IMPROVED ACID-FAST STAIN AND HISTOLOGY METHOD FOR THE DETECTION OF MYCOBACTERIA IN TISSUE SAMPLES

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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.

INTRODUCTION: In the early 19th century scientists and clinicians began suspecting a relationship between the microscopic forms of life and the diseases which afflicted the population. 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 bacteria on a glass slide to better see and distinguish them under the microscope.(1,2) This allowed him to systematically associate several diseases with microorganisms.(1) His focus soon turned to tuberculosis and its causative agent, leading him to develop the stain which was later adapted into the known acid-fast Ziehl-Neelsen stain by many scientists.(1) He described Mycobacterium tuberculosis for the first time and introduced bacteriological microscopy into clinical diagnosis.(2)

Tuberculosis (TB) has afflicted humanity for millions of years and it became a serious concern after the industrial revolution, when cities where overpopulated and sanitary conditions were poor.(3) Today it infects almost a fourth of the population and over 10 million people contract the disease each year. It is the single most deadly infectious disease in gross number of deaths and It has been reported that an infectious dose of only 1 CFU could be enough to cause infection.(4,5) Although in Portugal the incidence for recent years has been lower than the average of the European region, there were over 1700 reported cases in 2017 alone, of which about 500 were cases of extra-pulmonary infection.(6)

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.(7) If the infection is disseminated in the lung, the number of bacteria is much lower in sputum samples.(7) When it is the case of EPTB, biological samples for microbiology and histopathology are difficult to obtain yet essential for confirmation.(8) Furthermore, the methods used for pulmonary TB are not efficient in detecting TB on these kinds of samples, specifically biopsies, for several reasons.(8–12) The problems found in these situations are precisely the focus of this project.

The most common and important microbiology diagnosis of TB can be divided into four main types: microscopic observation, culturing, nucleic acid amplification tests (NAAT's) and serological tests.(7,13) Although culturing is the reference-method and the only way to unequivocally diagnose a mycobacterium infection, it requires special conditions such as biosafety level 3 laboratory, specific media and incubation conditions.(7) In a clinical setting it can become expensive and laborious, as specialized equipment is necessary to monitor the large amount of cultures. It is common in most developed countries but only in certain larger healthcare centers and it is very rare in poorer countries with the highest incidences of TB. NAAT's are a promising technology. Unlike culturing which may take several weeks, they can be done in hours. They can accurately identify TB caused by the M. tuberculosis complex and some can even screen for antibiotic resistance genes.(7) The pitfall of these methods is how they are technically more complex, more expensive and are not as effective for tissue samples.

Staining of biological sample smears is the most traditional method. It offers both quick results, simplicity of application, inexpensiveness and satisfying sensitivity. On top of all, the advantage of this method is its applicability in any settings of diagnosis, whether in developed countries or low-resource laboratories. The reference-method for acid-fast staining is the ZN stain and its variations, such as the "cold ZN" or Kinyoun method. The main disadvantage of the ZN stain is its reported low sensitivity, lower than culture and lower than NAAT's, which can vary from 20 to 80%. Sensitivity is dependent on the quality of the sample, smear and

microscope, the expertise of the technician and his fatigue. (14,15) Fatigue is the result of the need for the technician to thoroughly evaluate each smear with an amplification of 1000x, with a 100x oil immersion lenses. To confidently determine the presence of bacilli, it is important to expend a minimum of 4 minutes per smear, although the recommended time can be as high as 15 minutes.(16) This increases the workload to unreasonable times for laboratory staff, leading to rushing and fatigue in the observations and consequent lower sensitivity. An alternative to ZN is the auramine O method (AuO). Auramine O is a fluorescent diarylmethane dye which can replace fuchsin in the ZN stain and originate samples which can be observed under the fluorescent microscope. Its advantage is that it makes bacilli more easily seen, being that they become fluorescent yellow/green to a dark background. (17) This contrast allows for observations at lower magnifications of 100 or 200x, drastically reducing observation times and fatigue.(16,18) Several studies have found AuO method to yield higher sensitivity in diagnosis than ZN.(19) Although inexpensive, AuO staining is hindered by the fact it requires a fluorescence microscope, however, nowadays alternative portable LED fluorescence microscopes are available, which are cheaper and even recommended by the WHO.(20)

