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Improved Acid-Fast Stain And Histology Method For The Detection Of Mycobacteria In Tissue Samples

Pedro Miguel Festas Marinho

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Supervisor: Professor Doctor Thomas Hanscheid

Co-Supervisor: Professor Leonilde de Fátima Morais Moreira

Examination Committee

Chairperson: Professor Jorge Humberto Gomes Leitão

Supervisor: Professor Doctor Thomas Hanscheid

Members of the Committee: Doctor Tânia Gilot Mendes de Carvalho

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Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Preface

The work presented in this thesis was performed at the Instituto de Microbiologia, Faculdade de Medicina, University of Lisbon (Lisbon, Portugal), during the period July 2018 – October 2019, under the supervision of Prof. Doctor Thomas Hänscheid. The thesis was co-supervised at Instituto Superior Técnico by Prof. Leonilde Moreira.

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Resumo

A tuberculose (TB) continua a ser uma das doenças com maior impacto a nível mundial com o maior número de mortes. Métodos de diagnóstico ineficiente, especialmente em países de maior incidência, são um dos principais problemas. A coloração ácido-resistente com auramina O (AuO) é um método aplicável em vários contextos, inclusive laboratórios com poucos recursos. Oferece um diagnóstico definitivo, sensibilidade relativamente alta, facilidade de utilização e custo reduzido. No entanto, não há investigação para a optimização do método para melhor deteção, segurança e redução do custo. Adicionalmente, o diagnóstico histológico da tuberculose não é eficaz; sendo as colorações ácido-resistentes o método de referência, é necessário identificar possíveis causas e soluções. Neste projecto, é demonstrado o potencial de redução do tempo e concentração do primeiro passo da coloração AuO. Também se verificou que será possível remover o fenol da solução, utilizando um passo de aquecimento a 52ºC para permeabilização. O efeito negativo do xilol para desparafinação na deteção de mycobacteria foi confirmado e vários métodos alternativos foram criados e testados. Um novo método baseado na projeção de ar quente na amostra (PHAD) foi desenvolvido, originando resultados consistentemente superiores ao método tradicional. Numa instância, os bacilos tinham uma fluorescência 142% mais intensa em média. Estes resultados mostram o potencial de optimização e adaptação destas metodologias para melhorar o diagnóstico da tuberculose.

Palavras-Chave: Tuberculose; Mycobacterium; Extrapulmonar; Deparafinação; Auramina; Xileno; Optimização; Alvo; Coloração

Abstract

Tuberculosis (TB) remains one of the most impactful infectious disease in the world, especially concerning the number of deaths. Inefficient diagnosis, especially in high incidence countries, is one of the main reasons for this. Acid-fast (AF) auramine O (AuO) staining is a promising method in many settings, including low-resource laboratories. It provides a definitive diagnosis, relatively high sensitivity, ease of use and low cost. However, little has been done to optimize the method for higher detection, increased safety and lower costs. Importantly, histological diagnosis of TB is not efficient; AF stains being the reference-method, possible reasons must be identified and solved. In this project we show that there is potential for the reduction of concentration and/or staining duration of the AuO primary step. It was also found that phenol could be eliminated from the AuO solution if it is heated to 52°C immediately before application, yielding better results. The negative effect of xylene (used in histology) on detecting mycobacteria was further confirmed and several alternative methods were tried for xylene free deparaffinization. A method relying on the application projected hot air on the section for deparaffinization (PHAD) was developed, yielding consistently superior results. In one instance the bacilli found fluoresced 142% more on average. These results demonstrate the potential in optimizing and modifying these methodologies to help improve the diagnosis of TB and other diseases.

Key Words: Tuberculosis; Mycobacterium; Extrapulmonary; Deparaffinization; Auramine; Xylene; Optimization; Stain; Target

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List of Acronyms

AF – Acid-Fast
AFI – Arbitrary Fluorescence Intensity
AG – Arabinogalactan
AIDS – Acquired Immunodeficiency Syndrome
AuO – Auramine O
BCG – Bacillus Calmette-Guérin
BF – Bright Field
CM – Cell Membrane
CO_2 – Carbon Dioxide
DAB – 3,3'-Diaminobenzidine
DMSO – Dimethyl Sulfoxide
DNA – Deoxyribonucleic acid
ELISA – Enzyme-Linked Immunosorbent Assay
EPTB – Extrapulmonary Tuberculosis
FFPE – Formalin Fixed Paraffin Embeded
FIND – Foundation for Innovative New Diagnostics
H&E – Haematoxylin & Eosin
HCL – Hydrochloric Acid
HIER – Heat Induced Epitope Retrieval
ID – Identification
IUPAC – International Union of Pure and Applied Chemistry
LED – Light-Emitting Diode
LOC – Liquid Organic Cleaner
MA – Mycolic Acid
MB – Methylene Blue
NAAT – Nucleic Acid Amplification Test
PCR – Polymerase Chain Reaction
PG – Peptidoglycan
PP – Potassium Permanganate
PHAD – Projected Hot Air Deparaffinization
RCF – Relative Centrifugal Force
RNA – Ribonucleic Acid
RT – Room Temperature
TB – Tuberculosis
TEM – Transmission Electron Microscopy
TM – Trademark
UV – Ultraviolet
ZN – Ziehl-Neelsen

I – Introduction

1 – *Mycobacterium tuberculosis*: an acid-fast pathogen

1.1 – History of Infectious Disease Diagnosis and Identification of *M. tuberculosis*

The history of acid-fast staining for microbiology diagnosis, in particular of mycobacteria, is very closely linked with the very birth of clinical bacteriology and it dates to the 18th and 19th centuries when microbiology was taking its first steps. The beginning step of medical microbiology arguably took place with the first observation of microorganisms by Anton van Leeuwenhoek in the 17th century.(1) Microscopic life, curiously coincident with disease, was found by many scientists following this event. Moreover, the important notion that these agents were acquired from the environment and not just spontaneously generated in the body or from *miasma* gradually became stronger.(2)

During the 18th century, the germ theory of disease lost steam because the microscope technology was scarcely explored, and lenses were still weak and rare.(2) Diseases as a result of infection by different agents, causative of different pathologies and outcomes only became a topic of discussion in the early 1800s, when patient's body and cadaver observation became more popular of a practice in diagnosis.(2,3) Henle in 1840 (4) first categorically asserted that different diseases had different infectious agents as causes and that these were living and reproducing in the body, as cited by Foster WD. (2), bringing forth the germ theory of disease first theorized in the renaissance era by Girolamo Fracastoro (5), as cited by Pesapane F.(6). In that century much progress was achieved in establishing the idea of infectious agents. In 1874 Armauer Hansen was the first to describe a bacteria causing infection, the leprosy bacillus, a disease until then thought as hereditary. (7)

In the 1870-1880s Robert Koch boosted microbiology diagnostics immensely by developing a staining method. He was who introduced the technique of making a smear, fixing and staining a bacteria culture on a glass slide to better see and distinguish microorganisms under the microscope.(2,8) He was the first to employ aniline dyes, now so commonly used in diagnostic laboratories. Koch was also responsible for further showing association between a specific organism and a disease, 6 of them in fact, being that until that point only anthrax was proven to be caused by bacteria by Casimir Davaine in the 1860's.(2,8,9) It was in the trail of that work that he formulated the famous Koch postulates and established once and for all the existence of various microorganisms responsible for different diseases.(2,8,10) Koch was then determined to uncover the cause of one of the greatest afflictions of society at the time, tuberculosis. This led the scientist to recover samples from contaminated material, smear them on coverslips and employ his stain as he had done previously.(2,8) This staining method was unsuccessful, so he began to modify it and found that one of his previous discoveries proved most useful for these samples. The process of overstaining and later decolorizing the smear finally allowed him to observe the bacilli. (2,8) Eventually, in his work, he was able to show that these bacilli caused tuberculosis (8,11) and this was a tremendous milestone for microbiology, arguably birthing clinical bacteriology by introducing the practice of smear and stain preparation as an important tool for diagnosis. This was also the first basis for the later creation of an acid-fast staining procedure.

1.2 – Tuberculosis Disease

1.2.1 –History and Epidemiology

It is thought that the genus *Mycobacterium* first appeared 150 million years ago and the first infection of a hominid by a *M. tuberculosis* ancestor may have occurred in Africa, some 3 million years ago.(12) Tuberculosis disease (TB) has been mentioned in records throughout history and even found in Egyptian mummies.(13) In the middle ages, it was mentioned as the "King's evil", for they believed the king's touch could heal it.(14) It was well present in various empires and time periods. In the 18th century, during the industrial revolution, it was known by "the robber of youth" and the "white plague". Over the years, several notorious figures in art perished from tuberculosis: Emily Brontë, Frederic Chopin, George Orwell, Carlos Santana and many others.(15) It was in 1882 that Robert Koch presented his work in isolating the bacilli and in the following decades, skin tests, the BCG (Bacillus Calmette-Guérin) vaccine and antibiotics were discovered against TB (tuberculosis disease).(11,14) This led to a stark decrease in the prevalence and incidence of the disease in developed countries. (14) Today it still has a high incidence in low income countries and overpopulated areas (figure 1).

Although it is thought by many as a disease of the past, TB is still one of the top ten causes of death worldwide, causing about 1.6 million deaths in 2017 alone.(16) According to the WHO, up to 11.1 million people fell sick with this illness in that year, about as many people as the population of Portugal.(16) 1.7 billion people worldwide are thought to be latently infected by *Mycobacterium tuberculosis*, nearly a fourth of the world's population, and between 5 to 10% of those will develop an active infection later in life. (16) It has been reported that an infectious dose of only 1 CFU could be enough to cause infection.(17) It is the deadliest disease caused by a single infectious agent today, in terms of gross number of deaths, and it is most prevalent in countries whose diagnostic and treatment options are still reduced by socioeconomic limitations.(16)

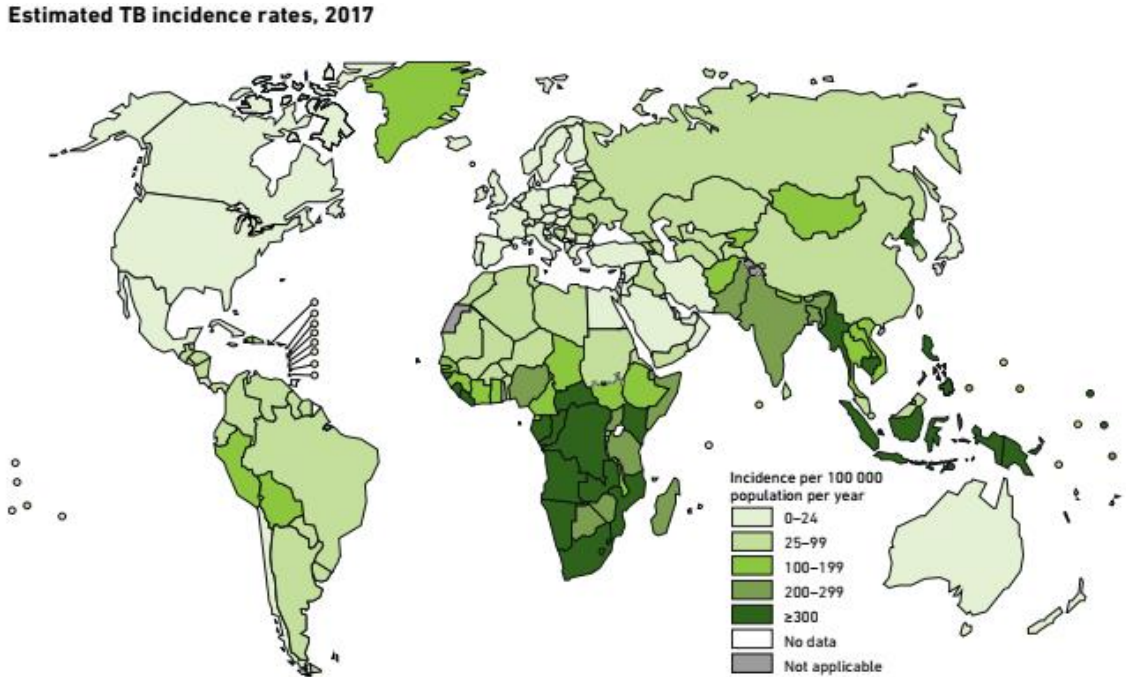


Figure 1 – Estimated TB incidence rates in 2017. (16)

The most recent estimates for the Portuguese incidence rate for 2017 is lower than the average of the European region, being around 20 compared to 30 (per 100 000 hab.), which represents a reduction of about 40% in the incidence of the disease in the last 10 years for the country.(18) There was a total of 1800 reported cases, with most of those reported in the two largest cities, Lisboa and Porto.(18,19) Pulmonary TB accounted for 71,5%.(18,19) Of all extrapulmonary TB cases, those of the pleura and extra-thoracic lymph-nodes were the most common (18). The number of deaths due to TB disease was estimated to be around 210 people in 2017, 250 if accounting for cases of HIV-positive individuals. (16,19)

On the Portuguese report, regarding the year 2015, more comprehensive data was shared, as illustrated in figure 2. (20) A similar percentage of cases was of pulmonary TB (71,6%) although the total number of cases was considerably higher. Of 2135 cases, over 600 were of EPTB and only about 30% of those were confirmed by a microbiology laboratory. The number of cases which were not confirmed was higher for EPTB than pulmonary TB.(20) It was also highlighted that the most common co-morbidities were with HIV and diabetes, while the highest social risk factors were alcohol and illegal-drug abuse.(20) However, the highest incidence of the disease was found among incarcerated individuals in Portuguese prisons, reaching a value of about 426 per 100 000 individuals. In figure 2 it is possible to see the incidence of the disease in the country for 2015, per district.(20)

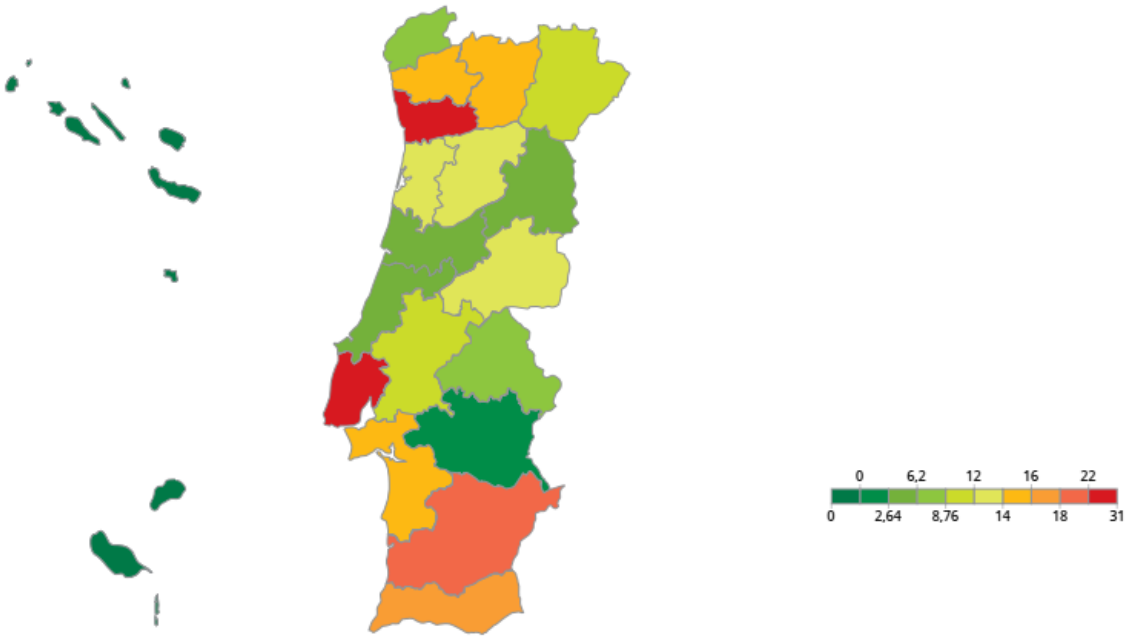


Figure 2 -Incidence rate of Tuberculosis in Portugal 2015, per 100 000 habitants. Source: A Saúde dos Portugueses, 2016 (20)

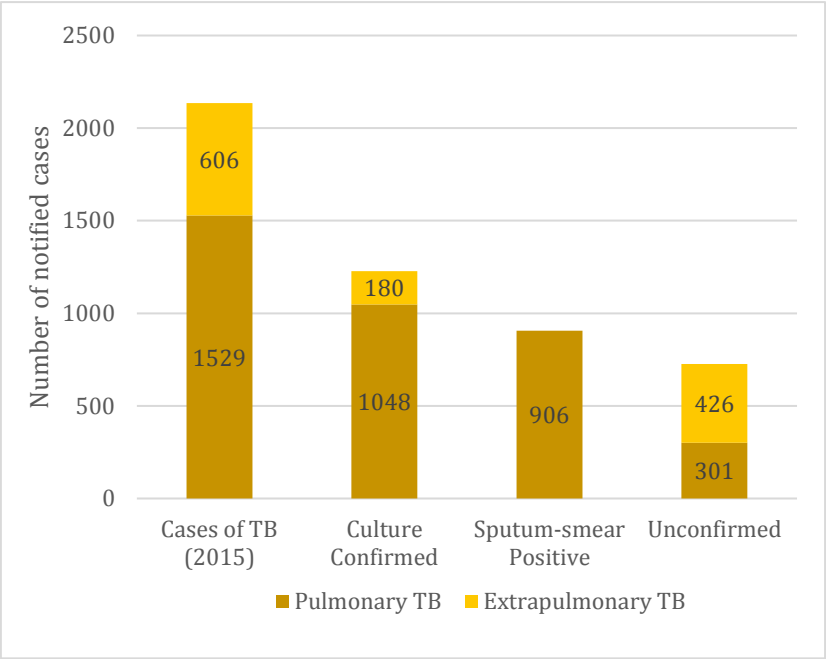


Figure 3 – Reported and confirmed cases of TB in Portugal (2015) – Graph representing the total number of reported cases of TB in 2015 in Portugal (2135), specifying extrapulmonary infection and pulmonary infection. It is also shown the reported number of cases confirmed by culture and confirmed by sputum-smear samples in the case of pulmonary TB. Finally, it is shown the number of cases not confirmed in microbiology laboratories. No data was found regarding histopathology. Data source: A Saúde dos Portugueses, 2016 (20).

1.2.2 – Microbiology and Pathogenesis

The genus *Mycobacterium* includes several pathogens causative of severe disease in humans and animals. It includes the *Mycobacterium tuberculosis* complex, comprised of several species capable of causing tuberculosis in humans (e.g. *M. tuberculosis, africanum* and *bovis* (rare)) or infecting other animals. (21) It also includes *M. leprae*, agent of leprosy, and *M. ulcerans,* causative of Buruli ulcers. Other mycobacteria are opportunistic saprophytes, causative of disease but not as common or severe, and include *M. avium.*(22–24)

Mycobacteria are rod-shaped bacteria which are primarily intracellular pathogens and obligate aerobes, able to survive and multiply inside macrophages in the lung. Another typical characteristic of mycobacteria is their slow growth.(25) They are divided into slow-growing and fast-growing mycobacteria. This division is corroborated by 16S RNA phylogenies.(26) Fast-growing species form colonies in about 7 days while the slow varieties can take up to weeks or months. *M. tuberculosis* and *M. bovis,* including the BCG strain, are of the slow-growing variety, making them harder to study.(26) *M. tuberculosis* takes to 20 days or more to be detected in culture.(27) The difference in times of growth has been suggested to be due to the presence of porin-like molecules on the wall of fast-growing species, which facilitate the uptake of hydrophilic nutrients.(28)

The wall structure of mycobacteria is extremely complex, it was illustrated in figure 5 based on several hypothetic descriptions of the wall found in literature. (29–32) Above the cytoplasmic membrane there is a layer of peptidoglycan (PG) similar to that of *E. coli* but with modifications in the residues of muramic acids. It is followed by the arabingalactan layer which a very large heteropolysacharide polymer covalentely linked to PG and the mycolic acid layer which rests above it (MA).(30) The MA layer is the outer-most layer, only topped by interspersed glycolipids and surface proteins. Mycolic acids are composed of α-alkyl-β-hydroxyl fatty acids with very long chains which can reach 80 carbons, being that *M. tuberculosis* is rich in C54 to C63 chains with C22 to C24 side chains.(31) The outer glycolipid layer is implicated in the aggregation of the bacteria, possibly through lectin-carbohydrate interactions, which manifests as a cord-like clustering of the bacteria (figure 4). (33)

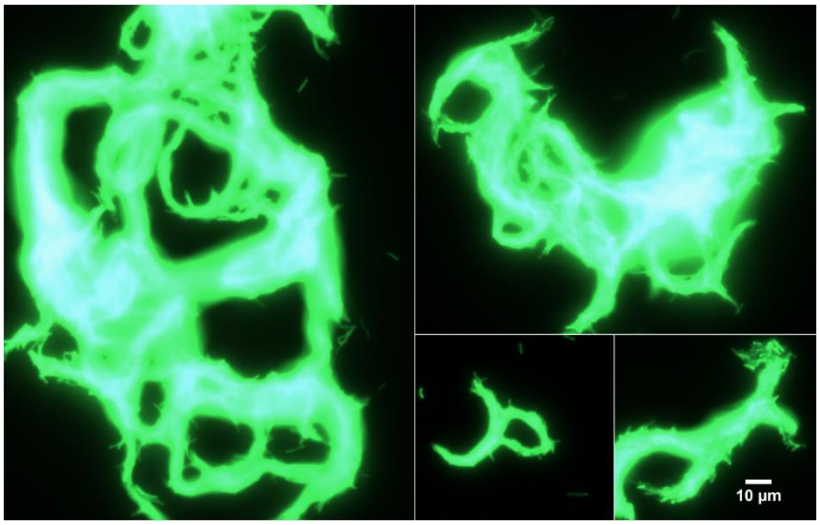


Figure 4 – *Mycobacterium bovis* (BCG) culture stained with Auramine O, observed by fluorescence microscopy at 1000x amplification, displaying a cording phenotype, with formation of typical aggregates, similar to cords.

