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COLLEGE OF BASIC AND APPLIED SCIENCES

AUTOPSY CHARACTERIZATION OF LUNG MICROBIOME OF HIV-POSITIVE PATIENTS IN A TERTIARY REFERRAL HOSPITAL IN GHANA

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A THESIS SUBMITTED TO THE DEPARTMENT OF BIOCHEMISTRY, CELL AND MOLECULAR BIOLOGY, UNIVERSITY OF GHANA, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF PHILOSOPHY DEGREE IN MOLECULAR CELL BIOLOGY OF INFECTIOUS DISEASES

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DECLARATION

This is to certify that this thesis is the result of research undertaken by Pheonah Badu towards the award of Master of Philosophy in Molecular Cell Biology of Infectious Diseases in the Department of Biochemistry, Cell and Molecular Biology, University of Ghana.

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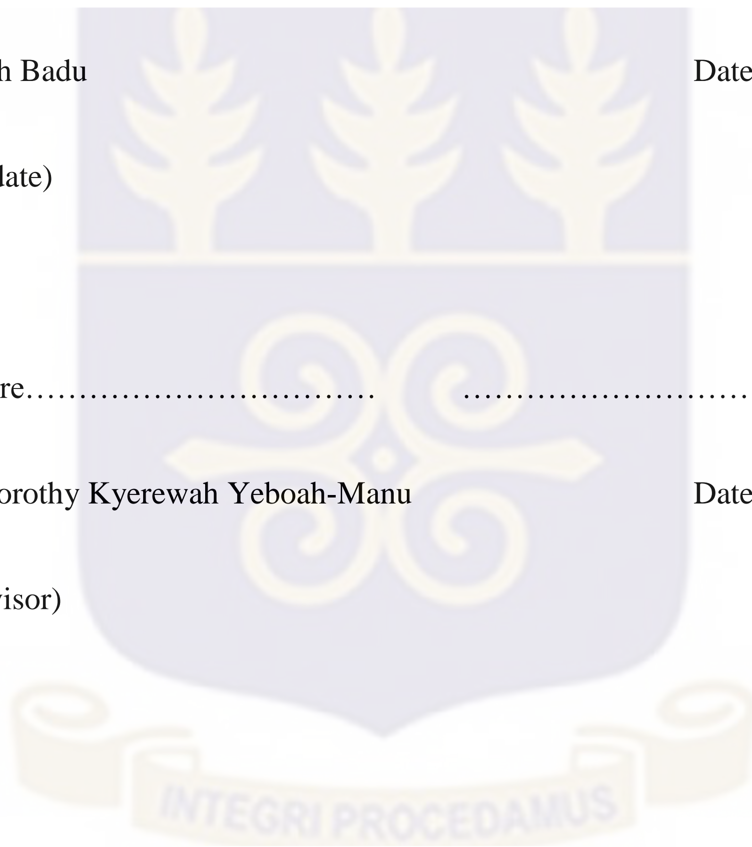

Signature…………………………… ……………………………………..

Prof. Dorothy Kyerewah Yeboah-Manu Date

(Supervisor)

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ABSTRACT

Pulmonary infections are the underlying cause of high morbidity and mortality amongst Human Immunodeficiency Virus (HIV) infected persons. Notwithstanding, there is limited data on pulmonary co-infecting pathogens and their susceptibility to commonly used antibiotics. Knowledge on these pathogens is therefore critical for implementing effective interventions and treatment guidelines especially, in highly burdened countries with scarce diagnostic facilities. The aim of this study was to characterize the lung microbiome of post-mortem biopsy samples of HIV/AIDS (Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome) patients in Ghana.

A total of 102 lung biopsies from HIV/AIDS decedents from the Korle-Bu Teaching Hospital in Ghana were examined for mycobacteria, other bacteria, fungi and viruses. The techniques utilized included; culture, Gram/Ziehl Neelsen (ZN) staining, Matrix Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) mass spectrometry and Polymerase Chain Reaction (PCR). The drug susceptibility pattern of *Mycobacterium tuberculosis complex* (MTBC) isolates and other bacteria were subsequently determined using Genotype MTBDR*plus* and disc diffusion assays respectively. Clinical data and autopsy causes of death were also retrieved for 86 cases and inferences were made by correlating the data to that of the laboratory investigations.

From the clinical data, 42 (48.8%) of the cases were males and 42 (48.8%) were females with the mean ages of 40.4 (±10.6) and 37.1 (±11.5) respectively. HIV type was defined in 39 (45.3%) cases and co-infections with tuberculosis (TB), pneumonia, oesophageal candidiasis and/or cryptococcal disease occurred in 12 (14.3%) cases.

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Characterization of mycobacterial isolates yielded, 25 MTBC and 5 *M. abscessus*. In addition, an acid-fast isolate was identified as *Norcardia farcinica*. Drug susceptibility testing of the MTBC isolates showed 1 isoniazid mono-resistance and 3 rifampicin mono-resistance. Other bacteria isolated were 217 (83.8%) with *Enterococcus species* (61), *Staphylococcus species* (35), *Escherichia coli* (28) and *Klebsiella pneumoniae* (23) dominating. Of the 217, 75 Gram-negatives and 117 Gram-positives were profiled for drug sensitivity. Gram-negative isolates were most susceptible to cefoxitin and gentamicin (45.3% each) but highly resistant to cefuroxime sodium (84.0%). The Gram-positives were also fairly susceptible to levofloxacin (58.0%) but highly resistant to oxacillin (81.2%) and flucloxacillin (75.2%). Also, nine (9) culturable fungi; *Candida species* (6), *Cryptococcus neoformans* (1), *Pichia occidentalis* (1) and *Yarrowia lipolytica* (1) were identified whereas PCR detected *Pneumocystis jiroveci* in 43 samples. Viruses detected in the samples included *Cytomegalovirus* (67); a DNA virus and RNA viruses; *Parainfluenza-2* (1) and *Enterovirus* (1).

Findings from this study calls for the introduction of comprehensive definitive laboratory investigation rather than empirical clinical diagnosis to identifying the exact underlying pathogens that cause pulmonary infections in managing HIV patients in Ghana.

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DEDICATION

I dedicate this work to the Almighty God for being with me throughout the periods of my course work and research work and to my parents for their care and support.

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LIST OF ABBREVIATIONS

HIV	Human Immunodeficiency Virus
AIDS	Acquired Immune Deficiency Syndrome
WHO	World Health Organization
UNAIDS	United Nations Programme on HIV/AIDS
MDR	Multi-Drug Resistance
MTBDR*plus*	*Mycobacterium tuberculosis* drug resistance
INH	Isoniazid
MTBC	*Mycobacterium tuberculosis* complex
MTBss	*Mycobacterium tuberculosis* sensu stricto
MAF	*Mycobacterium africanum*
NTMs	Non-tuberculous Mycobacteria
NTP	National Tuberculosis Control Programme
PCR	Polymerase Chain Reaction
RIF	Rifampicin
TB	Tuberculosis
LAV	Lymphoadenopathy-Associated Virus
HTLVV-III	Human T-cell leukemia/lymphoma virus
SIV	Simian Immunodeficiency Virus
ART	Anti-Retroviral Therapy
HAART	Highly Active Anti-Retroviral Therapy
LTNP	Long-term Non-progressors
CDC	Center for Disease Control and Prevention
LHMP	Lung HIV Microbiome Project
CMV	Cytomegalovirus

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PCP	*Pneumocystic carinii* pneumonia
PJP	*Pneumocystic jiroveci* pneumonia
TMP-SMX	Trimethoprim-sulfamethoxazole
NIH	National Institute of Health
MPV	*Meta-pneumovirus*

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Acquired Immune Deficiency Syndrome (AIDS), caused by Human Immunodeficiency Virus (HIV) is an infectious disease that greatly burdens global health. Currently, there are 36.7 million HIV-reactive patients globally, representing a prevalence of 0.8% with differences existing across regions, gender, age and special groups (e.g. sex workers). This high global incidence is attributed to the cumulative effect of old infections (as a result of longer patient survival time since the introduction of Anti-retroviral therapy (ART)) and new infections (Maartens *et al*., 2014; Benito *et al*., 2012). In 2015 alone, new HIV infection and death estimates were 2.1 million and 1.1 million respectively (UNAIDS, 2016). Furthermore, the HIV problem is exacerbated by the frequent occurrence of pulmonary infections such as tuberculosis (TB) and pneumonia, which occur in 70% of all HIV patients. These diseases mark the terminal stage of HIV infection referred to as AIDS and have been identified as the most common cause of hospitalization and death in HIV patients worldwide (Afessa *et al*., 1998; Benito *et al*., 2012; Kyeyune *et al*., 2010). HIV/AIDS is disproportionately distributed among men and women with respective rates of 49% and 51%. Even though, the incidence of HIV in children has declined by 70% in the last 15 years, 150,000 children were newly infected and 110,000 died in 2015 (UNAIDS, 2016).

Low and middle-income countries are the most endemic zones of HIV/AIDS and this is mainly due to the ineffective preventive, diagnostic and treatment implementation in these

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countries (Sobrino-Vegas *et al*., 2009; Mugavero, 2008). Sub-Saharan Africa which has the highest disease burden houses approximately 25.5 million cases (70%) out of the global estimate of 36.7 million (UNAIDS, 2016; WHO, 2016). Within the region, East and South Africa record the greatest burden of 19 million cases and 470,000 deaths, whiles Middle-east and North Africa bear the least; 230,000 infections and 12, 000 deaths (UNAIDS, 2016). HIV/AIDS is also present in Ghana and in 2015, 270,000 people were reported living with the virus and 13,000 AIDS-related fatalities were reported (UNAIDS, 2016). The disease affects more women than men and the trend among these groups closely mirrors the global distribution with approximately 56% of women living with HIV and children under 15 years contributing about 7% to the total rates (UNAIDS, 2016).

The causative agent for HIV belongs to the family *Retroviridae* and genus *Lentivirus*. Two main infecting strains of the virus exist; HIV-1 and HIV-2.Whilst HIV-1 has been disseminated globally since its emergence; HIV-2 is mostly restricted to the West African sub-region, however the mechanisms for this geographic restriction is still unknown. Moreover, HIV-1 is further classified into the sub-groups M, N, O and P based on genotype diversity, virulence and rate of infectivity (Worobey *et al*., 2008). Besides these, new genotypes are currently been rapidly identified as a result of the evolving nature of the virus (Lau & Wong, 2013; Shriner *et al*., 2004; Jetzt *et al*., 2000; Mansky & Temin, 1995).

The destructive effect of HIV infection is mainly due to the tropism of the virus for immune effector cells, especially for active CD4+ T lymphocytes. Upon entry into host cell, HIV integrates its genetic material into the host genome and takes over the replication

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machinery for its survival (Abbas, 2000). Thus, the host cell and viral RNA transcription and translation are synchronized to produce multiple copies of viral genetic material. Eventually, viral proteins produced are assembled into the new viral particles by the viral protease enzyme within the host cytoplasm. These egress by budding out of the host cell, expending the host cell membrane through formation of viral envelopes in the process, causing cell death in the end (Abbas, 2000; Pantaleo & Fauci, 1996). In addition, CD4+ T cells are depleted in HIV infection through other mechanisms such as direct killing by cytotoxic CD8+ T cells, pyroptosis and apoptosis of uninfected bystander cells (Doitsh *et al*., 2014; Garg *et al.,* 2012*;* Kumar *et al.,* 2012). As the levels of CD4+ T cells decline, cell-mediated immunity is gradually lost and this predisposes the infected individual to various opportunistic infections of bacterial, fungal or viral etiology such as pneumonia, tuberculosis, non-tuberculous mycobacterial infections, oesophageal or lung candidiasis and cryptococcal infections (CDC, 2016).

In most cases, localized opportunistic infections occur within the lungs and cause pulmonary complications in HIV patients. However, the incidence of specific pulmonary diseases differ with prevalence and geographical location. For example, due to the high prevalence of TB in Africa, it is the most common cause of pulmonary disease in HIV patients living in the area as compared to *Pneumocytic jiroveci pneumonia* (PJP) which is rather predominant in Western Europe (Daley *et al.*, 1996; Van Oosterhout *et al.*, 2007; Fisk *et al*., 2003). Besides immune suppression, another factor that possibly influences the incidence of opportunistic infections especially in the lungs of HIV patients is alteration of

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the microbiota as, stable microbial communities normally exist in healthy individuals (Practice, 1998).

Previously, the lung was considered sterile until recent data from Microbiome studies in both healthy and HIV patients disputed this fact. Major findings from this work show that the lung microbiome of healthy and HIV-infected individuals with preserved CD4+ T cell levels are comparable (Beck *et al*., 2015; Iwai *et al*., 2014). Nevertheless, significant differences in microbiome diversity and distribution existed between HIV-infected persons with advanced disease and uninfected controls. With these observations, severe immunosuppression has been associated with alterations in the lung microbiome and hence implicated in respiratory infections though this postulate is subject to further investigations (Twigg *et al*., 2017).

Even though HIV/AIDS is incurable, the introduction of combination anti-retroviral therapy (comprising of three or more anti-retroviral agents) has rendered the otherwise fatal disease chronic by extending life expectancy and reducing ailments and deaths (Palella *et al.*, 1998; Aberg *et al*., 2014). In essence, combination ARTs (cART) are the standard treatment drugs used as transmission preventive therapy for high risk HIV-negative groups in addition to its therapeutic use in HIV-positive persons (Kalapila & Marrazzo, 2016). Drugs included in cART target the different steps of viral life cycle providing constant viral suppression below detection limits whilst, simultaneously preventing the emergence of resistance. These drugs are distributed among five classes namely; nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (nNRTI), protease inhibitors (PI), entry inhibitors (CCR5 antagonist and fusion inhibitors) and

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integrase inhibitors. Examples of recommended drugs that fall in each respective class are Zidovudine (AZT), Efavirenz (EFV), Darunavir (DRV), Maraviroc (MVC), and Raltegravir (RAL) (Kalapila & Marrazzo, 2016; Pau & George, 2014).

Despite massive improvement in HIV management with cART, treatment is often hampered by poor adherence due to pill burden, drug-related toxicities, adverse drug-drug interactions particularly in patients with co-morbidities as well as some physical and psychological factors (Rudy *et al*., 2010; Agwu & Fairlie, 2013). Efforts to address some of these challenges include the introduction of an ART arsenal with greatly reduced toxicities and formulation of multiple fixed dose (FDC) tablets which also reduces the pill burden for patients (Cohen *et al*., 2011; Charlebois *et al*., 2011). Compliance to recommendations for administering ART in HIV-infected individuals with opportunistic infections is also necessary to prevent adverse events.

1.2 Rationale

Pulmonary infections cause high morbidity and mortality in HIV-infected persons. The current prevalence rate of pulmonary diseases in HIV patients is 70% (Benito *et al*., 2012) with a 25 fold chance of contracting lower respiratory tract infections as compared to uninfected persons (Feikin *et al*., 2004). The annual incidence of hospital admissions that results is about 20 to 25 per 100 episodes (Benito *et al*. 2001, Benito *et al*., 2004). These figures emphasize the severity of respiratory infections among HIV-infected populace.

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The anatomical location of the lung makes it prone to infection; it is usually the first internal organ of exposure to pathogens (Chen & Kolls, 2013). To this effect, the lung in a healthy person has a complex immunity comprising of various cells predominantly alveolar macrophages, which mount the appropriate response against invading pathogens. In HIV-infected individuals, due to the massive decline of CD4+ T cells associated with increased viral replication within these cells, the intricate lung immune system becomes deficient and dysfunctional, causing immune abnormalities which predispose infected individuals to opportunistic pulmonary infections (Chen & Kolls, 2013). The high predisposition to lung infections consequently causes high disease and death tolls among HIV/AIDS patients.

Notwithstanding, there are limited studies on lung microbiome and pulmonary infections in HIV populations especially in Africa where the HIV/AIDS pandemic is hardest hit. Moreover the few conducted studies hardly explored in detail the full spectrum of etiological agents in the lungs and their prevalence. Such investigations are usually silent on the mechanism by which the infections are established and antibiotic susceptibility patterns of the microbes. In Sub-Saharan Africa the few studies that have been conducted had an added challenge with the use of inconclusive methods such as clinical diagnoses and verbal autopsies. Only a few isolated studies have been reported in literature where microbiological methods were used in conjunction with clinical procedures (Cox *et al*; 2010; Karat *et al*., 2016; Bates *et al*., 2015). The sole reliance on clinical diagnoses for determining disease causing pathogens often times does not address the specific cause of the infection due to similar and overlapping clinical presentations exhibited by the various

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microorganisms. As such, cases of multiple infections can elude diagnosis and misdiagnosis can also occur easily leading to case mismanagement (Cox *et al*., 2010).

There is therefore an urgent need to characterize these pulmonary microorganisms to give insights into the predominant species in HIV patients and their antibiotic susceptibilities patterns. This is critical for implementing effective interventions and treatment guidelines in addition to, providing an improved prognostic approach in the clinical management of HIV-positive patients in Ghana.

1.3 Research Questions

- Which pathogenic bacteria, fungi and viruses occur in the lungs of HIV patients?
- Are there multiple concurrently infecting microorganisms in the lungs of HIV patients?
- What is the antibiotic susceptibility pattern of the bacteria isolated?

1.4 Aim

The aim is to characterize the lung microbiome in post-mortem biopsy samples of HIV/AIDS patients.

INTEGRI PROCEDAMUS

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1.5 Specific Objectives

1. To isolate cultivable bacteria and fungi from post-mortem lung tissues using conventional culture methods.

2. To characterize culture isolates using MALDI-TOF MS and PCR.

3. To determine the antibiotic sensitivity profile of the identified bacteria.

4. To characterize viral pathogens present in autopsy samples by real time PCR.

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CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Origin and historical trends of the HIV/AIDS Epidemic

The Human Immunodeficiency Virus (HIV) is alleged to have emerged from Kinshasa in the Democratic Republic of Congo between 1884 and 1924 after evolving from Simian Immunodeficiency Virus (SIV) (Worobey *et al*., 2008). Chimpanzees, specifically the subspecies *Pan troglodytes,* which are natural reservoirs of SIV, have been presumed to be the source of HIV-1 infection (Gao *et al*., 1999). The probable route for this zoonotic infection has been attributed to the hunting and eating of chimpanzees by indigenes of Kinshasa at the said period (Hahn *et al*., 2005). It is postulated that the virus then spread rapidly yet silently, until in the 1980's when increasing cases of severe immune deregulation, aggressive cancers (such as the Kaposi's sarcoma) and opportunistic infections mainly, *Pneumocystic jirovecii* pneumonia were reported among homosexual men as disease symptoms (CDC, 1981).

In the early epidemic season, most of the cases were reported in the United States of America and the term Acquired Immune Deficiency Syndrome (AIDS) was used for the first time by the CDC in September, 1982 explaining that this is "a disease at least, moderately predictive of a defect in cell mediated immunity, occurring in a person with no known case for diminished resistance to that disease" (CDC, 1982). The first viruses implicated as causes of AIDS were Lymphoadenopathy-Associated Virus (LAV) and the Human T-cell leukemia/lymphoma virus (HTLV-III) which were later confirmed to be same as the HIV virus in 1983 (Barre-Sinoussi *et al*., 1983; Laurence *et al*., 1984).

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The viral spread relentlessly continued and by the end of 1985, at least one case had been reported in each region in the world with a total global estimate of 20,303 (Veronika, 2013). Hardly hit regions in the early outbreak period included Africa, Americas, Europe, Oceania and Asia with infection estimates of 2,323, 31,741, 3,858, 395 and 84 respectively (Bureau of Hygiene and Tropical Diseases, 1986). The rapid spread of the infection prompted the establishment of intervention strategies such as introduction of the anti-retroviral therapy including Zidovudine (James, 1995), the Highly Active Anti-Retroviral Therapy (HAART) (AIDSinfo, 1987), approval of various specimen for HIV tests and test kits (The Henry J. Kaiser Foundation, 2014; Jon Cohen AIDS Research Collection, 1992) as well as dispelling the stigma infected persons faced in the ensuing years of the epidemic (The New York Times, 1991).

Extensive studies on SIV, the evolutionary ancestor of HIV have shown that, there are about 40 diverse African primates-adapted strains (Figure 2.1). Surprisingly, while inhabiting their natural hosts, these SIV strains remain non-pathogenic despite their phylogenetic relatedness to HIV (Sharp & Hahn, 2011). However, an exception to this observation occurs in macaques (Asian primates) in which, the presence of SIVs are associated with immunodeficiency. This information seemed controversial initially but, further studies confirmed that, macaques were certainly not natural reservoirs for SIV therefore their presence led to AIDS related pathologies. A historical review of events shows that, the first incidence of the SIV in macaques was as a result of cross species transmission triggered by an experimental mishap at the California National Primate Research Center. In this experiment termed "Kuru experiment", macaques were inoculated

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with blood or tissue from sooty mangabeys (natural carriers of SIV) (Apetrei *et al*., 2005, Apetrei *et al*., 2006). Although the intended aim of the Kuru experiment was to model prion diseases in different primates it turned out as the origin for AIDS-related simian infection in macaques (Apetrei *et al*., 2006).

Despite the high degree of SIV species-specific transmission that occurs in primates, few instances of cross species transmission have been documented and these have generated recombinant and super-infectious naturally occurring SIVs. An example of such recombinant SIV is the type found in chimpanzees. This was acquired from two smaller primates; the greater spot-nosed monkey (*Cercopithecus petaurista*) and the red-capped mangabey (*Cercocebus torquatus*) (Sharp & Hahn, 2010). According to Hahn, “Chimpanzees also acquired their infection like humans did, by hunting and consuming naturally infected smaller primates” (Hahn *et al*., 2005).

