THE UTILITY OF BLOOD BUFFY COAT SMEARS IN DIAGNOSIS OF MYCOBACTERIUM TUBERCULOSIS

THIS PROJECT HAS SUBMITTED IN PART-FULFILLMENT FOR THE MASTERS OF SCIENCE , MEDICAL MICROBIOLOGY, UNIVERSITY OF NAIROBI

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DECLARATION

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Dr. Peter Mwathi Mwangi

H56/70789/2007

Signature _____ Date_____/February/ 2011

This project has been submitted for examination with my approval as supervisor.

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Signature_____ Date_____/February/ 2011

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Dedication

Dedicated to my family which encouraged me throughout the duration of my Master of Science course.

ACKNOWLEDGEMENTS

To my God who gave me strength and good health all through.

To my supervisor , Prof. Omu Anzala. Who was a constant help and kept his office doors open to me for every consultation and advice.

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To laboratory technicians at KNH, Mbagathi hospital and Mediplan Consultants clinic. Your commitment , teaching and sacrifice I will always remember.

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To all patients who give their time, sputum and blood to make this project successful. Thank you.

ABBREVIATIONS

AFB---------- Acid Fast Bacilli

AIDS ---------- Acquired immune deficiency Syndrome

CDC ---------- Centers for Disease Control, USA

DNA---------- Deoxyribonucleic acid

ESR---------- Erythrocyte Sedimentation Rate

HIV---------- Human immune deficiency virus

IFN---------- Interferon

KNH---------- Kenyatta National Hospital

MS---------- Microsoft

PCR--------- Polymerase Chain Reaction

RBC--------- Red Blood Cells

TB---------- Tuberculosis

WBC--------- White Blood Cells

WHO------- World Health Organization

ZN---------- Zeihl- Neelsen Stain

CHAPTER I

ABSTRACT

Introduction : Tuberculosis remains a big burden in sub – Saharan Africa. Despite much progress on how TB infection occurs, its physiology and immunological interactions, definite diagnostic method is still out of reach. It is in this context that we needed to evaluate the usefulness of blood buffy coat AFB ZN stain.

Methods: In a cross – section study conducted at KNH and Mbagathi hospitals' TB clinics between August and October, 2010, we evaluated the outcome of sputum and blood buffy coat AFB ZN Stain smears. Patients with clinical symptoms and CXR suggestive of tuberculosis were eligible. In the study,114 patients were recruited. A questionnaire was completed and complete blood count was also done for each patient. The data collected was entered into MS Excel and later analyzed using STATA 10 and SPSS.

Results: 11(9.6%) of all patients had positive AFB buffy coat smear. 40(35%) had positive AFB sputum smear. All buffy coat positive smears also were sputum smear positive. Significant factors for outcome of buffy coat smear were age(p=0.0322) , sputum smear(p=<0.0001) and HIV status(p=0.01). In two- way table, compared to sputum smear, buffy coat smear had sensitivity of 27.5%, specificity of 100%, positive predictive value of 100%, and negative predictive value of 71.8%.

Conclusion: Even though buffy coat smear could diagnose AFB(100% specificity), its sensitivity was low at 27.5% compared to sputum smear . Therefore, it cannot be used with confidence as an alternative for sputum for it will miss most positive sputum smear. It can be used to rule in tuberculosis due to high predictive value. To increase it sensitivity we need to evaluate molecular methods and culture involving blood buffy coat .

BACKGROUND

Despite much progress in medicine and its related fields of microbiology and immunology in the 20th and 21st century, tuberculosis remains a big burden. This is due to its resurgence since 1980s largely attributable to drug resistance and HIV co-infection [1]. Eighty percent of tuberculosis cases are found in 22 high-burden countries of which Kenya is 13th in rank of burden[2]. The challenge has made scientists to research into better ways of prevention, diagnosis and treatment. This involves better logarithmic and novel diagnostic procedures, either new or existing, which can be synergistic so as to reduce missed opportunities in first health facility contact [3]. Even though there has been new and exciting tuberculosis diagnostic methods in the molecular field, they largely remain out of reach to developing nations due to lack of resources [3,4]. Therefore, there is need to evaluate the usefulness of blood buffy coat AFB ZN Stain which is simple, cheap and readily available method. Blood is universal to all patients and its quality is not affected as is the case with sputum. In a study conducted in Iran, it was found that blood buffy coat can be used to increase capture of new tuberculosis due to its high specificity and predictive value [16]. Therefore, there is need to have a study on utility of blood buffy coat in our local set-up to evaluate whether it can enhance detection of tuberculosis .This is in the background of low efficiency and effectiveness of newer molecular tests which are expensive and complex [24]; and end up supplementing- rather than replacing – traditional methods[27].

CHAPTER II

LITERATURE REVIEW

The major burden of disease in developing nations is accounted by tuberculosis, malaria ,HIV/AIDS ,diarrhoeal diseases and other respiratory infections . Sub-saharan Africa accounts for one-third of all tuberculosis cases in the world, two-thirds of people living with AIDS and 90% of malaria deaths globally [1]. Tuberculosis, a highly contagious disease caused by a bacillus *Mycobacterium tuberculosis*, has remained a great challenge despite BCG vaccine coverage, better treatment regimens and newer diagnostic methods. It has caused great constraints on the developing nations resource allocations due to high burden with few and scattered gains [2,3]. It is these challenges that call scientists, healthcare givers and healthcare managers to innovate better and novel ideas to diagnose , manage and prevent tuberculosis spread and mortality associated with it in the community[3.]

New inventions in tuberculosis have lead the scientists to go back to conducting studies to understand the basic structure, microbiology and immunology associated with it and the host and how these interactions can help us get new ways and methods to overcome tuberculosis infections through early diagnosis , treatment and vaccine development[4].

Mycobacterium tuberculosis is an intracellular pathogen, that is, it lives and multiplies intracellularly within endosomes of mononuclear phagocytes [5]. These include monocytes and macrophages. To defend the host against the infection , the macrophages release reactive oxygen metabolites, which are highly reactive atoms with unpaired electrons[6] . Although there is this release of metabolites it is weak in killing the bacilli and it has been shown that neutrophils are more efficient killers of mycobacterium without oxygen radicals and also mobilizes macrophages to migrate to the site to contain the spread of bacilli [7]. Once the *Mycobacterium tuberculosis* bacillus

is transmitted to a person from an active patient through droplet infection, it reaches the alveolar where it invades the alveolar macrophages and starts to multiply in their endosomes. This initial site of infection is called the Ghon focus [8]. Thereafter , the bacilli are picked up by dendritic cells which allow no multiplication of bacilli and are transported to the mediastinal lymph nodes. It can spread further through bloodstream to other body tissues and cause secondary tuberculosis lesions in other parts of the lungs away from primary site, peripheral lymph nodes, brain, pericardium , kidneys and bone [9].

Tuberculosis infection lesions are granulomas formed of lymphocytes and fibroblasts surrounding infected macrophages. They help in preventing the dissemination of bacilli and also provide a localized environment for immune cells to work on the bacilli. However , the immune cells do not always eliminate the bacteria in the granuloma and this leads to latent bacilli. This state is called latent TB. It is the most common form of tuberculosis accounting for 90% of global TB cases[2,3,]. Another important feature is necrosis in the centre of tubercles forming white cheese –like material, a process called caseous necrosis[10]. The *Mycobacterium tuberculosis* bacilli can gain access to the bloodstream from the granulomas via damaged tissue and the bacilli can disseminate to many tissues in the body and form tubercles in them. This is called miliary tuberculosis and is almost 100% fatal if untreated[11].

Once a person is infected, the above processes start and the patients exhibits varied symptoms and signs such as general malaise, night sweats, lymph node enlargement, cough, weight loss and at times chest pains. These symptoms in the early stages of TB infection can be confused with many other tropical infections and can lead to misdiagnosis of TB and missed opportunity in early diagnosis of TB[2,3]. Apart from medical examination, diagnosis of tuberculosis has offered a big challenge due to many tests involved especially where sputum Acid Fast Bacilli (AFB) test is negative or good quality sputum cannot be collected due to status of the patient [3]. This challenge has made scientists and clinicians to focus their research in two(2) main areas:

1. To improve and broaden the conventional existing methods of laboratory tests globally and especially in developing nations so as to reduce missed opportunities and capture more active tuberculosis patients.
2. To invent new methods with better specificity and predictive values for definite diagnosis of active, secondary and latent tuberculosis.

This is in line recommendations by tuberculosis surveillance unit which advocated that the most important strategy of tuberculosis control is early identification and treatment of infectious cases.If 70% of new sputum smear positive cases were detected and 85% cured, substantial impact on prevalence of tuberculosis , and thus its transmission and future incidence would decrease significantly[12,13]. Optimizing early diagnosis of tuberculosis involves increasing the sensitivity and specificity of acid fast smear test either in sputum, biopsies or needle aspirates or in blood[13,14]. Even in sputum microscopy, the limitations are many. These include sputum quality and sensitivity being low especially in HIV leading to high drop out rate before definite diagnosis and treatment due to diagnostic series of three sputum samples collection over three(3) days. Therefore, there is an urgent need to review all methods available for tuberculosis diagnosis . This will help capture negative acid-fast sputum and non-expectorating TB patients[14,15]. One of those methods is the acid-fast smears from blood buffy coat. It has been shown to have high specificity and high predictive value but low sensitivity when compared to sputum smear examination . Therefore , it may be a better diagnostic test in non- expectorating patients where culture and PCR are not readily available[16].

