

**THE DEVELOPMENT OF A “NEW” STAIN AND ITS  
COMPARISON WITH CURRENTLY AVAILABLE  
STAINS FOR THE EVALUATION OF  
MYCOBACTERIA IN PROCESSED TISSUE**

**BY**

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## Abstract

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Tuberculosis (TB) is an important global disease with the highest incidence rates in Africa with an annual rate of increase of 6%. In recent years, with the advent of AIDS, the number of patients infected by *Mycobacterium* (including *M.tuberculosis*), continues to rise. The histological diagnosis of TB hinges on the identification of acid/alcohol fast bacilli using the Ziehl-Neelsen (ZN) stain. As small numbers of organisms can generate an exuberant granulomatous response, searching for these bacilli can be a tedious and time consuming process. The auramine rhodamine (AR) stain is a fluorescent staining method used for detecting mycobacteria in cytological preparations (unprocessed tissue); this has been successfully applied to paraffin embedded sections in the past. The Papanicolaou (Pap) stain is used widely in cytology and reports exist of it being used to detect mycobacteria when examined under blue fluorescent light.

The aims of this study were to develop a novel fluorescent detection method, based on the Pap stain, for demonstrating mycobacteria in paraffin embedded (processed) tissue and to compare the results of this 'new' stain with two of the most widely used stains for the identification of tuberculosis in processed tissue, namely the Ziehl-Neelsen (ZN) and auramine rhodamine (AR) stains, with specific regard to (i) the ease of interpretation, (ii) the safety of the staining methods and (iii) the cost of the staining methods and equipment.

It was confirmed that the Pap stain induced fluorescence in mycobacteria in paraffin embedded tissue. The Pap stain was modified to optimise its staining characteristics in paraffin embedded tissue. The component that induces fluorescence was identified.

Comparison of the modified Pap stain with the AR and ZN stains showed that the time taken to identify mycobacteria was significantly shorter when using the two fluorescent stains than with the ZN stain. The modified Pap was the most difficult to interpret due to the abundant background staining but proved to be the easiest to prepare and was the safest of the three methods. It was, however the most expensive with regard to reagents, but likely to be the least expensive with regard to cost per slide.

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# Chapter 1 Introduction

## 1.1 A Brief Introduction to Tuberculosis

The human disease tuberculosis (TB) is caused by the organism *Mycobacterium tuberculosis*. Mycobacteria belong to the genus Mycobacterium, which is the single genus within the family of Mycobacteriaceae, in the order Actinomycetales (1). There are many types of mycobacteria that infect a wide range of species from invertebrates to higher mammals. Four broad groups exist, namely: the group that infects cold-blooded vertebrates such as reptiles and amphibians; the avian group causing disease in birds; the bovine group causing disease in a broad range of mammals, but predominantly seen in cattle; and the human group (2). The species *Mycobacterium tuberculosis* (*M.tuberculosis*) may be subdivided further into at least 3 major phage types, A, B, and C; and into 2 subtypes, Ax and A2 (3).

The human diseases caused by mycobacterial infections may be divided into three categories: tuberculosis, non-tuberculous mycobacterioses (or mycobacteria other than tuberculosis (MOTT's)), and leprosy. Tuberculosis may be pulmonary (limited to the lungs) or extra-pulmonary (systemic), where it may involve other organ systems, most notably the lympho-reticular, genito-urinary, gastro-intestinal and skeletal systems. It is caused by *Mycobacterium tuberculosis complex* (*M.tuberculosis*, *M.bovis*, *M.africanum*, and *M.microti*). Non-tuberculous mycobacteria are opportunistic pathogens that occur in patients with underlying disease or immunosuppression (including acquired immune deficiency syndrome (AIDS)). *M.avium complex* (*M.avium* and *M.intracellulare*) and *M.kansasii* are the most common causes of non-tuberculous mycobacterial diseases in humans. *M.leprae* is the causative agent of leprosy, an infection of the skin, mucous membranes, and peripheral nerves (4).

## **The Current Global Picture**

Tuberculosis is currently an enormous global health problem. In the year 2000, as the world looked out on the new millennium, it was estimated that one third of the world's population was infected with the disease (5). The thought is staggering, but to put this in figures, the World Health Organization (WHO) estimated that in that year, there were 8.3 million new TB cases and an estimated 1.8 million deaths due to the disease (6, 7)

The burden of the disease is carried by 'developing' countries, predominantly in sub-Saharan Africa, Central and South America whilst, in contrast, tuberculosis case rates are low in 'developed' countries such as Western Europe and North America (8).

### **1.1.1 'Developed' Countries**

In spite of the AIDS pandemic, the last decade has seen a steady decline in the number of tuberculosis cases in the majority of industrialised countries including western and central Europe, North and South America and the Middle East (9). The main reasons for this are: improved living conditions, lack of poverty, good prevention programs and access to adequate (supervised) treatment.

It has been stated that even in industrialised countries, the majority of tuberculosis cases occur in foreign-born individuals and in marginalized populations, including the homeless, prisoners, drug users, and people with HIV infection or AIDS (8).

The notable exception are those countries belonging to the former Soviet Union, where there has been economic decline, failure of general health provision and breakdown of TB control programs since the fall of the Berlin wall in 1991. There has been a marked decrease in the general standard of living and rising unemployment (7, 10). Tuberculosis

follows poverty. In addition, these countries have also been hard hit by the AIDS pandemic and have the greatest number of HIV/AIDS cases in all of Europe (11).

The recent advent of highly active anti-retroviral therapy (HAART) has had a major impact on the management of HIV positive patients. It has been shown that patients receiving HAART are not as severely immunocompromised as their counterparts not receiving treatment. Patients on HAART have been proven to be less susceptible to developing tuberculosis (12).

Since the advent of HAART, a condition called immune reconstitution inflammatory syndrome (IRIS) has emerged. A significant number of IRIS cases are as a result of an exuberant response to underlying tuberculosis (13-17).

### **1.1.2 'Developing' Countries**

'Developing' countries have borne the brunt of both the TB and HIV/AIDS pandemics. In Africa the annual rate of increase in the number of TB cases is 6% (6), largely fuelled by the HIV pandemic and poor public health infrastructure. Thirty-one percent of all new TB cases in adults (aged 15-49 years) in Africa are attributable to HIV infection and co-infection prevalence rates equalled or exceeded 5% in 8 African countries. In South Africa alone there were 2 million co-infected adults identified in 2003 (6). Over 25.8 million of the estimated 40.3 million HIV-positive adults worldwide are in sub-Saharan Africa (18).

Unlike 'developed' countries, where transmission of HIV is predominantly through homosexual sex and sharing contaminated needles when using intravenous drugs, transmission in the developing world is primarily through heterosexual sex. The WHO estimates that 71% of cases worldwide are transmitted by heterosexual sex, 7% through injections or drug use, 5% by blood transfusion, and 15% through homosexual behaviour. (18, 19)

It is clear that control of tuberculosis in Africa is closely linked to the control of HIV and AIDS (8, 20, 21), and attaining control of these two diseases, has been the topic of many conferences. The solution obviously lies in the giving to the developing world that which the developed world possesses, namely improved living conditions, lack of poverty, good prevention programs, access to adequate (supervised) treatment of both diseases in the form of directly observed therapy (DOT) and highly active anti-retroviral treatment (HAART), as well as changing the sexual practices of the populace. All this needs to be achieved in countries with large populations, poor infrastructure and few monetary resources (18).

Davies et al. (22) astutely predicts that not only is the incidence of tuberculosis on the rise globally, but is set to continue on its upward trend over the next decade. He gives three reasons for this. Firstly, tuberculosis is most prevalent in those areas which are currently experiencing the highest population growth, in particular, Central Africa and South Asia. Secondly, these same areas are seeing a massive increase in HIV infection rate. Thirdly, as AIDS devastates the economically active portion of the population, we are likely to see an increase in poverty in these regions. Poverty, overcrowding and poor living conditions favour the spread of tuberculosis - the disease risk will be compounded (22). In July 1996, it was announced by the WHO that South Africa had approximately 350 cases of tuberculosis per 100,000 population, the highest in the world (23, 24).

### **1.2 Detection Methods for Identifying Mycobacteria (including *M. tuberculosis*)**

The diagnosis of tuberculosis rests on the identification of the mycobacterium in samples taken from an infected patient. Various methods are used to achieve this and these fall into three broad categories: culture, microscopy and molecular methods (9, 25-27).

Firstly, culture entails the incubation of material taken from the patient in an appropriate culture medium followed by a process of identification. Various culture methods are available today, from the solid Lowenstein-Jensen medium to liquid-based techniques and automated radiometric, and non-radiometric systems (27, 28). Culture is highly specific and sensitive (more so than microscopy). It is, however, a process that requires a significant amount of time as mycobacteria are slow-growing and difficult to culture. Up to eight weeks are required for a definitive result using Lowenstein-Jensen medium (26, 29). The use of liquid media has decreased the turn-around time but these assays can still take 10 to 14 days (30). High-performance liquid chromatography is a technique developed in the last decade which can identify mycobacteria within a few hours (31). A major advantage is that sensitivity testing can be performed to determine which anti-tuberculosis agents the organism is sensitive (or resistant) to (30).

The second identification technique, microscopy, takes many forms but can be divided into those performed on fresh preparations in the form of smears, imprints and sections cut from fresh frozen tissue, or those performed on processed tissue that has been fixed in a fixative medium and then processed and embedded in a support medium allowing sections to be cut.

Fresh preparations are used routinely in microbiology and cytology laboratories for diagnosing mycobacteria (25, 32). The specimens are usually sputum samples, bronchial brushings, lavage or aspiration preparations. These specimens are either air-dried or fixed with an alcohol based fixative (e.g. Cytifix). Smears are the most widely used method for the identification of mycobacteria as they provide a rapid diagnosis (an answer can be given in a matter of minutes), are easy to process, require very little equipment and are thus inexpensive (9). The major disadvantage is that although they have good specificity, their sensitivity is low. Studies have shown that only up to 50% of pulmonary and 25% of

extrapulmonary tuberculosis cases are diagnosed on smear preparations, using the conventional Ziehl-Neelsen (ZN) stain (26, 33). Various stains have been used in an attempt to increase the sensitivity, however the only stains to have found their place in routine microscopy are the auramine based fluorescent stains (auramine-rhodamine/auramine O/Kuper May fluorochrome stains) (25, 34-45).

In Anatomical Pathology laboratories the majority of specimens are received in a fixative such as formaldehyde based solutions. These are then taken through a dehydrating process and ultimately embedded in paraffin wax to enable thin sections to be cut for staining and examination. The stain routinely used is the haematoxylin and eosin stain (H&E). Mycobacteria are not identifiable on H&E sections, therefore, when tuberculosis is suspected, special stains are applied, the most widely used being the ZN stain (25, 31, 44). The advantages of formalin fixed/wax embedded (processed) tissue specimens are:

- They are not as infectious as fresh preparations as the formalin is bactericidal.
- The specificity with certain stains (such as the ZN method) is high
- Tissue blocks and slides can be stored indefinitely.
- Multiple, near identical sections can be cut from a single wax block
- Multiple stains can be applied to these sections
- Tissue can be used for molecular studies (46, 47).

The main disadvantages are:

- Processing, cutting and staining usually take a minimum of six to eight hours.
- The sensitivity is low and the process of searching for mycobacteria is laborious and time consuming (29, 46).

Fresh preparation specimens are used as an ancillary technique in some Anatomical Pathology laboratories where they are typically only performed when fresh tissue is submitted for examination (usually as part of a study). These take the form of frozen sections and smear or imprint preparations. Smear and imprint preparations are identical to those used in cytology. Frozen sections (histologic sections cut from snap frozen tissue) have the advantage of allowing for a rapid diagnosis, but their use is dissuaded in the majority of diagnostic laboratories as there is a significant risk of infection of laboratory staff and necessitates sterilization of the cryostat which is a laborious task. The use of frozen sections for the identification of TB bacilli is generally limited to experimental laboratories.

The third group of identification processes to be elaborated is the molecular techniques. These are becoming more important in the identification of many pathological processes including those caused by infective agents. These take the form of in-situ hybridisation (ISH), RNA sequencing, polymerase chain reaction restriction enzyme assays (PCR) and proteomics (28, 48). Numerous studies have proven the worth of these techniques in identifying mycobacteria in both fresh and processed tissue in experimental and routine diagnostic settings (37, 49-57). PCR in particular has been applied widely and has been proven to be effective in identifying mycobacteria in fresh tissue, air-dried smears and paraffin embedded sections (33, 47, 56, 58-62).

Much research is currently being aimed at identifying genomic mutations that can be used as markers for drug resistance and virulence (28, 29, 31, 57, 63, 64). Molecular methods are very sensitive; however false positives are a significant problem, largely due to contamination, particularly in laboratories that deal with a high number of tuberculosis cases. Sensitivity also drops markedly when applied to non-respiratory specimens (28,

50). Molecular techniques require skilled personnel and costly equipment thus limiting their use to research or large reference laboratories (25, 27).

Taking into account the amount of research that is being done in developing novel molecular methods for the identification of TB, DNA chip-based hybridization assays will be available in the near future (58).

Novel diagnostic tests developed in recent years include Mycobacteriophage assays, interferon assays and antibody tests (48). Mycobacteriophage assays use a technique whereby clinical specimens are incubated in a solution containing a mycobacterium-specific phage (virus). A viricidal agent is then added to eliminate residual free phage. This ensures that only the phages that have entered mycobacteria present in the sample will survive. The specimen is then plated onto a petri dish covered evenly with a 'lawn' of laboratory grown, rapidly proliferating mycobacteria. This is then incubated for 24-48 hours. If mycobacteria were present in the original specimen then the phage will infect and kill the mycobacterial 'lawn' resulting in a patchy appearance to the growth on the petri dish (65).

The interferon assay is an *in vitro* test of how the patients' circulating mononuclear cells respond to purified protein derivatives of *M. tuberculosis* (48). This test cannot distinguish between latent and active infection and is of little use in a population with a high incidence of tuberculosis. The test may also not work satisfactorily in immunocompromised individuals.

Antibody tests, although promising, have not performed well in clinical trials and have proven to be of little use.

## Staining Methods

Many infectious agents require the application of special stains to make them visible on examination with conventional light or fluorescent microscopy (66). A number of stains have been developed for the identification of *Mycobacterium tuberculosis* and are discussed below:

### 1.2.1 Ziehl-Neelsen (ZN)

In 1882 a professor of bacteriology from Lübeck, Germany, Franz Ziehl described a method for the identification of mycobacteria that made use of the ability of the tubercle bacillus to retain the red dye carbol fuchsin when exposed to acid alcohol. The phenol in the carbol fuchsin solution acts as a detergent to allow the red basic fuchsin dye to bind to negatively charged groups within bacilli. The mycolic acids (amongst other cell wall components) present a barrier to the elution of the dye by strong acid alcohol solutions which bleach or decolourise the rest of the slide. Adding a blue counter stain such as methylene blue highlights the red mycobacteria (67).

This method was published in a handbook of staining methods the following year by Friedrich Karl Adolf Neelsen, a German pathologist (68). For the past century the Ziehl-Neelsen stain has been the most widely used stain for the identification of TB in both fresh and processed tissue preparations (25, 44). The stain is used in conventional light microscopy, is highly specific for mycobacteria, is easy to perform and interpret and has proven to give reliable and reproducible results (38). It is a cheap stain and can be performed in a rudimentary laboratory.

The major criticisms of the stain are: firstly, that it makes use of the highly toxic reagent phenol (69), and secondly has a low sensitivity (26, 33, 70).

The stain method has changed little over time. The method used in this study appears in Appendix 3.

### **1.2.2 Ellis and Zabrowarny (EZ)**

In 1993 Ellis and Zabrowarny published their modified fuchsin stain in the Journal of Clinical Pathology (69). This method excluded the highly toxic substance phenol. In its place, a basic fuchsin solution is used, in combination with a lipidophilic agent (such as L.O.C. High Suds (Amway)) to overcome the hydrophilic barrier presented by the waxy mycolic acids of the mycobacterial cell wall.

The results of the stain are practically identical to those of the ZN stain in that mycobacteria stain red against a light blue background.

The stain has proved specific for mycobacteria, but studies have yet to be done to evaluate its sensitivity. A second minor disadvantage is that the lipidophilic agent is more expensive than phenol, making the stain marginally more expensive than the ZN.

### **1.2.3 Auramine Based Fluorochrome Stains**

The auramine rhodamine fluorochrome stain was first described in 1960 by Kuper and May (71). Since then, it has found its niche in the examination of sputum smears (34-36, 38-45, 72). It has proven to be more sensitive for detecting mycobacteria than the ZN stain (40, 41, 45), but a number of studies have shown that the specificity is low leading to an increased number of false positive tests (38, 39, 42, 43, 72). It is for this reason that the WHO recommends that all cases labelled as positive for mycobacteria on an AR stain have this confirmed with a ZN stain (73).

The auramine rhodamine method has been successfully applied to paraffin embedded sections in the past (74).

The AR stain method used in this study may be found in Appendix 4.

#### **1.2.4 Papanicolaou (Pap)**

George Nicholas Papanicolaou, hailed as the father of modern cytology, described a stain for use in vaginal smears in 1942, which was subsequently modified by himself in 1954 and later in 1960 (75, 76). In collaboration with Dr. Herbert Traut from the department of Obstetrics and Gynaecology at Cornell Medical College in New York City, he used this technique in a test for the detection of carcinoma of the female genital tract. Their findings and conclusions were published in 1943 in the famous monograph, *Diagnosis of Uterine Cancer by the Vaginal Smear* (77). This diagnostic procedure was named the Pap test and has gained wide support and acclaim as a simple screening method for the early detection of pre-malignant and malignant lesions of the cervix and lower female genital tract.

In 1995 Kupper et al described the induction of fluorescence in mycobacteria, in cytology smears stained with the Pap stain (78). A later study by Wright et al performed at the University of the Witwatersrand confirmed this finding (79). No studies have investigated why the Papanicolaou stain induces fluorescence in mycobacteria and whether the same pattern of fluorescence is seen in processed specimens.

The Pap stain is composed of three dyes, namely: haematoxylin (which stains nuclei blue), OG-6 (composed of the yellow dye that stains keratin, orange G, and phosphotungstic acid) and the red-pink cytoplasmic counterstain eosin A (EA). This stain clearly defines the cell nucleus, highlights keratinized cells and lightly counter stains the cytoplasm, allowing

single and overlapping cells to be examined (75, 80, 81). Two forms of haematoxylin are commonly used: Harris' (as originally used by Papanicolaou himself) and Gill's.

The 'OG' of OG-6 is for the dye, Orange G; the 'G' is for 'gelb' meaning yellow and the '-6' designates the concentration of phosphotungstic acid.

Various forms of EA have been used, namely EA 36, EA 50 and EA 65. 'EA' is not an abbreviation, but is the label given to the combination of eosin Y, light green SF yellowish, Bismark brown Y and phosphotungstic acid, while the numerical values indicate different proportions of these reagents. Eosin is derived from the Greek "eos" for dawn and is a red dye that stains cytoplasm, nucleoli, cilia and red blood cells. The 'Y' is for yellow. Light green SF is a non-specific cytoplasmic counterstain and the 'SF' designation is for "saurefarbstoff" meaning acid dye. Bismark brown is a yellow-brown counterstain that has been proven to be of little additional benefit to the Pap stain. It and has the added problem of causing phosphotungstic acid to precipitate out, thereby shortening the shelf-life of EA. It is for these reasons that it is increasingly being left out of Pap stains (80, 82).

The various Pap stain methods used in this study may be found in Appendix 2.

### **1.2.5 Other Stains**

Other stains have been developed for identifying mycobacteria and include:

- Kinyoun stain (45, 83, 84)
- Dieterle stain (85)
- Silver-methenamine stain (36)
- The fluorescent Acridine Orange stain (39, 86).

The only one of these that has found widespread acceptance in routine diagnostic work is the Acridine Orange stain which is sometimes used as a substitute for the AR stain.