Both SIV and HIV are lentiviruses that suppress immune function in their pathogenesis (Worobey *et al*., 2010). It is worth noting that HIV-1 is similar to the SIV strain found in chimpanzees whereas HIV-2, is closely related to the sooty mangabey SIV strain (Figure 2.1) (Sharp & Hahn, 2011). The origin of HIV-2 has been traced to West Africa since sooty mangabeys from which they evolved are natural inhabitant of the wild in West Africa (Figure 2.2) (Hirsch *et al*., 1989). HIV-2 is therefore highly prevalent among individuals in West African countries including Ghana, Nigeria, Sierra Leone, Mali, Senegal, Cote d’ivoire and Liberia (Sharp & Hahn, 2011; Fischer & Madden, 2011; Beaupre *et al*., 2006).

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The HIV-1 on the other hand, originated from the Democratic Republic of Congo as shown in Figure 2.2.

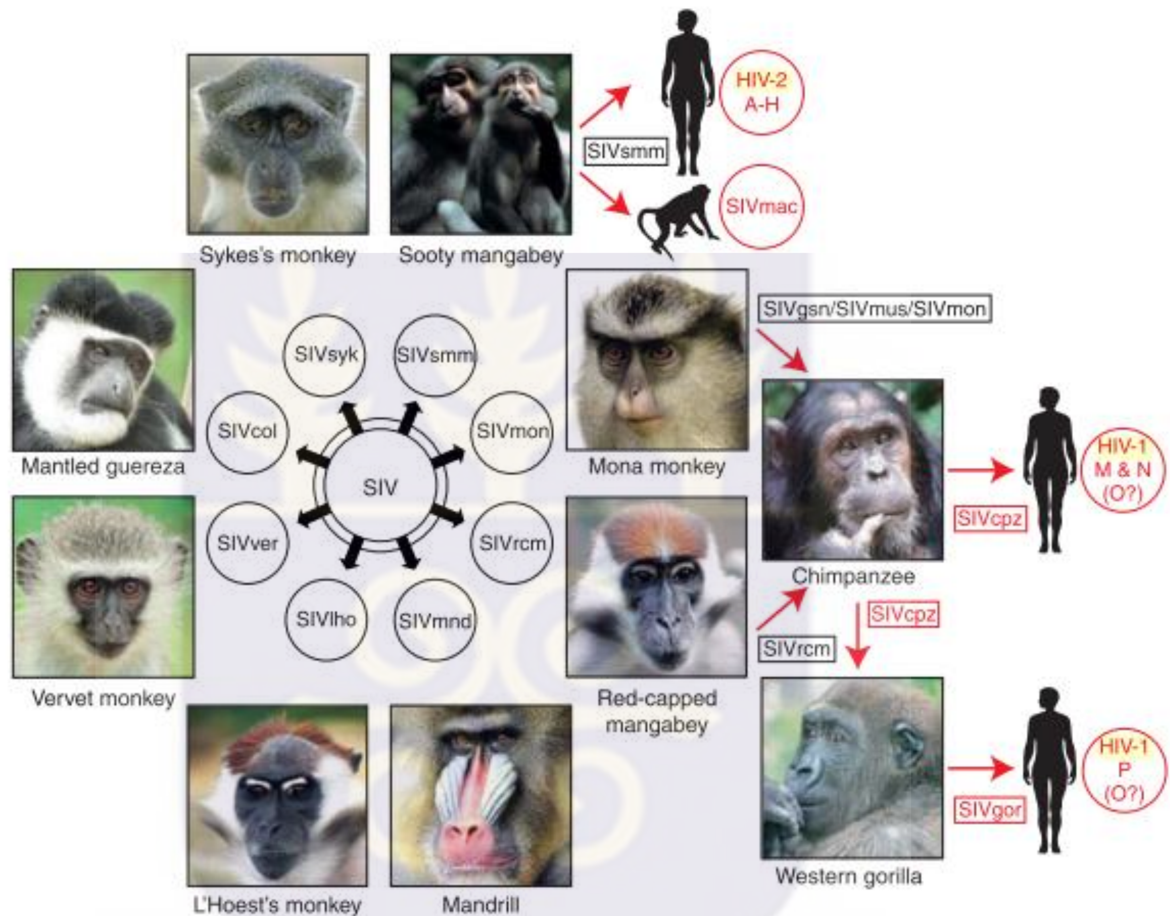

Figure 2.1: Origins of Human Immunodeficiency Virus. *African primates-adapted strains of SIV and their respective natural reservoirs are depicted in this figure. HIV-1 (particularly subtypes M, N & P) are believed to have evolved from the recombinant SIV strain in chimpanzees acquired from two smaller primates. HIV-2 together with the macaque SIV strain also evolved from SIV from sooty mangabey (Sharp & Hahn, 2011).*

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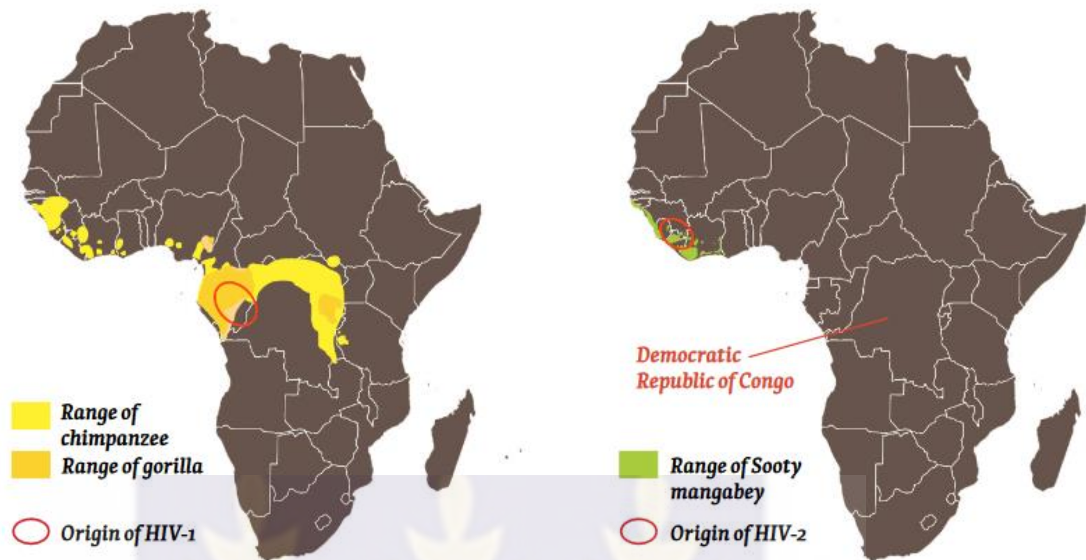

Figure 2.2: Regions where HIV-1 and 2 originated. *The red rings on the maps above show the approximate areas in which the two forms of HIV are thought to have originated. These areas coincide with the distribution of primates carrying SIV, which is genetically similar to HIV (Fischer & Madden, 2011).*

2.2 The Global HIV/AIDS Burden

HIV/AIDS is a disease that threatens global health. Significantly, HIV has infected 70 million people and claimed 35 million lives since its emergence (WHO, 2016). The current trends still suggest high disease burden although much efforts have been made towards detection, prevention, and management of this epidemic especially, with the advent of Anti-retroviral therapy (ART). As reported by both the World Health Organization (WHO) and the Joint United Nations Programme on HIV/AIDS (UNAIDS), there were 36.7 million infected persons worldwide at the end of 2015 (UNAIDS, 2016; WHO, 2016) signifying an upsurge from the 33.3 million estimate for the year 2010 (UNAIDS, 2016). This rise is ascribed to the accumulation of new infections (although this occurs at a declined rate) and old infections due to the extended survival time provided by ART

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(Maartens *et al*., 2014). In 2015, 2.1 million new infections and 1.1 million HIV-related deaths were reported (Wang *et al*., 2016, UNAIDS, 2016; WHO, 2016). HIV infects people of all ages and gender with higher disease prevalence among women (17.8 million) and an estimated 1.8 million children (under 15 years) harbouring the infection (UNAIDS, 2016). The main transmission route is by heterosexual intercourse and the gender-associated disparity in disease burden is due to anatomical differences between the male and female genital organs. The biological vulnerability of women is because semen usually contains higher concentrations of HIV than vaginal secretions (WHO, 1993). The vagina also provides the desirable environment for viral entry and multiplication unlike the male genital which is external and for that matter, constantly exposed to soap and cold environment (Healy, 1995). The global prevalence of HIV infection in adults is 0.8% but variations exist across countries depending on the level of endemicity (Figure 2.3) (WHO, 2016).

Across the globe, sub-Saharan Africa has the highest disease burden, with nearly 25.5 million infected persons representing 70% of people living with HIV worldwide and 3,000 deaths recorded daily (WHO, 2016). The Southern and Eastern parts together with the Western and Central African regions are the greatest contributors to the alarming regional estimate with 19 million and 6.5 million cases respectively (UNAIDS, 2016). Aside Africa, Asia and the Pacific are the next severely affected regions accounting for nearly 8.5% of new infections and 180,000 deaths annually (Wang *et al*., 2016). In spite of this burden, a sharp decline has occurred in the number of new infections in children since 2010 from

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290,000 to 150,000 in 2015. This progress was achieved through the active prevention and eradication of mother-to-child transmission of HIV (UNAIDS, 2016).

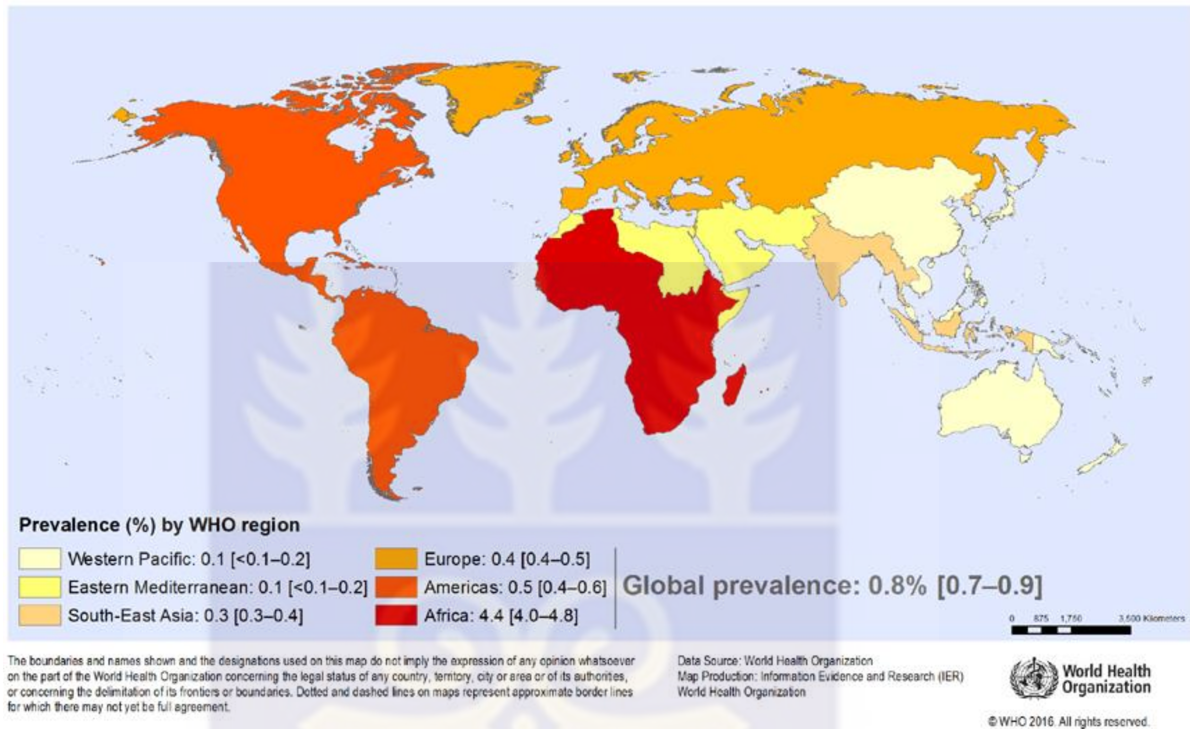

Figure 2.3: Adult HIV prevalence by WHO region for 2015 (WHO, 2016)

2.3 HIV/AIDS in Ghana

Ghana lies in the endemic belt of HIV-2 infection and so both HIV-1 and 2 are present in the country. The first incidence of AIDS in Ghana was recorded in March 1986 in Accra (Neequaye *et al*., 1987; Ampofo, 2005) and the transmission progressed with increasing infection rates at the turn of each New Year. In 1987, 107 confirmed HIV-positive cases were reported and this rose to 333 in 1988 and 2, 744 by April, 1990, out of which 1, 226 individuals had AIDS-related morbidities (Library of congress, CIA, 1994). Due to the rapid spread of the virus and the high infection estimates, various intervention strategies

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and programs were put in place to help combat the disease. This mainly involved setting up surveillance systems by the Ministry of Health with funding from the World Health Organization and creating awareness of the disease nationwide, with the aim of tracking and preventing spread of the virus in both high and low risk groups. These intervention programs facilitated massive improvements in case detection in the country with 12,500 cases recorded in the 1994, placing Ghana in the second rank for the infection in West Africa at the time (Biritwum 2014).

During the early phase of the epidemic in Ghana, Eastern Region had the highest disease prevalence and this was linked to the return of natives who travelled to neighbouring endemic countries such as Cote d'Ivoire (Neequaye *et al*., 1987). Recent reports suggested that the Volta and Brong Ahafo regions had the highest prevalence of 2.7% whiles Northern region had the lowest prevalence (0.7%) (www. ghanaids.gov). In terms of distribution of HIV infection with age in the country, the highest prevalence was recorded within the 45-49 years age group, whiles the lowest (0.8%) was within the 15–19 years age group (Ghana Health Service, 2013).

In Ghana, the infection is defined as an "established low level generalized epidemic" mostly with higher occurrence among certain communities and special groups (Ghana Health Service, 2013). The special groups, comprising of female sex workers and homosexual men recorded a prevalence of approximately 4.5% and 2.6% respectively in 2013. According to the UNAIDS 2016 report, 270,000 people had the infection and 13,000 resultant deaths were recorded in 2015. Also, the adult prevalence in 2015 was reported as

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1.6% and the distribution among male and female was 43% and 57% respectively. HIV-1 accounted for 92% of all HIV cases, HIV-2 was responsible for 0.5% of the cases and dual infections with HIV-1 and HIV-2 formed 7.4% of all reported HIV cases in the country (UNAIDS, 2016).

Furthermore, anti-retroviral therapy (ART) was introduced in Ghana in 2003 with the initial establishment of three ART sites. The number of ART sites has since increased significantly to 160 centers across the country and by 2011, a total of 65,342 people had been enrolled (Ghana Health Service, 2011). Currently, the ART regimen recommended for treatment in Ghana is based on rational selection of drug regimen, maximizing adherence to selected regimen, preserving future treatment options and frequently testing for HIV drug resistance in selected clinical settings (National HIV/AIDS/ STI Control Programme, 2010). Ghana utilizes triple combination of antiretrovirals (Highly Active Antiretroviral Therapy) thus, mono or dual therapy are not used under any circumstances for the treatment of people living with HIV. The recommended regimen should include; 2 Nucleoside/tide Reverse Transcriptase Inhibitors (NRTIs) and 1 NonNucleoside Reverse Transcriptase Inhibitor (NNRTI) or 2 NRTIs and 1 boosted Protease Inhibitor (PI) (National HIV/AIDS/ STI Control Programme, 2010).

Other achievements in HIV control in the country aside ART coverage include; creation of disease awareness, reducing stigmatization, HIV Testing and Counseling (HTC) and prevention of mother-to-child HIV transmission. The ultimate goal of the country toward HIV control aligns with that of the UNAIDS 90-90-90 target to ensure that, 90% of HIV

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infected persons know their HIV status, 90% of people aware of their HIV status have access to therapy and 90% of these have repressed viral loads, especially among adolescents, and much efforts are being channel to that end (UNAIDS, 2016).

2.4 Pathogenesis and Clinical Presentation

2.4.1 The HIV Virus

HIV belongs to the family retroviridae, genus lentivirus and is a spherical RNA (ribonucleic acid) virus with a diameter of 100 nm and a genome size of approximately 9.8 kilo base pairs (Castro-Nallar *et al.*, 2012). The genome consists of two identical copies of positive sense RNA strands, encoding nine (9) genes (thus; *LTR, gag, pol, vif, vpr, vpx/vpu, rev, env tat*and *nef*) (Figure 2.5) which express about 2000 proteins. The viral structure comprises of an outer bilayer lipid envelop from which the transmembrane trimeric glycoproteins gp41 projects with gp120 as a knob (Figure 2.4). These two proteins, mostly involved in cell attachments interact non-covalently (Modrow *et al.*, 1987) and are encoded by the *env gene,* first expressed as gp160 homotrimer glycoprotein and subsequently cleaved by furin protease on the cell surface into gp120 and gp41 (Allan *et al.*, 1985; Cleghorn *et al.*, 2005). Underlying the outer envelope is a matrix protein p17 and this surrounds the core protein antigen p24; a capsule containing the RNA strands, associated nucleocapsid protein 7 and enzymes (reverse transcriptase (p66), protease (p11) and integrase (p32)) (Figure 2.4). The *gag* and *pol* genes express all internal proteins and enzymes respectively (Abbas, 2000; Rajarapu, 2014) whereas *tat* (transactivator of transcription), *vpr* (viral protein R), *rev* (regulator of viral protein expression), *vif* (viral

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infectivity protein), and *nef* (negative regulatory factor) function as regulatory and immuno-modulatory genes.

The viral structure as described above is similar in HIV-1 and 2 however, disparities exist at the genome level. The viral protein U (*vpu*) gene is exclusive to HIV-1 whereas viral protein X (*vpx*) is found only in HIV-2 (Figure 2.5) (Cleghorn *et al*., 2005). High sequence variability also occur in the gene encoding aspartic acid protease enzymes, essential for viral maturation in the two viral strains and causes varying enzyme-substrate affinities. Aside these differences, the genomes of the two HIV types have sequence homology of 58%, 59% and 39% in the *gag, pol* and *env* genes respectively. In addition, both viruses have similar terminal repetitive nucleotide sequences referred to as the Long Terminal Repeat (LTR). These serve as sticky ends and facilitate insertion of viral genome into that of the host by the integrase enzyme, as well as function as promoters and enhancers of transcription once insertion is successful (Clavel *et al.,* 1986).

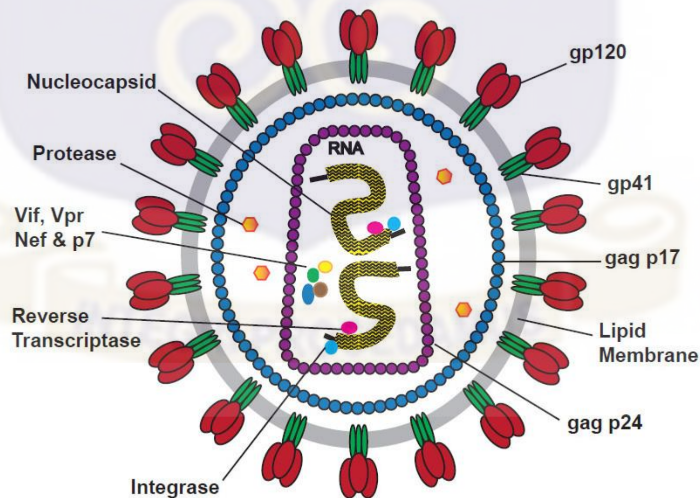

Figure 2.4: Viral structure of HIV (www.eenzyme.com/HIVResearchTools-2.aspx)

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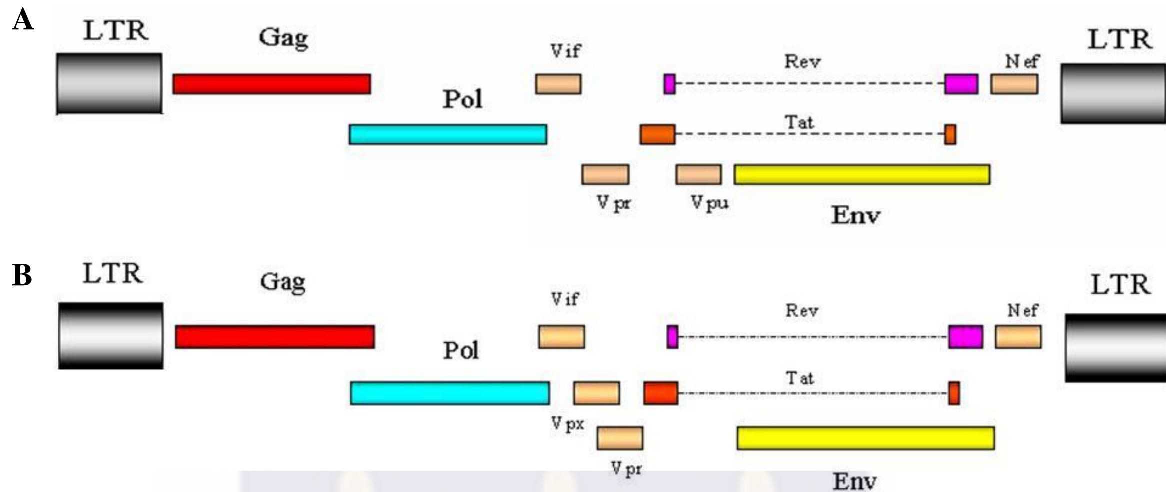

Figure 2.5: Genome layouts of HIV-1 and 2. *(A) HIV-1 genome showing 9 shared genes with HIV-2 and one exclusive gene (vpu) (B) HIV-2 genome showing 9 shared genes and one unique HIV-2 gene (vpr) (www.emedicine.medscape.com/article/965086-overview)*

2.4.1.1 HIV Sub-Types

HIV-1 is further classified into 4 groups namely, major (M), outlier (O), non-M/non-O (N) and the recent putative group identified (P) (Figure 2.6). The different groups represent distinct transmission events arising from different primates and have distinctive geographical boundaries of circulation with N, O, and P usually confined to the West African region whiles M is globally distributed. Group P infection in particular has only being reported in Cameroon but with very low prevalence (0.06%) (Vallari *et al*., 2011).