Both in culture and in some cases of infection, it is possible to see two phenotypes of mycobacteria through microscopy diagnosis: single cell and cording aggregates. (34) The latter results in the formation of typical elongated aggregates of bacteria cells, aligned parallel to one another, in a structure like cords. (figure 4).(35,36) When present, this phenotype is strong evidence of TB and suggests that the infection is possibly caused by a more virulent strain (34) and it may lead to specific immune responses. (36)

Mycolic acids, together with free lipids, grant mycobacteria several beneficial characteristics; such as resistance to acids, alkali and many chemicals lethal to most non-sporulated bacteria. They can survive for months in surfaces protected from the sun and heat and can survive freezing and desiccation. Even more their extremely impermeable surface protects them from antimicrobial agents, such as antibiotics or even from the host's immune response.(37) This also means that mycobacteria are in general resistant to staining with common stains, such as Gram's stain. It is necessary to employ special staining procedures to be able to observe the bacteria under the microscope, such as the Ziehl-Neelsen stain. They are, for this reason, acid-fast bacteria.(38)

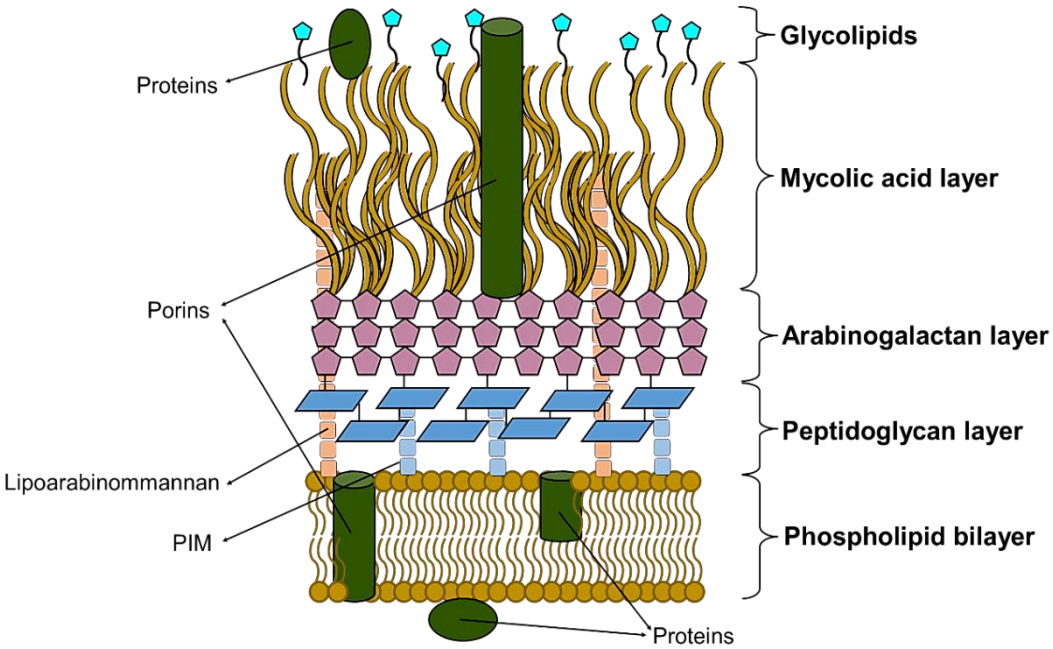


Figure 5 - Scheme of the cell wall structure of *Mycobacterium tuberculosis*

1.2.3 – Infection and Diagnosis

Humans are the only natural reservoir of *M. tuberculosis*.(25) Infection happens most commonly by the inhalation of aerosols from an infected person. Because of the body's immune response granules are formed in the lungs, resulting from the surrounding of the infection focus by several immune-system cells, which may contain the infection indefinitely if the host's immune's system is capable, resulting in latent infection, or if not, progressing to more severe infections.(39) The pulmonary cavities accumulate a high count of bacilli, which is then discharged in sputum, making this the usual biological sample for later culture and diagnosis. (25) After commencing treatment, patients quickly become non-infectious, which can be assessed by the decrease in number of bacilli found per sputum smear.(25)

The symptoms of pulmonary tuberculosis infection are intense coughing, bloody sputum, trouble breathing and chest pain. However, if the infection is disseminated, fever, night sweats, and weight loss occur due to the inflammatory response, but no coughing or generation of sputum is present and cavities are not found, being the miliary form of the disease.(25)

A good and quick diagnosis is crucial in the fight against TB. In general medical microbiology, several types of diagnostic methods and tests/assays can be applied to better help the clinician confirm what kind of infection is in hand: Direct examination techniques which include microscopy, immunofluorescence, other immunoassays and nucleic acid probes; Culturing of isolated specimens in selective media or conditions; Speciation/Microbe identification which includes biochemical and enzymatic tests, immunoassays and gene probes; Serodiagnosis which is the determination of the titer of specific antibodies; Susceptibility tests to understand which drugs are effective against the specific strain of the pathogen.(40)

In table 1, some of the most commonly used methods in the microbiological diagnosis and identification of tuberculosis are listed, mentioning the advantages of each method. Approved by

regulatory entities, recommended by the WHO and having been scaled-up and largely implemented are only a few of the relatively recent technologies. The BacT/Alert™ and Bactec MGIT ™ systems are widely used in high resource countries for liquid cultures and make the detection of growth easy, even in large numbers, as they are automated. (41,42) The GeneXpert™ MTB/RIF test is the most popular nucleic acid amplification test available because it comes as a cartridge based PCR system which allows minimal and easy manipulation of samples, capable of detecting rifampicin resistance genes and yielding sensitivities higher than that of microscopy. (43) In microscopy, the WHO has recommended that LED microscopes should gradually replace regular epifluorescence microscopes with the use of fluorescence stains, which have higher sensitivity. (44)

FIND reports several new coming products early in development and many others in late stages or already completed and awaiting scaling-up or market implementation, most being focused on molecular detection and drug susceptibility (45). Some of these tests are in transition to be adopted by national diagnosis networks, having already passed the approval of regulatory entities such as the WHO, FDA, etc.(42) A system based on the LAM immunoassay is already used in some places to rapidly test urine but is not fully endorsed by the WHO because it has low sensitivity and is only useful for HIV-related TB cases.(41,46) An improvement for Xpert™, called Xpert™ Ultra, is already approved by the WHO and in process to be distributed. This new Xpert™ Ultra is reportedly 17% more sensitive than the previous version.(42) Another important technology which is transitioning into the market is the Loopamp™MTBC system, a NAAT based on an isothermal DNA amplification, although reports have shown it to not be an adequate replacement to Xpert™ or even other NAAT's, due to its lower sensitivity and specificity.(42,47) The WHO suggests it could be used to replace standard BF microscopy or to follow up sputum-smear-negative cases.(45) Additionally, there are reports of attempts in automation of microscopy image analysis. In early development, the TBDx system, is reportedly capable of automatically reading up to 200 slides in 16 hours, with results "comparable to the most experienced microscopists". (45,48) Outside of microbiology diagnosis, X ray imaging is resurfacing as an important method to detect active TB cases due to the development of machine learning and computer-aided image analysis.(41,49) This technology is also currently being examined by the WHO (42), however, It is expected that culture, microscopy and NAATs should all remain important tools in TB diagnosis in the foreseeable future.

In a 2013 report it was revealed that in the European region, only 280 clinical laboratories for TB diagnosis used GeneXpert™ MTB/Rif compared to 9077 laboratories using microscopy and 1807 culture. (50) In Portugal, the number of laboratories using microscopy and culture are 54 and 52 respectively, while only 1 has access to GeneXpert™ MTB/Rif.(50)

Although in the 6 years since 2013 the landscape might have changed slightly, the importance of microscopy for TB diagnosis is clear and it remains the most widespread and commonly used method in routine diagnosis. The understanding and improvement of those methods continues to be an important field of research simply due to the scale and reach of their use and how they directly impact millions of people.

Table 1 – Diagnostic, identification and drug susceptibility methods for *Mycobacterium.* (25,51,52)

Method	Advantages	Disadvantages
Bright Field (BF) Microscopy with Acid-Fast stain	- Quick, results on the same-day - Simple to perform - Inexpensive - Still considered by the WHO as the primary diagnostic approach - Allows grading infection severity	- Low sensitivity (10 000 org./ml to detect), especially in patients with HIV - Unable to distinguish species of mycobacteria - Use of harmful or dangerous chemicals
Culture on solid media (Lowenstein-Jensen egg-based or Middlebrook agar-based media)	- It is the reference-method for isolation. - Detects as low as 10 organisms/ml - Allows for colony morphology observation and detection of mixed infections - Further possibility of speciation, strain ID and testing of antibiotics	- Growth can take up to 8 weeks - Requires biosafety level 3 laboratory
Culture in Liquid-broth	- Automated systems for detection of growth - Similar sensitivity and specificity as solid cultures - Further possibility of speciation, strain ID and testing of antibiotics - Growth in 1 to 3 weeks	- Inability to observe colonies or detect mixed infections - Automated systems are very expensive - Requires biosafety level 3 laboratory
Fluorescence Microscopy (Auramine O)	- Quick, results on the same-day - Simple to perform - Inexpensive - Increased sensitivity compared to BF - Observation at lower magnification - Allows grading infection severity	- Requires more expensive equipment (although with cheaper alternative if using LED Microscopy) - Use of harmful or dangerous chemicals

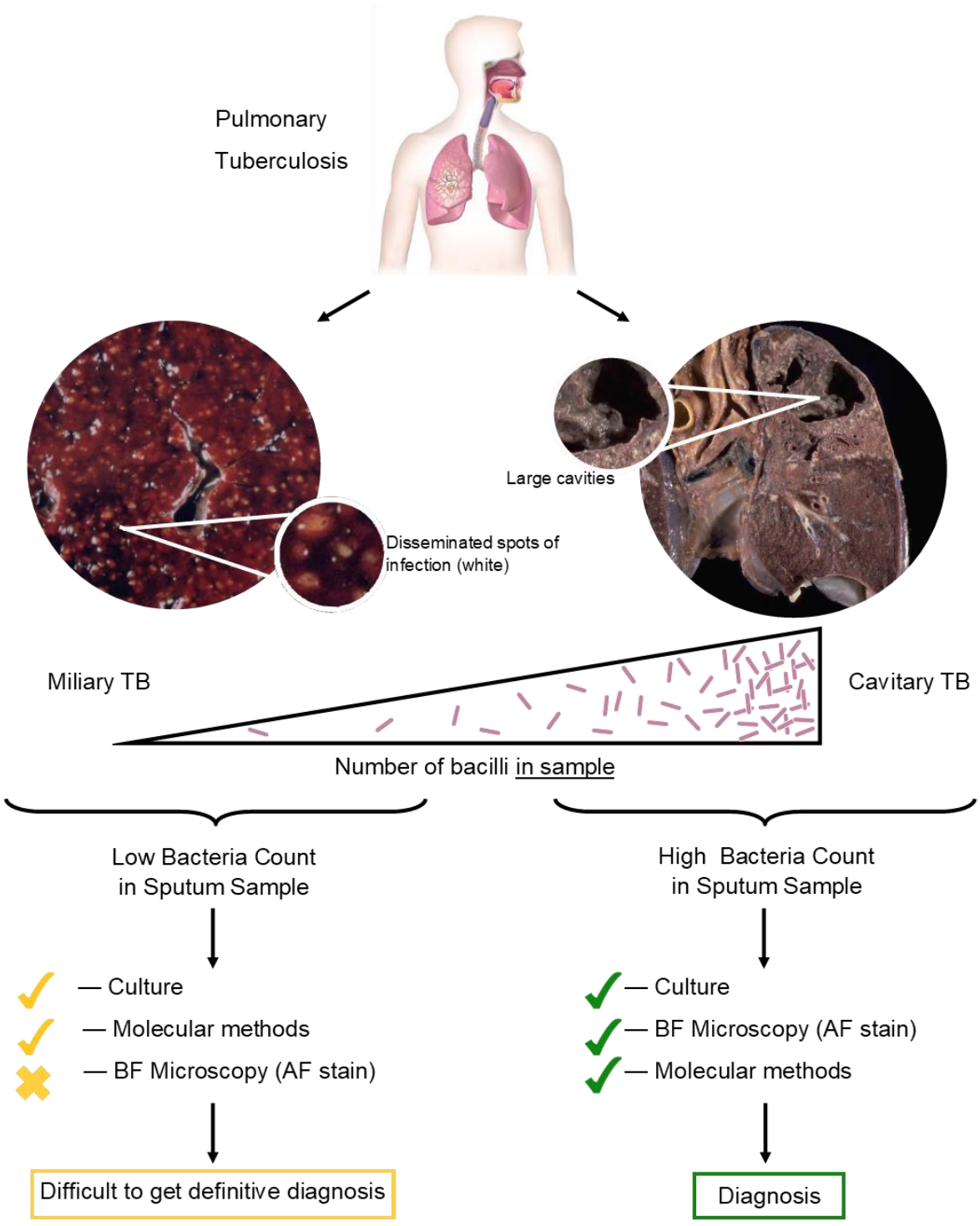
Mantoux skin test	- Gold standard for the diagnosis of latent infection	- Very prone to misleading results: False-negatives in very young or old people and false-positives in BCG vaccinated individuals
Nucleic Acid Amplification Tests (NAAT's)	- Quick, results on the same-day - Can be more sensitive than microscopy. - Distinguishes *M. tuberculosis* complex members from others - Can screen for Rifampin resistance genes	- Much more complex methodology - Does not distinguish viable or dead cells - The products based on this technology, which have been scaled up, are extremely expensive (e.g. Xpert™) - Many of the products created have failed preliminary tests and can be less sensitive and specific (e.g. Loopamp™MTBC)
Interferon-Gamma Release Assays	- Relatively high sensitivity - Can detect latent infection and recent infections - Requires only one clinic visit - In the future, could quantify infection load	- Expensive - Better than most other tests for immunodeficient patients
Urinary antigen detection (lipoarabinomannan)	- Possibility of point-of-care testing - Relatively high sensitivity in patients with untreated severe AIDS	- Very low sensitivity in other situations
High-performance liquid chromatography	- Results on the same-day - Highest sensitivity and specificity - Can identify species	- Very complex and expensive technology - Requires specialized technician just to interpret - Requires pure culture - Impractical in a clinical laboratory
Nucleic acid probes	- Very high sensitivity and specificity - Results on the same-day - Does not require amplification	- Need for high concentration of pure culture - Cannot speciate

Although some of these more advantageous methods are being adopted all around the world, delayed diagnosis is still a reality and it jeopardizes the success of the treatment as well as prolongs the exposure of the infectious individual to the population, effectively increasing chances of transmission. This delay is seen both in patients seeking medical care and in health care providers diagnosing the disease. This may be due to a number of different reasons, but in Portugal for example, the changes in the incidence of TB have been shown to be linked to socioeconomic fluctuations, rather than the better implementation of anti-TB measures.(53,54) This means that factors such as access to food and hygiene, wealth, education, alcoholism or drug abuse and even nationality or immigration can determine the quickness of diagnosis of a person. This is then reflected in the delay in patients.(55)

The delay of diagnosis by clinicians is of more interest to reflect upon in analyzing how the disease is diagnosed. If we think about TB we understand that in the scope of lung diseases it is one of the least common, which means that a clinician when confronted with the typical symptoms of a cough, trouble breathing or chest pain, might think of other illnesses such as lung cancer, bronchitis or emphysema, before TB, if the patient is not part of a risk group. Studies even show that, naturally, in patients from higher-incidence countries, diagnosis is quicker, showing that clinicians' awareness of the possibility of TB is fundamental in their investigation of the disease. Furthermore, if a patient is, for example, a smoker or has had previous lung issues, it becomes even less likely to think about a TB infection.(56,57) In addition to the relative rarity of lung TB, extrapulmonary TB (EPTB) is even more uncommon and with more generalized symptoms. As an aggravation to the diagnosis of EPTB, it often requires biopsies and histological examination. As a clinician, all possibilities of diagnosis will be exhausted before a biopsy is done.

Through continued efforts, the diagnosis of pulmonary TB has increasingly become more efficient and the future implementation of new alternatives, as explained in this section, will surely continue this trend, however it is in the case of EPTB diagnosis where development is slow and the existing methods insufficient and ineffective. This together with the inherent delay mentioned above, means that most cases of EPTB go unconfirmed, or worse, undetected. In the flowcharts on figure 6 it is shown a comparison between the different manifestations of the disease and what they imply for diagnosis.

As explained previously, pulmonary TB can present itself as a more localized infection, with varying severity of cavity formation. In this type of infection, it is relatively easy to detect and identify the pathogen, because the more severe it is the more bacteria are discharged in sputum and the easier to detect through imageology.(25) If the infection is disseminated in the lung, the number of bacteria is much lower in sputum samples.(25) When it is the case of EPTB not only are there the problems mentioned previously but biological samples for microbiology and histopathology are difficult to obtain. Furthermore, the methods used for pulmonary TB are not efficient in detecting TB on these kinds of samples, especially biopsies, for several reasons discussed in greater detail in section 3. The problems found in these situations are precisely the focus of this project. In figure 6-B it is represented the steps in the diagnosis of EPTB and how they seldom result in a diagnosis of TB.


Pulmonary
Tuberculosis
Large cavities
Disseminated spots of infection (white)
Miliary TB
Cavitary TB
Number of bacilli in sample
Low Bacteria Count in Sputum Sample
— Culture
— Molecular methods
— BF Microscopy (AF stain)
Difficult to get definitive diagnosis
High Bacteria Count in Sputum Sample
— Culture
— BF Microscopy (AF stain)
— Molecular methods
Diagnosis

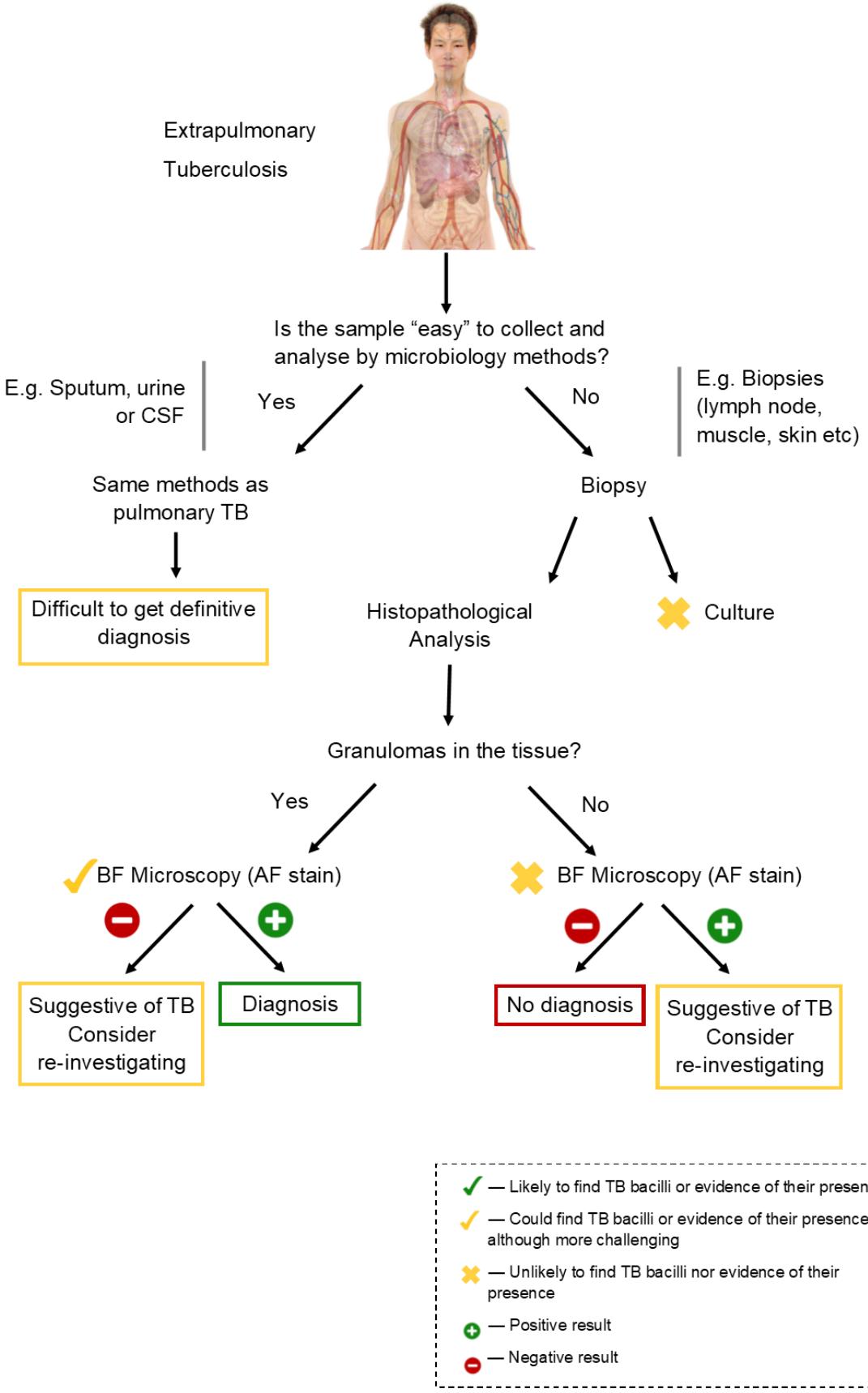


Figure 6 – Diagnosis of Tuberculosis Flowcharts – Flowcharts representing the process of the diagnosis of the different manifestations of TB, including the relative difficulty for each step and possible outcomes. In figure 6-A is represented the process of diagnosis of pulmonary TB and in figure 6-B of EPTB and the legend.

The issue with diagnosis of EPTB through biopsies is firstly that culture often is negative, as are NAAT's, which leaves histopathological analysis and AF stains as the alternative. In this case if no typical TB lesions are found, not only is it unlikely to find the bacilli, but if they are found, they will be in small counts, not confirmative of TB. This will require further re-investigation, dismissal of TB or possibly the need of new samples, which is difficult. The same occurs if lesions are found in the tissue but no bacilli are detected. Only when both the typical TB lesions and AF bacilli are found can it be considered a confident diagnosis of TB. In some cases, with only suspicions of the disease, treatment might commence without a confirmed diagnosis, because obtaining further biopsies might be too great a risk and cumbersome for the patient. The improvement of the sensitivity of AF stains for these instances will result in the detection of more cases and give stronger evidence of infection, preventing unneeded treatment but most importantly detecting cases which might have been dismissed. This issue is discussed further in part I section 3 – Diagnosis of Extrapulmonary Tuberculosis.

A Note on Coccidia (*Cryptosporidium* and *Cystoisospora*)

Another group of organisms to which AF techniques are invaluable diagnosis tools are Coccidia, in particular, *Cryptosporidium* and *Cystoisospora.* Typically present in the intestine, these infections are detected by analysis of stool samples which are normally preserved in formalin to stop the growth of other organisms abundant in stools and also to preserve the oocysts of the Coccidia.(58) Diagnosis by microscopy can often require concentration of the stool sample to increase sensitivity (reportedly 10 fold). In the case of *Cystoisospora*, the oocysts are large (around 20 µm to 30 µm length) and relatively easy to discern from debris, but *Cryptosporidium* may be overlooked due to its smaller size (3-8 µm), requiring concentration. For immunocompromised patients, the load of infection is usually high enough that such is not necessary.(58)

For the diagnosis of Coccidia, stains such as Giemsa or Jenner's stain were employed but eventually, a modified version of ZN was implemented which yielded better results, originally in 1981.(59) Of course the similar Kinyoun stain had already been described much earlier, in 1914, and the terms Kinyoun stain or modified ZN are now often used interchangeably.(60) The diagnosis reference-method is the modified ZN stain or the safranine stain although, in more resourceful countries, immunoassays, PCR-based methods, immunoserological assays, and direct fluorescence antibody assays are also performed.. Another stain, popular and successful for the diagnosis of these organisms is auramine O.(58,61) In the diagnosis of coccidia, an added complication is that only the interior sporozoites become stained, while it doesn't seem like the stain adheres to the "body" of the oocyst. This results in oocysts with variable degrees of staining and even some "empty" oocyst bodies, easily overlooked by the microscopist.(58) The acid-fast nature of *Cryptosporidium* could be explained by a waxy hydrocarbon layer contained in its wall, whose impermeability is dependent on temperature, and makes the oocyst resistant to decolorization, much like the mycolic acid layer in *Mycobacterium*.(62) One study found that *Mycobacterium* walls contain the fatty acids 16:0, 16:1, and 18:1 and glycopeptidolipids containing C28 to C24 which is a composition very much similar to that found in *Cryptosporidium*. (62,63)

2 – Acid-fast Stains in Microscopy Diagnosis of TB

2.1 – History and Principle of Acid-Fast Stains

Staining as a means of improving microscope observation in the distinction of bodies in the sample was a common practice for histology, to diagnose illnesses of the tissue. The transition of staining practices into microbiology was the doing of Karl Weigert, who believed that only by staining the small cells observed could one differentiate them and identify them unequivocally, among other similarly shaped debris in samples.(64) At that time, in the mid-19th century, scientists like Weigert, Wagner, Billroth, Ehrlich and many others were dedicated to finding the ideal staining methods. One of the greatest contributors to the field was, as discussed previously in this document, Robert Koch. After Koch first managed to successfully stain an Acid-fast organism, several scientists took interest in improving as well as simplifying this method, for it quickly became important to clinicians in diagnosing patients with tuberculosis.

Besides Koch, it could be argued that Ehrlich was the most important contributor to the acid-fast stain. He figured out the importance of the alkaline quality of the primary stain to penetrate the tubercle cell wall and found that aniline water as a carrier was more efficient, for it allowed a more aggressive decolorization without removing the stain from the cells, hence better differentiating the bacilli from the background.(64) Funnily enough, today the stain is known as the Ziehl-Neelsen stain, even though both Franz Ziehl and Friedrich Neelsen introduced only small improvements to the method later on.(2) The stain used today was, therefore, a product of numerous modifications by several scientists. The main modifications since the Koch discovery are summarized in table 2.

Table 2 – The beginning stages of the acid-fast stain Ziehl-Neelsen in chronological order.(64–66) After Koch's method, subsequent modifications introduced are underlined.

Scientist	Stain	Decolorizing Solution	Counterstain	Appearance
Robert Koch – 1882 (11)	10 parts Alcoholic Methylene Blue in 0.2 parts potassium hydroxide (10%) - Dipped in solution 1h at 40ºC (or 24 RT)	Rinsed in aqueous solution of vesuvin (bismarck brown)		Blue bacilli in brown background
Paul Ehrlich – 1882 (67)	11ml Methyl Violet or Fuchsin in 100 ml Aniline Oil - 15 to 30 minutes - Dipped in heated solution	30% nitric acid	yellow or blue dye	Pink/violet bacilli in a yellow/blue background
Franz Ziehl – 1882 (68)	Methyl Violet in Carbolic Acid 5% - 15 to 30 minutes - Dipped in heated solution	30% nitric acid	yellow or blue dye	Pink/violet bacilli in a yellow/blue background

Rindfleisch – 1883 (69)	Methyl Violet or Fuchsin in Aniline Oil - 15 to 30 minutes - Heated slide with solution	30% nitric acid	yellow or blue dye	Pink/violet bacilli in a yellow/blue background
Friedrich Neelsen – 1883 (70)	Fuchsin in Carbolic Acid 5% - 15 to 30 minutes - Heated slide with solution	30% sulphuric acid	yellow or blue dye	Pink/violet bacilli in a yellow/blue background

Koch's methodology of smear preparation and the ZN staining technique were both revolutionary at that point in history, for society was plagued with tuberculosis, and other infectious diseases and a simple diagnostics tool was of dire need. Hence it became common practice and the reference-method at the time. Kinyoun in 1914 introduced the "cold" ZN stain, by increasing the concentration of fuchsin and therefore making heating unnecessary.(60) This stain, as mentioned before, is now the gold-standard for *Cryptosporidium* and *Cystoisospora* diagnosis.(60) Later several modifications were introduced to allow better visualization of other AF organisms. Fite, in 1947, created a method which better stains the elusive *M. leprae* in tissues, by using a gentler method of mounting.(71) Stamp et al in 1950 developed the Stamp method for Brucella and Bartonella (72) and in 1926, Dorner created a method to detect bacterial endospores.(73) A method which was not adopted in general laboratories but has an interesting approach was that of Ellis and Zarowarny, who in 1993 removed phenol from the protocol and used LOC detergent instead.(74) It wasn't until the first half of the 20th century when a new development arose in acid-fast staining, besides ZN and variants.

