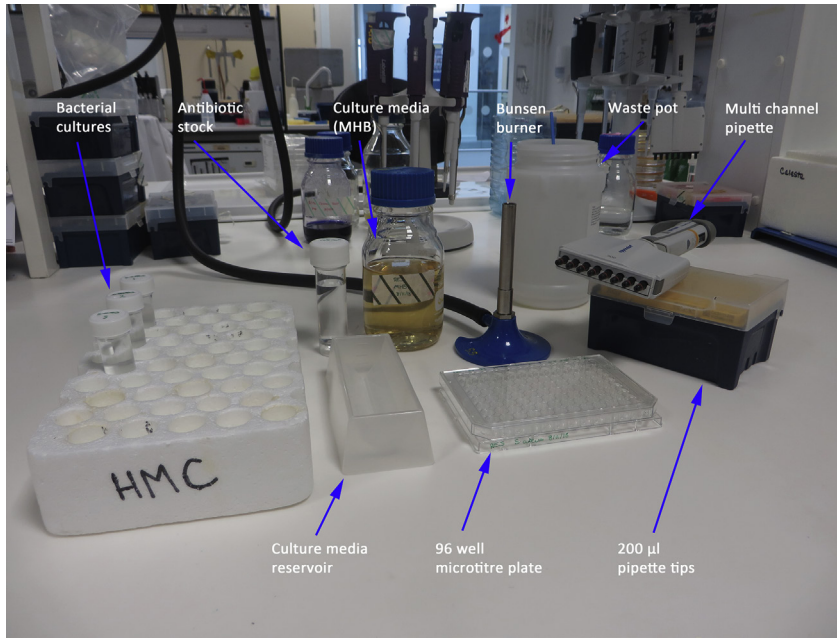

Figure 4.8 Typical setup used for minimum inhibitory concentration testing with all of the equipment needed arranged close by on the bench. *MHB*, Mueller–Hinton Broth.

an initial stock of the highest concentration needed in universal tubes or Falcon tubes. Then, this can be pipetted into the microtitre plates. Briefly, you would need to create a stock solution of antibiotic (e.g., 512 mg/L antibiotic). Pipette 3 mL of this into 3 mL sterile culture broth. Mix thoroughly (this gives you 6 mL 256-mg/L antibiotic). Pipette 3 mL of the new solution into 3 mL sterile culture broth and mix thoroughly. Keep doing this until you have created the range of antibiotic concentrations that you want to test. The amount you pipette between tubes will depend on how many wells you need to fill, which will depend on how many bacterial samples you are testing. Work out the volume of antibiotic you will need before you start this process.

A stock of the highest concentration of antibiotic can also be made in a universal or Falcon tube, but then the doubling dilutions can be carried out in the microtitre plate by filling each test well with 100 µL sterile culture media before adding 100 µL of the top concentration to the first set of wells, mix thoroughly, and then pipetting 100 µL from that well into the next and continuing in that way until you get to the final well. A total of 100 µL from the final test well will need to be pipetted into a waste bin or the volumes

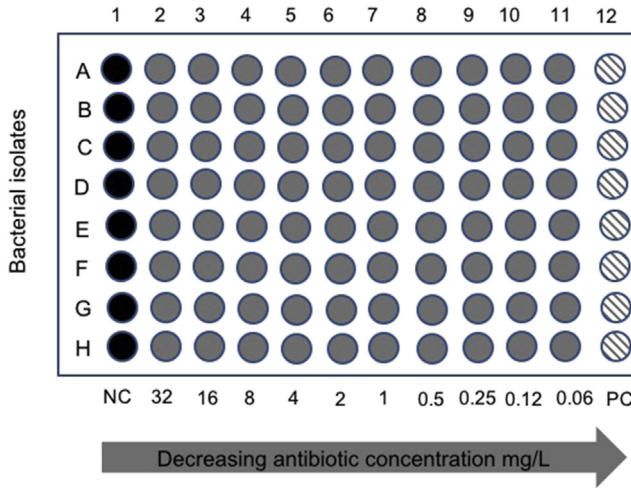

Figure 4.9 Microtitre plate displaying a horizontal layout allowing a wide range of concentrations (32–0.06 mg/L) to be tested. In this setup, if testing in triplicates, only two full sets of triplicate isolates will fit onto the plate. The third replicate of the third isolate would need to go into another plate. Alternatively, different isolates can be tested within the plate and multiple plates can be used to run the triplicates. *Black wells* represent negative controls (NC), *striped wells* represent positive controls (PC), and *grey wells* represent test wells. Each column contains a different concentration of antibiotic.

in the plate will be wrong. Make sure that none of the antibiotic is pipetted into either the positive or negative control wells; only the test wells should contain the test antibiotic.