The major (M) group has further evolved into 9 genotypically distinct sub-types (or clades) denoted as, A, B, C, D, F, G H, J, K and 55 circulating recombinant forms (CRFs) (Figure 2.6) (Lau & Wong, 2013). These sub-types have genetic variability of 30% and 15% in the *env* as well as the *pol* and *gag* genes respectively (Worobey *et al*., 2008; Hemelaar *et al*.,

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2006). Specific HIV-1 sub-types are usually associated with distinct risk groups; injection drug users and homosexuals have marked sub-type B infection and heterosexual individuals are associated with infections by HIV sub-types other than B (Mastro *et al*., 1997; van Harmelen *et al*., 1997; Louwagie *et al*., 1993). Moreover, genetic divergence of about 10% also occurs within each of the given sub-types yielding numerous sub-subtypes designated with numerals (eg. A3 or B2) (Meloni *et al*., 2004).

Conversely, circulating recombinant forms (CRFs) are formed when the genomes of two distinct sub-types infecting the same cell recombine during replication (termed as 'viral sex') and create a hybrid (Burke, 1997; Noble, 2006, Lau & Wong, 2013). These hybrids are often short-lived and so the few that succeed in infecting at least three epidemiologically unrelated individuals acquire the term 'Circulating recombinant form" (Noble, 2006; Hemelaar *et al*., 2011). Unique circulating forms (UCFs) of the virus which do not cluster genotypically with well characterized types of the virus and occur in isolated cases also exist (Hemelaar *et al.* , 2011).

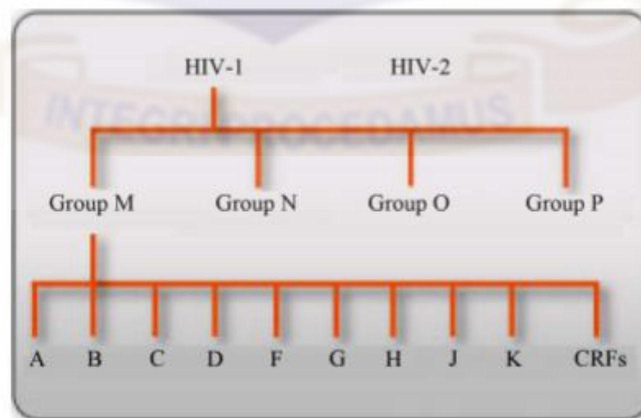

Figure 2.6: HIV Types, Groups and Clades (Agbelusi *et al*., 2013)

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2.4.2 Pathogenesis of HIV

The virus is transmitted primarily through sexual intercourse, injection drug use, perinatal means, transfusion of infected blood and occupational injuries (especially health workers) (Hankins *et al*., 2002). Transmission through the oral mucosa has been demonstrated but occurs at very low frequency even when plasma viremia is elevated due to the hypotonic environment and the presence of anti-HIV proteins (such as gp340 and SLPI proteins which play roles in innate immunity) and molecules (Malamud, 2010; Levy & Greenspan, 1988; Ho *et al.,* 1985; Barr *et al*., 1992; Xie *et al*., 2006).

Research by Adler in 2001 reports that in resource limited regions, vaginal sex accounts for 70-80% of all HIV incidences whereas perinatal and injection drug use contribute 5 to 10% each. HIV transmission is also generally influenced by factors such as age, sex, viral load, sexual risk (number of sexual partners), circumcision in males and host factors like the presence of shared class I HLA-B alleles in sero-discordant heterosexual partners (Quinn *et al*., 2000; Buchbinder *et al*., 2005; Dorak *et al*., 2004).

On entry, the virus migrates to the site of infection by an unknown mechanism speculated as probably through transcytosis, direct contact with intraepithelial dendritic cells (DCs) or migration through intercellular spaces in the epithelium making contact with underlying mucosal Langerhans cells and CD4+T cells (Lai *et al*., 2009; Cohen *et al*., 2011). Once the preferential site is reached, the first step in pathogenesis involves binding to specific host cell receptor CD4+ and any of the co-receptors CCR5 and CXCR4 on the target cell surface. The choice of co-receptor type is dependent on the type of infecting viral strain. Cells that express these receptors include immune cells, CD4+ T lymphocytes, monocytes,

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macrophages and dendritic cells as well as non-immune cells such as glial cells in the brain, chromaffin cells in the intestines and Langerhans' cells in the skin (Marr, 1998). Interestingly, there are recent evidence indicating the chance of HIV infection in non-CD4+ receptor bearing cells (Chen *et al*., 2011; Liu *et al*., 2004).

Various experiments have demonstrated that macrophage (M) and dendritic cell tropic viral strains engage CCR5 and additional co-receptors; CCR2 and CCR3 in the early stages of infection (asymptomatic stage) (Murdoch & Finn, 2000). In contrast, T-cell tropic strains use CXCR4 co-receptor (mostly expressed by T-cells) later in the infection (symptomatic stage) and are considered more pathogenic (Connor *et al*., 1997). Infection of a cell with T or M-tropic strains of HIV is also possible with the presence of co-receptor CCR8 on the preferred cell surface. Dual tropic strains capable of using more than one co-receptor have also been recognized (Murdoch & Finn, 2000).

HIV binds to its preferred host receptor and co-receptor using its surface glycoproteins gp120 through the creation of irreversible conformational changes which exposes co-receptor binding sites (Ray & Doms, 2006; Klatt, 2016). After a firm attachment is established, the viral envelop and host cell membrane fuse together and through pore formation (Ogle *et al*., 2005), the viral cell contents comprising of two single strands of positive sense RNA, tRNA primers, viral protease, reverse transcriptase, and integrase are released into the host cell cytoplasm. Reverse transcriptase enzyme then catalyses the reverse transcription of the viral RNA into complementary DNA (cDNA) using recruited host nucleotides (Coffin *et al*., 1997). A second complementary strand is produced by the

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same reverse transcriptase enzyme and the two strands hybridize to form a double-stranded (ds) DNA referred to as the pro-viral DNA (Abbas, 2000). The newly synthesized pro-viral dsDNA is then integrated into transcriptionally active domains of the host genome by integrase and the host DNA repair enzymes (Schroder *et al.*, 2002; Mitchell *et al*., 2004), marking the second step of the viral life cycle. The major host factor that mediates this step is the lens epithelium-derived growth factor (LEDGF/ p75), an integrase binding host factor (Ciuffi *et al*., 2005).

After integration, the provirus becomes part of the host genetic material and viral RNA transcription and translation are juxtaposed with that of the host cells. Viral proteins are then expressed using the host cell machinery and processed by the protease enzyme. Viral RNA and proteins are assembled into new viral particles within the host cytoplasm. The new viral particles egress from the cell by budding out via the vesicular sorting pathway (ESCRT-I, II, III) which mediates budding of endosomes into multi-vesicular bodies (Martin-Serrano *et al*., 2003). The progeny viruses acquire some cytoplasmic molecules and cell surface lipid bilayer from the host cell which forms its envelope as it egresses. These host components affect the phenotypes (such as the mode of replication) of the virions produced in the next infection (Cantin *et al*., 2005). A schematic presentation of the life cycle of the HIV virus is shown in Figure 2.7.

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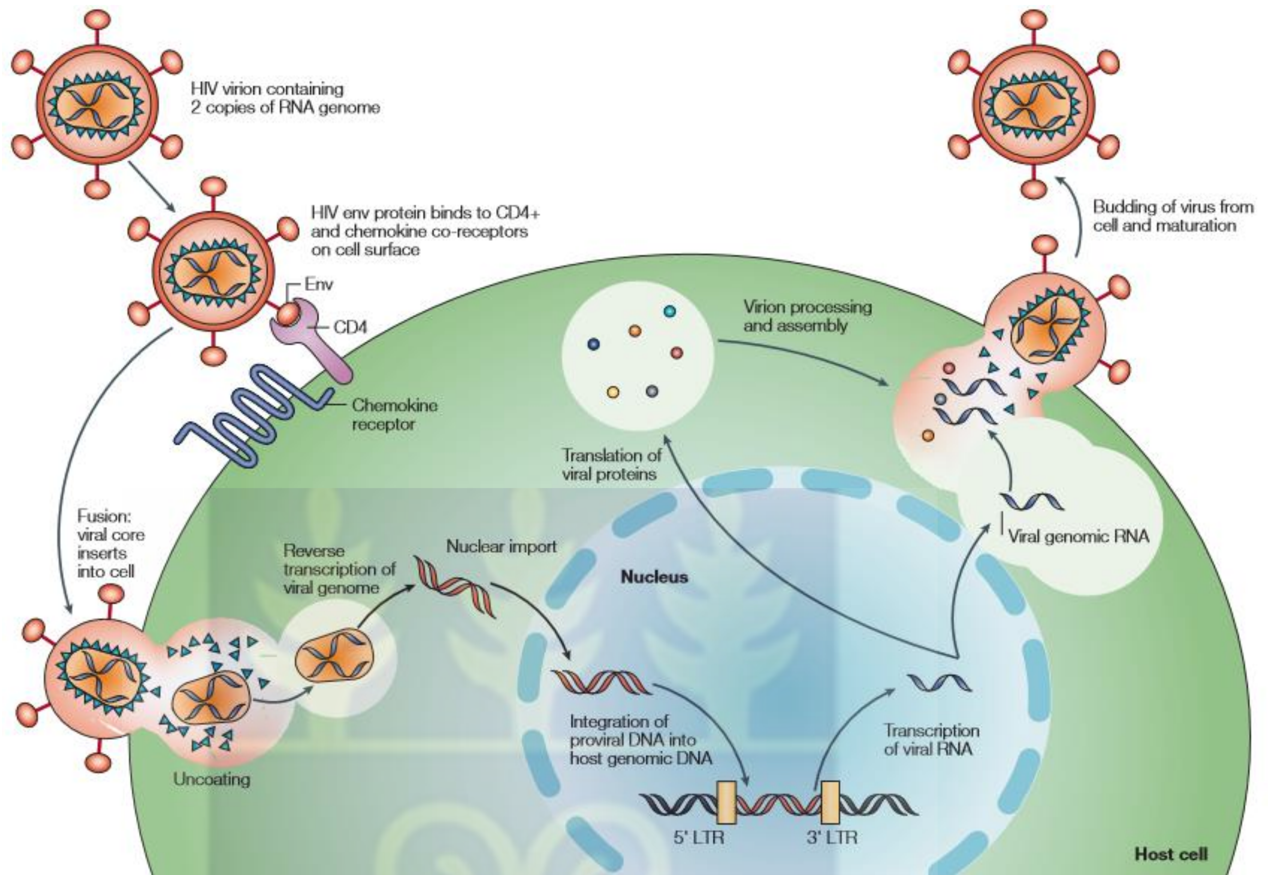

Figure 2.7: HIV Life Cycle. *The steps involved in HIV life cycle include receptor binding, fusion of viral envelope with host cell membrane and entry of viral core into host cytoplasm. Viral RNA is reverse transcribed by reverse transcriptase and replicated to form a dsDNA which is inserted into host DNA by integrase enzyme. Viral proteins are expressed using the host cell machinery and processed by the protease enzyme. Viral RNA and proteins are assembled into new viral particles within the host cytoplasm which egress from the cell by budding out (Rambaut et al., 2004).*

2.4.3 HIV Disease Progression

A successful viral infection progresses through the acute, chronic, early symptomatic and the AIDS disease phases. The World Health Organization classifies these phases as primary HIV infection and clinical stages I to IV (WHO, 2005) and, progression from primary to the symptomatic stages takes several years. The acute phase or primary phase of HIV

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infection spans the first 2 to 4 weeks of infection (Figure 2.8). It is characterized by rapid increase in viral loads and presents a highly infectious window for transmission (Weston & Marett, 2009). The high viremia is accompanied by no or few symptoms of headache, fever, swollen glands and rash collectively called acute retroviral syndrome (ARS) which could be misdiagnosed as a flu infection (www.aidsinfo.nih.gov). The high viral loads reduce the defense capacity of the immune system but overtime, the viral load reverts to a stable level termed the set point and detectable levels of HIV-specific antibodies are produced. This is termed as sero-conversion and usually occurs from the 4th to 10th week after exposure though, roughly 95% of patient sero-convert within 6 months (Coutlee *et al.,* 1994; Simmonds *et al*., 1988; Sheppard *et al.,* 1993). A slight increase in CD4+ cell counts is later observed and a small fraction of the infected population elicits important immune responses and viral containment (Madec *et al*., 2005).

The infection then advances to the chronic or clinical stage I phase which is marked by high rates of viral replication with no accompanying symptoms except for the development of "Persistent generalized lymphadenopathy" (PGL) (defined as enlargement of at least two noncontiguous sites of lymph) in patients with high levels of extracellular latent HIV forms in the lymph nodes (Pantaleo *et al*., 1993; Wei *et al*., 1995).

After clinical latency phase as shown in figure 2.8, the infection transitions into an early symptomatic clinical stage II phase. This was previously referred to as the Class B by the 1993 CDC classification scheme (Emmett *et al*., 2010) and "AIDS-related complex" as reported by WHO, (2005) with the manifestation of numerous non-AIDS indicating

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diseases. Case definition for this stage included moderate unexplained weight loss of less than 10% of patient's body weight, herpes zoster, recurrent bacterial respiratory infections, angular cheilitis, recurrent oral ulcerations, fungal finger-nail infections, papular pruritic eruptions and seborrhoeic dermatitis (WHO, 2005).

Transition to Clinical stage III infection is usually characterized by severe weight loss of more than 10% of patient's body weight, severe anaemia, persistent fever, chronic diarrhea, oral candidiasis, oral hairy leukoplakia, tuberculosis and other bacterial infection and acute necrotizing ulcerative gingivitis periodontitis (WHO, 2005). Whiles the terminal stage of HIV infection referred to as AIDS or clinical stage IV is mainly marked by severe immune deficiency with CD4+ T cell count below 200 cells/mm^3 and predisposition to a large number of opportunistic infections and related cancers (WHO, 2005; WHO, 2016).

In general, disease pathogenesis and progression are the same for both HIV-1&2 except for the slower progression of HIV-2 infection, infectivity and viral burden (O'Donovan *et al*., 2000; Adjorlolo-Johnson *et al*., 1994).

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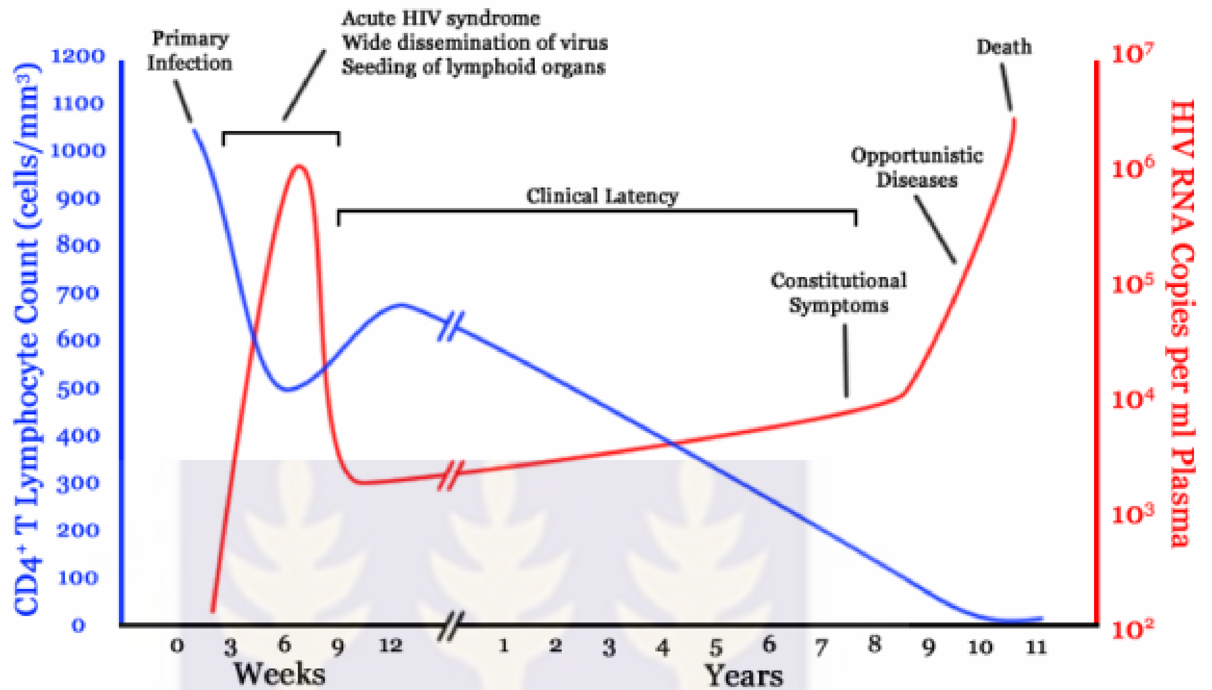

Figure 2.8: Progression of HIV infection. *Correlation of CD4+ T cell levels and viral load with the course of HIV infection* (Naif, 2013).

In reality, a rare exception to the regular HIV disease progression has been reported among some HIV-infected cohorts, grouped as the elite, viremic controllers and the long-term non-progressors (LTNP) (Palacios *et al*., 2012). Elite and viremic controllers have an estimated prevalence of 0.35%-0.80% and 2.83%-3.91% respectively (Okulicz *et al*., 2009). These individuals are capable of spontaneous viral suppression in plasma in the absence of ART (Deeks & Walker, 2007; Lambotte *et al*., 2009) with recorded viral loads less than 50 copies/mL and between 50-2000 copies/mL respectively for the elite and viremic controllers (Okulicz *et al*., 2009). Although viral titre quantification is the main characterization criteria for the elite and viremic controllers' groups, high CD4+ T cell counts (350 cells/µL for elite controllers) with low risk of AIDS and death are common

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phenotypes (Okulicz *et al*., 2009). On the other hand, long term non-progresses are defined as infected persons with sustained high CD4+ T cell counts for at least 13 years in the absence of ART. Various studies have suggested the prevalence of LTNP in the range of 2 to 15% (Okulicz *et al*., 2009; Madec *et al.,* 2009; Sheppard *et al*., 1993). The CD4+ T cell counts in these cohorts are usually greater than 600 cells/mm^3 and these numbers are sustained for about 5 years resulting in the preservation of the lymph node structure with the absence of clinical symptoms (Hughes *et al*., 1994; Gottlieb *et al*., 2002).

2.5 The lung immunity in HIV

The lung has a localized complex immune system comprising mainly of alveoli macrophages that is critical to defense against invading pathogens. In HIV infection however, the lung is severely affected because the abundant resident alveoli macrophages rather house and favour replication of the HIV virus (Fels & Cohn, 1986). Earlier studies suggested that, macrophage tropism of the virus within the lung is due to the presence of specific amino acid signature sequences in the virus which renders them CCR5-tropic and results in their compartmentalization within the lung (Itescu *et al*., 1994; Schuitemaker *et al*., 1992).

Persistent loss of CD4+ T cells also occur in the lung in a similar fashion as what happens in the systemic immune system in infected persons. The presence of the virus in the lungs leads to macrophage and dendritic cell activation with associated secretion of increased interferon- gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α). These chemokines/cytokines recruit other monocyte subsets to the site of inflammation. In

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addition, there is an influx and accumulation of activated CD8+ T cells in the lung compartment resulting in T-cell alveolitis together with a marked decrease in the CD4:CD8 T cell ratio. Along with the decreased in number, T cells become dysfunctional and mount abnormal host responses to T-cell–dependent antigens. The response of alveolar macrophage to bacteria through Toll-like receptor- 4 signaling via the myeloid differentiation factor 88 dependent pathway is also altered in HIV infection (Tachado *et al*., 2010). These abnormalities predispose patients to opportunistic infections such as influenza, pneumonia and tuberculosis (Huang & Crothers, 2009).

2.6 The lung microbiome

Until recently, there was the notion that the lung environment was sterile. The dubious nature of this claim considering the high adaptability of bacteria to extreme environmental conditions, high bacterial loads in inhaled air and the favourable growth conditions in the lungs led to lung microbiome investigations (Horikoshi & Grant, 1998; Beck *et al*., 2012). Microbial communities in the lungs of both healthy and diseased persons are now being explored and bacterial populations have attracted the most focus compared to viral and fungal populations (Beck, 2014).

The ultimate purpose of these studies is to enhance understanding and clinical outcomes of lung diseases (Beck *et al*., 2012) since, the microbiome has significant impact on physiology and immunology (Mitreva, 2012). To fulfill this mandate, the Lung HIV Microbiome Project (LHMP), currently the largest ongoing study on the lung microbiome was formed. The project is funded by the National Heart, Lung, and Blood Institute (U.S.A)

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to investigate the lung microbiome in HIV-infected and uninfected individuals with and without underlying lung diseases, such as chronic obstructive pulmonary disease (COPD) and pulmonary arterial hypertension.

Although this field is quite new, bulk of the data obtained from the few investigations demonstrates that lung microbiome is highly heterogeneous and the relative abundances of microbiota at different sections of the same lung may show great disparity, a phenomenon termed microgeography (Erb-Downward *et al*., 2011). Despite differences arising from various published data on the subject, frequently identified microbes belong to the phyla *Proteobacteria, Firmicutes, Actinobacteria* and *Bacteroidetes* (Hilty *et al*., 2010; Huang *et al*., 2011; Pragman *et al*., 2012; Morris *et al*., 2013). Characterization to the genus level by some studies also identified *Pseudomonas, Streptococcus, Prevotella, Fusobacteria,* and *Veillonella* as the dominant microbes of the lungs, with subdued occurrence of potential pathogens like *Haemophilus and Neisseria*. The differing results could be caused by non-standardized data analysis approaches across laboratories (Beck, 2014). These studies have also revealed the close resemblance of the lung and oropharynx microbiomes as compared to that of the nasopharynx or gastrointestinal tract (Venkataraman *et al.,* 2015; Bassis *et al.,* 2015; Morris *et al*., 2013; Segal *et al*., 2013).

