Unit 2: Working with Bacteria

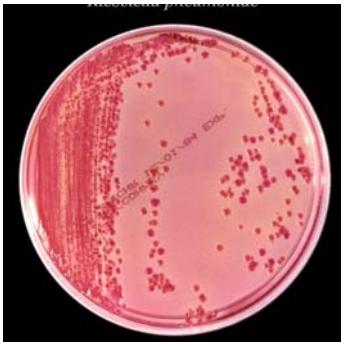
Introduction

When we pick our culture from the incubator, we will have a grown culture. Growth appears as colonies on the plate surface. Once we have obtained any growth, work flow in the Microbiology laboratory enters a second stage. And now we are expected to find out what kind of bacteria they are and which antibiotics are useful to destroy them.

Lesson 2.1 – Identification of Bacteria

Identification of bacteria consists of assigning a genus and a species to the bacteria grown on a culture. We say that bacteria grown on a culture plate are a **strain** of the species to which the strain belongs.

genus (sg)	genera (pl)
species (sg)	species (pl)



It is based on different diagnostic tests (cultural, morphological and biochemical) that have been selected on the basis that they provide discriminating information.

In lesson 2.1 we will learn about strategies and about cultural and morphological tests and in lesson 2.2 we will learn about biochemical tests.

Activity 1 – Strategies for identification of bacteria

Read this text in order to complete the activities below:

*We follow a systematic step-by-step approach to identification. Study of **cultural** and **morphological characteristics** is the first one and allows us to place the bacteria in one of the main groups of medical importance. This step is called **preliminary identification**.*

***Biochemical tests** are the second step. We have to perform several tests, sometimes more than ten and different for each bacterial group. It is to differentiate cells through their*

*metabolism or **biotype** as the rest of their features may be similar. This step is called **speciation**, because we differentiate **species**.*

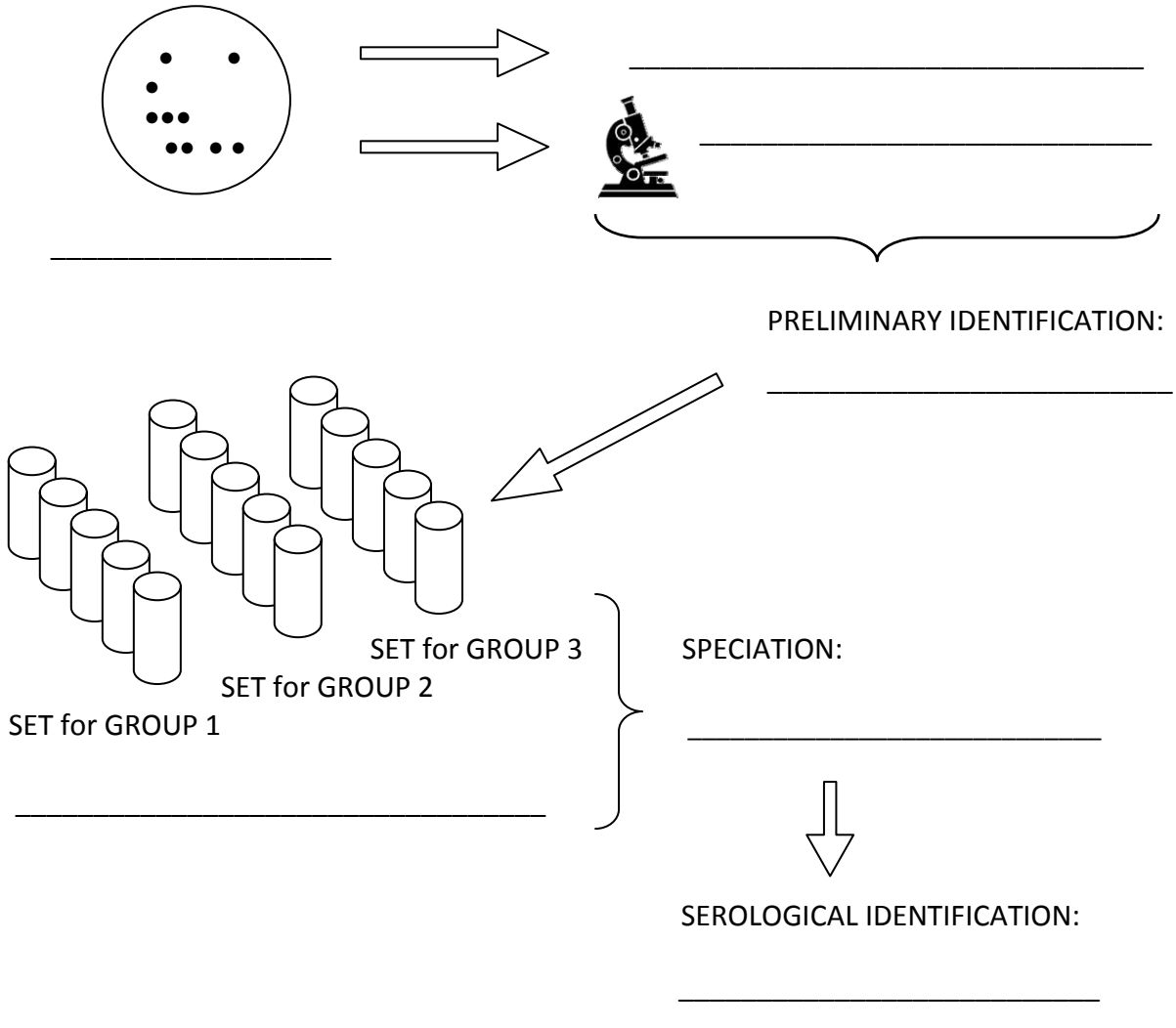
*There is a third step for especially pathogenic bacteria: **serological identification** according to their immunological features. We differentiate **serotypes** of a species. It is most useful for epidemiologic purposes with especially pathogenic bacteria.*

