Procedures

Part A: Comparing the effects of different antimicrobial substances on growth of bacteria in a lawn plate culture (semi-quantitative)

In this practical, you will wet filter paper discs with a range of chemicals. You may also use discs containing antibiotics. You will use aseptic technique to prepare bacterial lawn cultures, and will place the filter paper and/or antibiotic discs on the bacterial lawn. After incubating the plates, zones of inhibition around the discs indicate the effectiveness of each chemical dilution.

Student sheet

You are provided with the following:

- Chinagraph pencil or other marker, or sticky labels
- impervious plastic-coated sheet (A4 size) on which to work. This sheet has been placed in verified disinfectant (*Virkon* is usually used). Immediately before you start the aseptic procedure, remove the sheet from disinfectant, and blot the surface with a paper towel.
- small glass (McCartney) bottle containing a broth culture of bacteria (E.coli (K12) is often used)
- Bunsen burner and heat proof mat
- a discard pot (beaker or screw top bottle containing strong disinfectant (eg 1% *Virkon*))
- Petri dish containing sterile nutrient agar
- sterile plastic, glass or metal spreader (do not unwrap until point of use)
- sterile plastic or stoppered glass 1cm^3 Pasteur pipette (do not unwrap until point of use)
- rubber/plastic teat to fit glass Pasteur pipette where used
- forceps
- adhesive tape
- Antibiotic multi-test ring (*Mastring* / *Multodisc*), or separate antibiotic- containing discs.

You should read these instructions carefully before you start work.

Preparing lawn plate cultures

1. Place the Bunsen burner close to the edge of a heat proof mat and light it (yellow flame).
2. Place the disinfected plastic sheet on the work surface in front of the Bunsen burner.
3. Place the bottle of broth culture, the spreader and pipette next to the Bunsen burner.
4. Write your name, the date and the name of the bacteria on the **underside** of the Petri dish.

   Also divide the base of the plate into sections and write the name of an antimicrobial for test in each section.
5. Change the airhole on the Bunsen burner, so that it has a blue flame (hot)
6. Wash your hands thoroughly using liquid hand-wash/soap. Dry your hands using paper towels.
7. Unwrap a sterile 1cm^3 pipette and hold it in the air close to the Bunsen flame. If you are using a glass pipette, attach the teat to the pipette.
8. Remove the lid of the McCartney bottle and, without putting the lid down, move the neck of the bottle quickly through the Bunsen flame. This helps to prevent microbes from the air entering.

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9. Squeeze the teat of the pipette, place the tip into the broth culture, and release the squeeze on the teat to remove a small volume of the culture (approximately $0.3cm^3$).
10. Flame the neck of the bottle and replace the lid.
11. Lift the lid of the agar plate at an angle facing the Bunsen burner with your non-dominant hand. With your dominant hand, squeeze the teat of the pipette to release 2-3 drops of culture onto the surface of the agar.
12. Replace the lid of the Petri dish and immediately place the pipette into the discard pot.
13. Unwrap a sterile spreader.
14. Take the sterile plastic spreader in your dominant hand. Facing the Bunsen, lift the lid of the agar plate with your non-dominant hand and use the spreader to make sure that the bacteria are evenly spread around the surface of the agar.
15. Replace the lid of the Petri dish with culture (now called a lawn plate), immediately place the spreader into the discard pot of disinfectant.
16. Leave the lawn plate for 5-10 minutes, so that the broth is absorbed into the surface of the nutrient agar. Use the lawn plate immediately after this "rest" period.

Placing filter paper discs containing antimicrobial substances

1. Use forceps to dip filter paper discs into a test solution, so that the disc is wet. Shake the discs to remove drops of moisture before placing them onto the lawn plate culture.

   Students may wish to measure small volumes of solutions onto the discs, using $1cm^3$ pipettes or syringes.
2. Use forceps to place the disc with antimicrobial substance onto the bacterial lawn in the appropriate section of the plate.
3. Carefully flatten the Antibiotic multi-test ring or separate discs onto the surface of the plate, using forceps. If the forceps touch the lawn of bacteria on the surface of the agar, place them in the bottle of 70% ethanol provided by the teacher. Forceps that have not touched the bacterial lawn do not require disinfection. Do not place the forceps in *Virkon* disinfectant, as this will cause corrosion.
4. Fasten the lid of the plate in place using two pieces of adhesive tape.
5. Place the plastic-coated work-surface in the tray of *Virkon* disinfectant.
6. Wash your hands thoroughly using liquid hand-wash/soap.