Even though there is less focus on lung virome, few studies have characterized the lung virome to include DNA viruses such as *Anellovirus, Hepadnaviridae, Parvoviridae, Herpesviridae, Papillomaviridae* together with the RNA viruses like *Flaviviridae, Picornavirus and Retroviridae* (Twigg *et al*., 2017). Fungal composites of the lung have

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also been determined by few studies and include; *Ceriporia lacerata, Saccharomyces cerevisiae*, and *Penicillium brevicompactum* which have been implicated as opportunistic (Chowdhary *et al.,* 2013; Tawfik *et al.*, 1989).

The underlying factors that determine the composition of the lung microbiome include migration, elimination of microorganisms from the respiratory tract and existence of favourable growth conditions that facilitate multiplication of microbes (Lighthart *et al*., 2000; Gleeson *et al*., 1997). Disturbances in these conditions by environmental exposure, host immune system and genetics causes changes in the microbiome in both health and disease states (Dickson & Huffnagle, 2015; Kiley & Caler, 2014). Microbial passage into the lungs primarily arises from micro-aspiration of upper respiratory tract, inhalation of air and dispersal along the mucosa. Elimination of microorganisms is through mucociliary clearance (Munyard & Bush, 1996) and host immune system whereas, environmental conditions entail nutrients availabilty, optimal temperature and pH as well as oxygen tension within the lung (Dickson & Huffnagle, 2015). In healthy individuals, these factors are hostile to the growth of microbes hence, microbial reproduction is reduced and a steady state is also maintained between the immigration and elimination of these microbes (Dickson *et al*., 2014; Dickson *et al.,* 2015; Venkataraman *et al*., 2015). Nevertheless, changes associated with the fundamental conditions of the microbiome create selective niches for discrete microbial reproduction and these in turn overwhelm the immigration and elimination of microbial equilibrium in disease (Dickson & Huffnagle, 2015).

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2.7 Lung Microbiome in HIV

HIV suppresses immune function and may subsequently alter the lung microbiome causing various HIV- associated pulmonary complications (Twigs *et al*., 2016). However, a recent publications suggest that, microbiomes of HIV-infected and uninfected persons are similar (Beck *et al*., 2015). Another study also demonstrated that, treatment naïve HIV-infected populations and those on ART with CD4+ T-cell count of 668 cells/mm^3 plus those on ART with CD4+ T-cell count of 618 cells/ mm^3 respectively had similar microbiomes whiles, advanced HIV disease, was associated with a decline in microbial richness and diversity (lower alpha diversity) (Twigg *et al*., 2016), although the taxa structure differed greatly (beta diversity) between infected and uninfected HIV cohorts.

In people living with HIV, growing literature has shown increased colonization of *Tropheryma whipplei* in the lungs however, the abundance decreased with ART (Lozupone *et al*., 2013). Organisms belonging to *Actinobacteria, Bacteriodetes, Prevotella* and *Firmicutes* were detected in HIV-positive patients in another study by Iwai *et al*., (2012). Also, *Streptococcus species* are abundant in the lungs of advanced HIV patients and are implicated in pneumonia (Janoff *et al.*, 1992).

The lung virome of HIV-infected persons has been less studied. The scanty data available includes one in which virome in lung transplant recipients and 3 HIV-infected controls were examined. Viruses detected in broncho-alveolar lavage (BAL) samples from the study participants and controls were anelloviruses. The viruses differed amongst the groups only on the basis of their abundance with the lung transplant recipient group having higher

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viral abundance. Bacteriophages were also predominant in the lungs of the study samples (Young *et al*., 2015). Preliminary results from another study involving 3 HIV-positive patients and 6 health controls also detected *Lymphocryptovirus* only HIV-positive persons (Twigg *et al*., 2017). Epstein-Barr virus, an established persister in HIV patients falls within the *Lymphocryptovirus* genus (Stevens *et al*., 2002). Large study participants are required for confirmatory experiments to be performed.

The lung mycobiome is also an aspect of the lung microbiome studies. The mycobiome of HIV positive individuals is different from that of healthy people. Confirmed fungi components of the mycobiome in HIV exhibit high abundance compared to those in HIV-negative individuals. The taxa includes the pathogen *Pneumocystis jirovecii* and *Ceriporia lacerata* plus, HIV patients are usually infected with *Candida* and *Cryptococcus species* (Cui *et al*., 2015).

2.8 Opportunistic Infections in HIV

Opportunistic infections occur frequently in individuals with advanced HIV infection because of the opportunity presented by the state of immune suppression and the possibility of an altered microbiota in HIV-infected people. Although various treatments and prophylaxis exist, not all of them have universal coverage due to their high costs (Practice, 1998).

There are two main underlying factors that promote opportunistic infections and malignancies in HIV infected persons. One is immune-suppression caused by the virus and the other is the presence of a microbial environment within the body (Practice, 1998). From

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the first reported HIV cases in homosexually active men in 1980 to present reports, AIDS-defining opportunistic infections include bacterial infections (such as tuberculosis, *Mycobacterium avium* complex disease, pneumonia and septicemia), protozoan infections (toxoplasmosis, microsporidiosis and cryptosporidiosis), fungal infections (*Pneumocystic jiroveci* pneumonia, candidiasis and cryptococcosis), viral infections (example: herpes simplex virus, cytomegalovirus (CMV), and herpes zoster virus) as well as various malignancies (Kaposi sarcoma, lymphoma and carcinoma) (Sá *et al*., 2007; de Arruda *et al.,* 2015).

It is important to note that, the above mentioned examples of the various opportunistic infections are not exhaustive. The incidence of these infections vary greatly across countries due to genetic, environmental and social differences that exist (Kim *et al*., 2016). Numerous studies have identified tuberculosis as a major opportunistic infection in HIV patients living in Africa, followed by bacterial pneumonia but, the incidence of *Pneumocystic jirovecii* (formerly *Pneumocystic carinii* pneumonia –PCP) is rare within the region (Fisk *et al*., 2003; Van Oosterhout *et al*., 2007). On the other hand, PCP has high occurrence in Western Europe (Serraino *et al.,* 2003). The table below extracted from Perriëns (1994) depicts differences in the prevalence of opportunistic infections in HIV/AIDS patients in different countries.

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Table 1: Prevalence of AIDS-defining opportunistic diseases in six countries

Opportunistic disease or malignancy	Côte d'Ivoire	Brazil	Mexico	Thailand	USA	Zaire[2]	Infrastructure needed [3]
Aspergillosis	3%		3–7%				advanced
Atypical mycobacteriosis	4%		5–6%	2%	4%		advanced
Bacteraemia	7%			4%			advanced
Candidiasis	24%	5%	30%	11%	13%	minimal	
CMV	26%	5%	65–69%	4%	5%	13%	advanced
Cryptococcosis	5%	5%	7–11%	2%	7%	19%	medium
Cryptosporidiosis – Isosporiasis	4%	14%	8%	4%	6.2%	<2%	advanced
Enteritis, non-specific [4]	12%					13%	minimal
Herpes (systemic)	6%		5%	10%	4%		minimal
Histoplasmosis	3%		5–10%	8%		<2%	advanced
Kaposi sarcoma	13%	5%	30–43%		21%	16%	medium
Lymphoma	4%	4%	10%		0.7%		advanced
Nocardiosis	5%		<2%				advanced
Penicilliosis				4–25%			advanced
Progressive multifocal leukoencephalopathy (PML) or HIV encephalitis	6%	11%		7%	0.6%		advanced
Pneumocystis carinii pneumonia	4%	22%	24%	26%	64%	<2%	medium
Pneumonia	5%	16%				34%	advanced
Toxoplasmosis	21%	14–34%	17%	2%	3%	11%	advanced
Tuberculosis	54%	41%	28%	20%	3%	41%	medium
Others	9%			9%			

(Perriëns, 1994).

Additionally, autopsy studies conducted have revealed a decline in incidence of respiratory infections in HIV positive subjects from 100% in the pre-HAART to 70% in the current HAART era (Afessa *et al*., 1992; Masliah *et al*., 2000). Another study in Sub-Saharan Africa which investigated the causes of death in HIV-positive subjects using autopsy found that, approximately 66% of the pathologies were caused by pulmonary infections (Cox *et al*., 2010). It has been estimated that about 70% of HIV-infected populace, representing the majority have pulmonary complications caused by infectious etiological agents (Miller, 1996). In a current research involving 762 HIV/AIDS patients, pulmonary infections was found as the most prevalent infections (Huang *et al*., 2010). From various studies, it has been projected that, 65% of HIV-positive persons are prone to manifest pulmonary

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complication at the initial stage of the infection with about 80% of these patients manifesting symptoms of pulmonary disease as the disease progresses (Suffredini & Masur, 1988). The outlined estimates show the burden of opportunistic infections on the lungs especially in HIV-infected individuals.

2.8.1 Opportunistic Pulmonary Infections

2.8.1.1 Bacterial Infections

Opportunistic infection is the major cause of death in patients with HIV, with a hospital and 4 weeks discharge mortality rate ranging from 2.6-27% (Baril *et al*., 1998). Bacterial infections are the most common pathogenic infections and can affect HIV-positive patients at all stages of the disease with varying incidences relative to CD4 cell numbers (Hirschtick *et al*., 1995; Gordin *et al*., 2008). An HIV-infected person is 10 times more prone to bacterial pneumonia than a healthy individual (Feikin *et al*., 2004; Morris *et al*., 2000) and the annual incidence ranges from 5.5 to 29% in HIV-positive patients compared with 0.7 to 10% in HIV-negative people (Caiaffa *et al*., 1993; Hirschtick *et al*., 1995; Kohli *et al.,* 2006). This is further increased in patients who smoke or are injection drug users (Gordin *et al*., 2008; Miquez-Burbano *et al*., 2003).

The most common etiological agent responsible for these AIDS-defining bacterial pneumonia are *Streptococcus pneumoniae, Haemophilus influenza*, *Staphylococcus aureus*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa* (De Gaetano *et al*., 2000; Sullivan *et al.,* 2000). These microbes are similar to those that cause community-acquire pneumonia (NIH, CDC & HIVMA/IDSA, 2009). Among these bacteria, *Streptococcus*

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pneumoniae has been most implicated with a prevalence of 20% (Feikin *et al.,* 2004). The second ranked pneumonia causing bacteria in HIV persons is *Haemophilus influenzae* with a rate of 10–15% reported (Benito *et al.,* 2001; Benito *et al*., 2004). *Haemophilus influenzae* however, is frequently detected in patients with advanced disease whiles *Pseudomonas aeruginosa, Klesiella pneumoniae* and *Staphylococcus aureus* are community- acquired pathogens with higher frequencies persons with HIV infection (Afessa & Greene, 2000; Levine *et al*., 1990).

Treatment guidelines for HIV infected persons with bacterial pneumonia are the same as those for non-HIV infected persons. The only difference is that, macrolide monotherapy is replaced with beta-lactam plus macrolide for treatment of HIV-infected patients because of the increased risk of drug resistant *Streptococcus pneumoniae* (NIH, CDC & HIVMA/IDSA, 2009). Antibiotic therapy administered in HIV/ bacterial pneumonia co-infected individuals include; Amoxycillin, Azithromycin, Levofloxacin, Amoxycillin/Clavulanate (β -lactam/ β -lactamase inhibitors), Erythromycin, Ceftriaxone (3rd generation Cephalosporins), Gentamicin and Ciprofloxacin (National antibiotic policy, 2008).

2.8.1.2 Mycobacterial Infections

Tuberculosis is an underlying cause of most fatal pulmonary infections among HIV-postive patients. In 2015, an estimate of 11% (1.2 million) of all new active TB disease occurred in HIV-positive individuals and HIV-related death resulting from TB disease was 0.4 million (35%) (WHO, 2016). The greatest burden of TB/HIV co-infection falls on the

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WHO African region with about 71% case incidence report (WHO, 2005). Further proof of the burden of TB/HIV co-infection in Africa was highlighted in a review which attributed active TB disease as the leading cause of hospitalization among HIV-infected adults (18%) and children (10%) (Ford *et al*., 2015). In Ghana, the overall TB incidence is 160 in 100 000 people but the estimated TB case detection rate is as low as 33% (WHO, 2016; WHO, 2014).

Worsening the TB/HIV crisis is the fact that a third of the world's population have latent TB infection and serve as hidden reservoirs from which active disease can develop especially among people living with HIV (WHO, 2016). Moreover the major protective immune cells, CD4+T cells and macrophages, are depleted in HIV infection/disease resulting decrease in production of effector molecules such as interferon gamma. Thus TB can occur at all stages of HIV infection and there is an inverse correlation between its incidence and CD4 levels. Hence, in the lifetime of people without HIV infection, risk of reactivation of latent TB infection is 5% per year and this however doubles in HIV-infected persons (Havlir & Barnes, 1999).

Symptoms of TB are similar among healthy HIV-positive and HIV-negative persons. Defined ones include; productive cough, chest pain, fever, shortness of breath, night sweats, hemoptysis and/or weight loss (Lawn & Zumla, 2011). Nevertheless, as CD4 levels decline, HIV/TB co-infected patients show atypical symptoms with associated hilar lymphadenopathy, subtle infiltrates and pleural effusions other forms of extrapulmonary

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tuberculosis at CD4 counts of less than 200 cells/mm^3 in nearly 50% of patients. Continuous decline of CD 4 levels to below 75 cells/mm^3 is also allied with disseminated tuberculosis, shown as nonspecific, chronic febrile illness affecting a number of organs and causes high rates of early mortality (von Reyn *et al*., 2011). Not all symptoms may occur together in some HIV patients and others experience less specific symptoms depending on the interplay between the strength of host immunity and pathogen virulence.

Not only is HIV a risk factor for TB but, TB also augments replication of HIV and increases progression of HIV infection by enhancing the prodution of cytokine (TNF-α) (von Reyn *et al*., 2011).

2.8.1.3 Non-tuberculous mycobacterial infections

Mycobacterial species other than *Mycobacterium tuberculosis complex* and *M. leprae* are referred to as non-tuberculous mycobacteria (NTM). These organisms are environmental microrganisms that are ubiquitous in nature and found in various environments althoguh their presence vary in different regions. These organism are capable of colonizing the respiratory tract causing pulmonary infection which is emerging as a global heath problem, especially among immunocompromised individuals (Andrejak *et al*., 2007; Prevots & Marras, 2015). For instance, infections with *Mycobacterium avium complex* (MAC) is common in U.S but rare in Africa (Benito *et al*., 2010). In the U.S, the prevalence for adults who are 65 years and above, increases at a rate of 8.2% (Adjemian *et al*., 2012). Patients with NTM infections tend to have other pulmonary co-morbidies. This is baceause, NTMs are capable of forming biofilms (McGarvey & Bermudez, 2002). A current research on NTM among HIV-infected individuals starting ART in Ghana showed a prevalence of

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8% and the species identified were *M. avium complex, M. chelonae complex, M. simiae, M. fortuitum complex, M. kansasii, M. flavescens, M. terrae, M. arupens*. MAC however dominated (Bjerrum *et al*., 2016).

2.8.1.4 Fungal Infections

HIV-associated fungal infections are caused by *Pneumocystis jiroveci*, *Cryptococcus neoformans, Candida species* and endemic mycoses such as *Aspergillus species, Histoplasma species and Penicilliosis species* (Armstrong-James *et al*., 2014).

Pneumocystis jiroveci infection (PCP) was the major defining fungal illness for AIDS during the earlier epidemic season. With the introduction of PCP prophylaxis and combination therapy (HAART), the incidence of PCP fell significantly although it remains one of most severe opportunistic fungal infections among HIV infected persons. (Worodria *et al*., 2003; Fisk *et al*., 2003). In AIDS patients with PCP, the mortality rate is 10-20% (Randall *et al*., 2000) and many of these cases occur when CD4+ T-cell counts are below 200 cells/μL (Thomas & Limper, 2004). Pneumocytis is non-culturable therefore, culture independent methods like microscopy and radiological methods are employed in diagnoses. These methods lack high sensitivity and result in empirical therapeutic approaches especially in developing countries. The limitation in diagnosis of PCP is also responsible for the underestimated disease burden in Sub-Saharan Africa (Lowe *et al*., 2013). Therapy for PCP relies on trimethoprim-sulfamethoxazole (TMP-SMX) as the first line treatment 21 days. Options to TMP-SMX include intravenous pentamidine, oral atovaquone and clindamycin plus primaquine (Thomas & Limper, 2004). TMP-SMX

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(160mg/800mg or 80mg/400mg daily) is also recommended as primary and secondary prophylaxis therapy.

Currently, cryptococcosis is the most fatal AIDS-defining fungal infection (Park *et al.,* 2009), mostly associated with advanced stages of the infection usually when CD4+ T-cell counts are below 100 cells/mm^3. *Cryptococcus neoformans*; an exogenous etiological agent for this infection, gains entry into host through inhalation and remains latent in the alveolar macrophages of a healthy individual but reactivates and disseminates during immune suppression as in the case of HIV infection. Pathologies include lung infections, meningoencephalitis and increased intracranial pressure leading to death especially in Sub-Saharan Africa (Park *et al.,* 2009; Armstrong- James *et al*., 2014). The global incidence estimates per annum is 950,000 out of which 720,000 cases occur in Sub-Saharan Africa annually with case fatalities ranging between 9% to 70% for developed and Sub-Saharan Africa respectively. The treatment of crytococcosis entail the use of amphotericin B and flycytosine anti-fungal drugs (Day *et al.,* 2013).

Candida species are opportunistic fungal pathogens frequently associated with HIV infection (Delgado *et al.,* 2009; Thompson *et al.,* 2010). These pathogens can infect soft and hard palates, oro-pharyngeal tract, tongue and buccal mucosa (Thompson *et al.*, 2010). Disseminated infections also occur seldomly and accounts for mortality rates between 40%-100% (Vazquez *et al*., 2003). An estimated prevalence of 10% has been reported for Asia, Africa and Latin America (Kwamin *et al*., 2013). ART greatly reduces the incidence of candidiasis and the therapy can be re-inforced fluconazole (Patel *et al*., 2012).

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Also, some endemic fungi have been implicated as opportunistic and manifest in HIV infection, especially at the advanced stages. In Africa, *Histoplasma duboisi* is one such endemic fungi associated with systemic infection in HIV patients with an incidence rate of 2% (Lofgren *et al*., 2012). Treatment of histoplasmosis involves itraconazole and amphotericin B (Wheat *et al*., 2001). Another example of an endemic fungi that causes life threatening fungal infection in HIV patients is *Aspergillus species*. Aspergillosis infection although rare, is associated with poor survival in HIV patients (Holding *et al*., 2000).

2.8.1.5 Viral infections

Viruses have also been observed as the basis of pulmonary infections affecting HIV patients especially pneumonia. Notwithstanding, there is limited literature on the causes, risk factors and outcomes of respiratory viral infection amongst HIV positive individuals. There is also the assumption that, the respective role of each viral pathogen may differ in different environments and populations (Garbino *et al*., 2008). Viral pneumonia in HIV patients has been traced to several viruses for example adenovirus (Zampoli & Mukuddem-Sablav, 2017).

Aside pneumonia, influenza is also a major pulmonary illness related to HIV infection. It is the cause of febrile respiratory symptoms in HIV-positive persons in spite of being vaccinated against the virus (Penaranda *et al*., 2007) and the etiological agents responsible include influenza and parainfluenza viruses (Cohen *et al*., 2015).

Meta-pneumovirus (MPV) infection has also been recently described in association with HIV as the underlying cause of acute febrile respiratory illness after influenza (Klein *et al*.,

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2010). Until recently, this virus was not recognized as a respiratory viral pathogen (Fouchier *et al*., 2005). The exact roles and mechanism utilized by MPV in adult HIV infected persons is not clearly defined. Gathering from reports of MPV infection in non-HIV infected persons, it usually causes nonspecific symptoms, such as fever and myalgias (Boivin *et al*., 2002). Other symptoms suggestive of lower respiratory infection such as cough, dyspnea, and wheezing also prevail in HIV/MPV infected patients (Klein *et al*., 2010). Research on this pathogen is gradually advancing and the first vaccine candidates have been developed (Fouchier *et al*., 2005).

Cytomegalovirus (CMV) is another respiratory tract pathogen that occurs in HIV-infected people and CMV infection was one of the AIDS-defining condition before the advent of ART (Drew, 1992). It causes pneumonitis and pneumonia in infected HIV patients with CD4 cell counts of 50 cells/ mm^3. The common clinical outcome of CMV infection is retinitis, gastrointestinal and central nervous system diseases. Clinical presentation of CMV pneumonitis consist of pulmonary interstitial infiltrates, presence of CMV inclusion bodies, specific cytopathic changes in the lungs and the absence of other pathogens that are more commonly associated with pneumonitis in this population (Benito *et al*., 2010).