An additional problem with acid-fast stains is their use is histology diagnosis of extra-pulmonary TB (EPTB). The results for histology diagnosis are less sensitive than regular smear staining.(9) NAAT's have been reported to successfully identify mycobacterium in tissue, however there have also been reports of its reduced sensitivity, even compared to staining, not to mention extra costs and specialized labour.(10–12) ZN is still the reference-method for this case and for that reason it must be understood why sensitivity is not on par with smear staining. It has been hypothesized that the aggressive treatment of tissue samples for staining could be responsible for fragilizing the wall of bacteria cells in the tissue, diminishing their acid-fastness. Fukunaga *et al.* studied the effect of xylene and formalin on bacilli and suggested their potential destructive action on bacteria cell wall, causing loss of acid-fastness in reduced cell count and fluorescence intensity.(21)

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?

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 which are the ideal concentrations of reagents, times of the steps and composition of the solutions. Are they the same for tissue?

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,(22,23) but evidence is dubious and evidence which supports other targets has been published by several sources.(16,24–28) 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?

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.

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). **Tissue Sections** preserved in paraffine 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: BCG culture in formalin 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 the fixed with methanol at -18°C.

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: The smear 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: The smear 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.

Slide Preparation and Mounting for Observation: Glass slides with tissue sections were mounted with a coverslip for observation using *BacLight*™ 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*™ 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*™ (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. 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 or the whole schizont with merozoites 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 to resolve fragmented organisms and wrongly identified bodies.

Auramine O Staining Optimization: For all experiments of this section the methods used were as described before, having only manipulated the concentration of dye in solution or the times of application, compared always to the standard. The composition of the solutions used in the solvent and solubilizer replacement experiment was summarized in table 1.

Table 1 – Auramine O staining solutions used in the "Solvents and Solubilizers" experiment

	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: 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, 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 collected to 1% formvar coated copper slot grids, stained with 2% uranyl acetate in 70% methanol and Reynold Recipe lead citrate.

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 direct culture was diluted with xylene by adding 200 µl to 800 µl of culture and after 10 minutes smeared, air dried and fixed. 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.

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 (Rapid 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).

RESULTS AND DISCUSSION:

Optimization of the Auramine O stain for TB diagnosis: Several comparisons were carried out 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. 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. Reducing the primary staining step duration from 15 to 5 or 1 minute resulted in proportionally lower arbitrary fluorescence intensity values.

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 1) 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.

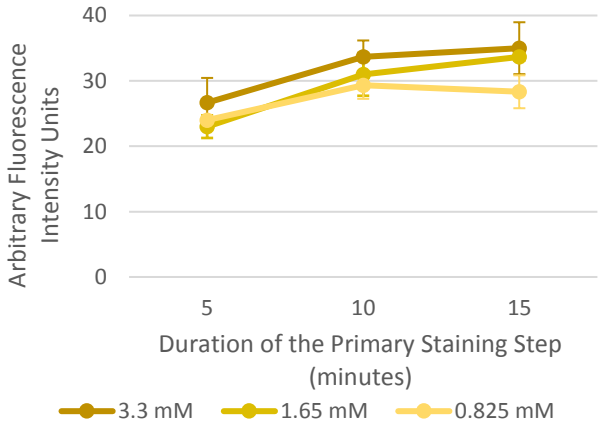

Figure 1 - Effect of the duration and concentration of the primary staining step – *AFI of bacilli stained with AuO at different concentrations: 3.3mM (standard, dark yellow), 1.65mM (half the standard, light yellow) and 0.825mM (a fourth of the standard, gray). Three durations of staining were tested 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.

A comparison between the two commonly used counterstains in the AuO staining method revealed a difference between potassium permanganate (PP) and methylene blue (MB). 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. According to our results it appears that potassium permanganate could quench less-fluorescent cells and, on the other hand, be advantageous in samples with more background and other fluorescent bodies, which it hides better.