Your plate should be placed upside down in an incubator at 25°C-30°C for 24-72 hours, or until zones of inhibition can be clearly seen.

After the plates have been incubated, the diameter of the zone of inhibition indicates the potency of each chemical.

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Extension Activity: Comparing the effects of different concentrations of an antimicrobial substance on growth of bacteria in a lawn plate culture (semi-quantitative)

In this experiment, you will investigate the effectiveness of different concentrations of an anti-microbial chemical.

Use laboratory glassware or syringes to prepare accurate dilutions of antimicrobial chemicals. Moisten filter paper discs with small volumes of each dilution and place them on the bacterial lawn.

After the plates have been incubated, the diameter of the zone of inhibition shows the effectiveness of the chemical dilution.

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After incubation:

Caution - plates must not be opened after they have been incubated

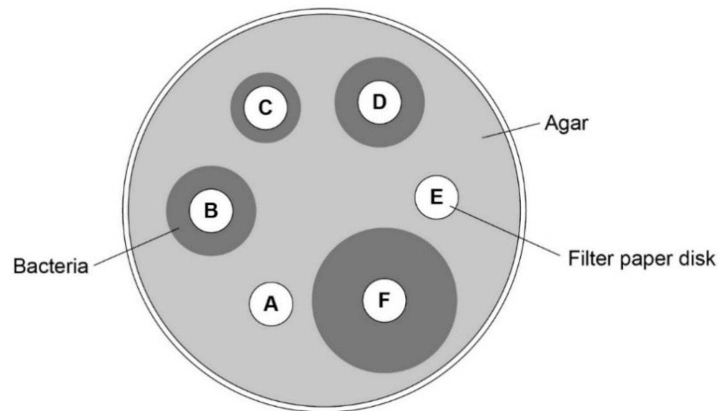
Plates made with an Antibiotic multi-test ring

A typical Antibiotic multi-test ring plate, showing clear areas where bacteria have been inhibited.

Each disc contains a different anti-bacterial agent (A-F).

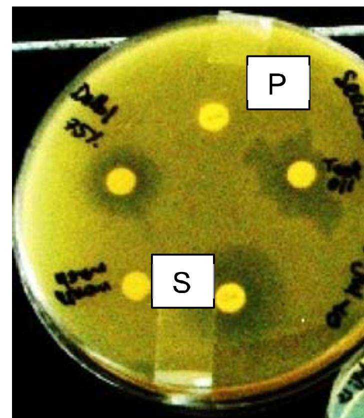
These clear areas are called **zones of inhibition.**

The situation is similar for single antibiotic discs.



Plates made with discs containing a range of chemicals

This plate shows typical zones of inhibition around discs that contained various chemicals including the antibiotics Streptomycin(S) and Penicillin (P).



Plates made with discs containing different concentrations of a chemical

This plate shows an E.coli (K12) lawn around filter paper discs that contained *Dettol* concentrations ranging between 25-100%.

The central disc contained sterile water (0% *Dettol*).

Obtaining data

Use a ruler to measure the diameter of the zones of inhibition.

Area of zone = πr^2 (Use 3.14 as π)

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Part B: Measuring the effects of antimicrobial substances on the growth of bacterial populations in a broth culture (fully quantitative).

In this practical, you will use aseptic technique to make serial dilutions of a broth culture of bacteria that has been incubated with an antimicrobial substance.

The broth culture will have been prepared by a teacher or technician, using quantities and concentrations based on the results of Part A experiments or your research.

You will use aseptic technique to prepare bacterial lawn cultures for your serial dilutions of the antimicrobial treated broth culture.

After incubating the plates, some of the plates will show discrete colonies. Each colony has been formed from a single bacterium that divided by binary fission. The number of colonies is the same as the number of bacteria that were inoculated onto the surface of the agar.