In the 1930's the tuberculosis epidemic in Europe and America was far past its peak. Although still a serious concern, the introduction of better sanitation, diagnostics and treatment options had made the incidence of the disease subside. In a report from that time it was observed that from all of the smears sent for analysis, no more than 15% were positive for *M. tuberculosis.* For each sample, the microscopist had to spend at least 15 minutes scanning the smear in the microscope, due to the necessity of using oil-immersion lenses with amplification of 1000x total. This meant that only a very small portion of the sample was seen at once and given the fact that a patient's sample can be positive but contain relatively few bacilli, it was imperative to scan the entire smear. This created a heavy workload for the microbiologist in the laboratory.(75) In the first decades of the 20th century, there was a growing interest surrounding a new technology. During the previous century, scientists had documented time and again the curious phenomenon of light emitting from objects. Physicists studied and uncurtained this mystery and by 1913 the Zeiss and Reichert companies were putting out in the market the first fluorescence microscopes. (76)

Fluorescence of organic materials had already been shown by biologists and curiosity about possible applications of this technology were increasing. Soon, dyes with fluorescent properties were being used to study all kinds of tissue in men. There are reports from 1917 of Kaiserling observing tubercle bacilli in the fluorescent microscope, due to their intrinsic fluorescence. However, it was in 1937 that fluorescence microscopy by use of dyes in microbiology was first published. Hagemann showed he could easily make viruses visible with primulin and berberine to visualize lepra bacilli. He later applied the same method to tubercle bacilli and found that it was possible to detect them much more easily, with an amplification 5 times smaller than needed for the traditional ZN stain.(75,77) The dye used by Hagemann to visualize *M. tuberculosis* was not berberine, instead, he used auramine O. This was the first ever protocol for auramine O staining of sputum, represented in figure 7.(75) Besides the higher concentration of phenol, changes in the decolorizing solution and absence of counterstain, it resembles what is still done today.

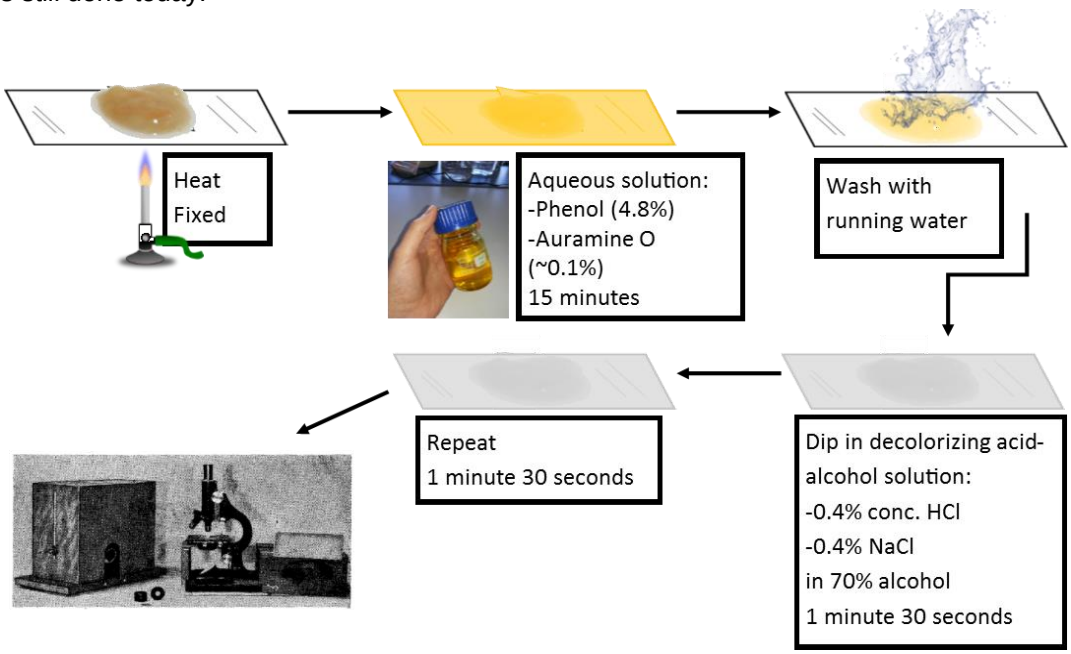


Figure 7 - The first described protocol for the use of auramine O as a fluorescent stain for sputum of a tuberculosis case. (Microscope photography from article 75 in reference).

Hermann in 1938 described the technique he used with auramine O, which much resembles what Hagemann had done, save for some differences in concentrations and times but most importantly with the use of a counterstain, potassium permanganate and methylene blue. (78) The observations of Bacilli in the fluorescence microscope was described as very bright green/yellow rods with a beady interior.(75) Later another fluorescent stain method with Auramine was proposed by combining it with Rhodamine B, known as the Truant stain, although Truant only proposed this method in 1962 and there are reports of it being used long before that, in 1946 and following years by Hughes, Cruickshank, Gray, and Wilson.(79,80) Graham in 1942 even mentioned Rhodamine B but never applied it in practice. The supposed advantage of Rhodamine B is only that it stains the target with a reddish tint, while artifacts fluoresce only in green/yellow. (78) Nowadays there are automated machines for the staining of smears, both for ZN and AuO and even Gram's stain, which further makes these "old" methods more advantageous in clinical settings.

Microbiologists have documented several organisms and biologic materials which are acid-fast (AF), or which have some AF characteristic useful for their identification. This work mainly centers around *Mycobacterium tuberculosis*, but there are several biologic materials and organisms which are AF (81): coccidia (58,82); spores of many fungi (83); Spores of *Bacillus* (84); human sperm (85); embryophores of *Taenia saginata* (86); hooklets of *Taenia echinococcus* (87); *Corynebacterium* or their inclusions (88); Nocardiaceae (89,90); DNA (81); keratin after in situ oxidation.(91)

Today there are more alternatives for fluorescent staining. Nucleic acid fluorescent dyes, such as SYBR Gold, DAPI, the TOTO family and others, which are cyanine dyes, are particularly good for fluorescence studies because they have a high affinity for DNA or RNA, emit very bright light and are very well characterized. Other DNA/RNA dyes can be Phenanthridines, Acridines, Indoles and Imidazoles.(92) The main disadvantage of these dyes is the extremely high price, which makes them impractical for any application other than research.

The basic principle of an AF stain of mycobacteria is the application of a primary dye, such as fuchsin, which by heat or use of solubilizers such as phenol, penetrates the mycobacteria and dyes them pink. Then, after a washing step to remove excess dye, an acidic alcohol solution is applied which very effectively removes the dye from all the sample with the exception of mycobacteria, whose wall resists penetration by this solution. This characteristic, as was said previously, is what grants them the name of acid-fast. In the end, and optionally, a counterstain like methylene blue can be applied which will dye all debris and other organisms blue, increasing the contrast between the pink mycobacteria and blue objects. In figure 8 is represented a simplified ZN staining procedure, showing the basic principle of AF stains.

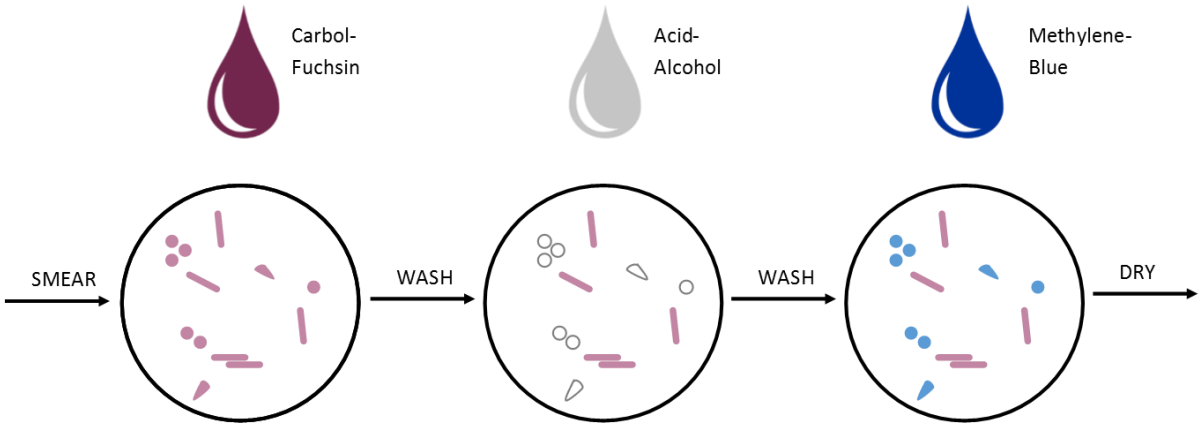


Figure 8 – Simplified scheme of the ZN stain, illustrating the basic principle of an AF stain. Certain steps, concentrations and specifications are omitted.

2.2 – Characteristics of auramine O

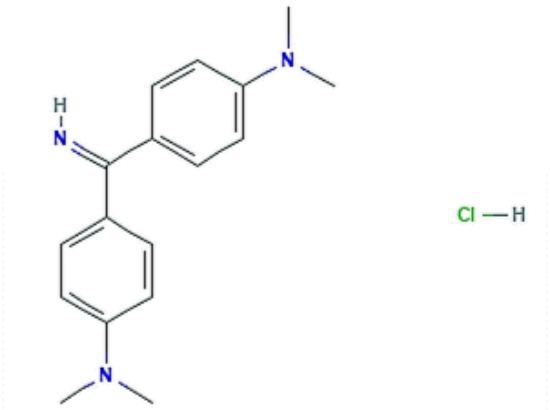


Figure 9- Chemical structure of Auramine O (IUPAC: 4-[4-(dimethylamino)benzenecarboximidoyl]-~{N},~{N}-dimethylaniline;hydrochloride)) as defined in PubChem.

Auramine O is a diarylmethane dye, also known as basic yellow 2 (figure 9). It was first synthesized in the 19th century, in 1883 by Caro and Kern, who took 4,4'-bis(diamethyldiamino)benzophenone and heated it at 160ºC with ammonium chloride and zinc chloride, introducing an imido group, originating the auramine base.(93) Only the auramine hydrochloride salt has a yellow color and it was first used as a textile dye, a paper dye and in burning incense, although it has since been linked to increases in metastatic capacities of lung cancer.(94,95) Its color is the result of atomic resonance of the 7 atoms in each of the dimethylamine chains connected by the amino group. If acetylated, the resonance takes up 11 atoms and creates a deep violet color.(94)

Regarding its spectral behavior, auramine has an excitation wavelength of 438 nm in water, emitting at about 505 nm. Its absorption spectrum has two peaks, at around 370 nm and 432 nm, as shown on figure 10.

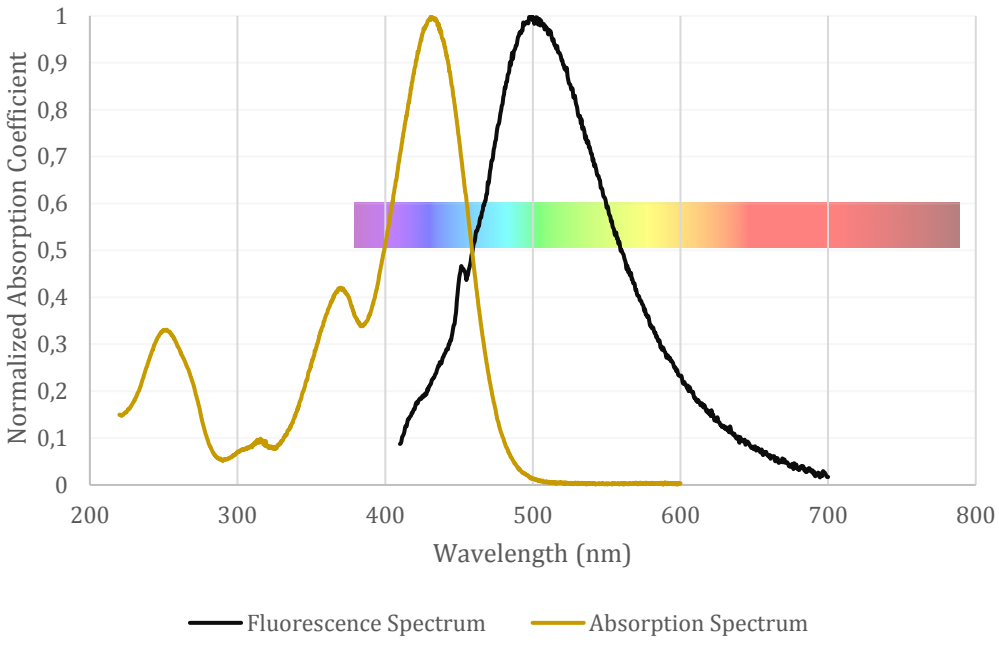


Figure 10 – Absorption and emission spectra of auramine O in water and glycerol, respectively.(96,97)

2.3 – Acid-Fast Stains Binding Targets

Traditionally the target of AF stains was believed to be mycolic acid. In 1941 Oscar W. Richards published in Science magazine a report of the process of extracting and staining mycolic acids, first done by Stodola *et al.* in 1938.(98) They first found that the only acid-fast material of tubercle bacteria, of all that they had extracted, which stained with AuO and resisted decolorization "was a hydroxy acid of very high molecular weight which was designated by the term 'unsaponifiable wax'". The same resistance to decolorization was not found for ZN. (99) From that point until today, most people believe AuO is a mycolic acid stain. However, there have been many publications disputing that fact or at least correcting it. There have been reports of AF stains binding to the mycolate, even on a mole to mole ratio, but also that that binding does not account for the overall fluorescence.(81)

It is known that the chemical composition of the mycolate and its order on the wall influence staining. For example, acetylation of the hydroxyl groups destroys acid-fastness. This may seem like supporting evidence of the mycolate theory.(100) It has also been discussed and shown how physical damage to the wall, compromising its integrity, result in loss of acid-fastness. If the wall were responsible for the staining, damage to it should not remove the stain entirely. This evidence suggests the stain is contained in the interior of the cell by the wall and, in particular, mycolate.(101) It has been suggested that mycolic acids act not as the target for binding but as a barrier which blocks the decolorizing solution from removing the stain. Furthermore, it has been hypothesized that a formation of a complex between the dye and mycolate further impermeabilizes the cell. (81) This might explain why fuchsin in ZN, although it scarcely stains the mycolate, still stains *Mycobacterium* and may also account for why other bacteria with mycolate on their walls don't resist decolorization because the complex structure is different. (81) More recent studies have shown that mutants with the disruption of KasB and KasA genes, involved in mycolate synthesis, result in loss of acid-fastness, explained as a result of the loss of compactness of the mycolate in the wall. Several other genes have been studied to similar results. (102) This is a clear indication of mycolate importance for this phenomenon. It seems therefore that mycolic acid, although not directly responsible for the staining of the cells, is, in fact, responsible for the acid-fast characteristic of resisting acid-alcohol decolorization. Even so, the question remains of what truly is the main binding target of fuchsin and AuO inside the cell, if any.

One report suggests that the dye does not particularly adhere to one specific target and merely is dispersed in the cytoplasm, occasionally forming the typical beaded structure on the interior. The same report also denotes that this effect may be reversed which indicates that no cell structure is causing it. (83) This is contradicted by reports which show auramine O as binding to DNA/RNA and proteins. These suggest even that AuO binding to DNA increases its fluorescence moderately and binding to horse liver alcohol dehydrogenase increases it dramatically.(103,104) Another study used AuO as probe to access the binding of drugs to a human protein(105). Hänsheid *et al.* have shown that stained cells have a non-uniformly stained pattern, already previously seen, but that regions of the wall were largely unstained.(106) The fact that auramine O is also shown to have carcinogenic abilities suggest it is binding to DNA. Still direct and concrete evidence that AF stains bind nucleic acids is still lacking.

2.4 – Auramine O *Versus* Traditional Stains and Other Methods

Any sputum sample collected in the hospital when there is a suspicion of TB is sent for culture and for smear/stain preparation. The WHO states that the positive predictive value of acid-fast stains for TB is dependent on the prevalence of the disease, and in low incidence countries culturing makes up for the lower specificity.(107) With the advent of fluorescent AF stains, such as auramine O, many comparisons were drawn between it and the classic ZN. In 1939, Didion compared ZN, AuO, culture and animal inoculation and found that inoculation was the most sensitive while the values for culture, AuO and ZN were 92%, 87.2% and 82.2% respectively. Many subsequent studies reported similar results, almost always placing AuO above ZN.(78) A study from 1944 states that in 300 smears, none positive by ZN was negative by AuO, on the contrary, 3 which were positive by AuO were negative by ZN even after repeated evaluation.(75)

Factors influencing the sensitivity of the ZN stain are the quality of the microscope, reagents and sample and the expertise in preparing and observing the smears. All of these factors generate greatly variable sensitivities, reported to fluctuate between 20 and 80%.(102,108) It has been shown that ZN stains can be observed under the fluorescent microscope to increase sensitivity.(109) The "cold" ZN stain, Kinyoun, has the advantage of making heating unnecessary, although it has been reported to lower specificity, or yielding more false-positives.(102) The most reported and indisputable difference, which must not be ignored, is the great reduction in time of observation with the use of AuO. (78) To exemplify this, a practical demonstration is drawn below. For demonstration purposes we will consider that positive results are found immediately upon observation, meaning that the remaining smears are observed for the recommended time.

Table 3 – Practical example of the benefits of AuO in saving observation time.

	Number of Smears	Number of positives (15%)	Amplification	Min. recommended time of observation (106)	Total time spent
ZN	50	8 (42 negative)	1000x	4 minutes	2h48min
AuO			200x	1 minute or less	42 min

These values are based on what is the typical amount of smears analyzed on a daily basis in a Lisbon hospital, as reported by Hänsheid *et al.*(106) Not only does laboratory staff have limited time to observe smears, meaning the minimum recommended time of observation is rarely met, but also they become more subject to fatigue the longer they are on the microscope, which reduces sensitivity.(108) Thus, it is logical that two extra hours of observation, in the given example, will not only reduce the quality of the diagnosis but also impede the furthering of other tasks.

Although not routinely done in all diagnosis laboratories, the AuO stain for fluorescence observation of *Cryptosporidium* has also been proven to be largely more sensitive than the modified ZN.(82,110) In conjunction with the fact that this method allows much quicker and easier observation, it ought to be a staple in the diagnosis of the disease. In addition, it has been suggested that AuO has a

higher affinity for the oocysts, seeing as it resists decolorization for much longer, while fuchsin in ZN is removed more easily.(110)

All in all, AuO seems like the better option for all settings, it can even be employed by color-blind people, unlike ZN. In a review from 2006, Steingart *et al.* took 45 studies and concluded that overall AuO is more sensitive and has similar specificity to ZN.(111) Even so, it comes with some disadvantages. Background and debris staining can reportedly generate more false-positives, although the use of a counterstain can mitigate this issue. Another issue is the use of toxic phenol and the reported carcinogenic capacity of auramine O.(95)

Other methods, summarized previously on table 1, draw several advantages over traditional stains. This includes nucleic acid amplification tests and immunoserology tests or assays. Many of them have higher sensitivities and are less dependent on user performance. Others are far more adequate at diagnosing immunocompromised patients and some can even be done at point-of-care. There are even methods which already speciate and do susceptibility tests simultaneously. However, those methods are often much more expensive to conduct, either in each application or because of the devices required to analyze each sample. Also, these tests often require more specialized personnel. This is an issue for low-resource laboratories. It is important to distinguish here that by low-resource laboratories one must not understand simply laboratories from poor countries. Often in poorer countries the funding for the fight against TB is much higher, for the incidence and prevalence also are higher, and that is where philanthropic foundations act. Those laboratories can many times afford methods such as GeneXpert™. Portugal is a great example of a developed country, which although it cannot be called a rich country, certainly isn't poor. Regardless, there are no significant foreign investments in TB diagnosis laboratories in hospitals and public funding is limited which means they are often constrained to more classic methods such as staining, culturing and in some cases simple PCR. This is the case for many countries, but it is also true that there are many places in sub-developed poor countries with access only to ZN staining, which are likely the majority. It is in these two cases where AuO could have the largest impact.

2.4.1 – Costs and ease of use of auramine O stain

It is necessary to understand if it is feasible to bring the AuO stain as an alternative to ZN in places where it is most needed. The price for a typical PCR or ELISA method can range anywhere from 2 to 10 euros or more, meaning it is extremely impractical to implement in any clinical pathology lab, let alone in a low resource country, long term and as the first line of diagnosis. That being said, staining, including the glass slide, is only around 2 cents.(112) Another advantage in the implementation of AuO is that nowadays there are automated staining machines which, in the case of low-resource laboratories of countries which are not poor, can be purchased with only the need for an investment on the machine and maintenance. After that, the only expense is the smear and stains. This is the case of Portugal where this technology is used, for example, in the Santa Maria Hospital from Lisboa and the Centro Hospitalar Universitário from Algarve. The machines save time and labor for the laboratory staff who can work on other tasks while the staining cycle is ongoing.

In terms of ease of use for laboratories without automated systems, AuO is not a complicated protocol. In general, for each run of the protocol, the time spent can be around 20 to 30 minutes, depending on the number of smears being stained, the type of fixation and drying and also the expertise of the microbiologist. The biggest inconvenience might be only the pungent smell of phenolic AuO solution, the need to dispose of the noxious chemicals and that it should be done in protection from sunlight and then the smears kept in the dark.

2.4.2 – Fluorescence Microscopes and The Inexpensive Alternative (LED Microscopy)

A potential disadvantage of the use of AuO could be that for the examination of the smears a special type of microscope is needed. Being that it is a fluorescent stain, a regular optical microscope does not suffice. The cost of a fluorescence microscope can go up to tens of thousands of euros, not to mention the price of the lamp. Most fluorescent microscopes use a lamp to generate a wide spectrum light which is then narrowed by filters. These lamps are usually mercury or xenon arc lamps, which can cost up to 300 euros and have a lifetime of only 200 hours. (113) Additionally, these gases have hazardous effects if freed on the environment and they also produce strong UV light. Finally, these lamps are sensitive and have a warming period and a cooling period, meaning they cannot be used reliably until after about 15 minutes of being turned on and can only be re-lit after an hour of being shut down. These maneuvering periods can represent in practical terms a great percentage of use, within the lifetime of the bulb, and are also inconvenient in the routine of a diagnostic laboratory. Nonetheless, these microscopes are present in most major health centers in developed countries and are an essential part of diagnosis and research.

For underdeveloped countries, however, the use of these microscopes is unrealistic. Not only are the costs too high to withstand, but they are also very sensitive devices which cannot be used in the field without special care and conditions. The alternative is a technology which is widespread in all areas of electronics, the LED. Today there are many LED epifluorescence microscopes available in the market. LED as a light source achieves basically the same quality of images and produces light in the desired wavelengths. Furthermore, these microscopes can be smaller, lighter and more robust, being more easily used in the field. Besides, the LED has a lifespan that can reach 30 000 hours and can be used cordless, with only a battery, and turned on or off as needed. These instruments may still be relatively pricey, but there exists a simple, mountable device, capable of attaching to a regular optical microscope which contains an LED lamp and effectively transforms the basic microscope into an epifluorescence one (figure 11).(113) With the adoption of the LED epifluorescence microscope, it could be possible to take AuO staining to any region.