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. In figure 2 is a histogram where the bars where omitted and only the trend curves are represented, showing the effect of substituting/removing water as a solvent. 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. 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. The solution containing glycerol also performed poorly, generating many, but 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 2, 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.

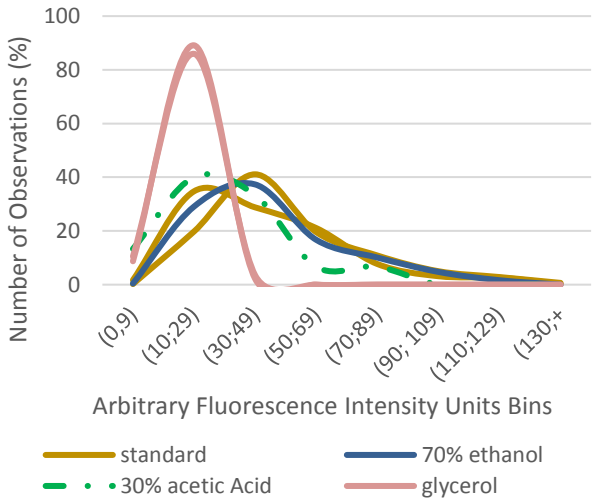

Figure 2 – Effect of alternative solvents in the auramine O stain - *Distribution of AFI of BCG bacilli according to the frequency of cells in each bin of values on the X axis. Conditions tested are of bacilli stained with an auramine O solution of a standard composition (yellow) and variants where: water was replaced by 70% ethanol (blue), 30% acetic Acid (green - dashed) and glycerol (pink). 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.*

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 while DMSO performed poorly (not illustrated).

Cellular Target of the Auramine O Stain: 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, 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.

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 3).(29) 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.(29) 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.

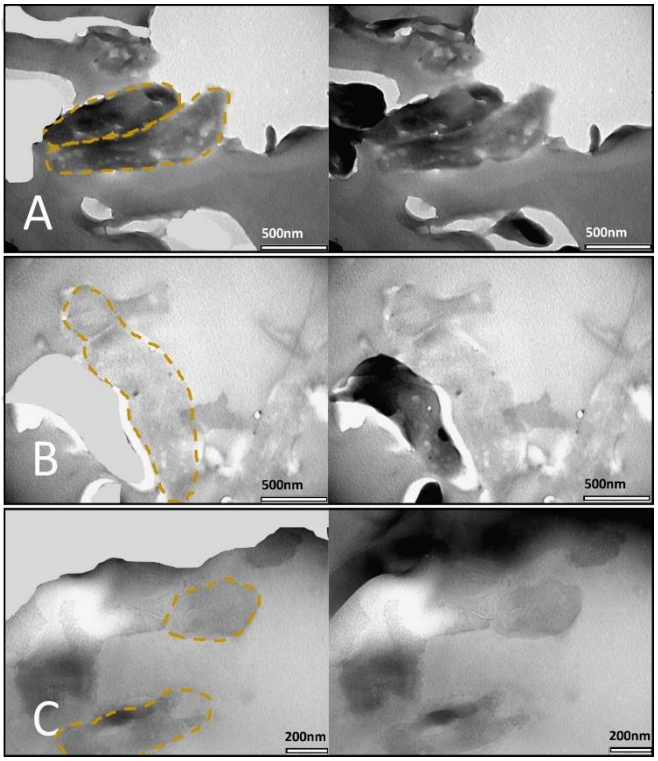

Figure 3 – Micrographs of mycobacteria by ChromEMT – Mycobacterium bovis *BCG stained with AuO and contrasted with DAB (A), stained with AuO but without DAB (B) and the control, unstained but contrasted with DAB (C). On the left are micrographs where mycobacteria were outlined and artifacts were covered, 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 3 it is possible to see that the bacteria stained with AuO and contrasted with DAB (3-A) became darker compared to the background, which did not happen with the not contrasted sample (3-B) and only slightly with the unstained control with DAB (3-C), indicating polymerization of DAB on the fluorophore attached to some internal structure. Interestingly, in the micrographs of stained and contrasted cells (3-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 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.