For example, when testing the sensitivity of *Staphylococcus aureus* to linezolid in which an MIC of ≥4 mg/L is resistant and an MIC of <4 mg/L is sensitive, there is a choice of two antibiotic concentration ranges that could be prepared in the plate, depending on how the plate is orientated and how many isolates need to be tested. In the first format, in which the antibiotic gradient runs horizontally across the plate, a negative and positive control as well as a range of 32–0.06 mg/L would be possible (Fig. 4.9).

If the gradient runs vertically, a negative and positive control as well as a range of 16–0.5 mg/L would be possible (Fig. 4.10). Which format you choose to use will depend on how many isolates you are testing.

Whichever way the dilutions are carried out, the final volume of antibiotic in each well before the bacteria are added should be 100 μL. When the antibiotic concentration range has been set up in the plate, the bacteria can be added. Moreover, once 100 μL of bacteria is added to each well, the concentration of antibiotic in each well will halve.

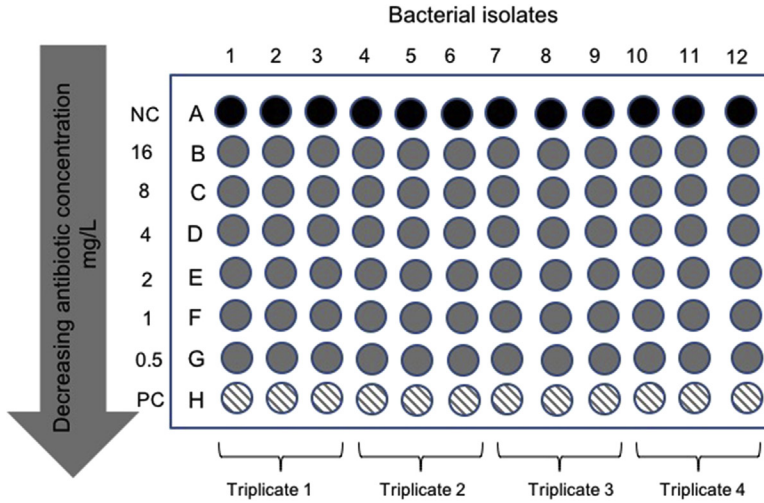

Figure 4.10 Microtitre plate displaying a vertical concentration layout that has a narrower range of antibiotic concentrations (16–0.5 mg/L). This setup allows complete sets of triplicate isolates to be tested in the same plate. *Black wells* represent negative controls (NC), *striped wells* represent positive controls (PC), and *grey wells* represent test wells. Each row contains a different concentration of antibiotic.

Once the control wells have been prepared, the test wells will need to have the bacterial inoculum added. Start by aseptically picking a few bacterial colonies from an overnight agar plate culture and suspend these in sterile saline to give a density equivalent to a 0.5 McFarland standard. Depending on which bacteria you are testing, there may be a further dilution step at this point, so check standardised methods for guidance. Once appropriately diluted, 100 µL of the culture will need to be pipetted into each well, including the positive control wells but excluding the negative ones.

When the plate is set up, there should be 100 µL of antibiotic (varying concentrations) plus 100 µL bacterial cells in each test well. The positive control wells will contain 100 µL of broth plus 100 µL bacterial cells and no antibiotics. The negative control wells will contain 200 µL broth with no antibiotics and no bacteria.

When preparing these plates, ensure that all the work is done aseptically and that the correct volume and concentration of antibiotic go into each well. All wells should contain 200 µL when the plate is completed. If using a multiwell pipette, check when drawing up liquid that the same amount is in each pipette tip. If you bend the tips while drawing up liquid or push the tip too hard against the slide of the container, the pipette may not draw up the right amount of liquid in each tip. It is good practice to note in

your laboratory notebook what is in each well of the plate and anything that happens during the preparation which could affect the results (e.g., if you think you might have pipetted twice into one well, missed a well or accidently contaminated something). This will allow you to pick up on any errors or issues more quickly when observing the results.