You will count the colonies on the plate and then calculate the density of the bacterial population in the culture that was treated with the antimicrobial substance.

Student sheet

You are provided with the following:

- Chinagraph pencil or other marker, or sticky labels
- impervious plastic-coated sheet (A4 size) on which to work. This sheet has been placed in verified disinfectant (*Virkon* is usually used). Immediately before you start the aseptic procedure, remove the sheet from disinfectant, and blot the surface with a paper towel.

For making serial dilutions of the broth culture

- Small glass (McCartney) bottle containing a broth culture of bacteria (eg E.coli (K12)), that was treated with an antimicrobial substance (eg 50% *Dettol*) and then incubated for 24 -72 hours.
- 5x sterile screw top bottles, or boiling tubes stoppered with non-absorbent cotton wool, each containing 9cm^3 of sterile nutrient broth.
- 5x sterile plastic or stoppered glass 1cm^3 Pasteur pipette (do not unwrap until point of use).

Note: If glass Pasteur pipettes are used, a syringe adapter is necessary to accurately measure small volumes. This is a 1cm^3 syringe that has a small piece of tubing attached to the nozzle.

The adapter is placed onto the stoppered end of the glass pipette, and the syringe can then draw up small volumes of broth into the pipette. The volumes are accurate to the smallest gradation on the syringe (eg 0.01cm^3). The syringe adapter does not need to be sterilised before or after use, as the cotton wool stopper in the glass pipette protects the culture from contamination.

Making the sterile dilutions

Use a sterile pipette to transfer 1cm^3 from the broth culture to the first dilution bottle to give a 1/10 dilution. Repeat the process for this dilution to give a 1/100 dilution for the second bottle, continue until all the bottles have been used. (1/100000 dilution). Please make sure you gently shake each dilution, to mix the culture with the broth, before you make the next dilution.

Preparing quantitative lawn plate cultures

- Use the materials and method for making lawn plates given in part A.
- Make lawn plate cultures using all your serial dilution broths.
- Secure the plates using two small pieces of sticky tape.

Your plate should be placed upside down in an incubator at 25°C -30°C for 24-72 hours, or until colonies can be clearly seen.

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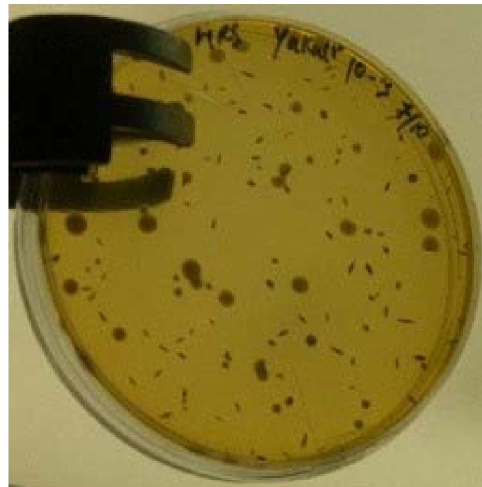
After incubation:

Caution - plates must not be opened after they have been incubated

The plates below show typical results from the 1/100 and 1/1000 dilutions of a broth (*Yakult*) culture.



Obtaining data

Choose a plate that shows a suitable number (eg 20-100) of non-overlapping colonies and count the number of colonies on the plate. Each colony was formed from one initial bacterium.

Calculate the number of bacteria in each cm^3 of the broth dilution used to inoculate the plate.

Calculate the number of bacteria in each cm^3 of the original (undiluted) broth culture that was incubated with an anti-microbial chemical. This is the population density.

The population density of untreated broth culture should also be estimated, to see the effects of the anti-microbial chemical.

The activity could be extended if each group in a class carried out serial dilution of broth cultures that had been treated with antimicrobial chemicals in different ways.

Potential investigations

A wide range of different parameters could be investigated, for example:

- species of bacterium
- different (possibly new) antimicrobial substances
- concentration and volume of broth/antimicrobial
- timing of introduction of antimicrobial in relation to the “age” of the initial broth culture
- addition of more than one antimicrobial substance to the broth culture.

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