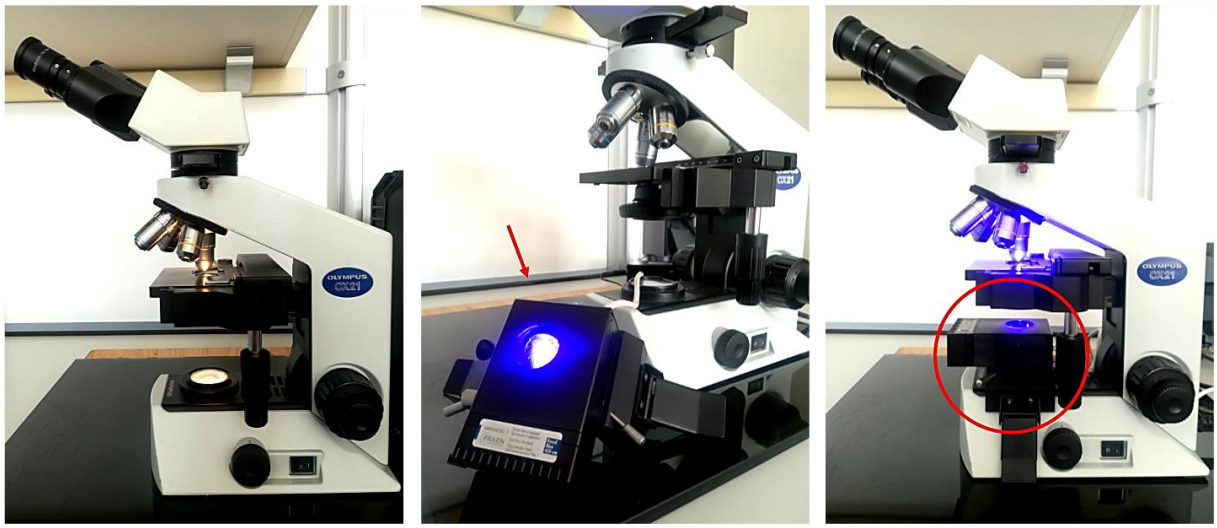


Figure 11 –Mountable LED microscope lamp - Photograph of a regular binocular optical microscope (LEFT) with an LED lamp mount disassembled (MIDDLE, red arrow) and assembled (RIGHT, red circle).

3 – Diagnosis of Extrapulmonary Tuberculosis

The case of diagnosis of a mycobacteria infection in histology is very particular in its process, thus it will be discussed individually. Not all Mycobacteria infect the lungs and cause the typical disease manifestation, as was mentioned before. Extrapulmonary Tuberculosis (EPTB) is more common in immunosuppressed individuals but it occurs in healthy people as well. Although more commonly found in the pleura and lymph nodes, mycobacteria can infect any tissue from the eyes to the kidneys.(31,114) It accounts for about 15 to 20% of all TB cases and only 20 to 25% of suspected EPTB cases are explicitly confirmed. Many treated patients begin treatment without proper confirmation of TB.(115) Diagnosis is challenging for several reasons but mostly because of the paucibacillary nature of the infection and because it manifests in several different ways depending on where it occurs.(115)

In the case of infection of tissues, it is often required the collection of biopsies for further microbiological analysis and for histopathological analysis. Often these can require invasive procedures to collect the necessary tissue or liquid. (115) Upon collection, the tissue becomes immediately exposed to deterioration and to combat this it is placed in a fixative solution which penetrates and preserves it.(116) There are several options depending on the purpose for the sample, such as glutaraldehyde, osmium tetroxide and formaldehyde.(117) The latter is most commonly used in processing tissue for paraffin embedding. It is usually available in a phosphate buffered solution with neutral pH (10% formalin), ideal for preserving proteins' structure and immunological epitopes but which does not interact with lipids and therefore is not ideal for fixing membranes. (118) It could be speculated that formalin allows the mycobacterial lipidic wall to lose its integrity over time, but it could also be argued that it does not itself degrade the wall. The sample is left in the fixative solution for 6 to 24 or even 48 hours, which ensures the preservation of the tissue in several ways: ending cell metabolism, preventing self-degradation and enzymatic lysis, hardening by cross-linking and protein denaturing and finally by killing all cellular pathogens and inactivating viruses.(116,118,119) In the case of EPTB, that also means it effectively kills mycobacteria. Then tissue is either cryopreserved or preserved in a waxy substance, paraffin. For the latter, a very aggressive process is employed which involves alcohol and xylene to remove water from the cells and allow paraffin to penetrate the matrix. In the end, this generated block of tissue is usually cut in 3 to 5 micrometers thick sections in a microtone, variable depending on the objective, and placed on special glass slides which are positively charged for better adherence.(119) The sections have to then go through the inverse process to remove the paraffin and after that, they can be stained and observed. (figure 12) (116)

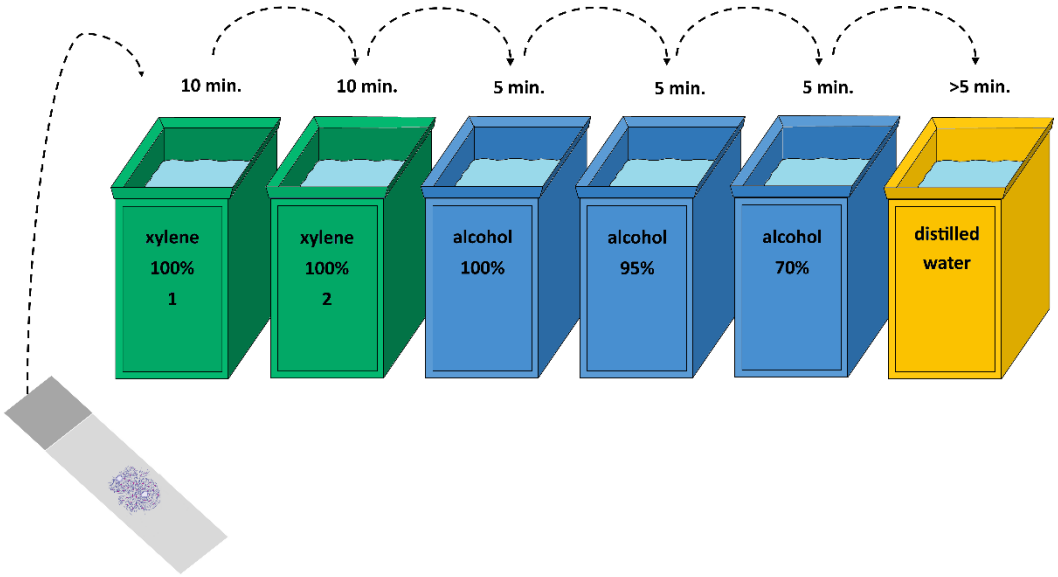


Figure 12 – Xylene deparaffinization steps in histological processing.

In the suspicion of an EPTB infection, there is direct diagnosis through culture and ZN, often unsuccessful, requiring processing of the tissue for histopathological analysis and acid-fast staining.(115) In general, the methods described previously on the section 1.3.1 can be applied also to the diagnosis of EPTB, but their usefulness does not always hold up in such cases. Histopathological analysis is the overall reference-method for diagnosing EPTB, but often it isn't possible to collect biopsies, or the risk of the procedure is too great. Furthermore, the granules which appear from an EPTB infection can be mistaken from other pathologies.(115) Cytology from fine-needle aspirates is oftentimes used instead because it is easier to collect this kind of sample, but it has a low specificity.(115) The ZN stain is considered to have a low sensitivity in tissue, which is a result of the need of a bacterial dose of above 100 cells per gram of tissue.(115,120) The Truant and regular AuO stains are considered more sensitive, but as mentioned, an issue with histology diagnosis of TB is not just the low number of bacilli usually present in the tissue, but that unlike in sputum, they are localized in certain regions in the tissue, not evenly dispersed, and may be harder to find.(121,122) Culture requires unfixed tissue and also it demands that there should be a decontamination processing of the tissue, which in turn may also compromise the few mycobacteria in a sample.(115) There have been several reports indicating the success of methods such as PCR and Immunohistochemical techniques, but these always come with extra costs, labor and many times can't fully replace staining.(121,123,124) One more recent study from 2015 showed clearly that PCR does not deliver sensitivity nor specificity above the traditional methods and should not replace them in histology diagnosis.(125) In America, PCR is never recommended as a sole method and should only be used as further confirmation in positive cases. (115,126) The British National Health System also recommends PCR to not be used singularly in samples other than sputum. (115,127)

It is important to understand why it is so difficult to obtain good and reliable results in the diagnosis of EPTB through microscopy, so that improvements can be made. One study points to formalin and xylene, used in the treatment of tissue, as the culprits for the low sensitivity of AF stains.

Fukunaga *et al.* suggest, as has been suspected previously, that xylene, a powerful organic solvent, and formaldehyde could be destroying mycolate, which they assume is the target of staining. The researchers indicate the higher sensitivity of the Fite stain, in comparison to ZN, as supportive evidence, since it uses less xylene in the mounting process.(128)

II – The Problems and Objectives

The overall mission of this work was to contribute to the improvement of the diagnosis of TB, in particular EPTB, and possibly of other diseases or research which indirectly benefits from improvements on AF stains and improvements on histology procedures.

This project is concerned with three main issues: what are the optimal staining conditions for auramine O and can it be improved for TB diagnosis?; which cellular component of mycobacteria, if any, does auramine O target and stain?; what is the cause of the low success of EPTB diagnosis with AF stains in histology, can something be changed to improve it?

What are the optimal staining conditions for auramine O?

As discussed in section 2.1 of the introduction, the methodology of the AuO stain has remained mostly unchanged since the 20th century. Although it presents as a better alternative for the diagnosis of TB through AF stains, there hasn't been any work done on trying to adapt and optimize the stain further for this purpose and for economic and safety purposes. Because microscopy is still extremely important and not replaceable by other methods, especially for EPTB diagnosis, we believe it is important to research this topic.

We intend on exploring the AuO stain in all its steps. What are the ideal concentrations of reagents, times of the steps and composition of the solutions? Are they the same for mycobacteria and Coccidia? Are they the same in histology sections?

What is the auramine O binding target?

To be able to improve and pinpoint the issues that might arise in working with AuO in TB diagnosis, one must understand completely how the stain itself works. It has been a longstanding belief that AF stains target mycolic acids on the bacteria cell wall, but evidence is dubious and evidence which supports other targets has been published by several sources. Regardless, no consensual answer has been reached.

It is our objective to further explore the binding target of auramine O, to truly understand the role of mycolic acid and the nature of AuO stain. Is it a mycolic acid and/or nucleic acid stain?

What are the causes of the low success of EPTB diagnosis?

The main objective of this project is both to understand the causes of the low success of EPTB diagnosis and to devise a solution for those issues. Microscopy through AF stains is extremely important and often the only way to diagnose EPTB, which makes this the most crucial part of this research project.

We mean to further test the hypothesis that both xylene and formalin could have negative effects on the structural integrity of mycobacteria, which in turn would mean the bacteria in infected tissues processed in histology become damaged and more difficult to detect. Furthermore, we raise a second issue, which is to understand if the thickness of tissue sections created in histology could also be damaging some of the mycobacteria in the sample and making them undetectable, further lowering sensitivity of later diagnosis.

Having learned which are the main issues in the processing of tissue samples, then alternative methods will be explored, with the objective to mitigate the negative effect on mycobacteria diagnosis and potentially to originate improved methods for general histology.

III – Materials and Methods

Mycobacteria Culture and Samples: ***Mycobacterium bovis****,* BCG strain (Bacillus Calmette-Guérin), were cultured from a frozen stock at -80°C. The stock was thawed at 37°C and inoculated in Midlebrook 7H9 medium (Difco™ – Detroit, MI, USA) supplemented with pooled human blood serum at a 15% concentration. The blood serum was inactivated at 50°C for 30 minutes. The culture was incubated in culture flasks (EasYFlask™ 75cm^2 Nunclon™, *ThermoFischer Scientific* – Waltham, MA, USA*)* at 37°C, 5% CO_2, until log-phase. Culture was then preserved in half formalin 10%(v/v) neutral buffered (*Enzifarma* – Lisboa, Portugal). **Stool samples** infected with coccidia were donated by the clinical pathology laboratory of the Hospital Santa Maria, Lisbon. The samples were preserved and homogenized in formalin 10% (v/v). **Tissue Sections** preserved in paraffin infected with mycobacteria were donated by Faculdade de Medicina Veterinária from the Universidade de Lisboa. They were processed by the histology department and fixed in histology grade glass slides (Superfrost© Plus, *ThermoFischer Scientific* – Waltham, MA, USA). These were preserved in the dark at RT. The hydrated tissue was immediately stained after treatment.

Smear Preparation: Before preparing the smear, the BCG culture was physically homogenized by forcefully passing up and down through a 0.5mm needle 15 times. For every smear, 20 µL of culture was applied in a regular glass slide. The smear was left to air-dry and then fixed with methanol. Stool sample smears were prepared by homogenizing the sample and collecting it with an inoculation loop.

Stock Staining Solutions: **Auramine O solution (3.3mM)** was prepared by dissolving 0.5 g of auramine O powder (*Merk* – Darmstadt, Germany) in 50 ml of 70%(v/v) ethanol. This is then mixed in a solution with 15g of dissolved phenol crystalline (*AppliChem* – Darmstadt, Germany) in 420ml of distilled water. The flask is kept in the dark at room temperature. **Carbolic Fuchsin (9.2x10^{-5}mM)** was prepared by dissolving 4 g of Basic Fuchsin powder (*Sigma-Aldrich*® – St. Louis, MO, USA) in 20ml of 70%(v/v) ethanol. This is then mixed in a solution with 8g of dissolved phenol crystalline in 100ml of distilled water. The flask is kept in the dark at room temperature. **Decolorizing acid-alcohol solution 0.5%(v/v)** was prepared by adding 0.5ml of concentrated hydrochloric acid (HCl) to 100ml of 70% ethanol(v/v). **Counterstains Methylene Blue 1%(w/v) and Potassium Permanganate 1% (w/v)** are both prepared by dissolving 2g of the respective powders in 200ml of distilled water each. Methylene blue was purchased from Methylene Blue (*Alfa Aesar* – Karlsruhe, Germany) and Potassium Permanganate was obtained from the Hospital de Santa Maria, Lisbon.

Auramine O Staining Protocol: After the smear is prepared it is flooded with the auramine O solution for 15 minutes and then rinsed gently with running tap water applied on the edge of the slide and never on top of the smear. Then the decolorizing solution is applied drop by drop until no more color is visible on the smear. Again, the smear is rinsed and then flooded for 2 minutes with the counterstain, rinsed with tap water and left to air-dry.

Kinyoun or Cold Ziehl-Neelsen Staining Protocol: After the smear is prepared it is flooded with the Carbolic-Fuchsin solution for 2 minutes and then rinsed gently with running tap water applied on the edge of the slide and never on top of the smear. Then the decolorizing solution is applied drop by drop

until no more color is visible on the smear. Again, the smear is rinsed and then flooded for 2 minutes with the counterstain, rinsed with tap water and left to air-dry.

Hematoxylin and Eosin Staining Protocol (H&E): The protocol for the H&E stain consists of submitting the sample to the following steps: flooding with Harris Hematoxylin (*Bio-Optica* – Milano, Italy) for 3 minutes; washing with running water for 5 minutes, applied not directly to the sample; one dip in 70% ethanol; four dips in alcoholic eosin (*Sigma-Aldrich*® – St. Louis, MO, USA); flooding with 70% ethanol for 30 seconds; flooding with 95% ethanol for 30 seconds; flooding with 100% ethanol for 30 seconds; repeating previous step with clean 100% ethanol; mounting slides.

Slide Preparation and Mounting for Observation: Glass slides with tissue sections were mounted with a coverslip for observation using *BacLight*^{TM} mounting oil (*Invitrogen* – Eugene, OR, USA). For permanent mounts, tissues were dehydrated as described in steps 6 through 8 of the H&E method or left to air dry completely if using the ZN stain; they were flooded with xylene (*Leica Biosystems* – Nussloch, Germany) for 10 minutes and then two drops of *Quick-D* mounting medium (*Klinipath* – Duiven, Netherlands) were put on the edges of the glass slide, the coverslips were dipped in xylene and placed carefully on top of it. Air bubbles were gently pushed out and the mounted slide was dipped in xylene to clean excess mounting medium. The slides were left for 24h to dry and solidify. Smear samples were observed directly, not mounted.

Imaging and Measurements: Every smear and tissue section stained with AuO were analyzed under a DM2500 epifluorescence microscope (*Leica Microsystems* – Wetzlar, Germany) with a 11500325 100W mercury lamp (*Leica Microsystems* – Wetzlar, Germany). About 20 photographs were taken of different fields for each sample with a DFC480 camera (*Leica Microsystems* – Wetzlar, Germany). For observations with an oil immersion objective of 100x amplification, *Immersol*^{TM} immersion oil (*Zeiss* – Oberkochen, Germany) was used. For each type of sample, the settings of the camera were adjusted to better capture the cells. They were standardized and unchanged between smears in comparison. (Annex, table 1) To measure fluorescence two different programs were used: Image J (version 1.52a) and *CellProfiler*^{TM} (version 3.2.1). All bacilli measurements were done with Image J's measure tool by selecting individualized and focused cells and clumped cells only if stacked laterally without being superimposed. The maximum value was taken for each cell. The value is calculated as a result of converting the colour images to grayscale using the formula gray= 0.299red + 0.587green + 0.114blue and selecting the highest value, in a scale from 0 (black) to 255 (white). In cell profiler two pipelines were developed to automatically measure both *Cryptosporidium* and *Cystoisospora.* They were designed to select the bright areas corresponding to the sporozoites in *Cryptosporidium* and sporoblasts in *Cystoisospora,* which are the main stained parts of the parasites. Then, the pipeline calculated the area and total fluorescence of all pixels and calculates de mean fluorescence value. Thresholds were defined so that the largest number of parasites possible was detected without the program wrongfully detecting debris such as fat micelles. The results were then manually curated.

Duration and Concentration of Staining Experiments: To assess the effect of the duration of the primary staining step and of the counterstain step several durations were tested for influence on fluorescence intensity, independently and in duplicate. For the duration of the auramine O primary step,

five conditions were tested: the standard 15 minutes, 10 min., 5 min., 1 min. and 10 seconds. The counterstain durations tested were: 10 min., the standard 2 min. and 10 seconds. In a second experiment, several durations of the primary staining step were cross-tested against varying concentrations of the AuO solution, as summarized in table 4.

Table 4 – Summary of the experimental conditions tested, crossing the durations of time of the primary staining step with auramine O of several different concentrations to assess its effect on AFI.

First Step	AuO at 3.3 mM	AuO at 0.33 mM	AuO at 0.033 mM
Durations tested (minutes)	30	30	30
	15	15	15
	7	7	7
	3	3	3
	1	1	1
Second Step	Decolorization solution 0.5% (v/v) HCL		
Third Step	Counterstain Methylene Blue 1% (w/v)		

To further confirm the results obtained, the previous experiment was repeated with different durations of staining and concentrations, which were thought would wield the best results from what had been observed. The concentrations of 3.3mM, 1.65 mM and 0.825 mM were cross-tested with the durations of staining of 15 minutes, 10 min. and 5 min.

The Effect of Different Counterstain: Smears of BCG and stool samples containing Cryptosporidium were stained with AuO and counterstained with either Methylene Blue or Potassium Permanganate. Standard protocol was applied.

Optimization of auramine O Solution Composition: The standard described AuO staining procedure was employed, however the composition of the AuO solution was changed. Several solutions were prepared where either water was replaced with other solvents or phenol was replaced by other solubilizers. All solutions prepared are summarized in table 5. In the case of solution H phenol was eliminated and instead the solution was first heated to 52°C and then immediately applied to the smears.

Table 5 – Summary of the experimental conditions regarding the preparation of auramine O solutions with different compositions, where N is the standard solution, E corresponds to the substitution of water by more 70% ethanol, A by Acetic Acid and G by glycerol; solution D where phenol was substituted by DMSO and in H by heating the solution to 52ºC. 70% ethanol corresponds to the first solvent, universal to all solutions.

	70% ethanol	AuO	Solubilizer	Second Solvent
N	10ml	0.1 g	3g phenol	84ml dH_2O
E	94ml		3g phenol	----------
A	10ml		3g phenol	84ml acetic acid
G	10ml		3g phenol	84ml glycerol
D	10ml		3ml DMSO	84ml dH_2O
H	10.34ml		“Heat”	86.68ml dH_2O

Transmission Electron Microscopy: BCG in suspension was centrifuged at 3000 RCF for 15 minutes and fixed overnight at 4 ºC in 0.1 M sodium cacodylate buffer, pH 7.4, containing 2.5% (v/v) glutaraldehyde. Then they were washed and centrifuged and the pellets containing bacteria were post-fixed with 2% osmium tetroxide (*EMS* – Hatfield, PA, USA) for 30 min, and stained *en bloc* with 1% Millipore-filtered uranyl acetate (*Agar scientific* – Essex, UK). Samples were dehydrated in increasing concentrations of ethanol, infiltrated and embedded in EMBed-812 hard (*EMS* – Hatfield, PA, USA). Polymerization was performed at 60°C for 24 hours, and ultrathin sections were obtained in a UC7 ultramicrotome (*Leica Microsystems* – Wetzlr, Germany) collected to 1% formvar coated copper slot grids (*Agar scientific* – Essex, UK), stained with 2% uranyl acetate in 70% methanol and Reynold Recipe lead citrate (Sigma-Aldrich® – St. Louis, MO, USA). For the ChromEMT experiment, homogenized BCG suspension smears were prepared on 22x22mm glass coverslips and fixed with methanol. They were then carefully stained with the AuO protocol and examined under the fluorescence microscope; one coverslip was left unstained for control of the experiment. The coverslips were then placed inside a 6 well plate submerged in 0.1 M sodium cacodylate buffer, , pH 7.4, containing 2.5% (v/v) glutaraldehyde. Then they were washed and dyed with 0,5mg/ml DAB with 0.03% H_2O_2 in sodium cacodylate buffer. Coverslips were screened under the stereoscopic microscope for color change, then washed and post-stained in 1% osmium tetroxide for 1 hour. The bacteria adhered to the coverslips were dehydrated in increasing concentrations of ethanol, infiltrated and embedded in Durcupan resin (*EMS* – Hatfield, PA, USA) using upside down bottle neck BEEM capsules (*Agar scientific* – Essex, UK). Polymerization was performed at 60°C for 24 hours, and ultrathin sections were obtained in a UC7 ultramicrotome (*Leica Microsystems* – Wetzlar, Germany) collected to 1% formvar coated copper slot grids(*Agar scientific* – Essex, UK), stained with 2% uranyl acetate in 70% methanol and Reynold Recipe lead citrate (Sigma-Aldrich® – St. Louis, MO, USA).

Sections were examined in a H-7000 transmission electron microscope (*Hitachi* – Chiyoda, Tokyo, Japan) at an accelerating voltage of 100 kV. Digital images were obtained using a Megaview mid mount digital camera (*Olympus* – Shinjuku, Tokyo, Japan). The sections were systematically analysed using iTEM© software and several high and low magnifications were acquired, bacteria cross and longitudinal orientation were individually measured using iTEM© measurement tool.

Formalin and Xylene Experiments: To test the effects of formalin 10% (v/v), smears were prepared from direct BCG culture diluted in half with formalin 10% (v/v) and another aliquot diluted in half with Midlebrook 7H9 medium. These were then stained with auramine O standard concentration 3.3mM, 0.33mM and 0.033mM. Photographs were taken on the fluorescent microscope on day 0, day 2 and day 7. The preparation of more smears under the same premises but only at 3.3mM auramine was done to allow counting of cells. 30 photographs were taken in a straight horizontal line from one edge of the smear inwards, without observing the optical field upon stopping of scrolling each time, in order to avoid interference from the user on selecting fields with more or less bacilli. This was done in triplicate.

Regarding the effects of xylene, an inital essays was carried out: Direct culture was diluted with xylene by adding 200 µl to 800 µl of culture and after 10 minutes smeared and air dried. Alternatively, fixed smears were submerged in xylene for 10 minutes and then rinsed. These were prepared in extra

adherent glass slides (CELL-BOND™, *AlphaTech*® – Vancouver, WA, USA) and prepared in 4 replicates. To further confirm the results, two more essays were done in triplicates: **1st** – Culture fixed in formalin 10%(v/v) was distributed in Eppendorf tubes and centrifuged for 5 minutes at 12300 RCF. The supernantant was discarded and xylene dilutions were applied: 100%, 50%, 10% and 0%. These were vortexed and incubated for 20 min. Finally, each sample was smeared, fixed and stained. **2nd** – Culture preserved in formalin 10%(v/v) (1:1) was smeared and fixed in extra adherent slides (CELL-BOND™, *AlphaTech*® – Vancouver, WA, USA). The same xylene dilutions were applied over the smear, discarded after 20 minutes and left to air dry. Then, each smear was stained.

Depparafinization Methods: Several tissue sections with thicknesses of 4 or 10 micrometers were deparaffinized with the standard xylene method, the novel PHAD method, a water bath method, a dry heat oven, the Lab Vision™ PT Module (*ThermoFischer Scientific* – Waltham, MA, USA) method for immunohistochemistry and centrifugation in a high humidity and high temperature atmosphere. **The standard deparaffinization** protocol using xylene is as follows: xylene 100% for 10 minutes; xylene 100% for 10 minutes; Alcohol 100% for 5 minutes; Alcohol 95% for 5 minutes; Alcohol 70% for 5 minutes and finally distilled water for 5 minutes immediately followed by staining. Other sections were left in a dry heat **oven at 68ºC**, standing vertically, overnight, then placed in distilled water for 5 minutes immediately followed by staining. **Water bath** deparaffinization consisted in the submersion of the tissue sections in distilled water, each in individual containers, for a duration of 20 minutes, whose temperature was kept at around 72ºC, but occasionally raised or dropped in the range of 70-75ºC. After this time, the slides were immersed in distilled water at RT for 5 minutes immediately followed by staining. **The Lab Vision™ PT Module™** is a device where the tissue sections are submerged in a buffer of various pH, in this case pH 6, with a composition including alternative solvents to xylene. This bath consisted in 3 stages of 20 minutes each, first heating to 65ºC, then to 95ºC and finally cooling to RT. **Centrifugation** in a high humidity and high temperature atmosphere consisted in fixing glass slides vertically, facing outwards, on a rotor of a large centrifuge of a domestic appliance; the rotor is placed inside an enclosed chamber. Water is heated to boiling point and poured into the chamber to about a centimeter bellow the rotor. The centrifuge is immediately turned on for a cycle lasting 5 minutes which reached 68ºC of air temperature. At the end of the cycle, the glass slides are submerged in distilled water at RT for about 5 minutes.