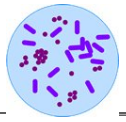
*Furthermore, **molecular biology techniques** for detection of specific genes may be used too.*

1.a – Classify the different sort of tests into speciation in compulsory and optional

Steps that are always compulsory	Steps that depend on purpose

1.b – Complete the scheme about systematic steps in identification explained in the text above:





Activity 2 – Preliminary identification

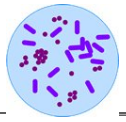
Read the information below in order to complete the visual organizer. There are some clues from previous lessons to help you:

This step involves a few single tests. We have to examine plates for cultural and morphologic characteristics as the following:

- *The **type of culture media** where the strain has grown because some bacteria has specific media designed for their growth (lesson 1.3)*
- *The **atmospheric conditions** of growth because that enables us to classify bacteria according to their respiratory metabolism (lesson 1.4)*
- *The **microscopic characteristics** of cells (lesson 1.1)*
- *The **macroscopic appearance** of colonies that sometimes is typical as haemolysis on blood agar media in Streptococci cultures*
- *And finally, if there is some doubt about the likely family or genus, we perform some easy biochemical reactions such as **oxidase test** and **catalase test**, that can be read under naked eye conditions in a few seconds.*

Give examples of each category of cultural and morphologic characteristics and transfer them to the table. Choose them from the word bank below and use the former text and your own resources to complete the missing ones

	Category	**Examples**
1	Media of growth	
2	Macroscopic characteristics of culture	
3	Respiratory metabolism	
4	Gram stain	



5	Basic Biochemical tests	

haemolysis	cells shape	selective media	oxidase
colonies shape	atmospheric conditions		colonies size

Activity 3 – Information exchange: haemolysis, catalase and oxidase

You are going to read a text that may help you to understand one of the sections below. Your teacher has three different texts and will give you one text. Read it and fill the gaps in the appropriate text frame below to explain the test. Then answer the questions related to your text.

Your colleagues will do the same with the other two texts. When you are ready, speak to two of your colleagues to get more information and complete all the sections. Check pronunciation of any words you need in the online dictionaries you know.

3.a – Haemolysis

▶ Text frame: explaining the test

The name of the test is _____

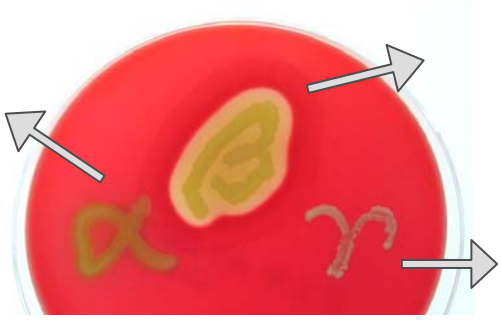
We have to use _____ to observe this feature. When it appears, this means that bacteria _____ red blood cells in blood agar.

Macroscopically, colonies may present three appearances:

1. _____ around the colonies, which means lysis of RBC is complete and we call this _____.
2. _____ around the colonies, which means lysis of RBC is incomplete and we call this _____.
3. _____ around the colonies, which means there is no lysis of RBC and we call this _____.

It is an important observation for _____.

▶ Identify in the image below the three kinds of haemolysis (see ppt for coloured version):



3.b - Catalase test

▶ Text frame: explaining the test

The name of the test is _____

This enzyme _____ hydrogen peroxide into _____ and _____. We demonstrate this by _____ a colony in a drop of hydrogen peroxide. If there is the enzyme, _____ will appear.