The blood buffy coat is the fraction of anticoagulated blood sample after density gradient centrifugation that contains most white blood cells and platelets. It is a thin layer which is white-green in colour and seen between the plasma(clear top layer) and red blood cells(red bottom layer)[16]. It has many uses in diagnostic laboratory from conventional methods to molecular DNA techniques in diagnosis. In DNA separation and incubation the buffy coat isolation is important due anucleate nature of red blood cells. Thereafter the white blood cells are used for various tests as in cell culture, diagnosis and crime solving techniques[17,18]. It is also used for diagnosis of malaria through an improved method called Quantitative Buffy Coat test using a capillary tube

coated with a fluorescent dye, acridine orange, to demonstrate malaria parasites. It is more sensitive method than the conventional blood smear for malaria parasite demonstration[19]. Another area of its use with excellent outcome has been in diagnosis of streptococcal pneumonia in infants leading to early treatment and reduction in mortality [20].

Blood buffy coat has many uses in microbiology laboratory where microscopy and molecular methods are cornerstones of diagnosis of infectious diseases. It is an important tool in diagnosis of blood and tissue parasites such as Plasmodia, Babesia,Trypanosoma, Brugia, Mansonella and Wuchereria[21].In tuberculosis research it is used in isolation of monocytes in studies searching for the effect of *Mycobacterium tuberculosis* on tissue apoptosis and necrosis[22]. There has been studies of enzymes released in tuberculosis infection using buffy coat[23]. Other areas of use has been in isolation of activated T lymphocytes in peripheral blood of patients with tuberculosis and its use as sample for culture in tuberculosis and in bacterial disseminated infections or septicaemia[24,25].

In diagnosis of tuberculosis, the interest of buffy coat came into the fore between 1920s and 1940s after discovering the reaction of erythrocyte sedimentation rates were high in active tuberculosis infection[20,26]. After the acid-fast tests using sputum were well developed, the issue of buffy coat and tuberculosis was ignored until 1980s after the advent of HIV/AIDS and resurgent of negative acid –fast sputum tuberculosis [27,28]. With development of new molecular methods such as PCR , buffy coat was and is more used to increase the sensitivity by using it instead of whole blood. It is now method used to concentrate and isolate white blood cells for studies and incubation in molecular tests requiring DNA[17,18]. Despite molecular techniques being developed, they are generally e out of reach for the poor developing nations due to high costs in price and equipment. In addition, there is often a lack of qualified laboratory technicians with required expertise[29]. It is through seeking optimization of existing conventional tests that there has been a renewed interest in acid-fast smear tests in developing nations with high burden of tuberculosis and missed opportunities in tuberculosis diagnosis so as to capture most of tuberculosis infections.In this respect, opportunities for synergestic combinations of interventions such as sputum and buffy coat smears are encouraged[12,

[30] .In comparison with blood culture for mycobacteria and the direct buffy coat smear the sensitivity was shown to be almost the same[30]. With very high prevalence of sputum smear negative pulmonary tuberculosis among patients suspected of tuberculosis in developing nations, we need to develop cost –effective approaches for the diagnosis of tuberculosis. In our settings there is need to improve screening and diagnostic methods such as chest x rays, specimen collection and processing , smear microscopy , culture and laboratory quality control[31].

RATIONALE

Tuberculosis remains one of the most challenging disease to diagnose despite much newer diagnostic methods due to low specificity and sensitivity of most tests. Some of these tests are not affordable in developing nations because of financial and trained labour costs. It is in this view that conventional diagnostic tests,which are readily available and cheap, are being re-evaluated so as to cut down on missed opportunities. One of this test is buffy coat AFB smear which we need to review its utility in our local set up. This will help us find out its merits on whether it can be a regular routine test for tuberculosis diagnosis.

HYPOTHESIS

Acid fast smear of blood buffy coat can be diagnostic tool for *Mycobacterium tuberculosis* Infection in developing countries .

RESEARCH QUESTION

What is the role of blood buffy coat acid- fast smear in diagnosis of Mycobacterium tuberculosis?

AIM

To establish whether blood buffy coat Acid Fast Bacilli ZN smear has a significant role in diagnosis of Mycobacterium tuberculosis in patients suspected of tuberculosis.

GENERAL OBJECTIVE

To describe the role of blood buffy coat smear in diagnosis of Mycobacterium tuberculosis.

Specific Objectives

1. To determine the proportion of AFB positive buffy coat smears in AFB positive sputum patients.
2. To determine the proportion of AFB positive buffy coat smears in AFB negative sputum patients suspected of Tuberculosis.
3. To determine the outcome of AFB positive buffy coat smears in relation to the white blood cell levels .

CHAPTER III

METHODOLOGY

Study site and population

The study was conducted at the Kenyatta National Hospital(KNH) and Mbagathi District hospital. KNH is located in Upper Hill area of Nairobi and is 3 kilometers from city centre. It is the main teaching and referral hospital in Kenya.

Mbagathi is the main district hospital for Nairobi and mostly handles suspected TB patients referred from city council and private clinics. It is located at Ngumo area 1.5 kilometer from KNH.

Their main sources of patients were Nairobi and surrounding areas of Kiambu, Thika , Machakos and Kajiado areas. The patients are from low and mid- income areas. Patients from high income areas tend to visit other private and high-cost hospitals in Nairobi.

Patients with suspected tuberculosis attending these hospitals were approached to participate in the study. The participants included patients referred to microbiology laboratory for sputum smear acid fast test from KNH and Mbagathi hospital casualty departments and the clinics and the wards .

Study design

The study design was a cross-section study carried out over the project duration between August 19th 2010 and October 12th, 2010. The patients were included sequentially as they were referred to TB clinic and laboratory over this time.

Sample size

The sample size was 114 patients suspected of tuberculosis calculated using the formula below[33].

$$n = \frac{z^2 \times p\hat{}(1-p\hat{})}{m^2}$$

where , n is sample size calculated ,114

z= z statistic at 95% confidence interval

m= degree of precision taken at 5%

p^= proportion taken at 8% buffy coat smear positivity outcome for similar set-up in Iran(Zohreh Aminzadeh et al)[16].

The study was chosen because it was done in a referring hospital and specifically was examining the buffy coat , AFB outcome and culture.

Sampling method

Patients referred to the clinic for tuberculosis work up involving AFB ZN staining and microscopy were included sequentially till the sample size was reached.

All participants in the study were required to give their written informed consent prior to being enrolled into the study. Ethical approval to conduct the study was obtained from Kenyatta National Hospital / University of Nairobi ethical committee prior to starting the study(Appendix IX).

Inclusion criteria

All patients referred for tuberculosis work up during the study duration were eligible.

Exclusion criteria

1. All patients who did not sign the informed consent were excluded. This was due to voluntary refusal, patients with altered mental status unable to understand and sign, and unaccompanied children.
2. All patients with bleeding disorders which had not been controlled.

Definition of Suspected tuberculosis patient

This involved the suspected tuberculosis patient sent to the TB clinic. These were patients with the following:

1. Cough of more than 2 weeks
2. Fever of 1 month or more
3. Weight loss with above symptoms
4. Haemoptysis
5. Tuberculosis contact with any of above symptoms
6. Suggestive Chest X-ray(s) findings of tuberculosis.
   Suggestive Chest X-ray consist of x-rays with nodular infiltrates and cavitations of any size, milliary picture, and pleural effusions.

Procedures

The procedures used in the study included Questionnaire and laboratory procedures.

1. *Questionnaire (Appendix I)*

The questionnaire was used to gather the information on patients particulars and other data used to analyze the sputum and buffy coat smears results.

The patients were approached at the TB clinic where a room was allocated for questionnaire interview. The patients were informed of the study and an informed consent *(Appendix II)* signed only voluntarily after detailing orally the procedures in Kiswahili or English for better comprehension. She/he was informed that all information she/he provided orally and in the questionnaire was confidential and so were the results which could only be shared to the attending physician for treatment or other management modalities. The patient was informed by me, TB officer or the nurse that he/she was required to fill the questionnaire and would take upto 15 minutes. The patient was also informed that she/ he would give 3(three) samples of sputum as usual for acid fast smear for diagnosis of TB causing bacilli. All patients were informed that they would be required to give 10mls of blood for erythrocyte sedimentation rate (ESR) and blood counts as done to assist in TB diagnosis work-up and the same blood was used for acid fast smear of its buffy coat.

The patient were informed that the same samples will be used for diagnosis of TB as routinely done and no added delay would come due to the study and no added costs other than the one usually paid to KNH or Mbagathi hospital for the tests. Also they were informed of the outcome of acid fast smear of buffy coat without any added costs. .

Ethical Considerations

The study was approved by UON/KNH Ethics Committee (Approval P111/02/2010 dated 19th August, 2010) in addition to authorization by KNH respiratory diseases department and Mbagathi Hospital administration.

The patients attending the TB clinic were approached by myself, TB officer and the study nurse.

The patients were informed of the study at the TB clinic waiting bay and doctors' consultation rooms.Those interested were guided into a room allocated for the study. The purpose of the study was explained to each patient individually. She /he was informed that the information given and materials such as questionnaire and laboratory forms would confidential. The procedures involved which included blood withdrawal and expectoration of sputum were explained. The patient were encouraged to ask any lingering questions and were answered appropriately . The languages used were English and Kiswahili and where necessary the mother tongue was used.

Children below 18 years were explained the procedure together with their parents or guardians and all their questions answered.