PHAD (Projected Hot Air Deparaffinization): A common hair dryer (*SilverCrest*® SHTK 2000W B1, Lidl Stiftung & Co. KG – Neckarsulm, Germany) was set up on a grid, with the mouth of the hairdryer at about 21 cm elevation, perpendicular to a glass slide rack, at a distance of approximately 22 cm. Maximum speed and temperature settings of the hairdryer were found necessary for at least 20 minutes, reaching an air temperature of about 72.5ºC . After 20 minutes the slides were immediately submersed in RT distilled water until staining and for 5 minutes minimum. Temperature was measured with a mercury thermometer every 2 minutes, for 20 minutes plus additional measurements in the first minute and after shutting off the hair dryer and submerging in distilled water (Annex – table 8). Duration of the process was chosen by conducting the deparaffinization at several different periods of time: 10, 20, 30 and 40 minutes (shorter times were not tested because they proved inefficient in initial attempts).

IV – Results and Discussion

1 – Optimization of the Auramine O stain for TB diagnosis

Duration of staining: The effect on the duration of the staining time and counterstaining time was accessed by measuring the relative fluorescence of bacilli, expressed as arbitrary fluorescence intensity units (AFI - explained in section III – Materials and Methods). It was found that of the four durations tested, that of 15 minutes yielded the higher average AFI of above 50 and the duration of 5 minutes of around 40 (figure 13). Both these durations had more bacilli at higher AFI values. Interestingly between the shorter two durations tested not much difference was seen in terms of AFI, despite only staining for 10 seconds compared to a minute.

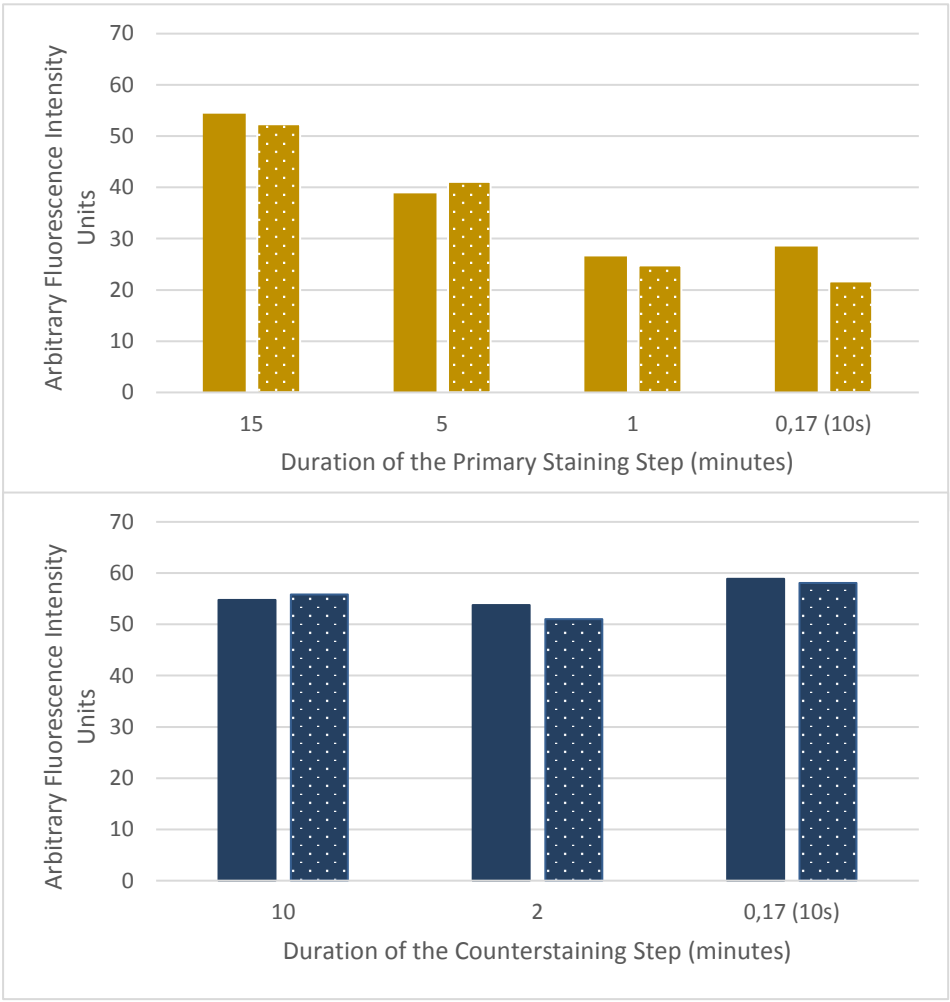


Figure 13 – Effect of the duration of the primary and counterstaining steps – AFI of bacilli stained with AuO for different durations of time: 15, 5, 1 minute and 10 seconds (TOP) or of which Methylene Blue counterstain was applied for different durations of time: 10 and 2 minutes and 10 seconds (BOTTOM). For each condition both duplicates are represented in se same color, with dots.

The duration of the counterstaining step was also tested to verify if it would influence the AFI of bacilli; in this case, of methylene blue. It was found that between the standard of 2 minutes or 10 minutes, little difference could be seen in terms of AFI, as all the measurements were around 50 to 55 arbitrary units.

This is relevant because, occasionally and due to large amounts of samples to process, technicians can surpass the recommended time unintentionally, but as the results suggest this has no influence in the fluorescence.

However, at 10 seconds of duration, a very slight increase of AFI to almost 60 units was seen (figure 13, BOTTOM) through which one can speculate that the counterstain has a small quenching effect on the fluorescence of the bacilli themselves and not just on the background as intended. This could be a factor to consider in cases of low infectious doses in samples with little background fluorescence and debris, where perhaps a counterstain should not be used at all.

Following these experiments, it was carried out several comparisons with the intention of seeing the relationship between longer and shorter durations of the primary staining step against the concentration of dye in the stain, to understand if the standards used are the most optimized conditions and to further confirm the previous findings. It was proposed that the 3.3mM standard concentration used, is rather high for typical cell stains, specifically those which target nucleic acids, which prompted these tests. In experiments not illustrated it was found, as expected, that the cells stained with the standard concentration of 3.3mM yielded higher values of fluorescence, although with a ten-fold reduction to 0.33mM it was still possible to easily detect bacilli. However, at 0.33mM bacilli were found in lower density per optical field, which may indicate that more fragilized and less AF bacteria could retain too little stain to be detected. Knowing this it was thought that it might still be possible to reduce the concentration of stain to about only half without needing to prolong staining and still retain similar values of AFI, which was tested.

A final experiment done in triplicate supported this hypothesis, as the values of AFI for 15 minutes at standard concentration or at 1.65mM are identical. (figure 14) Furthermore, at 10 minutes and 15 minutes, the values of AFI for the standard concentration are identical and the 1.65mM values only slightly lower at 10 minutes. Additionally, the values obtained at 5 or 10 minutes for the 0.825mM concentration are still close to the other two conditions, however at the duration of 15 minutes there is an unexpected drop.

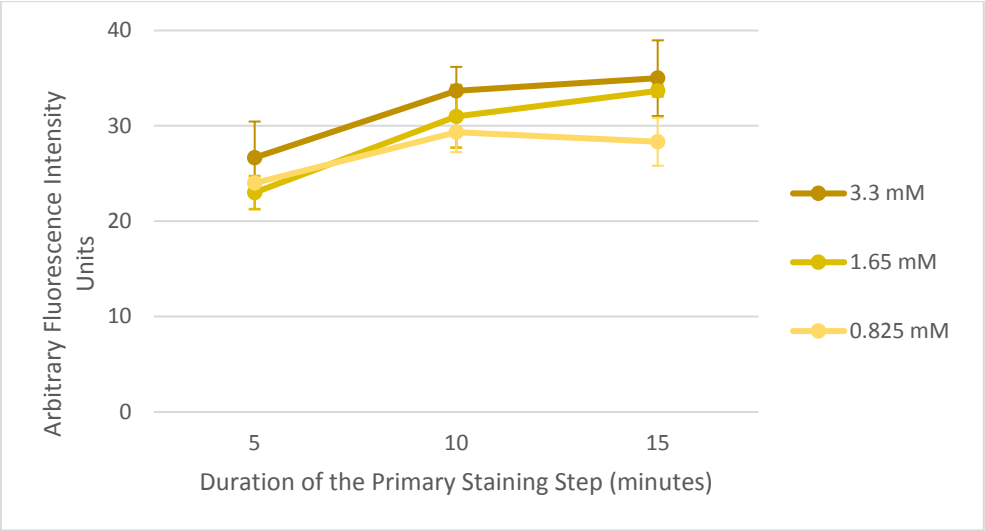


Figure 14 – Effect of the duration and concentration of the primary staining step – AFI of bacilli stained with AuO at different concentrations: 3.3mM (standard, dark yellow), 1.65mM (half the standard, light yellow) and 0.825mM (a fourth of the standard, gray). Three durations of staining were tested for each condition: 5, 10 and the standard of 15 minutes.

This result suggests that in typical applications the AuO stain could be applied in only half the standard concentration, or possibly less, which represents a long-term reduction in the cost of the technique. Furthermore, the reduction in the duration of the primary staining step could have important implications for clinical settings, where a large volume of samples is processed each day, not only for a possibly change in used protocols, but also because it means delays or early interruptions of the staining by a technician, by error, shouldn't have considerable implications for the diagnosis success. For this to be adopted as a standard condition, however, sensitivity tests must be performed with comparisons to the current standard and culture.

Counterstain comparison: Methylene blue (MB) and potassium permanganate (PP) counterstains were used to assess if the use of one or the other would result in changes of AFI of the sample. The results seem to suggest that PP as a counterstain originates lower AFI. The average values of AFI for both MB smears were 48.6 and 55.9 while for PP they were 37.1 and 40.9. This is reflected in a shift of MB values slightly to the right, into higher value bins (figure 15).

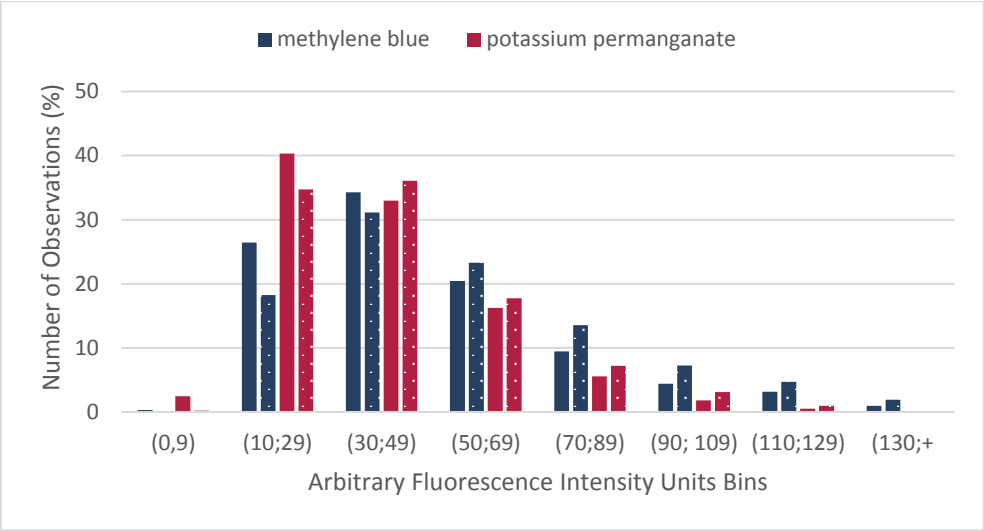


Figure 15 – Comparison of Two Counterstains – Distribution of AFI of BCG bacilli counterstained with MB or PP alternatively according to the frequency of cells in each bin of values on the X axis. The bars in dotted blue correspond to the duplicates stained with methylene blue and in dotted magenta to those stained with potassium permanganate.

The same assay was performed with fecal samples from a cryptosporidiosis case seeing that counterstaining is more relevant with this kind of sample, and it was found that also in that case, the samples stained with MB had slightly higher average AFI (0,00096 and 0.001) while PP stained smears had lower AFI (0.00069 and 0.00089). In the case of fecal samples, it was also very noticeable that the background and debris, such as fat micelles, were less fluorescent when PP was used.

According to these results one must consider the possibility of potassium permanganate quenching less-fluorescent cells and, on the other hand, its advantage in samples with more background and other fluorescent bodies. Potassium permanganate is the most commonly used counterstain for AuO stains, being referenced by the WHO as the counterstain of choice for fluorescence microscopy along with acridine orange.(129) PP is, however, highly restricted for general commercialization and usage, due to its involvement in the manufacturing of explosives and drugs.(130) A study reported MB to be as adequate as PP in fluorescence microscopy observations, having a higher sensitivity.(130) They also report a slightly smaller specificity, which could be a result of PP having a stronger quenching effect as was found in the results of this project. In the study mentioned above the comparison was only done in terms of number of positive or negative smear observations, so the results of this project complement those findings with direct evidence of PP's stronger quenching of bacilli fluorescence and potential benefit in fecal samples or high-background samples.

Solvents and solubilizers: Several modifications were done to the typical AuO staining solution mainly to gauge the effects the elimination of water or phenol could have on AFI. The intent of removing water was for its reported quenching effect on AuO, considering the potential increase in fluorescence of AuO in less polar solvents, like acetic acid, or more viscous solvents, such as glycerol.[22,23] Phenol was substituted because, as a toxic chemical, it is undesirable, especially if it must be used routinely such

as in tuberculosis diagnosis laboratories. It has a pungent smell, irritating effect in mucous membranes and is lethal if ingested. (LD50 50-500 mg/kg)[24,25].

It was thought to select solvents which would in theory enable a higher fluorescence from the AuO molecule; those would be less polar, more viscous or those already reported to cause said increase in fluorescence by having higher reported quantum yields. In figure 16 is a scheme representing the theoretic influence of the chosen solvents on AuO based on said physical properties.

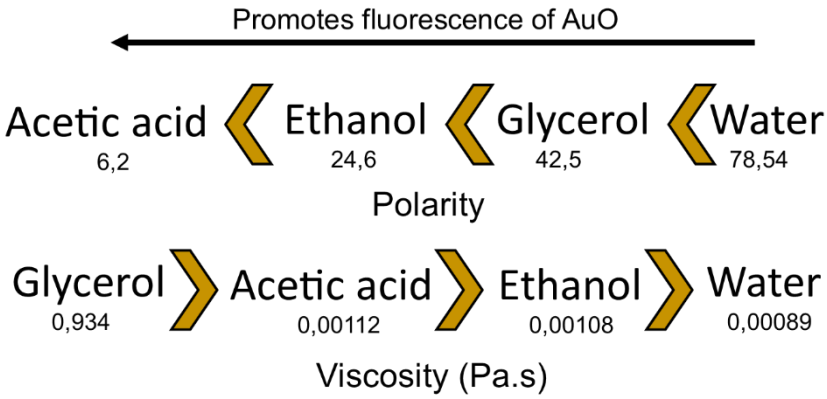


Figure 16 – Scheme representing the theoretical adequacy of solvents for an AuO solution based on physical properties of polarity (dielectric constant) and viscosity. (135-138)

In figure 17 are two histograms where the bars where omitted and only the trend curves are represented, showing the effect of substituting/removing water as a solvent (TOP) and substituting/removing phenol as a solubilizer (BOTTOM). Of those solutions where water was replaced, 30% acetic acid performed the worst, being that almost no bacilli were found in one of the duplicates and none in the other. Curiously, some of the bacilli found had strong fluorescence. It might be possible that the acidic quality of the solution does not promote the containment of the dye in the cell, in cells whose wall is most fragilized, despite possibly promoting AuO fluorescence enhancement. The solution containing glycerol also performed poorly, generating many barely visible bacilli, which might be explained by the overly viscous quality of the solution, hindering penetration of the cell. This resulted in nearly all mycobacteria registering in lower value bins of AFI in figure 17 - TOP, visible as tall curves to the left of the graph. The solution containing only 70% ethanol performed nearly identically to the standard, however one of the duplicates' results had to be eliminated. Regardless, the result can be preliminarily indicative of the behavior of the solution. The average AFI values for the standard solution were 43.1 and 49.6 while the value for ethanol was 44.9.

Regarding the substitution of phenol, heating the solution appears to be able to generate AFI higher or at least on par with the standard for BCG smears (figure 17 – BOTTOM) while DMSO performed poorly. It is worth mentioning that these solutions were also tested in coccidia samples and it was found that for *Cystoisospora* the heated solution gave the best results while DMSO and ethanolic solutions matched the standard (not illustrated).

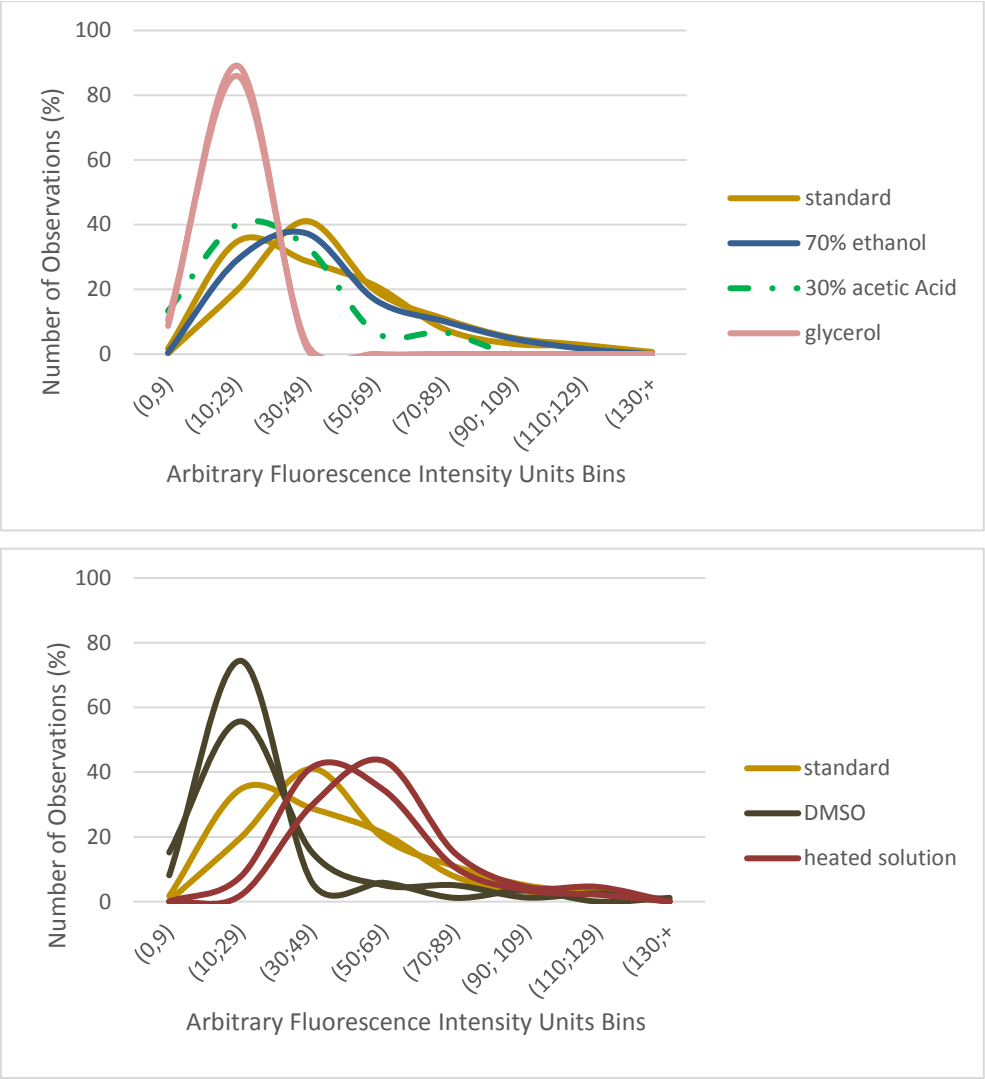


Figure 17 – Effect of alternative solvents (TOP) or phenol alternatives (BOTTOM) in the auramine O stain – Distribution of AFI of BCG bacilli according to the frequency of cells in each bin of values on the X axis. Conditions tested are of bacilli stained with an auramine O solution of a standard composition (yellow) and variants where: water was replaced (TOP) by 70% ethanol (blue), 30% acetic Acid (green - dashed) and glycerol (pink); phenol was replaced (BOTTOM) by DMSO (gray) and by heating the phenol-free solution to 52˚C (red). Note: Curves tending to the right of the graphs represent conditions which generated higher fluorescence values. The height of the curve represents only the percentage of cells which are in a bin for each condition. The graphs do not represent absolute bacteria counts, only proportions.

Experiments with tissue sections showed that all substitutions of water resulted in much worse AFI or even undetectable bacilli. It isn't clear the reason for the failure of staining in this case, but it could be due to the permeability of the tissue to the different solvents For the heated and DMSO-containing solutions the results among duplicates was inconsistent because one yielded higher or similar AFI than the standard, while the other was lower with a distribution tending to lower value bins. The number of bacilli detected was generally lower, which is likely to be a result of worse staining or a product of observation variation. However, it appears that both phenol substituents are capable of generating AFI values similar to the standard, which would need to be further tested to be able to make a conclusion.

These results are only preliminary and should be interpreted as an initial attempt at manipulating the composition of the staining solution. Through this experiment it seems ethanol could potentially be

an equivalent replacement to water as a solvent and this should be tested with solutions of a higher concentration of ethanol than 70%. It might also be possible to mix some of these substances for better results, which could be tested in future experiments. Overall, no result showed a benefit to substituting water as a solvent, but it is my belief that further experiments in this subject could provide a simple means to improving stain quality for mycobacteria detection.

Regarding the substitution of phenol, although also only initial results were produced, it seems that it could be possible to substitute phenol as a dye mordant, using instead a heating step. This could be a great benefit for the safety of the use of the stain but also because phenol could potentially be damaging to the wall integrity of mycobacteria. This is only speculative, but since it is capable of permeabilizing the cell wall, it might be that its effect is permanent and reduces its resistance to later decolorization. It would be important to test for this potential effect.

2 – Cellular Target of the Auramine O Stain

To understand why different factors influence mycobacteria staining with AuO, in particular what reduces its fluorescence, it's important to know if AuO is binding to the cell wall or if indeed it is a nucleic acid stain. In that sense it was attempted to understand the binding target of auramine O. As discussed previously, the distribution of the dye in the bacilli, typically either in a homogeneous fashion or by taking a beaded appearance inside the cell, suggests the dye is in fact not binding to the wall, or at least not only to the wall. By observing the staining of other organisms, it is possible to make similar observations. Throughout this project it was possible to observe the behavior of the AuO dye in terms of distribution in the cells of mycobacterium but also in the oocysts of coccidia. (figures 18 and 19).

In figure 18 it is possible to see that AuO regularly localizes inside the bacilli in a non-homogeneous way. It is often seen that at the extremes of the bacilli the fluorescence intensity is higher than in the rest of the cell, appearing as beads on its edges. In some bacilli, such as the one at the bottom right, a very clear segregation of the dye is seen to only some parts of the cell, as if it were adhering to internal structures. In the case of bacteria, nucleic acids are not compartmentalized within a membrane, but are also not evenly distributed across the cell, which could account for why the AuO dye accumulates in certain parts of the cytoplasm, possibly by binding to the nucleic acids there located.