Catalase may exist in _____ that produce _____ as a result of oxygen metabolism.

We may obtain false positives if we use _____ to pick up the colony or a colony from a _____ plate.

The test is important to differentiate GPC: *Staphylococci* are _____ and *Streptococci* are _____.

▶ Questions:

(i) Complete the chemical reaction that involves catalase:

catalase

$\longrightarrow$

(ii) How will we report the catalase test on the image and why?

3.c - Oxidase test

▶ Text frame: explaining the test:

The name of the test is _____

This enzyme takes part in aerobic respiration helping _____ to accept hydrogen and produce _____. We demonstrate its presence by mixing a colony with a drop of _____. This is used by bacteria as hydrogen acceptor instead of _____. The reduced reagent develops a _____.

Oxidase may exist in _____.

We may obtain false positives if we use _____ to pick up the colony or a colony from media containing _____.

The test is important to differentiate GNB: *Pseudomonas* are _____ and Enterobacteriae are _____.

▶ Compare both images.

Image 1 – Swab method: oxidase +

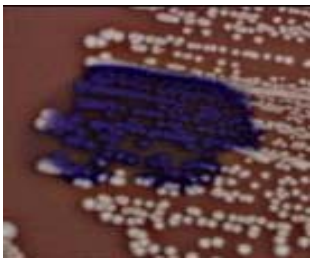


Image 2 – Oxidase +

(i) Deduct how the oxidase test in image 2 was performed and the name of the method.

(ii) Do we have to take any precaution when using this second method?

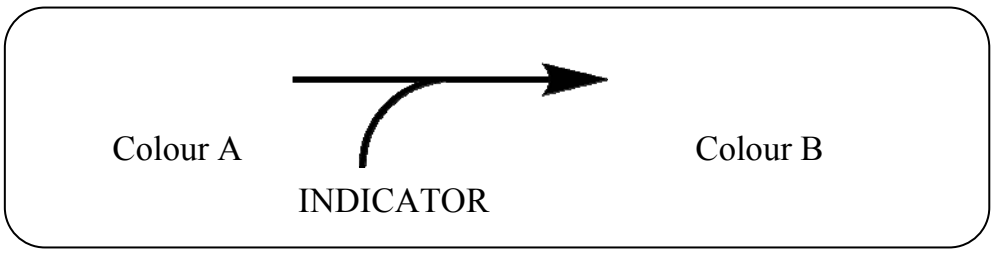
Lesson 2.2 – Biochemical tests

Prokaryotes have a poor morphological diversity, but instead they have a wide metabolic diversity. They have the same basic metabolism pathways as eukaryotes plus a lot of other pathways that doesn't exist in eukaryotes. This feature allows them to live in all kinds of habitats on earth.

Activity 1 – Fundamentals: what is in a biochemical test?

The diagram below represents the relationship between the basic elements in a biochemical test. Complete it with the words in the text:

> *When identifying bacteria, we inoculate a series of different identification media designed to demonstrate a particular biochemical feature in the inoculated bacteria. These features are based on enzymes. Usually, they are liquid media where we emulsify some bacterial colony. After incubation, we observe the media to see if there is some macroscopic change.*
>
> *As a rule, an **enzyme** modifies a **substrate** to convert it into a **final product**. Biochemical tests search for the presence of specific enzymes involved in energy-generating metabolism and sometimes search for a whole biochemical pathway. Consequently, enzyme action consists of breaking down or hydrolysing the substrate. There may be more than one final product, but only one is detected by the indicator.*
>
> *Identification media contain a high concentration of one **substrate** and an **indicator** which usually changes colour in the presence of the final product of the reaction. Sometimes the indicator comes apart and we have to **reveal** the test by adding manually a particular reagent that reacts specifically with the final product giving a visible result. Sometimes the final product is coloured itself and it is called a **chromogenic reaction**.*

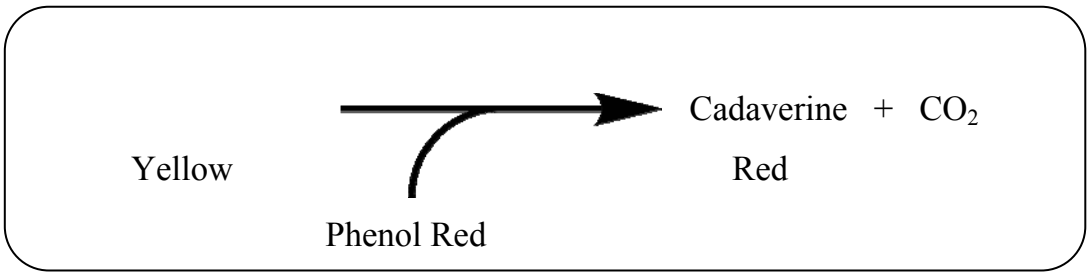


To ensure accurate identification, a wide range of biochemical tests must be employed. This group of inoculated tests is called "**battery**" and the group of its results constitutes the "**biochemical profile**" of the inoculated bacteria.