The patient were informed of risks involved in blood withdrawal such as mild pain and site discomfort. They were informed there was no financial reimbursement and the benefit that may be accrued by the participant was that the test may help diagnose TB. Once the consent process was explained and reading of the written informed consent done, the patient was requested to sign the consent in duplicate and one copy given to back(appendix II).

Through the study and consequent remission of sputum to the laboratory the patient was encouraged to ask any questions arising.

The patient was informed that the study involvement was fully voluntary and could withdraw at any time without any consequences.

The HIV results in this study are the ones the patient disclosed to us after being tested at CCC and HIV centres at the request of TB officers or previously done elsewhere in other health facilities. It was fully voluntary to disclose (appendix II).

2. Laboratory procedures

The procedures in the laboratory included blood counts and acid fast smear of sputum and blood buffy coat.

I. BLOOD COUNTS AND ESR

Blood collection:

1.10ml of venous blood was collected from the patient into a well labeled non-coagulating EDTA tube and mixed thoroughly.

2.The blood was used on the same day of collection and at room temperature for buffy coat smears preparation and not stored in fridge so that the WBCs and monocytes would not be destroyed.

Blood counts:

1. The blood counts were done by an automatic full blood count machine giving at least Haemoglobin level white blood count and differential of neutrophils, lymphocytes, monocytes and eosinophils and platelets level .
2. The patient's particulars were typed and entered on the machine screen and then 1ml of blood put into input tube and run for results.
3. The results were be recorded into the standard form*(Appendix III)* and reconfirmed well and the result slip given to the patient or passed to the clinic as appropriate.

II. ERYTHROCYTE SEDIMENTATION RATE

Was done with automatic analyzer and results recorded against the patients particulars in the standard form for records (as in Laboratory procedure I) .

III. ZN ACID FAST SMEAR PROTOCOL FOR SPUTUM

1. Sputum collection

- Three specimens of sputum were expectorated: the first on day 1 at the hospital, second was early morning sputum on day 2 done at home and a third expectoration on remitting the 2nd sputum at the hospital on day 2.
- The sputum should was expectorated deeply after a deep breath in and out.
- A wide necked container (a polypot) with a well-fitting cover was used to collect the sputum. It was clearly labeled with patients name and identification number .
- Request forms *(Appendix IV)* in duplicate were filled as per collection and results. On final collection and result, a copy was given to the patient to take back to the TB clinic's attending TB officer/doctor.
- The sputum specimen was receipted at the TB laboratory and labeled with laboratory / identification number.

2. *Sputum processing*

Part of sputum was processed to kill the bacilli and concentrate it if present. Not all sputum was processed incase culture and sensitivity was required.
Processing was done using 5%v/v Sodium hypochlorite (NaOCL) , that is bleach and then centrifuged.

Procedure:

1. 1-2 ml of sputum was be transferred to a screw-cap universal bottle of 15-20 ml capacity.
2. An equal volume of bleach was added , shaken and mixed well(when well screw covered).
3. It was left at room temperature for 15 minutes shaking at intervals to break down mucus,

4. 8 ml of distilled water was added and mixed well.
5. The whole mix was centrifuged at 3000g for 15 minutes
6. The supernatant fluid was discarded using glass Pasteur pipette or plastic bulb pipette. The remaining sediment was mixed well.
7. A drop of well –mixed sediment was transferred on to a scratch free glass slide. Spread evenly but thick and allowed to air dry.
8. It was labeled with the date and patient's name and number using led pencil
9. The smear was heat fixed carefully by passing it rapidly , smear uppermost, over spirit lamp flame.

3.Ziehl-neelsen staining technique

The above heat fixed smear will be stained using ZN technique.

1. The smear was covered with carbol fuchsin stain (Appendix V) .
2. The stain was heated until vapour began to rise without further overheating. The heating was done using small flame.
3. The heated stain was allowed to remain on the slide for 5 minutes then washed off with clean running tap water
4. The smear was covered with 3% v/v acid alcohol(Appendix VI) for 5 minutes until well decolorized to pale pink then washed off with clean water.
5. Then, the smear was covered with methylene blue (Appendix VII) stain for 1-2 minutes and washed off with water.
6. The slide was then wiped on the back and let air-dry.
7. It was then examined microscopically using 100x oil immersion objective systematically and results be reported as per WHO AFB reporting system(Appendix VIII).

Quality Control for ZN stainining

At regular intervals, that is, once a week and always when a new batch of stain was started , two sputum smears of known high and low AFB positivity were stained with the

study / routine smears to check that the Carbol fuschsin , staining method and microscopic examination of smears were satisfactory.

Also on expectoration of salivary sputum , the patient was advised and encouraged to get another deeper mucoid sputum.

IV. ZN ACID FAST SMEAR PROTOCOL FOR BUFFY COAT

[a] Buffy coat separation

1. 10 ml of Venous blood was collected. The sample was split into two; 2mls for full blood counts and ESR and 8ml was used for buffy coat separation.
2. The 8ml sample was put in centrifuge tubes and centrifugated at 2500 xg for 10 minutes at room temperature
3. The buffy coat layer, the white-green stratum between plasma(top) and RBC(lower) layer was identified
4. Using autopipette set at 10μl , the buffy coat was aspirated and 2 thick smears made on clean, clear, glass slides.
5. The slides were let to air dry.

[b] ZN staining of buffy coat smears

1. The technique and quality control were same as the ones detailed above in sputum ZN staining.
2. Reporting was done and recorded after an agreement with two(2) microbiology laboratory technicians.

Data Analysis Plan

Data was collected and entered sequentially in a code- secured MS Excel spreadsheet by myself and the study nurse. It was cleaned continually as the study progressed. The variables were entered patient by patient. Dependent variables were the AFB outcomes of buffy coat and sputum smear. Independent variables were age, sex, occupation fever, haemoptysis, weight loss, haemoglobin, RBC count , WBC count and its differential count, ESR levels, lymph node swelling CXR reports and HIV.
For analysis, the MS Excel spreadsheet of the data collected was imported into SPSS and STATA 10 statistical software.
In STATA 10, bivariate comparisons of continuous symmetric characteristics such as age, haemoglobin, WBC count and ESR were be performed using t-test. Chi square test was be used for comparison of categorical characteristics such as sex, fever, CXR report and haemoptysis. Factors associated with AFB outcome status in sputum and buffy coat smears were assessed using logistic regression .
Summary graphs were done using SPSS and MS Excel.

Study limitations

1. One of the limitations that arose was inadequate buffy coat. Most buffy coats are of little depth due to low counts of white blood cells. Therefore , appropriate and adequate centrifuging was carried out. In case, this did not increase the depth of the buffy coat, then more blood (20ml) was requested from the patient for further centrifuge. Cautious separation of buffy coat was done to get the most of the available coat for smears.
2. Another limitation of the study is that we cannot differentiate the *Mycobacterium tuberculosis* and atypical mycobacteria such as *Mycobacteria avium complex(MAC)* especially in immune-compromised patients. This will require a further study involving culture and species differentiation using molecular or chemical reactions methods.

CHAPTER IV

RESULTS

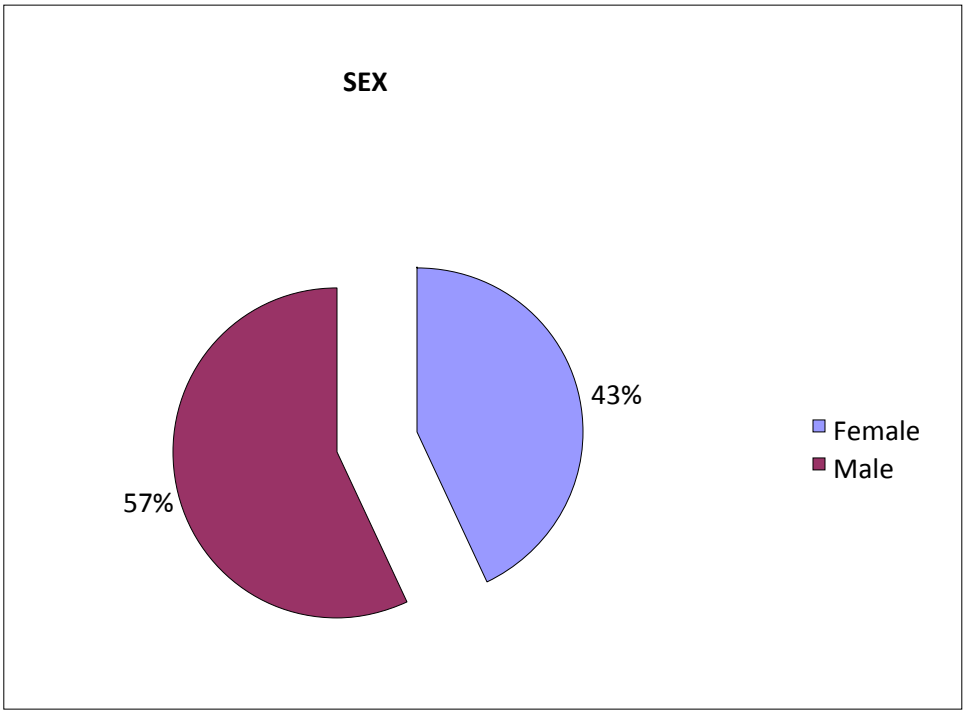
In this study, one hundred and fourteen (114) new patients suspected of tuberculosis were evaluated. They all had a productive cough of sputum and suggestive CXR of pulmonary tuberculosis. The males were 65 and females were 49. The population characteristics are as summarized below in the table and graphs.

