Experiment 9: Acid-Fast Staining

Aim
To explain the chemical basis of the acid-fast stain.
To perform the procedure to differentiate bacteria into acid-fast and non–acid-fast groups.

Principle
A few species of bacteria in the genera *Mycobacterium* and *Norcardia,* and the parasite *Cryptosporidium* do not readily stain with simple stains. However, these microorganisms can be stained by heating them with carbolfuchsin. The heat drives the stain into cells. Once the microorganisms have taken up the carbolfuchsin, they are not easily decolourised by acid-alcohol, and hence are termed acid-fast. This acid-fastness is due to the high lipid content (mycolic acids) in the cell wall of these microorganisms. The Ziehl-Neelsen acid-fast staining procedure (developed by Franz Ziehl, a German bacteriologist, and Friedrich Neelsen, a German Pathologist in the late 1800s) is a very useful differential staining technique that makes use of this difference in retention of carbolfuchsin. Acid-fast microorganism will retain this dye and appear red. Microorganisms that are not acid-fast, termed non-acid-fast, will appear blue or brown due to the counterstaining with methylene blue after they have been decolourised by the acid-alcohol. A modification of this procedure that employs a wetting agent (Tergitol No 7) rather than heat to ensure stain penetration is known as the Kinyoun staining procedure (developed by Joseph Kinyoun, a Germany Bacteriologist, in the early 1900s).

Application
The acid-fast stain is a differential stain used to detect cells capable of retaining a primary stain when treated with an acid alcohol. It is an important differential stain used to identify bacteria in the genus mycobacterium, some of which are pathogens (e.g., m. Leprae and m. Tuberculosis, causative agents of leprosy and tuber - culosis, respectively). Members of the actinomycete genus nocardia (n.Brasiliensis and n. Asteroides are opportunistic pathogens) are partially acid-fast. Oocysts of coccidian parasites, such as crypto sporidium and isospora, are also acid-fast. Because so few organisms are acid-fast, the acid-fast stain is run only when infection by an acid-fast organism is suspected. Acid-fast stains are useful in identifying acid-fast bacilli (AFB) and in rapid, preliminary diagnosis of tuberculosis (with greater than 90% predictive value from sputum samples). It also can be performed on patient samples to track the progress of antibiotic therapy and determine the degree of contagiousness. A prescribed number of microscopic fields is examined, and the number of AFB is determined and reported using a standard scoring system.

Specimen
Mucopurulent sputum

Apparatus / Materials / Reagents
- Smear of *Mycobacterium spp* on microscope slide
- Ziehl-Neelsen carbolfuchsin
- 3% Acid-alcohol v/v
- methylene blue (0.5% w/v)

- bright field Microscope, Lens paper and lens cleaner
- Immersion oil, staining racks.

Procedure

Ziehl-Neelsen (Hot stain) Procedure

1. Place the fixed sputum smear on a staining rack and flood it with Ziehl-Neelsen's carbolfuchsin.
2. Heat the slide from the underside for 3 to 5 minutes or until vapour just begins to rise. Do not allow the slide to dry out and avoid excess flooding. Also, prevent boiling of the stain.
3. Leave the heated stain on the slide for at least 10 minutes.
4. Gently rinse off the stain with clean water and drain off the water from the slide (If your tap water is not clean use distilled water).
5. Decolourise by flooding the slide with 3% acid-alcohol for 3 minutes.
6. Gently rinse the slide with clean water for 2 to 4 min and drain off the water from the slide.
7. Flood the slide with methylene blue for about 1 to 2 min.
8. Gently rinse the slide with clean water and drain off the water from the slide.
9. Blot dry with a filter paper.
10. Examine the slide under oil immersion (x100) and record your results in your report. Acid-fast organisms stain red, the background, and other organisms stain blue or brown.

Kinyoun (Cold stain) Procedure

This may be used instead of or in addition with Ziehl-Neelsen procedure.

1. Place the smear on a staining rack and flood it for 5 min with carbolfuchsin prepared with Tergitol 7 (heat is not necessary).
2. Decolourise with acid-alcohol and wash with tap water. Repeat this step until no more colour runs off the slide.
3. Counterstain with alkaline methylene blue for 2 minutes. Wash and blot dry.
4. Examine under oil immersion (x100). Acid-fast organisms stain red, the background and other organisms stain blue.

Quality Control

Known positive slide and a known negative slide should be included in the procedure.

Results

For main results present the following information:

For each method indicate if the acid-fast bacterium (AFB) were seen and give a quantitative report of the results for Acid fast bacilli microscopy (see **Appendix 4** for guide). Draw a representative field for each bacterium stained slide for both staining results and provide information on the size, shape, colours, and arrangement of organism seen if present (See **Appendix 2** for guide).