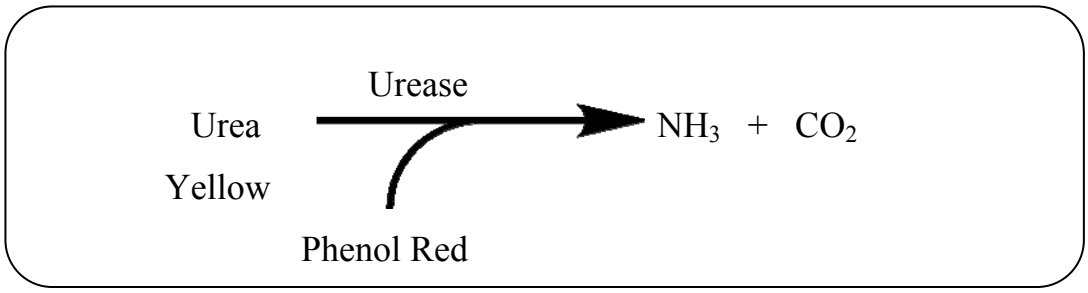
Activity 2 – Taking biochemical tests to pieces

2.a - The diagrams below represent different biochemical tests used in Gram-negative bacilli identification batteries. Complete the diagrams using the words in the word bank at the bottom.

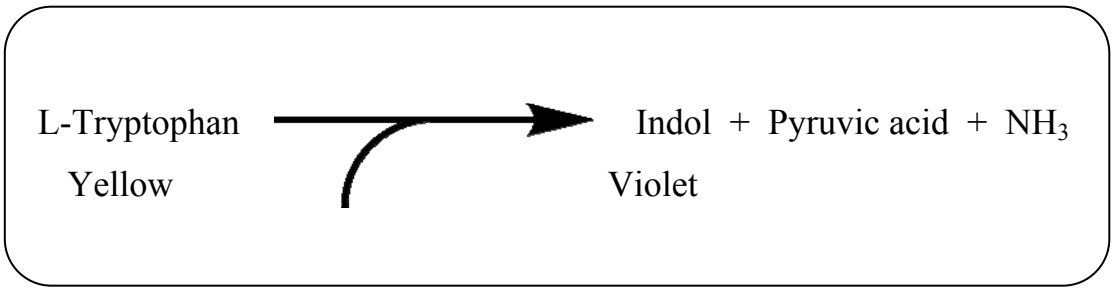
A) Lysine Decarboxilase Test



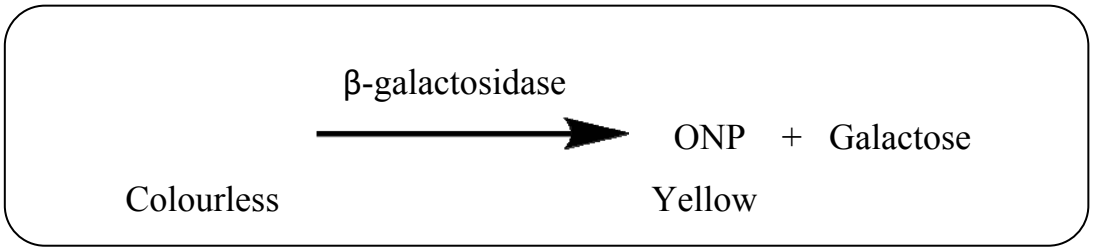
B) _____

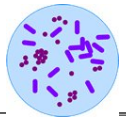


C) _____



D) ONPG Test





Kovac´s Indol Reagent	Lysine	Tryptophanase	ONPG
Lysine Decarboxilase	Urease test	Indol Production Test	Red

2.b – Analyse the diagrams above about biochemical tests and answer the questions:

1	Which one is an example of a chromogenic reaction?
2	Which one is an example of a test that has to be revealed?
3	Which are the different strategies followed to name the tests?
4	Which is the strategy for indicators used in the reactions above and why is it useful?