Table 1: Characteristics of the study population:

Characteristics		**n**	**%**
Sex	Female	49	43
	Male	65	57
Marital Status	Single	43	38
	Married	68	60
	Widowed	3	2
Occupation	Self Employed	55	48
	Student	21	19
	Unemployed	7	6
	Employed	31	27
Education	No formal education	3	3
	Primary	15	13
	Secondary	55	48
	College/University	41	36
Age(years)	Median (IQR)	32(24-44)	

Graphical representation:

Figure 1: SEX DISTRIBUTION



In this study 60% of the patients were married, 38% were single and 2% were widowed.

On their sources of their incomes, 75% were employed, 19% were students and 6% were unemployed .

Figure 2: Marital Status

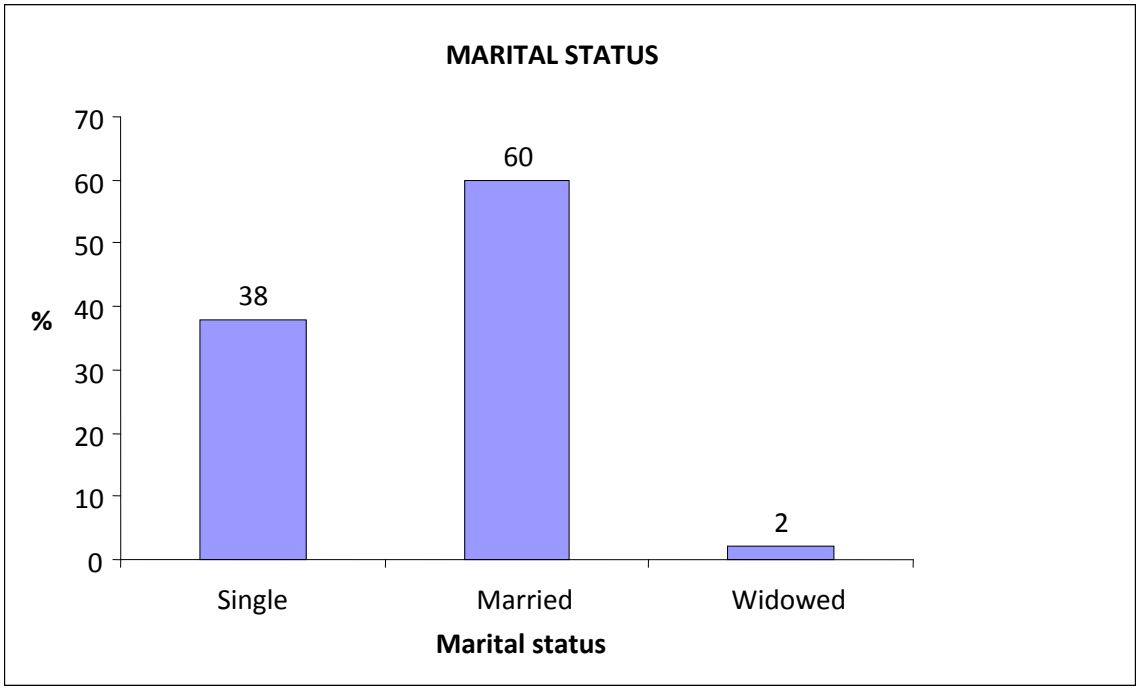


Figure 3: Occupation

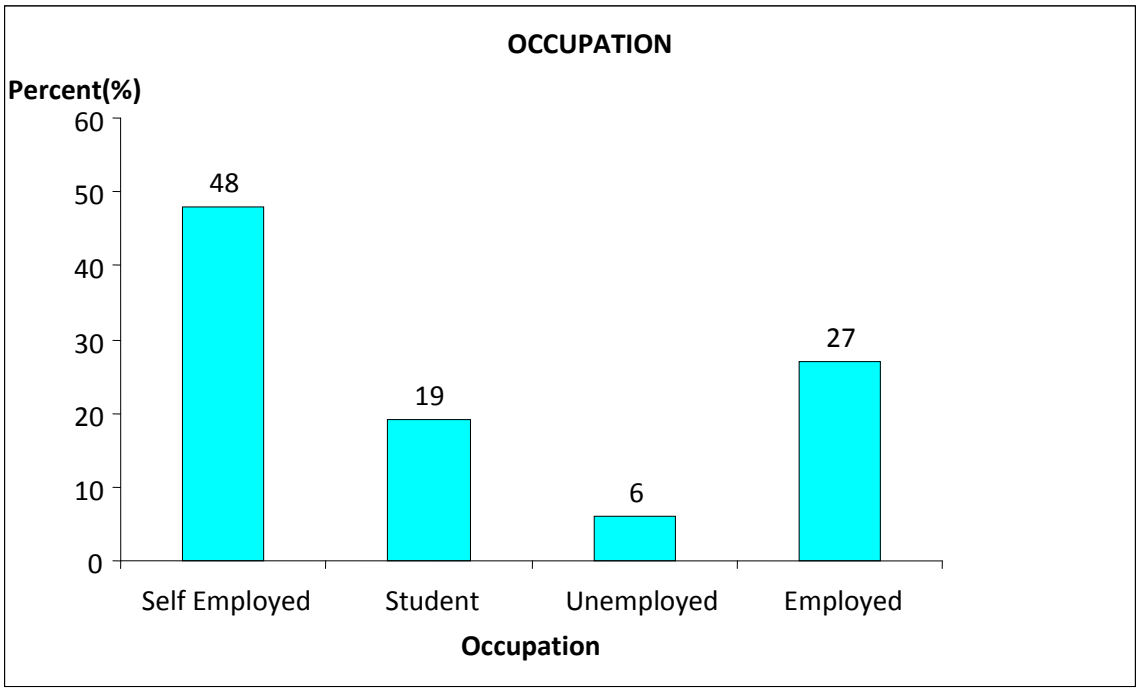
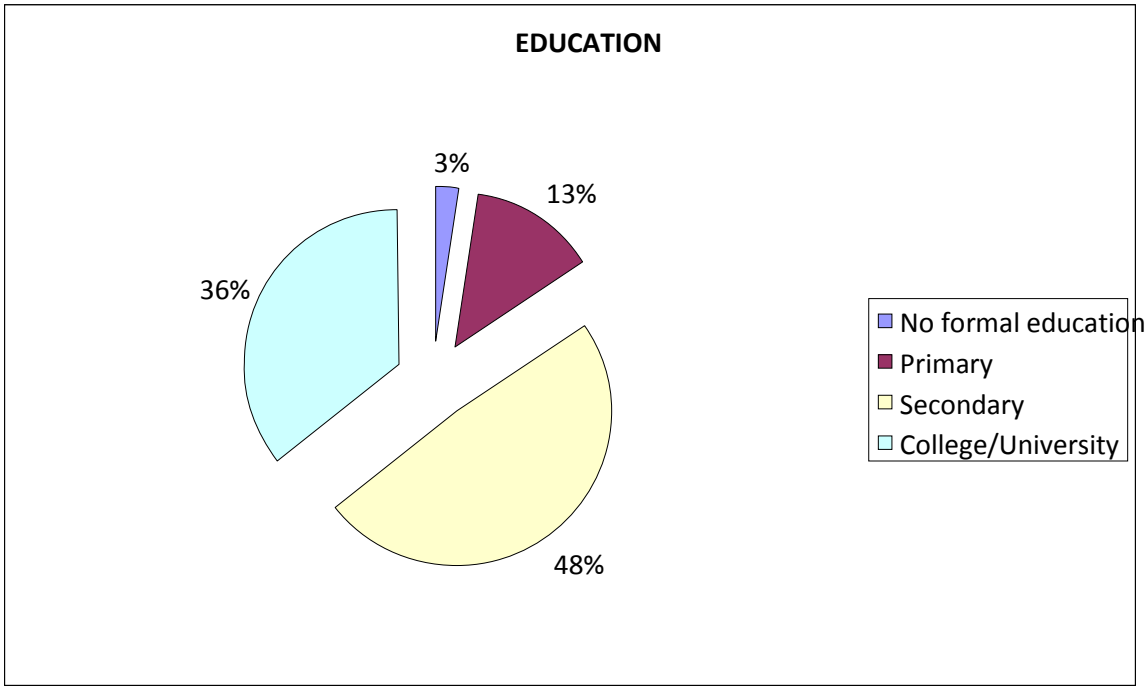
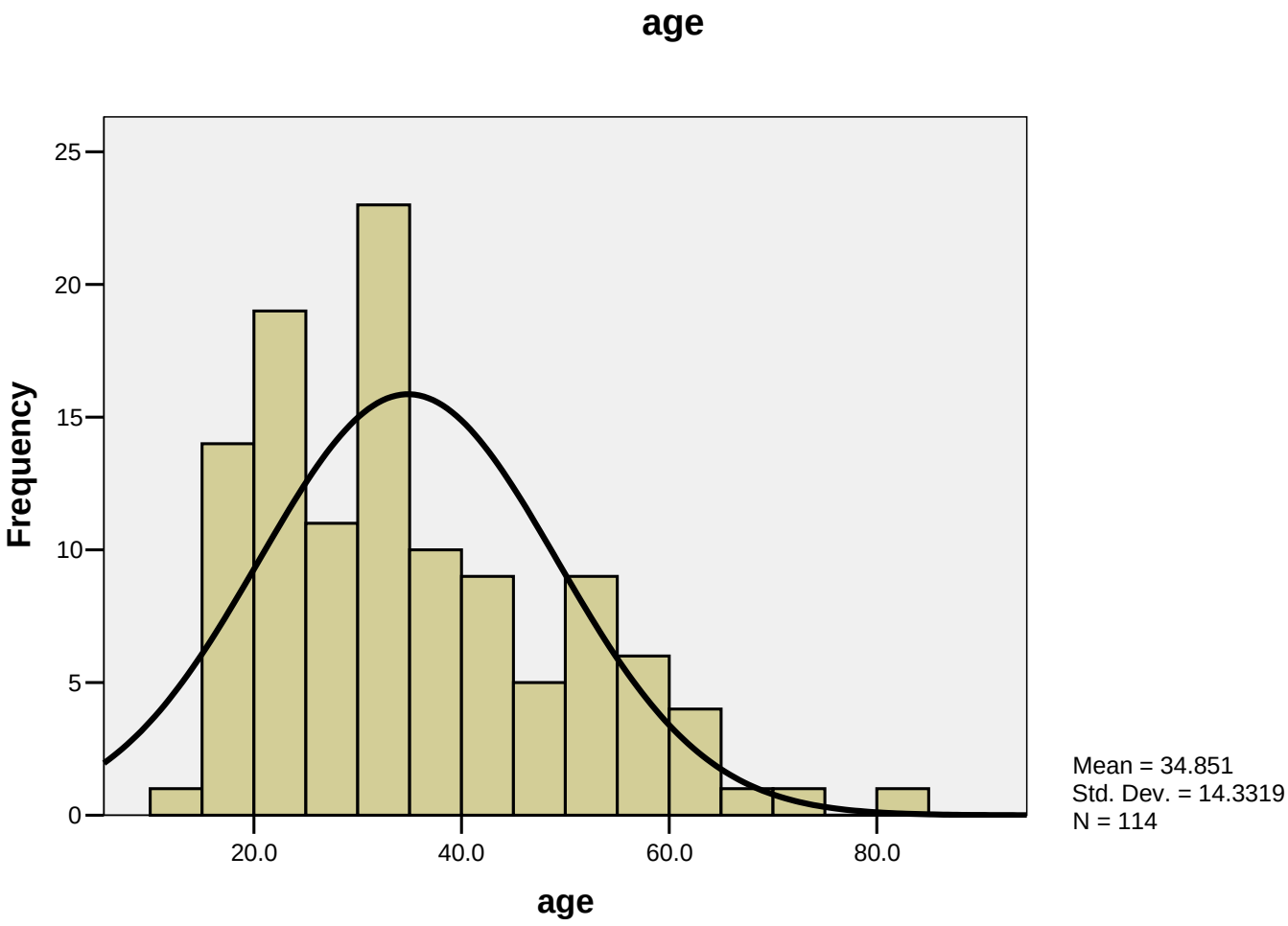


