PATTERN OF OPPORTUNISTIC INFECTIONS AND CYTOLOGICAL CHANGES IN SPUTUM SPECIMENS FROM HIV INFECTED PATIENTS AT MBAGATHI DISTRICT HOSPITAL, NAIROBI, KENYA.

A dissertation submitted to the University of Nairobi in part fulfillment of the degree of Master of Science in Clinical Cytology

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I, Titus Kamau Karuga declare that this dissertation is my original work under the guidance of the supervisors listed below and has not been submitted to the University of Nairobi or any institute of higher learning

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Dedication

I dedicate this book to my fiancée Nancy Wanjiku Mbugua and my family for their support immense love.

Acknowledgment

I thank God for his strength and grace that has brought me this far.

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List of abbreviations

AFB	Acid Fast Bacilli
AIDS	Acquired Immune Deficiency Syndrome
ART	Anti-Retroviral Therapy
ASCUS	Atypical squamous cells of undetermined significance
BAL	Bronchoalveolar Lavage
CMV	Cytomegalovirus
DPX	Dibutylphthalate Xylene
EA	Eosin Azure
ERC	Ethical Review Committee
HIV	Human Immunodeficiency Virus
HAART	Highly Active Antiretroviral Therapy
FNA	Fine needle aspirate
HSV	Herpes Simplex Virus
H&E	Heamatoxylin Eosin
KAIS	Kenya Aids Indicator Survey
MDH	Mbagathi District Hospital
OG	Orange G stain
OI(s)	Opportunistic Infection(s)
PJP	*Pneumocystis Jiroveci* Pneumonia
PCR	Polymerase Chain Reaction

PTB	Pulmonary Tuberculosis
SOP	Standard Operating Procedures
SPSS	Statistical Package for Social Scientist
TB	Tuberculosis
UON	University of Nairobi
WHO	World Health Organization
ZN	Ziehl Neelsen stain

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ABSTRACT

Background information: Patients with advanced stages of HIV infection are vulnerable to secondary infections that are generally termed as opportunistic infections (OIs). This is because the microorganisms take advantage of the opportunity offered by a weakened immune system.

Respiratory opportunistic infections commonly associated with HIV includes pneumococcal pneumonia, *Pneumocystis jiroveci* pneumonia and tuberculosis. Sputum is one of the specimens that can be utilized in making a rapid diagnosis of opportunistic infections.

Objective: To describe the pattern of opportunistic infections and cytological changes in sputum specimens from HIV infected patients from Mbagathi District Hospital (MBH), TB Clinic, Nairobi, Kenya.

Study design: A cross-sectional descriptive study.

Setting: This study was conducted at MDH TB Clinic, Nairobi, Kenya, between February and June 2013.

Study population: Adults 18 years and above attending MDH TB Clinic, who submitted sputum specimens for diagnosis of OIs diagnosis.

Main Outcome Measures Age, gender, clinical information (coughing, chest pain, difficulty in breathing and loss of weight), correlation (CD4+ Lymphocytes), laboratory results (presence or absence of opportunistic infections and epithelial cellular changes)

Material and methods: Demographic and clinical information was collected by direct interview of the patients. Two sputum samples per patient were obtained, pick and smear technique was used to prepare smears which were stained using Papanicolaou and Heamatoxylin and Eosin stain. The remainder of the sample was processed using bleach centrifugation method and stained with Ziehl Neelsen stain.

Result: A total of 100 HIV infected patients were studied. Majority of the patients, (51%) were female, while remainder were male (49%). The mean age was 38.98 years (± 10), with a median (IQR) of 38years (32, 45). A total of 80% patients were on HAART medication and 20% had

defaulted on HAART. The mean CD4+ lymphocyte count was 207 cell/mm^3, (±114.9), with a median (IQR) 193.5 cell/mm^3 (134.3, 269.5). Inflammatory changes were seen in 57% of patients, atypical squamous cells of undetermined significance (ASCUS) in 4%, negative findings in 34%. and unsatisfactory smears were 5%. The commonest opportunistic pathogen was *Mycobacterium* species (30%) and *Candida* species (14%).

Conclusion: Majority of these HIV infected patients had inflammatory changes in the sputum, with *Mycobacterium*, *Candida* and *Aspergillus* species the pathogens that were detected.

Recommendations: Sputum cytology should be used for preliminary diagnoses of opportunistic pathogens before confirmatory test.

1.0 INTRODUCTION

Currently, an estimated 35.3 (32.2-38.8) million people are living with Human Immunodeficiency Virus (PLWHA) (1). The first known case of HIV in humans occurred in a person who died in Congo in 1959, later confirmed as having HIV infection from his preserved blood samples (2). The first recognized cases of acquired immune deficiency syndrome (AIDS) occurred in the USA in the early 1980's after a number of gay men in New York and California suddenly began to develop rare opportunistic infections (OIs), and cancers that seemed stubbornly resistant to any treatment.

The first case of HIV/AIDS in Kenya was reported in 1984; the prevalence in adults has stabilized at 5.6% (3). Heterosexual intercourse is the primary form of transmission in Kenya, and epidemic varies greatly across demographic groups (4).

The current HIV pandemic has changed considerably, as infected people are now living longer with chronic HIV infection due to highly active antiretroviral therapy (HAART). With HAART therapy, the major cause of death in the later stage of AIDS is malignancy, rather than infection. Patients who have responded to HAART and have sustained increase in CD4+ lymphocyte count appear to be well protected from OIs. However, individuals unlikely to be on HAART therapy and those with poor adherence are still presenting with OIs, such as tuberculosis, fungi, and parasites.

Opportunistic infections (OIs) and co-morbidity are a major cause of death amongst HIV infected patients and are dependent upon time of initiation of HAART, risk factors, type of exposure and geographic region (5).

Respiratory cytology can be useful in diagnosis of a variety of benign and malignant diseases and plays an important role in accessing of OIs in the immunocompromised hosts such as patients with AIDS and transplant recipients (6). The cytological specimens that can be used in diagnosing OIs include sputum, bronchial washing and aspirates, bronchial brushing and bronchoalveolar lavage (BAL).

Pneumocystis jiroveci and *Mycobacterium tuberculosis* have become 2 of the most common etiologic agent of OIs associated with AIDS. A specific diagnosis of *Pneumocystis jiroveci* requires demonstration of organisms in respiratory specimens, and Bronchoalveolar Lavage (BAL) is favored over sputum for *Pneumocystis jiroveci* investigation. Sputum offers a specificity of nearly 100% and sensitivity of 55% while BAL alone has a diagnostic yield of 97–100% (7). In general, a more invasive procedure (e.g., BAL) used to collect specimens will provide better diagnostic yield. BAL and/or biopsy is preferable in difficult clinical settings, for example an uncooperative patient or a patient too dyspneic (7).

The gold standard for diagnosing pulmonary tuberculosis (PTB) is culture of sputum on Lowenstein-Jensen medium. However, due to lack of access to culture facilities and the long turn-around times involved with sputum culture, direct Ziehl-Neelsen microscopy for detection of acid-alcohol fast bacilli (AAFB) in sputum smears is utilized. This technique, when performed optimally, has reported sensitivities ranging from 61.8% to 70% compared with the gold-standard. Sensitivity is reduced if samples are of poor quality, which is often the case in children and in people who are HIV infected, since sputum expectoration is more difficult (8).

Sputum is the most common specimen for pulmonary cytology. Sputum is composed predominantly of mucoid substances as well as variable numbers of inflammatory and epithelial cells (9). Variation in the number of macrophages, neutrophils and epithelial cells can yield significant insight into underlying pathologic process. The sputum specimens stained by Papanicolaou technique and other special stains can provide a rapid preliminary diagnosis which will enable the clinician to begin therapy before definitive diagnosis is provide by more sensitive methods which include culture and polymerase chain reaction (PCR).

2.0 LITERATURE REVIEW

Patients with advanced stages of HIV infection are vulnerable to secondary infections that are generally termed as opportunistic infections. This is because the microorganisms take advantage of the opportunity offered by a weakened immune system (10).

Kenya has an estimated 1.5million people living with HIV, around 1.2million children have been orphaned by AIDS and in 2009, about 80,000 people have died from AIDS related illness (11). Kenya's HIV prevalence among adults aged 15 to 64 years decreased nationally from 7.2%, as measured in KAIS 2007 to 5.6% in 2012. HIV prevalence among children aged 18 months to 14 years was 0.9% (3). This decline is thought to be partially due to increase in education and awareness and high death rates (3).

Opportunistic infections (OIs) have been recognized as a common complication of HIV infection due to immune deficiency which deteriorates both the quality of life and life expectancy of those infected people. One of the main reasons behind hospitalization and substantial morbidity in HIV infected patients is due to OIs.

Several toxic and expensive therapies are required as part of the treatment regimen. This ultimately leads to shortened survival of people with HIV infection. The decrease in the CD4+ count in HIV infected persons is partially responsible for these OIs (12). Since the introduction of HAART, a decline in OIs and AIDS progression has been observed.