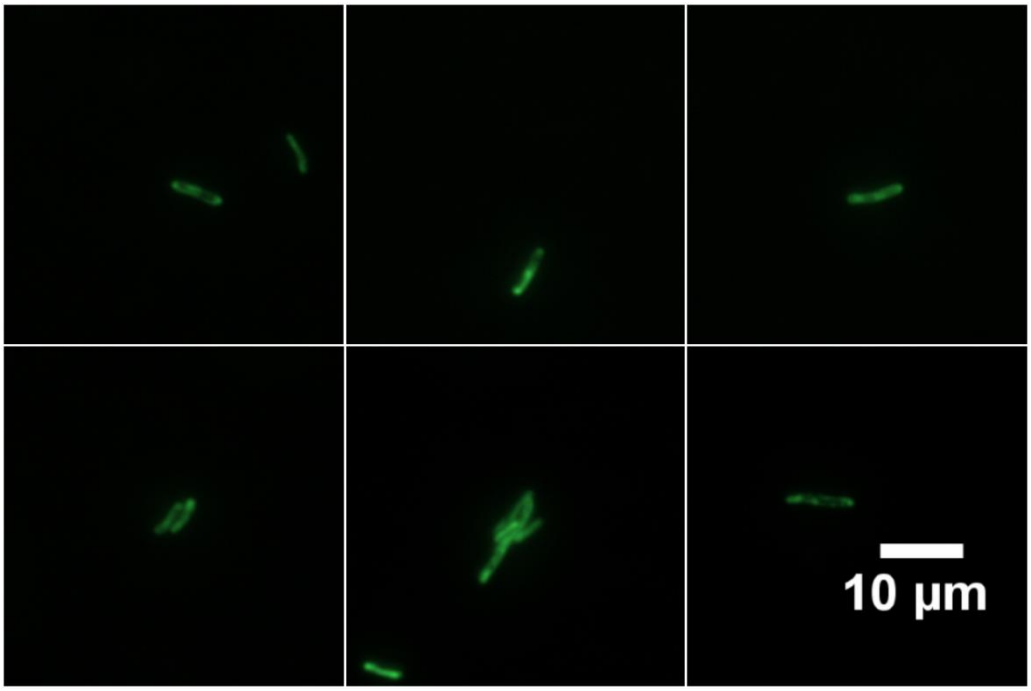


Figure 18 – Beaded appearance of tubercle bacilli – *Mycobacterium bovis* (BCG) stained with Auramine O, observed in the fluorescence microscope at 1000x magnification, displaying a non-homogeneous distribution of dye within the bacilli.

In figure 19 internal distribution of the AuO dye is discernable, concentrating inside the parasites in an apparent coincidence with structural features. Although possible to see faint surrounding membranes, both in *Cryptosporidium* and *Cystoisospora* the greater part of the dye, and therefore the most fluorescent areas, are the merozoites and sporoblasts, respectively. In figures 19-A and B it is possible to see that in two of the *Cryptosporidium* oocysts only about half of oocyst is strongly fluorescent, leaving half of the body translucent. It is also particularly clear in picture 19-C but also in D

that the dye is concentrated inside the merozoites within the oocyst, displaying crescent-shaped structures with a stronger fluorescence. In figures 19-E and G it is possible to see two *Cystoisospora* oocysts with sporozoite-containing sporoblasts. These sporoblasts are very fluorescent compared to the translucent body of the oocyst. In the case of 19-F, which represents an oocyst with a disrupted sporoblast, the fluorescence is distributed within the entire body of the parasite, but it is also a heterogeneous distribution, with more concentrated areas.

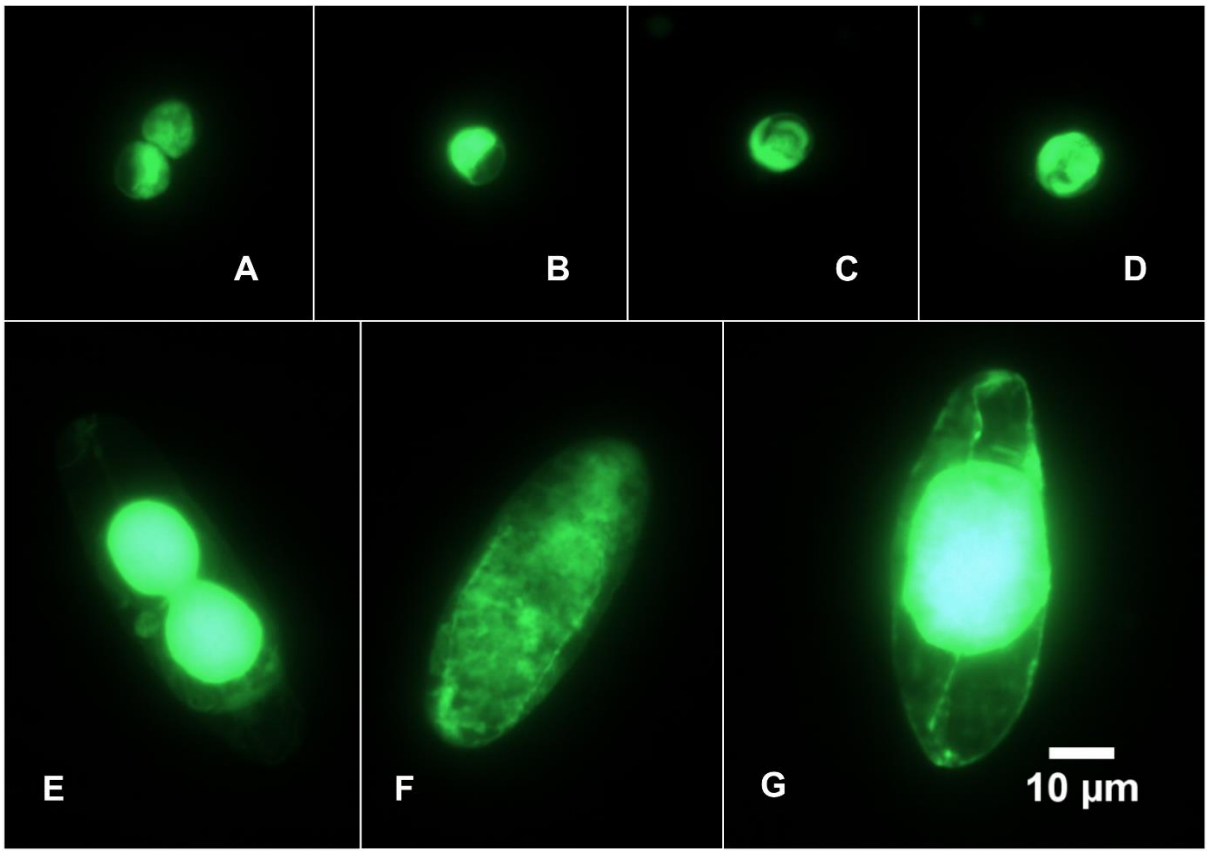


Figure 19 – Coccidia stained with auramine O – *Cryptosporidium* (A-D) and *Cystoisospora* (E-G) observed in the fluorescent microscope at 1000x amplification. Regarding the morphologies of *Cystoisospora* represented: E - Oocyst with two sporoblasts; F – Oocyst which might have been damaged or aged and has a disrupted sporoblast; G - Oocyst with one sporoblast.

The sporoblasts of *Cystoisospora* and merozoites of *Cryptosporidium* contain the bulk of nucleic acids and many proteins of the parasites, which could explain the concentration of the dye within these structures, if we assume that auramine O does bind nucleic acids and/or certain proteins. In the disrupted oocyst of *Cystoisospora* the internal material of the sporoblast became distributed across the entire body of the parasite, possibly accounting for the more evenly distributed fluorescence.

Samples stained with AuO were prepared in order to assess its binding to DNA (figure 20) and it was observed a difference between stained and unstained samples with transmission electron microscopy.

In figure 20 (TOP), corresponding to unstained bacteria, it was possible to see some structures that could potentially be nucleic acids, but are faint and hard to identify. On the other hand, in figure 20 (BOTTOM), corresponding to bacteria stained with AuO, it was possible to find several fibrillar structures

and electronically dense regions corresponding to nucleic acids. This could be a result of the binding of AuO to nucleic acids, effectively making the region more electronically dense and darker in micrographs. It is also noticeable that the dark granules of various sizes, which are accumulates of several elements, including phosphate, calcium, magnesium and potassium (135), become also larger and darker. This may indicate that AuO binds to other materials and not just nucleic acids and proteins, as was described in the past, and this could possibly be partially responsible for the beaded appearance of the bacteria cells. Consequently, both these observations reinforce the idea that AuO stains not just the mycolic acids in the cell wall but interior components as well, nucleic acids in particular.

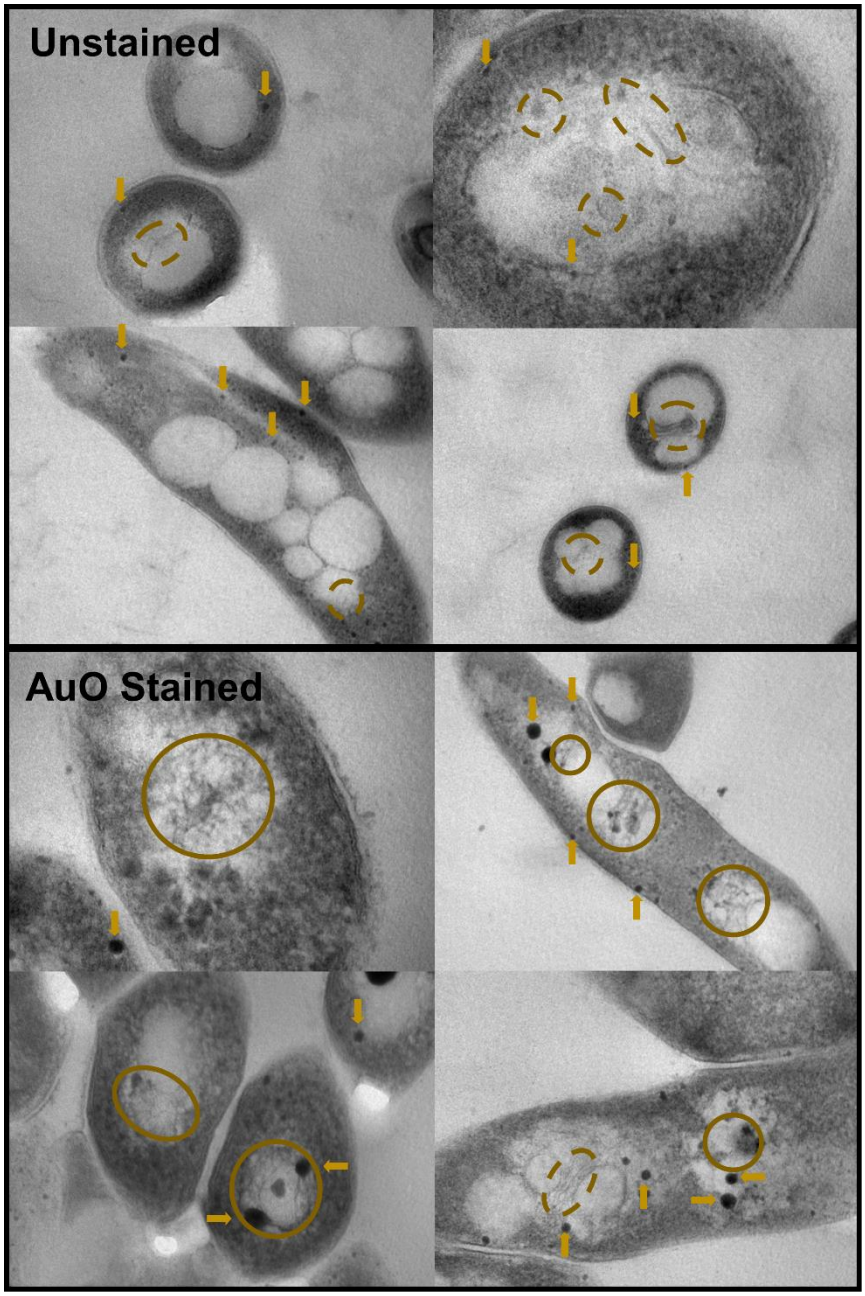


Figure 20 – TEM micrographs of mycobacteria – *Mycobacterium bovis* BCG unstained (TOP) or stained with AuO (BOTTOM). In dashed circles are internal structures that are hard to identify but could be nucleic acids. Full circles point to fibrilar structures likely to be nucleic acids. The dark granules of variable sizes correspond to polyphosphate granules which also accumulate other micronutrients (some exaples shown with arrows).

Using a technique recently described in literature, ChromEMT, used to visualize the 3D ultrastructure of chromatin in eukaryotic cells, we prepared samples stained with AuO and contrasted with DAB (diaminobenzidine) (figure 21).(136) In the literature it is explained that through this technique the binding of the fluorophore to DNA and subsequent polymerization of DAB on its surface, creates dark precipitates in the fluorophore target, enabling visualization.(136) Although a different fluorophore was used originally, it was attempted to reproduce the described effect with AuO and it was observed a difference between stained and unstained samples and the control.

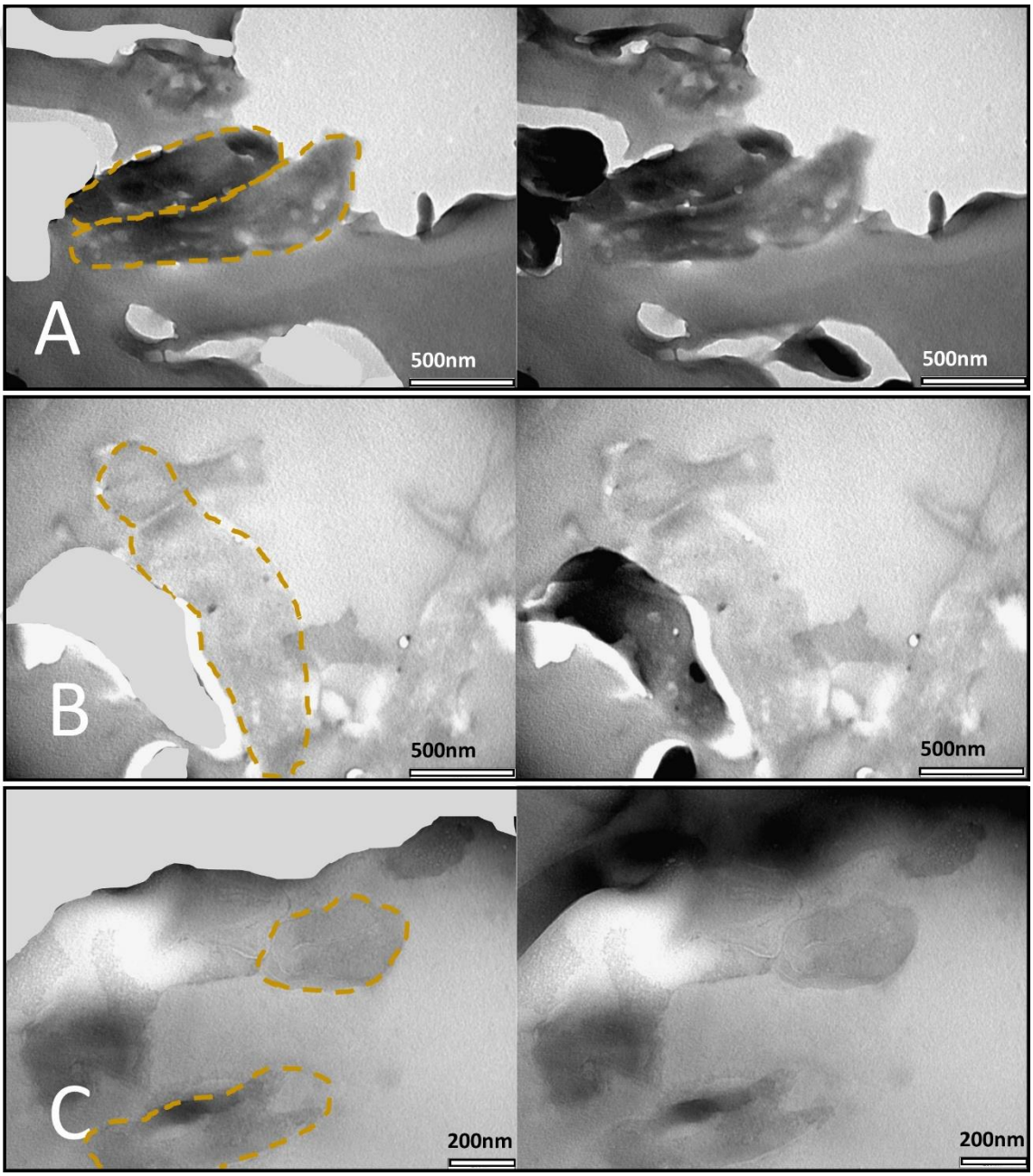


Figure 21 – Micrographs of mycobacteria by ChromEMT – *Mycobacterium bovis* BCG stained with AuO and contrasted with DAB (A), stained with AuO but without DAB (B) and the control, unstained but contrasted with DAB (C). On the left are micrographs where mycobacteria were outlined and artifacts were covered, corresponding to bacteria which were detached from the matrix and originated dense regions independent of conditions tested. On the right are the original micrographs unaltered.

In figure 21 it is possible to see that the bacteria stained with AuO and contrasted with DAB (A) became darker compared to the background, which did not happen with the not contrasted sample (B) and only slightly with the unstained control with DAB (C), indicating polymerization of DAB on the fluorophore attached to some internal structure. Interestingly, in the micrographs of stained and contrasted cells (A), it is possible to see that the electronically denser regions are not homogeneous throughout the cell with a distribution like that observed of AuO in fluorescence microscopy. These observations are also indicative of the possible binding of AuO to internal components, possibly nucleic acids.

In a subsequent experiment we were not able to reproduce these observations and due to logistic and time constraints it wasn't possible to explore this further, therefore these results should be cautiously interpreted as they represent a one-time observation from a preliminary attempt at using the chromEMT technique.

These observations strengthen the idea that the cell wall is essential for the process of acid-fast staining for diagnosis of mycobacteria, not because the wall itself is stained, but because it contains the dye. If the stain is retained inside the cell, regardless if bound to any specific target, this means that the cell wall is responsible for retaining the stain against acid-alcohol decolorization. It also means that damage to the wall can leave the interior vulnerable to the removal of the dye. Therefore, it is very important to analyze the steps of processing of clinical samples to understand if in anyway they could be causing damage to the wall.

3 – The Effect of Histological Processing on Mycobacteria in Infected Tissue

The effect of formalin (tested in culture samples): The effect of formalin on the AFI of BCG from a culture sample pointed to formalin not only generating higher AFI values but also preserving them for a longer period. These effects were however not observed at lower concentrations of AuO than the usually used 3.3mM. In figure 22 it is possible to see that cells fixed in formalin yielded a distribution of AFI values which tends more to the right than smears of not-fixed culture, which means most of the population of bacteria cells have higher fluorescence values. More interestingly, the average AFI values of non-fixed culture at day 0 were 37.8 and 43.9 while those of formalin fixed culture were 53.0 and 45.4; on day 7, the values for non-fixed culture were reduced to 39.2 and 37.4 while those of formalin fixed culture were 57.0 and 48.0. The counting of cells revealed an average of 235 cells in non-fixed culture smears and 245 in fixed culture smears, showing that the higher AFI is not a result of the inability to see less bright cells who could potentially be damaged by formalin, as was suggested by Fukunaga *et al.*[19]

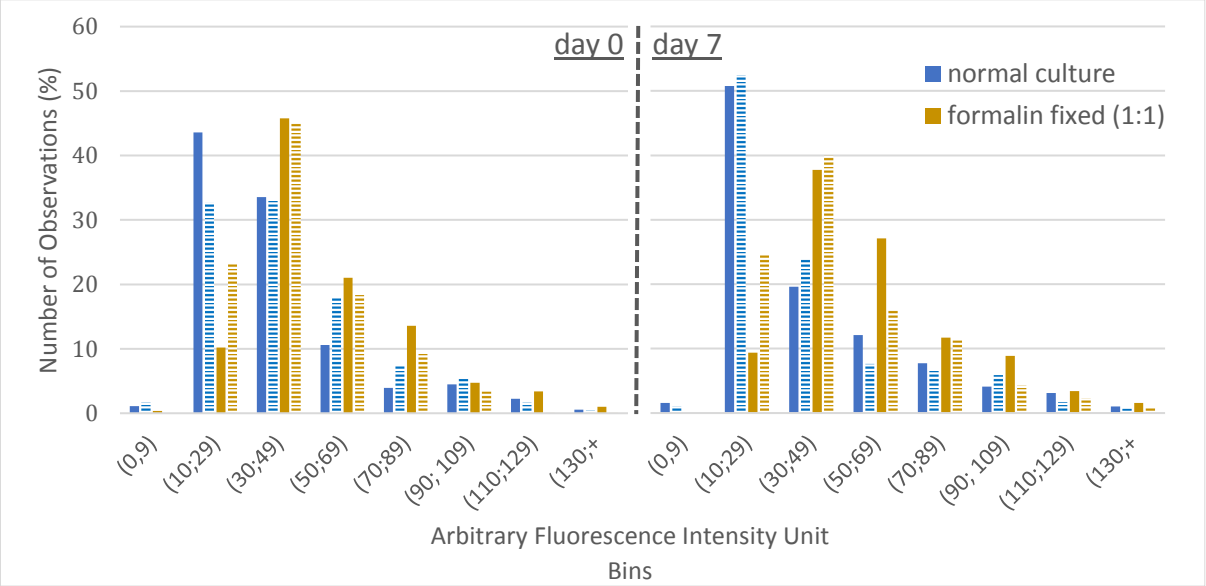


Figure 22 – Effect of formalin on fluorescence – Distribution of BCG bacilli according to the frequency of cells in each bin of AFI values on the X axis. In blue are represented the duplicate conditions of non-fixed culture stained with AuO and in yellow of those fixed with formalin (1:1) beforehand. Replicates are represented as dashed bars. Measurements were taken at two time points: Day 0 (left) and day 7 (right).

The effect of xylene (tested in culture samples): In figure 23 are represented the results first obtained when testing the effect of xylene on mycobacteria fluorescence. The bacilli not treated with xylene have higher values of AFI while both the bacteria pre-incubated as well as the those treated with xylene post-fixation to the glass slide have lower values of AFI. There is, however, another key difference upon microscopic observation. The pre-incubated cells, which in this case were fully immersed in 20% xylene for 10 minutes, and which normally display a cording phenotype even if homogenized, appear to “melt”. The typical cord-like structures are seen disintegrating into “pools” of bacilli which is illustrated in figure 24. This is a clear indicator that xylene has some effect on the integrity of the mycobacterial cell wall, resulting in the destruction of inter-cell aggregation. This may or may not compromise the acid-fastness of the cells, which had to be further tested.

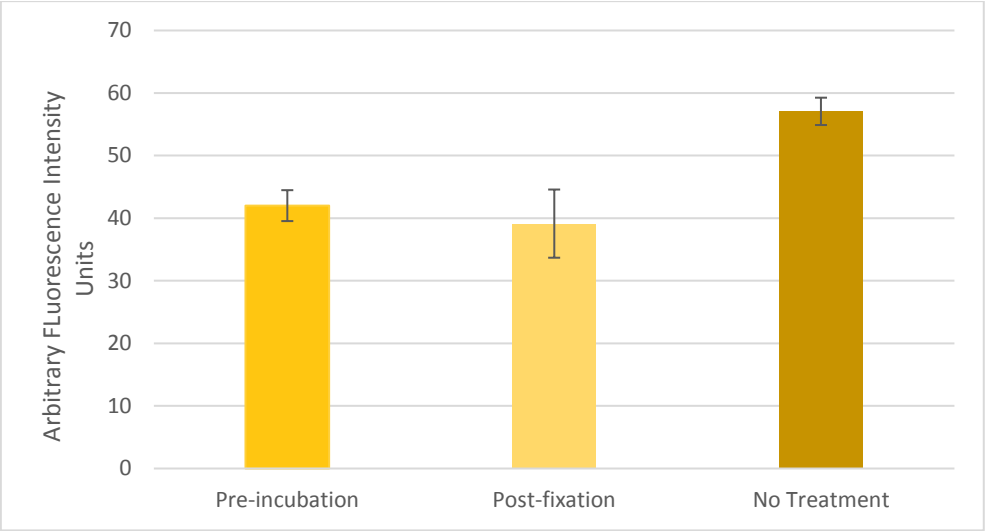


Figure 23 – Effect of xylene on fluorescence: pre-incubation and post-fixation – AFI of BCG bacilli exposed to treatment with xylene, either by pre-incubation or after fixation, compared to the control which had no treatment before staining. The error bars represent the standard deviation of the average of the four replicates' average AFI value.

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Further experiments were carried out, as an attempt to match the exposure time between pre-exposed and post-fixation treated bacteria as well as to the time they are exposed to xylene in typical histology procedures. Curiously, an apparently contradictory result was consistently obtained when at first it was tested a treatment of xylene to bacteria in suspension, before fixation to the glass slide. Bacteria incubated with 10 and 50% xylene fluoresce as much as non-treated bacteria, but bacilli incubated with 100% xylene concentration fluoresced most, compared to any of the other concentrations (figure 25). After further inspection of these samples by microscopy it was concluded that this effect was not due to an actual increase in fluorescence, but an artifact of the "melting" effect described previously. The bacteria incubated with xylene prior to fixation, but especially those at 100% concentration suffer a more intense "melting" effect and their aggregates disintegrate more thoroughly, releasing many more individual cells. It was speculated that these newly released bacilli are healthier, because they had been able to form cell-to-cell aggregates, and for that reason their acid-fastness is more preserved. Because measurements are only taken of individual bacteria and not aggregates, this accounts for the increase in AFI.