There are more complex strategies for indicators. For example to detect **hydrogen sulphide (H_2S) production**: this gas is generated from sulphur containing amino acids. To trap it, we use its chemical properties as an acid. With media containing an **iron salt**, the acid gas reacts and produces a **black precipitate** of iron sulphide (Fe_2S_3).

What will happen if there is no trap for H_2S in the medium? If there is no trap ...

Activity 3 – Information exchange: commercial kits

The tests may be performed in commercial miniature plastic kits. They usually consist of a **plastic strip** with **wells** containing different dehydrated identification media. The number of tests per strip may range from 7 to 15.

Image: API 20E strip

Each well is inoculated with a **bacterial suspension** and media become re-hydrated at a time. We incubate overnight and check for spontaneous colour changes and reveal the tests which require the addition of reagents, just as in manual procedures.

We read and record the results in a particular order that summarizes the profile as a **numerical code**. Then we just look for the code in a list where we find the corresponding species for each code.

▶ You are going to read a text about how to inoculate a commercial kit. Look at the chart first and then read the text. Underline what is relevant to the chart. Finally, transfer the information into the chart to complete what you can. Check meaning and pronunciation of any words you need in the online dictionaries you know.

There are three different texts for three different kits. Form a group of three with students that have read the other texts. Explain your text to each other and exchange information to fill in your charts.

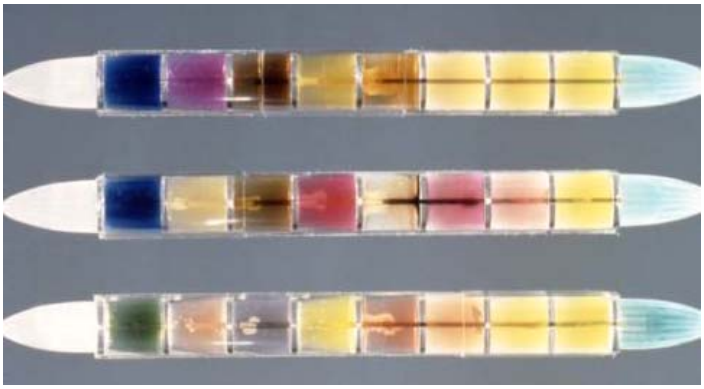


Image: Enterotube II

	Enterotube II	Api 10 S	Diatabs
Description of the kit	Sealed tube with 12 compartments containing media, ...	Strip with wells containing media and	
How to prepare inoculums			
How to inoculate the tests			
Assuring aerobic conditions		Nothing special. All the wells are open.	Nothing special. Tubes are used for aerobic incubation.
Assuring anaerobic conditions	When you reinsert the wire, it closes		

Lesson 2.3 – Variability of Species

Bacteria vary mainly in their metabolic properties and we have to use **biochemical tests** to differentiate species. Usually, one species gives a particular reaction to a test. But it is not so easy.

Variability is a biological characteristic of living beings and that means there are slight differences which distinguish the individuals of the same species. So, amongst bacteria, there may be some individuals that react differently from what is expected of their species.

Activity 1 – Limits for variability

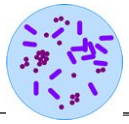
Microbiologists decided to set limits to variability. So, one species is considered **positive** for a test if 85% or more of the strains give a positive reaction, and **negative** if 85% or more of the strains give a negative reaction. That means that we may have up 15% of unexpected reactions. And in addition, when a species doesn't reach the 85% to be considered positive or negative for a test, the tests result is considered **indeterminate**.

We have a table below where we consider some Enterobacteria species. The table shows the percentage of strains that have a positive reaction to some biochemical tests. We have **in rows** the percentages for each species and **in columns** the tests considered. The names of the species appear in the column **on the left hand** and the acronyms for the biochemical tests in the row **at the top**.

SPECIES	ODC	H_2S	URE	TDA	IND
Citrobacter freundii	0	65	1	0	1
Escherichia coli	32	0	2	0	50
Enterobacter aerogenes	99	0	2	0	0
Proteus mirabilis	98	83	99	98	2
Aeromonas hydrophila	0	0	0	0	85

Pronunciation of Latin names: http://www.hyms.ac.uk/learning_resources/bacteria

The text below reflects the meaning of variability. Fill the gaps of the missing words approximating numbers. Use this word bank below. There may be some word left over and some repeated.



almost	nearly	under	less than
over	about	around	half

1.- When percentages are 98 or 99, that is _____ 85% and it means that _____ all the isolated cultures will give the same result and we may consider the species is positive to the test, but even so, we will find some strain will be negative. These tests have high significance because statistically they have quite sure results.

2.- On the other hand we have some percentages that are not so clear. Percentages _____ 85% may be considered as positive reactions of the species, but accepting this means that _____ 15% of negative strains. The one that has only 65% will be considered indeterminate for the referred test and that means that the test in this case has no significance.