The majority of these infections occur when the CD4+ lymphocyte cell count falls below 500cells/mm^3.These include pneumonia, pulmonary tuberculosis, and herpes zoster. Levels lower than 200cells/mm^3 are associated with *Pneumocytis jiroveci*, toxoplasmosis, miliary and extra pulmonary tuberculosis. Levels lower than 50cells/mm^3 are associated with disseminated cytomegalovirus and *Mycobacterium avium* complex infection. Sputum product of the respiratory tract is the result of interaction between mucocilliary apparatus and the immune system of host and between the animate and inanimate invaders from the environment. It is the most frequently examined specimen from the respiratory tract (6)

Criteria for diagnosis of opportunistic infections and cytological changes in sputum specimen from HIV infected patients.

Inflammatory changes

Inflammatory changes can be of two types acute and chronic inflammation. Acute inflammation is characterized by presence of neutrophils, histiocytes, debris, and necrosis, including pneumonia, abscess, and purulent bronchitis, resulting in tissue destruction.

Chronic inflammation is characterized by presence of an abundance of immature lymphocytes and plasma cells. Immature lymphocytes should not be mistaken for malignant lymphoma. The key to this differential diagnosis is the same as for follicular cervicitis in the Pap smear and consists in finding a range of maturation of the lymphoid cells and tingible body macrophages.

When inflammation is severe, a protein-rich exudate, with deposition of fibrin and finely granular precipitate, may occur. In addition, mucus, bacteria, inflammatory cells, and lysed cells may cause the "dirty background" of inflammation to closely resemble a tumor diathesis (13).

Atypical squamous cells of undetermined significance (ASCUS)

These are squamous abnormalities that are more marked than those attributable to reactive changes, but that quantitatively or qualitatively fall short of a definitive diagnosis of a squamous intraepithelial lesion. Atypical repair can be included in the category of ASCUS (13).

Unsatisfactory

These are sputum samples without alveolar macrophages. The presence of alveolar macrophages confirms origin of the sample from pulmonary alveoli hence sputum specimens are rarely of diagnostic value in their absence. Marked acute inflammation is a unique case in which a sputum specimen may be from the deep lung i.e., adequate without the presence of alveolar macrophages (13).

Negative

Negative sputum sample have normal squamous, intermediate, bronchial cells with alveolar macrophages.

Bacteria

Cytopathology is limited in bacterial identification in morphology and gram stain characteristics. Routine stains of cytologic specimen can reveal bacteria. Although bacteria may be stained red or blue by pap stain, this cannot be correlated to gram positive and negative staining patterns (6).

***Candida* species**

Small budding yeast 3 to 4 um and pseudohyphae are typically identified as *Candida* species. This organism is probably the most frequently encountered fungus in cytologic specimen. When encountered in the lower respiratory tract, it should be considered as a possible pathogen.

***Aspergillus* species**

Aspergillus species contain hyphal structures that measure between 3-6um in diameter, with regular dichotomous branching at 45^0. These organisms are opportunistic pathogens usually identified in respiratory specimen and rarely found in other sites. Occasionally when fungus cavity is exposed to the air fruiting bodies with conidia can be detected in cytologic specimens (13).

Mycobacteria

Mycobacteria are slender, aerobic rods that grow in straight and branching chains. They have a waxy mycolic cell wall that makes them retain carbol fuchsin stain even on attempt to destain with acid alcohol. *Mycobacterium tuberculosis* is responsible for most cases of tuberculosis.

Globally, laboratory diagnosis of *Mycobacterium tuberculosis* is based on direct demonstration by light microscopy and fluorescent microscopy, isolation of the organism by culture, antibody detection by enzyme immunoassay, immunochromatographic, latex agglutination assay and cytomorphology (14).

Some of the most common opportunistic infection in the respiratory tract include:

2.1 Bacterial infections

Tuberculosis

Pulmonary tuberculosis (PTB) is a chronic granulomatous disease caused by the bacteria *Mycobacterium tuberculosis*. Infection with the bacterium usually involves the lungs but may affect any organ or tissue in the body.

HIV is the most important risk factor that promotes progression to active TB in people with *Mycobacterium tuberculosis* infection. Tuberculosis can occur at any point in the course of HIV infection. An individual infected with HIV has 10 times more risk of developing TB when compared with a person's without HIV (5). The prevalence of TB has increased 3-5 times in sub-Saharan Africa in the last decade and HIV seroprevalence has been reported to be about 75% among these patients (14).

Kenya has a large and rising TB disease burden and is ranked among the twenty-two countries that collectively contribute about 80% of the world cases. The TB notification rate rose from 51 to 326 per 100,000 populations between 1987 and 2009. Similar to the rest of sub-Saharan Africa this large increase in TB is attributed primarily to the Human Immunodeficiency Virus (HIV). Other factors that may be contributing to the increasing TB disease burden in Kenya includes the high poverty levels with consequent socio-economic deprivation (15).

Mycobacteria are slender rods which are 2-4micrometre in length and are "acid alcohol fast bacilli" (AAFB) meaning once stained by aniline dye, such as carbol fuchsin, they resist decolorization with acid and alcohol due to their thick walls containing mycolic acid. This property allows the detection of AAFB in specimen by using Ziehl-Neelsen (ZN) staining technique widely used in sub-Saharan African countries. Patients with PTB are the most important source of infection. Infection occurs by inhaling droplets with infectious particles of respiratory secretions usually less than 5micrometers which contain tubercle bacilli. These are spread into the air by coughing, sneezing, talking and spitting and they remain suspended in air for long periods of time.

Microbial culture of *Mycobacterium tuberculosis* from respiratory specimens most commonly utilizes expectorated or induced sputum for a definitive diagnosis of PTB. In addition detection

of AAFB in smear made from concentrated sputum is of considerable clinical and epidemiological value and remains the most widely used rapid diagnostic test for TB. The use of two consecutive sputum specimen identifies the vast majority (95-98%) of smear positive for TB (16). Smear for AAFBs can be directly prepared or prepared after concentrated and gravity sedimentation of sputum. In a study to compare direct and concentrated acid fast smear, direct smear detected *M.tuberculosis* in 81% of the patients whereas smears made from concentrated samples detected 91%(17). The use of a single AAFB smear showed a sensitivity of 53.3% and specificity of 89.5%(18).

Smear based microscopy is rapid, specific and reasonably easy to perform, WHO recommends its use for screening patients with cough lasting for more than two weeks. However the technique has low sensitivity, detecting only 20-43% of culture –positive cases (19).

2.2 Fungal infections

Pneumocystis jiroveci Pneumonia (PJP)

Pneumocystis jiroveci, previously known as *Pneumocystis carinii* is an extracellular organism usually found in alveolar spaces of the lungs and causes pneumonia. It has become the most common opportunistic infection in HIV/AIDS patients with a CD4+ count < 200cell/mm^3 (20).

In Kenya, a study done in Mbagathi District Hospital *Pneumocystis jiroveci* was positive in 27% and 37% of the 51 patient studied using toluidine blue and immunofluorescent stains respectively (21). According to a review PJP was the primary cause of death in 0 - 2% of HIV-1 infected patients in autopsy series from Kenya and Cote d'voire (22). Among HIV-1 infected patients with respiratory disease the prevalence of PJP was 1 - 4% in Tanzania studies, 5% in Burundi and 22% in a study from Zimbabwe(22). When stained with Papanicolaou stain, the organism are seen as green, foamy alveolar casts that are more circumscribed than debris or lysed red blood cells. The cysts are visualized with Grocott's methamine silver stains (GMS) and Periodic acid Schiff (PAS). They are cup shaped, measure 5 -7um in diameter, and often have a central dark zone (6).

Other diagnostic methods include fluorescein-conjugated monoclonal antibodies, immune fluorescence and real time PCR which is the gold standard. New techniques that detect messenger RNA (mRNA) have been proposed as surrogate markers for organism viability. The

rationale is that mRNA is less stable than DNA. Therefore, if the patient is not actively infected with viable organisms, the mRNA should be largely degraded and no longer detected. In contrast, DNA is far more stable and may still be present even if the organisms are dead. Recent data from HIV patients with suspected *Pneumocystis* pneumonia using reverse-transcriptase PCR targeting a heat shock protein of *Pneumocystis* mRNA (*Phsb1*) have yielded a diagnostic sensitivity and specificity of 100% and 86%, respectively, in BAL specimens (23).

2.3 Viral infections

Cytomegalovirus

Cytomegalovirus, a beta herpes virus, is the major cause of non-Epstein Barr virus infectious mononucleosis in the general population and an important pathogen in immunocompromised hosts, including patients with AIDS and transplant recipients (24). The risk of exposure to CMV increases with age. The CMV remains latent in the infected host throughout life and rarely reactivates to cause clinical illness except in the immunocompromised individual. Infection with CMV is more prevalent in populations at risk for HIV infection, approximately 75% of injection drug user and > 90% of homosexual who are infected with HIV have detectable IgG antibodies to CMV(25). Higher prevalence rates among homosexual men correlate with the increased risk of exposure associated with receptive anal intercourse (25). In addition, high prevalence rates of CMV IgM antibody in longstanding seropositive homosexual men suggest that this group is frequently re-exposed (and at least sometimes re infected with) differing exogenous strains of CMV. Viral cytopathic changes including cytomegaly, large nuclear and small basophilic cytoplasmic inclusions are found in bronchial cells, pneumocytes macrophages and endothelial cells.