Once the plates are completed, they will need to be read in a spectrophotometer to provide a 0-h reading. Place the microtitre plate into the spectrophotometer and ensure it is in the right orientation. Set the spectrophotometer to the wavelength needed: for example, 600 nm. With many software packages, there is an option to label the wells on-screen to match those in the plate. Once the wavelength is set and the template has been set to measure the optical density (OD) in all of the wells that are required, read the plate. Make sure the data generated are saved under a name that can be found again. The spectrophotometer might be used by many people or you might be reading multiple plates. If the reading is saved as something such as the date, it might be difficult to find again when it is looked for it among all of the readings performed in a day. Ensure that there is enough information to be able to identify each reading back to the original plate. Most spectrophotometers will allow data generated to be saved or exported into a programme such as Excel. This will make it easier to analyse the data at a computer not connected to the spectrophotometer.

Once the 0-h reading has been completed and saved, incubate the plates at approximately 35°C for 16–20 h. Once the incubation period is over, look at the plate by eye to pick up obvious problems or contamination issues and then read it again at the same wavelength at which the 0-h reading was carried out and save the data. The data are then ready to be analysed.

It is good practice to run these tests using three biological replicates and three technical replicates for each of biological replicate (this will generate nine data points for each well tested). Ensure that a control organism with known sensitivities is used to verify that the results are reliable and not caused by an issues with the method or media.

Once the data have been exported from the spectrophotometer, they will need to be analysed and presented in a way that is easy to read, such as a line graph. It is usually a good idea to subtract the 0-h and negative control data from the 20-h test data. If each test well has been done in triplicate or multiples, the mean value will need to be calculated. For example, when working with tetracycline, the values for each concentration would be added together and divided by the number of replicates. This will generate one mean value for each concentration of tetracycline. Once there is a mean value for each concentration, those figures can be plotted onto a graph. The concentration at which there is no turbidity (growth) is the MIC. The standard deviation

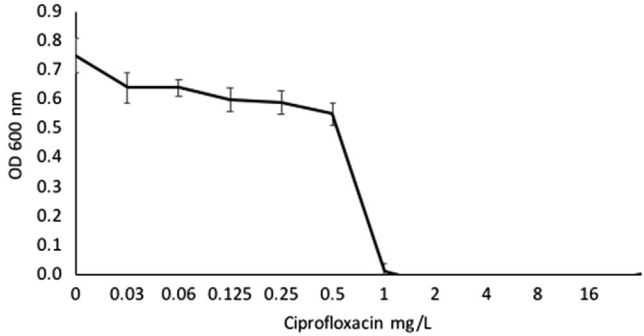

Figure 4.11 Example of how to present minimum inhibitory concentration (MIC) data from a broth dilution experiment. The MIC (1 mg/L) of ciprofloxacin is shown when tested against an isolate of *Staphylococcus aureus*. Error bars represent the standard deviation of the mean. *OD*, optical density.

should also be calculated for each concentration to ensure that replicates are similar and can be added to the graph as error bars (Fig. 4.11).

The standard deviation can be calculated in a programme such as Excel by selecting an empty cell, pressing Equals, selecting standard deviation from the Functions option bar (often at the top left in Excel and displayed as STDEV or similar). Then, select all of the replicates for the concentration of interest and press Return. This process can be repeated for each concentration. Once the standard deviations have been generated and plotted onto the graph, they should provide an easy way to seeing whether your replicates were similar or whether there is a lot of variation between samples. A large range of variation with samples could indicate that the experiment needs to be repeated. For some antibiotics, there might not be complete reduction of OD to 0. If there is no MIC for the antibiotic tested, it is possible to present the lethal dose (LD) as 50 or 90, which is the amount of antibiotic needed to kill 50% or 90% of cells and is often represented as LD50 or LD90. It can be calculated for any proportion of cell death, but 50 and 90 are commonly used.