Figure 4:Education level



Of the participants 36% had reached college/ university education. 48% had secondary education. This better education levels could be due to the source of patients in the two hospitals which are mostly referral for further tuberculosis investigations(CXRs and blood) which require more monies and therefore add costs. Therefore , there could be referral bias all the way down from the private, government and city council clinics.

Figure 5: Age Distribution

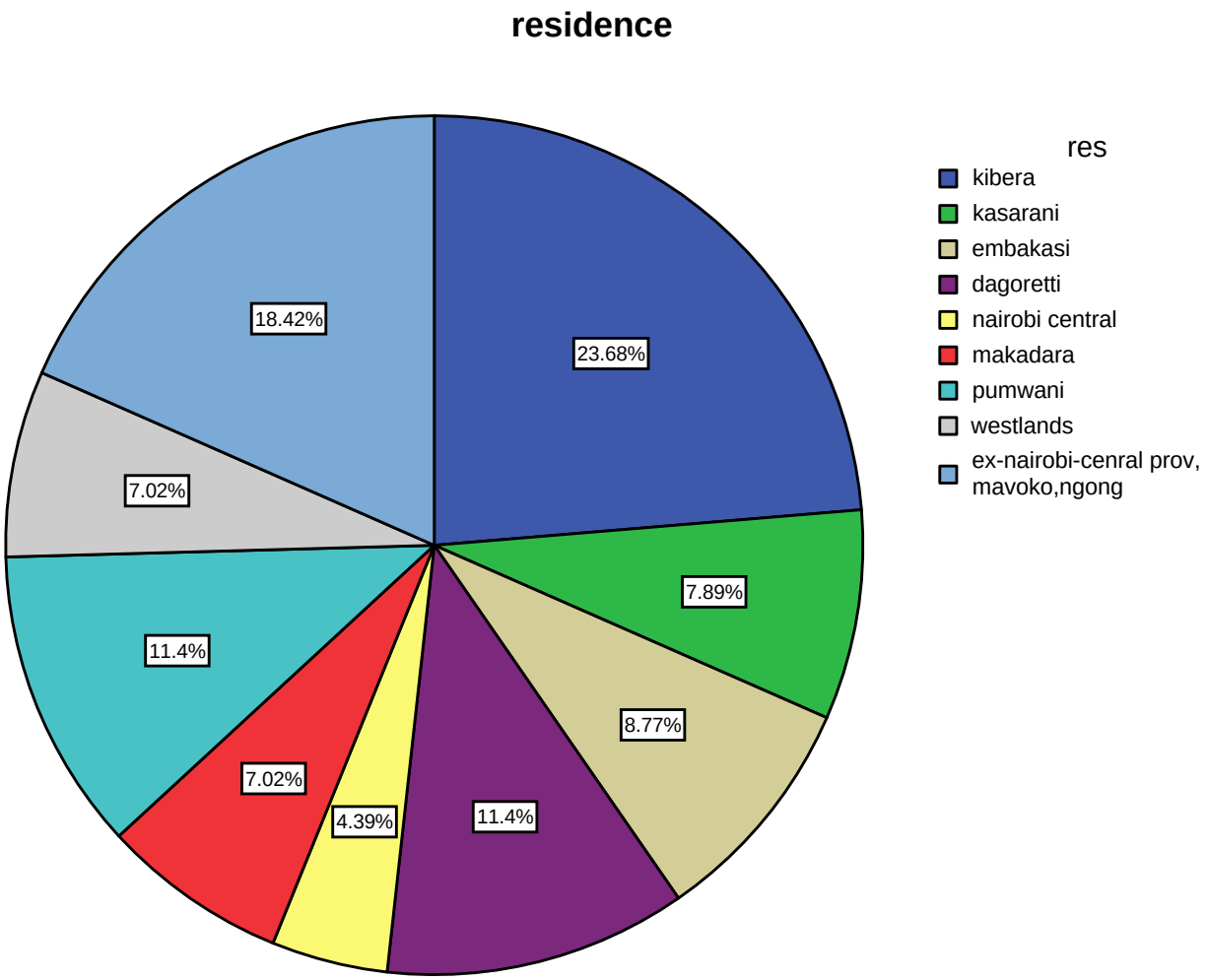


age distribution

The age distribution ranged from eleven(11) to eighty- five(85). The majority were between sixteen and fifty years.

The residential areas were included as administrative divisions of Nairobi and another category of outside Nairobi. Kibera had the highest number at 23.7%.

Figure 6 : Residence

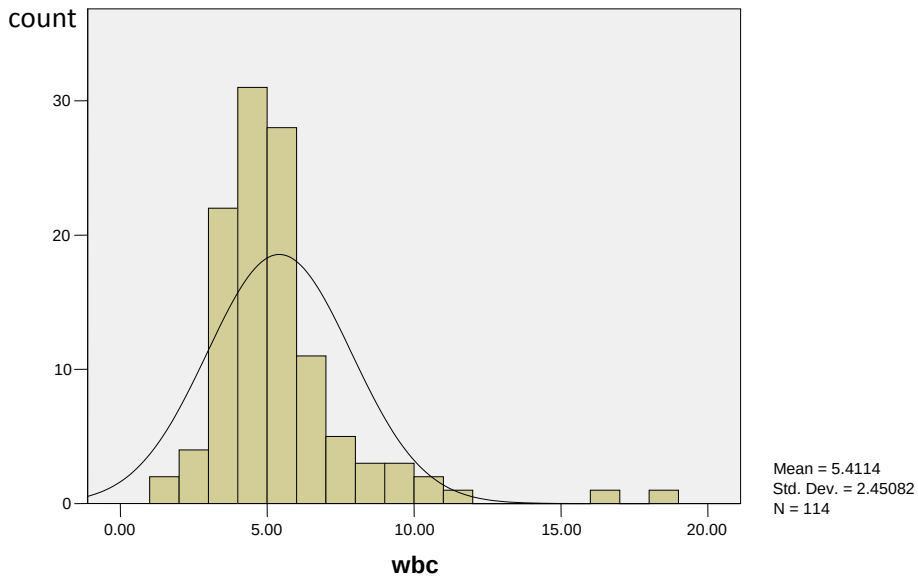


The distribution of haemoglobin, RBCs, WBC and its differential, ESR is as represented below.

ESR was generally above the normal limits of 20mmhr^{-} and only six(6) patients had normal ESR. The WBC counts were mostly normal ranges. However, the differential count percentages showed a high monocyte differential of more than 10%.

Haemoglobin levels and red blood cells were generally normal with mean of 12g/dl(SD =2.5) and 4.7(SD=0.98) respectively.