In a study comparing centrifugation culture (CC), in situ hybridization(ISH), and nested-primer polymerase chain reaction (npPCR) techniques on bronchoalveolar fluid for the diagnosis of respiratory CMV infection. Sensitivity, specificity, positive and negative predictive values were as follows: 86%, 86%, 90%, and 80% for the CC; 5%, 100%, 100%, and 41% for ISH; and 86%, 57%, 75%, and 73% for npPCR. CC appeared to be the best of the three techniques compared in this study for diagnosis of respiratory CMV infection in HIV-infected patients. The sensitivity and predictive values of DNA-DNA ISH were very poor. Results with npPCR were acceptable,

and this technique may be considered in situations when rapid diagnosis of CMV infection is necessary (23).

Herpes simplex virus

Herpes simplex virus (HSV) pharyngitis and pneumonia most commonly affect patients and neonates who are immunocompromised. HSV-1 is the most common serotype to involve the respiratory tract. It is acquired in childhood and causes herpes infections on the mouth and lips (6).

HSV-2 is transmitted sexually and causes anogenital ulcers. Among HIV-1 infected individual HSV-1 and HSV-2 infections are common, with prevalence that approximate or exceed those of the general population. In recent years a number of studies have focused on the prevalence of HSV-2 among HIV-1 co-infected individual, finding seroprevalence of 50-90% in some populations, significantly higher than among those without HIV (26). The highest prevalence of HSV-2 HIV-1 co-infected individual is in heterosexual women and men in sub-Saharan Africa and men who have sex with men in the America (26). Herpetic inclusions can be seen in metaplastic squamous cells (when inflammation is centered around airways) or multinucleated giant cells (within necrotic inflammatory debris with the interstitial form of infection cells,

Diagnosis can be confirmed by viral culture, immunohistochemistry or in situ hybridization and PCR.

Adenovirus

Human Adenovirus causes numerous diseases which include respiratory tract infections, ocular and gastrointestinal tract disorders. The virus usually causes mild, self limiting respiratory illness primarily in children, due to normal host responses which include natural immune response involving induction of cytokines and activation of effective leukocytes (6). However, potentially fatal disseminated disease in highly immunocompromised patients has been reported. The virus causes two types of nuclear inclusions. A smudge cell in which a large basophilic inclusion usually fills the entire nucleus and obscures chromatin detail. The other is an eosinophilic inclusion that resembles the Cowdry A inclusion of herpes virus infection (6, 13).

Measles Virus and Respiratory Syncytial virus

Measles is a highly contagious self limited disease caused by the rubeola virus (6). Measles pneumonia occurs as an opportunistic complication in children who are immunocompromised as a result of premature birth malignancy or immunologic disorder. Infection causes a giant cell pneumonia characterized by multinucleated cells with cytoplasmic and nuclear inclusions. Similar findings are seen with infections by respiratory syncytial virus. The diagnosis is usually confirmed by detecting syncytial virus antigen in bronchoalveolar lavage (BAL) specimen (6, 13).

2.4 Anatomy and Cytology of the Respiratory Tract

The respiratory tract may be roughly divided into three portions. The cranial portion is supported by the bones of the skull and the cervical vertebrae; it comprises the nasal cavity and the paranasal sinuses, the buccal cavity and the pharynx. The intermediate portion is composed of larynx, trachea and the main bronchi, and it stretches from the larynx to the hilus of each lung. The third portion is the lung proper composed of lobar, segmented and similar bronchi and alveolar with its extraordinarily rich blood supply (13).

Contrary to the squamous epithelium which desquamates easily and well represented in all exfoliated samples, the normal respiratory epithelium does not desquamate freely. Consequently, cells derived from the bronchial epithelium are uncommon in sputum and are typically seen in specimen obtained by bronchial brushing.

The cytologic appearance of squamous cells in respiratory cytology is identical to their appearance in Pap smear. There is usually a predominance of superficial cells. Anucleate squames and intermediate cells may be found. Benign pearls and occasionally spindle squamous cells may be seen.

The most characteristic feature in glandular cells is the presence of cilia on the apical surface, anchored into a terminal bar or plate. Cilia stain bright red and the terminal bars are seen as a distinct thickening of the apex. Goblet cells which are mucus producing cells are approximately the same length but usually wider than the ciliated cells with a basal placed nucleus and a

distended supranuclear cytoplasm that is tightly packed with faintly basophilic vacuoles representing packages of mucus (6).

Basal or Germinative cells form the source of epithelial regeneration and normally form a single layer of cells in the basement membrane. In response to inflammation or injury these cells may proliferate and form several layers resulting in basal or reserve cell hyperplasia (13).

Pneumocytes are flat cells with keratin and long cell process that form 90-95% of the alveolus.

Alveolar macrophages are bone marrow derived histiocytes. They neutralize foreign material in the alveoli. They are of great importance in evaluating cytologic material from the respiratory tract. Their presence confirms origin of the sample from pulmonary alveoli hence sputum specimens are rarely of diagnostic value in their absence (11).

2.5 Different cytologic sampling methods

Sputum

Sputum is expectorated or artificially induced and is the simplest sample utilized in investigating the respiratory tract. Sputum consists of a mixture of cellular and non cellular elements that are cleared by the mucocilliary apparatus. The adequacy of a sputum specimen is determined primarily by the presence of alveolar (pulmonary) macrophages indicating that the specimen obtained is a deep cough specimen from the lower airways. It is relatively easy to obtain and causes little if any discomfort. The optimum number of specimen to submit for diagnosis is between three and five to exclude cancer. The use of two consecutive sputum specimens identifies the vast majority (95-98%) of smear positive for TB (16).

For patients unable to expectorate sputum, a sputum sample can be induced by having the patient breath in either a nebulized solution of 15% saline with or without 20% propylene glycol or plain 3-8% saline.

The most popular techniques are the ‘‘pick-and-smear’’ technique and the Saccomanno methodology. The ‘‘pick and-smear’’ technique avoids the use of carbowax and begins with visual inspection of fresh specimens for strands or flecks of solid or bloody material. Experience is essential to select appropriate areas for processing. These are selected along with random samples, and prepared as direct smears for immediate fixation (95% ethyl alcohol or spray fixation. If the material is abundant, paraffin-embedded cell blocks may be prepared, sectioned,

and stained with Heamatoxylin& Eosin. This may increase the diagnostic yield but at added expense (13).

In Saccomanno technique the specimen is broken up in a food blender and smears are prepared from the centrifuged button. The sputum is usually collected in Saccomanno solution, tissue fragments, fungal hyphae, and secretory vacuoles may also be disrupted using the Saccomanno technique. In addition, problems with infection of laboratory personnel, due to aerosolization of infectious agents, have occurred with the Saccomanno technique (13).

The commonly used fixatives are 70% ethanol and Saccomanno solution (50%ethanol and 2% polyethylene glycol or carbowax (6)

Bronchial brushing

Cell samples are obtained with a small brush threaded through a separate channel in a fiberoptic bronchoscope guided to a selected site under visual control. The entrapped cells are either smeared onto a glass slide or rinsed in a collection medium for thin layer or cell block preparation (6). A satisfactory specimen should contain a large number of well-preserved, optimally stained ciliated bronchial epithelial cells and macrophages.

The best potential use of bronchial washings is for diagnosing pneumonia caused by strictly pathogenic organisms, such as *M. tuberculosis* and endemic systemic fungi, particularly in patients for whom the BAL return volume is inadequate (27).

Bronchial aspirates and washings

Introduction of the bronchoscope in the lower respiratory tract enables the examiner to obtain specimen by means of suction apparatus that aspirates secretions. Aspirates and washing samples provide information on the status of the respiratory tract in small bronchi beyond the reach of the bronchoscopic brush (6).

Washings from the visualized areas may also be collected by instilling 3-5ml of a balanced salt solution through the bronchoscope and aspiration of resulting material. Direct smear can be made with immediate fixation in 95% ethyl alcohol.

The incidence of mucosal hemorrhage is therefore slightly higher after this procedure.

Because the usual bronchial brush is not protected from contamination during passage through the bronchoscope channel, it is inappropriate for bacterial cultures. In contrast, cells are obtained from the airway walls, specimens from a cytology brush are appropriate and accurate for the diagnosis of cytopathic changes or viral inclusion bodies in airway cells (27).

Bronchoalveolar lavage

During collection of bronchoalveolar lavage, the bronchoscope is wedged into position as far as it will go, and distal airways are flushed with several aliquots of sterile saline. The first aliquot is representative of cellular material from larger airways, whereas subsequent aliquots reflect the alveolar compartment (6).