The MBC can now be determined from the original MIC plate. After reading the plate in the spectrophotometer, pipette 10 μL from every well that has no growth in it onto an agar plate. Wait for these spots of liquid to dry in the plate, and then incubate the agar plate at 35–37°C overnight. After incubation, inspect the plate for growth. The lowest concentration where there is no bacterial growth is the MBC. The antibiotic is normally considered to be bactericidal if the MBC is not more than four times the MIC. If the MBC is more than four times the MIC, the antibiotic is usually considered to be bacteriostatic.

The MIC can also be determined using an agar dilution method. This is similar to the broth dilution method in that an antibiotic would be added to agar

in a range of concentrations before the agar has set. This can be carried out in a Petri dish. The size of the dish does not matter, but the volume required to fill it is important. The volume of antibiotic that needs to be added to the agar has to be calculated based on the volume of agar used to create the agar plate. Ensure that the agar is not too hot when adding the antibiotic. If possible, wait until the agar has cooled to at least 50°C before adding the antibiotic. To ensure that there is an even distribution of antibiotic throughout the agar, swirl the Petri dish once the agar has been added. When swirling the agar in the Petri dish, make sure the agar does not spill out over the side of the plate or up into the lid. Leave the agar to cool and set. Once the agar is set, a known quantity of bacteria can be spotted on the surface. Adding a standard inoculum to each agar plate allows reproducibility between tests. After the bacteria have been added and have dried in the plate, incubate the inverted plates at 16–20 h at about 35°C. After incubation, inspect the plates for growth. The minimum concentration will be the plate containing the lowest concentration of antibiotic that displays no growth. This method can be useful if you have a series of bacterial isolates to test, because they can be spotted onto different areas of the same agar plate. Unlike the broth dilution, it is impossible to determine an MBC from these plates.

4.1.5 Synergy

The use of combined antibiotics to enhance the activity of one or all of antibiotics that have been combined is an approach that has become popular since increasing antibiotic resistance has been observed. Combining antibiotics to treat infection can reduce the amount of each antibiotic needed, reducing associated host toxicity and potentially leading to quicker eradication of infection. Whether a particular antibiotic will improve the activity of another one will depend on the antibiotics being combined and the bacteria being tested. Being able to identify correctly which antibiotic combinations are likely to improve efficacy is important. Some ways in which you can check antibiotic interactions are described next.

The methods described in Sections 4.2–4.4 can be adapted to determine whether there are interactions among the antibiotics being tested. Interactions among antibiotics or antibiotics and other compounds are important because they can highlight where compounds inhibit one another or where they increase the efficacy of a compound beyond what you would expect when they are used alone. The antibiotics could have different interactions, including;

Antagonism: when the combined effect of two or more antimicrobial agents reduces the efficacy of those antimicrobial agents compared with their use alone;

Synergy: when the combined activity of two or more antimicrobial agents is greater than the expected activity of the antimicrobial agents when added together; and

No interaction: when the combined antibiotics cause no increase or decrease in the efficacy of the other antibiotic.

Disc diffusion is the first method which can be used to determine whether there are interactions between antibiotics. The disc tests can identify both antagonistic and synergistic interactions between antibiotics. The plates should be prepared as described in Section 4.2 and the discs of interest should be placed next to each other on the plate, approximately 20 mm apart. Incubate the plate as outlined in Section 4.2. When examining the plates the following day, if antagonism is present, the zone of inhibition between the discs will display a blunted shape of one or both of the zones involved (Fig. 4.12). This indicates that the antibiotic that shows a blunted zone of inhibition is no longer as effective as it was when used on its own.

If synergy occurs between antibiotic discs, there will be an increase in the zone of inhibition on the side of the zone nearest the antibiotic disc with which it is interacting. This interaction indicates improved activity of the antibiotic when it is used in conjunction with the second antibiotic (Fig. 4.13).

To looks for interactions using a broth method, you will need the same equipment as for the MIC broth dilution, so ensure that you have a bacterial culture, a Bunsen burner, a 96-well microtitre plates (preferably with a lid),