### **1.2.6 Immunohistochemical and Immunofluorescent Methods**

A number of monoclonal and polyclonal antibodies to antigens expressed on the cell wall of mycobacteria have been developed and are commercially available. Some react with several mycobacterial species, whilst others are species specific (e.g. NCL-MTSS and NCL-MT available from Novocastra Laboratories Ltd.). These stains have been applied to fresh smears, frozen sections and processed tissue sections with both avidin-biotin and fluorophore labels, but with limited success. They have not been widely used in the diagnosis of mycobacterial infections (48).

### **1.3 The Cost of Investigations**

There is marked disparity in the funds available for health care in developed and developing countries and yet, as highlighted in section 1.3.2, the brunt of both the TB and HIV/AIDS pandemics are borne by the developing world. The diagnosis of tuberculosis rests on the identification of mycobacteria in samples taken from infected individuals. Culture is the most sensitive and specific method for achieving this, but it is costly and time-consuming. Over the years, examining sputum smears with the ZN stain has proven to be cheap and easy to use, even in developing countries, but its low sensitivity is a major drawback (33). In support of this, a study performed in the Nairobi City Council Chest Clinic in Kenya, compared the efficiency and cost-effectiveness of the ZN and AR methods. Using the two staining methods, they detected 92% of culture-positive cases. Not surprisingly, the AR proved more sensitive than the ZN ( $P < 0.001$ ), and the cost was lower at 40.30 US dollars for AR compared to 57.70 US dollars for the ZN (87). The more modern techniques such as the molecular technologies (e.g. PCR) and the more novel methods such as Mycobacteriophage assays are sensitive, specific and fast, but they are expensive and require expertise not available in the third world setting (59, 63). The search is on for a simple, cheap technique that can easily be applied in underdeveloped

countries at a clinic level that is sensitive enough to be used as a screening test, but specific enough to render a definitive diagnosis with few false positives.

As stated above in section 1.2.4 (pg 13), The Pap stain is a cheap, widely available stain that has been shown to induce fluorescence in mycobacteria in cytological preparations. This study was undertaken in an attempt to produce a fluorescent mycobacterial stain for processed tissue, using as its base the Pap stain.

University of Cape Town

# Chapter 2 Development of the Modified Pap: Aims, Materials, Methods and Results

This study was approved by the Human Ethics Committee, Groote Schuur Hospital / University of Cape Town.

## 2.1 Aims

1. To develop a novel fluorescent method to detect mycobacteria in formalin fixed, processed, paraffin embedded tissue, using the Pap stain as a base.
2. To compare the results of this 'new' stain with two of the most widely used stains for the identification of tuberculosis in processed tissue, namely the Ziehl-Neelsen (ZN) and auramine rhodamine (AR) stains, with specific regard to:
  - the ease of interpretation reflected by the time taken to identify the organism
  - the safety of the staining methods
  - the cost of the staining methods and equipment.

The results of each of the studies that follow are included in this chapter for the sake of continuity and clarity, as each result influenced the design of subsequent studies.

## 2.2 Testing the Pap stain

**AIM:** To test the hypothesis that *M.tuberculosis* bacilli fluoresce under blue light on a Pap stained slide of processed tissue.

**MATERIAL AND METHOD:** Two positive control cases and a negative control case were selected. One of the positive controls was taken from the archives of Division of

Anatomical Pathology, Department of Laboratory Medicine, University of Cape Town, whilst the other was processed tissue taken from a rabbit that had been infected with *M.tuberculosis* (referred to hereafter as human control (HC) and rabbit control (RC)). The HC was a section of colon from a patient who had culture proven pulmonary and gastrointestinal tuberculosis on and was selected as there was abundant tissue available. The RC was subcutaneous tissue taken from a rabbit inoculated with *M.tuberculosis*. Both positive controls contained numerous acid-fast bacilli when stained with a ZN stain, allowing bacilli to be readily identified. The negative control (NC) was tissue taken from a non-infective surgical case. This was a section of myometrium taken from a leiomyomatous uterus.

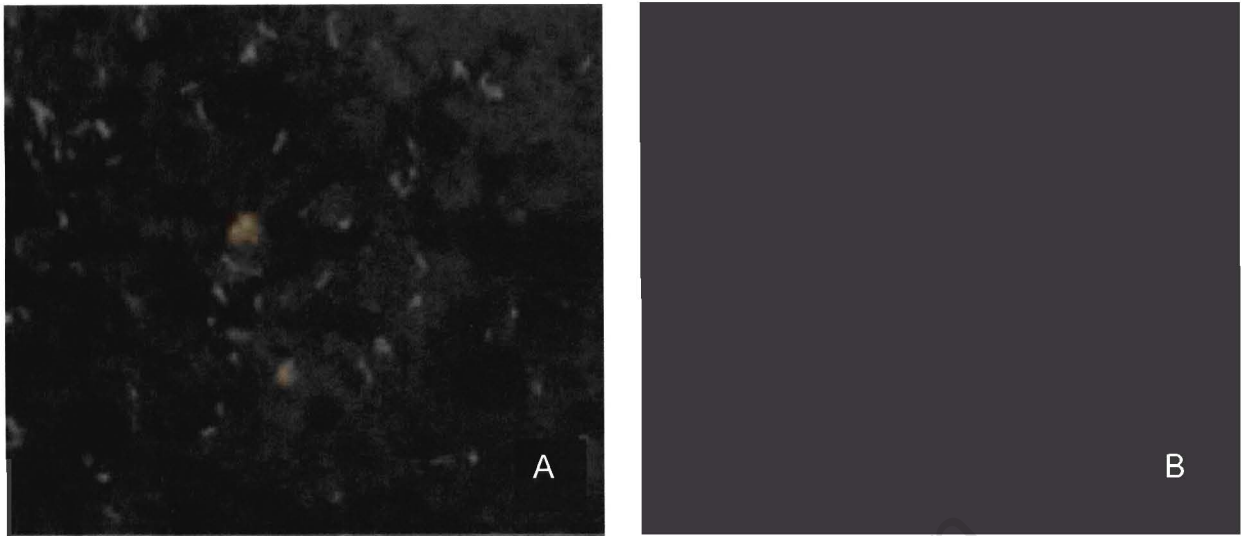
These three blocks had been fixed in formalin, processed in the standard fashion and embedded in paraffin wax. Sections were cut on a standard microtome to a thickness of 3-4 microns. These were then stained with a conventional Pap stain, an AR stain, a ZN stain and a haematoxylin and eosin (H&E) stain. Unstained sections of all three cases were also produced. (The methods for the Pap, ZN an AR stains may be found in Appendices 2, 3 and 4 respectively).

All stained and unstained sections were then examined under blue light (400nm) using a Zeiss Axioscope binocular fluorescent microscope. The ZN, Pap, H&E stained and unstained sections were examined using conventional (bright field) light microscopy on a Zeiss Axioscope binocular microscope.

## **RESULTS:**

- TB bacilli were readily identified on all sections of the two positive cases stained with the two fluorescent stains (Pap and AR), fluorescing yellow-green on the Pap stain

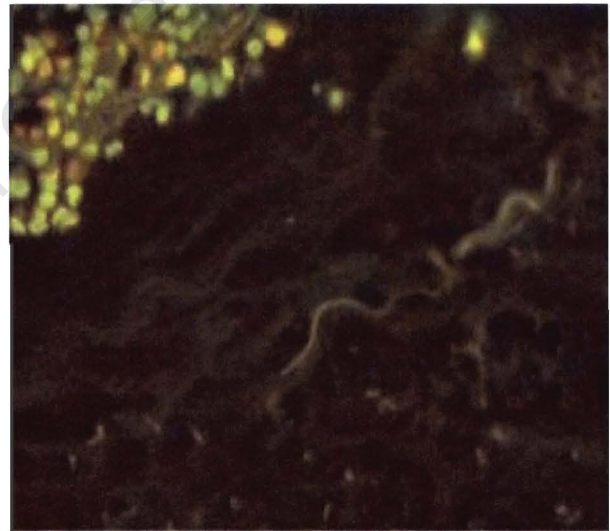
(Fig. 2.1) and bright golden-yellow against a dark background on the AR stain (Fig. 2.2).



**Fig 2.1** Mycobacterium tuberculosis bacilli on a smear preparation as seen under blue fluorescent light after staining with a Papanicolaou stain. A) Medium power (200x); B) Oil emersion (1000x) True-colour images.



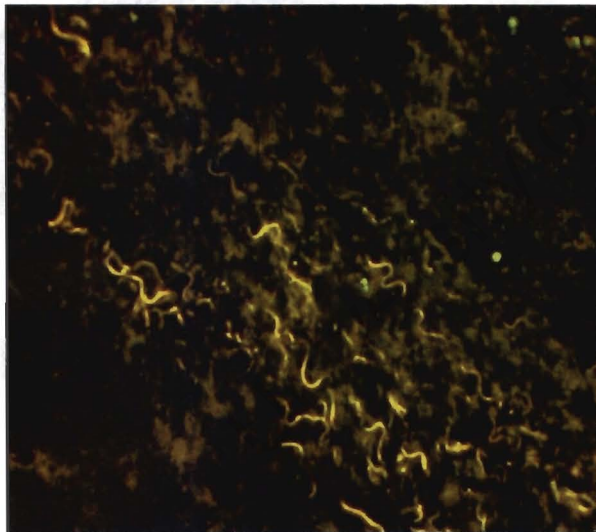
**Fig 2.2** Mycobacterium tuberculosis bacilli in a processed tissue section as seen under blue fluorescent light after staining with an auramine rhodamine stain. Medium power (200x)



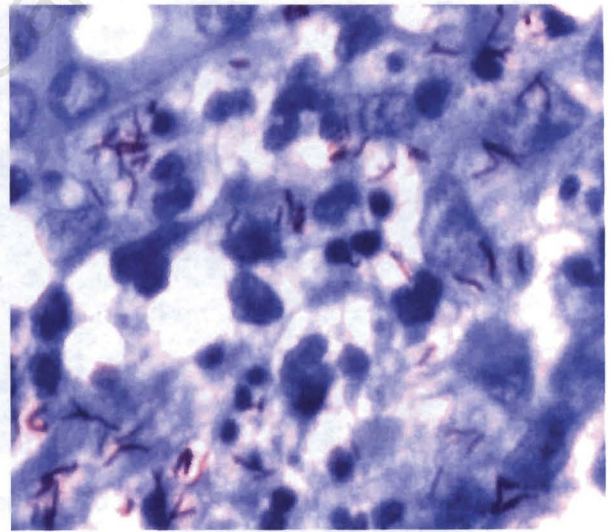
**Fig 2.3** Mycobacterium tuberculosis bacilli (bottom right) with associated elastin fibres (centre) and red blood cells (top left) on a modified Papanicolaou stain. Seen at medium power (200x). True-colour image.

- The Pap stain induced a moderate degree of fluorescence in the background tissue, with red blood cells and elastin fibers fluorescing brightly (Figs. 2.3 and 2.4). This made identification of the bacilli more difficult than on the AR stained sections in which the background did not fluoresce or fluoresced only weakly.

- No bacilli were identified on the Pap stained sections of the two positive control cases when they were examined using conventional light microscopy.
- Bacilli were easily identified on the ZN stain of the two positive control cases using bright-field microscopy, appearing red against a light blue background (Fig. 2.5). No fluorescence was induced under blue light.
- No bacilli were seen on the H&E stained sections on bright-field microscopy, but bacilli were noted when the positive control cases were examined under fluorescence. There was, however, marked background staining that made identification of the bacilli very difficult.
- No bacilli were identified on the unstained sections by either fluorescent or bright-field microscopy.
- No bacilli were found on the sections of the negative control case.



**Fig 2.4** Elastin fibres on a Papanicolaou stain. Medium power (200x). True-colour image.



**Fig 2.5** Mycobacterium tuberculosis bacilli as seen on a Ziehl Neelsen stain. High power (400x).

**CONCLUSION:** The Pap stain does induce fluorescence in *M.tuberculosis* bacilli in processed tissue sections.

In view of the above finding, all AR, Pap and modified Pap stained sections in the studies that follow were examined with fluorescent microscopy under blue fluorescent light and all ZN stained sections were examined using conventional light microscopy.

### **2.3 Testing the Various Pap Methods**

Having shown that the Pap stain did induce fluorescence in TB bacilli, and knowing that a number of variations of the Pap stain exist, the next question to be addressed was: do all Pap staining methods induce fluorescence in TB bacilli? To answer this, the following study was undertaken:

**AIM:** To determine whether three variants of the Pap stain (the first using Harris' haematoxylin, EA 50 and Bismark brown, the second, similar to the first, but excluding the Bismark brown and the third using Gill's haematoxylin and EA 65) induce a fluorescent signal in *M.tuberculosis* bacilli in sections of processed tissue.

**MATERIAL AND METHOD:** Sections were again cut from the same three control cases and stained with three variations of the Pap stain (see Appendix 2 for the methods). In addition an AR and ZN stain were also performed on the three cases (HC, RC and NC) the first two as positive controls and the third as negative controls. All stained sections were examined and the brightness of the TB bacilli and the amount of background staining were noted on the Pap stained sections.

#### **RESULTS:**

- TB bacilli were readily identified in the AR and ZN stained positive control sections.
- TB bacilli were evident in all sections from the two positive controls stained with the three Pap methods. There was little difference in either the brightness of the bacilli or

the degree of background staining. The TB bacilli fluoresced moderately and the background fluoresced brightly.

- No bacilli were found in the sections from the negative control.

**CONCLUSION:** All three tested variations of the Pap method induced a fluorescent signal in the positive cases with little appreciable difference in the fluorescent properties of the stains. The methods 2 and 3 were chosen for the remainder of the study for the following reasons:

- Bismark brown had no appreciable effect on the fluorescence inducing properties of the Pap stain
- Method 2 could be broken down into its various components as the reagents were prepared in the laboratory and not bought as ready-mixed solutions as in method 3.
- The ready-mixed solutions of method 3 were readily available, easy to use, had a long shelf-life and were consistent from batch to batch.

#### **2.4 Testing the Components of the Pap**

The next question raised was which component of the Pap stain induced fluorescence in the bacilli? In order to determine this, the following study was performed:

**AIM:** To determine which component of the Pap stain induced fluorescence in TB bacilli.

**MATERIAL AND METHOD:** For this phase of the study sections were cut from the three control blocks (HC, RC and NC) and were stained as follows:

- ZN
- AR
- Unstained section

- Harris's haematoxylin (HH)
- HH with differentiation
- HH with differentiation and 'blueing'
- Orange G (OG) 6
- Eosin A (EA) 50
- Conventional Pap

The brightness of the staining/fluorescent signal of the bacilli and background were scored using a four tiered scoring system listed in Table 2.1.

**Table 2.1 Scoring system used to indicate intensity of stain/signal**

Intensity of stain/signal	Score
No stain/signal	-
Weak	+
Moderate intensity	++
Strong	+++

The method can be found in Appendix 5.

## RESULTS:

**Table 2.2 Results of the staining pattern induced by the components of the Pap stain**

	Human control (HC)		Rabbit control (RC)		Negative control (NC)	
	TB	Bg	TB	Bg	TB	Bg
ZN	+++	-	+++	-	-	-
AR	+++	-	+++	-	-	-
Unstained	-	+	-	+	-	+
HH	-	+	-	+	-	+
HH with differentiation	-	++	-	++	-	++
HH with 'blueing'	-	+	-	+	-	+
OG 6	-	+	-	+	-	+
EA 50	+	+++	+	+++	-	+++
Pap	+	+	+	+	-	+

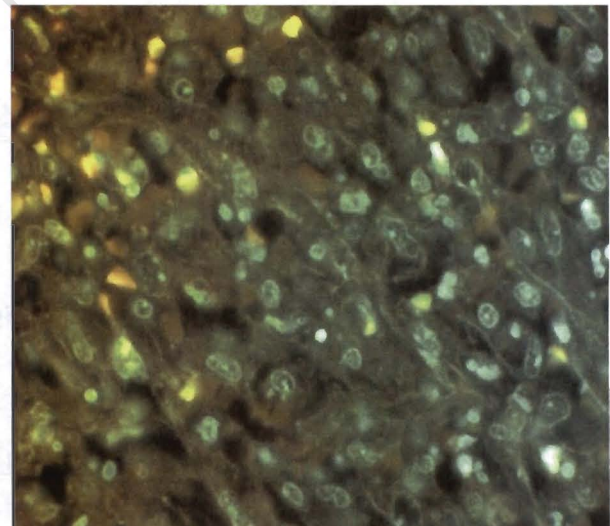
TB = TB bacilli; Bg= Background

The main findings were as follows:

- Bacilli were readily identifiable in the AR, Pap and ZN stained sections from all positive control cases (HC and RC)
- No bacilli were noted in any of the negative control sections (NC)
- EA 50 was the only component of the Pap to induce fluorescence in the bacilli (Fig.2.7) in sections from HC and RC. This signal was weak (+) and comparable to that of the Pap stained sections.
- EA 50 induced marked fluorescence in the background (Fig. 2.8), making the bacilli difficult to identify. The structures that fluoresced strongly included red blood cells, elastin, collagen, cell nuclei, cell membranes and intracytoplasmic structures/granules.
- Weak to moderate background fluorescence was induced by HH, particularly once it had undergone differentiation.



**Fig 2.7** Mycobacterium tuberculosis bacilli (circle) in an EA 50 stained section. High power (400x). Monochrome image.



**Fig 2.8** Background fluorescence on an EA 50 stained section. Medium power (200x). True-colour image.

**CONCLUSION:** The component in the Pap stain responsible for inducing fluorescence in TB bacilli is EA 50.

## 2.5 Testing the Components of EA 50

The next step was to determine which of the EA components induced the fluorescence.

The components of EA 50 are as follows:

- Eosin Y
- Light green
- Phosphotungstic acid
- Glacial acetic acid
- Alcohol and methanol

The following study was performed:

**AIM:** To determine which component(s) of EA 50 induced fluorescence in TB bacilli.

**MATERIAL AND METHOD:** Sections of the three cases (HC, RC and NC) were stained with the following stains:

- ZN, Pap and AR as control stains
- The individual EA 50 components as listed above.

The method for staining with the individual EA 50 components may be found in Appendix 6

**RESULTS:** The bacilli fluoresced weakly when stained with Eosin Y, but not with any of the other components. As in the EA 50 stained sections, there was marked background staining induced by Eosin Y and light green.

**CONCLUSION:** Eosin Y is the component of EA 50 that induces fluorescence in TB bacilli.

## 2.6 Modifying the Pap stain

To have a stain that competed with either the AR or ZN stains, the brightness of the bacilli needed to be enhanced and the background staining dampened. At this point, as all three Pap methods had worked equally well in inducing fluorescence in the bacilli, it was decided to use the commercially available ready mixed reagents for all further studies as this would save on preparation time.

### 2.6.1 Recombining the Pap Components

Knowing that Eosin Y induced weak fluorescence in the bacilli but marked background fluorescence, the next question that needed addressing was which component reduced the background stain? The following study was performed in an attempt to answer this.

**AIM:** To determine which component of the Pap stain decreased the background staining when recombined with EA 65.

**MATERIAL AND METHOD:** Sections of the three cases (HC, RC and NC) were stained with the following stains:

- ZN, Pap and AR as control stains for TB bacilli
- Gill's haematoxylin (GH), OG 6 and EA 65 as control stains for comparison of the background staining
- EA 65 combined with GH.
- EA 65 combined with OG 6
- The brightness of the staining/fluorescent signal of the bacilli and background were scored using a four tiered scoring system listed in Table 2.1.

The method for recombining the Pap components appears in Appendix 7.