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CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

Sterile cotton tipped swabs (Technical Service Consultants Ltd., England), forceps (Quick Medical Ltd., USA), sterile petri dish, sterile disposable inoculating loops (Thermo Scientific, USA), 4mm diameter glass beads (Fisher Scientific UK limited), powder free nitrile examination gloves (Polyco-Bodyguard, UK), powdered latex examination gloves (Digamed, South Africa), Bench guard (Sterilin Ltd., England), sterile single wrapped disposable Pasteur pipette (Alpha Laboratories, UK), disposable laboratory coats (Medline, France), 1.5 mL sterile microfuge tubes (Sarstedt, Germany), 2 mL sterile microfuge tubes, sterile DNase free PCR tubes, autoclave bags (Greiner Bio-One, Germany), 15 mL sterile falcon tubes (Greiner Bio-One, Germany), flat-bottom flask (Pyrex, USA), 50 mL sterile falcon tubes (Greiner Bio-one, Germany), Plate cushions for miniblotter (Isogen Life Science, Maarssen, The Netherlands), Hyperfilm (X-ray films) Enhanced Chemiluminiscence (18 x 24 cm), (Amersham Biosciences, UK), Exposure cassette (24 cm X 30 cm) (SIGMA, USA).

3.1.1 Reagents

Antibiotic discs (Oxoid, USA), Middlebrook OADC (Becton, Dickinson and Company, USA), Middlebrook 7H9 broth (Becton, Dickinson and Company, USA), Glycerol (Timstar Laboratories, UK), Primers for PCR (Macrogen), HotStarTaq *Plus* DNA polymerase (Qiagen), Blood Agar base (Oxoid, USA), MacConkey Agar (Oxoid, USA), Nutrient Agar (Oxoid, USA), Potato dextrose Agar (Oxoid, USA), Schaedler Anaerobic

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Agar (Oxoid, USA), Muller-Hinton Agar (Oxoid, USA), 20 x SSPE, (Gibco BRL Life Technologies Inc.), SDS (BDH Laboratory Supplies), Streptavidin-POD-conjugate (Boehringer), ECL detection liquid (Amersham International), Amplification mixes A and B(Hain Life Sciences GMBH, Germany), Quick RNA mini-preps plus kits (Zymogen), QIAamp DNA Mini Kit (Qiagen), Allplex respiratory assay (Panel 2) (Seegene), Formic acid (Sigma-Aldrich), MBT Galaxy HCCA Matrix GPR (Bruker Daltonik, Germany).

3.1.2 Equipment

Heratherm Incubator (Thermo Scientific, USA), Twincubator (Hain Lifesciences, Germany), Thermal cycler (Applied Biosystems 2720, USA), Vortex mixer (Scientific Industries, USA), Heating block (Heat Labor Consult, Germany), Digital coagulator (Hirasawa works, Japan), Weighing balance (A&D Instruments, Japan), Water bath (Grant Technical, England), Autoclave (TOMY SEIKO CO. LTD., Japan), Hybrigen oven (Bibby Scientific Ltd, UK), Class 2 Safety cabinet (Air Tech, UK), Class II Biosafety cabinet type A (Dalton), PhoenixSpec Calibrator kit (Becton, Dickson and Company, U.S.A), PhoenixSpec Nephelometer (Becton, Dickson and Company, U.S.A), CFX-96 Touch^{TM} Real-Time PCR Detection System (Bio-Rad), Digital coagulator (Hirasawa works, Japan), Olympus CH30 Light Microscope (Olympus Optical Co.LTD, Japan), Weighing balance (A&D Instruments, Japan).

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3.2 Methods

3.2.1 Ethical Statement

Ethical approval for the study and its protocols was obtained from the Institutional review board of the Korle-Bu Teaching. Informed consent from participants was obtained from their respective families and the postmortem procedures followed national due process.

3.2.2 Study Design and Participant Inclusion

This was a cross-sectional study design with the objective to characterize the lung microbiome of post-mortem lung biopsies from HIV/AIDS patients at a tertiary referral teaching hospital with a 1,600-bed capacity in Accra, Ghana. Clinical and demographic data were retrieved from patient hospital records with the consent of the subjects' relatives. The inclusion criteria for subject participation was all HIV/AIDS cases with autopsy requests and suspected HIV cases that were confirmed at autopsy. Poorly preserved HIV/AIDS cadavers were excluded from the study. Standard autopsy procedure was adhered to and was carried out within 24-48 hours after death. The weight of each lung was determined before the lungs were serially sectioned at 10 mm intervals. The final biopsy samples used in the experiment were two representative specimens (1 cm^3) from each lobe of the lungs. The samples were placed in 50 mL falcon tubes containing sterile PBS and stored at 4°C prior to transportation to the Noguchi Memorial Institute for Medical Research (NMIMR) which was done within 24-48 hours of collection.

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3.2.3 Sample Size

The annual average autopsy cases handled by the Pathology department are approximately 2,300, out of which about 120 are HIV/AIDS cases. The sample size calculation for this study was determined based on these estimates using an online sample size calculator (www.surveysystem.com/sscalc.htm) and the minimum size was 92 with respective confidence limit and interval of 95% and 5.

3.2.4 Media preparation

3.2.4.1 Media preparation for Bacteria and Fungi cultures and Antibiotic Sensitivity Test

- Blood Agar:

Blood agar is an enriched media that facilitates the growth of fastidious bacteria. The media was prepared as indicated by the manufacturer as follows; 40 g of the agar base (which contains a protein source for example tryptones, soybean digest, sodium chloride and agar) was suspended in 1 L of distilled water and dissolved completely by shaking and then boiling briefly. This was sterilized by autoclaving at 121°C for 15 minutes. It was allowed to cool to 50 ^{o}C and 5% sterile sheep blood warmed to room temperature was added aseptically and mixed thoroughly by gently rotating the media bottle whiles avoiding bubble formation. A volume of 20 mL of the molten blood agar was dispensed into labelled sterile glass petri plates to obtain a depth of 4mm. The plates were allowed to solidify and incubated overnight at 37 ^{o}C to check for sterility before used. Plates that were not used immediately were stored upside down at 4 ^{o}C in a sealed plastic bags to prevent moisture loss and reduce chances of contamination (Cheesbrough, 2006).

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- Chocolate Agar:

Chocolate agar is a variant of blood agar in which the red blood cells are lysed to release factors such as hemin and NAD required for the growth of some fastidious bacteria. The media was prepared according to manufacturer's instructions by weighing 40 g of the agar base and suspending in distilled water. The media was dissolved by boiling briefly and then sterilized by autoclaving at 121°C for 15 minutes. After cooling to about 70 ^{o}C, 5% sheep blood was added. The heat lyses the red blood cells giving the media a chocolate appearance. The molten media was dispensed into labelled sterile glass petri dishes after cooling to 50 ^{o}C and incubated overnight for ensure sterility. Plates with growths on media surfaces were not used. Plates were stored at 4 ^{o}C in sterile plastic bags with the media side of the plate upward for later use (Cheesbrough, 2006).

- MacConkey Agar:

This is a differential medium used for the isolation of Gram-negative bacteria and also helps to distinguish between lactose and non-lactose fermenters. Fifty–two grams (52 g) of the agar base was added to 1 L distilled water and completely dissolved by heating to boil. The mixture was sterilized at 121°C for 15 minutes with an autoclave and allowed to cool to 50 ^{o}C in a water bath. This was in accordance with the manufacturer's instructions. The molten agar was mixed thoroughly and 20 mL was aliquoted into each sterile petri dish. The plates were incubated at 37 ^{o}C overnight after gelling (Cheesbrough, 2006).

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- Schaedler Anaerobic Agar:

For the isolation of anaerobic bacteria, Schaedler anaerobic agar was used. This was prepared by suspending 40 g of the agar base in 1 L of distilled water and dissolving completely by boiling briefly. The media was then sterilized using an autoclave at a temperature of 121 ^{o}C for 15 minutes as indicated by the manufacturer. After cooling to 50 ^{o}C in a water bath, the media was mixed and poured into sterile petri dishes (20 mL per plate). The plates were incubated upside down at 37 ^{o}C for 15-24 hours to ascertain the sterility of the media before use. For isolation of fastidious anaerobes, the prepared Schaedler media was supplemented with 5% sheep blood before aliquoted into sterile petri plates (Cheesbrough, 2006).

- Nutrient Agar:

Nutrient agar was used for sub-culturing non-fastidious bacteria isolated from MacConkey agar (primary culture media) to obtain pure bacteria and also expand the number of cells. The agar was prepared by weighing 28 g of agar base and suspending it in 1 L distilled water. This was dissolved completely by heating to boil and sterilizing at 121 ^{o}C for 15 minutes using an autoclave. The agar was also allowed to cool to 50 ^{o}C in a water bath, mixed gently and poured into sterile petri plates (20 mL per plate). The plates were left to stand for about 15 to 20 minutes to solidify and incubated at 37 ^{o}C overnight (Cheesbrough, 2006).

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- Potato Dextrose Agar:

Fungi were cultivated using potato dextrose agar. As specified in the manufacturer's instructions, 39 g of the agar base was added to 1 L of distilled water and allowed to dissolve completely by heating to boil. The agar was sterilized by autoclaving at 121 ^{o}C for 15 minutes. Afterwards, it was cooled to 50 ^{o}C in a water bath and 20 mL was dispensed into each petri dish after being well mixed. The agar was allowed to solidify in each plate and incubated overnight at 37 ^{o}C whiles the plates were inverted (Cheesbrough, 2006).

- Muller-Hinton Agar:

This was used for the antibiotic sensitivity test and was prepared according to the manufacturer's instructions by suspending 38 g of the dehydrated agar base in 1 L of distilled water. Sterilization of the media was achieved by autoclaving at 121 ^{o}C for 15 minutes. The media was cooled to 50 ^{o}C in a water bath, mixed and 20 mL of it was dispensed into each sterile petri plate. The solidified media were incubated overnight at 37 ^{o}C whiles turned upside down (Cheesbrough, 2006).

- Storage Media for bacteria isolates:

Pure bacteria isolates from sub-culture media plates were stored in 10% skimmed milk solution supplemented with 15% glycerol as a cryoprotectant. This media favours long-term preservation of isolates at temperatures of ≤ -20 ^{o}C and protects against cell death in cases of elevated temperatures. The media was prepared by uniformly mixing 10 g of dehydrated skimmed milk powder with 85 mL distilled water and sterilizing at 121 ^{o}C for 10 minutes in an autoclave together with 15 mL glycerol aliquoted into a separate flask.

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After sterilization, the milk solution was aseptically mixed with glycerol whiles the two mixtures were still warm to prevent curdling of the milk. The media was aliquoted into cryo-vials and used to store viable isolates (Cody *et al*., 2009; Fahy, 1986; Meryman, 1971).

3.2.4.2 Lowenstein-Jensen media preparation for Mycobacteria growth

Lowenstein-Jensen (L-J) media was prepared for the culture of mycobacterial species. Clean glassware were sterilized by heating at 160 ^{o}C in a hot air oven for 1 hour. L-J media mineral salts were weighed (Appendix 1) and added to 100 mL of distilled water in flat-bottomed flask and sterilized at 121 ^{o}C for 15 minutes. After cooling to room temperature, 167 mL filtered egg homogenate was added to the L-J suspension. In order to inhibit the growth of fast growing bacteria, malachite green solution was filtered and added to the media. About 5 mL of the media was aliquoted into glass tubes and slanted in a coagulator to solidify. Once set, the freshly prepared L-J slants were incubated overnight at ambient temperature to check for sterility. The sterile media slants were then used to culture the homogenized tissue. One set of the L-J media contained glycerol but not pyruvate for the growth of *Mycobacterium tuberculosis sensu stricto* (MTBss) whereas another set contained pyruvate but not glycerol to improve isolation of *M. africanum* (MAF) and *M. bovis* (Grange *et al*., 1996).

- Storage Media for Mycobacteria:

One liter (1 L) storage media was prepared by aseptically adding 4.9 g of Middlebrook 7H9 powder dissolved in 900 mL distilled water and sterilized at 121 ^{o}C for 10 minutes to

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ADC enrichment in a 1:9 ratio. The media was used to store viable culture isolates at -20 ^oC and -80 ^oC temperatures for future use (Middlebrook & Cohn, 1958; Becton Dickinson Manual, 2006).

3.2.5 Tissue Homogenization

Sectioned lung biopsies were homogenized in a sterile petri plate using sterile scissors and scalpels, to enhance the recovery of microorganisms from the tissue samples as described by Anaissie *et al*., 2003.

3.2.6 Isolation of Microorganisms from Homogenized Tissue

3.2.6.1 General Bacteria Isolation

Tissue homogenates were inoculated onto sterile Blood, Chocolate and MacConkey agar plates by streaking using a 10 µL loop. After streaking in one direction across the top of the media surface from one end to the other, the plate was rotated 60 ^oC and streaking was done again. The plate was rotated thrice and in the last streaking a zigzag line well separated from each other was made for growth of distinct colonies. The plate was incubated aerobically at 37 ^oC for 18-24 hours upside down to prevent condensed water from falling back onto media surface and causing contamination. Schaedler anaerobic agar was also used as medium for isolating anaerobes. The incubation condition was under anaerobic atmosphere created in an anaerobic jar with an anaerobic gas pack at 37^oC for 4-5 days.

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The mixed colonies on the primary culture plates were purified and multiplied by sub-culturing onto Nutrient agar by streaking. The plates were incubated upside down under aerobic atmosphere at 37 ^{o}C for bacteria isolated under aerobic conditions. Sub-cultures from anaerobically subjected primary culture plates were also incubated under anaerobic conditions (Cheesbrough, 2006).

3.2.6.2 Fungi Isolation

Tissue homogenate was inoculated by streaking on Potato Dextrose Agar plates using a 10 μL loop. The plate was rotated at 60 ^{o}C after each streaking across the top of the media surface. The rotation was done three times and the last streaking was done across the remaining unstreaked media surface in a zig-zag fashion to allow the growth of distinct colonies. The plate was incubated with the media side up and incubation was done at 28 ^{o}C for 18-24 hours (Cheesbrough, 2006).

3.2.6.3 Mycobacteria Isolation

Culture of tissue homogenates for mycobacteria was done in a class II bio-safety cabinet in Biosafety level 3 laboratory. The homogenized sample was first decontaminated using 5% oxalic acid to suppress the growth of fast growing microorganisms to allow the growth of mycobacterium (which is slow-growing) if present. The method was adapted from Yeboah-Manu *et al*., 2004. The method involves a 30-minutes incubation of 5% oxalic acid with an equal volume of the sample in a 50 mL falcon tube with intermittent vortexing. The decontamination reaction was stopped by adding Phosphate buffered saline (PBS) up to the 45 mL mark. The suspension was then pelleted by centrifuging at 3800 rpm for 30

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minutes. The tubes were left to stand for 5 minutes before opening to allow aerosols formed during centrifuging to settle. The supernatant was decanted and the pellet was re-suspended in 2 mL PBS. After standing for a few minutes, approximately 250 µL of the suspension was directly inoculated in duplicate onto pyruvate based and glycerol based L-J slants.

The inoculum was spread evenly on the entire media surface by slightly tilting the media tubes and incubated at 37 ºC in tilted positions on a wooden rack. Smears for Ziehl-Nelson (ZN) staining and microscopy were also prepared from the decontaminated samples by placing 2 drops of the suspension on a labeled slide and spreading evenly to cover the central area of the slide in order to evenly disperse the bacilli. The cultures were observed daily for the first week and once a week subsequently for growth. Culture readings entailed the observed colony morphology, degree of growth and time of observed macroscopic growth for each tube. Culture positivity was confirmed with Acid fast bacilli (AFB) positive results from smear microscopy. Culture Positives isolates with confluent growths were sub-cultured on L-J for their purification and multiplication. Tubes with no growth after 12 weeks of incubation were declared culture negative and contamination was defined by overgrowth of non-acid fast bacilli on an L-J slant.

3.2.7 Bacterial and Fungi Isolate Identification

3.2.7.1 Ziehl-Nelson Staining

The prepared smears for ZN staining were allowed to dry at room temperature in the biosafety cabinet to reduce contamination and risk of infection. The slides were then heat-fixed by passing it slowly through a Bunsen burner flame about 3-4 times to denature

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bacterial proteins to allow the cells to stick to the slide. The staining procedure followed is that described by the Standard Operating Procedure for tuberculosis microscopy in Ghana and the tuberculosis microscopy laboratory manual for Ghana (Ghana Health Service, 2011; National Tuberculosis Programme, 2012). The heat fixed slides were arranged on a rack with spaces in between them to prevent cross contamination. The smears were covered with carbol-fuchsin stain and the undersides of slides were heated to about 60 ^{o}C until vapour rose from the stain to create pores in the mycolic acid mycobacterial cell wall, to allow the uptake of the stains. After 5 minutes the stain was washed off gently with tap water to remove any residual dye. The smears were then covered with 20% H_2SO_4 solution for 5 minutes to decolorized non acid-fast cells of the carbol-fuchsin stain. The decolourizer was gently washed off with tap water and the smears were counterstained with 0.1% methylene blue for 1 minute. This was then gently washed off and the slides were allowed to air-dry. The stained smears were examined microscopically using the 100x oil immersion objective lens by scanning at least 100 fields systematically. Non acid-fast microorganisms were observed to have the counter stain (blue) and were graded as AFB negative whereas AFB positives were identified as bright reddish-pink stained bacilli. Depending on the numbers observed, the AFB were graded as 1+, 2+, and 3+ in accordance with the International Union against Tuberculosis and Lung Diseases (IUATLD) guidelines.

3.2.7.2 Gram staining

A heat-fixed smear was flooded with the primary stain, crystal violet solution and rinsed off after 1 minute with running water. The slides were then covered with Lugol's iodine

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solution for 1 minute and washed off under running water. The iodine acts as a mordant and helps in the retention of the primary stain. The smear was then decolorized for 1-5 seconds with acetone-alcohol followed by another washing step. The smear was counterstained with neutral red for 2 minutes and washed with running water. The slide was then allowed to air-dry in an upright position and then examined microscopically first under 40x objective lens, then the 100x oil immersion objective lens. At least 100 fields were scanned and the Gram reaction of the cells observed was recorded as positive indicating purple stained cells or Gram-negative for pink or red stained cells (Cheesbrough, 2006).

3.2.8 DNA Extraction

A loop full of mycobacterial isolate growing at the log phase of pure sub-cultures (*Mtb* usually attain the log phase of growth on the 2nd week of sub-culture on L-J media (Kent & Kubica, 1985)) was suspended in 0.75 mL of sterile distilled water. The mycobacterial isolates were then inactivated by heat killing on a heating block at 95 ^{o}C for 1 hour in order for the mycobacterial cell wall to be lysed for the release of DNA into suspension. The DNA was stored at -20 ^{o}C until further use.

3.2.9 Molecular Characterization Experiments

3.2.9.1 MTBC Detection by IS*6110* Polymerase Chain Reaction (PCR)

All AFB positive isolates were confirmed as MTBC by PCR detection of the insertion sequence *6110* as described by Yeboah-Manu *et al*., 2001. The PCR mix consisted of 16.2 µL nuclease-free H_2O, 5 µL PCR 10x buffer, 10 µL Q Solution, 2.5 µL $MgCl_2$ (25 mM),

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1 µL dNTP (10 mM), 2.5 µL forward primer (primer F) (10 pmol/µL), 2.5 µL reverse primer (primer R) (10 pmol/µL), 0.3 µL HotStarTaq *Plus* DNA polymerase (5 U/µL), 5 µL 10x Coral dye and 5 µL of DNA template to give a final volume of 50 µL. The *Mycobacterium tuberculosis* reference strain H37Rv was used as template in the positive control and nuclease-free water as template in the negative control. The primers sequences were TB284: 5′-GGA CAA CGC CGA ATT GCG-3′ and TB85: 5′-TAG GCG TCG GTG ACA AAG GCC AC-3’. The amplification process was then carried out in a PCR thermal cycler with cycling conditions as follows;

Table 3.1: Cycling Conditions of *IS6110* PCR

Temperature (°C)	Duration (minutes)	Number of Cycles
96	5	1
95	1	35
65	1	
72	1	
72	10	1

The PCR amplicons were then resolved on 2% agarose gel at 80-120 V for 30 minutes, viewed under the UV trans-illuminator of the Gel Logic Imager and sized using a 100 bp molecular marker. IS*6110* positive isolates had an expected band size of 550 bp.

3.2.9.2 Mycobacteria Identification by *hsp65* Sequence

Isolates confirmed to be acid fast but IS*6110* negative were further identified by amplification of mycobacterium genus specific *hsp65* gene followed by sequencing. The PCR master mix consisted of 14.8 µL of nuclease-free water, 3 µL of Q Solution, 1.8 µL

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of $MgCl_2$ (25 mM), , 3 µL of 10x PCR buffer, 0.6 µL of dNTP (10 mM), 1.8 µL of TB11 primer (10 pmol/µL), 1.8 µL of TB12 primer (10 pmol/ µL), 3 µL of 10x Coral dye, 0.2 µL of HotStarTaq *Plus* DNA polymerase (5 U/µL) and 5 µL of DNA template. The final reaction volume was 35 µL and the primers sequences; TB11: 5'-ACC AAC GAT GGT GTG TCC AT-3' and TB12: 5'-CTT GTC GAA CCG CAT ACC CT- 3'. The cycling conditions used for successful amplification are presented in the table below.

Table 3.2: Cycling Conditions of *hsp*65 PCR

Temperature (^oC)	Duration (minutes)	Number of Cycles
95	5	1
96	1	35
60	1	
68	1	
72	10	1

The PCR amplicons were then resolved by agarose gel electrophoresis as previously indicated. Positive isolates for *hsp65* showed a band with a size of 439 bp (Telenti *et al*., 1993). The amplicons were then sequenced by outsourcing and the sequences were imported for analyses.