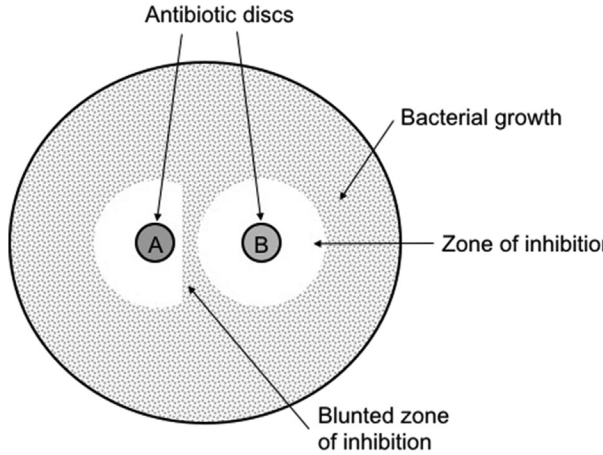

Figure 4.12 An antagonistic interaction is seen between two antibiotics (A and B) in this diagram of antibiotic disc testing. Blunting in the zone of inhibition for Antibiotic A is seen because interaction with Antibiotic B has reduced the efficacy of Antibiotic A compared with Antibiotic A used alone.

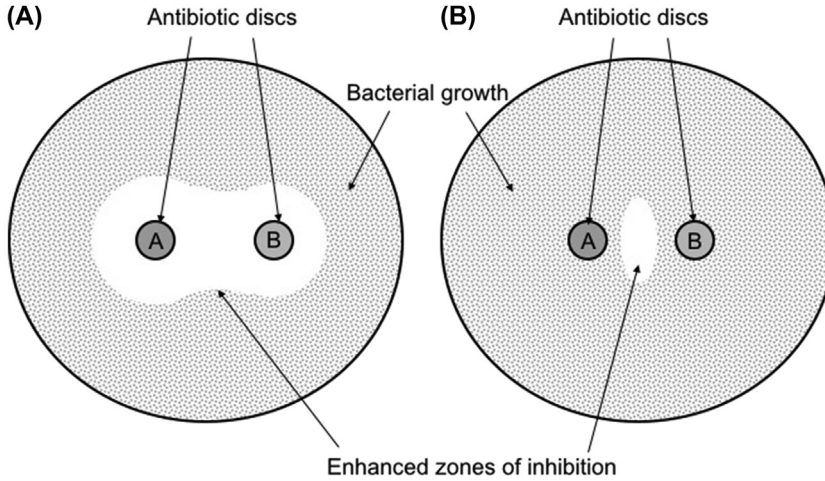

Figure 4.13 (A and B) Synergistic interactions. Increased zones of inhibition are seen in (A), where the two antibiotics interact, indicating that this combination of antibiotics is synergistic. (B) A zone of inhibition has appeared in the area between the two discs, indicating a synergistic interaction. The antibiotic discs do not inhibit the bacteria when used alone, because no zone of inhibition is seen around the rest of the disc.

inoculating loops, sterile saline and sterile broth (usually Mueller–Hinton Broth), the antimicrobial agents being tested, a low-volume pipette and tips (100–200 µL) and a multichannel pipette if you have one available.

Before working out whether there are interactions between antibiotics, an MIC will need to be established for each antibiotic alone. This can be done in the plate in which the synergy experiment is being carried out, because there will be an antibiotic-alone series of test wells for each antibiotic. However, it is probably better to establish the antibiotic MIC using a standard broth dilution experiment as described earlier, so that when preparing the synergy plate, you have already established the range needed to give several concentrations of antibiotic both above and below the original MIC.

Once the series of antibiotic concentrations has been prepared, it will need to be pipetted into the 96-well plates. Pipette 50 µL of each antibiotic into the wells. You can set up the plate as shown in the template in Fig. 4.14. Once the antibiotics have been added, 100 µL of bacteria (prepared as described in Section 4.4) can be pipetted into all of the test wells and positive control wells. Do not add bacteria to the negative controls.

The concentration of antibiotics going into the well will halve in wells where you are adding both antibiotics, and it will halve again when the bacteria are added. For example, when adding 50 µL of Antibiotic