In the first experiment this confounding effect was not detected, likely because bacteria were incubated with only 20% xylene and for only 10 minutes, not releasing many individual cells. In figure 24 it is possible to observe that the pools of bacilli in the 100% xylene treated conditions are much more dispersed than in other conditions and many more individual bacteria are originated.

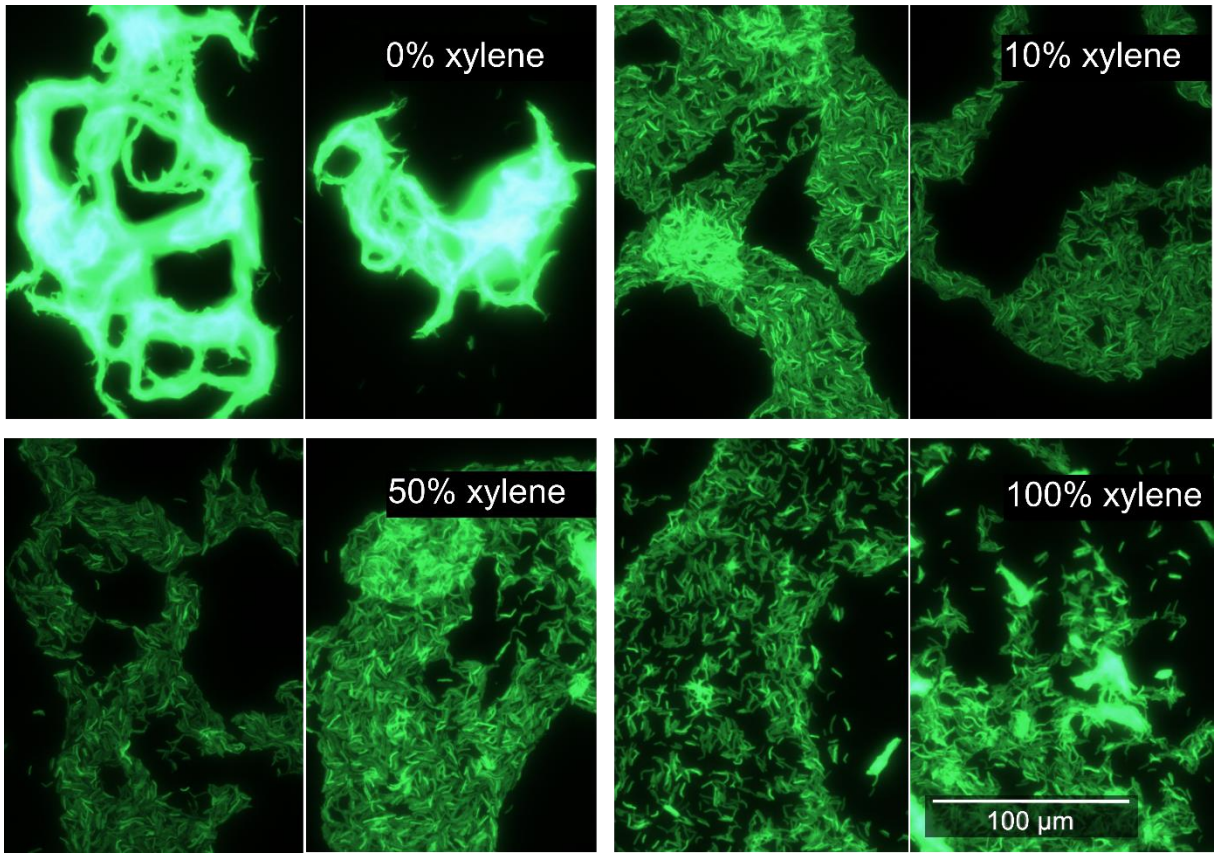

Figure 24 – The "melting effect" – Photographs of BCG bacilli incubated with xylene at several concentrations for 30 minutes, prior to fixation. Pictures taken at 1000x amplification with an oil-immersion objective of 100x power

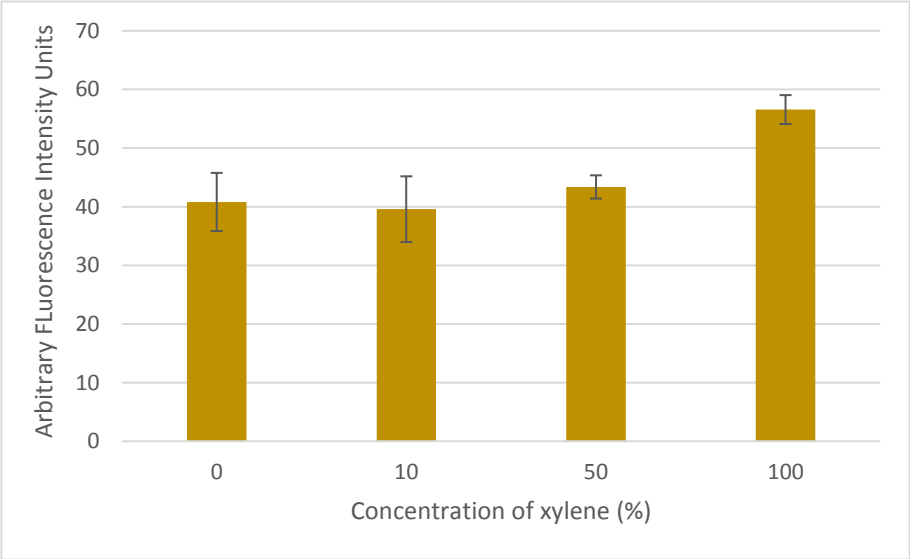

Figure 25 – Treatment with xylene pre-incubation – Values of AFI of BCG bacilli treated with xylene at several concentrations before fixation. The error bars represent the standard deviation of the average of the three replicates' average AFI value.

It was possible to confirm this observation by treating the bacteria post-fixation. Because they are already fixed to the glass slide, increasing the concentration of xylene should not release many individual cells and if it did, most would be washed away in subsequent steps. Indeed, it was then confirmed that with an increase in xylene concentration, a decrease in AFI could be seen (figure 26). Athough the difference in AFI across conditions is not as accentuated in this experiment, it should also

be noted that the overall scale of values is lower than in other experiments. It could be a problem with the condition of the staining solution, however the trend is still clear and corroborates previous results.

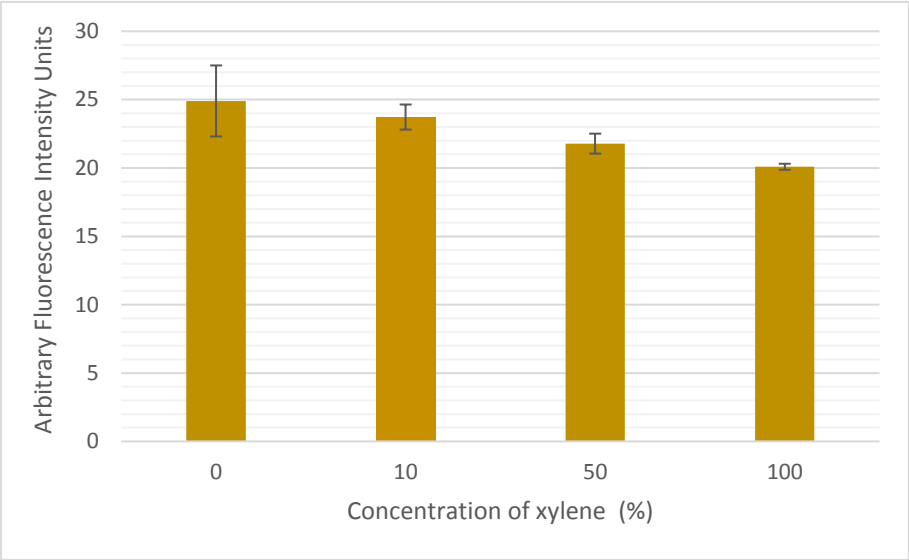

Figure 26 – Treatment with xylene post-fixation – Values of AFI of bacilli treated with xylene at several concentrations after fixation. The error bars represent the standard deviation of the average of the three replicates' average AFI value.

Deun, *et al.* (1999) had discussed a suspicion of the negative effect of exposure of mycobacteria to xylene in the number of countable bacilli with the ZN stain, in a study evaluating routine procedures' effect on reproducibility of examination. (137) In this case they used xylene as a clearing agent to de-stain and later stain again several smears. What they found however, was that xylene did not reduce the number or intensity of AF bacilli in thicker smears, but it did in thin ones. (137) Fukunaga, *et al.* published results showing a marked effect of xylene and formalin in reducing the cell count of both ZN and AuO stained smears. Although xylene probably did have an effect, this study had an incubation in xylene of 4h and several centrifugation and resuspension steps, which in no way mimic the conditions used in histology procedures. Furthermore, sensitivity was calculated on the basis of an estimation of total cells in which no mention of the cording-phenotype/homogenization nor "melting" effect with xylene was made, although these are factors which greatly influence cell counts. (128)

There have not been many published articles on the effect of xylene on mycobacteria cell integrity, but those that exist support the results found in this project. The experiments described above show a clear negative effect of xylene both visually in the disintegration of clusters and in terms of the intensity of staining.

There have been many other publications searching for alternatives to xylene, such as different solvents or oils, based solely on the fact that xylene is a very toxic chemical, showing that finding an alternative to it is a concern for many scientists, not just for the protection of the sample, but also of the health of histology laboratory users. (138–140). For histological diagnosis of the bacteria there is the prime concern of increasing the sensitivity of the stains after deparaffinization.

The effect of section thickness: It was speculated that the thickness of the sections of tissue could influence the number of bacilli found. This is because mycobacteria cells are heterogeneous in length and can range up to 10µm. The thickness of the sections used in histology is variable but usually around 5µm. If we assume that the AuO stain is contained inside the bacteria cell and retained by the cell wall, damaging the wall, for example by slicing it, would expose the interior to the removal of the stain. If mycobacteria cells are vertically oriented in a section, it is plausible to think that sectioning would not only cut the tissue but also all bacteria which exceed the thickness of the section in length, or which are positioned at the edge of the section. If this is true, thicker sections should increase the number of bacilli found, therefore facilitating diagnosis (figure 27).

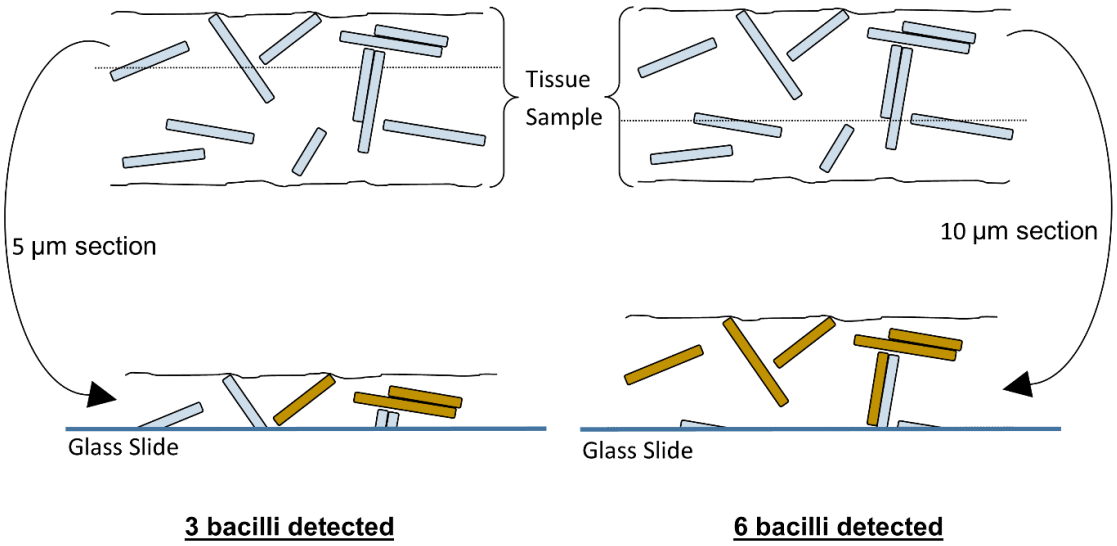

Figure 27 – Section thickness effect on EPTB diagnosis – scheme representing the hypothisis that the thickness of tissue sections used for EPTB diagnosis influences the sensitivity of the stains. In light blue are unstained bacteria and in yellow are stained bacteria, which are those that remain intact after tissue sectioning.

To test this effect was difficult because of the irregular distribution of bacteria across the tissue, making it impossible to perform cell counts which truly represent the global variation in number of bacteria between tested thicknesses. In that sense, it was only possible to qualitatively infer the influence of section thickness.

It was found that the thicker the section the more fluorescent bodies or debris, which cannot clearly be identified as bacilli, were found; background was also more fluorescent and, naturally, the depth of field is higher, meaning it is harder to focus on many cells at once, having to analyze each optical field also along the z axis of the plane to find additional bacilli which are initially out of focus. Furthermore, histology personnel reported the difficulty of obtaining quality sections when choosing higher thicknesses, which can result in sample waste. Regardless of technical difficulties, it is as easy to find bacilli in a 10µm thick section as in a 4µm section and the number of bacilli found was not noticeably different. However, because it is possible to find more bacilli in different focal planes at 10µm, it can be possible that more bacilli are present. The debris reported could, however, lead to false positives.

Overall, it is my belief that section thickness does play an important role in the sensitivity of AF stains, for it seems logical that damaging the cells in such a way as shown in the illustrations above would influence the number of bacteria which retain stains. Assuming this is true, the reason it was not possible to show such effect is the nature of the tissue infection distribution itself. Were it possible to create a matrix where bacilli were evenly and randomly distributed in the space and which could later be sectioned, I am confident a significant difference in cell counts would be found. Such a matrix was imagined, possibly by the use of a gelatin and mycobacteria culture, but then it would be necessary to solve the issue of cell aggregates and, most importantly, of sedimentation while the gelatin would solidify. Such experiments could not be conducted in the scope of this project, due to time constraints.

4 – Alternative Deparaffinization Methods

Preliminary comparisons of xylene deparaffinization and other methods: Having confirmed the negative effect of xylene, it was important to look for alternative methods of deparaffinization to the standard xylene method. Several ideas were preliminarily tested, to screen for potential candidates. The key aspect considered was that if organic solvents such as xylene were to be avoided, heat would have to replace them in order to physically remove the paraffin, which melts at above 50˚C. It was initially tested four candidate methods:

Table 6 – Four candidates for a novel deparaffinization method

Method	Principle	Concerns
Hot static air in a dry heat oven:	- Leaving the slides vertically at a high temperature, overnight, could be enough to melt the paraffin and for it to gradually drip off the tissue by gravitational force;	- Gravity would not suffice to remove the paraffin.
Centrifugation in a high humidity and high temperature atmosphere:	- The high temperature vapor should be enough to melt the paraffin while preserving the tissue integrity, while the centrifugation would expel the paraffin off the slide and sample;	- The tissue sample being removed from the glass slide and lost.
Wet heat by means of a water bath:	- The heated water would slowly melt and remove the paraffin which would float upwards on the water;	- The tissue sample being removed from the glass slide and lost..
Hot air projected on the sample:	- The projected hot air would both melt the paraffin and simultaneously blow it off the sample and glass slide;	- The very high temperature and dryness of the air would cause too much damage to the sample, by desiccation.

It was found that leaving the formalin-fixed paraffin-embedded tissue sections overnight at 68˚C in a dry heat oven was not effective and upon observation the sections became overexposed in fluorescence microscope observations, not allowing the discernment of any kind of structure, meaning the paraffin had not been removed. Centrifuging the slides in a high humidity and high temperature atmosphere showed relative success, although only preliminary tests with few samples were processed. In a glass slide containing several sections of tissue, only parts of the tissues were properly stained with ZN. Upon brightfield microscope observation several regions of the tissue were either completely transparent and unstained, probably still covered in paraffin and others retained too much fuchsin. However, parts of the tissue, even entire sections, were properly stained and appeared undamaged. This method would have to be further tested to achieve optimal conditions, but it appears to have

potential. Because other methods showed better potential immediately, this method was not further explored.

The method of projecting heated air (PHAD – Projected Hot Air Deparaffinization) resulted in promising observations. While in the xylene treated samples few bacilli were found, in those treated with PHAD many more were detected (figure 28). The samples submitted to the water bath also appeared to have a higher number and more fluorescent bacilli than the xylene treated samples. It was carried out a comparison between the three methods, to quantify these observations.

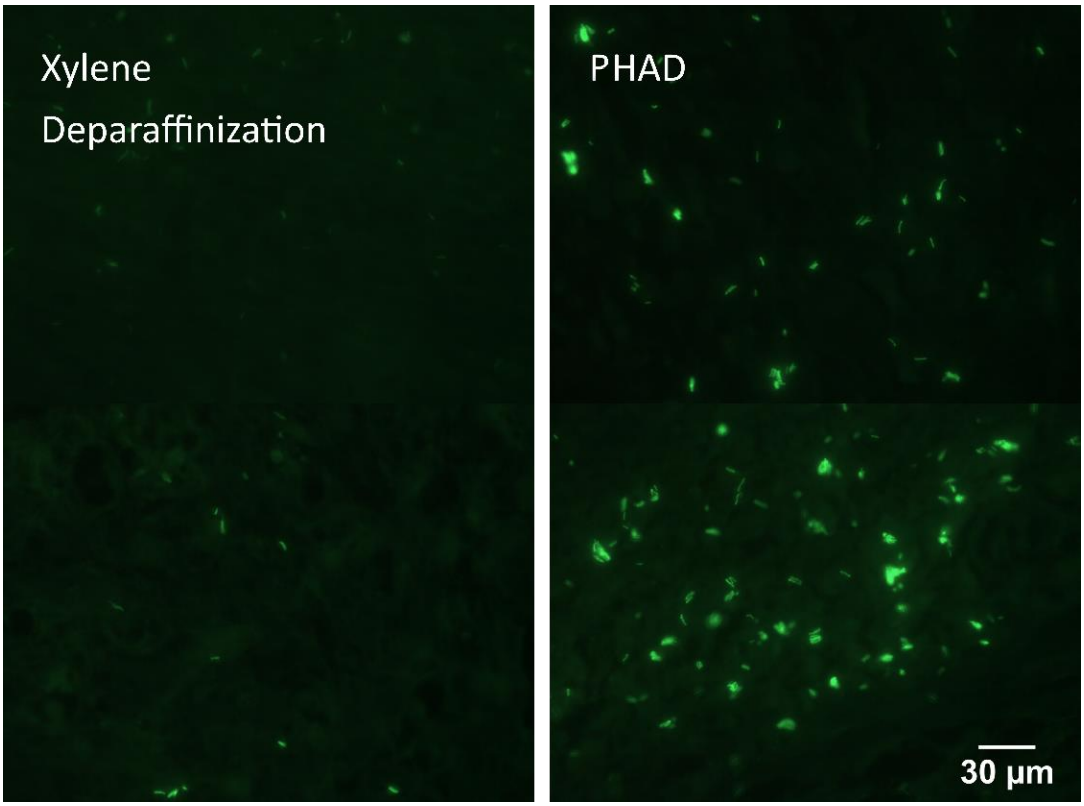

Figure 28 – Micrographs comparing PHAD to the xylene method – Fluorescence microscopy micrographs, taken with an oil-immersion lens at 1000X amplification. On the left the photographs were taken from samples deparaffinized with xylene and stained with AuO, while those on the right were deparaffinized with the PHAD method.

In terms of AFI measurements, the difference between the PHAD and water bath methods compared to xylene was considerable (figure 29). It was found that the samples treated in the water bath had an average fluorescence of bacilli of around 80 AFI units, while those treated with the PHAD of about 100 AFI units. It was further noticed that still, in these two cases, more bacilli were usually seen per field of observation and were more easily found, although not possible to count the bacteria comparatively. The samples treated with the standard xylene method had on average about 40 AFI units of bacilli fluorescence, half or less than the other methods, and the bacteria still appeared less frequently and comparatively faintly, such as those illustrated on figure 28.

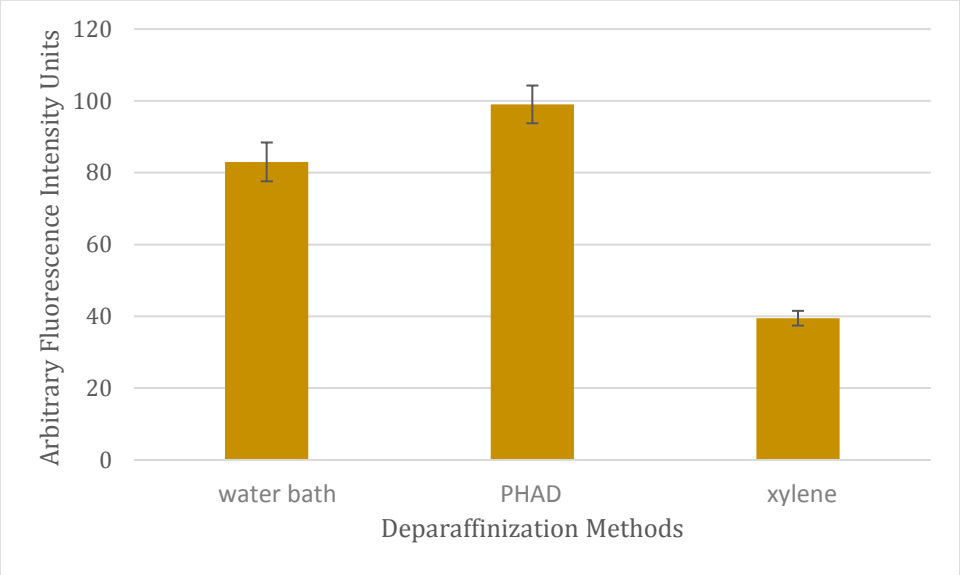

Figure 29 – Evaluation of two novel deparaffinization methods – Comparison of the AFI values obtained for the standard xylene method compared to the water bath and PHAD methods. These were tested in an intestinal lymph node sample. The error bars represent the standard deviation of the average of the three replicates' average AFI value.

These results seem to support the idea that removing paraffin from the sample using heat is an adequate approach. There already exist other methods in histology procedures which employ heat to remove paraffin, such as those used in heat induced epitope retrieval systems (HIER), which are similar to the water bath method here employed. However, to the best of our knowledge, dry heat applied on the sample has not been used or tested as a viable approach for deparaffinization, although here it appears to be most successful. It was still necessary to further test and improve the method and furthermore to understand if is useful for any histology application, or if it could possibly damage the morphology of the tissue, meaning it would only be useful from a microbiology perspective.

Because the PHAD method appeared superior to the water bath deparaffinization, it was decided to invest more time in exploring it, in detriment of the latter, although both showed the best results. Firstly, the PHAD method was further tested against the already established protocol of xylene deparaffinization and a method used in immunohistochemistry. Lab Vision™ PT Module™ is a heat induced epitope retrieval system used to both deparaffinize the sample and to simultaneously recover antigens for subsequent immunohistochemistry staining. Although it is not used for microbiology diagnosis, it could serve our purpose of eliminating xylene by using heat as the means to remove paraffin, even if the preservation of tissue antigen integrity is not relevant.

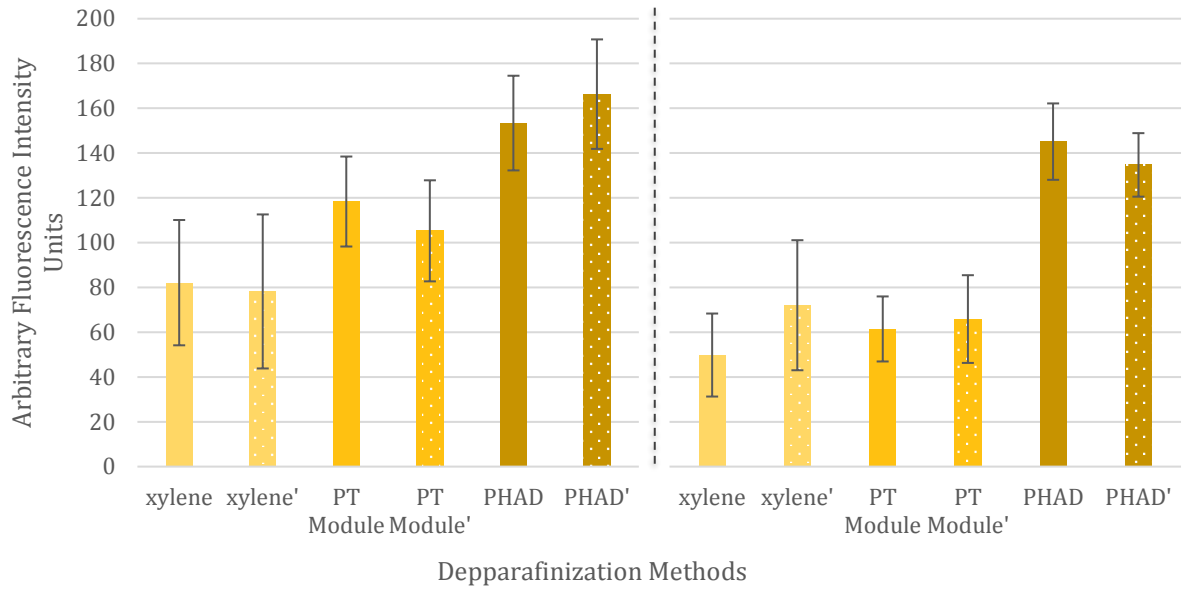

Figure 30 – PHAD deparaffinization compared to common methods - Comparison of the AFI value obtained for several deparaffinization methods, including the standard xylene method. These were tested in different samples: muscle (LEFT) and intestine + lymph node (RIGHT). Replicates are signaled with an apostrophe and their bars dotted. The error bars represent only the standard deviation of AFI values of individual bacilli for each condition and replicate.