The Effect of Histological Processing on Mycobacteria in Infected Tissue: The effect of formalin on the AFI of BCG pointed to formalin not only generating higher AFI values but also preserving them for a longer period. 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)

It was found that the bacilli not treated with xylene have higher values of AFI while both the bacteria pre-incubated (42 avg. AFI) as well as the those treated with xylene post-fixation(39 avg. AFI) to the glass slide have lower values of AFI than none treated cells (57 avg. AFI). It was also observed that culture incubated with xylene caused the cord-like cell aggregates to disintegrate more intensely with increasing xylene concentrations. This is further evidence of the effect it exerts on the integrity of the cell wall. 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 show a clear negative effect of xylene both visually in the disintegration of clusters and in terms of the intensity of staining.

The effects of tissue section thickness were not quantifiable and no definitive answer is proposed, however qualitative observations on the experiments conducted support the idea that thicker sections include more detectable bacteria.

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 paraffine, which melts at above 50˚C. It was initially tested four candidate methods: Hot static air in a dry heat oven; Centrifugation in a high humidity and high temperature atmosphere; Wet heat by means of a water bath; Hot air projected on the sample.

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 paraffine 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 paraffine 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, and due to time constraints, 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 4). The samples submitted to the water bath also

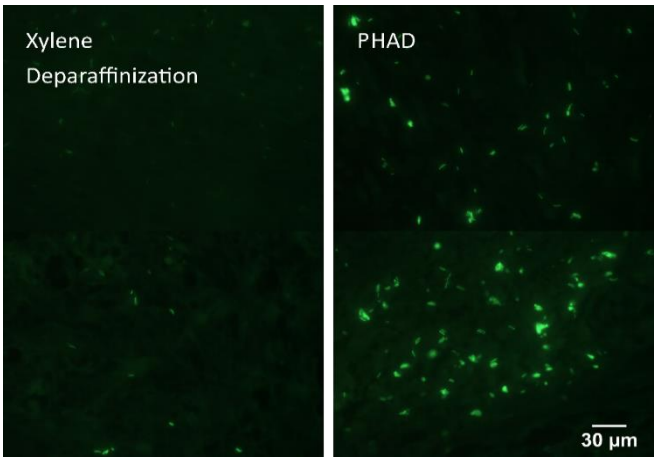

Figure 4 – Micrographs comparing PHAD to the xylene method – *Fluorescence microscopy micrographs, taken with an oil-immersion lens at 1000X amplification. On the left the photographs were taken from samples deparaffinized with xylene and stained with AuO, while those on the right were deparaffinized with the PHAD method.*

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.

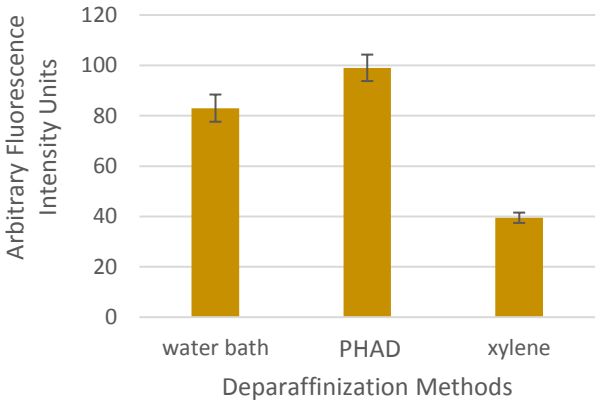

Figure 5 – Evaluation of two novel deparaffinization methods – *Comparison of the AFI values obtained for the standard xylene method compared to the water bath and PHAD methods. These were tested in an intestinal lymph node sample. The error bars represent the standard deviation of the average of the three replicates' average AFI value.*

In terms of AFI measurements, the difference between the PHAD and water bath methods compared to xylene was noticeable (figure 6). 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 4.