The BAL specimen is particularly useful for diagnosis of OIs in patients who are immunocompromised. The specimen can be examined cytologically and a portion also submitted for microbiologic studies in patients with acquired immune deficiency syndrome (AIDS).

BAL cytology serves as an effective and easy technique to diagnose *Pneumocystis* pneumonia. A careful cytological examination for foamy alveolar casts along with the use of special stains which includes Gomori methenamine silver and both the cresyl violet confirms the diagnosis of *Pneumocystis jiroveci* (27).

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2.6 JUSTIFICATION

Local data on the prevalence of OIs is not readily available despite Kenya having an estimated 1.5million PLWHA. OIs are the major cause of mortality among PLWHA. The incidence of many OIs is decreasing because of the advances in HIV therapy. The decrease is attributed to successful OIs prophylaxis and use of HAART. However, millions of PLWHA in resource poor communities /countries do not get access to HAART and in these cases especially, occurrence of OIs is very common. Even where ART drugs are available they do not entirely remove the need for preventing and treating OIs. Because of poor adherence, drug resistance or other factors, measures to prevent and treat OIs become essential if HAART become ineffective.

Timely intervention of OIs not only helps HIV positive persons to live longer better quality of lives but it also helps to prevent transmission of OIs to others in the community.

Sputum cytology is a rapid, accurate and minimally invasive method which can be used to diagnose OIs prior to confirmation by microbiological techniques. Determination of OIs in HIV infected patients will be used in management of patients and forms the basis for further research on OIs.

2.7 RESEARCH QUESTION

What is the pattern of opportunistic infections and cytological changes in sputum specimens from HIV infected patients attending Mbagathi District Hospital TB Clinic?

2.8 STUDY OBJECTIVE

Broad Objective

To determine the pattern of opportunistic infections in sputum cytology in HIV infected patients attending Mbagathi District Hospital TB Clinic.

Specific Objective

1. To determine cytological changes associated with opportunistic infections in sputum specimens of HIV/AIDS infected patients attending Mbagathi District Hospital TB Clinic.
2. To identify the type of pathogen(s) in sputum cytology in HIV infected patients attending Mbagathi District Hospital TB Clinic using specials stains, (Ziehl-Neelsen stain and Periodic Acid Schiff).

3.0 MATERIAL AND METHODS

Study design

This was a cross sectional descriptive study.

Study area

The study was conducted at Mbagathi District Hospital (MDH) TB Clinic and the University of Nairobi Histology & Cytology Laboratories. Mbagathi District Hospital is located on Mbagathi Way, Nairobi. The catchment area includes the Kibera slum in Nairobi one of the largest informal settlements in Africa.

The TB Clinic is open between Monday to Friday and serves approximately 120 HIV infected patients every month. Data on the number of OIs present was not available since the TB clinic specializes in diagnosis of TB only.

Study population

Adult patients 18 years and above attending MDH TB Clinic, who provided sputum specimens for diagnosis of OIs

Selection criteria

Inclusion criteria

- Adult patients 18yrs and above who had HIV and CD4+ lymphocyte counts $<500 cells/mm^3$.
- Those who provided informed consent

Exclusion criteria

- Those with known respiratory tract cancer or TB and on therapy
- Patients who were unable to expectorate sputum

Sampling method

Recruitment

Potential patients were recruited and group counselled on the importance of the study by the research assistant (nurse) at the triage room at MDH TB Clinic. The patients were then screened for the selection criteria through direct interview. Those who fulfilled the selection criteria were informed about the study and a written informed consent was obtained from those who agreed to participate in the study by the attending doctor. (**APPENDIX VII**)

Data collection procedures

The patient's socio demographic and clinical data were obtained both by direct interview and review of the patient's file.

The data collected from each patient was captured in a pre-designed structured questionnaire (**APPENDIX I**).

Specimen collection

A good wide mouthed sputum container, which was disposable, made of clear thin plastic, unbreakable and leak - proof material was used for specimen collection. Patients were given instructions to deeply inhale 2-3 times and cough deeply from the chest and spit into the sputum container by bringing it close to the mouth. Patients were given another sputum container with laboratory number on it for an early morning specimen and advised to rinse their mouths with plain water before expectorating the sputum.

Sample processing

Four smears were prepared, two were fixed with 95% alcohol and two air dried. The pick and smear technique was used to prepare smears which were stained with Papanicolaou and Heamatoxylin and Eosin stains (**APPENDIX II, IV**).One air dried smear was prepared using bleach centrifugation method and stained with Ziehl Neelsen (ZN) stain (**APPENDIX VI**), the other air dried smear was not stained since no organism in the study was recommended for any other special stains.

Laboratory procedures

Pick and smear technique

Two consecutive samples were pooled into a Petri dish after shaking for 30sec. Bloody, distorted or solid particles were selected and a small portion of each particle placed on three glass slides. With a clean glass slide the particle of sputum on each of the three slides was crushed with a rotary motion, with overlapping horizontal strokes the material was spread evenly over the slides.

Bleach method

The remainder of the sample after pick and smear technique was transferred into 10ml tube with equal volume of commercial bleach. The mixture was then homogenized by shaking and incubated for 15min at room temperature. The mixture was then centrifuged at 2000rpm for 15min.The supernatant was decanted and smears made from sediments.

Quality Assurance

For ZN, positive and negative AFB slides smears were included in every batch of the staining process for quality check of the stains and processing procedure. All reagents were prepared in accordance with Standard Operating Procedures used in UON Histology & Cytology laboratory (**APPENDIX VI**).The smears were read independently by the principal investigator and signed out with the three supervising pathologists. Every tenth smear and 10% of the negative smear were read by an independent pathologist and any discrepant smears were given to a tie breaker pathologist and the diagnosis made was taken as the final diagnosis.

Biosafety

Participants were instructed accordingly on the importance of good quality sample collection for good results and salivary and leaking samples were rejected. The use of personal protective equipment which included gloves, masks and laboratory coats was observed during processing of samples and hand washing immediately after every procedure. Samples were processed in a biohazard hood. All sputum samples were collected and transported in leak proof container. The containers used for collection of sputum samples were disposed by treating in 2% freshly prepared hypochlorite solution and then incinerated.

Data Handling

Sample size

$$n = \frac{Z^2 P\,(1-P)}{d^2}$$

n= sample size (n=**100**)

Z= Z statistic for a level of confidence or normal distribution critical value set at 1.96 which corresponds to 95% confidence interval(Z = 1.96)

P=expected prevalence or proportion estimated to a particular characteristic 52% of population

d^2=degree of precision, usually set at 0.05 (using d ±10%)

$$n = \frac{1.96^2 x 0.52 x 0.48}{0.1 x 0.1}$$

n= 100

Variables

Independent variable

Demographic characteristics- age, gender

Clinical data- coughing, loss of weight, difficulty in breathing, chest pains

CD4+ Lymphocytes count

Dependent variable

Presence of opportunistic infection, inflammatory and epithelial cellular changes

Data Analysis plan

The data collected using structured questionnaires was entered into Ms Excel database, cleaned and verified, then imported into SPSS (v.20) statistical software for analysis.

Descriptive statistics on socio-demographic characteristic were presented using percentages and frequency for categorical or nominal data. Continuous variables were presented using means (standard deviation) if normally distributed and medians (inter-quartile range) for non-normally distributed variables. Tables and appropriate charts were used to display results.

Correlation analysis to access for any linear association was done using Pearson correlation coefficient for the continuous and categorical variable.

A p-value of **<0.05** was considered significant.

3.10 Ethical consideration

Approval for the study was obtained from Kenyatta National Hospital Ethical and Research Committee prior to commencement of the study **(P646/11/2012).** Permission to carry out the study at MDH TB clinic was obtained from the Medical Superintendent at MDH. The patients were carefully taken through a consent process that informed them about the study, benefits and risks involved and what was required of them.

Any information obtained from the patients was kept strictly confidential and used solely for achieving the study objectives.

Patients were informed of their results in accordance with the established clinical practice and referred for further management at Mbagathi District Hospital when indicated.