Ziehl-Neelsen's (hot stain)

Kinyoun (cold stain)

Magnification: ___________

Magnification: _____________

Interpretation (Discussion)

Discuss every aspect of your results as much as possible and give:

- **scientific interpretations**, mention if your results are consistent or different from what is known in **literature** about that topic so far (e.g., in books or other studies) and discuss what that might mean.
- Discuss the **clinical significance** of some of your findings etc. **Put in-text citations (Harvard standard).**

Experiment 10: Fluorescence based Acid-Fast bacteria (AFB) microscopy.

Aim
To understand the biochemical basis of Fluorescence based Acid-Fast bacteria (AFB) staining.
To perform a fluorochrome stain procedure using auramine-O
To differentiate bacteria into acid-fast and non-acid-fast groups

Principle
Another special method used to microscopically examine acid fast bacteria is the fluorochrome procedure using auramine-O dyes. This method generally operates under the same principle for staining of acid-fast bacteria. The acid-fast bacteria are first stained with auramine-O which is a special fluorochrome dye that penetrates and stains the nucleic acid of the cell. An acid-alcohol decolouriser is then added but these bacteria retain the auramine-O due to their acid fastness while the surrounding materials become decolorized. The counter stain Potassium Permanganate is then added to stain the background to provide contrast between the stained bacterium and a darkened surrounding. The counter stain also acts to quench fluorescence in the background. The stained bacterium can then be seen using the fluorescence microscope which uses ultra-bright LED (light emitting diode) technology. This microscope transmits focused light of a specific wavelength which interacts with the auramine-O on the stained bacteria cells causing the bacteria cells to fluoresce (glow) and hence the acid-fast bacteria can be seen against a dark background. Fluorescence microscopy is 10% more sensitive than the conventional light microscope especially for low positives.

Application
Fluorescence microscopy uses fluorescence and phosphorescence to examine the staining chemical properties, structural organization, size, and spatial distribution of samples. It is particularly used to study samples that are complex and cannot be examined under conventional transmitted-light microscope. Fluorescence microscopy images helps to study substances present in low concentrations where high sensitivity is crucial to detect them.

Specimen

Apparatus / Materials / Reagents
- Smear of *Mycobacterium spp* on microscope slide
- Filtered 0.1% Auramine O
- 0.5% acid-alcohol
- 0.5% potassium permanganate
- LED Microscope
- Lens paper, lens cleaner, and staining racks

Procedure

Auramine-O Staining Procedure

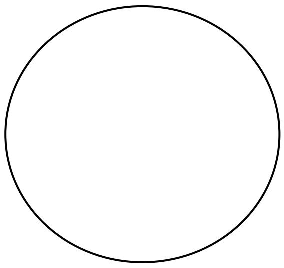
1. Place the smear on a staining rack and flood it with Filtered 0.1% Auramine O.
2. Do not heat.
3. Keep the staining reagent (the Auramine O) for at least 20 minutes.
4. Rinse with water and drain.
5. Flood the slide with decolorizing solution, 0.5% acid-alcohol, for 3 minutes.
6. Gently rinse with water until the macroscopically visible stain has been washed away and drained.
7. Flood the slide with 0.5% potassium permanganate solution for 1 minute. This is critical because counterstaining for a longer time may quench the acid fast-bacilli fluorescence.
8. Gently rinse with water and drain
9. Air dry on a slide rack.
10. Examine the slide under **x40** magnification (No immersion oil) and record your results in your report. Acid-fast organisms stain bright yellow green, the background and other organisms stain dark.

Quality Control

Results

For main results present the following information:

Draw a representative field for the bacterium seen and provide information on the size, shape, and arrangement, and colours of organisms seen if present (See **Appendix 2** for guide). Also, give a quantitative report of the results for Acid fast bacilli microscopy (see **Appendix 4** for guide).



Magnification: ___________

Interpretation (Discussion)

Discuss every aspect of your results as much as possible and give:

- **scientific interpretations**, mention if your results are consistent or different from what is known in **literature** about that topic so far (e.g., in books or other studies) and discuss what that might mean.
- Discuss the **clinical significance** of some of your findings etc. **Put in-text citations (Harvard standard).**

Experiment 11: Molecular testing for Mycobacterium tuberculosis (GeneXpert MTB/RIF)

Aim

To understand the molecular basis of real time PCR and MTB/RIF testing.
To perform MTB/RIF testing using the GeneXpert technology.
To interpret and explain the results of a GeneXpert MTB/RIF test.