It was found that the PHAD method outperformed the other methods in all samples whereas PT Module™ was better than the standard xylene method only in the muscle sample (figure 30). In both tissue samples, bacilli detected in the PHAD deparaffinized tissue fluoresced in average at above 130 AFI, up to 160, while the xylene deparaffinized averaged at most at around 80. It is also noteworthy that the population of bacilli where the xylene method was employed, was apparently less homogeneous than in the other two methods, resulting in larger standard deviations, represented by the error bars.

The wider variation of AFI values within each replicate of the xylene method, could reflect the fact that many of the more fragilized bacilli were damaged and dipped to lower values of AFI while the most resistant were not as impacted by the xylene. Some variation is expected always, because not all bacteria will have the same degree of acid-fastness, or structural integrity, but this degree of variation should be somewhat constant among methods unless the method itself were further impairing the cells, as appears to happen with xylene.

Histological Evaluation of the PHAD Method: To assess if the application of the PHAD method causes damage to the tissue sections, because of the long exposure to dry air at high temperatures, a comparison was drawn between PHAD and xylene deparaffinized tissues stained with H&E and ZN. The samples were submitted to a histology specialist for evaluation of the conservation of tissue morphology, tissue damage and detection of mycobacteria.

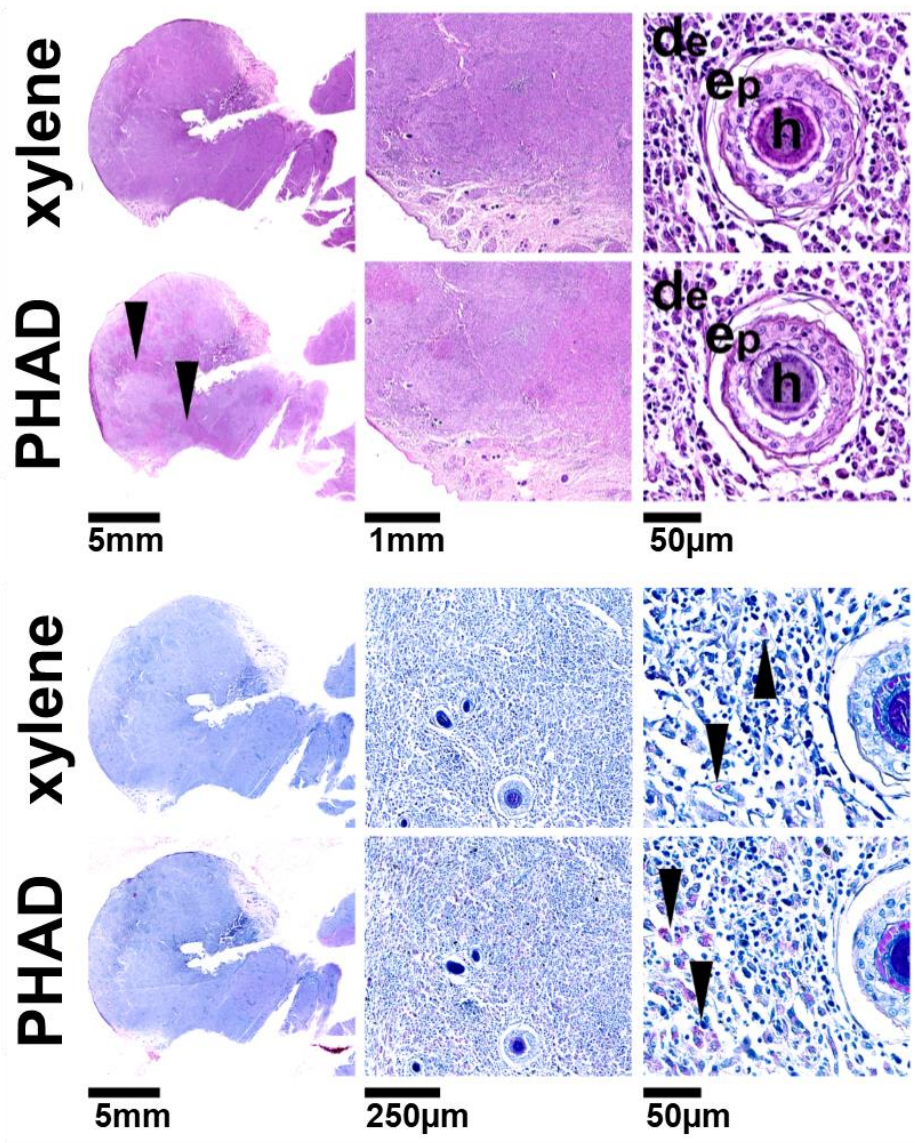

Figure 31 –Representative microphotographs of H&E-stained (TOP) and ZN-stained (BOTTOM) 5µm sections of a cutaneous mycobacterial granuloma, deparaffinized with xylene or PHAD. Compared to xylene, heat deparaffinization followed by H&E stain (TOP) results in light-colored and patchy unevenly stained sections (arrowheads). Original magnifications 5x, 2.5x and 40x. (de, dermis; ep, epidermis; h, hair). Ziehl-Neelsen-stained sections of heat deparaffinized tissue (TOP) show higher number of intracellular acid-fast bacilli, faintly visible in the same region of interest, with xylene deparaffinization (arrowhead). Original magnifications 5x, 10x and 40x.

Regarding the morphology of the tissue, it was seen that the cell structures and organization were unchanged between methods and no damage to the tissue was observed (figure 31- TOP – right column). However, it was noted that the H&E staining of PHAD deparaffinized tissue sections was uneven and lighter, compared to xylene deparaffinized samples. It could be possible that residues of paraffin are left in the tissue sections, preventing proper staining of the cellular matrix of the tissue. Upon evaluation it was described many more visible mycobacteria bacilli in the same comparative regions of the ZN-stained tissue sections deparaffinized with PHAD than in those deparaffinized with xylene, where bacilli appeared fainter (figure 31 – BOTTOM).

The report from this expert evaluation is further confirmation of the potential of the PHAD method for EPTB diagnosis, showing that both in traditional ZN stains in addition to AuO stains, a higher number of bacilli and with a stronger pink contrast or fluorescence intensity are seen, which facilitates detection and diagnosis. Although regions of the tissue appear to not be fully deparaffinized, the fact that the heat and air application do not damage the tissue allows further optimization in finding conditions for complete paraffin removal and therefore for future expansion to other histology applications. For either of these applications, however, it would be necessary to scale up the device to allow for processing of larger number of samples.

5 – PHAD (Projected Hot Air Deparaffinization)

Optimization and standardization of the PHAD method: Having preliminary results showing a good performance from the PHAD method, it was necessary to define optimal and standard parameters for its application.

During the very first tests with this method, it was important to construct a set-up which could be recreated. The hair dryer used was mounted on a tower at measured distances from the base and the glass slide support rack (figure 32), further characterized in section III (Materials and Methods).

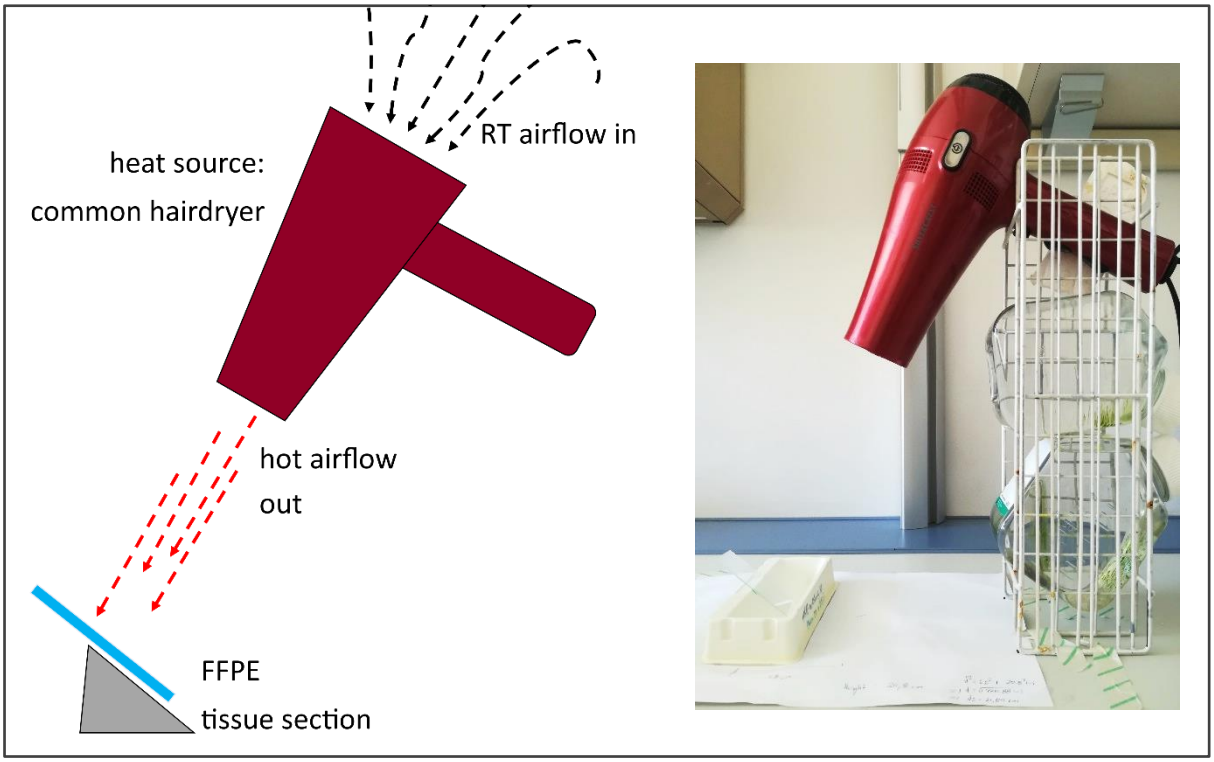

Figure 32 – PHAD method experimental set-up – On the left a representation of the working method, on the right a photograph.

Initially upon the first attempts at using PHAD, 5 minutes was tried as a duration for heat application followed by direct staining. The resulting slide was overexposed in fluorescence microscope observations. Later 20 minutes were adopted as the standard for the preliminary tests, followed by immediate dipping in distilled water at RT for about 5 minutes, followed by staining. It was then decided to optimize this duration to carry out further tests.

It was found that between 10 and 20 minutes not much difference could be seen in terms of AFI, although 20 minutes showed a slightly higher AFI and, although no formal cell count could be carried out, per field of observation more bacteria were found (figure 33). In this case it appears that 20 minutes is a good duration for the application of heat, although perhaps 10 or 15 minutes could be as adequate. For practical reasons it was decided to continue using 20 minutes, as it allowed a continuous flow of sample deparaffinization and subsequent AuO staining, in the circumstances of these experiments in our lab.

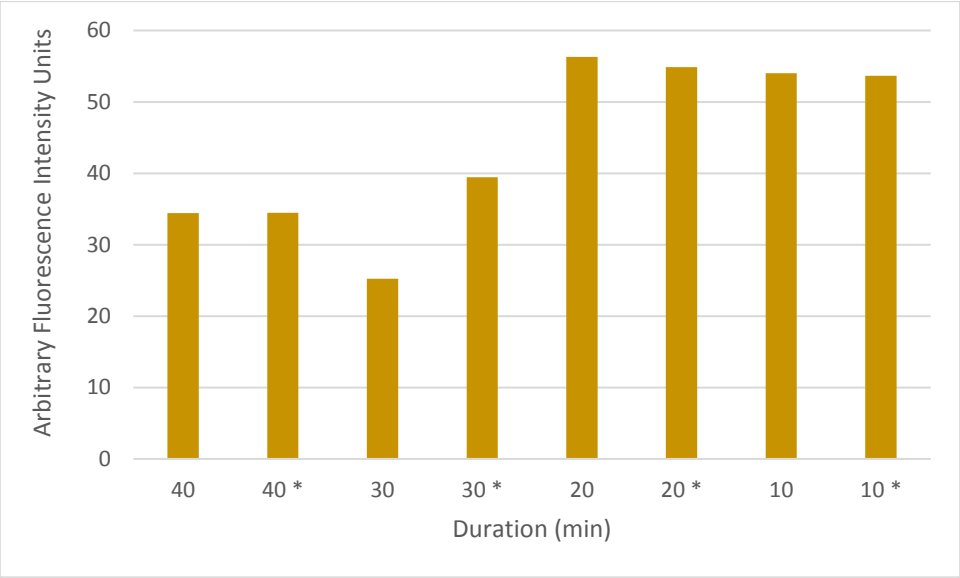

Figure 33 – Duration of Heat Application (PHAD) – Comparison of several durations of heat application in the PHAD method, regarding its effect on AFI. Replicates are represented with an asterisk.

The next step was to assess the temperature range of the hair dryer used as heat source. It was observed that the sample reaches the histology grade paraffin fusion point of 56-57˚C (119) within less than 40 seconds of the starting point. (figure 34) At around 2 minutes it reaches temperatures around 70˚C and it maintains an average of approximately 72.5˚C until the heat source is shut down. Once placed in water the temperature of the sample decreases greatly from 65˚C after shut-off to 27˚C in 40 seconds, crossing the paraffin fusion point in about 5 seconds. The apparent success of the method suggests that the heat reached, coupled with the air speed employed, is enough to melt the paraffin and blow it off the sample, while the immediate immersion in water assures that the tissue is rehydrated after the paraffin is removed and solidified. Time points and temperature measurements can be found in Annex- table 8.

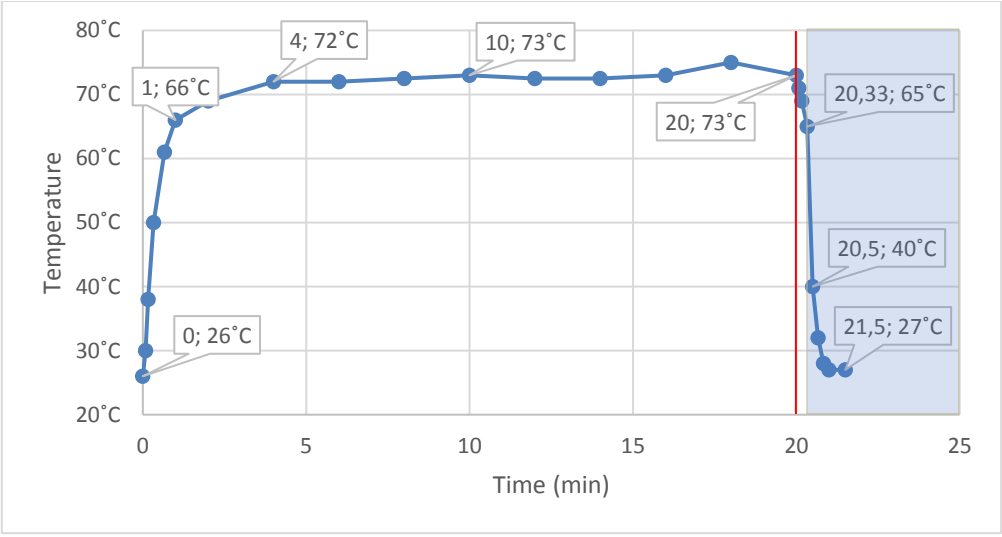

Figure 34 – Temperature Variation (PHAD) – Temperature variation of the sample under the heat source of the PHAD method. The red line represents the shut-off time point and the blue rectangle represents the immersion of the sample in water.

A final comparison was carried out with five replicates per condition and using the same tissue samples. (figure 35) The results still show PHAD outperforming the standard xylene method, although this time the differences were not as accentuated in the muscle sample. Also, the variation of AFI values within each replicate in this experiment (not illustrated) does not corroborate what was described earlier, being that the xylene method yields similar variation of bacilli AFI to PHAD.

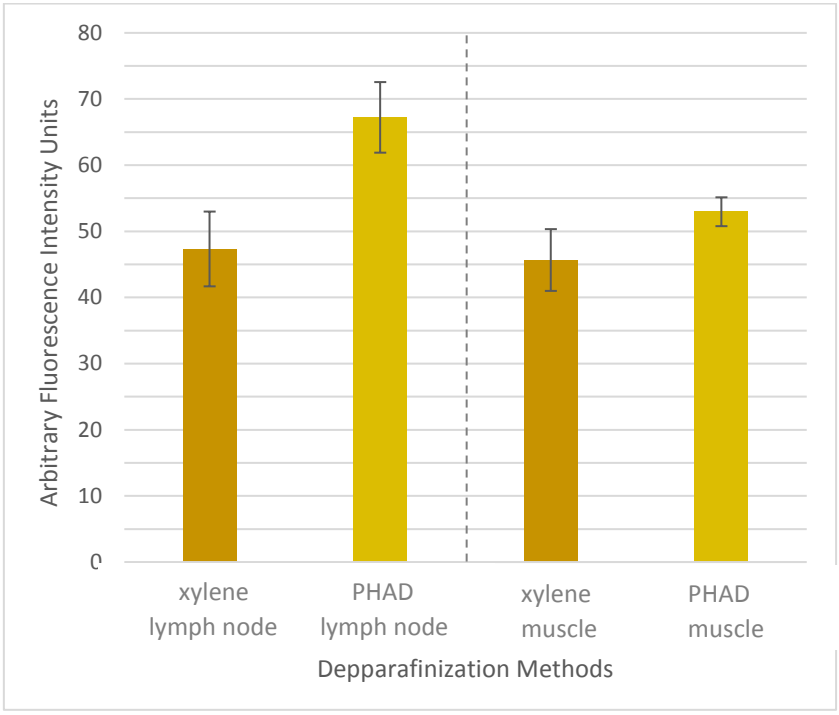

Figure 35 – Comparison of xylene and PHAD methods – Comparison of the AFI value obtained for the standard xylene method and the PHAD method. These were tested in different samples: muscle and lymph node. The error bars represent the standard deviation of the average of the five replicates' average AFI value.

It is possible that these observations and the lower overall scale of AFI results could be due to the ageing of the AuO solution, which results in weaker fluorescence and a narrower window of values. The fact that the gap of values between xylene treated and PHAD treated samples is reduced in the muscle sample and not the lymph node, could potentially indicate that the PHAD method could produce variable results depending on the nature of the tissue. This seems like a plausible effect since tissues have different principal components, which respond to heat differently.

V – Conclusion and Future Perspectives

Through the development of this project, several aspects of the diagnosis of the tuberculosis disease, in particular of the extrapulmonary kind, were dissected, explained and improved. As this project closes it was shown that typical histology procedures for EPTB diagnosis are damaging to the sample and a new method for the diagnosis of TB in tissue sections was developed as well as two more potential alternatives which can be explored in the future. Furthermore, possible improvements on the auramine O stain were suggested and new contributions to the debate of the AuO staining target were put forward.

The auramine O acid-fast stain was tested under various conditions to find that the standard in routine uses is perhaps not optimized for the purpose of TB diagnosis. In this subsection, several aspects of the protocol were manipulated and studied in order to improve both the practicality, safety and cost of the auramine O staining technique, but also to possibly further perfect it for application on extrapulmonary TB diagnosis on tissue sections. It was found that it is possible to reduce the duration and concentration of the primary stain, perhaps simultaneously, without compromising the success of the technique in smear samples. This simple alteration can have important implications in practical applications. It was also found that it is possible to remove phenol, a toxic constituent, in favor of safer and better alternatives, in culture smear samples. The implications for diagnosis of EPTB aren't clear but it seems that further investigating the composition of the stain for this application could yield good results and improve the diagnosis of these forms of the disease, especially by manipulating the mordant, phenol. It would also be interesting to determine the effects of duration and concentration of the stain in tissue samples.

For future works, it would be important to study the use of other nucleic acid stains, manipulating their protocol in terms of durations and concentration, in the sense that it might be possible that the manufacturer's recommended concentrations are in excess of what is necessary, as occurs with AuO. For example, SYBR® gold has been shown to increase sensitivity beyond AuO staining, based on its much stronger fluorescence, resistance to fading and fluorescence enhancement upon binding to nucleic acids.(108) These experiments could be applied to stains like SYBR® gold, perhaps showing that they could be used in lower concentrations, reducing their current high cost for clinical application.

Regarding the research on the auramine O staining target this project contributed further suggestive and preliminary evidence which indicates that AuO is not simply a mycolic acid stain. It was shown that stained mycobacteria and coccidia accumulate the fluorophore in their interior and that it seems to organize in co-localization with internal structures. Through TEM these observations were reinforced and observations that AuO perhaps links non-specifically to other cellular components were documented.

It would also be interesting to find a way of removing or manipulating the amount of nucleic acids within the bacteria and attempt to stain them, which would give direct proof of if nucleic acids are the target of the AuO stain for mycobacteria.

It was found through this work, that subjecting tissue samples containing mycobacteria to the fixative formalin in no way hinders later detection of the pathogen unlike what had been described.(128) In fact, it was shown that it might be possible that formalin-fixed cells, in some way, better retain or enhance the fluorescence of AuO. However, the experiments on the influence of xylene further confirmed those published in the literature.(128) Xylene decreases the fluorescence of AuO stained cells, possibly by damaging the cell wall. The observation of the "melting" of mycobacteria clusters is new evidence confirming an influence of this compound on cell integrity. Furthermore, these observations contribute to the theory of the target of the stain being inside the cell wall. The attempts at studying the effect of tissue section thickness were not quantifiable and no definitive answer is proposed, however the experiments conducted gave some qualitative observations supporting the idea that thicker sections include more detectable bacteria, which in turn also supports the hypothesis of the staining of the interior of the cell.

Future work could focus on finding a way to quantify the influence of tissue section thickness; for example, as mentioned, creating a gelatin like medium, immobilizing mycobacteria in a matrix, randomly distributed, allowing counting. I believe this is an important subject, for it could be another simple modification in the diagnosis process which could have large implications for its success.

Finally, but most importantly, this project originated a new method for the deparaffinization of FFPE tissue sections for EPTB diagnosis. It was found that heat applied through projected dry air results in the detection of many more and much brighter mycobacteria in tissue sections compared to those treated with the regular xylene deparaffinization. This method was named PHAD for projected hot air deparaffinization. Not only was the new method PHAD proposed, but the water bath and centrifugation methods were shown as potential candidates in which future work could find improvements. It is also noteworthy that the HIER method using PT Module™ also was shown as more adequate than xylene deparaffinization for EPTB diagnosis, although not as successful as PHAD.

For implementation, it is necessary to adapt PHAD into a device which could process samples at a larger scale and be routinely used by clinical histology laboratories. This, however, must be preceded by studies on its influence on sensitivity of diagnosis compared to culture and NAAT methods and will require further tests with more samples, preferably of cases of human TB. It is also important that in the scaling up process, optimizations are carried out in order to achieve complete paraffin removal, maintaining conditions which do not damage the tissue and preserving its advantage in mycobacteria detection.

It is my belief that the PHAD method is promising not only for clinical diagnosis of EPTB, by allowing an easier detection of bacteria, faster and cheaper diagnosis, with possibly a higher throughput, but also because it shows potential for further applications in histology and research.

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Annex

Table A.1 – Camera settings used in the epifluorescence microscope image capturing across several different experiments, depending on the sample and staining type.

Sample Type	Tissue Sections (4um), AuO	Tissue Sections (10um), AuO	Tissue Sections, Bright Field	Smear: ZN	Smear: AuO
Exposure	1.7s	6.0s	51ms	1.5s	580ms
Gain	1.7x	1.0x	1.0x	1.0x	1x
Levels	0; 2.0; 223	0; 2.0; 223	0; 1.7; 255	0; 1.7; 255	0; 2.0; 223
Color	Light Red	Light Red	Pinkish-Red	Red	Light Red
Saturation	80%	80%	100%	100%	80%

Table A.2 – Timepoints of measurement of the temperature of the sample in the PHAD method. From 20.08 minutes to 20.33 the hairdryer was shut off and at 20.5 submerged in distilled water, represented with gray and blue tonalities, respectively.

Time point (min.)	Temperature (ºC)	Time point (min.)	Temperature (ºC)	Time point (min.)	Temperature (ºC)	Time point (min.)	Temperature (ºC)
0	26	2	69	14	72.5	20.33	65
0.08	30	4	72	16	73	20.5	40
0.17	38	6	72	18	75	20.67	32
0.33	50	8	72.5	20	73	20.83	28
0.67	61	10	73	20.08	71	21	27
1	66	12	72.5	20.17	69	21.5	27