3.2.9.3 Spoligotyping (spacer oligotyping) of Confirmed MTBC Isolates

Spoligotyping is a PCR-based method used for the simultaneous detection and typing of *Mycobacterium tuberculosis complex* species based on the number of spacers within the direct repeat locus in the genome which is unique for different strains of MTBC. The

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experiment was carried out as described by Kamerbeek *et al*., 1997, using approximately 10ng of genomic mycobacterial DNA purified from cultured cells. The direct repeat locus was amplified and the reaction mixture comprised of 34.5 µL of dH_2O, 5µL of 10x buffer, 0.4 µL dNTPs, 4 µL each of the forward and reverse biotinylated primers (DRa: 5'-GGT TTT GGG TCT GAC GAC-3 and DRb: 5'-CCG AGA GGG GAC GGA AAC-3'), 0.1 µL HotStarTaq *Plus* DNA Polymerase enzyme, and 5 µL DNA template. H37Rv together with *M. bovis* and the master mix without DNA template were used as positive and negative controls respectively. The details of the cycling conditions used are shown in table **3.3**.

Table 3.3: Cycling Conditions of PCR for spoligotyping

Temperature (°C)	Duration (minutes)	Number of Cycles
96	3	1
96	1	35
55	1	
72	1	
72	5	1

At the end of the PCR, 25 µL of the amplicon was added to 145 µL of 2xSSPE/0.1% SDS solution and heated to 99 ºC for 10 minutes to denature the double stranded DNA. The mixture was immediately transferred onto ice to prevent re-annealing of the separated strands and the working concentrations of the different buffers used in subsequent hybridization steps of the assay were prepared. The buffers including 450 mL 2xSSPE/0.1% SDS, 850 mL 2xSSPE/0.5% SDS and 450 mL 2xSSPE were pre-warmed at 60 ºC, 60 ºC or 45 ºC and room temperature respectively in a Hybrigen oven. The

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membrane for the assay which has a set of 43 immobilized oligonucleotides (each corresponding to one of the unique spacer DNA sequences within the DR locus) was also prepared by washing in 250 mL of 2xSSPE/0.1% SDS solution for 5 minutes at of 60 °C. Afterwards it was placed in a miniblotter, rightly oriented with a support cushion and residual wash buffer was aspirated from the slots of the miniblotter. A 130 µL volume of the denatured amplicons were then dispensed into each slot on the membrane and incubated for 60 minutes at 60 ºC to allow hybridization of the amplicons to the immobilized membrane oligonucleotides. Afterwards, residual solution in the slots were aspirated and the membrane was washed twice in 250 mL 2xSSPE/0.5% SDS solution at 60 °C for 10 minutes. The washed membrane was then placed in a rolling bottle and a mixture of 5 µL streptavidin-peroxidase conjugate (Boehringer) and 10 mL 2xSSPE/0.5% SDS was added once temperature was low to preserve the integrity of the peroxidase enzyme. This was incubated for 45 to 60 minutes at 42 °C as the rolling bottle rotated.

The membrane was washed twice in 250 mL 2xSSPE/0.5% SDS solution for 10 minutes at 42 °C and rinsed twice again with 250 mL of 2xSSPE solution for 5 minutes at room temperature. Detection of hybridized DNA was achieved by incubation of the membrane in chemiluminescent ECL (Amersham) detection liquid (this was covered with a transparent plastic sheet for uniform distribution on the membrane surface) for 1 minute, followed by exposure to X-ray film for 5 minutes in a dark room. The membrane was then developed using a developer solution, washed in water for 1 minute and subsequently placed in fixer for another minute.

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3.2.10 Identification by MALDI-TOF MS

Bacteria and fungi colonies at the log phase of growth were picked with a 1 µL loop and well spotted on the MALDI-TOF 96-spot Anchorchip target plate (Bruker Daltonics, Inc., Bremen, Germany) The sample was allowed to dry at room temperature and 1 µL 70 % formic acid was added to each spot to lyse the cell wall of the microorganisms and expose the ribosomal proteins in the cytoplasm for enhanced identification. This method is termed as extended direct. Finally, the dried sample spot was overlaid with 1 µL of HCCA (cyano-4-hydroxycinnamic acid) MALDI matrix solution and allowed to dry. The target was then placed in the MALDI-TOF and measurement was initiated using the FlexControl software (version 3.3.108.0). A unique spectrum of the relative abundance of ribosomal proteins was generated and this was compared to the reference database of MALDI-TOF to identify the organism based on a score. A score of 2.0 and above showed reliable identification, scores between 1.6 and 2.0 gave genus level identification and a score below 1.7 was considered low identification or unreliable. The procedure followed was in accordance to the manufacturer's instructions (Bizzini *et al.,* 2010; Haigh *et al*., 2011).

3.2.11 Detection of Non-culturable *Pneumocystis jirovecii*

3.2.11.1 DNA Extraction

DNA was purified from the lung biopsy samples using the commercial DNA extraction kit QIAamp DNA Mini Kit, a silica membrane-based nucleic acid purification technique. The procedure followed as described in the manufacturer's protocol is detailed as follows. First, 100 µL Buffer ATL and 20 µL proteinase K were added to 150 µL of the homogenized tissue in a 1.5 mL microcentrifuge tube, mixed by vortexing and incubated at 56 ºC on a

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rocking heating block for 3 hours to ensure efficient destruction of nucleases and digestion of proteins. After incubation, the microcentrifuge tubes were briefly centrifuged to remove any drops that settled on the lid. A volume of 200 µL Buffer AL was then added to the sample and pulse-vortexed for 15 seconds to yield a homogenous mixture which was incubated at 70 ºC for 10 minutes to ensure cell lysis. After incubation, 200 µL of 99.8% ethanol was added to the sample to concentrate the DNA in solution to facilitate its binding to the silica membrane when transferred into the spin column. The mixture was then briefly centrifuged and carefully transferred into the QIAamp Mini spin column without wetting the rim. This was centrifuged at 8,000 rpm for 1 minute and the speed was increased to 14,000 rpm if the solution did not completely pass through the filter in the spin column. This step was to ensure separation of the DNA from the remaining lysate. The flow-through was discarded and the QIAamp Mini spin column containing bound DNA was placed in a clean 2 mL collection tube. This was followed by two washes with 500 µL each of Buffers AW1 and AW2 each with a centrifugation step (14,000 rpm for 3 minutes) to remove residual proteins, salts and contaminants. The DNA is finally eluted into a clean 1.5 mL microcentrifuge tube with addition of 200 µL Buffer AE which hydrates the DNA causing its release from the membrane and elusion on centrifuging at 8,000 rpm for 1 minute.

3.2.11.2 Detection of *Pneumocystis jiroveci* by Nested PCR

Detection of *Pneumocystis jirovecii* was achieved using a nested PCR primer set that targets the mitochondrial large-subunit (mtLSU) ribosomal RNA. The external primer set for the first PCR round were pAZ102-E: 5’-GAT GGC TGT TTC CAA GCC CA-3’ and pAZ102-H: 5’-GTG TAC GTT GCA AAG TAC TC-3’ and PCR reaction volume was 30

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μL. This consisted of 10.7 μL nuclease-free H_2O, 5 μL 10x PCR buffer, 2 μL $MgCl_2$ (25 mM), 1 μL dNTP (10 mM), 2 μL primer pAZ102-E (10 pmol/μL), 2 μL primer pAZ102-H (10 pmol/μL), 0.3 μL HotStarTaq *Plus* DNA polymerase (5 U/μL) and 7 μL of DNA extracted from lung biopsies. The second round PCR reagents included; 10.7 μL nuclease-free H_2O, 5 μL 10x PCR buffer, 2 μL $MgCl_2$ (25 mM), 1 μL dNTP (10 mM), 0.3 μL HotStarTaq *Plus* DNA polymerase (5 U/μL) and 2 μL of DNA template (amplicons of first round PCR) to give a final volume of 25 μL. The second round primers sequences were pAZ102-X: 5'-GTG AAA TAC AAA TCG GAC TAG G-3' and pAZ102-Y: 5'-TCA CTT AAT ATT AAT TGC GGA GC-3'.

For both rounds, DNA from a sample with confirmed clinical diagnosis of Pneumocystis pneumonia ante-mortem was used as template in the positive control and nuclease-free water as template in the negative control. The amplification process was then carried out in a conventional PCR thermal cycler with the cycling conditions for both PCR rounds shown in table 3.4.

Table 3.4: Cycling condition for *Pneumocystis jiroveci* Nested PCR

<table>
<tr><th colspan="3">Round 1 PCR</th><th colspan="3">Round 2 PCR</th></tr>
<tr><th>Temperature (°C)</th><th>Duration (minutes)</th><th>Number of Cycles</th><th>Temperature (°C)</th><th>Duration (minutes)</th><th>Number of Cycles</th></tr>
<tr><td>96</td><td>5</td><td>1</td><td>96</td><td>5</td><td>1</td></tr>
<tr><td>94</td><td>1</td><td rowspan="3">35</td><td>94</td><td>1</td><td rowspan="3">35</td></tr>
<tr><td>58</td><td>30 seconds</td><td>58</td><td>30 seconds</td></tr>
<tr><td>72</td><td>1</td><td>72</td><td>2</td></tr>
<tr><td>72</td><td>5</td><td>1</td><td>72</td><td>5</td><td>1</td></tr>
</table>

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3.2.12 Drug susceptibility testing

3.2.12.1 Disc Diffusion Assay (Kirby bauer method)

The standard Kirby-Bauer disc diffusion test was used to determine the antibiogram of distinct isolates on Mueller-Hinton (MH) agar according to European Committee on Antimicrobial Sensitivity Testing (EUCAST) and British Society for Antimicrobial Chemotherapy (BSAC) guidelines (British Society for Antimicrobial Chemotherapy, 2015; European Committee on Antimicrobial Sensitivity Testing, 2015; Brown *et al*., 2016). The testing strain suspension was prepared by using a sterile swab to pick a log phase pure colony and emulsify in sterile saline until turbidity equivalent to 0.5 McFarland Standard was achieved. Already prepared MH plate was inoculated by swabbing the entire surface evenly with the suspension. The surface was allowed to dry for about 5 minutes and using a pair of forceps, antibiotic discs were placed aseptically on the agar equidistant from each other and around the periphery with one in the center (not too close to the edge of the plate to obtain fully rounded zones for all drugs). The plates were then incubated upside down at 37 ^{o}C and the plates were examined after 16 to 18 hours of incubation (20 to 24 hrs. for *Streptococcus spp., N. meningitidis* and *N. gonorrhoeae*). A full 24 hours of incubation was adhered to as recommended for *Staphylococcus aureus.* For fastidious organisms such as *Streptococcus spp*, the MH agar was supplemented with 5% Sheep Blood. The antibiotics used were grouped into those potent against Gram positive and Gram negative organisms. Gram positive antibiotics used were gentamicin, oxacillin, flucloxacillin, erythromycin, ciprofloxacin, sulfamethoxazole trimethoprim, levofloxacin and vancomycin whiles antibiotics used against Gram negatives included; gentamicin,

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cefoxitin, cefuroxime sodium, ceftriaxone, amoxycillin/clavulanic acid, sulfamethoxazole trimethoprim, cefotaxime and ceftazidime.

Following incubation, a metric ruler was placed across the zone of inhibition, at the widest diameter and measured from one edge of the zone to the other whiles the plate was held to light. In an instance where there was no zone at all, it was recorded as zero (0). The recorded zone of inhibition was compared with standard breakpoint of the different antibiotics for each given organism and graded as sensitive or susceptible (S), resistant (R), or intermediate (I) (BSAC, 2015; EUCAST, 2015).

3.2.12.2 Anti-TB Drug susceptibility testing using Genotype MTBDR*plus*

The susceptibility pattern of MTBC isolates to the most important first line anti-TB drugs; rifampicin and isoniazid were determined using Genotype MTBDRp*lus* version 2.0 (Hain lifescience). The assay involved two major steps; first PCR amplification of specific gene targets and secondly, hybridization of amplicons to immobilized probes for the detection of drug resistance associated mutations. The PCR was carried out by adding a 45 µL mixture of two reagents; AM-A (Amplification mix A-10 µL) and AM-B (Amplification mix B- 35 µL) to 5 µL DNA template followed by amplification in a conventional thermal cycler in parallel with a negative (nuclease free water) and positive control (H37Rv). The PCR cycling conditions are presented in table 3.5;

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Table 3.5: The cycling condition for Genotype MTBDRplus PCR

Temperature	Duration	Number of cycles
95^{0}C	15 min	1 cycle
95^{0}C	30 sec	20 cycles
65^{0}C	2 min	
95^{0}C	25 sec	30 cycles
50^{0}C	40 sec	
70^{0}C	40 sec	
70^{0}C	8 min	1 cycle

For hybridization to be achieved, the PCR amplicons were first denatured by a 5 minutes incubation with 20µL denaturation solution (DEN) at room temperature. One milliliter of pre-warmed hybridization (HYB) solution (containing 10% anionic tenside and dye) was added to wells with denatured amplicons and mixed carefully by tilting the trough up and down to obtain a uniform colour. Labelled strips containing the mobilized single-stranded DNA probes were then placed in the wells with the aid of tweezers and incubated in an automated shaking Twincubator for 30 minutes at 45 ^{o}C.

After the hybridization process, the HYB was completely aspirated and 1 mL of pre-warmed stringent (STR) wash solution (containing 25% of a quaternary ammonium compound, <1% anionic tenside and dye) was added to each test well. This was also incubated for 15 minutes at 45 ^{o}C to remove all non-specific bound DNA. Residual stringent solution was completely aspirated and the strips were washed with pre-warmed

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rinse (RIN) solution (consisting of buffer, <1% NaCl, and <1% anionic tenside) for 1 minute in the Twincubator. Excess RIN solution was completely removed and 1 mL diluted conjugate (containing streptavidin-conjugated alkaline phosphatase, dye, buffer, 1% blocking reagent and <1% NaCl) was incubated with the strips for 30 minutes. The conjugate was aspirated and washed twice using 1 mL RIN solution, then once with 1 mL distilled water for 1 minute each. The strips were again incubated with the 1 mL diluted substrate solution (<70% dimethyl sulfoxide, <10% 4-nitro blue tetrazolium chloride, <10% 5-bromo-4-chloro-3-indolyl phosphate, buffer, <1% MgCl2 and <1% NaCl) for 10 minutes at room temperature and the reaction was finally stopped by rinsing twice with distilled water. The strips were removed with the aid of tweezers and placed between two layers of absorbent paper to dry.

The developed bands on the strips were evaluated by pasting strips in designated fields on an evaluation sheet in alignment with the conjugation control band (CC-which indicates successful conjugation process), amplification control band (AC-which indicates successful amplification) and the other gene designated lines on the sheet (figure 3.1). In total, a strip has 27 immobilized probes (highly specific) which give specific banding patterns on the strip following successful hybridization and are evaluated against a template to determine susceptibility or resistance to rifampicin, isoniazid or both. Drug resistance was therefore determined by the absence of wild-type band, presence of mutation band or both. The interpretation was only done when bands intensities were comparable to that of the universal control (band labelled as TUB), samples with faint bands were repeated (figure 3.1).

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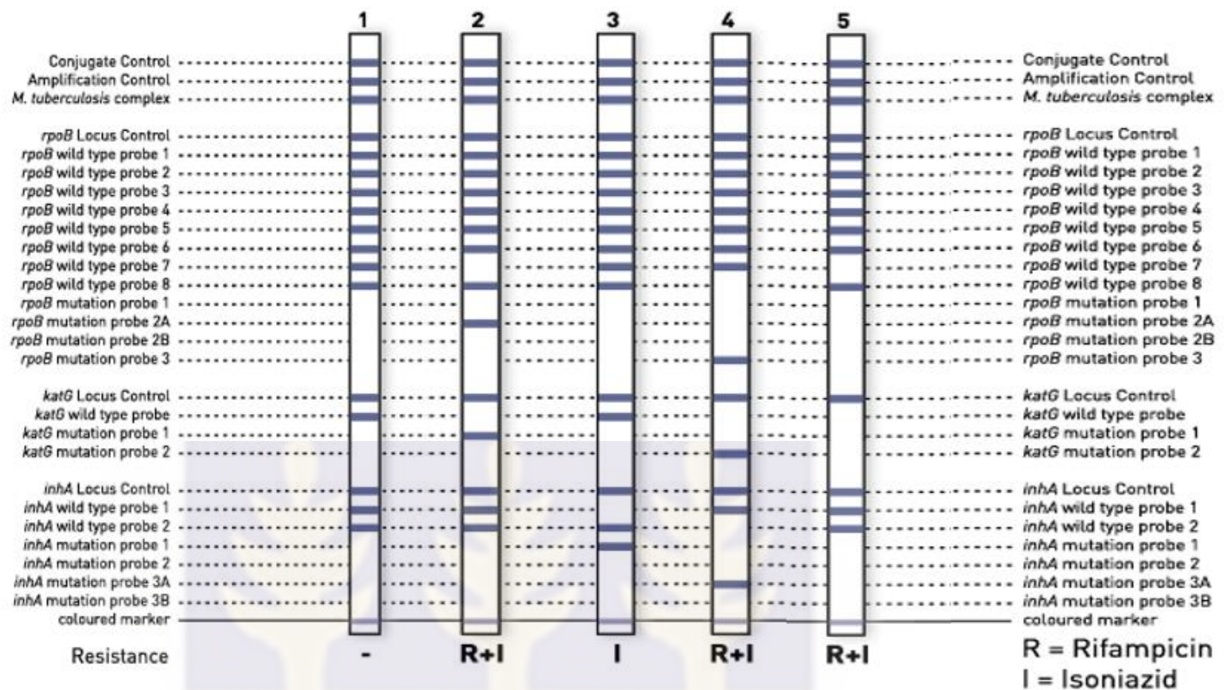

Figure 3.1: Interpretation of MTBD*Rplus* results

3.2.13 Viral Detection

3.2.13.1 RNA Extraction

To enable detection of RNA viruses present in the lung biopsies, RNA extraction was performed using a commercial RNA extraction kit (Quick-RNA MiniPrep Plus, Zymo Research) in accordance with the manufacturer's instructions. The main steps included; protein digestion, cell lysis, DNA digestion and removal, and finally elution of the RNA extract. The eluted RNA was stored at ≤-70ºC for use in subsequent experiments.

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3.2.13.2 RNA Viruses Detection by Reverse Transcriptase Real-Time PCR (Allplex Respiratory panel 2 Assay)

Seven different RNA viruses including Adenovirus, Enterovirus, Metapneumovirus and Parainfluenza 1-4 were the targets of the multiplex RT-qPCR (Allplex assay). The reaction mixture comprised of; 5 µL 5X RP2 MOM (containing all primer sets and fluorescent probes), 5 µL RNase-free water, 5 µL 5X Real-time One-step Buffer, 2 µL Real-time One-step Enzyme and 8 µL RNA template.

The PCR was carried out in the CFX-96 Bio-Rad thermal cycler set up to detect specific fluorophores (FAM, HEX, Cal Red 610, and Quasar 670) at designated steps (steps 4 and 5) with positive (containing all viral gene targets as the template) and negative controls (containing nuclease-free water instead of the template). The reaction conditions and fluorophore indices are presented in tables 3.6 and 3.7 respectively.