**RESULTS:**

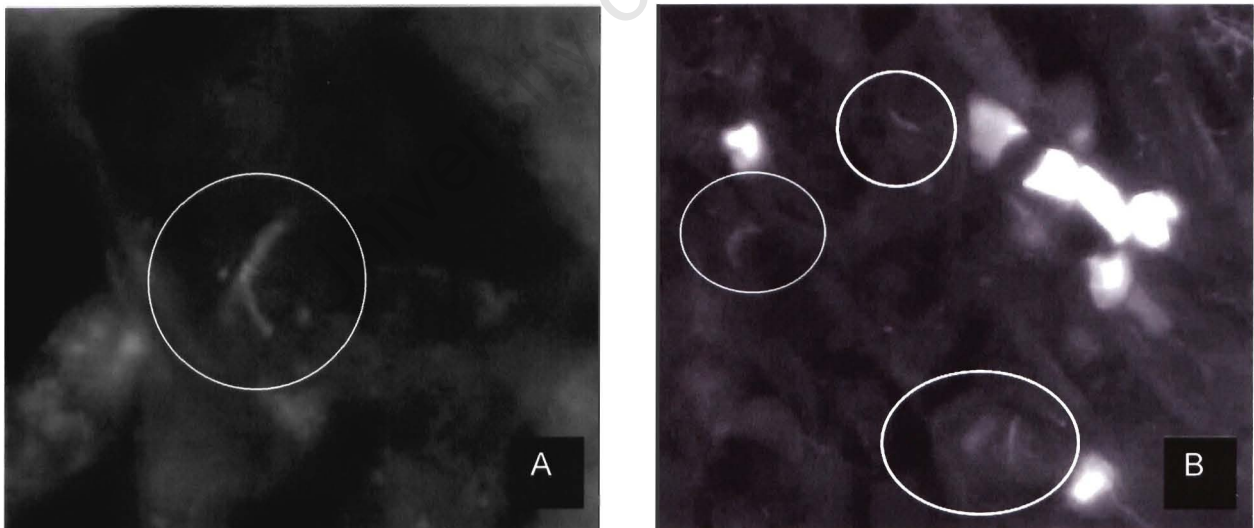
**Table 2.3 Staining profile of the recombined Pap components**

	HC		RC		NC	
	TB	Bg	TB	Bg	TB	Bg
ZN	+++	-	+++	-	-	-
AR	+++	-	+++	-	-	-
Pap	+	+	+	+	-	+
GH	-	+	-	+	-	+
OG 6	-	+	-	+	-	+
EA 65	+	+++	+	+++	-	+++
EA 65 and OG 6	++	++	+++	+++	-	+++
EA 65 and GH	++	+	++	+	-	+

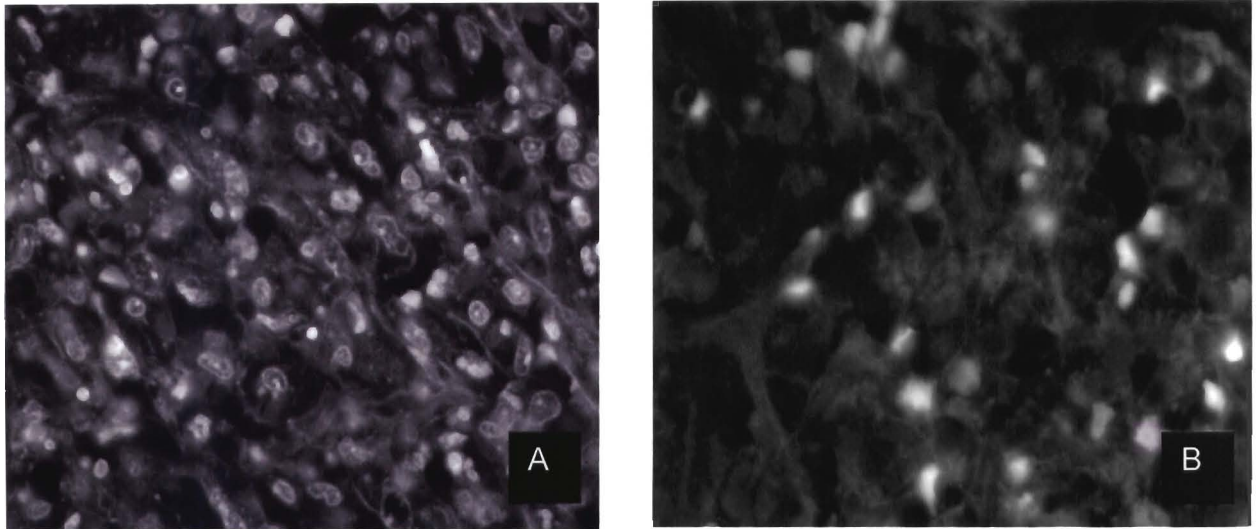
TB = TB bacilli; Bg = Background

The main findings (as listed in Table 2.3) were:

- Both the bacilli and the background fluoresced moderately (++) to strongly (+++) when EA 65 was combined with OG 6.
- The bacilli fluoresced moderately (++) when EA 65 was combined with GH (Fig. 2.9) and there was a marked reduction in the background staining (Fig.2.10).



**Fig 2.9** Mycobacterium tuberculosis bacilli (circles) in sections stained with a combination of EA 65 and Gill's haematoxylin. A) High power (1000x) and B) Medium power (400x). Monochrome images.



**Fig 2.10** Background fluorescence in sections stained with A) EA 65 only; and B) both EA 65 and Gill's haematoxylin. Medium power (200x). Monochrome images.

**CONCLUSION:** GH was the reagent responsible for the reduction in the background staining when recombined with EA 65.

### 2.6.2 Increasing the exposure to EA

The first step in the modification of the Pap stain was to determine the effect of an increased exposure to EA 65. The following study was performed:

**AIM:** To determine whether lengthening the exposure time to EA 65 increased the fluorescent signal of the TB bacilli and/or background.

**MATERIAL AND METHOD:** Sections of the three cases (HC, RC and NC) were stained with the following stains:

- ZN, Pap and AR as control stains for TB bacilli
- EA 65 as control stains for comparison of the background staining
- Pap stain with the exposure time to all reagents doubled.
- Pap stain with the exposure time to EA 65 doubled.

- The brightness of the staining/fluorescent signal of the bacilli and background were scored using a four tiered scoring system listed in Table 2.1.

The method followed appears in Appendix 8.

## RESULTS:

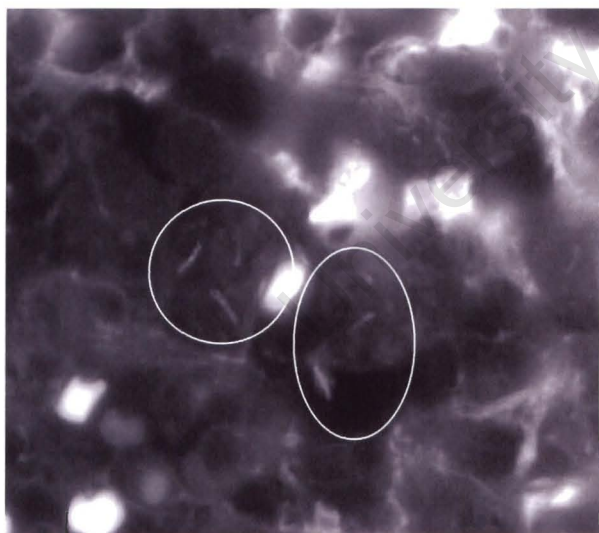
The main results appear in Table 2.4 below:

**Table 2.4 Results of lengthening the exposure time to EA 65**

	Human control (HC)		Rabbit control (RC)		Negative control (NC)	
	TB	Bg	TB	Bg	TB	Bg
EA 65 control	+	+++	+	+++	-	+
Pap stain with exposure time to all reagents doubled	+++	++	+++	++	-	+
Pap stain with exposure time to EA 65 doubled	+++	+	+++	+	-	+

TB = TB bacilli; Bg = Background

Figure 2.11 shows the increased positivity for bacilli when the exposure time to EA 65 was doubled.



**Fig 2.11** Mycobacterium tuberculosis bacilli (circles) in a section stained with a Pap stain with double exposure to EA 65. High power (400x). Monochrome image.

**CONCLUSION:** Doubling the exposure time to EA 65 increased the fluorescent signal of the TB bacilli and decreased the background fluorescence.

### **2.6.3 Quenching the Background Fluorescence**

Fluorescence can be quenched in one of two ways: by staining with a blue dye (88), or by the addition of heavy metals (89-93). The addition of a weak solution (0.5% aqueous) of toluidine blue is a good example of the former, whilst sodium borohydride, mercury and osmium tetroxide are examples of the latter. Both these techniques were applied to the Pap stain in an attempt to decrease the background fluorescence.

The following two studies were performed in an attempt to use the above two methods to quench the background fluorescence:

#### **2.6.3.1 'Blueing' the Pap**

**AIM:** To determine whether the blue dyes methylene blue and toluidine blue would quench the background fluorescence.

**MATERIAL AND METHOD:** Sections were cut from CH, CR and NC, and stained as follows:

- Control Pap, AR and ZN stained sections
- Pap stain with the addition of methylene blue counterstain (as used in the ZN stain)
- Pap stain with 0.5 % toluidine blue counterstain.

(The methods followed appear in Appendix 9)

**RESULTS:** The results were disappointing in that, although there was a marked decrease in the background fluorescence on the two Pap stains to which blue counterstains had been added, the signal from the TB bacilli was also completely quenched.

**CONCLUSION:** The blue dyes methylene blue and toluidine blue quench the background fluorescence, but also completely blot out the signal from the TB bacilli, making the stain useless for the identification of TB bacilli.

### 2.6.3.2 The Addition of Heavy Metals

As stated in the initial observations in section 2.2, red blood cells and elastin fibres are the two components of the background fluorescence that can mimic bacilli and cause a false positive result. In the study that follows, an attempt was made to quench the fluorescence of elastin by combining an elastin van Gieson (EVG) stain with the Pap stain, as the silver of the EVG should in theory quench the emissions for elastin.

**AIM:** To determine whether the addition of silver to the Pap stain would successfully quench the signal from elastin.

**MATERIAL AND METHOD:** The following stains were performed on the three control cases:

- Control Pap, AR and ZN stained sections
- Combination EVG/Pap stain

The method used may be found in Appendix 9.

**RESULTS:** The elastin did not fluoresce on the combination Pap/EVG stained section, producing an almost black background, but there was also a marked reduction in the number of bacilli seen on the sections from the HC and RC cases when compared to the corresponding Pap, AR and ZN stained sections.

**CONCLUSION:** Adding silver to the Pap stain successfully quenched the signal from elastin but also dampened the signal from the TB bacilli, resulting in a less reliable stain.

#### 2.6.4 Etching with Acid Alcohol

Acid alcohol (AA) is a potent bleaching agent (82). TB bacilli are acid-alcohol fast, hence the name acid (alcohol) fast bacilli (AFB). This is a property exploited by the phenol based stains. In the study that follows an attempt was made to use this property to 'lift' the fluorescence of the bacilli from the background induced by the Pap stain.

**AIM:** To determine the effect of etching the Pap stain with AA.

**METHOD:** The following stains were performed on the three control cases:

- Single control ZN and AR stained sections
- Six stained sections of each of the following stains were made:
  - Pap stain with double exposure time to EA 65 (as listed in Appendix 8 Technique 2)
  - EA 65 stained section with 2 min exposure time to EA 65 (as listed in Appendix 7 Technique 3)
  - EA 65 stained section with 4 min exposure time to EA 65 (as for the preceding stain but with 4 min exposure to EA 65)
- One of each of these sections was then exposed to 1% AA for the following time periods:
  - 20 min (as for ZN protocol)
  - 5 min
  - 2 min
  - 1 min
  - 10 dips.
  - No exposure to AA

- The brightness of the staining/fluorescent signal of the bacilli and background were scored using a four tiered scoring system listed in Table 2.1.

## **RESULTS:**

The results are listed in Table 2.5 below.

The main findings were:

- There was marked reduction in the background fluorescence in all sections exposed to AA, with little variation between those exposed for 20 min and those exposed for only 10 dips.
- Bacilli were not visible on any sections exposed to AA for longer than 2min.
- Bacilli were weakly positive on the sections exposed to AA for 2 min or less.
- The type of Pap/EA 65 stain used made little difference.

**CONCLUSION:** The addition of AA reduced the signal from both the background and the TB bacilli. The longer the exposure to AA, the weaker the fluorescent signal from the bacilli.

**Table 2.5 Results of etching the Pap with varying exposures to acid alcohol**

	Exposure to AA	HC		RC		NC	
		TB	Bg	TB	Bg	TB	Bg
ZN		+++	-	+++	-	-	-
AR		+++	-	+++	-	-	-
Pap	20 min	-	+	-	+	-	+
	5 min	-	+	-	+	-	+
	2 min	+	+	+	+	-	+
	1 min	+	+	+	+	-	+
	10 dips	+	+	+	+	-	+
	nil	+	++	+	++	-	++
Pap, dbl EA	20 min	-	+	-	+	-	+
	5 min	-	+	-	+	-	+
	2 min	+	+	+	+	-	+
	1 min	+	+	+	+	-	+
	10 dips	+	+	+	+	-	+
	nil	+	++	+	++	-	+++
2min EA	20 min	-	+	-	+	-	+
	5 min	-	+	-	+	-	+
	2 min	+	+	+	+	-	+
	1 min	+	+	+	+	-	+
	10 dips	+	+	+	+	-	+
	nil	+	+++	+	+++	-	+++
4 min EA	20 min	-	+	-	+	-	+
	5 min	-	+	-	+	-	+
	2 min	+	+	+	+	-	+
	1 min	+	+	+	+	-	+
	10 dips	+	+	+	+	-	+
	nil	+	+++	+	+++	-	+++

TB = TB bacilli; Bg = Background

#### 2.6.4.1 Etching with short exposures to Acid Alcohol

As bacilli were only identified on those sections exposed to AA for less than 2 minutes, a second study was performed, in which only EA 65 stained sections were used. These were exposed to AA for very short periods of time. This study was as follows:

**AIM:** To determine the effect of etching the EA 65 stain with short exposures to AA.

**METHOD:** The following stains were performed on the three control cases:

- Standard EA 65 stain (as listed in Appendix 7 Technique 3)
- EA 65 stain with 2 min exposure to EA 65 with 5 dips in AA

- EA 65 stain with 2 min exposure to EA 65 with 2 dips in AA
- EA 65 stain with 4 min exposure to EA 65 with 5 dips in AA
- EA 65 stain with 4 min exposure to EA 65 with 2 dips in AA

## RESULTS:

The main finding was that the background was moderately reduced in all sections exposed to AA with little dampening effect on the fluorescence of TB bacilli.

**Table 2.6 Results of etching EA 65 stained sections with acid alcohol**

	Exposure to AA	HC		RC		NC	
		TB	Bg	TB	Bg	TB	Bg
EA	Nil	++	+++	++	+++	-	+++
2 min EA	5 dips	++	++	++	++	-	++
	2 dips	++	++	++	++	-	++
4 min EA	5 dips	+	+	+	+	-	+
	2 dips	++	+	++	+	-	+

TB = TB bacilli; Bg= Background

**CONCLUSION:** Short exposures to AA dampen the background signal but do not affect the signal from the TB bacilli.

### 2.6.5 Effect of Heat, Light and Time on the Pap

The final attempt to modify the Pap stain was to determine the effect of heat, light and time (post staining) on the Pap stain. The following study was performed:

**AIM:** To determine the effect on the Pap stain of exposure to sunlight and heat.

**MATERIAL AND METHOD:** Three sets of sections were cut from CH, CR and NC, and stained with the Pap stain:

- One set was not exposed to light or heat
- One set was exposed to sunlight for 30 min

- One set was exposed to heat for 30 min in an incubator set to 40 degrees Celsius.

## **RESULTS:**

- All NC sections were negative for bacilli.
- The bacilli fluoresced brighter on those HC and RC sections exposed to heat than on those sections exposed to light.
- There was little (if any) reduction in background staining.

No formal study was conducted on the effects of time on the Pap stained sections, however certain important observations can be noted, as many of the Pap stained sections were reviewed days to months (up to 4 years) after staining. These are as follows:

- The fluorescence induced in bacilli by the Pap stain did not appear to fade to any significant extent with time; but rather appeared to increase after a few days. This curious effect was noted when stained sections were re-examined a number of days after being stained and examined.
- The background fluorescence also appeared to brighten with time
- By comparison, fluorescence induced by the AR stain reduces markedly with exposure to heat, light and with time, fading to nothing after 2-3 days (depending on the storage conditions).

**CONCLUSION:** The fluorescent signal induced in the TB bacilli and the background by the Pap stain does not appear to fade over long periods of time.

## **2.7 The Final Modified Pap**

After the myriad of manipulations, the final modified Pap (mPap) stain used in the larger study (for comparison with the AR and ZN stains) was a Pap stain using the commercially

available prepared reagents from Polychem United Scientific (Fig.3.2). The stain included Gill's Haematoxylin, excluded OG6 and increased the exposure time to EA 65 to 4 minutes. The method may be found in Appendix 10.

University of Cape Town

## Chapter 3 Comparison of the Modified Pap with AR and ZN Stains: Aim, Material and Method and Results

**AIM:** To compare the mPap stain with the ZN and AR stains, with specific regard to -

1. the ease of interpretation reflected by the time taken to identify the organism
2. the safety of the staining methods
3. the cost of the staining methods and equipment.

**MATERIAL AND METHOD:** As an indicator of the ease of interpretation of the three stains (mPap, AR and ZN) a study was constructed using archival material from the files of the Division of Anatomical Pathology, Department of Laboratory Medicine, University of Cape Town. All cases reported as positive for acid/alcohol fast bacilli on a ZN stain in a one-year period from 1999 to 2000 were selected and the histology reviewed. This produced 61 cases. The blocks on which ZN stains had been performed were then identified, which gave a total of 78 blocks.

These 78 blocks (of various tissues) were then cut and stained with the mPap, AR and ZN stains and examined using the appropriate microscopy modality, namely, fluorescent microscopy for the mPap and AR stains and conventional light microscopy for the ZN stain.

The time taken to identify mycobacteria was recorded using a conventional stopwatch, and tabulated. The time was taken from when the slide was placed on the stage to when a definitive mycobacterium was identified. To be called positive for TB bacilli, one or more bacilli had to meet the following criteria:

- On all stains, the organism had to be of appropriate size (2-4 um in length and 0.2-0.5 um in width), curvilinear in shape and situated within areas of inflammation (granulomatous or acute) or in necrotic debris.
- For the mPap stain, the organism had to fluoresce bright yellow-green under blue light and there had to be no elastin fibers in the near vicinity (within the high power (x400) field)
- For the AR stain, the organism had to fluoresce bright golden-yellow under blue light.
- For the ZN stain, the organism had to stain deep red.

If the entire section had been thoroughly examined and no bacilli were found that met these criteria, the case was recorded as being negative for bacilli. No time limit was set for a negative call and the decision to call a case negative was dependent only on the thorough examination of the tissue on the section.

If a case was designated as being negative, the original (archival) ZN was reviewed and the presence or absence of bacilli noted. The majority of the archival ZN stains were still well preserved with only a few having faded.

If a stained section in the current study was found to be of poor quality, the section was re-cut and re-stained.

**STATISTICAL METHODS:** Statistical analyses were performed on the resultant data. These included: cross-tabulation, calculation of specificity, sensitivity, accuracy and the K-value for the positive/negative values of the three stains as well as cross-tabulation, the standard deviation and statistical significance of the time values of the three stains. The latter was performed using the Wilcoxon method for evaluating matched pairs.

Additional information was obtained on the ease of preparation of the stains, their safety profile and the cost of reagents and equipment required for preparation or interpretation of the stain.