4.0 RESULTS

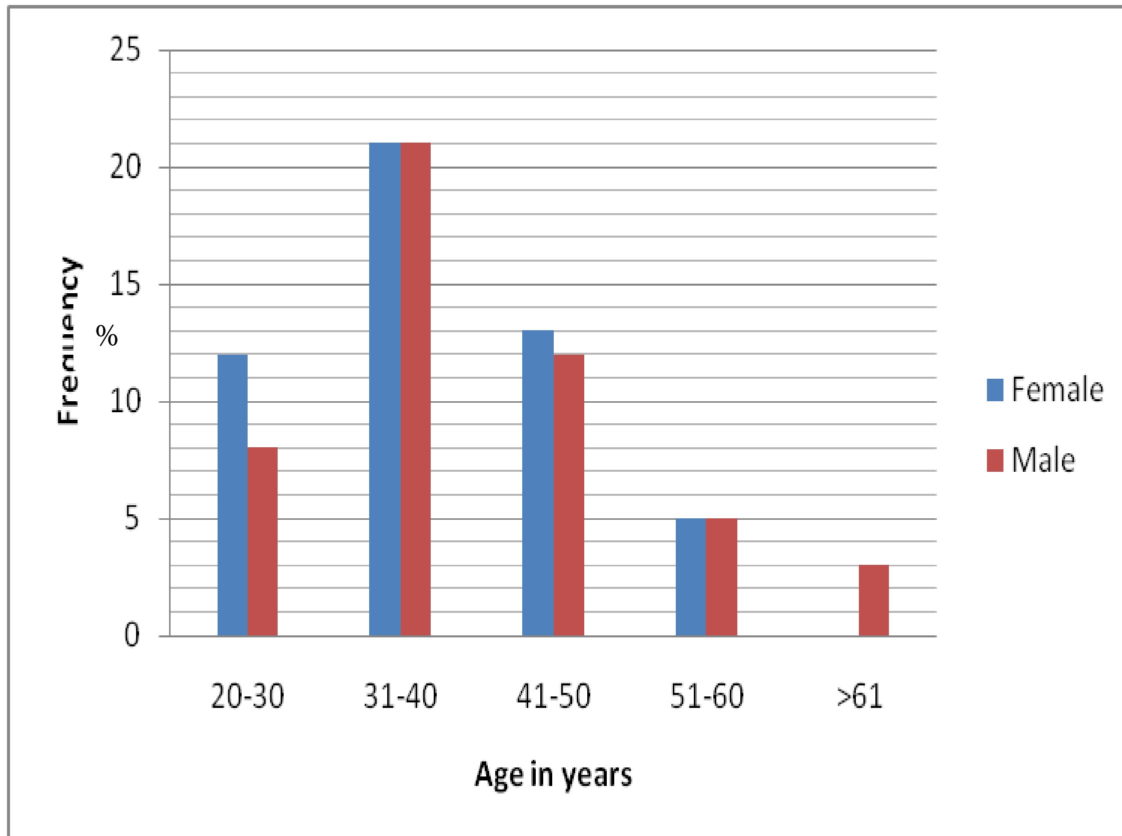
Socio-demographic characteristics

A total of 100 HIV infected patients were consented and enrolled into the study. Of these 49% were males and 51% were females. About 41% of the patients enrolled had primary level education, 32% had secondary level, 15% had no formal education and only 12% has tertiary level education. There were two main occupation types of which 33% were in various forms of employment and 26% were in small scale businesses. Majority of the patients, 41% were unemployed.

Age distribution by gender

The mean age of the 100 HIV infected patients was 38.98 years (± 10), with a median (IQR) of 38years (32, 45).There were three peak ages groups for females, those aged 31 to 40 years (21%), followed by 41 to 50years (13%) and those aged 20 to 30 years (12%). There were three peak ages for males, those aged 31 to 40 years (21%), followed by 41 to 50 years (12%) and those aged 20 to 30 years (8%). The 51 to 60 years age group had 5% female and male, while the over 61 years age group had 3% males only (Figure 1).

Figure 1: The age distribution of study participants (n=100)



Clinical characteristics

The common presenting signs and symptom included cough, chest pains, loss of weight and difficulty in breathing as indicated in Table 1

Table 1: Presenting signs and symptoms

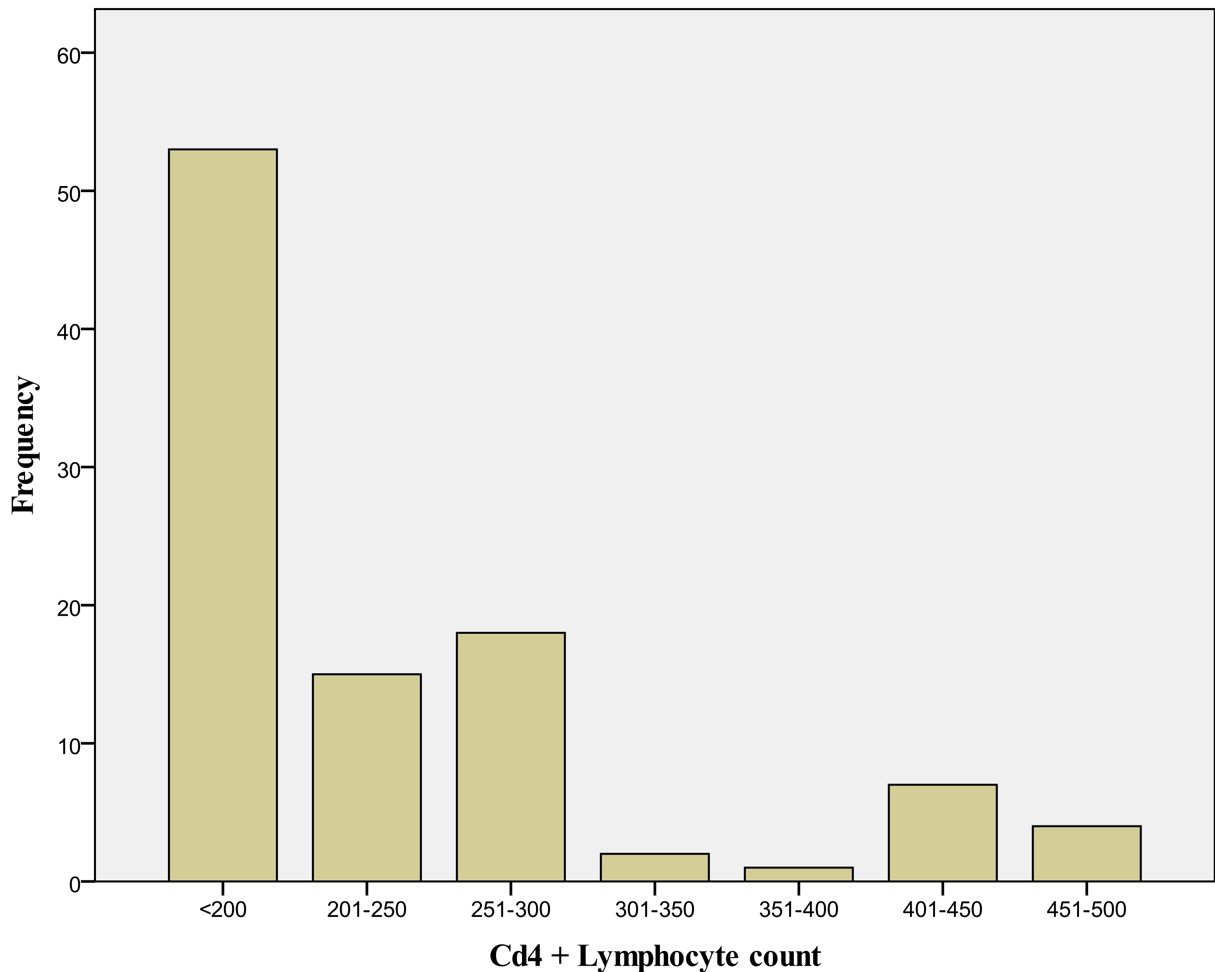
Presenting symptoms	n (%)
Cough	71(32.3%)
Chest pains	40(18.2%)
Loss of weight	30(13.6%)
Difficulty in breathing	11(5.0%)
Others*	50(30.9%)

*Other symptoms- night sweats, vomiting, fever, headache, pleural effusion

HAART use and distribution of patient CD4+ lymphocyte count

A total of 80% patients were on HAART medication and 20% had defaulted on HAART. The mean CD4+ lymphocyte count was 207 cell/mm^3, (±114.9), with a median (IQR) 193.5 cell/mm^3 (134.3, 269.5).

Figure 2: Distribution of patient CD4+ lymphocyte cell count



Of the 53% study participant with less than 200cell/mm^3, 22% had *Mycobacterium* and *Candida* species and 36% had inflammatory changes.

Cytological changes associated with opportunistic pathogens in HIV infected patients

Various cytological changes were observed in the sputum samples. Inflammatory changes were seen in 57% of patients, atypical squamous cells of undetermined significance (ASCUS) in 4%, negative findings in 34% and unsatisfactory smears were 5%. The cytological changes associated with opportunistic pathogens in HIV infected patients are shown in Table 2 below.

Table 2: Cytological changes associated with opportunistic pathogens in HIV infected patients (n=41)

Pathogens (ZN and H&E)	**Inflammatory changes**	**ASCUS**	**Negative**	**Total**
***Mycobacterium* spp**	**18**	**0**	**12**	**30**
***Candida* species**	**10**	**0**	**4**	**14**
***Aspergillus* species**	**1**	**0**	**0**	**1**

Pathogens identified in sputum specimens

The 100 sputum specimens were stained using the Ziehl Neelsen stain of which 30% were positive for acid-fast bacilli and 70% were negative.

Heamatoxylin and Eosin (H&E) stain identified pathogens in 42 sputum samples. These were distributed as shown in Table 3 below.