3.- The most puzzling situation here is that of *E. coli* for Indol test: 50% means that _____ the isolated strains will be positive and _____ negative. The most indeterminate result we could ever have. For percentages _____ 50% we may apply the same reasoning that before for percentages _____ 65%.

Activity 2 – Making it clear

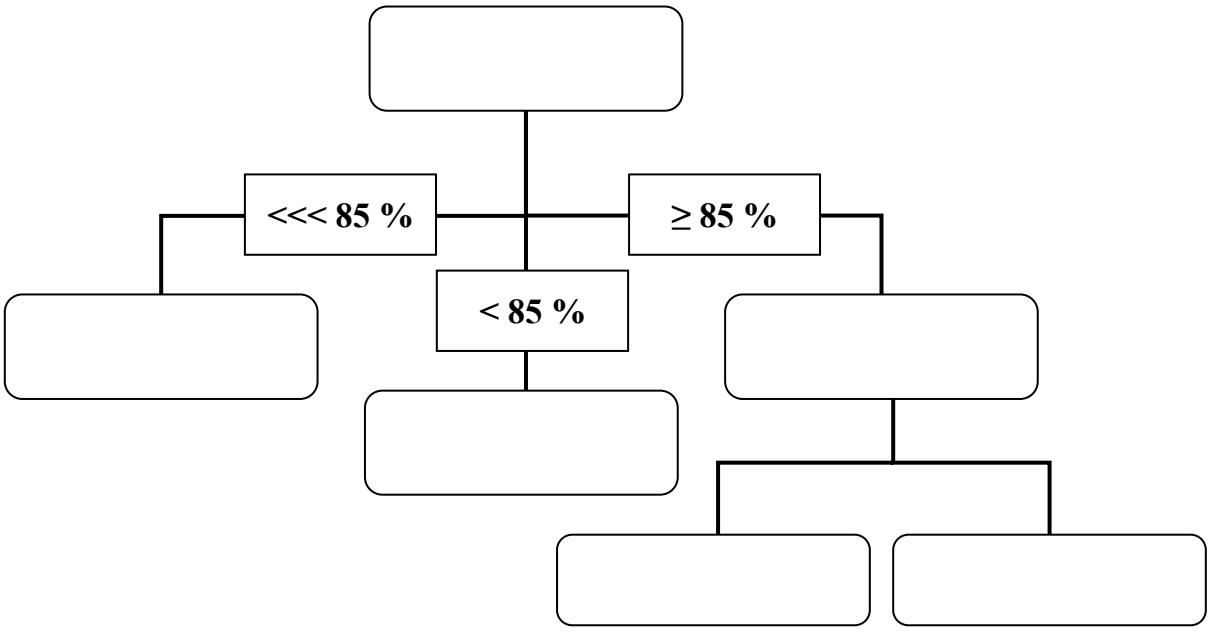
2.a Complete 5 sentences about the conclusions of the former text. Use this substitution table joining one expression from each column:

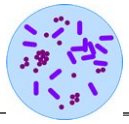
Test results with a low percentage probability	may lead us to	misclassify bacteria
Variability of species	is the cause for	unexpected results
Negative results with high percentage probability	have	indeterminate results for tests
		low significance
		high significance

1	
2	
3	
4	
5	

2.b Put the heading and complete the tree diagram about the concepts from the previous text. Use the words in the word bank:

indeterminate	no significance	positive
biochemical tests	high significance	negative





Activity 3 – Functional matrix

Now simplify the table on biochemical tests for some Enterobacteria, just writing **in the boxes** the expected result (+/-) to each species relating to their percentage probability.

SPECIES	ODC	H_2S	URE	TDA	IND
Citrobacter freundii					
Escherichia coli					
Enterobacter aerogenes					
Proteus mirabilis					
Aeromonas hydrophila					

This is the kind of table we usually use in the laboratory and look at the probability table only when our bacterial profile does not fit any of the profiles in the functional matrix.

Lesson 2.4 – Susceptibility Tests

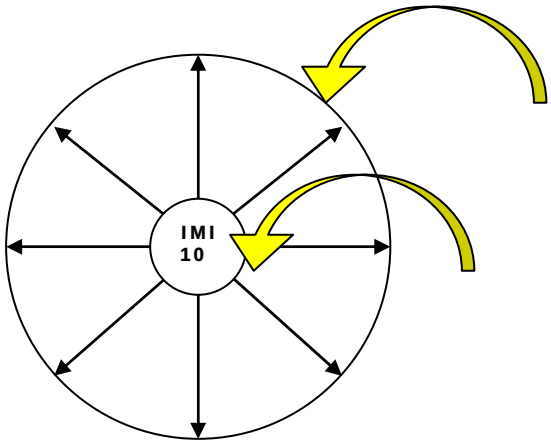
Once the causative agent of an infectious disease is isolated, susceptibility tests determine to which antibiotic it is susceptible, so that an effective treatment may be prescribed.