These results seem to support the idea that removing paraffine from the sample using heat is an adequate approach. There already exist other methods in histology procedures which employ heat to remove paraffine, 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 (figure 6). 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 paraffine, even if the preservation of tissue antigen integrity is not relevant.

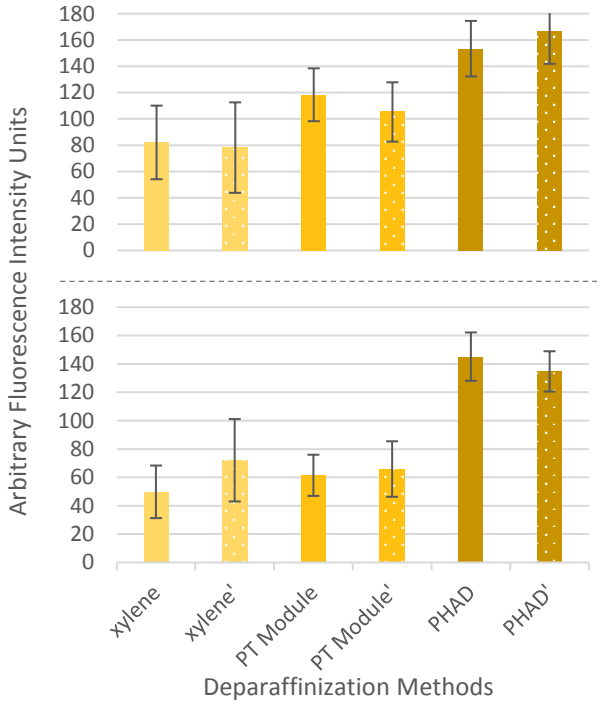

Figure 6 - PHAD deparaffinization compared to common methods *- Comparison of the AFI value obtained for several deparaffinization methods, including the standard xylene method. These were tested in different samples: muscle (TOP) and intestine + lymph node (BOTTOM). Replicates are signaled with an apostrophe 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 6). 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.

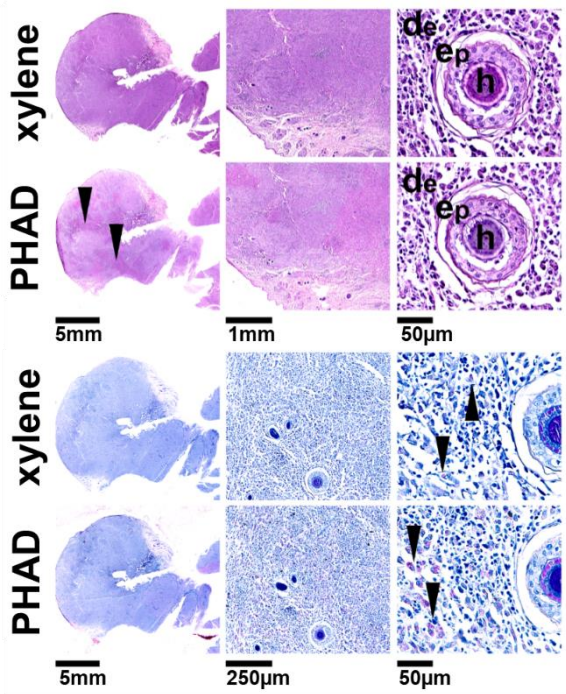

Figure 7 - Representative microphotographs of H&E-stained (TOP) and ZN-stained (BOTTOM) 5µm sections of a cutaneous mycobacterial granuloma, deparaffinized with xylene or PHAD*. H&E stain (TOP) results in light-colored and patchy unevenly stained sections (arrowheads). Original magnifications 5x, 2.5x 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 7- 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 7 – 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.

Optimizations and standardization of the PHAD method: 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 8), further characterized in section III (Materials and Methods).

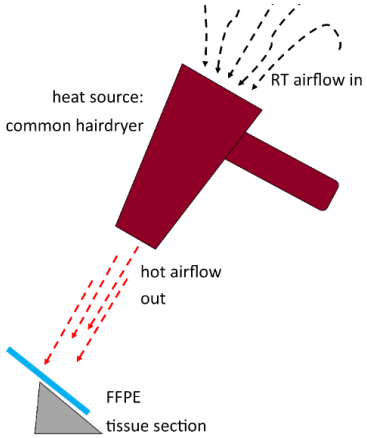

Figure 8– PHAD method experimental set-up.