Table 3: Types of pathogens (Heamatoxylin and Eosin)

Characteristic	**Frequency**	**(%)**
Bacteria	27	64.2
***Candida* species**	14	33.3
***Aspergillus* species**	1	2.38
Total	42	100

Photomicrographs showing cytological changes and pathogens identified (Leica)

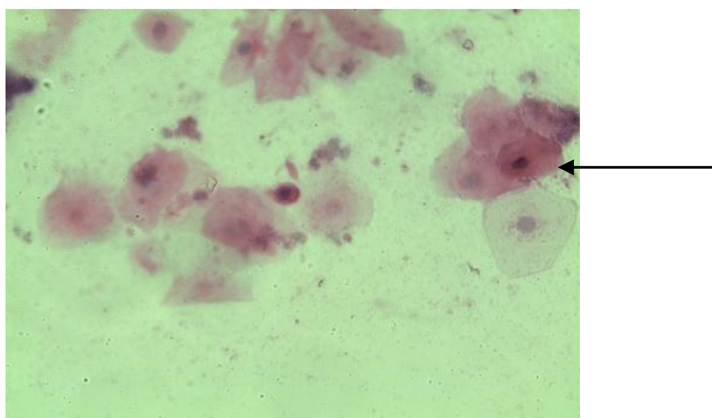


Figure. 3 **Squamous cells**. Sputum. Papanicolaou stain (x40)

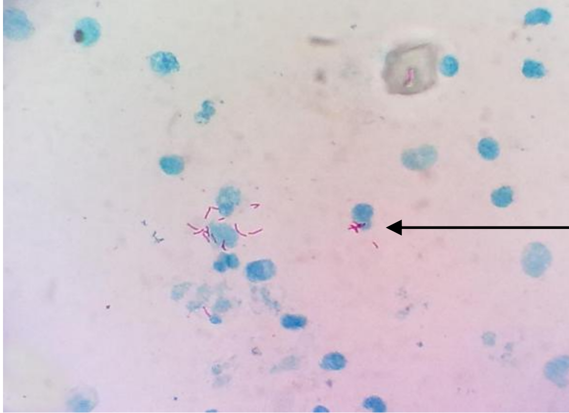


Figure. 4 **ZN+ for acid fast bacilli**. Sputum. ZN Stain (x100)

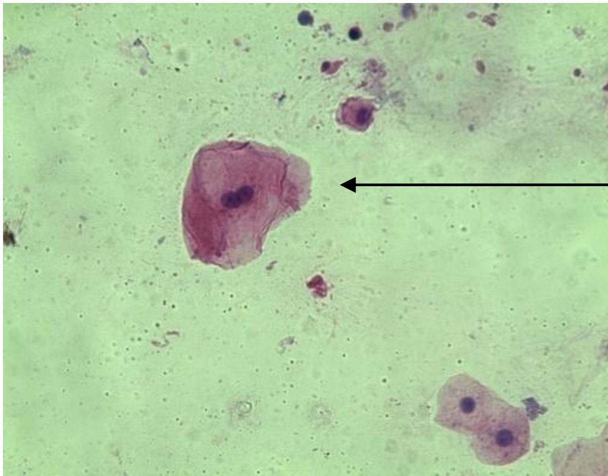


Figure. 5 **Atypical squamous cells of undetermined**

Significance. Sputum. Papanicolaou stain (x40)

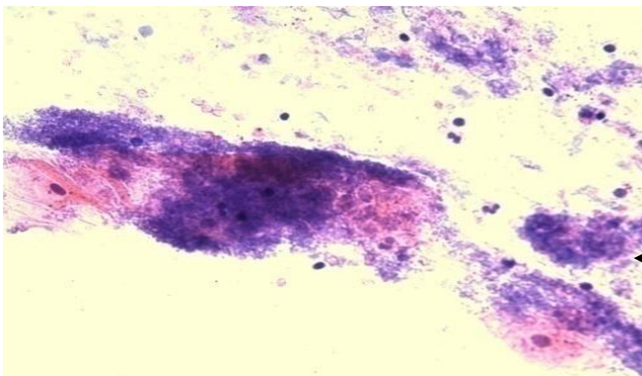


Figure. 6 **Bacteria colonies forming clue cells.** Sputum. H&E (x40)

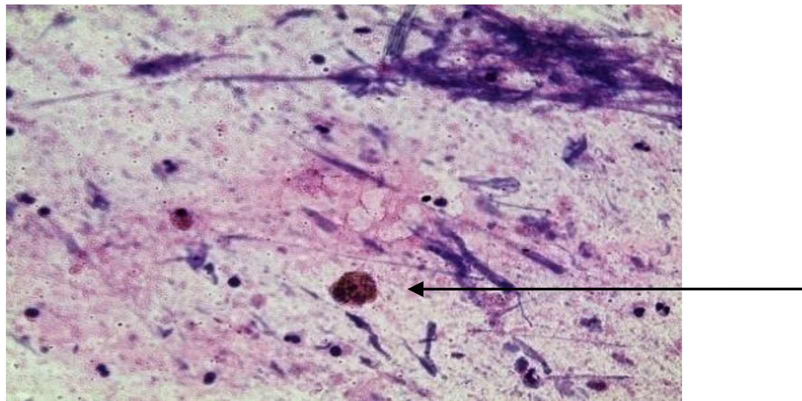


Figure. 7 **Carbon laden macrophages** Sputum. H&E (x40)

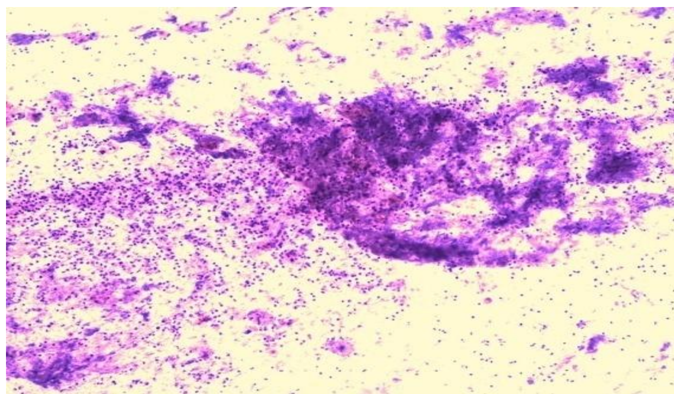


Figure. 8 **Inflammatory smear showing abundant neutrophils, bacteria colonies and debri.** H&E(X40)

Multiple co-infections were also found in some of the samples. These were distributed as shown in Table 4 below.

Table 4: Multiple pathogens in the HIV infected patients

Pathogen	**Frequency (n=13)**
***Mycobacterium* spp and other bacteria**	**8**
***Mycobacterium* spp and *Candida* species**	**4**
***Mycobacterium* spp, *Candida* spp and bacteria**	**1**
Total	**13**

5.0 DISCUSSION

Pulmonary cytology analysis using sputum was used in this study, a specimen that is easily accessible using a noninvasive procedure, simple to process, accurate, cost effective and multiple specimens can be collected for a variety of investigation.

A total of 100 HIV infected patients who attended Mbagathi District Hospital TB clinic aged 31-40 (42%) years were studied, similar to other studies done by Saha et al where the majority of the patients were 31-40 (43 %) years (5) and Ahmed et al (28) 31-40 (57%). This group consists of people who are sexually active and in their reproductive age and hence have an increased risk of acquiring HIV infection.

Majority of the patients, 41% had primary level education; this is consistent with Kirunga (29) from Uganda who reported 51% although much higher than this study. Naik et al (30) from India found that the highest risk for HIV infection was observed in those who only completed primary education. These patients abandon school for work and thus increasing their mobility which facilitates the spread of HIV.

Employment is a major factor in maintaining income levels and living conditions, especially among patients with long-lasting chronic diseases which HIV infection has become. In this study, 41% of the patients were unemployed with 59% in various forms of employment and small scale business.

Studies have reported persistent high unemployment rates among persons living with HIV/AIDS in the era of HAART, ranging from 45% to 65% which consistent with this study. Such unemployment rates are likely to be the result of a debilitating effect of HIV infection on workforce participation (31).

Cytological examination of sputum is accepted as a useful diagnostic tool in carcinoma of the lung (32). The most common cytological changes in this study were inflammatory changes in 57 %, four (4%) had atypical squamous cells of undetermined significance. Selvaggi reported mild inflammatory changes in AIDS patients with pulmonary infections (33). Epithelial cells of the respiratory tract undergo morphologic changes in response to injuries caused by various agents. These cytologic features should be correlated with clinical and radiologic finding to avoid any false positive findings (34).

The high number of inflammatory changes in this patients could be attributed to infections in the respiratory tract the commonest been bacterial pneumonia. Bacterial pneumonia has been reported to be 5-15 times more common in HIV infected patients than in HIV negative patients, and is the common cause of pneumonia requiring hospitalization in HIV infected patients (27).