## RESULTS:

### 3.1 Positive vs. Negative Stain Results

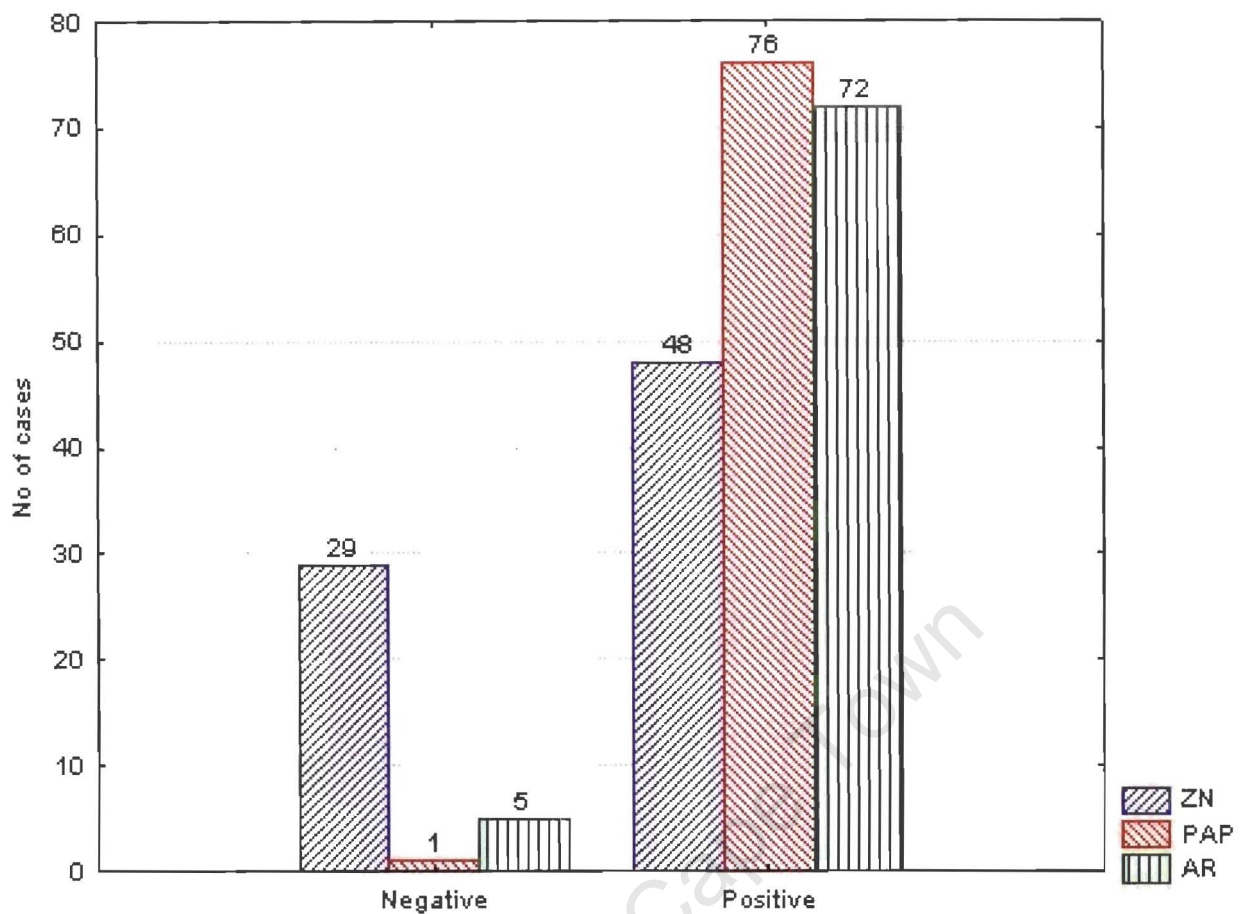
A total of 61 cases were identified with 78 blocks on which ZN stains had been performed. The tissue of one of these blocks had been cut away, thereby preventing further sections from being cut. This case was therefore excluded from the study, leaving 77 blocks from 60 cases from which sections could be cut and stained.

The results are presented in Table 3.1 and Fig.3.1.

**Table 3.1 Positive and negative results of mPap, AR and ZN stains**

	mPap	AR	ZN
Positive	76 (99%)	72 (94%)	48 (62%)
Negative	1 (1%)	5 (6%)	29 (38%)
Total	77		

Forty-seven (78%) of the 60 cases had only one block entered into the study whilst 10 (17%) had two blocks entered, 2 (3%) had three blocks entered and 1 case (2%) had four blocks entered. More than one block was examined in these 13 cases as it was not certain on which of these blocks TB bacilli were originally identified. Of the 60 cases, 15 (25%) were negative for bacilli on a ZN stained section from all blocks pertaining to that case, whilst only one case (2%) was negative on mPap stain, and one (2%) was negative on AR stain. No case was negative for all stains, or put another way, bacilli were identified on at least one stain in all cases. (See Table 1 in Appendix I for the table of raw data)

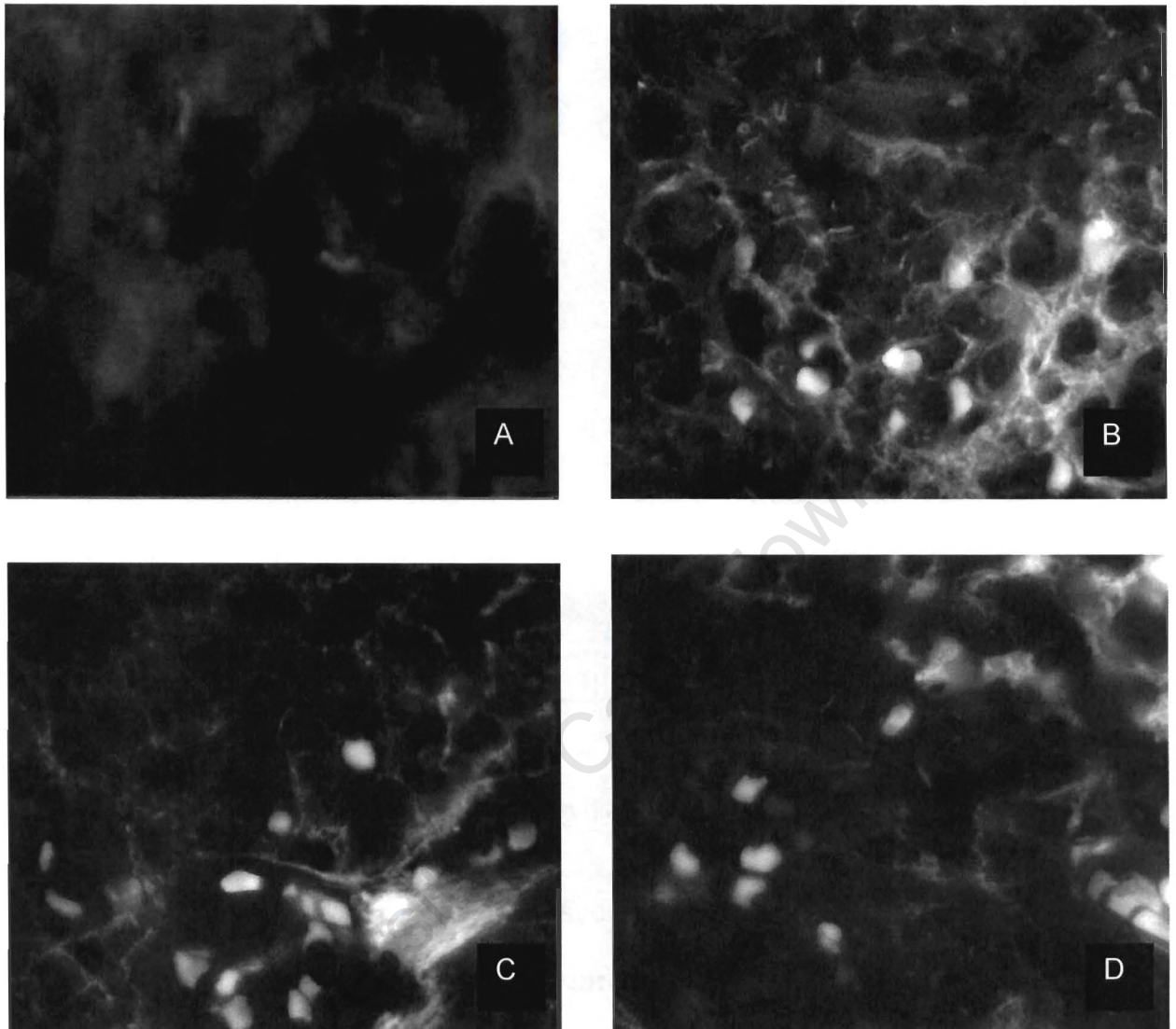


**Fig.3.1** Histogram of Negative and Positive Cases by Stain

The original ZN stained sections were available for 24 of the 29 blocks that were negative for TB bacilli in the current study. This represented 11 of the 15 negative cases. These 24 stained sections were reviewed and bacilli identified in 18 of them resulting in identification of TB bacilli in 10 of the 11 cases in which the original ZN stained sections were available.

In all stains, TB bacilli stained strongly and fulfilled the criteria listed at the beginning of this chapter (p38). On a ZN stain the mycobacteria stained deep red against a light blue background. Under blue fluorescent light, the organism fluoresced bright yellow-green when stained with an mPap stain (Fig.3.2). After staining with an AR stain, the bacilli fluoresced golden-yellow against a dark background under blue light. There was little background positive staining with the ZN stain. Red blood cells stained positive on the AR

stain and in addition to the red cells, elastin and nonspecific cytoplasmic staining was seen on the mPap stain.



**Fig.3.2** *M. tuberculosis* bacilli (circles) in a sections stained with the modified Papanicolaou stain. A) High power (400x); true-colour image. B – D) Medium power (200x), monochrome images

### 3.2 Statistical Analysis of Positive vs. Negative Stain Results

The numbers of positive and negative sections for the three tests (staining methods) were cross-tabulated and analysed for sensitivity, specificity, accuracy and K value. The results were as follows:

**Table 3.2 Cross-tabulation of ZN versus mPap**

		mPap		
		Negative	Positive	Total
ZN	Negative	0	29	29
	Positive	1	47	48
Total		1	76	77

The sensitivity was 61.80, the specificity 50 and the accuracy 61.54 with a K value of 0.015.

**Table 3.3 Cross-tabulation of ZN versus AR**

		AR		
		Negative	Positive	Total
ZN	Negative	3	26	29
	Positive	2	46	48
Total		5	72	77

The sensitivity was 63.89, the specificity 66.67 and the accuracy 64.10 with a K value of 0.108.

**Table 3.4 Cross-tabulation of mPap versus AR**

		AR		
		Negative	Positive	Total
mPap	Negative	3	26	29
	Positive	2	46	48
Total		5	72	77

The sensitivity was 93.4, the specificity 50 and the accuracy 92.30 with a K value of 0.22.

### 3.3 Time per Section Results

The total time spent examining all 231 sections was 19 hrs 46 min 53 s. The total amount of time spent examining the ZN stained sections was 9 hrs 44 min 4 s with an average time of 7 min 35s per slide and a median of 5 min 51s. The maximum time spent on a ZN section was 28 min 51 s and the minimum time 7 s. More time was spent on the 29 of 77 sections where no bacilli could be found – a total of 5 hrs 5 min 43 s with an average of 10 min 32 s per slide to a maximum of 28 min 51 s and a minimum of 2 min 30 s. As expected, less time was spent on the positive sections – a total of 4 hrs 38 min 21 s with

an average of 5 min 48 s per slide - half the time spent on the negative cases. The longest time taken to identify a bacillus was 19 min 56 s and the quickest a bacillus was identified was 7 s (as stated above) (Fig.3.3).

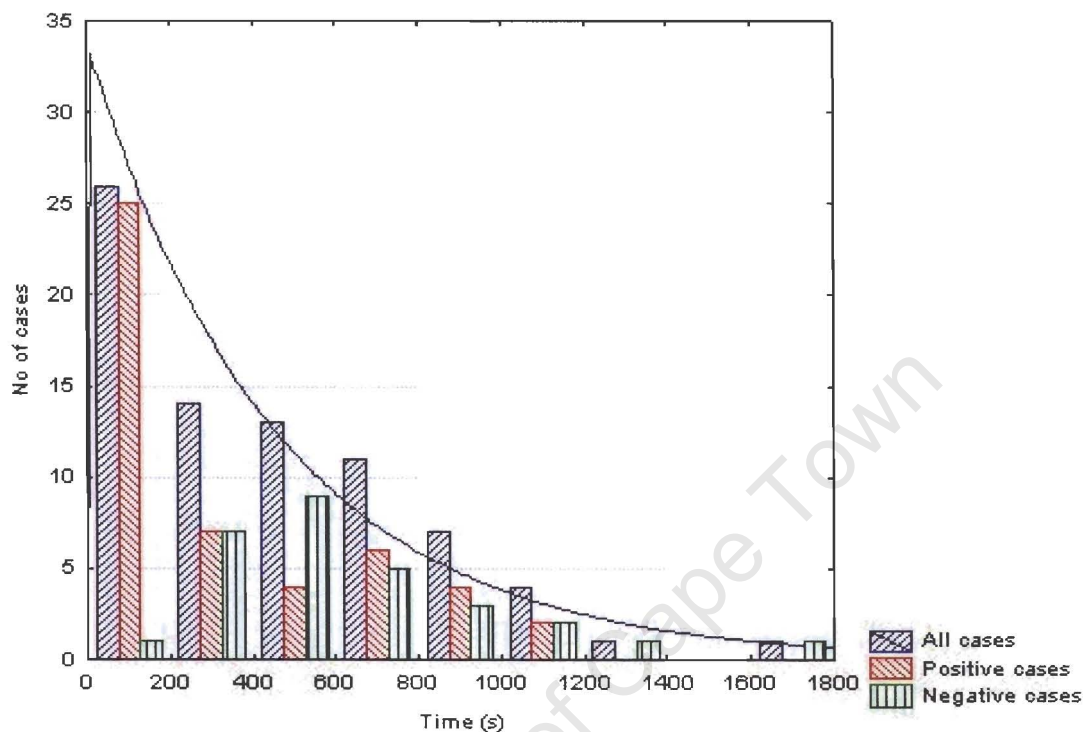
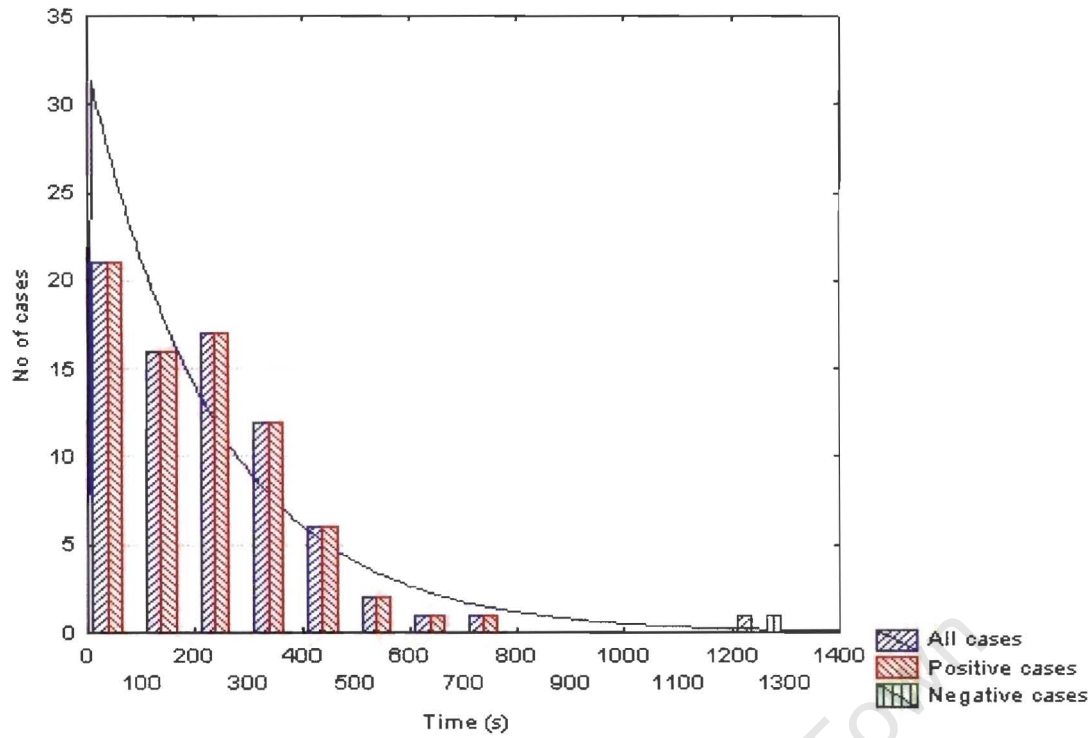


Fig.3.3 Histogram of ZN times.

In contrast, the total amount of time spent examining the mPap stained sections was 5 hrs 5 min 48 s with an average time of 3 min 58 s per slide with a median of 3 min 33 s. The maximum time spent on an mPap section was 20 min 21 s, this being the only negative case. The minimum time was 18 s. The total time spent on the positive sections was 4 hrs 45 min 27 s with an average of 3 min 45 s per slide. The longest time taken to identify a bacillus was 12 min 8 s (Fig.3.4).



**Fig.3.4** Histogram of mPap times.

The total time spent examining the AR stained sections was 4 hrs 57 min 1 s with an average time of 3 min 51s per slide with a median of 2 min 21s. The maximum time spent on an AR section was 34 min 18 s and the minimum time 2 s. As above, more time was spent on the 5 sections where no bacilli could be found – a total of 1 hr 3 min 45 s with an average of 12 min 45 s per slide to the maximum of 34 min 18 s quoted above and a minimum of 5 min. The total time spent on the positive sections was 3 hrs 53 min 16 s with an average of 3 min 14 s per slide to a maximum of 11 min 44 s and a minimum of 2 s as quoted above (Fig.3.5).

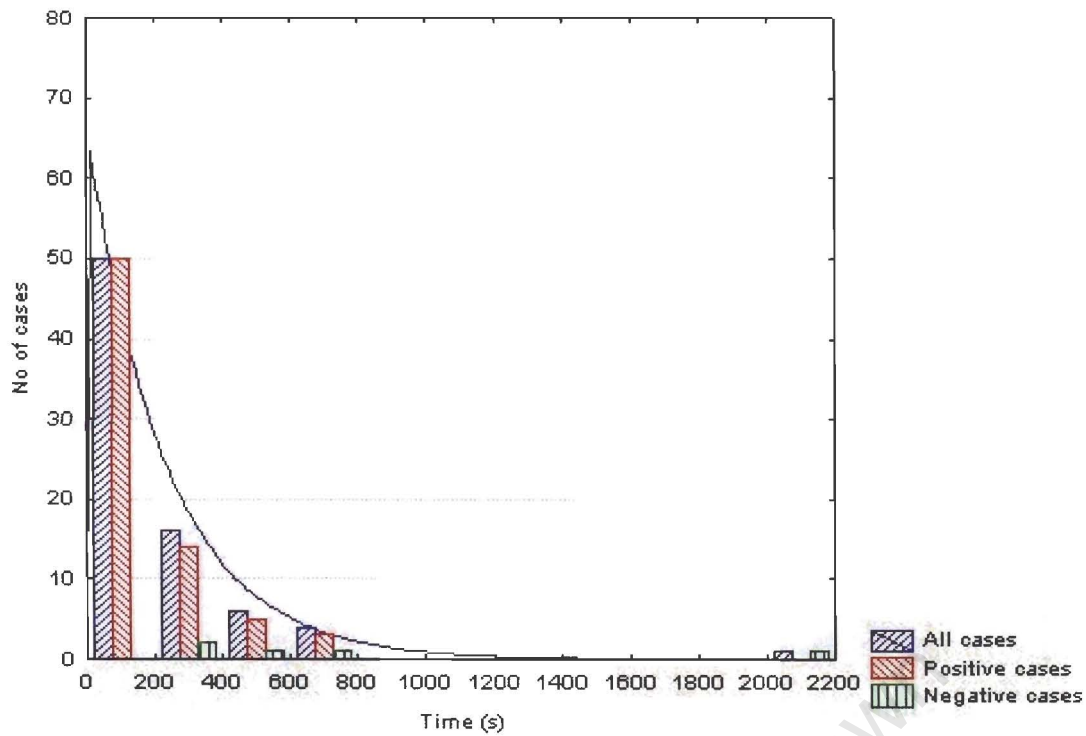


Fig.3.5 Histogram of AR times.