Tests were carried out both to define optimal application times and the average temperature reached. It was found that a deparaffinization time of around 10 or 20 minutes yielded very similar results in terms of AFI, although the latter was slightly higher and more bacilli seemed to be observable. Shorter and longer durations were not adequate. Considering this, 20 minutes was adopted as the standard procedure. It was observed that the sample reaches the histology grade paraffine fusion point of 56-57˚C (30) within less than 40 seconds of the starting point. 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, crossing the paraffine fusion point in about 5 seconds. It appears the heat reached is enough to melt the paraffine and blow it off the sample, while the immediate immersion in water assures that the tissue is rehydrated after the paraffine is removed and solidified.

A final comparison was carried out with five replicates per condition and using the same tissue samples. (figure 9) 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.

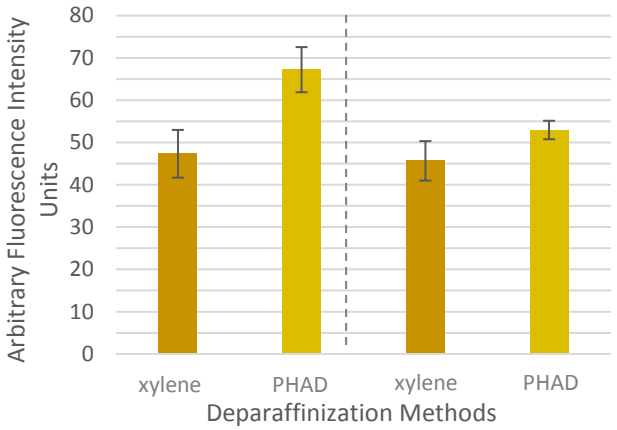

Figure 9 – Comparison of xylene and PHAD methods – *Comparison of the AFI value obtained for the standard xylene method and the PHAD method. These were tested in different samples: lymph node + intestine (LEFT) and muscle (RIGHT). The error bars represent the standard deviation of the average of the 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/intestine, could potentially indicate that the PHAD method could produce variable results depending on the tissue. This seems plausible since tissues have different principal components, which respond to heat differently. This must be further explored in future experiments.

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 and improved.

The manipulation of the stain concentration, composition and time of application executed in this study can have important implications in practice, reducing the time necessary to employ the method, reducing cost and increasing safety for routine clinical use. The implications for diagnosis of EPTB aren't clear but it seems that further investigating the composition of the stain for this application could mprove the diagnosis of these forms of the disease.

It would be important to study the use of other nucleic acid stains, manipulating their protocol, since it might be possible that the manufacturer's recommended concentrations are also in excess of what is necessary. 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.(15) 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.

This project contributed further suggestive and preliminary evidence which indicates that AuO is not simply a mycolic acid stain. Stained mycobacteria accumulate the

fluorophore in their interior and it seems co-localize with internal structures. Through TEM these observations were reinforced and observations that AuO perhaps links non-specifically to other cellular components were documented.

Unlike what had been described,(21) it was shown that that subjecting tissue samples containing mycobacteria to the fixative formalin might, in some way, cause a better retention or enhancement of the fluorescence of AuO. Experiments on the influence of xylene further confirmed those published in the literature.(21) A decrease in the fluorescence of AuO stained cells, possibly by damage to the cell wall and the observation of the "melting" of clusters is 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.

Future work could focus on finding a way to quantify the influence of tissue section thickness; for example, 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 for clinical histology laboratories. Studies on its influence on sensitivity compared to culture and NAAT methods and further tests with more samples, preferably of cases of human TB, are still necessary. It is also important that in the scaling up process, optimizations are carried out in order to achieve complete paraffin removal.

The advantages of the PHAD method could be an easier detection of bacteria, faster and cheaper diagnosis and possibly a higher throughput of samples. Also, it shows potential for further applications in histology and research.

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