Figure 7: wbc counts



wbc distribution

%

Figure 8:WBC DIFFERENTIAL COUNT

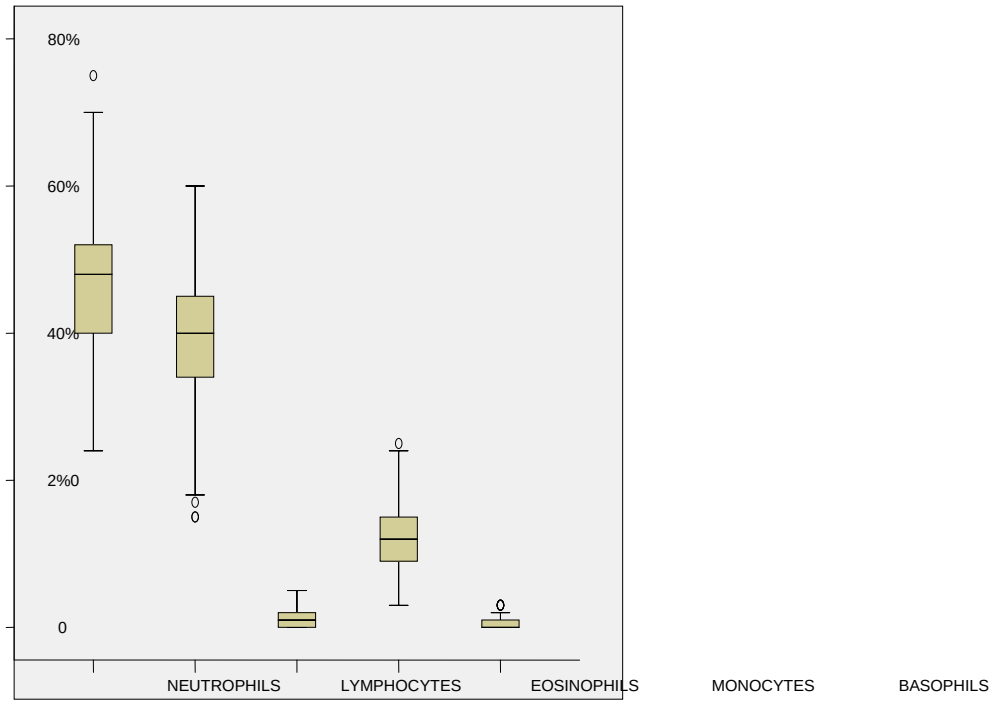
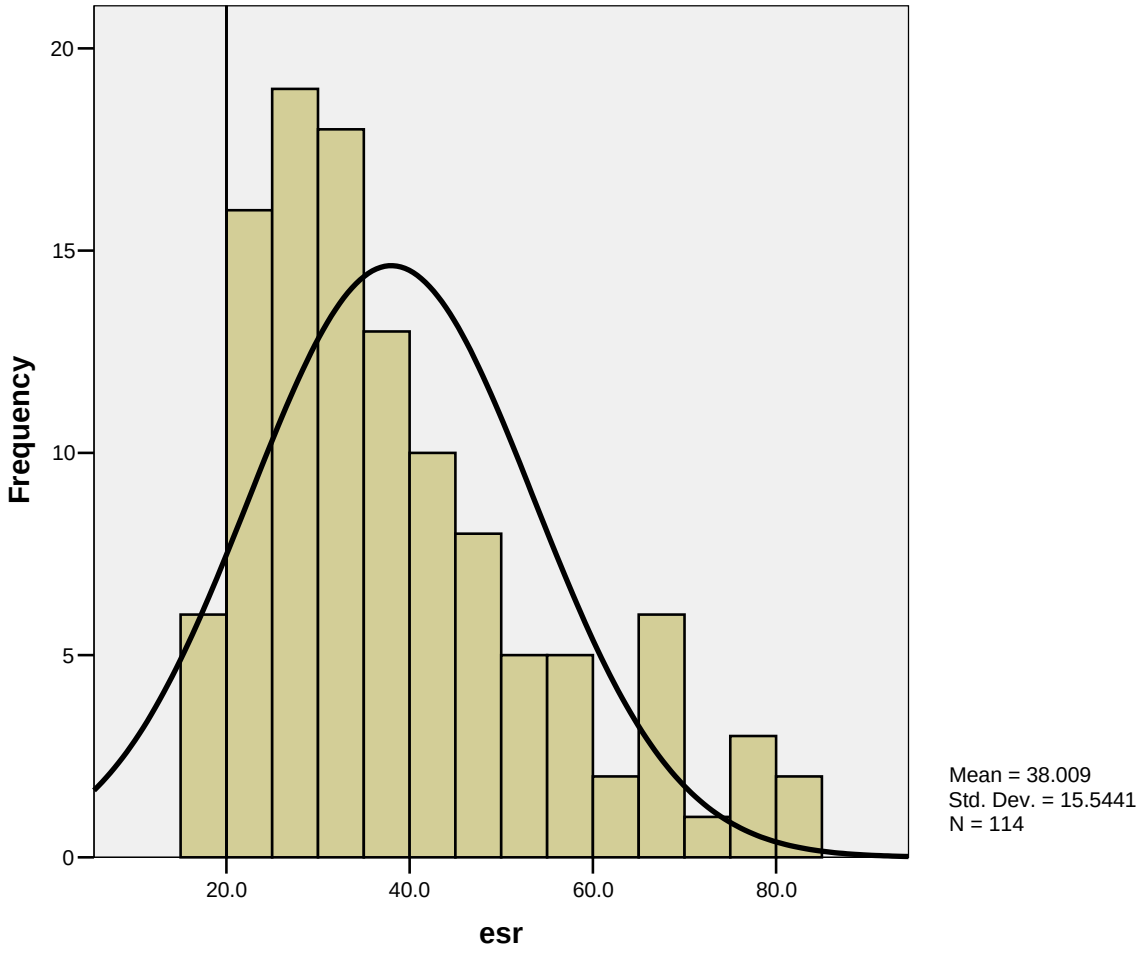


Figure 9: ESR DISTRIBUTION(with reference line at 20mmhr^{-})



Univariate Analysis :

The significance of buffy boat outcome on various independent variables is as tabulated below.

Table 2: Univariate analysis of independent variables

		Buffy Coat Smear		P-value
		Negative	***Positive***	
Sex,	Female,	45(92%),	4(8%)	**0.641**
	Male	58(89%)	7(11%),	
Age(years)	Median(IQR)	32(24-45)	26(18-35)	***0.0322***
Sputum Smear	Negative	74(100%)	0(0%)	***<0.0001***
	Positive	29(73%)	11(27%)	
HIV Status	Negative	49(83%)	10(17%)	***0.01***
	Positive	49(100%)	0(0%)	
	Declined test	5(83%)	1(17%)	
Marital Status	Single	39(91%)	4(9%)	**0.836**
	Married	61(90%)	7(10%)	
	Widowed	3(100%)	0(0%)	
Occupation	Self Employed	51(93%)	4(7%)	**0.365**
	Student	17(81%)	4(19%)	
	Unemployed	7(100%)	0(0%)	
	Employed	28(90%)	3(10%)	
Education	No formal education	3(100%)	0(0%)	**0.507**
	Primary	15(100%)	0(0%)	
	Secondary	49(89%)	6(11%)	
	College/University	36(88%)	5(12%)	
Fever	No Fever	5(100%)	0(0%)	**0.455**
	Fever Present	98(90%)	11(10%)	
Weight Loss	No	18(90%)	2(10%)	**0.953**
	Yes	85(90%)	9(10%)	

Cough Duration	2-4 weeks	20(83%)	4(17%)	**0.365**
	4-8 weeks	51(93%)	4(7%)	
	> 8 weeks	32(91%)	3(9%)	
Haemoptysis	Absent	83(90%)	9(10%)	**0.921**
	Present	20(91%)	2(9%)	

The age, sputum smear and HIV status were the only significant variables. The buffy coat smears were all negative in HIV positive patients.

The proportion of HIV positive patients with suspected tuberculosis was 43%.

The age significance is that all buffy coat smears positive patients were all below 40 years. This is in comparison with positive sputum smears which were more distributed between sixteen to sixty years as shown in pyramids below.

On buffy coat smear oucome, there was zero positive among the widowed, unemployed and in those with no or primary education However, the sample proportion of the widowed , unemployed and with education upto primary level was low compared to other categories in each group.