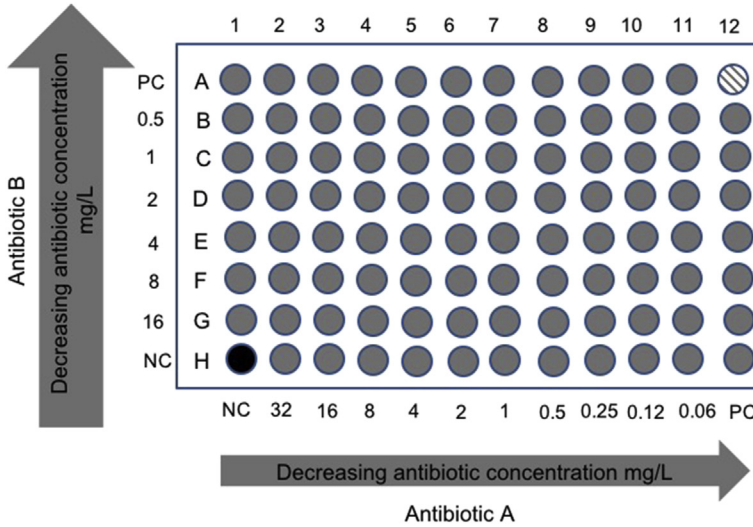

Figure 4.14 Sample plate layout that can be used for checkerboard testing of antibiotics (it can be adapted to test other antimicrobial agents). *Black well* represents the negative control (NC) well, *striped well* represents the positive control (PC) well and *grey wells* represent the test wells. Each grey well will contain different combinations of concentrations of the two antibiotics being tested.

A at 256 mg/L to 50 µL Antibiotic B at 256 mg/L, there will be 100 µL of Antibiotic A and B at 128 mg/L. When 100 µL bacteria is added, the well will contain 200 µL Antibiotic A and B at 64 mg/L. When marking the plate or writing in a laboratory notebook, make it clear whether the plate layout is describing the original concentration created in the dilution series or the final concentration in the well once the antibiotic and bacteria have been added.

The plate can now be read in the spectrophotometer at 0 h. Incubate at 35°C for 16–20 h and then read it in the spectrophotometer again. Analysis of these data is similar to that carried out on the microbroth dilution plate. Subtract the 0-hand negative control values from the 20-h values and work out what the new MIC is for each antibiotic. The new MIC for each antibiotic can be calculated for every well in which the second antibiotic is at a subinhibitory concentration. The fractional inhibitory concentration index (FICI), which indicates which type of interaction is occurring, can be calculated using the original and new MIC values for both antibiotics by using the calculation:

$$\mathrm{FICI} = \mathrm{FIC}\,(\mathrm{A}) + \mathrm{FIC}\,(\mathrm{B})$$

where FIC(A) = MIC Antibiotic (A) in combination/MIC Antibiotic (A) alone; and FIC(B) = MIC Antibiotic (B) in combination/MIC Antibiotic (B) alone.

For example, if:

$$\mathrm{FIC}\,(\mathrm{A}) = 0.5/16 = 0.06$$

$$\mathrm{FIC}\,(\mathrm{B}) = 0.5/2 = 0.25$$

$$\mathrm{FICI} = 0.06 + 0.25 = 0.3$$

In this example, the FICI value indicates a synergistic interaction between Antibiotics A and B. There are different cutoffs depending on which report you read, but values can be interpreted using Table 4.3, which gives FICI interpretations as suggested by Odds (2003). There are other interpretations of FICI values provided in the literature, such as those suggested by EUCAST (2000); however, the values given here allow for experimental variation.

4.1.6 Novel compound testing

To test novel or non-antibiotic antimicrobial agents that are not included in standardised methods but that can be dissolved in broth or phosphate-buffered solution, a couple of options are available to determine MICs, adapted from standardised methods described in Sections 4.2–4.4.

Blank paper discs are available for purchase from suppliers such as Sigma-Aldrich and Thermo Fisher Scientific. These can be used in a manner similar to the antibiotic disc testing method. Pipette a known concentration and amount of test material onto a blank paper disc and allow the liquid to soak into the disc. The disc can then be used in the same manner as the antibiotic discs described in Section 4.2. Keep the other parameters the same, including the bacterial inoculum, the temperature and time of incubation and the measurement of the zones of inhibition. Using the blank discs in this way will allow you to create reproducible zones of

Table 4.3 Interpretation of fractional inhibitory concentration index values, taking conservative approach to allow for testing variation suggested by Odds (2003).

Interaction	Value
Synergy	≤0.5
Indifference	0.5 to ≤4
Antagonism	>4

inhibition from the discs, and therefore comparable results. This should allow you to assess whether the novel compound can inhibit bacteria and also to compare effectiveness between different strains or species of bacteria. There are unlikely to be breakpoints available for a novel compound, but if the concentration and volume of compound are kept consistent, this adapted method should provide useful information about the activity of the compound.