Development of bacterial resistance makes it most useful. But it is important to standardised methods, otherwise the results cannot be interpreted correctly.

Activity 1 – Fundamentals of Disc Diffusion Test

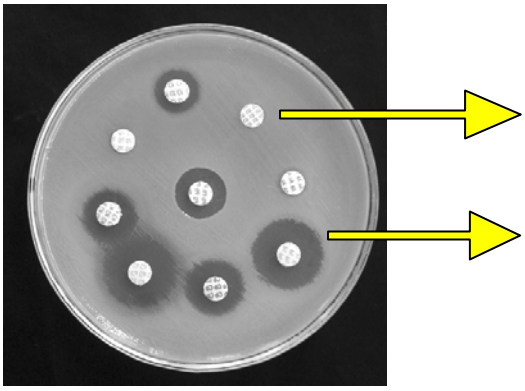
1.a **Paper disks** are soaked with a known quantity of antibiotic and are deposited on the surface of an inoculated plate of agar Mueller Hinton. We incubate overnight to let the **antibiotic spread** across the agar while the bacteria grow. As antibiotic spreads, it creates a **concentration gradient.**

The diagram below represents diffusion from a disk of Imipenem (10 µg). The inner circle represents the paper disk. The outer circle represents the farer distance that antibiotic has reached after incubation. Label maximum and minimum concentrations in the gradient:



1.b After incubation we may observe bacterial growth all over the plate and circular halos of no growth called **inhibition zones** around the disks. This indicates that bacteria perhaps are **susceptible (S)** to the antibiotic contained in the disk. We have to measure the zones to know how susceptible they are. If bacteria are **resistant (R),** there is no halo.

Look at the inhibition zones of this plate and qualify as S or R those signalled by an arrow.



Activity 2 – Kirby-Bauer Method

Read the text about the steps of the Kirby-Bauer method. Then listen to the explanation where you will find the words to fill the gaps in the text below:

1. ***Preparation of inoculum**:*

   Prepare a _____ with 3 to 5 well isolated colonies in distilled water. Adjust turbidity to 0.5 MacFarland standard.

2. ***Inoculation of plates**:*

   a) *Dip a swab in the suspension and remove excess liquid by _____ it against the inner side of the tube.*

   b) *Spread the inoculum _____ over the entire surface of the plate by streaking in three directions. Finally swab the _____ of the agar. Take care not to leave gaps between streaks. Allow the plate to dry.*

3. ***Application of disks**:*

Image: picking the disks one by one

Apply the disks to the surface of the plate using tweezers to pick them from the container. _____ the tweezers before taking each disk and _____ over the disk on the plate to ensure full contact. Put no more than 6 equidistant disks per plate of 9 cm.

Do not _____ disks once they hav touched the agar surface.

4. ***Incubation**:*

   Invert the plate and incubate overnight at 37ºC.

5. ***Measure of zones**:*

   With a ruler, measure the _____ of zones of complete inhibition around each disk from the _____ of the plate.

6. ***Interpretation of susceptibility**:*

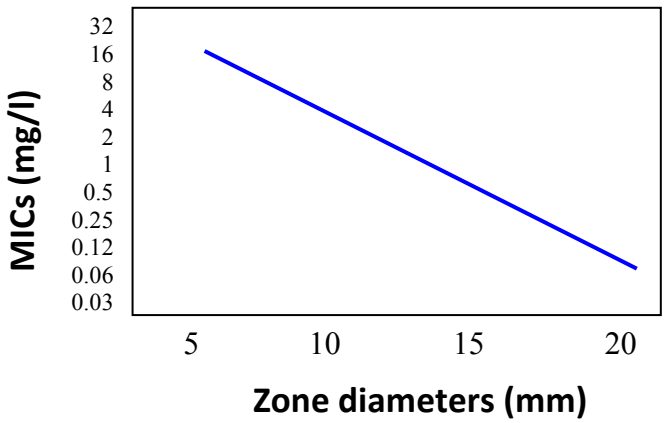
   Interpret diameters into _____ of susceptibility according to the **Breakpoint Tables.**

Activity 3 – Interpreting diameters

A large zone diameter means that the bacteria are more susceptible to the antibiotic, because the low concentration of antibiotic that the bacteria find far from the disk is enough to avoid its growth. A small diameter means that the bacteria are more resistant. Concentration of antibiotic required to be effective is high.