**Table 3.5 Statistical analysis of time values (in seconds) for ZN, Pap and AR stained sections.**

Time		ZN	Pap	AR
Total	Positive	4hr 38min 21s	4hr 45min 27s	3hr 53min 16s
	Negative	5hr 5 min 43s	20min 21s	1hr 3min 45s
	All	9hs 44min 4s	5hr 5min 48s	4hr 57min 1s
Average	Positive	5min 48s	3min 45s	3min 14s
	Negative	10min 33s	20min 21s	12min 45s
	Total	7min 35s	3min 58s	3min 51s
Median	Positive	3min 5s	3min 32s	2min 15s
	Negative	8min 37s	20min 21s	6min 52s
	Total	5min 51s	3min 33s	2min 21s
Maximum	Positive	19min 56s	12min 8s	11min 44s
	Negative	28min 51s	20min 21s	34min 18s
	Total	28min 51s	20min 21s	34min 18s
Minimum	Positive	7s	18s	2s
	Negative	2min 30s	20min 21s	5min 0s
	Total	7s	18s	2s
Std Dev	Positive	341.47	160.26	167.53
	Negative	359.17	Division by 0	739.44
	Total	372.69	195.49	273.91
95% confidence	Positive	96.60	36.03	38.70
	Negative	130.72	Division by 0	648.14
	Total	83.24	43.67	61.18

Standard deviations and 95% confidence intervals are presented in Table 3.5.

### 3.4 Statistical Analysis of Time per Section Results

Simple cross-tabulation was applied to the positive/negative status of the stains and time taken to identify bacilli. See Tables 3.6, 3.7 and 3.8.

**Table 3.6 Cross-tabulation of total time taken for negative and positive ZN and mPap stains**

ZN		mPap		Grand Total
		Negative	Positive	
Negative	ZN time	0s	5hr 5min 43s	5hr 5min 43s
	mPap time	0s	1hr 58min 11s	1hr 58min 11s
Positive	ZN time	15min 18s	4hr 23min 3s	4hr 38min 21s
	mPap time	20min 21s	2hr 47min 16s	3hr 7min 37s
Total ZN time		15min 18s	9hr 28min 46s	9hr 44min 4s
Total mPap time		20min 21s	4hr 45min 27s	5hr 5min 48s

**Table 3.7 Cross-tabulation of total time taken for negative and positive ZN and AR stains**

ZN		AR		Grand Total
		Negative	Positive	
Negative	ZN time	19min 0s	4hr 46min 43s	5hr 5min 43s
	AR time	46min 10s	1hr 24min 22s	2hr 10min 32s
Positive	ZN time	10min 4s	4hr 28min 17s	4hr 38min 21s
	AR time	17min 35s	2hr 28min 54s	2hr 46min 29s
Total ZN time		29min 4s	9hr 15min 0s	9hr 44min 4s
Total AR time		1hr 3min 45s	3hr 53min 16s	4hr 57min 1s

**Table 3.8 Cross-tabulation of total time taken for negative and positive Pap and AR stains**

		AR		
mPap		Negative	Positive	Grand Total
Negative	mPap time	0s	20min 21s	20min 21s
	AR time	0s	11min 14s	11min 14s
Positive	mPap time	25min 50s	4hr 19min 37s	4hr 45min 27s
	AR time	1hr 3min 45s	3hr 42min 2s	4hr 45min 47s
Total mPap time		25min 50s	4hr 39min 58s	5hr 5min 48s
Total AR time		1hr 3min 45s	3hr 53min 16s	4hr 57min 1s

The sections in which bacilli were identified (the positive stains) give an indication of the time required to identify bacilli using the various staining methods. Therefore, considering only these, it can be seen that the median time taken to identify bacilli using a ZN stain 5 min 48 s is far greater than when using either of the fluorescent stains (median time for mPap being 3 min 45 s and for the AR being 3 min 14 s). The times taken using the two fluorescent methods were similar, with the AR stain being the quicker of the two. The Wilcoxon method for evaluating matched pairs was used to determine the statistical significance of the difference in the times of all positive stains. The resultant values are as follows:

When applied to the 47 blocks that were positive on both the ZN and Pap stains the following values were generated:

T value = 388.00

Z value = 1.863

P value = 0.063

When applied to the 46 blocks that were positive on both the ZN and AR stains the following values were generated:

T value = 306.50

Z value = 2.556

P value = 0.010

When applied to the 71 blocks that were positive on both the Pap and AR stains the following values were generated:

T value = 1025.50

Z value = 1.2699

P value = 0.204

From the above it can be seen that there was a significant difference in the time taken to identify bacilli on the ZN stain compared with the Pap and AR methods (p values of 0.063 and 0.010 respectively) whilst there was no statistical significance between the two fluorescent methods (p value of 0.204)

**CONCLUSION:** The time taken to identify TB bacilli in the two fluorescent stains was significantly shorter than when using the ZN stain.

### **3.5 Ease of Preparation**

All three stains were readily reproducible by a number of technologists who participated in this study

The ZN stain is relatively easy to prepare and for this reason is widely used in both microbiology and histopathology laboratories. The staining preparation time is 20 minutes to half an hour but requires that the sections be heated either on a hot-plate or over an open flame. The solutions have a good shelf-life, but the carbol-fuchsin solution has to be prepared on site. The only technical faults specific to the ZN stain in the current study,

were a heavy (dark) blue counter stain and crystalline fuchsin deposits on the slide. Both faults are easily corrected by adhering to recommended staining times and using fresh filter paper when preparing solutions, respectively.

By comparison, the AR stain was the most technically difficult stain to prepare as the solutions have a short shelf-life, need to be stored in dark containers or away from light, and require a fume cupboard for preparation. This stain, as stated earlier, is used on a routine basis in microbiology laboratories around the world, but is not widely used in histopathology laboratories as the demand is on an ad hoc basis and often requires the preparation of fresh solutions.

The mPap stain was the easiest to prepare as it used commercially available pre-mixed solutions. The staining process took approximately 15 minutes. The background staining pattern did have a tendency to vary slightly from batch to batch, even in the hands of a single technologist. The stain lends itself to automated staining (as has been applied to the standard Pap stain in many cytology laboratories).

### **3.6 Safety of the Stains**

The ZN stain uses phenol which is highly toxic and may be absorbed through the skin. The phenol and ethanol solution needs to be prepared under a fume extraction hood while wearing gloves and eye protection. The carbol-fuchsin requires heating to approximately 60 degrees Celsius. In many laboratories this is done over an open flame. To avoid using a naked flame, sections may be placed in a coplin jar of preheated carbol-fuchsin (60 to 65°C) and left until the solution has cooled.

The AR stain also requires the use of phenol, but in addition uses Auramine O and Rhodamine B, both of which are toxic substances. With regard to the ZN, a fume

extraction hood is required for the preparation of the solutions and gloves and eye protection need to be worn. The Auramine-Rhodamine solution also requires heating to approximately 60 degrees Celsius. Many laboratories use a naked flame to do this, however, in the interests of safety, it is advisable to use a hot-plate or heated water bath instead.

The mPap stain uses only eosin and haematoxylin, both routinely used, safe reagents. The use of gloves is recommended. No heating is required. The ability to automate the staining procedure further limits staff exposure to reagents and makes this stain extremely safe to use.

### **3.7 Cost**

The cost assessment in this study has been divided into two components: firstly, cost of the stain and, secondly, equipment required in preparing and interpreting the stain.

#### **3.7.1 Cost of Reagents**

The prices of the various reagents used in the three staining methods appear below (see Table 3.8)

The additive cost of the reagents of the three stains amount to the following:

R 559.00 for the ZN stain

R 786.00 for the mPap stain

R 629.00 for the AR stain.

**Table 3.8 Cost of reagents broken down by stain**

Stain	Reagent	Quantity	Cost
Ziehl-Neelsen			
	Basic fuchsin *	25 g	R 99.00
	Absolute ethanol *	2.5 l	R 193.00
	Phenol crystals *	500 g	R 96.00
	Hydrochloric acid *	2.5 l	R 150.00
	Aqueous methylene blue *	2.5 l	R 21.00
Auramine Rhodamine			
	Auramine O *	25 g	R 143.00
	Rhodamine B *	25 g	R 178.00
	Glycerol *	500 ml	R 31.00
	Phenol crystals *	500 g	R 96.00
	Hydrochloric acid *	2.5 l	R 150.00
	Potassium permanganate *	500 g	R 31.00
Modified Papanicolaou			
	Gill's haematoxylin *	2.5 l	R 343.00
	Hydrochloric acid *	2.5 l	R 150.00
	Scott's tap-water substitute:		
	Sodium bicarbonate *	500 g	R 23.00
	Magnesium sulphate *	500 g	R 23.00
	Eosin 65	2.5 l	R 190.00
	Glacial acetic acid *	2.5 l	R 57.00

(\*as listed in the Merck catalogue 2005.)

Of interest is that the National Health Laboratory Service, the laboratory group in which the current study was performed, charges R 33.00 per section regardless of which of the three stains is performed.

### 3.7.2 Cost of Equipment

The majority of the equipment required for the production and interpretation of the three stains in this study is standard laboratory equipment. As such, the cost of items such as fume extraction hoods, staining racks, and the like, have not been taken into consideration. The only item that is not standard in the majority of laboratories is a fluorescent microscope. To give an indication of the cost of an entry-level fluorescent microscope, a quote for a Zeiss Axiostar with an attached fluorescent module, linear slider and three filters was obtained (see Appendix 11 for a complete list of the specifications). The total cost as of May 2005 amounted to R 70 550.00. The lifespan of a well maintained, good quality fluorescent microscope is over a decade.

Another consideration is that a fluorescent microscope should be standard equipment in referral laboratories as it is required for the interpretation of fluorescent stains performed on renal and skin biopsies.

**CONCLUSION:** With regard to the cost of reagents, the ZN is the cheapest, the AR is the second cheapest and the mPap is the most expensive stain. The ready mixed reagents used in the mPap stain drive the cost up. No additional equipment, (other than standard laboratory equipment) is needed for producing or interpreting the ZN stain. A fluorescent microscope is required for the interpretation of both the fluorescent stains, thereby adding to the expense. This expense is, however, negligible because the purchase of a microscope is a once-off expense as the lifespan is long.

University of Cape Town

## Chapter 4 Discussion and Conclusion

In 1995 Kupper et al described the induction of fluorescence in mycobacteria in cytology smear preparations that were stained with the Pap stain (78). The current study set out to ascertain whether the Pap stain would induce fluorescence in *M tuberculosis* bacilli in processed tissue sections. In this regard, the results are conclusive - the Pap stain does indeed induce fluorescence in *M tuberculosis* bacilli in sections of processed tissue. The next logical question was why this occurs. What in the stain induces fluorescence? No other published study has attempted to answer this question. Chapter 2, sections 2.2 and 2.3, describes the various methods for the Pap stain that were tested and broken down into their various components. From the results, it became evident that bacilli fluoresced no matter which method was used, and yet the fluorescence was not an innate property of the bacilli themselves, as they did not fluoresce in unstained sections.

EA (50 or 65) was identified as the reagent which induces fluorescence and when broken down into its various components (Chapter 2, section 2.4), eosin Y was found to be the responsible subcomponent. This is not surprising as eosin is known to induce secondary fluorescence (94, 95), however, the fluorescence is not selective to the bacilli. Sections stained with eosin show intense, diffuse background fluorescence of (amongst others) cell nuclei, cytoplasmic components, stroma (including intense staining of elastin) and red blood cells which practically obscure the bacilli.

Two forms of eosin are used in the various Pap staining methods, namely EA 50 and EA 65, both of which contain eosin Y. It is to be expected that bacilli will fluoresce no matter which Pap stain method is used. Eosin is a common dye, widely used in routine histology and in cytology as a component of the H&E and Pap stains respectively. Eosin Y, the water-soluble yellowish form, is the most widely used. Other forms available are eosin B (a bluish form) and eosin ethyl (an alcohol soluble form). Eosin is an anionic dye and binds to

cationic tissue components such as those listed above (82). When viewed under blue fluorescent light (400nm), a signal in the green-yellow spectrum (544 nm) is emitted (94).

The light green component of EA 50 and 65 appeared to have no effect on the fluorescence of the bacilli as there was no appreciable difference between those sections stained with EA 50 and those stained with eosin Y.

In order to determine which Pap stain components lifted the fluorescence of the bacilli from the diffuse background stain, the components were recombined in with EA (Chapter 2, section 2.5.1). EA 65 was used in this method as it is available in a pre-mixed form. It was found that Gill's haematoxylin was responsible for dampening the background fluorescence. The explanation for this is relatively simple; haematoxylin is essentially a blue stain formed by combining a blue dye with a metal, usually either aluminum (the alum forms) or iron (the ferric forms), which confer a net positive charge to the compound. This cationic dye-metal complex binds to anionic tissue components, such as nucleic acids, resulting in a blue stain (82). When viewed under blue fluorescent light, the resulting emission is in the red light spectrum (approximately 610nm) thereby 'blanking out' or quenching the background emissions, in particular those of the cell nuclei. It stands to reason that the anionic eosin Y binds to a component of the M tuberculosis bacilli which has a net positive charge, possibly to components such as peptidoglycans or arabinogalactans that are integral components of the cell wall core (96). The bacilli do not stain with the cationic haematoxylin and therefore, when excited with blue fluorescent light, stand out against the now quenched background.

Both Gill's and Harris' haematoxylin were used in the current study with surprisingly equivocal results. Gill's haematoxylin has the advantage of a shorter staining time, a long shelf-life and requires no filtering. Harris's haematoxylin has a tendency to stain

background tissue as well as nuclei and is more difficult to use than Gill's haematoxylin as it requires pre-use filtering and careful differentiation, but is reputed to give the most intense and precise staining (82). It would be expected that Harris's haematoxylin would result in a more diffuse quenching of background fluorescence due to staining of background tissues when compared to Gill's, but this did not appear to be the case.

A point of interest is that, when H&E stained sections were examined using fluorescent microscopy, bacilli were identified, but with extreme difficulty. In this case, the predominant stain is eosin, with relatively little 'blueing' by haematoxylin when compared to the Pap stain, thereby resulting in diffuse fluorescence of the background.

Various attempts were in this study made to either increase the brightness of the bacilli or dampen that of the background. These included:

- Increasing the exposure to EA
- Quenching the background autofluorescence.

Increasing the exposure to EA was noted to increase the fluorescent signal of the bacilli, but also increased the background signal.

Autofluorescence may be quenched either by the addition of blue dyes or exposure to heavy metals (88-93). As discussed already, haematoxylin is a blue nuclear dye and has a major effect on the background stain. An attempt was made to add additional blue dyes that have a less specific staining pattern. Two blue dyes were tested, namely, methylene blue and toluidine blue. The reason for the selection of these particular dyes was that methylene blue is used in the ZN stain and was selected on the supposition that it would not 'blue' the bacilli and toluidine blue is a dye regularly used to quench autofluorescence in unprocessed tissues or smears. Unfortunately these attempts failed as in addition to

successfully quenching the background fluorescence, they also effectively quenched the signal of the bacilli. The very reason for selecting methylene blue and toluidine blue was that they were relatively non-specific in their staining; it is probably therefore not surprising that they quenched the signal from the bacilli.

Silver is a heavy metal that is widely used in histochemical stains and, although it is not commonly used for quenching autofluorescence (osmium, mercury and sodium borohydride are more commonly used), it was selected because it is used in the elastin van Gieson (EVG) stain to stain elastin black. As the reduction of elastin fluorescence was the aim, it was decided to attempt to combine the EVG and Pap stains. Unfortunately the results were similar to those of the 'blueing' attempt in that the background fluorescence was successfully quenched, but the signal of the bacilli was also negated.

One of the properties of the mycobacterial cell wall is its relative resistance to acid alcohol, a potent bleaching medium – a property exploited by the fuchsin based stains, and in particular the ZN stain (67, 69, 82, 97). It was proposed that in EA 65 stained sections, a similar effect could be achieved after exposure to acid alcohol, namely, that the dye would be retained by the bacilli but bleached from the background. A fuchsin stained section is exposed to 0.5% hydrochloric acid in 70% alcohol for anywhere from 2 to 20 minutes in the ZN stain. In the current study the EA 65 stained sections were exposed to acid alcohol for time periods ranging from 20 minutes to as little as 2 dips. It was found that the bleaching effect was so strong that it markedly reduced the signal of the bacilli to such an extent that they fluoresced only weakly or not at all. Increasing the exposure to EA 65 prior to acid alcohol etching did not have any noticeable effect.

Stains are known to fade when exposed to heat and/or light. The final investigation in the development of the mPap stain was to assess what effect these had on the Pap stained

sections. Interestingly, when the Pap stains were exposed to heat (40 degrees Celsius for 30 min) the signal was enhanced. This is a phenomenon that is inexplicable to the author. Exposure to 30 minutes of sunlight had no effect. Neither heat nor light decreased the background signal.

Most stains fade with time. The H&E stain can fade over a period of months; the haematoxylin fading more rapidly than the eosin. ZN stained sections fade slowly allowing for reassessment months to years later. Fluorescent stains have a tendency to fade extremely quickly – the AR stain is virtually useless after 48 hrs (depending on storage conditions) (97). A useful feature of the Pap stain, mentioned anecdotally, is that the fluorescent properties do not appear to fade with time. Bacilli were identified in all positive sections examined after a considerable period of time (up to 4 years after staining). This allows for review of the fluorescent findings.

The final modified Pap stain compared in this study with the ZN and AR stains differed only slightly from the standard Pap stain. The important features being as follows:

- The commercially available, pre-mixed EA 65 and Gill's haematoxylin from Polychem United Scientific were used. The reasons for using the commercially available reagents were as follows:
  - The solutions are standard, and do not vary from batch to batch.
  - The reagents are used in the Cytology laboratory of the Division of Anatomical Pathology, University of Cape Town where this study took place and were therefore readily available for use.
  - These reagents are widely used in cytology laboratories around the globe.
- OG 6 was omitted from the protocol as it had been proven to have no effect on the fluorescent signal.
- The exposure time to EA 65 was doubled.

The AR and ZN stains were selected as 'gold standards' as they are the two most widely used stains for the identification of M tuberculosis (25, 34-36, 37 Kalich, 1979 #778, 38-45). The ZN is the gold standard in light microscopy and auramine based stains, such as the AR, are the most widely used (and studied) fluorescent stains. Fluorescent techniques are not widely applied to the identification of TB bacilli in routine histopathology practice, but a number of studies performed over the past three decades have shown that they dramatically improve the 'pick-up rate' of bacilli in both smears and processed tissue sections when compared to the ZN stain (25, 34-45).

Archival material from known tuberculosis cases, from the files of the Division of Anatomical Pathology, Department of Laboratory Medicine, University of Cape Town, was used as it was a ready source of processed tissue. A weakness of the study was limiting the cases to those in which acid-fast bacilli had been identified using a ZN stain. This immediately imparted a significant selection bias in that, when examining the stained sections, the investigator was aware of the fact that bacilli had been identified in the majority of sections. This had two effects - firstly, it lowered the threshold for a positive call, thereby increasing the risk of false positives, and secondly, it placed pressure on the investigator to identify bacilli. It would have been a better indicator of the strengths and weaknesses of the three stains had they been applied to a selection of cases which showed either granulomatous inflammation on light microscopy, or were clinically suspected of having tuberculosis. As this is essentially a pilot study into a previously un-researched area, it was deemed adequate to concentrate on ZN positive cases only.

Sixty one cases were identified, one of which was excluded due to there being insufficient tissue in the block for three sections to be cut. The blocks were again limited to those on which a ZN had been performed, resulting in 47 cases in which one block was entered, 10

cases in which two blocks were entered, 2 cases in which three blocks entered and 1 case in which 4 blocks were entered, resulting in 77 blocks. In practice, a case is reported as positive for M tuberculosis if TB bacilli are identified on at least one of the blocks pertaining to that case. It is likely that bacilli were not identified on all blocks from those cases in which a ZN was performed on more than one block, but as the pathology reports did not state which block the bacilli were identified it is impossible to know for certain which of these sections were positive. It is also not known how many ZN stained sections were examined from these blocks before bacilli were identified. The original ZN stained sections were not available on all cases making it impossible to correlate the results of the ZN stained sections in the current study with those of the original cases.