Tuberculosis is one of the most common types of OIs associated with HIV infection and this may be further compounded by socio- economic factors such malnutrition, poor hygiene, poverty and unemployment (5). Ziehl- Neelsen (ZN) method is recommended by the World Health Organization (WHO) for screening patients since it is simple, quick at picking most of the infectious patients (35). Though conventional ZN staining method for acid – alcohol fast bacilli (AAFBs) plays a key role in the diagnosis and monitoring of treatment in TB, its major disadvantage is low sensitivity, ranging from 20% to 43% (35). *Mycobacterium* culture is the reference method for isolation of tubercle bacilli, but it is time consuming and requires specialized safety procedures in laboratories.

This study utilized the bleach method in detection of AAFBs in sputum. According to a study done by Ongkhammy et al smear positivity increased from 12% by conventional ZN method to 16% by bleach method (36). Thirty percent of the HIV infected patients enrolled in this study had AAFBs detected in their sputum. This is consistent with similar studies from Kenya and South Africa where tuberculosis was diagnosed in 27-34% consecutive HIV-1 infected patients admitted in hospitals (22) and Saha et al (5) study which was at 35%.

In a study by Yassin et al (37) in Ethiopia the prevalence of TB was 19%, which was much lower than in this study. Dauda (38) from Nigeria, and Saini et al (39) in India found the prevalence of TB to be 38% and 44% respectively all of which are much higher than this study. Nigeria has been noted as among the leading countries burdened by TB and ranks 4th among the 22 countries that account for 80% of the world's TB cases, India has the largest number of tuberculosis cases in the world, this may explain the high prevalence in TB in studies done in both countries (38, 39)

Kenya is one of the countries identified as high TB burden this therefore explains the high prevalence of TB in this study. The incidence is still rising with an average increase of 16%

cases (all forms) notified to National Leprosy and tuberculosis Program (NLTP) in the last ten years. The fact that endogenous reactivation of TB occurs most commonly among persons with AIDS also accounts for the high prevalence of TB among HIV/AIDS as indicated by Githua et al (40).

The total number of bacterial infection other than *Mycobacterium* species found in this study was 27%.This is higher than the 7% prevalence reported by Ogba et al (41), but lower than 44% reported by Shailaja V V et al (42) and Mulla et al (43) at 32%. The high number of bacteria in these studies was because culture was used to isolate the bacteria as opposed to the current study that used Pap and H&E stain to identify the bacteria. Bacteria detected in this study were mainly coccobacilli and diplococci.

Candidiasis is the most common reported opportunistic fungal infection in HIV infected patients (44). In this study, 14% of the patients had *Candida* infection. This is in contrast with studies conducted by Saha et al (5) where Candidiasis was found to be 53%, Esebelahie et al (45) in Nigeria reported an overall prevalence of *Candida* colonization among HIV infected patients of 52%, Saini et al (39) reported 23% and Mulla et al (43) a prevalence of 26%. This study compared well with Ogba et al (41) in Nigeria at 14% and Shailaja V V et al (42) who reported 12%.

Local data on the prevalence of *Candida* in HIV infected was not available, studies used for comparison utilized culture to isolate *Candida* species as opposed to study that only used routine special stains to make a diagnosis. Culture is used as a gold standard in identification of fungal species thus increasing sensitivity of the test. Special stains which include Gomori Methanamine Silver (GMS) and Periodic Acid Schiff (PAS) can also be used as ancillary test to make a diagnosis.

Pulmonary aspergillosis has recently been described as an emerging infection in patients with AIDS, but the pathological changes have not been well documented (46). In this study one patient was reported to have *Aspergillus* species. Bharathi and Rani in India (47) reported 16% which was higher than this study.

Multiple infections were also found in some samples. The most frequent co-infections in this study population were *M. tuberculosis* and bacterial agents (eight patients), *M. tuberculosis* and *Candida* species (four patients) and one patient with *M tuberculosis*, bacteria and *Candida*. Many patients with TB usually have co-infection with *Candid*a species; this is due to increased use of broad spectrum antibiotic and immunosuppressive drugs and possibly as a result of TB in the background of the HIV epidemic (5).

Majority of the participants (53%) had a CD4+ lymphocyte count less than 200cell/mm^3. Takalkar et al (48) and Saha et al (5) reported 46% and 50% of the study participant had CD4+ lymphocyte count less than 200cell/mm^3 respectively.

5.1 CONCLUSION

Majority of the patients had inflammatory changes, with *Mycobacterium*, *Candida* and *Aspergillus* species the pathogens that were detected. No correlation was found between CD4+ lymphocyte count and opportunistic pathogens.

5.2 RECOMMENDATION

Sputum cytology should be used for preliminary diagnoses of opportunistic pathogens before confirmatory test.

5.3 LIMITATION

Special stains were not comprehensively used in identification of the opportunistic pathogens.

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APPENDICES

APPENDIX I: Face to face structured questionnaire

Pattern of opportunistic infections and cytological changes in sputum specimen from HIV infected patients at Mbagathi District Hospital.

This form is to be filled by the interviewer before sputum sample collection

Study identity number……………………..

Gender……………………………. Age………………..

1. What is the level of education you have attained?

0	None
1	Primary
2	Secondary
3	Tertiary

2. What is your marital status?

1	Single
2	Married
3	Separated
4	Divorced
4	Widowed

3. What are your chief complaints?

0	Coughing
1	Loss of weight

0

Difficulty in breathing

3 Chest pains

Others (specify)……………………

4. For how long?

0 Less than 3wks

1 More than 3wks

5. Are you on medication?

0 Yes

1 No

6. What is your occupation?

0 Small business enterprise

1 Employed

2 Unemployed

Others (specify)…………………

7. Last level of CD4 count? (Patient File) ……………………..

8. Are you on antiretroviral therapy? ....

PATHOLOGICAL REQUEST FORM

Study identity number……….

Cytological report

Sample adequacy

Macroscopic appearance

Muco-purulent ☐ Muco-Salivary ☐ Blood Stained ☐

Microscopic appearance

Acid Fast Bacilli Positive ☐ Negative ☐

Viral cytopathic changes (specify)……………………………………………………..

Fungal infection (specify)…………………………………………………………….

Others………………………………………………………………………………….

Conclusion

Comment

Pathologist name………………… ………Sign……………….Date………………..

Cytologist name…………………. ....Sign……………….Date………………….

APPENDIX II: Pap staining (13)

1. The smears will be fixed in 95% alcohol
2. The smear will be hydrated in descending grades of alcohol 80%, 70% and 50% alcohol.
3. The smears will be stained in Harris Heamatoxylin for 4 minutes.
4. The smears will be rinsed in tap water
5. The smears will be differentiated in 0.05% tap water 10 dips
6. The smears will be rinsed in tap water and Scott's tap water 10dips
7. The smears will be rinsed in ethanol 10 dips.
8. The smears will be stained in O G.6 for 1.5 minutes.
9. The smears will be rinsed in 95% ethanol for 10dips
10. The smears will be stained E .A 5O for 1.5 minutes.
11. The smears will be dehydrated in two changes of absolute alcohol
12. The smears will be cleared in three changes of Xylene 10dips each
13. Mount in DPX.

APPENDIX III: Ziehl Neelsen's Staining Technique (6)

1. Scratch-free new glass slide will be cleaned using gauze and labeled with patient's laboratory number using a diamond pencil or an ordinary pencil on the frosted end of the slide.
2. Smears will be prepared from the centrifuged sputum deposits in the middle of the slides using rotational movement with applicator stick. The smears will be left to air dry.
3. Smears will be fixed by passing over a flame 2-3 times, smear uppermost.
4. The slides will be placed on the staining rack across a sink and flooded with carbol fuchsin.
5. Using cotton wool around the end of applicator stick, wet with alcohol and lit with a flame, the slides will be heated from underneath until vapour rises from stain. The stain will be kept hot for 10 minutes without letting it boil or dry up.
6. Picking up each slide with a pair of forceps, the smears will be rinsed with a stream of clean water over a sink
7. The smears will be covered with 20% sulphuric acid for 2 minutes. Rinsed with water. If smears turn red, they will be decolorized further until properly decolorized and appear pale pink after rinsing with water.
8. The slides will be returned to the staining rack and counter stained by covering the smears with 0.1% methylene blue for 1 minute.
9. The smears will be rinsed in a thin stream of clean water and allowed to air dry at room temperature.
10. The stained smears will be mounted with DPX

Reporting of ZN smears

More than 10 AAFBs per oil immersion field………………..+++

1-10 AAFBs per oil immersion field…………………………. ++

10-99 AAFBs per 100 oil immersions fields…………………. +

1-9 AAFBs per 100 oil immersion fields……………………..record exact count

No AAFBs seen in 100 fields of oil immersion………………...Negative

APPENDIX IV: Harris Heamatoxylin and Eosin stain(6)

Staining technique

1. The smears will be fixed in 95% alcohol
2. Stained in Harris heamatoxylin for 5 minutes.
3. Rinsed in tap water.
4. Differentiated in 1% acid alcohol, 3dips.
5. Rinsed in tap water.
6. Blued in Scotts tap water for 30 seconds or in running tap water for 10 minutes.
7. Counter stained in Eosin for 5 minutes.
8. Rinsed in tap water to remove excess eosin followed by 70% ethanol to obtain the desired shades of red and pink.
9. Dehydrated in the 3 changes of absolute alcohol.
10. Cleared in 3 changes of Xylene.
11. Mounted with D.P.X.