Table 3.6: Cycling Conditions of RT-qPCR (Allplex assay)

<table>
<tr><th>Step</th><th>Temperature (°C)</th><th>Number of Cycles</th><th>Duration</th></tr>
<tr><td>1</td><td>50</td><td>1</td><td>20 min</td></tr>
<tr><td>2</td><td>95</td><td>1</td><td>15 min</td></tr>
<tr><td>3</td><td>95</td><td rowspan="3">45</td><td>10 sec</td></tr>
<tr><td>4</td><td>60</td><td>1 min</td></tr>
<tr><td>5</td><td>72</td><td>10 sec</td></tr>
</table>

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Table 3.7: Fluorophores Detection Indices

Fluorophore	Analyte (Graph 1)	Analyte (Graph 2)
FAM	Parainfluenza Virus 4	Metapneumovirus
HEX	Parainfluenza Virus 2	Parainfluenza Virus 1
Cal Red 610	Adenovirus	Enterovirus
Quasar 670	Internal Control (IC)	Parainfluenza Virus 3

3.2.13.3 DNA Virus (Cytomegalovirus) Detection by Nested PCR

Detection of Cytomegalovirus (CMV) was achieved using a nested PCR primer set that targets the CMV glycoprotein B gene. The primer set for the first round PCR were CMV-5A: 5'-TCA TGA GGT CGT CCA GA-3' and CMV-5B: 5'-TGA GGA ATG TCA GCT TC-3'. The PCR mix consisted of 12.5 µL nuclease-free H_2O, 3 µL 10x PCR buffer, 2 µL $MgCl_2$ (25 mM), 0.6 µL dNTP (10 mM), 2 µL CMV-5A primer (10 pmol/µL), 2 µL CMV-5B primer (10 pmol/µL), 0.3 µL HotStarTaq *Plus* DNA polymerase (5 U/µL) and 2.6 µL of DNA template (extracted from lung biopsy samples) to give a final volume of 25 µL. The second round PCR reagents included; 14.1 µL nuclease-free H_2O, 1.5 µL 10x PCR buffer, 2 µL $MgCl_2$ (25 mM), 0.6 µL dNTP (10 mM), 2 µL CMV-5C primer (10 pmol/µL), 2 µL CMV-5D primer R (10 pmol/µL), 1.5 µL 10x coral dye, 0.3 µL HotStarTaq *Plus* DNA polymerase (5 U/µL) and 1 µL of DNA template (first round PCR amplicons) to give a final volume of 25 µL. The second round primers sequences were CMV-5C: 5'-TCG TCC AGA CCC TTG AGG TA-3' and CMV-5D: 5'-CCA GCC TCA AGA TCT TCA T-3'. For both rounds, DNA from a sample with confirmed clinical diagnosis of CMV ante-mortem was used as template in the positive control and nuclease-free water as template in

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the negative control. The amplification process was then carried out in a conventional PCR thermal cycler with cycling conditions as follows;

Table 3.8: Cycling condition for CMV Nested PCR

Round 1 PCR			**Round 2 PCR**		
Temperature (^oC)	Duration (minutes)	Number of Cycles	Temperature (^oC)	Duration (minutes)	Number of Cycles
96	5	1	96	5	1
94	1		94	1	
52	30 seconds	35	58	30 seconds	25
72	1		72	1	
72	5	1	72	5	1

3.2.14 Data Analysis

Decedents' demographic and clinical data extracted from the hospital records and post-mortem reports were first entered into Microsoft Excel and then exported to STATA version 14.0 for analysis (Karat *et al*., 2016). Summaries and descriptive statistics of data were carried out on all decedents' information stratified by gender, type of HIV and TB infection diagnosed as well as TB/HIV co-infection. The ART status together with the point prevalence for the primary and contributory cause of death was also considered in the analysis. Frequencies were determined for the Gram-positive and negative isolates identified. The antibiogram of bacteria isolates stratified by Gram status was also interpreted according to the EUCAST and BSAC standards as resistant, intermediate or susceptible (British Society for Antimicrobial Chemotherapy, 2015; European Committee

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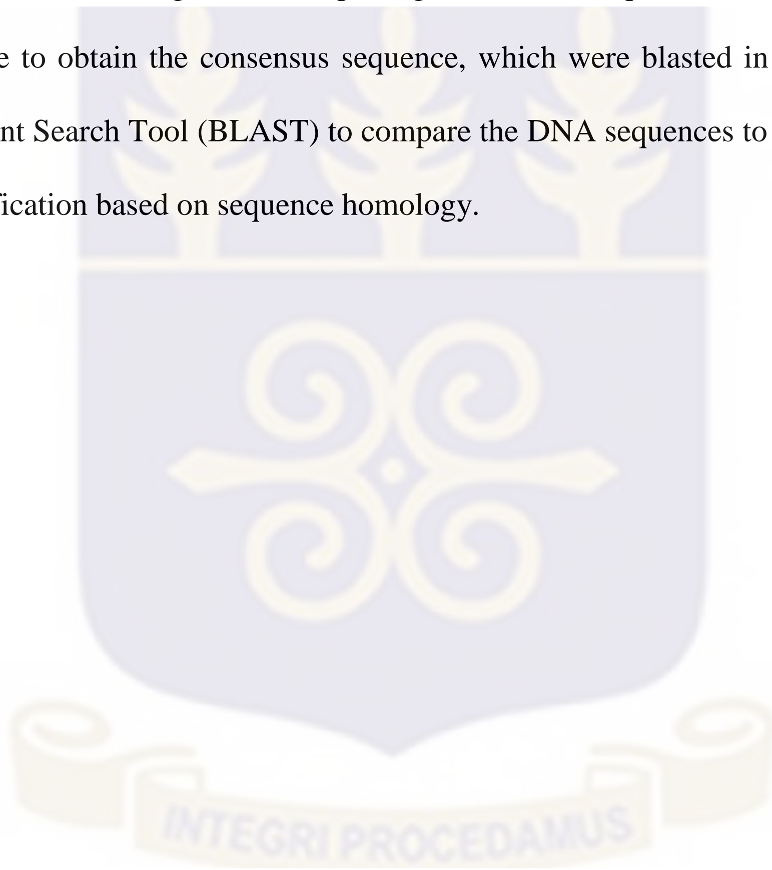
on Antimicrobial Sensitivity Testing, 2015; Brown *et al*., 2016) and the percentage resistance and susceptible were estimated. The frequencies of cases with multiple microorganisms and the various polymicrobial combinations were also determined using STATA.

The nucleotide sequence reads obtained from sequencing the *hsp65* PCR amplicons were cleaned and edited using the Staden package. The clean sequences were exported to Mega 5 software to obtain the consensus sequence, which were blasted in NCBI Basic Local Assignment Search Tool (BLAST) to compare the DNA sequences to sequence databases for identification based on sequence homology.



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CHAPTER FOUR

4.0 RESULTS

4.1 Demographic and Clinical data of Participants

A total of one-hundred and two post-mortem lung biopsies were collected following consent by subject's relative, however, biographical and clinical data were available for only 90 participants out of which 86 (95.5%) were HIV-positive. Of the 86 HIV positive cases, males were 42 (48.8%) and females were 42 (48.8%) with corresponding mean ages of 40.36 (±10.57) and 37.11 (±11.48); while the gender for two of the cases was not indicated. Among the HIV-positive cases, HIV type was defined only in 39 (45.3%) and as expected HIV-1 dominated [32 (37.2%)] with an equal distribution among males and females. Five of the cases (5.8%) had dual HIV-1&2 infection and 2 cases had HIV-2 as shown in Table 4.1. Data on duration of viral infection was also available for only 44 (51.2%) with 27(31.4%) diagnosed less than 6 months pre-mortem, 6 (7%) was between 6 months and 2 years before death and 11 (12.8%) had been diagnosed for more than 2 years before death. Antiretroviral therapy status was indicated for 51 (59.3%) and of these, 20 (23.3%) were on active treatment (males 11 and 9 females), 17 (19.8%) were ART naïve (males 8 and 9 females), and 14 (16.3%) defaulted (males 7 and females 6, 1 gender unknown) (Table 4.1).

Additionally, HIV/TB co-infection was indicated in 42 (48.8%) out of the 86 HIV-positive cases with an equal proportion among males and females. Three forms of TB were documented; pulmonary TB 17 (40.5%) with an approximate 2:1 ratio (males: females), followed by disseminated TB 11 (26.2%) and TB meningitis which was present in 6

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(14.3%) cases. In eight (19.0%) of the cases however, the form(s) of TB was not specified (Table 4.1).

Table 4.1: Biographical and Clinical History Participants

Characteristics	Gender			Total (%)	p-value[#]
	Male n (%)	Female n (%)	Unknown n (%)		
Mean Age[*]	40.36 (±10.57)	37.11 (±11.48)	-	-	1.00
HIV Positive	42 (48.8)	42 (48.8)	2 (2.3)	86 (100)	
HIV Type					
Not Defined	22 (46.8)	23 (48.9)	2 (4.3)	47 (54.7)	
HIV-1	16 (50.0)	16 (50.0)	-	32 (37.2)	1.00
HIV-2	1 (50.0)	1 (50.0)	-	2 (0.9)	
HIV-1&2	3 (60.0)	2 (40.0)		5 (5.8)	
HIV Duration					
Not Defined	19 (45.2)	22 (23.7)	1 (2.4)	42 (48.8)	
<6 Months	12 (44.4)	14 (51.8)	1 (3.7)	27 (31.4)	0.62
6 months- 2 years	4 (66.7)	2 (33.3)	-	6 (7.0)	
3-8 years	7 (63.6)	4 (36.4)	-	11 (12.8)	
Therapy					
ART	11 (55.0)	9 (45.0)	-	20 (23.3)	0.93
ART Naïve	8 (47.1)	9 (52.9)	-	17 (19.8)	
Defaults	7 (50.0)	6 (42.9.)	1 (7.1)	14 (16.3)	
Data Unavailable	16 (45.7)	18 (51.4)	1 (2.9)	35 (40.7)	
HIV/TB Co-infection (N=42)					
Pulmonary TB	11 (64.7)	6 (35.3)	-	17 (40.5)	
Disseminated TB	3 (27.3)	8 (72.7)	-	11 (26.2)	0.29
Meningitis	3 (50.0)	3 (50.0)	-	6 (14.3)	
Unspecified TB	4 (50.0)	4 (50.0)		8 (19.0)	

Not Defined (Information not available on case forms)

[*] ***The Mean ages were computed with mean ± SD***

[#] ***The unknown data were excluded from the statistical analysis***

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Analyses of autopsy reports indicated several causes of death with co-morbidities occurring in 78 cases. HIV/TB co-infection occurred in 24 cases whereas HIV/bronchopneumonia was reported in 22 cases. The cause of death for one case was unknown although HIV-positive status had been confirmed 2 years before death. Seventy-two of the cases had HIV as the underlying cause of death and tuberculosis related deaths were the most frequent autopsy diagnosis in 25 of the cases. In two of the 25 TB-infected decedents, diagnosis with TB was made only at post mortem for one and the other had multidrug-resistant (MDR) TB. The third most common cause of death reported was bronchopneumonia which was found in 24 of the cases. In addition to the afore-mentioned, other pulmonary complications were also reported (Table 4.2).

Besides pulmonary diseases, the other indicated causes of death were cerebral complications (15), severe anaemia/ diarrhoea (9), purulent leptomeningitis (7), renal complication (6), sarcoma (3), hypertension (3), severe wasting (2), gastro-intestinal (2), cardiac (2) and liver–associated diseases (1) (Table 4.2).

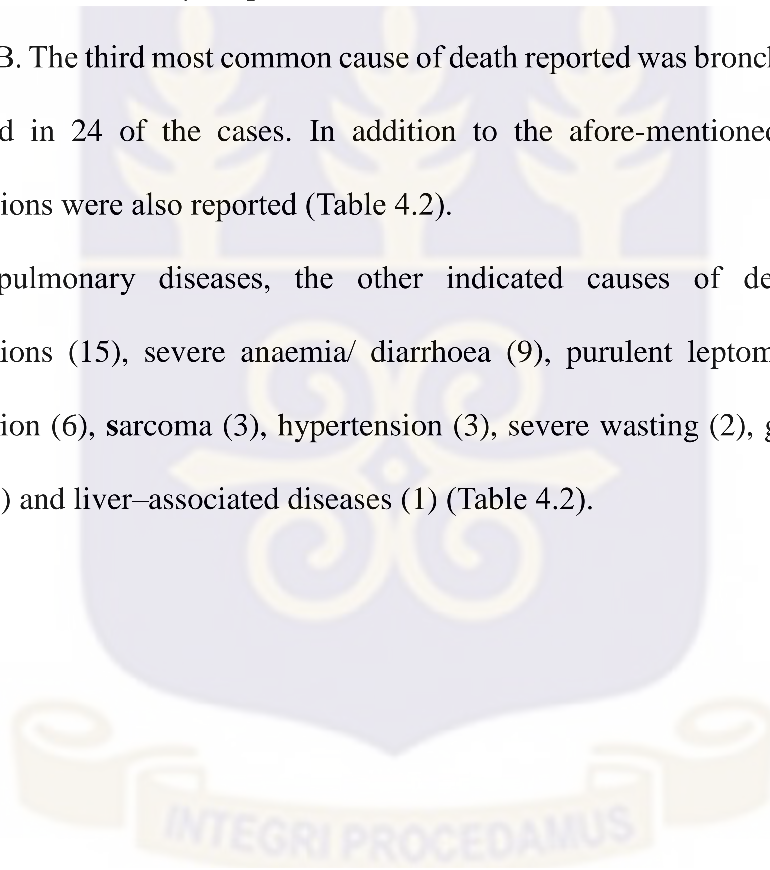

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Table 4.2: Main Post-mortem Diagnoses in Study Decedents

Autopsy Finding	Number of Cases
HIV/AIDS	72
Tuberculosis (pulmonary, disseminated & meningitis)	25
Bronchopneumonia	24
Other respiratory complications (Oedema, embolism, oesophagitis)	7
Purulent leptomeningitis	7
Sarcoma	3
Cerebral complications	15
Liver complications	1
Gastro-intestinal complications	2
Hypertension	3
Cardiac complications	2
Severe Anaemia/ diarrhoea	9
Severe wasting	2
Renal complications	6
Unknown	1

4.2 Microbial Isolates from Lung Biopsies

A total of one hundred and two (102) lung biopsies were screened for fungi, mycobacteria and other bacteria species (Figure 4.1). In 100 out of the 102 lung biopsies cultured, 375 microorganisms were isolated and the relative isolation frequency ranged from 1 to 7. The most common microorganisms isolated were bacteria (221) whiles fungal cultures had the lowest culture recovery with positivity of 3. 50%. Using PCR, 43 non-culturable fungi were detected increasing the fungal estimates to 52 (13.9%). Mycobacteria were isolated from 33 out of the 102 lung biopsies and 69 (18.4%) viruses were also detected (Table 4.3).

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Table 4.3: Different Microorganisms Detected

Type of microorganism Isolated	Number	Frequency
Mycobacteria species	33	8.8%
Other bacteria	221	58.9%
Fungi	52	13.9%
Virus	69	18.4%
Total	375	100%

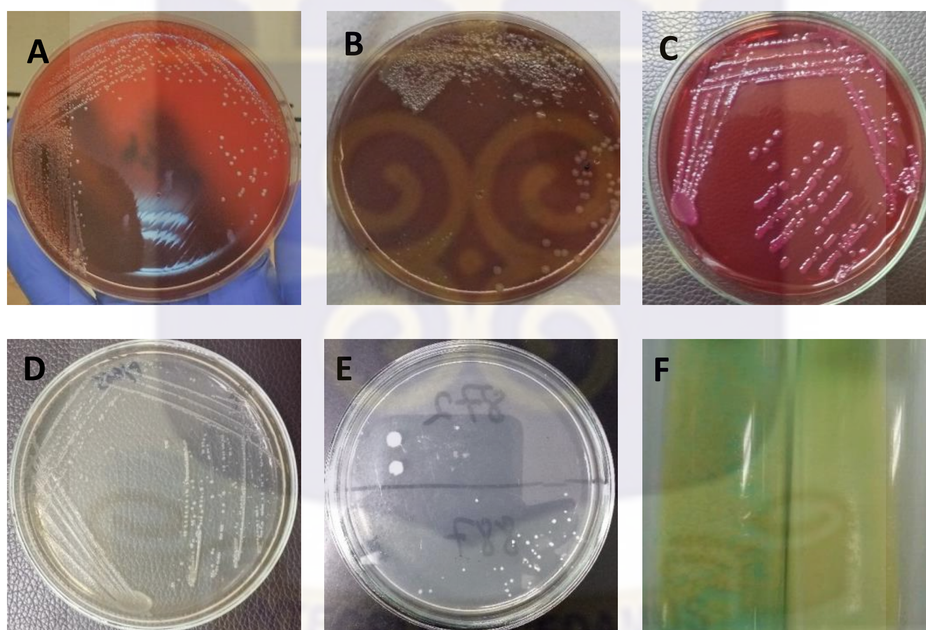

Figure 4.1: Bacteria, mycobacteria and fungal culture. *(A) Blood Agar plate with bacteria colonies (B) MacConkey Agar with bacteria colonies (C) Chocolate Agar plate with bacterial colonies D) Potato Dextrose Agar with mixed fungi colonies (E) Bacterial colonies on Nutrient Agar (F) Mycobacterial colonies on L-J slants*

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4.3 Fungal species Identification

The fungi obtained were Gram stained (Figure 4.2) and further identified using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS). Using MALDI-TOF MS, the fungal isolates were identified as *Candida glabrata (3), Candida tropicalis (2), Candida albicans (1), Cryptococcus neoformans (1)*, *Yarrowia lipolytica (1)* and *Pichia occidentalis (1).* Non-cultivable *Pneumocystis jiroveci* was also detected in 43 of the biopsy samples by nested PCR and visualized by gel electrophoresis (Figure 4.3). These are presented in the table below (Table 4.4).

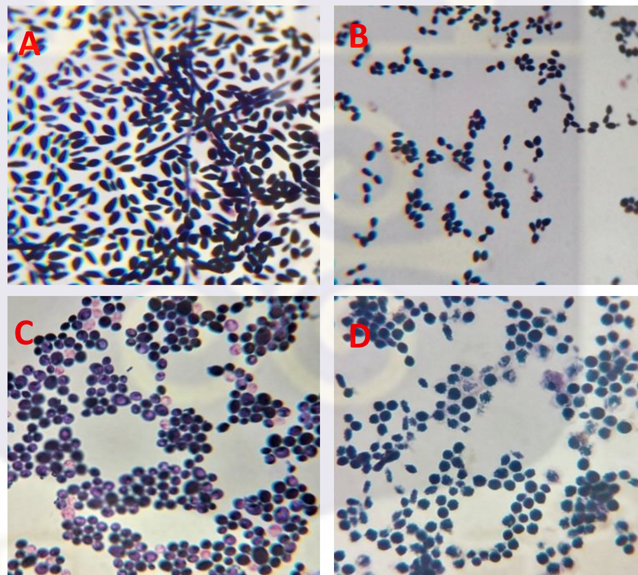

Figure 4.2: Gram-stained fungi species under x100 objective lens of a light microscope. *(A) Gram-positive hyphae forming fungus (B-D) Gram-positive budding yeasts*

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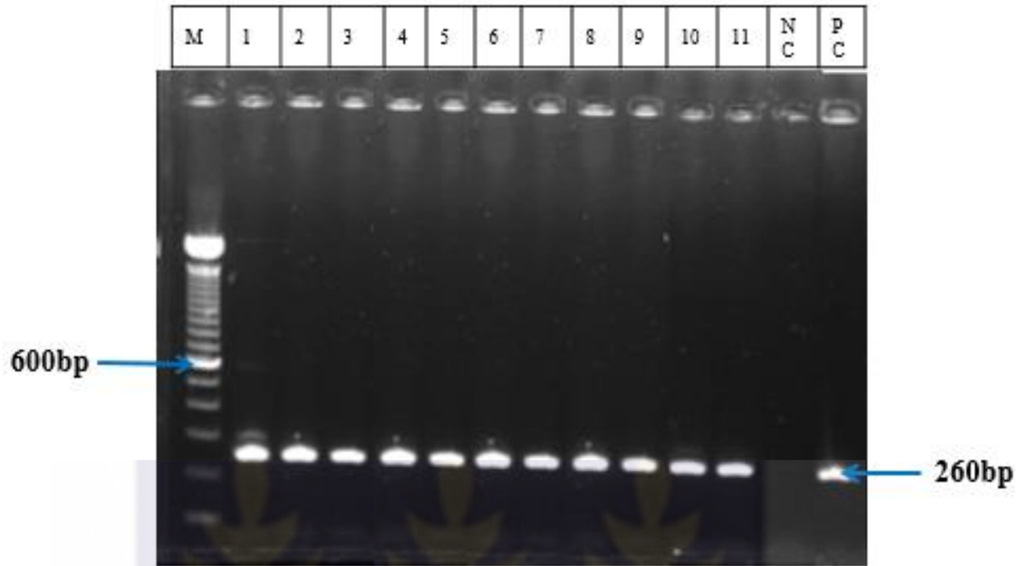

Figure 4.3: Electrophoresis gel showing *Pneumocystis jiroveci* positive samples. *M: 100bp Molecular weight marker, Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11: Pneumocystis jiroveci positive samples, NC: Negative control, PC: Positive control* (A known positive *Pneumocystis jiroveci* DNA sample)

Table 4.4: Identified Fungi species from lung biopsies

Fungal species Identified	Number
Pneumocystis jiroveci	43
Candida albicans	1
Candida glabrata	3
Candida tropicalis	2
Cryptococcus neoformans	1
Yarrowia lipolytica	1
Pichia occidentalis	1
Total	52

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4.4 Characterization of Bacteria Isolates

4.4.1 Bacteria Species Identified

The bacteria isolated were identified by Gram staining and MALDI-TOF MS as shown in figure 4.4 and 4.5. Gram positives constituted 59.8% whiles Gram negatives were 40.2%. MALDI-TOF MS classified the 221 bacteria isolates into 57 different species. The predominant species included *Enterococcus species* (63), *Staphylococcus species* (35), *Escherichia coli* (28), *Klebsiella pneumoniae* (23), *Bacillus species* (15), *Citrobacter species* (8), *Enterobacter species* (7), *Acinetobacter* species (7), *Pseudomonas species* (6), *Lactobacillus species* (5), *Kocuria rhizophila* (4), *Streptococcus species* (3), *Neisseria meningitides* (2) and *Stenotrophomonas maltophilia* (2).