Of the 77 blocks, tuberculous bacilli were identified in only 48 (62%) ZN stained sections. This is a surprisingly low percentage, given that all cases were originally identified as being mycobacterium positive. Put another way, the false negative rate for the ZN stain in the current study is high (38%). The possible reasons for this may be broken into factors related to the study design and factors relating to the investigator. The study was limited to the examination of a single ZN stained section per block. If bacilli were not identified on this section then the stain was deemed to be negative. This restriction is not applied in routine practice, where it is not uncommon to examine multiple stained sections (levels) from a block. Another weakness in study design was the fact that only one person examined the stained sections in this study – the sections were not re-examined by additional investigators. Factors related to the investigator include fatigue (no restriction was placed on the number of cases examined or time spent at a single sitting, or on the time taken to examine a single section); and possibly poor screening technique. In an attempt to lower the possibility of missing bacilli due to poor or incomplete examination of the sections, no section in the current study was deemed to be negative unless it had been examined twice on high power (400x). This was usually performed in the same sitting.

Of the 29 (38%) ZN stained sections that were negative 14 were from cases that had additional blocks whilst the remaining 15 had only a single block. The original ZN stained sections were available for 24 of the 29 blocks that were negative for TB bacilli - this represented 11 of the 15 negative cases. These 24 original ZN sections were reviewed and bacilli identified in 18 of them resulting in identification of TB bacilli in 10 of the 11 cases in which the original ZN stained sections were available. In other words, the presence of bacilli was confirmed in all but one case on the original ZN sections. The fact that the investigator could identify bacilli on these original ZN sections (under the same conditions as applied to the study) makes investigator error less likely. The fact remains, however, that the false negative rate on a single section in the current study remains high.

The fluorescent staining methods had a much higher positive rate with bacilli being identified in 76 (99%) mPap stained sections and 72 (94%) AR stained sections. Only 1 (<1%) mPap stained section and 5 (6%) AR stained sections were negative for bacilli - low false negative rates. Many studies performed on sputum or fine needle aspirate smears comparing the detection rates of ZN and fluorescent stains in culture or PCR positive TB cases have shown the fluorescent stains to be more sensitive for detecting mycobacteria than the ZN stain. These report a positive rate of between 61 and 75 percent (34, 40, 41, 45, 85, 87). This must be balanced with the fact that a number of studies have shown that the specificity of the fluorescent stains is low, giving rise to false positives (38, 39, 43, 72). It is likely that the false positive rate of the mPap will be comparable to the AR stain, if not even higher, but it is not possible to calculate the numbers of false positive cases in the current study as only ZN positive cases were selected.

In an attempt to limit the number of false positives, a suspected bacillus had to meet strict criteria (listed in Chapter 3) before the section was deemed to be positive. The more

rigorously these criteria are applied, the lower the false positive rate is likely to be, but this is at the risk of increasing the false negative rate.

Most published data are from studies comparing the pickup rate of the ZN stain with either mycobacterial culture or molecular techniques such as PCR. The majority were performed on routine histopathology specimens showing granulomatous inflammation. The percentage of confirmed *M.tuberculosis* cases detected on ZN stain alone is in the region of 45% (34, 87). These studies are not directly comparable to the current study as it is the ZN stain that is being used as a 'gold-standard' and not culture or molecular techniques, however a number of studies have used ZN and auramine based stains as standards when assessing a new stain (36, 39, 83, 85, 98). In the current study, the ZN has proven to be less useful in this regard than the other 'gold-standard' – the AR stain. The reason for this statement becomes apparent when one compares the positive and negative data for the three stains. When comparing the mPap to the ZN, the mPap stains sensitivity was 61.80, the specificity 50 and the accuracy 61.54. These figures are very poor, the main reason for this being the poor (62%) positive pickup rate with the ZN stain and not visa versa. When the exercise is performed using the AR as the standard the figures are very different: the sensitivity was 93.4, the specificity 50 and the accuracy 92.30m. The reason for the low specificity is that there was only one negative mPap section which was called positive on the AR section and 5 negative AR sections that were all called positive on the mPap. When the exercise was repeated with the ZN and AR stains, using the ZN as the 'gold-standard', the sensitivity was 63.89, the specificity 66.67 and the accuracy 64.10 – figures similar to the mPap sections. Thus the two fluorescent stains are comparable with each other, but do not compare well with the ZN.

Ease of interpretation is a subjective assessment. The best measure would be to expose a number of pathologists to the three stains performed on a number of cases and have them

assess the stains in a number of categories. This was beyond the scope of the current study. The subjective opinion of the author on the ease of interpreting the three stains, and the objective measure of the time taken to identify bacilli are presented below:

The ZN stain was the easiest to interpret as it required only light microscopy – a medium familiar to all pathologists. The mycobacterial bacilli stood out readily, staining deep red against a pale blue background. The only technical faults specific to the ZN stain that interfered with interpretation were a heavy (dark) blue counter stain and crystalline fuchsin deposits on the slide. The former may hide bacilli making them harder to find and increasing the false negative rate; and the latter may be misinterpreted as representing bacilli, thereby resulting in false positives. The short-fall of the ZN stain, as indicated in the introductory chapter, is that bacilli may be very scarce and one is required to scan the slide on high power to identify bacilli – a very time consuming process as indicated by the significantly greater time values for the positive ZN sections (discussed below).

The AR stain was easy to use, once one mastered the fluorescent microscope. Bacilli were readily identified on a scanning power of 200 times allowing the sections to be scanned more rapidly than for the ZN sections. The major shortcomings were the presence of fluorescent artefact in the sections that could be misinterpreted as bacilli; and the rapid loss of fluorescence (within 24 to 48 hours depending on the storage conditions). The former accounts for the high rate of false positives reported in numerous studies (38, 39, 42, 43, 72). In an attempt to limit misinterpretation, the criteria listed in Chapter 3 were adhered to.

The mPap stain was the most difficult to interpret as the background staining made identification of organisms difficult. It would be reasonable to expect that this would result in a reduction in the numbers of bacilli identified and in increased numbers of false

negatives. This did not appear to be the case as more mPap stained sections were interpreted as being positive than on either of the other two stains. On the other hand, the bright fluorescence of fragments of elastin and red blood cells in particular could mimic curvilinear organisms, which could lead to increased false positives. In an attempt to limit misinterpretation, the criteria listed in Chapter 3 were adhered to. As with the AR stain, the mPap stained sections could be scanned at 200 times magnification, however it was necessary to move to 400 times magnification to confirm the presence of bacilli.

As stated in Chapter 3, the time taken to identify bacilli is a measure of the ease of interpretation. As can be seen from the results presented in Chapter 3, section 3.5, the mPap and AR stains had significantly reduced times when compared with the ZN stain. The total amount of time spent examining the ZN stained sections was 9 hrs 44 min 4 s with an average time of 7 min 35s per slide compared with 4 hrs 57 min 1 s for the AR and 5 hrs 5 min 48 s for the mPap with average times of 3 min 51s and 3 min 58 s per slide respectively.

It is logical that more time was spent on those sections where bacilli could not be found, as these cases were examined twice before they were deemed to be negative. This is in contrast to the positive cases in which the time was recorded as soon as a bacillus was identified that fulfilled all criteria. This is highlighted by the fact that the average time spent on negative ZN sections (10 min 32 s per slide) was double the average for the positive sections (5 min 48 s per slide), whilst for the AR stained sections the average time of the negatives (12 min 45 s per slide) was almost four times that of the positives (3 min 14 s per slide) and for the mPap this went up to five-and-a-half times (20 min 21 s per slide compared with 3 min 45 s per slide respectively).

When the data sets of the time taken to identify bacilli on the positive sections for each of the three stains were compared using the Wilcoxon method for evaluating matched pairs, it could be seen that there was a significant difference between the ZN stain compared with both the Pap and AR methods (p values of 0.063 and 0.010 respectively), whilst there was no statistically significant difference between the two fluorescent methods (p value of 0.204). This proves that bacilli were identified significantly faster when either of the two fluorescent stains were used compared to the ZN stain. Although the times for the AR stain appear faster than those for the mPap stain, this difference has not proved to be statistically significant.

An interesting point to note is that 95% of the positive ZN cases were identified within 14 min 28 s and those of the AR and mPap within 7 min 50 s and 8 min 53 s respectively. This implies that if TB bacilli are present in a section the majority of cases will be identified within 15 minutes for a ZN, 8 minutes for an AR and 9 min for an mPap. The longest time spent examining a positive ZN section in this study was 19 min 56s, whilst for a positive AR the longest time was 11 min 44 s and for a positive mPap was 12 min 8 s. Assuming that all bacilli were identified (a great assumption), these times give an indication of the minimum length of time one should spend examining a stained section before calling it negative.

Holding to the assumption that the speed with which bacilli are identified is a measure of the ease of interpretation, it stands to reason that the two fluorescent stains were easier to interpret than the ZN stain.

The ease of preparation of the stains and safety of the various methods was examined. The ZN, AR and Pap stains are performed routinely in laboratories world-wide and are familiar to technical laboratory staff. The mPap differs little from a conventional Pap and is

readily performed in laboratories were the Pap stain is used routinely. Of the three stains, the mPap was the easiest to perform, with the ZN next and the AR the most technically difficult. This opinion is largely subjective, however, no heating is required in the mPap stain, the reagents are premixed and no fume cupboard is needed. The stain also lends itself to automation. By comparison, both the ZN and AR require heating and the use of a fume cupboard. Both stains require preparation of reagents in the laboratory. The reagents for the AR stain have a short shelf-life and require new solutions to be prepared frequently.

As far as safety is concerned, the mPap stain is the safest of the three as none of the reagents are particularly volatile or noxious and no safety precautions, beyond the routine use of gloves and goggles, are required. There is no need for heating or the use of an open flame. By comparison, the ZN stain requires the use of hydrochloric acid and phenol, whilst the AR stain uses auramine and rhodamine. All these reagents are either toxic or corrosive.

The cost of using a specific detection method for identifying TB bacilli is extremely difficult to ascertain. Costs can be analysed prospectively and retrospectively. Prospective analysis entails costing the equipment and reagents whilst retrospective analysis entails implementing a new staining technique and calculating the increased expenditure compared to expenditure prior to its introduction. The only realistic method is retrospective in nature. Both methods are fraught with difficulties. The current study has attempted the former method.

It is nearly impossible to accurately assess the cost of a single stained section as the quantity of reagents used varies from stain to stain, method to method and technologist to technologist. Amount of wastage (both during the staining procedure and due to expired

reagents) and staff hours (time it takes to mix reagents and perform the stain) need to be taken into consideration and are beyond the scope of this study.

In spite of all of these complicating factors some basic points can be made:

- The cheapest reagents (if purchased in standard quantities at 2005 prices) were those for the ZN stain amounting to R 559.00, whilst the most expensive were the premixed reagents of the mPap stain amounting to R 786.00, with those of the AR lying between the two and amounting to R 629.00.
- As stated above, the solutions and reagents of the AR stain have a limited shelf-life resulting in increased wastage due to the discarding of expired solutions, whilst the reagents for both the ZN and mPap have long shelf-lives.
- The mPap uses premixed reagents, is a quick stain and can be automated, resulting in less wastage and in decreased staff hours
- In practice, although the cost of the Pap stain reagents is greater, the cost of the stain per slide is infinitesimal since the reagents can be bought in bulk, a small quantity used for an extended period of time (up to a week) for staining batches of slides and can even be filtered and reused. This cannot be done with either the ZN or the AR stains.

As stated in section 3.8.2, the only equipment necessary for performing and interpreting the three stains that is not standard equipment in a basic histopathology laboratory was taken into consideration. No non-standard equipment was required by the ZN stain, whilst the two fluorescent stains require a fluorescent microscope for their interpretation.

A quotation was obtained for a Zeiss Axiostar with an attached fluorescent module, linear slider and three filters as this is a good example of an entry-level fluorescent microscope that is available internationally. The total cost as of May 2005 amounted to R 70 550.00.

This is a relatively minor expense when account is taken of the fact that it is a once-off purchase as a good quality microscope will last for many years, if given the proper care.

In summary, all three stains are relatively cheap to perform and the only major difference in cost is the fluorescent microscope which is a once off expense.

The current study should be viewed as a pilot study as it has major biases built into the study design. The most telling of these is that the stains were performed on cases that were known to be positive for acid-fast bacilli on a ZN stain. This made it difficult to assess the false positive rate. To remedy this, future studies could include the following:

A blinded study in which the stains are applied to a range of cases of granulomatous inflammation that include proven TB cases and cases proven to be negative for TB.

A range of routine surgical cases in which granulomatous inflammation is seen or in which tuberculosis is suspected for clinical reasons.

The ZN and AR stains were used as the 'gold-standards'. Neither stain is ideally suited for this purpose as the ZN is known to have a high false negative rate – a fact corroborated by the current study where the rate was 38%. This does not allow for accurate calculation of the sensitivity and specificity of the detection method under investigation. In contrast, the AR stain is known to have a high false positive rate, thereby adding a bias to any comparative study. The question of which detection method to use as a 'gold-standard' is a vexing one as no single method has proven to be entirely accurate. Many studies have used PCR as the standard with good results (33, 46, 49, 55, 60, 62, 99-103), but PCR is known to have a high false positive rate. TB culture is the most likely candidate as it is very accurate, but is time consuming (29, 31, 58, 99). An alternative route is to determine positive and negative TB cases based on the results of a number of investigations including microscopy (ZN), PCR and TB culture.

Further avenues of study raised by the investigation of the fluorescent properties of the Pap stain include:

- Refinements of the mPap stain such as the use of heat during staining with EA 65. Heat is used in both the ZN and AR methods to improve penetration of the dye into the bacilli (82). If the EA 65 solution is heated to 65°C then applied to the section for 10 minutes this could improve the retention of the dye by the bacilli during counterstaining or acid etching.
- Using eosin Y instead of EA 65, as it is the eosin in EA 65 that induces fluorescence and not the other components of the solution.
- Replacing haematoxylin with a general cationic blue stain, as haematoxylin is a cationic blue nuclear dye but does not stain other tissue components well. A cationic blue dye with a wider staining profile may blot out the background fluorescence to a greater extent.
- The use of heavy metals warrants further investigation as agents to blot out background fluorescence, as this was only dealt with very superficially in the current study.
- The use of filters to narrow the range of detected emission frequencies in an attempt to identify the frequency of emissions from the bacilli compared to those of the background components. The appropriate filters can then be applied to exclude emissions from background elements but not from the bacilli thereby 'raising' the bacilli from the obscuring background.
- The use of subtraction fluorescence to cancel out the background stain allowing the bacilli to fluoresce unimpeded. This is unfortunately only applicable to photography and cannot be used in screening.

- Apply Pap fluorescence to a range of organisms and tissue types to determine its usefulness in identifying structures other than TB bacilli, such as elastin (as noted in the current study).

*In conclusion*, this study has successfully proved that the Pap stain induces fluorescence in TB bacilli in processed tissue sections. Eosin Y has been shown to be the component of the Pap stain that induces fluorescence and haematoxylin has been identified as the reagent that quenches much of the background fluorescence. Various attempts have been made to modify the Pap stain in order to enhance this fluorescence and quench the background staining. The resultant mPap had the highest detection rate (most number of positive sections) and compared favourably with the AR stain. The ZN stain had a poor pickup rate (highest number of negative sections), bacilli being identified in only approximately two thirds of the potentially positive sections.

The time taken to identify bacilli using the fluorescent stains (mPap and AR) was (statistically) significantly shorter than when using the ZN stain. There was no statistically significant difference between the times of the two fluorescent methods.

The mPap was the most difficult to interpret due to the abundant background staining but proved to be the easiest to prepare and was the safest of the three methods. It was, however the most expensive with regard to reagents, but is likely to be the cheapest in practice. The cost of non-standard laboratory equipment (a fluorescent microscope) was negligible.

This study highlights the fact that further studies are warranted in an attempt to develop an inexpensive, reliable fluorescent stain for the detection of *Mycobacterium tuberculosis* using the fluorescent properties of eosin. Until such time as one is developed, the age old ZN and the more recent AR stains will have to suffice.

## Chapter 5 References

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# Appendix 1

Table 1 Table of Data for the comparison of the mPap with the ZN and AR stains.

Case	Block	ZN		Pap		AR	
		Time (s)	Status	Time (s)	Status	Time (s)	Status
PS003082V/00	A1	556	positive	188	positive	374	positive
PS008177D/99	A1	57	positive	106	positive	703	negative
PS010779R/99	A1	75	positive	684	positive	67	positive
PS011000T/99	A1	140	positive	728	positive	179	positive
PS001059A/00	A1	828	negative	324	positive	704	positive
PS001059A/00	A2	783	negative	497	positive	96	positive
PS000563V/00	A1		cut away		cut away		cut away
PS005216T/00	A1	110	positive	236	positive	55	positive
PS010071T/99	A1	185	positive	74	positive	116	positive
PS009963N/99	A1	1200	negative	50	positive	50	positive
PS009963N/99	A2	1196	positive	121	positive	190	positive
PS008742G/99	A1	165	positive	153	positive	72	positive
PS012156M/99	A1	340	negative	241	positive	161	positive
PS006745G/00	A5	80	positive	47	positive	76	positive
PS007198Y/00	A1	1071	negative	545	positive	56	positive
PS000458H/00	A1	150	negative	65	positive	18	positive
PS000458H/00	B1	301	negative	139	positive	100	positive
PS012942E/99	A1	11	positive	152	positive	170	positive
PS008899J/99	A1	433	negative	89	positive	210	positive
PS011869W/99	A4	420	negative	598	positive	2058	negative
PS011869W/99	A6	263	negative	339	positive	537	positive
PS011869W/99	A7	517	negative	110	positive	371	positive
PS011869W/99	A8	448	negative	87	positive	85	positive
PS010775M/99	A1	918	positive	1221	negative	674	positive
PS011771Z/99	A1	783	negative	192	positive	111	positive
PS006171F/00	A1	590	negative	402	positive	468	positive
PS011563V/99	A2	902	positive	308	positive	93	positive
PS013348A/99	A1	9	positive	45	positive	14	positive
PS013348A/99	B1	7	positive	68	positive	2	positive
PS008632C/00	A1	27	positive	213	positive	143	positive
PS007524Z/99	A1	747	positive	392	positive	66	positive
PS004065J/00	A1	157	positive	246	positive	110	positive
PS009566N/99	A1	185	positive	442	positive	631	positive
PS001140X/00	A1	764	negative	273	positive	101	positive
PS012898H/99	A1	1731	negative	401	positive	99	positive
PS003423R/00	A1	268	positive	285	positive	124	positive
PS000433E/00	A1	472	positive	358	positive	182	positive
PS007583C/99	C1	16	positive	40	positive	132	positive
PS010913A/99	A1	668	negative	283	positive	139	positive
PS010913A/99	B1	351	negative	272	positive	412	negative
PS010365K/99	A1	256	positive	43	positive	311	positive
PS008619B/00	A1	456	negative	24	positive	25	positive
PS002806P/00	A1	146	positive	106	positive	145	positive
PS011578X/99	A1	94	positive	31	positive	56	positive
PS005566J/00	A4	680	negative	150	positive	107	positive
PS009778J/99	A1	412	negative	55	positive	68	positive
PS011092N/99	A1	350	negative	329	positive	388	positive
PS009223S/99	A2	147	positive	18	positive	39	positive
PS005627X/00	A2	1179	positive	369	positive	267	positive
PS005627X/00	A3	706	positive	43	positive	51	positive
PS005627X/00	B1	944	negative	282	positive	108	positive
PS007652C/99	A1	547	positive	253	positive	352	negative
PS007652C/99	A2	567	negative	35	positive	26	positive