Principle

Molecular testing techniques such as Polymerase chain reaction (PCR) offer highly accurate and sensitive methods of detection and identification of microbial pathogens for clinical diagnosis and research. PCR is used to target and amplify specific segment of DNA through repeated thermal cycles to have enough copies easily detectable. *Mycobacterium tuberculosis* (MTB) has a unique *rpoB* gene and sometimes mutations in part of this gene causes the bacteria to be resistant to Rifampicin (a very important anti-Tuberculosis drug). The GeneXpert technology therefore uses real time PCR to target and amply this entire *rpoB* gene and it does this by amplifying five different segments of the gene at the same time. If *Mycobacterium tuberculosis* is present, then all these segments of the gene are amplified, and five unique fluorescent reporter molecules (probes) attach to the amplified products causing signals to be sent to the computer which reports that MTB has been detected. Each specific segment product (copy) and reporter molecule (probe) combination produces its own special signal for all the segments and the more copies made per cycle the stronger the signal. If the bacteria present is resistant to Rifampicin, then one specific segment of the *rpoB* gene will not be amplified as it is mutated and the special reporter molecule that is supposed to attach to the amplified products (copies) for that segment will not send its signal to the computer hence the computer reports that MTB is detected, and Rifampicin resistance is also detected. If **NO** *Mycobacterium tuberculosis* bacteria is present in the sample to begin with then no amplification occurs, and no signal is sent to the computer by any of the reporter molecules. This is reported as MTB not detected.

Application

Specimen

Apparatus / Materials / Reagents

- Sputum sample of not less than 1ml volume in sputum container.
- A separate empty sputum collection container for making mixtures.
- Sample reagent (SR)
- Xpert ® MTB/RIF cartridge
- Sterile disposable transfer pipettes
- GeneXpert device (thermal and sensor unit modules)
- Basin with disinfectant.

Note* MTB belongs to the biosafety level III class of organisms. Therefore, all biosafety level III safety, procedures, and laboratory requirements should be met for conducting this laboratory practical.

Procedure

MTB/RIF **testing using the GeneXpert technology.**

1. Bring the Sample Reagent (SR), Sputum sample, and the empty sputum collection container close together in one place.
2. label the Sputum sample as well as the empty sputum collection container with the sample ID or your student group number.
3. Using the transfer pipettes, Add 2 volumes of Sample Reagent (SR) to 1 volume of sputum in the empty sputum collection container. It does not matter what volumes of each you use but just make sure that the final mixture volume is not less than 2mls. Throw the used pipets in the basin with disinfectant.
4. Make sure you tightly close all the lids after the transfer.
5. Shake the sample mixture vigorously 10-20 times, you can also vortex it for about 30 seconds.
6. Incubate at room temperature for about 10 min.
7. Shake the sample mixture vigorously 10-20 times once more and incubate again at room temperature for 5 min.
8. At this point the sample mixture should be perfectly fluid before being processed, with no visible clumps of sputum. If still viscous and some clumps are still visible, wait 5-10 further minutes. The sample reagent causes lysis of the mucous strands hence this process results in a uniform homogenous solution.
9. Get the Xpert ® MTB/RIF cartridge and label it with the same sample ID (or your student group number) by writing on the left or right side. **DO NOT** label on the bar code.
10. Open the cartridge lid and pipet 2mls of the homogenised solution through the aperture provided in the corner. Throw the used pipet in the basin with disinfectant.
11. Close the cartridge lid. Once cartridge is ready, scan the cartridge bar-code and insert the cartridge in the GeneXpert device module and start the test. The test runs for 90 min.

Results

For main results present the following information:

Collect the printed results reports for all the sputum samples analysed in the practical (results for your group as well as the other students' groups). For each result write the sample ID and the full results given for that sample in terms of whether MTB is detected or not, how heavy the infection is, and Rifampicin resistance. You can present this in a table form.

Interpretation (Discussion)

Discuss every aspect of your results as much as possible and give:

- **scientific interpretations**, mention if your results are consistent or different from what is known in **literature** about that topic so far (e.g., in books or other studies) and discuss what that might mean.
- Discuss the **clinical significance** of some of your findings etc. **Put in-text citations (Harvard standard).**

Appendix 2

1) Bacteria sizes are described as:
    - Small
    - Medium (though usually silent in clinical reports)
    - Large

2) Basic shapes and arrangements of bacteria **figure 3**.

(a) Cocci.