Figure 10: Buffy Coat AFB Smear

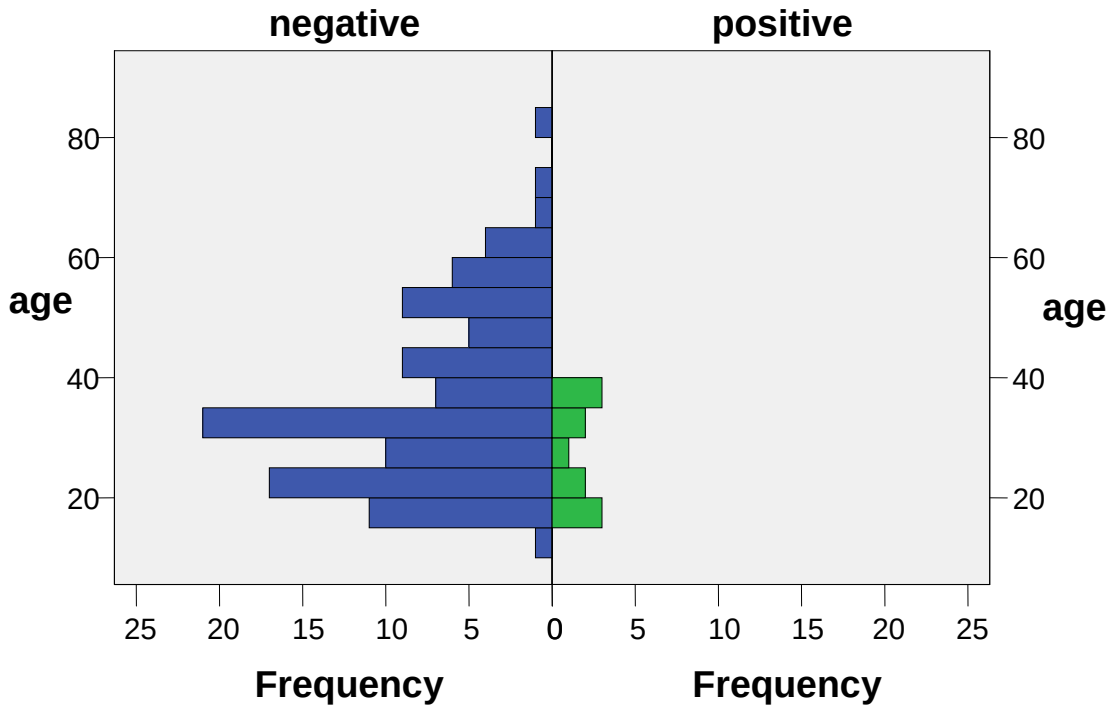
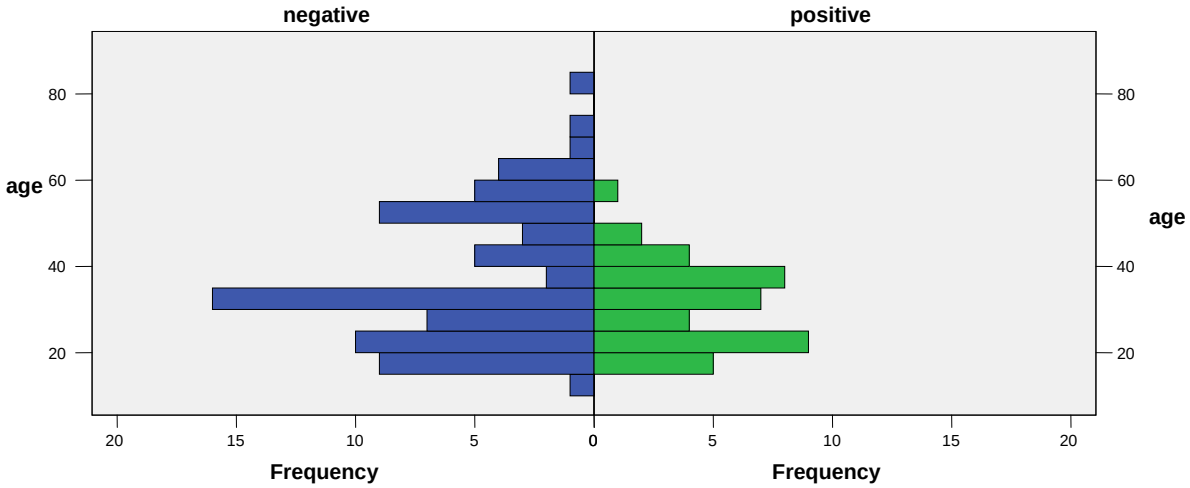


Figure 11:Sputum AFB smear



There were 7 patients below age 18 years and the youngest was 11 years. Sputum positive in this age bracket was 3(43%) and buffy coat positive was 2(29%). In adult patients, sputum smear positive were 37(35%) and buffy coat smear was 9(8%). Therefore, buffy coat was more efficient among the young patients than older patients. However, there were no patients below 10 years recruited in the study for it was difficult to expectorate voluntarily and TB diagnosis relied heavily on clinical score algorithms.

On CXR report, 8(11%) of patients with cavitations and nodular infiltrates suggestive of TB had positive buffy coat smears. 12.5% of military TB patients had positive buffy coat smear and zero(0) pleural effusion patients had positive buffy coats.

To determine the proportion of AFB positive Buffy coat smears in AFB positive sputum patients the following two-way table was run.

Table 3: Contingency table for Sputum and Buffy coat smears

		Sputum Smear		
		Negative	**Positive**	Total
Buffy Coat Smear	**Negative**	74	29	103
	Positive	0	11	11
	Total	74	40	114

The proportion of buffy coat smear positive in the whole sample population was 9.6%.

The proportion of buffy coat smear positive was 27.5% in the sputum smear positive patients and zero(0%) percent in sputum smear negative.

The buffy coat smear had sensitivity of 27.5% [11/29+11 ×100], specificity of 100% [74/74+0 ×100], positive predictive value of 100%[11/11+0 ×100] and negative predictive value of 71.8%[74/74+29 ×100].

Multivariate Analysis:

The variables that were significant, that is age, sputum smear and HIV status were run for multivariate regression analysis. The results were not significant. This was because in formula entry, some predictions were zero such as zero positive smears in negative sputum smears. Therefore, the significance of each variable was independent of each other.

Observations made on hospitals

During the collection of data, some observations were made on the hospitals and require a mention. At Mbagathi hospital laboratory, there was no biological safety cabinet. In both hospitals there were no expectoration booths which greatly exposes

the general public to TB infection. The patient flow for TB testing at KNH came was not well centralized and made it difficult to follow up patients.

DISCUSSION

In the quest to increase diagnosis of tuberculosis in the world, many tests have been developed in the last two decades such as PCR and interferon based tests. However, they still remain out of reach in developing nations due to labour and price costs. Therefore, conventional tests such as buffy coat smears need to be assessed for their role in developing nations with high burden of tuberculosis.

Out of 114 patients, only 11(9.6%) patients had AFB positive buffy coat smears with Ziehl Neelsen Staining. This is slightly higher than the results of Aminzadeh et al[16]. In Aminzadeh's study , only 8% of the sample population were buffy coat smear positive. This could be due to geographical ,tuberculosis prevalence and sample population differences . Aminzadeh's study was conducted in Iran where the tuberculosis prevalence is lower than Sub-Saharan Africa . Kenya has 47% prevalence, thirteenth(13^{th}) highest in the world[37]. The study sample population was fifty(50).

However, this is lower proportion than Sen et al study. In his study it had 55% positivity. This could be due to his participant's selection criteria which was patients who were "positive sputum smear pulmonary tuberculosis"[27] . In another study, Richter found all buffy coat smears negative but 13% were buffy coat *Mycobacterium tuberculosis* culture positive. The difference could be due to the selection of participants in the study who were HIV positive pulmonary TB patients[38].

In our study, all HIV patients and negative sputum smear patient's were buffy coat AFB smear negative. This finding does not mean that there is no Mycobacteria in blood or its buffy coat. This is because in Richter's study, culture showed that 13% of buffy coat negative had mycobacterial growth. Therefore, the problem may be in visualization by microscopy due to low concentration of mycobacterial in circulation whether free or in tubercles [38].

When compared to sputum, sensitivity of buffy coat AFB smear is low at 27.5%. The specificity of buffy coat is high at 100%. The positive predictive value of buffy coat is 100%. That is ,if buffy coat smear is positive then the patient's sputum AFB smear is 100% positive. The negative predictive value of buffy coat is 71.8%.This means that if

buffy coat smear is negative, there is 71.8% chance that sputum AFB smear is negative. Therefore, if buffy coat is to be used as alternative test for sputum, its efficiency in diagnosis would be quite low. When we view this in Baye's theorem [39], the predictive value is high and can be used to rule in tuberculosis in positive sputum smear. However, we are not in doubt about positivity of AFB positive sputum smear. Therefore, buffy coat smear application here is limited. Its negative predictive value at 71.8% is low to rule out tuberculosis in AFB sputum smear which is negative.

The proportion of HIV positive patients with suspected tuberculosis in this study was 43%. The Kenya's national proportion according to Division of Leprosy, Tuberculosis and Lung disease is 44% of 88% tested. This means that 12% decline HIV test. WHO estimates that 60% 0f tuberculosis patients are co- infected with HIV in Kenya[1].

Patients who had buffy coat smears had lung cavitations and military picture tuberculosis. Patients who had pleural effusions had no positive AFB buffy coat smears. This could be due to rate of uncontrolled multiplication of bacilli in cavitations and miliary tuberculosis which could have led to escape of bacilli in the blood circulation.

The role of leucocytes is pivotal in controlling every infection and the efficiency of their interactions determine the outcome of most infections to disease status and recovery. In tuberculosis infection, once the first small caseous lesion is established, two immune responses occur. The first is tissue damaging which is produced by delayed – type hypersensitivity (DTH) reactions to the tuberculin-like products of bacilli[40]. It is useful in destroying nonactivated macrophages within which the mycobacteria bacilli is multiplying. The second response is the macrophage – activating response which is a cell – mediated response that activates macrophages to kill and digest the bacilli ingested[41]. Both responses specific antigens locally stimulate T lymphocytes to produce cytokines that mobilize, attract and activate macrophages[42].

The mobilization and activation of monocytes and macrophages in tuberculosis disease is a known process in tuberculosis. The monocytes differential tend to increase. Some clinical signs such as fast wasting and unbearable night sweats are attributed to activated monocytes and macrophage cells. This is because they use tremendous

energy in formation of oxygen radicals, nitrous oxide(NO) and proteases which are toxic to pathogens and unfortunately to the host itself[43].

In this study, monocytes were often higher(mean =12.4%) compared to normal levels(upto 10%). However, the difference between the buffy coat positive patients' and buffy coat negative patients' monocytes was not statistically different(p value=0.141). This shows the mobilization of monocytes was same in both groups. The mobilization and activation of monocytes may not matter on the site of infection. This can be explained partially by immune defenses against mycobacterial infections of which the main is to try to contain the infection in a localized area with tubercle formation[44]. Also , monocyte and macrophage activation is a tightly controlled process by regulating the half – life of the mRNA encoding interferon(IFN) – γ. This may control extreme mobilization of monocytes from the bone marrow[45].