PS010722L/99	A1	694	positive	158	positive	59	positive
PS010722L/99	A2	760	positive	50	positive	105	positive
PS004115J/00	A1	278	positive	237	positive	141	positive
PS009464H/99	A2	919	positive	321	positive	247	positive
PS012273C/99	A1	311	positive	89	positive	364	positive
PS012175A/99	A1	76	positive	74	positive	151	positive
PS010111M/99	A1	109	positive	475	positive	531	positive
PS008065N/99	A1	1326	negative	453	positive	445	positive
PS008065N/99	A2	910	positive	140	positive	144	positive
PS009517P/99	A1	297	positive	247	positive	337	positive
PS006091C/00	A1	157	positive	299	positive	99	positive
PS009226N/99	A1	566	positive	134	positive	288	positive
PS007935W/00	A1	11	positive	292	positive	177	positive
PS007935W/00	B1	8	positive	211	positive	584	positive
PS009210J/99	B1	84	positive	299	positive	135	positive
PS006179X/00	A1	416	negative	324	positive	305	positive
PS006179X/00	A2	281	positive	110	positive	84	positive
PS006179X/00	A3	862	negative	121	positive	150	positive
PS010114R/99	A2	675	positive	340	positive	305	positive
PS011358F/99	A1	145	positive	81	positive	90	positive
PS007634L/00	A1	292	positive	329	positive	340	positive
PS001920J/00	A1	19	positive	150	positive	111	positive
PS010084M/99	A1	320	negative	90	positive	134	positive
PS010084M/99	B1	369	negative	321	positive	300	negative
PS006723L/99	A1	751	positive	251	positive	303	positive

## Appendix 2

### Papanicolaou Stain

#### Method 1: (Papanicolaou 1942)

##### Solutions:

1. Harris's haematoxylin
2. OG 6
  - i. Orange G 0.5 g
  - ii. Phosphotungstic acid 0.015 g
  - iii. 95% ethanol 100 ml
3. EA 50
  - i. 0.1% light green SF in 95 % ethanol 45 ml
  - ii. 0.5% eosin ws yellowish in 95 % ethanol 45 ml
  - iii. 0.5% Bismark brown in 95 % ethanol 45 ml
  - iv. Phosphotungstic acid 0.2 g
  - v. Saturated aqueous lithium carbonate 1 drop

##### Technique:

1. 95 % alcohol for 1 min
2. 70 % alcohol for 2 min
3. Rinse with distilled water for 3 min
4. Harris's haematoxylin solution for 2 min 30 s
5. Rinse in tap water for 1 min
6. 1% acid alcohol for 3 s
7. Rinse in tap water for 2 min
8. Ammoniated water for 1 min
9. rinse in tap water for 3 min
10. 70 % alcohol for 2 min
11. 95 % alcohol for 2 min
12. OG 6 for 1 min 30 s
13. 95 % alcohol for 2 min
14. 95 % alcohol for 2 min
15. EA 50 for 2 min 30 sec
16. 95 % alcohol for 2 min
17. Absolute alcohol for 1 min
18. Absolute alcohol for 1 min
19. Absolute alcohol for 1 min
20. Xylene for 2 min
21. Xylene for 2 min
22. Mount

#### Method 2: (Bancroft 1996)

##### Solutions:

1. Harris's haematoxylin
  - i. Haematoxylin 5 g
  - ii. Ethanol 50 ml
  - iii. Potassium alum 100g
  - iv. Distilled water 1000 ml
  - v. Mercuric oxide 2 - 5 g
  - vi. Glacial acetic acid 40 ml

2. OG 6		
vi. Orange G (10% aqueous)		50 ml
vii. Alcohol		950 ml
viii. Phosphotungstic acid		0 - 15 g
3. EA 50		
ix. 0.04 M light green SF		10 ml
x. 0.3 M eosin y		20 ml
xi. Phosphotungstic acid		2 g
xii. Alcohol		750 ml
xiii. Methanol		250 ml
xiv. Glacial acetic acid		20 ml
Filter all solutions before use.		

Technique:

1. 95 % alcohol for 2 min
2. 70 % alcohol for 2 min
3. Rinse with water for 1 min
4. Harris's haematoxylin solution for 5min
5. Rinse in tap water for 2 min
6. Differentiate in 0.5% aqueous hydrochloric acid for 10 s
7. Rinse in tap water for 2 min
8. 'Blue' in Scott's tap water substitute for 2 min
9. rinse in tap water for 2 min
10. 70 % alcohol for 2 min
11. 95 % alcohol for 2 min
12. 95 % alcohol for 2 min
13. OG 6 for 2 min
14. 95 % alcohol for 2 min
15. 95 % alcohol for 2 min
16. EA 50 for 3 min
17. 95 % alcohol for 1 min
18. Absolute alcohol for 1 min
19. Absolute alcohol for 1 min
20. Absolute alcohol for 1 min
21. Xylene for 2 min
22. Xylene for 2 min
23. Mount

**Method 3: (Based on recommendations of Polychem Scientific 2004)**

Solutions:

1. Gill's haematoxylin
2. OG 6
3. EA 65

Technique:

1. 70 % alcohol for 10 dips
2. 50 % alcohol for 10 dips
3. Rinse in tap water for 10 dips
4. Gill's haematoxylin solution for 1 min
5. Rinse in tap water for 10 dips
6. Differentiate in lithium carbonate for 1 min
7. 96 % alcohol for 10 dips

8. OG 6 for 1 min
9. 96 % alcohol for 10 dips
10. EA 65 for 2 min
11. 96 % alcohol for 10 dips
12. 96 % alcohol for 10 dips
13. Absolute alcohol for 10 dips
14. Absolute alcohol for 10 dips
15. Xylene for 10 dips
16. Xylene for 10 dips
17. Mount

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## Appendix 3

### Ziehl-Neelsen Stain: (Ziehl 1882)

#### Solutions:

1. Carbol fuchsin
  - i. basic fuchsin 1 g
  - ii. absolute ethanol 10 ml
  - iii. phenol crystals 5 g
  - iv. distilled water 100 ml
2. 0.5% hydrochloric acid in 70% alcohol
3. 0.1% aqueous methylene blue

#### Technique:

1. De-wax sections and rehydrate in distilled water.
2. On a stain rack, flood sections with filtered carbol-fuchsin, heat from below with a spirit flame until the solution begins to steam, leave for 10 minutes. Alternatively place the dye in a coplin jar, heat to 65°C then stain for 10 minutes. (If a coplin jar is used it is placed into cold water after 10 minutes. The slides remain in the staining solution until the dye has cooled).
3. Drain sections, blot dry and differentiate in acid alcohol.
4. Wash sections in tap water for 2 minutes.
5. Counterstain in methylene blue for 15 to 30 seconds.
6. Rinse in tap water.
7. Dehydrate and differentiate counterstain in absolute alcohol, clear in xylene and mount.

## Appendix 4

### Auramine Rhodamine Stain: (Kuper and May 1960)

#### Solutions:

1. Auramine rhodamine solution
  - i. Auramine O 1.5 g
  - ii. Rhodamine B 0.75 g
  - iii. Glycerol 75 ml
  - iv. Phenol (liquefied at 50°C) 10 ml
  - v. Distilled water 50 ml

Mix solution thoroughly and filter before use. The solution is stable for at least three months.

2. 0.5% hydrochloric acid in 70% alcohol
3. 0.5% aqueous potassium permanganate

#### Technique:

1. De-wax sections and rehydrate in distilled water.
2. Stain in auramine-rhodamine solution in a coplin jar for 10 minutes at 60° C.
3. Wash in running tap water for 2 minutes.
4. Differentiate in acid-alcohol for 2 minutes.
5. Wash in running tap water for 2 minutes.
6. Flood with potassium permanganate for 2 minutes.
7. Wash in running tap water for 2 minutes.
8. Blot slides dry, dehydrate rapidly in absolute alcohol, clear in xylene and mount.

## Appendix 5

### Methods for the testing the Pap components:

#### Solutions:

1. Harris's haematoxylin
  - vii. Haematoxylin 5 g
  - viii. Ethanol 50 ml
  - ix. Potassium alum 100g
  - x. Distilled water 1000 ml
  - xi. Mercuric oxide 2 - 5 g
  - xii. Glacial acetic acid 40 ml
2. OG 6
  - xv. Orange G (10% aqueous) 50 ml
  - xvi. Alcohol 950 ml
  - xvii. Phosphotungstic acid 0 - 15 g
3. EA 50
  - xviii. 0.04 M light green SF 10 ml
  - xix. 0.3 M eosin y 20 ml
  - xx. Phosphotungstic acid 2 g
  - xxi. Alcohol 750 ml
  - xxii. Methanol 250 ml
  - xxiii. Glacial acetic acid 20 ml

#### Technique:

1. **Unstained section:**
  1. De-wax
  2. Mount
2. **Harris's Haematoxylin**
  1. De-wax
  2. Rinse in water for 1 min
  3. Stain with Harris's Haematoxylin for 3 min
  4. Place in 95% alcohol for 2 min
  5. Repeat 95% alcohol for 2 min
  6. Mount
3. **Harris's Haematoxylin with differentiation**
  1. Repeat steps 1 to 3 above
  2. Differentiate in 0.5% aqueous hydrochloric acid for 10 sec
  3. Rinse in water for 2 min
  4. Place in 95% alcohol for 2 min
  5. Repeat 95% alcohol for 2 min
  6. Mount
4. **Harris's Haematoxylin with differentiation and 'blueing'**
  1. Repeat steps 1 to 5 above
  2. 'Blue' in Scott's tap water substitute for 2 min
  3. Place in 95% alcohol for 2 min
  4. Repeat 95% alcohol for 2 min
  5. Mount
5. **Orange G 6**

1. De-wax
2. Rinse in water for 1 min
3. Stain with OG 6 for 2 min
4. Place in 95% alcohol for 2 min
5. Repeat 95% alcohol for 2 min
6. Mount I

**6. Eosin A 50**

1. De-wax
2. Stain with EA 50 for 3 min
3. Place in 95% alcohol for 1 min
4. Mount

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## Appendix 6

### Methods for the testing the EA 50 components:

#### Solutions:

1. Eosin Y
2. Light green
3. Phosphotungstic acid
4. Glacial acetic acid
5. Alcohol and methanol

#### Technique:

Sections were stained with one of the above EA 50 components. The staining protocol for the individual EA 50 components followed these steps:

1. De-wax
2. Stain with EA 50 component for 3 min
3. Place in 95% alcohol for 1 min
4. Mount

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## Appendix 7

### Methods for recombining the Pap components:

#### Solutions:

1. Gill's haematoxylin (GH)
2. Orange G 6 (OG 6)
3. Eosin A 65 (EA 65)

#### Technique:

##### 1. Gill's haematoxylin

1. De-wax
2. Rinse in tap water for 10 dips
3. Gill's haematoxylin solution for 1 min
4. Rinse in tap water for 10 dips
5. Differentiate in lithium carbonate for 1 min
6. 96 % alcohol for 10 dips
7. 96 % alcohol for 10 dips
8. Absolute alcohol for 10 dips
9. Absolute alcohol for 10 dips
10. Xylene for 10 dips
11. Xylene for 10 dips
12. Mount

##### 2. Orange G 6

1. De-wax
2. 96 % alcohol for 10 dips
3. Stain with OG 6 for 1 min
4. 96 % alcohol for 10 dips
5. 96 % alcohol for 10 dips
6. Absolute alcohol for 10 dips
7. Absolute alcohol for 10 dips
8. Xylene for 10 dips
9. Xylene for 10 dips
10. Mount

##### 3. Eosin A 65

1. De-wax
2. Stain with EA 65 for 2 min
3. 96 % alcohol for 10 dips
4. 96 % alcohol for 10 dips
5. Absolute alcohol for 10 dips
6. Absolute alcohol for 10 dips
7. Xylene for 10 dips
8. Xylene for 10 dips
9. Mount
10. Place in 95% alcohol for 1 min
11. Mount

##### 4. Eosin A 65 combined with Gill's haematoxylin.

1. De-wax
2. Rinse in tap water for 10 dips
3. Gill's haematoxylin solution for 1 min

4. Rinse in tap water for 10 dips
5. Differentiate in lithium carbonate for 1 min
6. 96 % alcohol for 10 dips
7. EA 65 for 2 min
8. 96 % alcohol for 10 dips
9. 96 % alcohol for 10 dips
10. Absolute alcohol for 10 dips
11. Absolute alcohol for 10 dips
12. Xylene for 10 dips
13. Xylene for 10 dips
14. Mount

**5. Eosin A 65 combined with Orange G 6**

1. De-wax
2. 96 % alcohol for 10 dips
3. OG 6 for 1 min
4. 96 % alcohol for 10 dips
5. EA 65 for 2 min
6. 96 % alcohol for 10 dips
7. 96 % alcohol for 10 dips
8. Absolute alcohol for 10 dips
9. Absolute alcohol for 10 dips
10. Xylene for 10 dips
11. Xylene for 10 dips
12. Mount

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## Appendix 8

### Methods for increasing the exposure of the Pap components:

#### Solutions:

1. Gill's haematoxylin
2. OG 6
3. EA 65

#### Technique:

##### 1. Pap stain with the exposure time to all reagents doubled

1. De-wax
2. Rinse in tap water for 10 dips
3. Gill's haematoxylin solution for 2 min
4. Rinse in tap water for 10 dips
5. Differentiate in lithium carbonate for 1 min
6. 96 % alcohol for 10 dips
7. OG 6 for 2 min
8. 96 % alcohol for 10 dips
9. EA 65 for 4 min
10. 96 % alcohol for 10 dips
11. 96 % alcohol for 10 dips
12. Absolute alcohol for 10 dips
13. Absolute alcohol for 10 dips
14. Xylene for 10 dips
15. Xylene for 10 dips
16. Mount

##### 2. Pap stain with the exposure time to EA 65 doubled

1. De-wax
2. Rinse in tap water for 10 dips
3. Gill's haematoxylin solution for 1 min
4. Rinse in tap water for 10 dips
5. Differentiate in lithium carbonate for 1 min
6. 96 % alcohol for 10 dips
7. OG 6 for 1 min
8. 96 % alcohol for 10 dips
9. EA 65 for 4 min
10. 96 % alcohol for 10 dips
11. 96 % alcohol for 10 dips
12. Absolute alcohol for 10 dips
13. Absolute alcohol for 10 dips
14. Xylene for 10 dips
15. Xylene for 10 dips
16. Mount

## Appendix 9

### Methods for quenching the background fluorescence

#### 1. 'Blueing' the Pap

##### Solutions:

1. Gill's haematoxylin
2. OG 6
3. EA 65
4. Methylene blue
5. 0.5 % toluidine blue (Aqueous)

##### Technique:

#### 1. Pap stain with the addition of methylene blue counterstain

1. De-wax
2. Rinse in tap water for 10 dips
3. Gill's haematoxylin solution for 1 min
4. Rinse in tap water for 10 dips
5. Differentiate in lithium carbonate for 1 min
6. 96 % alcohol for 10 dips
7. OG 6 for 1 min
8. 96 % alcohol for 10 dips
9. EA 65 for 2 min
10. 96 % alcohol for 10 dips
11. Wash in tap water for 2 minutes.
12. Counterstain in methylene blue for 10 dips.
13. Rinse in tap water.
14. Absolute alcohol for 10 dips
15. Absolute alcohol for 10 dips
16. Xylene for 10 dips
17. Xylene for 10 dips
18. Mount

#### 2. Pap stain with 0.5 % toluidine blue counterstain

1. De-wax
2. Rinse in tap water for 10 dips
3. Gill's haematoxylin solution for 1 min
4. Rinse in tap water for 10 dips
5. Differentiate in lithium carbonate for 1 min
6. 96 % alcohol for 10 dips
7. OG 6 for 1 min
8. 96 % alcohol for 10 dips
9. EA 65 for 2 min
10. 96 % alcohol for 10 dips
11. Wash in tap water for 2 minutes.
12. Counterstain in 0.5% toluidine blue for 10 dips.
13. Rinse in tap water.
14. Absolute alcohol for 10 dips
15. Absolute alcohol for 10 dips
16. Xylene for 10 dips
17. Xylene for 10 dips
18. Mount

## 2. The addition of heavy metals (Combination EVG/Pap stain)

### Solutions:

1. Gill's haematoxylin
2. OG 6
3. EA 65
4. 5% haematoxylin solution
  - i. Haematoxylin 10g
  - ii. 100% ETOH 200ml
5. 10% aqueous ferric chloride (prepare fresh)
6. Weigert's iodine solution
  - i. Potassium iodide 2 g
  - ii. Iodine 1 gm
  - iii. Distilled water 100 ml
7. Verhoeff's staining solution (prepare fresh)
  - i. 5% alcoholic haematoxylin 20ml
  - ii. 10% ferric chloride 8ml
  - iii. Weigert 's iodine solution 8ml
8. 2% aqueous ferric chloride (prepare fresh)
9. 5% aqueous sodium thiosulfate.
10. 1% aqueous acid fuchsin 5ml
11. Saturated aqueous picric acid 100ml

### Technique:

1. De-wax
2. Hydrate slides to distilled water
3. Stain in Verhoeff's solution for 1 hour
4. Rinse thoroughly in tap water with 2 or 3 changes
5. Differentiate in 2% aqueous ferric chloride for 3 min (agitate slides gently)
6. Rinse in tap water
7. 5% sodium thiosulfate for 1 min
8. Wash in running tap water for 5 min
9. Gill's haematoxylin solution for 1 min
10. Rinse in tap water for 10 dips
11. Differentiate in lithium carbonate for 1 min
12. 96 % alcohol for 10 dips
13. OG 6 for 1 min
14. 96 % alcohol for 10 dips
15. EA 65 for 2 min
16. 96 % alcohol for 10 dips
17. Wash in tap water for 2 minutes.
18. Counterstain in 0.5% toluidine blue for 10 dips.
19. Rinse in tap water.
20. Absolute alcohol for 10 dips
21. Absolute alcohol for 10 dips
22. Xylene for 10 dips
23. Xylene for 10 dips
24. Mount

## Appendix 10

### The modified Pap stain:

#### Solutions:

1. Gill's haematoxylin
2. EA 65

#### Technique:

1. De-wax
2. Rinse in tap water for 10 dips
3. Gill's haematoxylin solution for 1 min
4. Rinse in tap water for 10 dips
5. Differentiate in lithium carbonate for 1 min
6. 96 % alcohol for 10 dips
7. EA 65 for 4 min
8. 96 % alcohol for 10 dips
9. 96 % alcohol for 10 dips
10. Absolute alcohol for 10 dips
11. Absolute alcohol for 10 dips
12. Xylene for 10 dips
13. Xylene for 10 dips
14. Mount

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## Appendix 11

### Specifications and Cost of the Zeiss Axiostar with Fluorescent Module



Description	Qty	Catalogue Number
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**Binocular microscope "Axiostar" plus  
for epi-fluorescence in combination with  
transmitted-light phase contrast**

"Axiostar" plus microscope stand with 4x revolving nosepiece W 0.8, power supply 100...240VAC/50...60Hz/ 65VA, mechanical stage 75x30 right with ceramic surface, covered X-guide positioned at bottom, right drive 135 mm (extendable by 15 mm) with friction setting, specimen holder with spring lever on the left, condenser carrier with drive on the left, luminous-field diaphragm insert with iris and filter clamping device, long-life halogen lamp 6V 20W, mains cable	1	000000-1169-149
Long-life halogen bulb 6V 20W		380079-9690-000
Contrast-enhancing blue filter, d=32x2 mm		000000-1220-224
Binocular tube 30°/20 for "Axiostar" for use with adapter for 30° tubes or reflected-light illuminator FL		000000-0428-271
Dust cover G		459306-0000-000

**Epi-fluorescence equipment**

Reflected-light FL illuminator for "Axiostar"		000000-0428-273
Filter set 09 shift free		488009-0000-000
Filter set 15 shift free		488015-0000-000
Reflector module FL P&C	2x	424920-0000-000
Antiglare screen for "Axiostar" plus		000000-1177-213

**HBO 50 illuminator**

Lamp housing HBO 50 with socket		447220-0000-000
Lamp collector HBO 50/SF 20		447271-0000-000
HBO 50 super-pressure mercury lamp		381619-0000-000
Transformer mbq52ac-z for HBO 50ac		000000-1113-833

**Optical equipment**

Objective CP-"Achromat" 5x/0.12 (a=11.2mm)	440920-0000-000
Objective CP-"Achromat" 10x/0.25 (a=5.1mm)	440930-0000-000
Objective CP-"Achromat" 40x/0.65 (a=0.3mm)	440950-0000-000
Objective CP-"Achromat" 100x/1.25 Oil (a=0.07mm)	440980-0000-000
Condenser 0.9/ 1.25 for "Axiostar"	000000-1029-023
Eyepiece E-PL 10x/20 Br.	444231-9901-000
Eyepiece E-PL 10x/20 Br.foc.	444232-9902-000

**Price exclusive of 14% VAT****R 70,550.00**

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## Appendix 12

The following was originally written as part of the introductory chapter, but has been moved to an appendix as, although being of interest from a historical perspective, it did not pertain directly to the study at hand, namely that of staining techniques for identifying *Mycobacterium tuberculosis* bacilli. It is presented here as a paper on the history of tuberculosis.