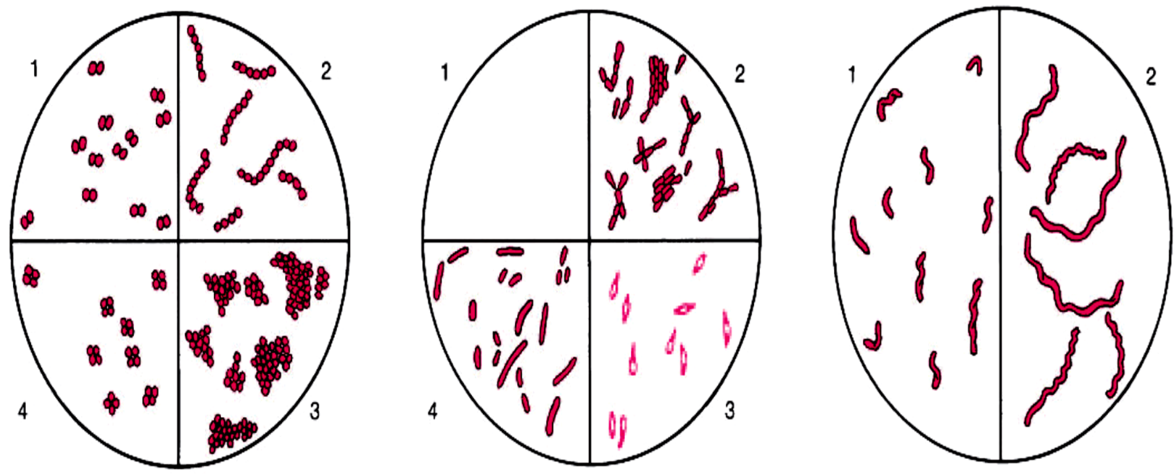
- 1. Diplococci (pairs).
- 2. Streptococci (chains).
- 3. Staphylococci (grapelike clusters).
- 4. Tetrads (packets of four).

(b) Bacilli (rods).

- 1. Non observed.
- 2. Palisades; V, X, and Y figures, clubbing.
- 3. Endospore-forming bacilli (note endospores as small, round, hollow, unstained areas, within or at one end of bacillary bodies);
- 4. A bacillus showing pleomorphism (note varying widths and lengths).

(c) Spirals.

- Spirilla (short curved or spiraled forms with rigid bodies);
- 2. Spirochetes (long tightly or loosely coiled forms with sinuous flexible bodies).



(a) Cocci

(b)

(c) Spirals

Fig 3.

Further examples: **Figure 4** showing bacilli in chains and **Figure 5** vibrios.

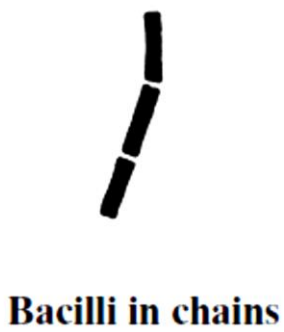


Fig 4.

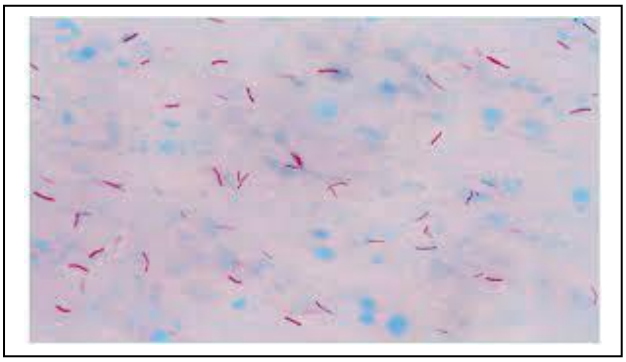
Fig 5.

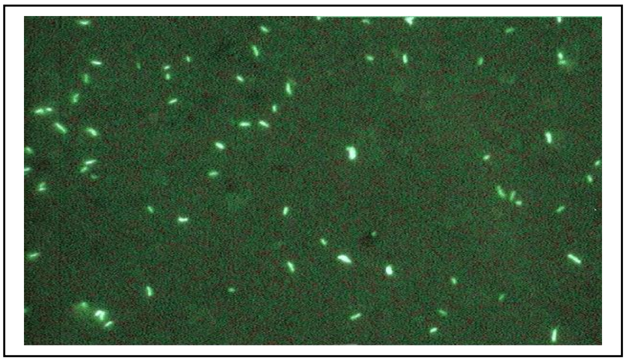
Appendix 4

Quantitative reporting for acid fast bacilli microscopy.

IUATLD/WHO scale (1000x field = HPF) Result	Microscopy system used	
	Bright-field (1000x magnification: 1 length = 2 cm = 100 HPF)	Fluorescence (400x magnification: 1 length = 40 fields = 200 HPF)
Negative	Zero AFB / 1 length	Zero AFB / 1 length
Scanty (Actual Numbers)	1–9 AFB / 1 length or 100 HPF	1–19 AFB / 1 length
1+	10–99 AFB / 1 length or 100 HPF	20–199 AFB / 1 length
2 +	1–10 AFB / 1 HPF on average	5–50 AFB / 1 field on average
3+	>10 AFB / 1 HPF on average	>50 AFB / 1 field on average

IMPORTANT!!!! For the Negative do not report it as negative but instead report it as No acid fast bacilli seen or (No AFBs seen).





Ziehl-Neelsen TB stain (x100 oil immersion)

Fluorescence TB stain (x40)