If the compound is soluble, it is also possible to adapt the microbroth dilution test described in Section 4.3. Replace the 100 µL of antibiotic with 100 µL of the antimicrobial compound (at a range of concentrations) and keep all other parameters the same, including the bacterial inoculum, incubation time and temperature and data analysis. The test for an MBC can also be used in the same way as described in Section 4.3. It is unlikely that there will be breakpoints available for novel antimicrobial agents, but adapting standard tests can provide a reproducible way to establish both an MIC and MBC.

When working with a material such as an antimicrobial wound dressing or antimicrobial surface, it may not be possible to test using these techniques. However, several variations on standard antibiotic testing can be used.

It is possible to assess the antimicrobial effect of exposure to a material or surface on test bacteria as calculated compared with a control. For example, if testing a new dressing impregnated with an antimicrobial agent, a protocol such as the one suggested here could be followed.

If testing one type of bacteria against one type of dressing, start with multiple pieces of dressing all cut to the same size (e.g., 2 cm^2). To test on agar, prepare a bacterial lawn plate as if preparing for a disc diffusion test (Section 4.3). Place the material or dressing directly onto the plate, incubate the plate at about 35°C for 16–20 h and measure the zone of inhibition with callipers as in Section 4.3. Like the blank disc method, this method will allow you to assess whether the novel compound can inhibit bacteria and also allow comparison of its effectiveness between different strains or species of bacteria. If the material or dressing is impregnated with an antimicrobial agent or has an antimicrobial coating, it is a good idea to run a test sample that does not have the coating, to see whether the material alone has a antimicrobial effect.

To test the novel material or dressing directly, create a bacterial standard with a known number of cells (such as the McFarland standard). Inoculate each piece of dressing with a set volume of the bacteria (e.g., 20 µL). Each piece of dressing can be placed in a sterile Petri dish or universal container.

Incubate the material with the bacteria for a set period. This time could be set to match the standardised testing or adapted to give a customised set of results. If possible, and if testing a completely novel material, it may be useful to inoculate enough pieces of test material for a time course. This would allow testing to occur at multiple time points (e.g., 1, 2, 4, 8, 12, 18 and 24 h).

At the given time point, place the test material into 5 mL of one quarter-strength Ringer's solution and vortex for 2 min to release any bacteria. Create a dilution series from this solution using the Miles and Misra technique outlined in Chapter 2, Section 2.5 and pipette 50 µL onto an agar plate. Incubate the inverted agar plate overnight at 35–37°C, count the colonies the following day and calculate the colony forming units per millilitre as outlined in Chapter 2, Section 2.5. This should allow you to see whether the material has an antibacterial effect and how long it takes to cause that effect.

As with all experiments, it is essential to run control samples alongside the test samples to ensure the reliability of results. If possible, when testing a novel dressing or material with an antimicrobial impregnated or coated onto the material, run a uncoated version of the material with the bacteria as a positive control. This should allow you to check whether the material itself has an impact on the bacteria being tested. Running a negative control with no bacteria added allows any contamination of the experiment to be identified.

This type of experiment could also be run by adding the novel material or dressing to a broth culture of bacteria of a known starting density and vortexing the broth and dressing together at the various time points, including a 0-h cell count. An aliquot from this broth can then be processed at each time point using the Miles and Misra technique. If testing the material in broth, a positive control using untreated or coated material can be run. A bacteria-only control (no material or dressing) could also be run to determine whether the dressing alone has an impact on the bacterial population. These types of methods can be adapted to suit the different types of novel antimicrobial agents (compounds, materials and surfaces) being tested. If you have to adapt away from standard methodologies, it is best to make sure that positive and negative controls are run whenever possible and to minimise variables. This may involve optimising the method, but it will allow the maximum amount of useful information to be gathered from testing.

Because of increasing antibiotic resistance seen in a wide range of bacteria, the ability to test bacteria for susceptibility to both conventional and novel antimicrobial agents is a fundamental skill needed by many microbiologists. This chapter has provided an overview of different methods by which this testing can be carried out and has described ways in which the data generated can be analysed and interpreted.

4.2 Notes page

Record observations and notes here.