### **Tuberculosis through the Ages: A Historical Perspective of the Epidemic**

#### **The Corridors of History**

Tuberculosis is an ancient disease that has dogged the footsteps of mankind down the corridors of history. It is not known when tuberculosis first affected mankind, but archaeological evidence places it in the realms of prehistory. The evidence takes two forms: skeletal changes associated with tuberculosis and depictions of morphologic features suggestive of tuberculosis in prehistoric art. However, this evidence can only suggest that tuberculosis was the underlying disease process, as there are a number of diseases that have similar skeletal and morphologic abnormalities to those seen in tuberculosis. These diseases include fungal infections, bone tumours and certain types of arthritis [1]. Tuberculosis of the skeletal system is rare in comparison to pulmonary and non-skeletal extra-pulmonary tuberculosis but internal organs are not as resistant to the ravages of time (even if mummified) as the skeletal system and are either no longer present or are too degenerate to supply convincing evidence of the disease. With this in mind, changes consistent with tuberculosis have been found in skeletons dating back to the Neolithic period, the oldest possible case being that of an adult male found near the German town of Heidelberg, which dates back about 7000 years to 5000 BC [2] [3]. Studies of Egyptian mummies [4] and skeletal remains from Jordan [5] indicate that tuberculosis was rife in the Middle East, particularly in the Dynastic period (starting 3400 BC). The earliest conclusive example of tuberculosis is the mummified remains of a five-

year old girl who had evidence of both pulmonary and spinal tuberculosis. Acid-fast bacilli were identified on microscopy [4]. In addition there is the famous mummy of Nesperehan, a young Egyptian priest who lived around 1000 BC, which shows both spinal changes and a large psoas abscess as a result of tuberculosis [1]. Mummified remains from Peru of a woman in her forties (dated 1300 to 1000 BC) and a Nazca boy (dated 700 CE) show evidence of tuberculosis not only of the skeletal system, but also of the lungs and other internal organs. Tuberculosis DNA was found in lung tissue from the former using polymerase chain reaction (PCR) [6].

### **Tuberculosis in Prehistoric Art**

Depictions of people with the typical 'gibbus' deformity, the sharp, angulated hunchback caused by collapse of the vertebral bodies as a result of spinal tuberculosis, are evident in Egyptian art from the Dynastic period (3400 BC) [6]. Similar depictions are seen in prehistoric art from the Pueblo and Inca cultures of Central and South America [7]. However, art is not considered definitive evidence of the existence of tuberculosis in a society, as there are many causes of kyphosis and depictions of 'deformities' may be a result of stylistic convention rather than representative of real pathology.

### **Early Written Accounts**

In the ancient writings evidence of the existence of tuberculosis is clouded by the fact that the concept of disease held by ancient societies is very different from our modern understanding. Diseases were diagnosed on the existence of symptom complexes [7]. The symptoms of tuberculosis vary widely, and range from a persistent cough to haemoptysis, night sweats, marked loss of weight, lymphadenopathy, dysentery and skeletal deformity, depending on the organ system involved. Terms used to describe some of these symptoms were galloping consumption, pthisis, tabes pulmonalis, tissic, hectic fever, gastric fever, scrofula, struma, laoping, yakshma and tabes mesenterica [6, 7]. The earliest

descriptions that fit with tuberculosis are found in ancient Indian literature dating back to 2000 BC in the form of a hymn to cure 'yakshma' and the *Ordinances of Manu* from 1300 BC [6]. References also occur in Egyptian, Greek, Roman and Chinese literature [6-8].

### **Where did TB Originate?**

Understanding the above, the question now arises: where did tuberculosis originate? The human pathogen *M. tuberculosis* is closely related to *M. bovis*, which causes tuberculosis in animals, particularly cattle, and humans. It has been proposed by some authors that *M. tuberculosis* is a mutant form of *M. bovis* and that transmission to humans occurred with the domestication of cattle during the Neolithic period [3]. Domesticated cattle played a central role in many of the ancient Mediterranean cultures with close contact between cattle and people. Milking and consumption of cow's milk is also believed to have started in 5000 to 4000 BC in this, the Fertile Crescent. These factors contributed to an ideal setting for the transmission of tuberculosis to humans. Evidence cited in support of *M. tuberculosis* representing a mutant form of *M. bovis* are the facts that the two organisms are genetically similar [1], and cause similar patterns of disease. Whereas *M. bovis* has a wide host range, *M. tuberculosis* does not, being confined largely to humans. The earliest evidence of tuberculosis in domestic animals comes from India where there are skeletons of domestic elephants from around 2000 BC showing changes consistent with TB [3].

### **Greeks and Romans**

The rise to pre-eminence of the ancient Greek and later the Roman civilisations saw the first steps taken towards the development of modern Western medicine, with the teachings of Hippocrates (born 430 BC), hailed by many as the father of modern medicine, along with the founding of the Medical schools of Cos and Alexandria (300 BC). These brought with them a new understanding of disease where emphasis was placed not on the cause of disease, but rather on a compilation of the patient's history of symptoms, clinical



indication of the prevalence of scrofula during this period, as King Charles II of Britain is said to have touched ninety-two thousand people suffering from the disease between 1662 and 1682, an average rate of 90 patients a week [6]. John Locke, a diarist, philosopher, statesman and physician reported that twenty percent of all deaths in London in 1667 were due to tuberculosis [10]. It was at this time that English writer John Bunyan called tuberculosis, “the captain of all these men of death” in his book *The Life and Death of Mr. Badman*.

The western world was not alone in its grief. The voyages of discovery in the 15<sup>th</sup> century undertaken by men such as Christopher Columbus heralded an influx of European explorers, traders and colonisers into the ‘New World’. They brought with them their diseases. Small pox, syphilis and ‘consumption’ swept through and devastated the indigenous populations of the Americas, Australia, Southern and Western Africa and the Far East. At the height of colonisation, it was noted that the indigenous peoples were particularly prone to developing ‘galloping consumption’ – rapidly progressive tuberculosis.

### **Urbanisation**

In both Europe and the colonies, with the advent of the industrial revolution, there was migration of people from rural areas to the cities. This tide of humanity swamped the ill-equipped cities, resulting in poor hygiene, malnutrition, over-crowding and general deprivation. Tuberculosis spread unchecked. The tuberculosis epidemic reached its peak in Europe in approximately the year 1815. It was at this time that Thomas Young estimated that one-fourth of the European population were suffering from ‘consumption’.

In 1857, at the peak of the tuberculosis epidemic in North America, the Channing Street shelter for tuberculosis patients was opened in Boston by Harriet Ryan. In 1859 Herman Brehmer, a Prussian physician, opened the first tuberculosis sanatorium in the Silesian

Mountains [6-8]. Others soon followed. As mentioned earlier, it was the Galen who had recommended dry elevated places for the treatment of tuberculosis as a result of similar beliefs held in the 19<sup>th</sup> and 20<sup>th</sup> centuries that saw sanatoria opening at locations that met these criteria. The age of sanatoria begun – these provided the mainstay of anti-tuberculous treatment until the 1950's.

### **Emerging from the Dark**

A vital step forward was the invention of the microscope, credited to Zacharius Jansen of Middelburg, Holland in 1590. This was considered by Galileo in 1610, and later by Antoni van Leeuwenhoek (1612 - 1723). Leeuwenhoek, a Dutch draper by training and self-taught microscopist, is considered the father of modern protozoology and bacteriology. He described ciliates that he termed *infusoria* (later termed protozoa) and, "graceful little animals numerous than all the people of the Low Countries," which he saw on a piece of food found between his teeth – probably bacteria [9]. Benjamin Franklin, an avid microscopist and a follower of van Leeuwenhoek's teachings, published *Theory of Consumptions: More Especially of a Phthisis or Consumption* in London in 1720, put forward the hypothesis that consumption was caused by 'animalcula' that could be transmitted from one person to another. This was proven out both in epidemiological studies and in laboratory experiments. The first documented case of tuberculosis in 1831 of the devastating effect tuberculosis had had on Pitcairn Island. The latter was proven when, in 1865, Jean-Antoine Villemin, a military surgeon, produced tuberculous lesions in rabbits by inoculating them with material from human tuberculosis patients [6].

In 1882 Robert Koch, a German pathologist, discovered the tubercle bacillus and demonstrated that this, *M.tuberculosis*, was the cause of the disease whose name it bears. It was in this paper, published in the April edition of *Berliner Klinische Wochenschrift*, which he put forward his criteria for establishing that a bacterium was the cause of a disease – today known as Koch's postulates. He stated: "it was necessary to isolate the bacilli from the body (of a diseased individual); to grow them in pure culture; and, by administering the isolated bacilli to animals, reproduce the same morbid condition which, as is known, is obtained by inoculation with spontaneously developed tuberculous material." It was for this work that he was later awarded a joint Nobel Prize in 1905 [6].

### **The Search for a Cure**

Surgery formed the first active treatment option in the war against tuberculosis. In 1696, Giorgio Baglivi, an Italian physician, reported that one of his 'consumptive' patients improved dramatically following a pneumothorax induced by a sword wound to his chest. Similar reports dotted the medical literature of the 18<sup>th</sup> and 19<sup>th</sup> centuries, but it was in 1894 that the first artificial pneumothorax was induced by Carlo Forlanini. He described a technique of injecting filtered air into the pleural cavity of a severely diseased lung. His first patient, a seventeen year old girl, tolerated the procedure and showed a remarkable recovery from her pulmonary tuberculosis. The procedure gained wide support and was used well into the 1950's.

On the pharmacological front, Streptomycin was isolated from the fungus *Streptomyces griseus*, a soil actinomycete, by Selman Waksman, Albert Schatz and Elizabeth Bugie in 1943. This substance was noted to have antibiotic activity against several bacteria including tuberculosis. It was first administered to a human subject in 1944 with good results – the patient was cured of her advanced pulmonary tuberculosis and remained clear of disease in a follow-up examination and chest radiograph performed ten years

later. The drug underwent rigorous clinical trials and gained massive support. Waksman received the Nobel Prize in 1952 for his discovery. By 1953, it is estimated that Streptomycin formed the subject of ten thousand scientific papers and twenty books [6]. The drug became fondly known as the *magic bullet*.

The magic did not, however, last for not only did streptomycin induce severe and often poorly tolerated side effects, but resistant strains of *M. tuberculosis* soon started to emerge. In the ensuing hunt for additional agents, thioacetazone and para-aminosalicylic acid (PAS) emerged. It was a derivative of these, isonicotinic hydrazide (isoniazid or INH) that soon proved to be ten times more effective against tuberculosis in laboratory studies than any drug described so far. In addition, it was an oral agent whereas streptomycin had to be administered via injection or infusion. In 1952 the trial of isoniazid got underway in Sea View Hospital, New York with dramatic results [11]. Isoniazid proved to be rapidly acting, well tolerated and extremely effective in the treatment of all forms of tuberculosis. It soon became the first-line agent for both the treatment of the disease and in TB prophylaxis.

In spite of the success of INH, the search continued for anti-tuberculosis drugs. The next breakthrough was in 1957 when rifamycins were isolated from the fungus *Streptomyces mediterranei*, at the Lepetit laboratories in Italy. These agents were modified into the oral agent rifampin. This too proved a highly effective agent [12, 13].

The oral agents were not only effective, they were easy to administer. In fact it is as a result of these agents that the sanatorium movement went into rapid decline [6, 7].

### **Prevention is Better than Cure**

In 1796 William Jenner used cowpox to perform the first vaccination for smallpox and, almost a century later, in 1885, Louis Pasteur successfully immunized Joseph Meister against rabies. Five years later, in 1890, Koch produced tuberculin, an extract of broth cultures of *M. tuberculosis*. This substance, he claimed, halted tuberculosis in Guinea pigs, and he postulated it would do the same in humans – a theory that was not borne out. However, tuberculin was used in 1906 in a diagnostic skin test for tuberculosis by an Austrian physician, Clemens Freiherr Baron von Pirquet. Mantoux modified this test two years later. In 1902, Edmond Nocard isolated another strain of mycobacterium, *M. bovis*, from the udder of a tuberculous cow. It was this strain that was used in 1913 by Leon Charles Albert Calmette and Jean Marie Camille Guérin to develop a vaccine that was effective in preventing bovine tuberculosis. This vaccine, known as the Bacille Calmette-Guérin (BCG), was first administered to a human subject in 1921. The League of Nations certified the BCG safe for human use in 1928. A number of trials of the vaccine were run in the inter-war years, including the Aronson BCG trial, but it was after the Second World War and the successful implementation of a country-wide vaccination program in Poland, that BCG started to be used more widely. In 1947 Johannes Holm implemented a joint UNICEF and World Health Organisation (WHO) tuberculosis control program for Europe in which the BCG vaccine played an important role. Following the success in Europe, many countries (not including the United States) started to include BCG in their vaccination campaigns [6].

The BCG vaccine had gained widespread support, but there was also widespread scepticism. A preliminary trial based in Georgia, USA, in which more than 11 thousand school children were vaccinated, showed no beneficial effect [14]. 1968 saw the start of the largest ever trial of the BCG vaccine which took place in the Tamil Nadu district of South India, in which more than a quarter of a million subjects were vaccinated. The results were less than encouraging, showing again that BCG offered no protective effect

[15]. Following the release of these results, a number of large meta-analyses were undertaken, one of the largest being by Colditz, in which it was shown that the BCG vaccine had a 50% efficacy rate for preventing tuberculosis in a vaccinated population [16]. In spite of these trials, the WHO endorsed the use of BCG in vaccination programs in 1973. The debate of its worth continues to this day.

### **The HIV/AIDS Era**

In 1981 the Centers for Disease Control (CDC) in the United States received reports of five patients in the Los Angeles area with *Pneumocystis carinii* pneumonia (PCP) – a rare form of fungal pneumonia seen only sporadically in severely immunosuppressed individuals prior to this date. A warning was issued in the CDC Morbidity and Mortality Weekly Report of 5 June of that year. It was noted that all five patients were young men and homosexual. Despite intensive treatment, two patients succumbed to their disease rapidly whilst the remaining three declined slowly over a period of months.

Four of these were also noted to have extensive mucosal candidiasis and multiple viral infections including cytomegalovirus (CMV). One patient developed Kaposi's sarcoma. All were noted to be lymphopenic with virtual elimination of the CD4 helper T-cell subset. It was thought at the time that cytomegalovirus infection was an important factor in the pathogenesis of this immunodeficient state and that it was due to a high level of exposure of male homosexuals to cytomegalovirus-infected secretions [17].

G. J. Gottlieb noted an increased incidence of disseminated Kaposi's sarcoma in the homosexual male population of Los Angeles [18]. Cases of severe immune deficiency, opportunistic infections and Kaposi's sarcoma were also noted in the male homosexual population of New York [19]. If you are going to quote figures for prevalence eg in a paper you need 2005 figures

A task team – the Kaposi's Sarcoma and Opportunistic Infections (KSOI) Task Force - was set up by the CDC to investigate this new epidemic that appeared to affect, almost exclusively, the gay population. Within three months they had identified 108 patients showing similar features [20].

This 'new disease' was recognised as an acquired immune deficiency, probably caused by a virus (thought initially to be CMV [17] or Epstein-Barr virus (EBV) [20] and was given the name Acquired Immune-Deficiency Syndrome (AIDS). By August 1983, over one thousand cases had been reported in the USA [6].

In 1983, a French team of virologists at the Pasteur Institute isolated a strain of retrovirus from a lymph node of a patient showing symptoms consistent with early AIDS Related Complex (ARC). The virus showed features similar to, but distinct from, the recently described Human T-cell Leukaemia Virus (HTLV) types I and II. It was concluded that this virus belonged to a general family of T-lymphotropic retroviruses that are horizontally transmitted in humans and may be involved in several pathological syndromes, including AIDS [21]. The virus was given the name lymphadenopathy associated virus (LAV) and later immune deficiency associated virus (IDAV). In 1984, an American group headed by Robert Gallo, identified a retrovirus they designated HTLV-III, in the saliva, serum, blood and lymph nodes of patients with AIDS/ARC [22], which was later proved to be identical to LAV. In May 1986 the virus was designated the Human Immunodeficiency Virus (HIV) by an international commission on virological nomenclature [23]. Following the identification of the virus, causation was soon proven and serological tests became available.

In 1985, Serwadda reported the emergence of a disease in Uganda that was affecting predominantly young, sexually active males and females, and presented with features similar to those described in AIDS cases from the USA [24]. This 'new' disease was termed 'slims disease' by local health workers. Serwadda noted that it was strongly

associated with HTLV-III infection (63 out of 71 patients). Evidence points to the presence of this disease in Central and Eastern Africa as far back as the early 1970's [6].

Tuberculosis, which was rarely seen in the large cities of the West Coast of the USA, was noted to occur with increasing frequency in those patients suffering from AIDS [25, 26]. In these cases, the tuberculosis was noted to present with unusual clinical features and follow a more rapidly progressive course [27, 28]. Very high tuberculosis infection rates were also noted in AIDS patients in underdeveloped countries, particularly Haiti, Tanzania, Zambia and Uganda [27, 29-31]. Cases of multi-drug resistant (MDR) forms of tuberculosis started to be reported with increasing frequency in the late 1980's. In 1990 high rates of MDR-TB were reported in AIDS patients [32-39].

In 1992 the WHO estimated that 4.5 million people were co-infected with HIV and TB [39] and this had risen to 11.5 million people by the year 2000, 70% of whom were in sub-Saharan Africa. [40, 41]

### **Drug Resistant Tuberculosis**

The emergence of a strain of tuberculosis that was resistant to multiple anti-tuberculosis drugs was first highlighted by the Centre for Disease Control (CDC) in 1992 [39]. The emergence of MDR-TB is blamed largely on the fact that less than 20 countries worldwide had implemented a sound tuberculosis control program [42]. Developing countries, particularly sub-Saharan Africa and the former USSR, were identified as 'hot spots'. The resurgence of tuberculosis in the developed world and its alarming rise in incidence in the developing world, coupled with the spread of the HIV/AIDS pandemic and the emergence of MDR-TB, lead the WHO to declare tuberculosis a global emergency in 1993. 1994 saw the launch of the WHO's five element DOTS treatment strategy. This strategy gained wide acceptance and, by the year 2000, was being used in 148 countries. This program alone

has had a major impact on the control of the spread of the disease. In spite of this, MDR-TB remains a major global problem [42, 43].

### **Conclusion:**

Tuberculosis is an ancient disease that has dogged the footsteps of humankind down the corridors of history. It appears to have been transmitted to humankind as a result of domestication of cattle. Tuberculosis is a chronic disease and as such has not been given the recognition of the rapidly fatal “plagues” such as the black death, smallpox, cholera, malaria and the like, but its relentlessly fatal course earned “consumption” the title of “captain of all these men of death” from John Bunyan in the 17<sup>th</sup> century.

Urbanisation and the colonisation of the New World increased the incidence of tuberculosis. In spite of the gains made in the treatment of the disease in sanatoria, with the advent of pharmacological anti-tuberculosis drugs and mass vaccination programs, the advent of the AIDS era has seen a massive surge in the numbers of individuals infected with the disease. It was estimated that in the year 2000, one third of the world's population was infected with the disease [44]. The main burden of this disease is carried by the developing countries and South Africa falls squarely into this category.

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