APPENDIX V: Periodic acid Schiff(6)

1. The smear will be fixed in 95% alcohol
2. The smear will be oxidized in 0.5 % periodic acid solution for 5min
3. The smears will be placed in Schiff reagent for 10minutes.
4. The smears will be rinsed in tap water.
5. The smears will be counterstained in Harris heamatoxylin for 4minutes
6. The smears will be rinsed in tap water.
7. The smears will be dehydrated in the 3 changes of absolute alcohol.

10. The smears will cleared in 3 changes of Xylene.

11. The smears will be mounted with D.P.X.

APPENDIX VI Standard Operating Procedure

Ziehl Neelsen's stain (6)

Reagents

1.3% Alcoholic Basic fuchsin solution

a. Basic fuchsin	3g
b.95% Alcohol	100ml

Procedure

100ml of 95% alcohol will be added to 3g basic fuchsin in a flask and shaken to dissolve. This will then be mixed with 55ml of aqueous phenol followed by continuous shaking and distilled water will be added to make 1000ml. This will be allowed to settle for 24hours and filtered before each staining procedure.

Aqueous phenol will be prepared by adding 10ml of distilled water to 100g of crystallized phenol. It will be boiled in water until complete dissolution and allowed to cool.

2. 0. 1% Aqueous Methylene blue Counter stain

Reagents

Methylene blue	1g
95% Alcohol	1000ml

1g of methylene blue will be dissolved by shaking in 100ml of alcohol and distilled water added to make 1000ml. The mixture will be allowed to settle for 24hrs and filtered before use.

3. 20% Aqueous Sulphuric Acid Decolourizer

Reagents

Concentrated Sulphuric Acid 20ml

Distilled water	980ml

Procedure

20ml of concentrated sulphuric acid will be added in small quantities while stirring in 980ml of distilled water.

Heamatoxylin and Eosin Stains

1. Harris' Heamatoxylin

Reagents

Heamatoxylin crystals	2.5g
Absolute alcohol	25ml
Distilled water	500ml
Alum (ammonium or potassium)	50g
Mercuric oxide	1.25g

Procedure

2.5 g of Heamatoxylin crystal will be dissolved in alcohol and 50g of alum will be dissolved in water by aid heating. The two solutions will then be mixed and heated to boiling and removed from heat and added mercuric oxide. As soon as the mixture turns dark purple it will be cooled quickly by plunging into water. When cool 2-4ml glacial acetic is added to enhance nuclear stain. The stained will be filtered before use.

2. Eosin Y 1% counterstain

Reagent

Eosin y	1g
95%ethanol	20ml
Distilled water	80ml
Total volume	100ml

1g of eosin will be dissolved in 20ml of water and then added 80ml of ethanol. The working solution will be diluted 1:2 with 80% alcohol.

3. 1% Acid alcohol

Reagent

Concentrated Hydrochloric Acid 10ml

70% ethanol 990ml

Procedure

With a pipette 10ml of Hydrochloric acid will be slowly added into 70% alcohol and shaken gently.

4. Scott's Tap Water

Reagents

Sodium Bicarbonate 3.5g

Magnesium Sulphate 20g

Distilled water 1000ml

3.5g of sodium bicarbonate and 20g magnesium sulphate will be dissolved in 1000ml of distilled water.

Periodic Acid Schiff

1. 0.5% Periodic Acid solution

Reagents

Periodic Acid 5g

Distilled water 100ml

5g of Periodic acid will be added to 100ml of distilled water.

2. Schiff's reagent

Reagents

Basic fuchsin 5g

0.5 sodium metabisulphate 10g

Distilled water 1000ml

Hydrochloric Acid 100ml

Activated charcoal 5g

Procedure

1gm of basic fuchsin will be dissolved in 200ml boiled distilled water and shaken for 5min

The mixture will be cooled to 50^0c and added 20ml of hydrochloric acid and then cooled to 25^0c.

1gm of sodium or potassium metabisulphite will be added, this will be stored overnight in a dark cupboard or fridge and should be straw coloured after 24hour.

2gms of activated charcoal will be added and shaken and well filtered

The reagent will be kept in dark bottles at $0\text{-}4^0$c

APPENDIX VII Informed consent

TITLE: Pattern of Opportunistic infections in sputum specimen from HIV/AID infected patients at Mbagathi District Hospital.

Researcher Statement

My name is Karuga T. Kamau a postgraduate student at the University of Nairobi. I wish to request you to participate in the study at your will. The purpose of this consent form is to give you information you will need to help you decide whether to be in the study or not. You are free to ask questions about the purpose of the research, your role in the study, the possible risk and benefits you will encounter, your rights as a volunteer and anything else about the research for clarification.

You are at liberty to be included in the study or not without any coercion. You are entitled to have a copy of this consent for your records.

Purpose and benefits

The purpose of this research study is to investigate opportunistic infections in sputum among HIV positive patients presenting with pulmonary conditions. The finding will aid in proper clinical management.

Risk, Stress or discomfort

Discomfort may be experienced during the interview due to the sensitive nature of the questions, but we will do our best to ensure that we make the procedure as comfortable as possible. We will discuss the questions with you in a language you are comfortable with. You may skip any questions that you are uncomfortable with. You may skip any questions that you don't want to answer and may terminate the interview at any time without consequence. You will also be free to withdraw from the study at any time you feel like. All records will be identified by serial numbers only, to maintain your confidentiality we will not record your names or the personal identifiers. Completed study forms will be kept in locked cabinets, in an access limited room at the study site.

Other information

You may refuse to participate and withdraw from the study at any time without consequences. You are also free to ask the investigator questions both before consenting to participate and anytime thereafter

Do you have any questions? …………………….

If any specify…………………..

Do you agree to participate in the study? ....

Name of researcher…………………. Signature of researcher…………………..

Date………………….

Subject statement

This study described above has explained to me and i volunteer to take part. If I have future questions about the research, I can contact the following,

CHAIRPERSON

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Participants name…………………………

Signature……………………….

Date……………………

Doctor Name ………………..

Signature……………………….

Date………………………………..

FOMU YA IDHINI

KICHWA CHA UTAFITI: UTAFITI WA MAGONJWA YANAYOAMBATANA NA UKIMWI.

Kwa majina naitwa Karuga T. Kamau, mwanafunzi kutoka chuo kikuu cha Nairobi. Ningependa kukujulisha kuhusu utafiti wa kuchunguza baadhi ya magonjwa yanayoambatana na ukimwi ambayo nakusudia kafanya. Madhumuni hasa ni kupata idhini kutoka kwako ili kutafiti na kujua kama umeadhirika au la. Ikiwa umeadhirika utapata matibabu yafaayo.

Kwa vyovyote vile waweza kuuliza maswali kuhusu utafiti huu uzuri wake na madhara ambayo yanaweza kukupata. Maswali yote ikijimbiwa kinaganaga waweza kushiriki au kukataa. Sitakabadhi hii ni maelezo ambayo waweza kujisomea mwenyewe ama kutafsiriwa.

Madhumuni aswa ya huu utafiti ni kuchunguza ikiwa una magonjwa yanayoambatana na ukimwi. Baadhi ya haya magonjwa ni kifua kikuuna saratani ya kifua na kadhalika. Haya madhara ya kigunduliwa mapema utapata matibabu.

Unapojiamulia kushiriki kuna maswali ambayo unapaswa kujibu kwa njia mwafaka. Baada ya hayo, muuguzi atakupa maagizo jinsi anavyo unatakikana utoe kikohozi. Kisha atakupa chombo kutia kikohozi cha asubuhi kabla ya kifungua kinyua.

Kulingana na takwimu za ukaguzi waweza kujipata au kugadhambishwa na yale mambo Daktari atakufanyia, ama pia maswali ambayo yanayodhuru roho yako. Maswali mengine waweza kuyaacha na kujibu yale ambayo yana kuridhishwa. Zitakabadhi zako zitahifadhiwa kwa njia inayofaa na hakuna mtu yeyote atarusiwa kuzisoma.

Ukiwa na maswali yeyote waweza kuuliza na ukiamua kutoshiriki waweza pia kujitoa kwa utafiti huu kwani hulamimishwi kufanya hivyo hakuna yeyote umefanya

Una maswali yeyote?

Umeamua kushiriki Kwa utafiti huu?

Njina la mtafiti ………………….   Sahihi……………………………..

Tarehe…………………

Uamuzi wa mshiriki

Nimeleezwa vyema juu ya huu utafiti,mimi mwenyewe nimeamua bila kushurutiswa na yeyote.Nikiwa na maswali juu ya huu utafiti nitamjulisha