The pulmonary tuberculosis infection was most common in ages between 16 and 50 years. This is the common tread in developing nations. The population at this age bracket is high and also most interactive in their day to day activities. It is also the age with the highest HIV infections in developing nations. In this study, 79.5% (39 out of 49 HIV positive patients) were between 16 and 50 years. Another factor thought to cause high TB rates in this group is the weaking of the BCG vaccine to almost zero protection after 15 years of age[46].

RECOMMENDATIONS:

1. **To Hospitals:**

    i. Both hospitals need to construct sputum expectoration booths urgently. It is not fitting and ethical to have patients expectorate sputa in public and non – designated areas.

ii. It would be proper to have Mbagathi District hospital install biological safety cabinet, especially Class II.

iii. It would be better to align the patient flow and initiation of TB treatment at KNH. It seems disjointed at KNH when compared to centralization of TB officer reviews and initiation of treatment at Mbagathi.

2. <u>For Research:</u>

i. There is need for further studies on monocytes and macrophage activation and the probable magnitude they contribute to the symptoms and signs in tuberculosis patient.

ii. Further studies to expand the existing conventional tests and upcoming molecular tests such as mycobacteria PCR studies and γ-interferon.

iii. Studies on cytokines and cellular interplay in HIV positive and negative tuberculosis patients especially in leucocytes mobilization.

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APPENDIX I : QUESTIONNAIRE

Study : Utility of Blood Buffy Coat Ziehl Neelsen Stain in Diagnosis of Mycobacteria Diagnosis

Date of filling questionnaire form : ___/_____/ 2010

Identification/ Registration no._____

A).Demography

1. Name: _____ _____ _____
2. Sex : male ☐ female ☐
3. Area of residence:_____
4. Marital status: Single ☐ married ☐ widowed ☐ separated ☐
5. Age _____ years
6. Occupation:_____
7. Education level:

   I. None _____
   II. Primary_____
   III. Secondary_____
   IV. College/university_____

B).Presenting Clinical Symptoms and Signs

I. Fever?
   1.Yes
   2 .No

II. Weight loss?
   1. Yes
   2 . No

III. Cough?
   1.Yes

2 .No

If yes, duration_____ weeks

IV. Sputum from cough?
1.Yes
2. No

V. Blood in sputum (haemoptysis) ?
1. Yes
2. No

VI. Swollen lymph nodes?
1.Yes
2. No

VII. Recurrent chest infections?
1.Yes
2.No

C).Diagnosis

I. Hospital / clinic of referral?_____

II. Tests done;

a) CXR report:
1. Diagnostic
2. Suggestive
3.No TB
4.Pneumonia
5.Other

b) Blood tests:
1. Hb _____
2. WBC Count and differential_____ _____ _____
3. ESR_____ ____
4. HIV TEST:
Yes ☐
No ☐ ☐ ☐

If Yes, Positive Negative

III. Other tests?

i. Sputum tests

1. Yes
2. No

If Yes, Results :Sample 1_____Sample 2_____
sample 3_____

ii. Lymph aspirate

1. Yes
2. No if yes Results_____

iii. Lumbar puncture CSF

1. Yes
2. No

If yes , results(indication of tuberculosis)_____

iv. Others

_____
__

D).Previous tuberculosis and contacts

i. Any previous tuberculosis infection yes no

a. If yes , site_____
b. Regimen of treatment_____

ii. Any history of TB contact

1. Yes
2. No

If yes, months since contact to start of symptoms_____

iii. Are you on any medications

1. Yes
2 . No

If yes which/ specify and duration (days) taken

_____
_____
_____

E). Any previous medical conditions .

1. Yes
2. No

If yes specify _____

Thank you for taking time to fill this form.

Signature(participant)----------

Signature(interviewer)----------

APPENDIX II: THE CONSENT FORM

The Utility of Blood Buffy Coat smears in Diagnosis of Mycobacterium tuberculosis

I , Dr. Peter Mwathi, will be studying the usefulness of blood buffy coat smears in diagnosis of Mycobacterium tuberculosis.

Mycobacterium tuberculosis is bacteria that causes a disease called tuberculosis which presents mostly with chronic cough, wasting, night sweats, chest pains , blood streaked sputum and at times swollen lymph nodes.

Buffy coat is a small white layer that forms when blood in a test tube is span in a centrifuge machine.

You are invited to participate in this study by giving blood on a voluntary basis, just once for the study and diagnosis.

All blood draws will be performed by qualified technicians at the laboratory, and 10 ml of blood will be withdrawn from a vein in your arm.

During the collection of blood, you may experience discomfort, bruising and pain at the collection site. To minimize this, you will be asked to sit down comfortably on a chair as the technician withdraws blood sample. If you feel faint, you should not sit up and should notify the technician and the nurse immediately.

You may benefit directly from participating in this study by helping make diagnosis of tuberculosis in your blood. However , the test is not an alternative to sputum tests or other tests requested by your clinician. It will also help make a contribution to the information known about diagnosis of tuberculosis and in future may benefit doctors learn about diagnosis of tuberculosis.

Even though you will be asked to disclose about your HIV status, the answer will be voluntary, and you are not under any obligation to reveal you HIV status in this study . Therefore, no HIV test will be done on your blood collected for this study.

All records of blood drawn and results will be kept in a secure database and in a secured locker at the department of Medical Microbiology at the university of Nairobi. Only the professional staff at the laboratory will know the identity of study patients.

There will not be any monetary compensation or incentive for being in the study. If you feel that you are injured as a direct result of this participating in this study, please

contact Dr. Peter Mwathi on telephone, 0733826325 and email ; petmwa@yahoo.com and the chairman of KNH/UON ERC on telephone, (020)726300-9 at any time.

Your signature on this form means that you understand the information presented , and you want to participate in the study. You understand that participation is voluntary, and you may withdraw from the study at any time without any negative consequences.

Signature of Participant: _____

Signature of researcher: _____

Dr. Peter mwathi.

Appendix III: Blood Results Reporting Form

Clinic/ Ward_____ Reg. No._____

Name_____ Age____ Sex_____

Address_____

_____

Date_____/_____/2010

Full haemogram:

Haemoglobin level(Hb)_____ MCV_____ MCHC_____

RBC Count_____ WBC Count_____

Differential WBC count:

Neutrophils _____% Lymphocytes _____% Eosinophils_____%

Monocytes_____% Basophils_____%

ESR Level_____$mmhr^{-}$

Blood Buffy coat ZN Smear results:

Smear slide	1	2
Negative		
Positive		

Appendix IV: <u>Sputum Results Report Form</u>

Clinic/Ward_____ Registration no._____

Name_____ Age____ Sex_____

Address _____

_____

New patient____ Follow-up examination _____ Requested by:

Date_____ Name _____

Signature_____

1. Appearance

Specimen	1	2	3
Blood stained			
Muco-purulent			
Saliva			

2. Microscopy

Specimen	1	2	3
Date			
Serial no.			
Negative			
Positive			
Examined by			

Appendix V: Preparation of Carbol fuchsin

To make 1115ml:

Chemicals;

Basic fuchsin----------10g

Ethanol, absolute---------- 100ml

Phenol---------- 50g

Distilled water---------- 1 litre

1. weigh the basic fuchsin on a piece of clean paper (preweighed) , and transfer the powder to a container of at least 1.5 litre capacity.
2. measure ethanol (ethyl alcohol) , and add to the bottle.mix at intervals until the basic fuchsin is completely dissolved.
   Caution: away from fire for these chemicals are highly flammable.
3. with care , weigh the phenol in a beaker. Measure the water , and add some of to the beaker to dissolve the phenol. Transfer to the bottle of stain, and mix well.
4. add the remainder of the water and mix well.
5. label, the name and date of preparation and store at room temperature. The stain is stable indefinitely.

Appendix VI: Preparation of Acid alcohol, 3% v/v

This is a 3% v/v hydrochloric acid solution in 70% v/v alcohol.

To make 1 litre

Chemicals:

Ethanol, absolute---------- 680ml

Distilled water---------- 290ml

Hydrochloric acid ,concentrated-- 30ml

1. with caution and away from fire , measure the ethanol and transfer to a 1 litre capacity leak-proof container.
2. measure the water, add to the alcohol, and mix.
3. with great care, measure 30ml of concentrated hydrochloric acid, add to the solution, and mix well.
4. label the bottle, and mark it FLAMMABLE. Store at room temperature in a safe place.the reagent is stable indefinitely.

Appendix VII: Preparation of Methylene blue, 3g/l

To make 1 litre
Methylene blue---------- 3g
Distilled water ----------to 1 litre

1.weigh the Methylene blue and transfer it to a leak – proof bottle.
2. Measure the distilled water and add the dye. Mix until the dye is completely dissolved.
3. Label the bottle and store it at room temperature.

The stain is stable for several months but for use transfer a small amount of the stain to a dropper bottle.

Appendix VIII: AFB Reporting System [36]

When any definite red bacilli are seen, the smear should be reported “AFB positive” and given the number of bacteria present as follows:

More than 10 AFB/field---------- Report +++

1-10AFB/ field ---------- Report ++

10-100AFB/100 fields---------- Report +

1-9 AFB/100 fields---------- Report exact number and if only 1 or 2 , do another specimen before reporting.