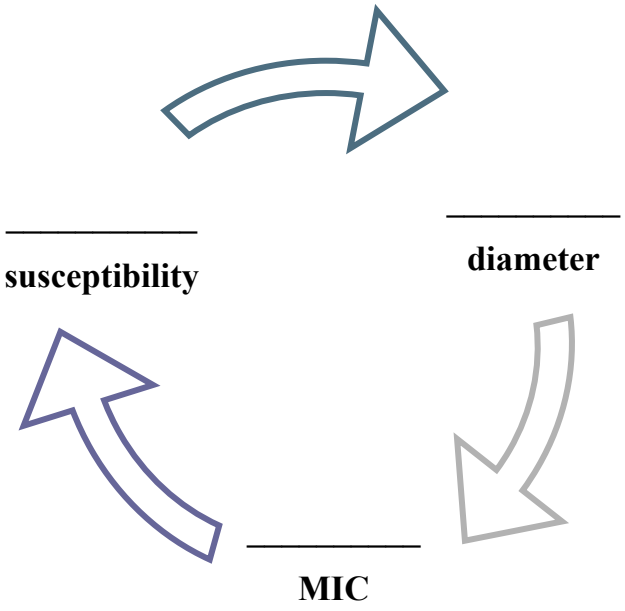
MIC or **minimum inhibitory concentration** is a characteristic that defines the relationship between a bacterial strain and an antibiotic. MIC is the lowest concentration of an antibiotic that is still effective. We measure it in mg/l.

3.a This is the graph that shows the **relationship between MICs and zone diameters**. The scale on the Y-axis is logarithmic. Read the text that describes the graph and circle the correct words:



This *bar graph / line graph* shows that MICs and zone diameters are *directly / inversely* proportional. That is, as diameter *increases /decreases*, MIC *increases / decreases.*

3.b Complete the diagram comparing diameter, MIC and susceptibility. Use the word bank to choose the adjectives.



Size word bank:

For numbers or figures
high / low

For three dimensions
big / small

For non physical conditions
great /small

For other cases
large / small

Comparative

low – lower – the lowest
high – higher – the highest
great – greater – the greatest
small – smaller – the smallest
big – bigger - the biggest
large – larger – the largest

Activity 4 – Using breakpoint tables

Based on standard dosages in patients and toxic effects of antibiotics, the **MIC breakpoints** have been established, that is the MIC limits to consider a strain susceptible or not. **Diameter breakpoints** are used in tables and are calculated from MICs breakpoints.

Zone diameters depend not only on the bacteria but on the antibiotic as well. The rate of diffusion of the antibiotic depends on its size, its concentration and its chemical properties. So, interpreting criteria for inhibition zones will be different for each antibiotic and for each bacteria. All these data are collected in the **Breakpoint Tables.**

Consulting these tables, we classify the microorganisms in one of three **categories of susceptibility** for each antibiotic:

- **Susceptible (S)**.
- **Intermediate (I)**.
- **Resistant (R)**.

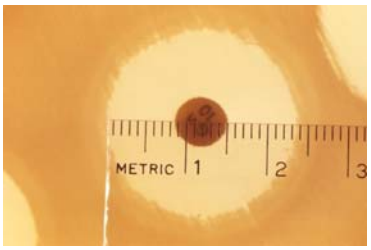
Match the columns: for each category choose its definition.

Susceptible	That means bacteria are not affected by this antibiotic at all or maybe they are but at too high concentration
Intermediate	That means bacteria are susceptible to low concentrations of it
Resistant	That means bacteria are susceptible to higher concentrations than that in usual dosages

Activity 5 – Real-life test

▶ We have performed a susceptibility test from a culture of *Pseudomonas aeruginosa*. The zone diameters measured are in the table below. Interpret them with the breakpoint table below.

Antibiotic	Diameter (mm)	Result
Piperacillin	22	
Aztreonam	24	
Imipenem	16	
Ceftazidime	16	
Gentamicin	0	
Ciprofloxacin	20	



Breakpoints of zone inhibition for *Pseudomonas sp.* (Source: EUCAST)				
Antibiotic	**Disk content (µg)**	**Zone diameter breakpoint**		
		R	**I**	**S**
Piperacillin	30	<19	-	≥19
Aztreonam	30	<16	16-49	≥50
Imipenem	10	<17	17-19	≥20
Ceftazidime	10	<16	-	≥16
Gentamicin	10	<15	-	≥15
Ciprofloxacin	5	<22	22-24	≥25