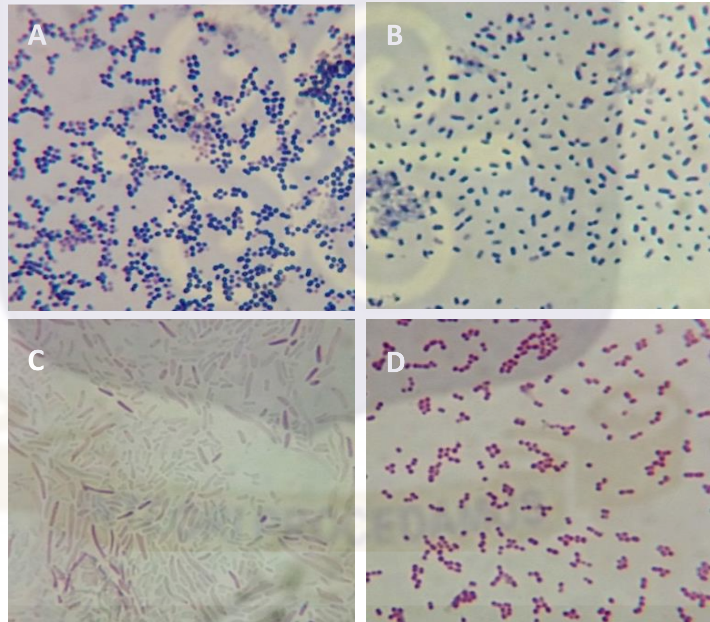

Figure 4.4: Gram-stained bacteria under x100 objective lens of a light microscope. *(A) Gram-positive cocci (B) Gram-positive rods (C) Gram-negative rods (D) Gram-negative cocci.*

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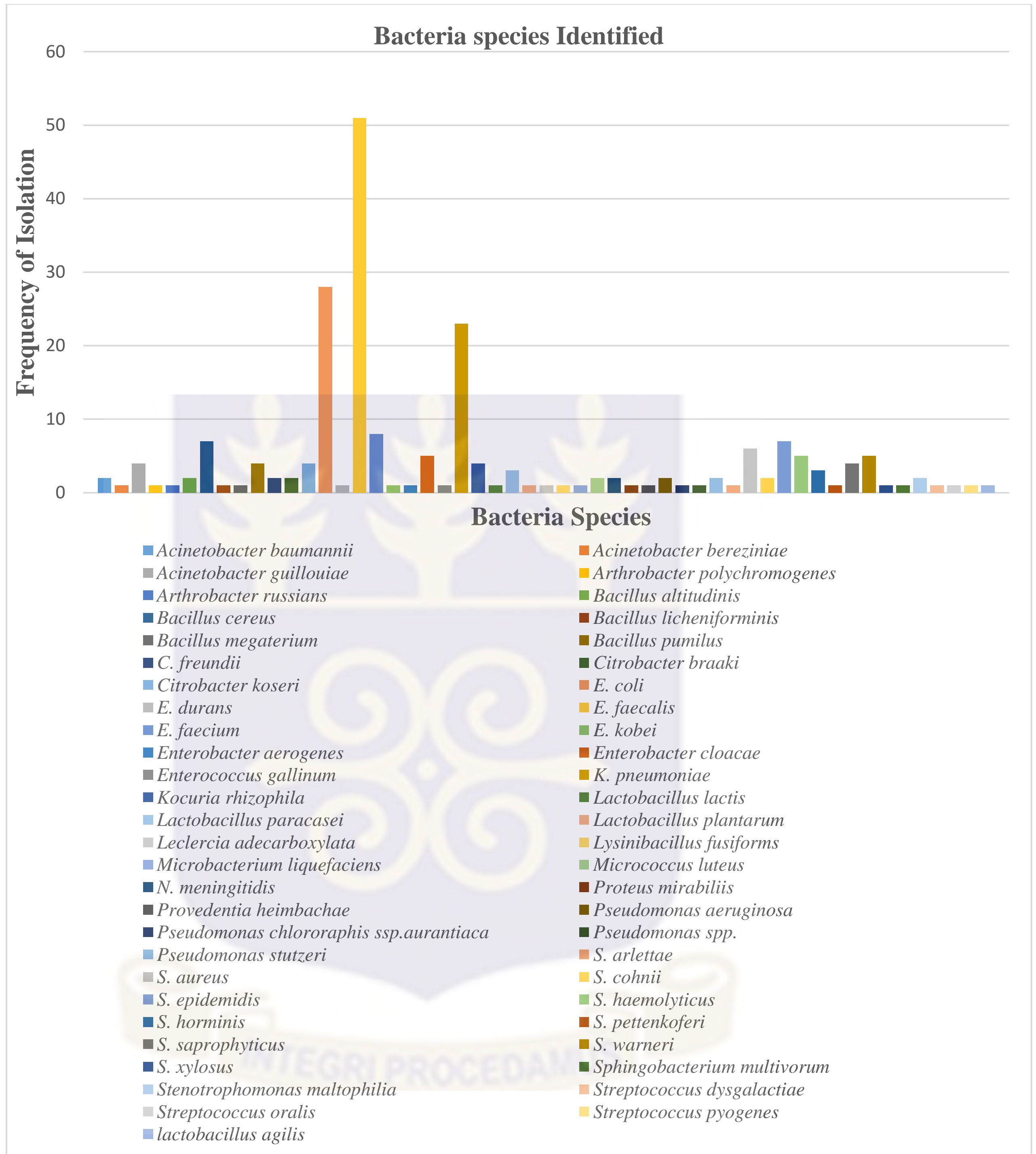

Figure 4.5: Identification of Bacteria species by MALDI-TOF MS

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4.4.2 Antibiotic Susceptibility Profile of Bacteria Isolates

Overall, 192 purified bacteria isolates comprising 117 Gram positives and 75 Gram negatives were profiled for drug susceptibility using the disc diffusion method (Figure 4.6). The proportion of all Gram positive bacteria that showed resistance to the various drugs were; oxacillin (81.2%), flucloxacillin (75.2%), sulfamethoxazole trimethoprim (60.6%), erythromycin (58.1%), vancomycin (47%), gentamicin (41.9%), levofloxacin (29.5%) and ciprofloxacin (38.5%) (Table 4.5). Among the tested drugs, levofloxacin had the highest potency (59.0%) followed by gentamicin (53.8%) with oxacillin (17.1%) having the least (Table 4.5). Likewise, among the Gram-negative isolates, resistance to the tested antibiotics were cefuroxime sodium (84.0%), cefotaxime (77.3%), ceftriaxone (70.7%), amoxicillin/clavulanic acid (70.7%), sulfamethoxazole trimethoprim (70.7%), ceftazidime (61.3%), cefoxitin (45.3) and gentamicin (44%). Cefoxitin (45.3%) and gentamicin (45.3%) exhibited the highest susceptibility, but cefuroxime sodium (8.0%) had the least susceptibility (Table 4.5).

Resistance rate for *Enterococcus sp.* to oxacillin 55/56 (98.2%), flucloxacillin 51/56 (91.1%) and erythromycin 42/56 (75.0%) was high whiles that against levofloxacin 23/56 (41.1%) was relatively low. The susceptibility for *Enterococcus sp.* to levofloxacin 26/56 (46.4%) and gentamicin 23/65 (41.1%), was also higher than oxacillin 1/56 (1.8%). Among the *Staphylococcus isolates,* the recorded resistances were, oxacillin 18/32, (56.3%), flucloxacillin 15/32, (46.9%), sulfamethoxazole trimethoprim 15/32, (46.9%), vancomycin 11/32, (34.4%), ciprofloxacin 11/32, (34.4%), levofloxacin 9/32, (28.1%) and gentamicin

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8/32, (25.0%). The most sensitive antibiotic was gentamicin 23/32, (69.7%) followed by levofloxacin 22/32, (68.8%) with oxacillin 9/32, (28.1%) giving the least susceptibility.

The *Streptococcus species* were fairly susceptible to all antibiotics with the exception of vancomycin to which complete resistance was recorded (2/2, (100%). The resistance rates for *Lactobacillus species* to flucloxacillin 5/6, (83.3 %), oxacillin 5/6, (83.3 %), vancomycin 4/6, (66.7%), erythromycin 4/6, (66.7%) were also higher than gentamycin 1/6, 16.7%. None of the *Lactobacillus species* were resistant to ciprofloxacin. Susceptibility to levofloxacin 6/6, (100%) and gentamycin 5/6, (83.3%) among *Lactobacillus species* were higher than vancomycin, oxacillin and flucloxacillin (Table 4.5).

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Table 4.5: Antibiotic susceptibility pattern of Gram-positive Bacteria

Antibiotics	**Gram Positive (n=117)**		***Enterococcus sp. (n=56)***		***Staphylococcus sp. (n=32)***		***Streptococcus sp. (n=2)***		***Lactobacillus sp. (n=6)***	
	R	*S*	*R*	*S*	R	S	R	S	R	S
Flucloxacillin	*88 (75.2)	26 (22.2)	*50 (90.9)	5 (8.9)	15 (46.9)	16 (50.0)	1 (50.0)	1 (50.0)	5 (83.3)	1 (16.7)
Oxacillin	*95 (81.2)	20 (17.1)	*55 (98.2)	1 (1.8)	*18 (56.3)	9 (28.1)	1 (50.0)	1 (50.0)	5 (83.3)	1 (16.7)
Vancomycin	55 (47.0)	43 (36.8)	*30 (53.6)	17 (30.4)	11 (34.4)	16 (50.0)	2 (100)	0 (0.0)	4 (66.7)	1 (16.7)
Erythromycin	*68 (58.1)	32 (27.4)	*42 (75.0)	5 (8.9)	14 (43.8)	17 (53.1)	0 (0.0)	2 (100)	4 (66.7)	2 (33.3)
Gentamicin	49 (41.9)	63 (53.8)	33 (58.9)	23 (41.1)	*8 (25.0)	23 (69.7)	0 (0.0)	2 (100)	1 (16.7)	5 (83.3)
Ciprofloxacin	45 (38.5)	51 (43.6)	*30 (53.6)	15 (26.8)	*11 (34.4)	19 (59.4)	0 (0.0)	2 (100)	0 (0.0)	3 (50.0)
Levofloxacin	*34 (29.1)	69 (59.0)	23 (41.1)	26 (46.4)	*9 (28.1)	22 (68.8)	0 (0.0)	2 (100)	0 (0.0)	6 (100)
Sulfamethoxazole Trimethoprim	*71 (60.6)	32 (27.4)	34 (60.7)	0 (0.0)	15 (46.9)	15 (46.9)	0 (0.0)	1 (50.0)	2 (33.3)	4 (66.7)

****Level of resistance was significantly different (p<0.05) from the level of susceptibility***

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The antibiogram were also determined for the Gram negatives and the resistance rates of *K. pneumoniae* to cefuroxime sodium 20/23 (87%), cefotaxime 20/23 (87%), ceftriaxone 19/23 (82.6%), ceftazidime 18/23 (78.3%) and sulfamethoxazole trimethoprim 18/23 (78.3%) were high as compared to gentamicin 13/23 (56.5%) and cefoxitin (6/23, 26.1%). On the other hand, *K. pneumoniae* was most susceptible to cefoxitin 13/23, (56.5%).

Among *E. coli* isolates, resistance was very high among the various antibiotics and the rates were: cefotaxime 23/26 (88.5%), sulfamethoxazole trimethoprim 23/26 (88.5%), ceftriaxone 22/26 (84.6%), cefuroxime sodium 22/26 (84.6%), and amoxicillin/clavulanic acid 20/26 (76.9%). Susceptibility of *E. coli* was fairly high for cefoxitin 13/26, (50.0%) (Table 4.6).

Equal level of resistance in *Enterobacter isolates* was observed for amoxicillin/clavulanic acid 6/7 (85.7%), cefuroxime sodium 6/7, (85.7%), cefotaxime 6/7, (85.7%) Cefoxitin, ceftriaxone and sulfamethoxazole trimethoprim also recorded similar resistance 5/7 (71.4 %). All the tested antibiotics had much lower susceptibility rates ranging from 14.8% to 28.6%. Resistance in *Pseudomonas* isolates was very high for cefoxitin and cefuroxime sodium 3/3, (100.0%) however, susceptibility was high for gentamicin, sulfamethoxazole trimethoprim and ceftazidime 2/3, (66.7%) (Table 4.6).

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Table 4.6: Antibiotic susceptibility pattern of Gram-negative Bacteria

Antibiotics	Gram Negative (n=75)		*Escherichia coli (n=26)*		*Klebsiella pneumoniae (n=23)*		*Enterobacter sp.* (n=7)		*Pseudomonas sp.* (n=3)	
	R	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Amoxicillin/Clavulanic acid	*53 (70.7)	16 (21.3)	*20 (76.9)	4 (15.4)	*16 (69.6)	4 (17.4)	*6 (85.7)	1 (14.3)	2 (66.7)	1 (33.3)
Cefuroxime Sodium	*63 (84.0)	6 (8.0)	*22 (84.6)	2 (7.7)	*20 (87.0)	2 (13.0)	*6 (85.7)	1 (14.3)	3 (100)	0 (0.0)
Ceftazidime	*46 (61.3)	21 (28.0)	*17 (65.4)	6 (26.1)	*18 (78.3)	4 (17.4)	4 (57.1)	2 (28.6)	2 (66.7)	0 (0.0)
Cefotaxime	*58 (77.3)	12 (16.0)	*23 (88.5)	1 (3.8)	*20 (87.0)	3 (13.0)	*6 (85.7)	1 (14.3)	1 (33.3)	1 (33.3)
Ceftriaxone	*53 (70.7)	16 (21.3)	*22 (84.6)	3 (11.5)	*19 (82.6)	3 (13.0)	5 (71.4)	1 (14.3)	1 (33.3)	1 (33.3)
Cefoxitin	34 (45.3)	34 (45.3)	*1 (3.8)	13 (50.0)	*6 (26.1)	13 (56.5)	5 (71.4)	2 (28.6)	0 (0.0)	0 (0.0)
Gentamicin	33 (44.0)	34 (45.3)	11 (42.3)	11 (42.3)	13 (56.5)	9 (39.1)	3 (42.9)	1 (14.3)	1 (33.3)	2 (66.7)
Sulfamethoxazole Trimethoprim (SXT)	*53 (70.7)	20 (26.7)	*23 (88.5)	2 (7.7)	*18 (78.3)	4 (17.4)	5 (71.4)	2 (28.6)	1 (33.3)	2 (66.7)

**Level of resistance was significantly different ($p<0.05$) from level of susceptibility*

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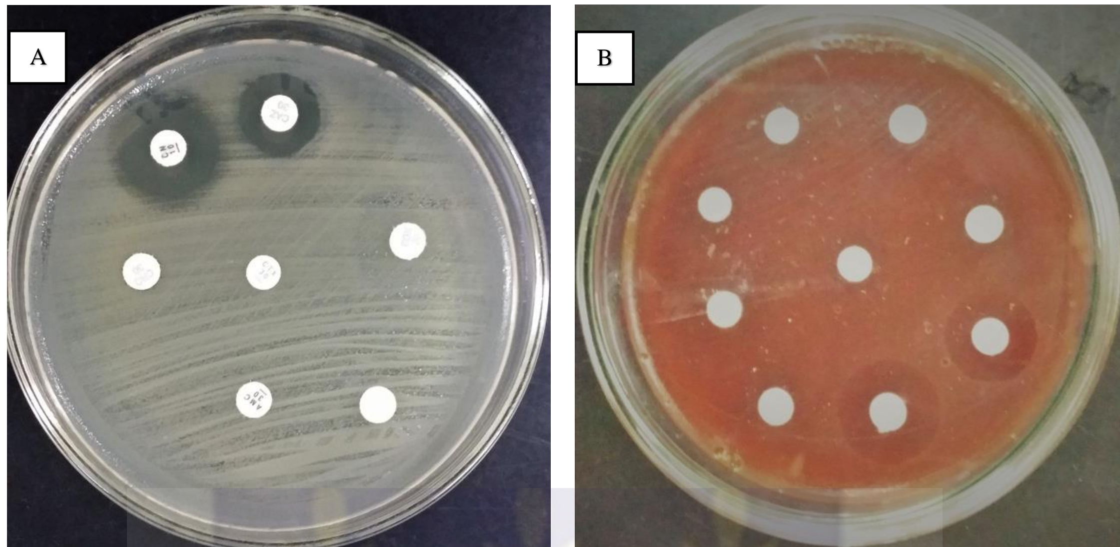

Figure 4.6: Muller-Hinton Agar plates showing zones of inhibition in the Kirby-bauer test. *(A) A representative Muller-Hinton Agar plate for testing the antibiotic sensitivity of Gram-negative bacteria (B) A representative Muller-Hinton Agar plate for testing the antibiotic sensitivity of fastidious Gram-positive bacteria*

4.5 Mycobacterial species identified and characterized

All the 32 mycobacterial isolates from lung biopsies were first confirmed as AFB by ZN staining (Figure 4.7A). Characterisation by IS*6110* PCR identified 25 as members of the MTBC (Figure 4.7B). Out of the 25 MTBC, 15 were further differentiated by spoligotyping as *M. tuberculosis senso stricto* of associated lineages, Cameroon 7 (41.2%), Ghana 4 (23.5%), Haarlem 1(5.9%), Lam 1(5.9%), Beijing 1(5.9%) and Delhi/CAS 1(5.9%) (Table 4.7). Three of the isolates were also characterised as *M. africanum* by spoligotyping and of these, two were characterised as West African 1 lineage whiles the remainder was West African 2 lineage. Six of the AFB positive isolates that were negative for IS*6110* PCR were further characterised by *hsp65* sequencing (Fig 4.7C). Sequenced data were cleaned and edited using Staden package (Figure 4.9A&B) and blasted using NCBI database. Upon

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blasting five isolates were identified as *M. abscessus* and one as *Norcadia farcinica.* The remaining two isolates were IS*6110* PCR negative but *hsp65* PCR negative. The aligned sequences of *hsp65* positive isolates are also shown in Appendix 4.

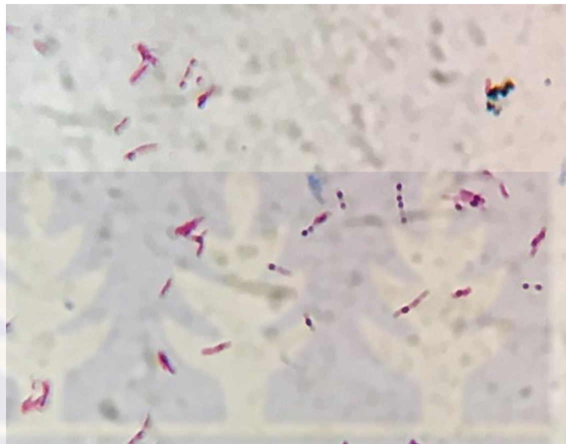

Figure 4.7A: Acid-Fast mycobacteria under x100 objective lens of a light microscope

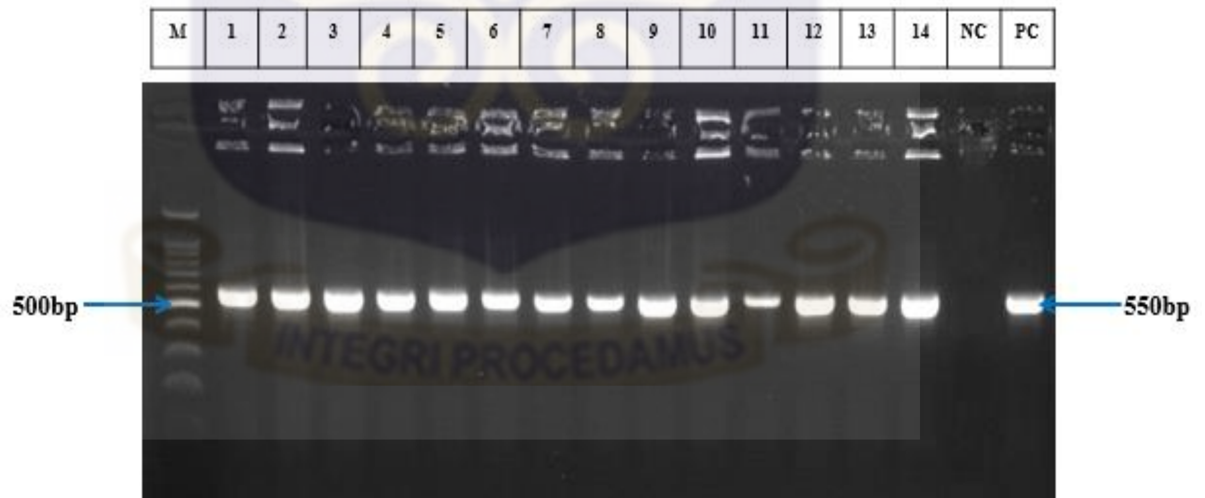

Figure 4.7B: Electrophoresis gel showing IS*6110* positive isolates. *M: 100bp Molecular weight marker, Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14:* IS*6110 positive isolates, NC: Negative control, PC: Positive control*

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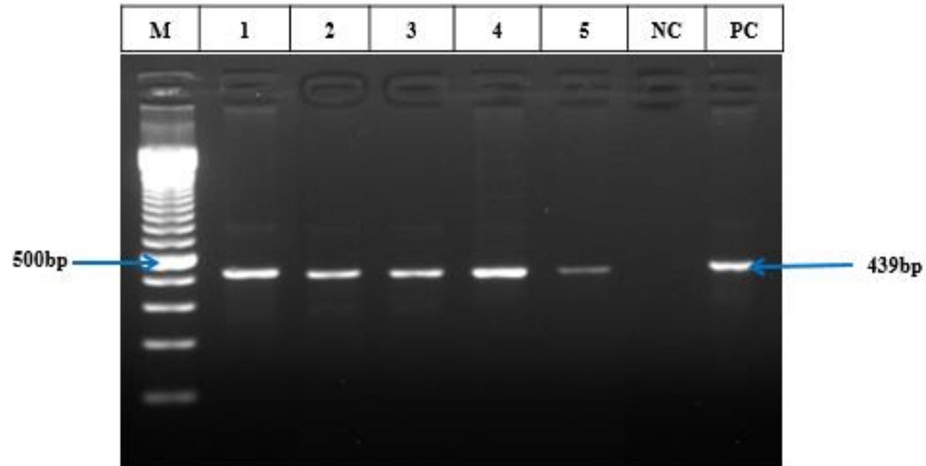

Figure 4.7C: Electrophoresis gel showing *hsp65* positive isolates. *M: 100bp Molecular weight marker, Lanes 1, 2, 4, 6, 8: hsp65 positive isolates NC: Negative control, PC: Positive control* (H37Rv)

Table 4.7: Characterized *Mycobacteria species* from autopsy lung biopsies

Mycobacteria species		**Lineage**	**Number (%)**
MTBC	*M. tuberculosis*	Cameroon	7 (38.9%)
		Ghana	4 (22.2%)
		Haarlem	1 (5.6%)
		Lam	1 (5.6%)
		Beijing	1 (5.6%)
		Delhi/CAS	1 (5.6%)
	M. africanum	West African 1	2 (11.1%)
		West African 2	1 (5.6%)
NTM	*M. abscessus*		5 (100%)

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	Sample ID	Species	Sub-lineages	Spoligotyping Profile
1	P/149	MAF	West African 2	
2	P/357	MAF	West African 1	
3	P/478	MAF	West African 1	
4	P/297	MTB	Ghana	
5	P/466	MTB	Cameroon	
6	P/N1	MTB	Cameroon	
7	P/194	MTB	Cameroon	
8	P/318	MTB	Delhi/CAS	
9	P/683	MTB	Ghana	
10	P/122A	MTB	Ghana	
11	P/172	MTB	Beijing	
12	P/175	MTB	Haarlem	
13	P/524	MTB	Cameroon	
14	P/625	MTB	Cameroon	
15	P/640	MTB	Ghana	
16	P/814	MTB	Cameroon	
17	P/872	MTB	Cameroon	
18	P/1198	MTB	LAM	

Figure 4.8: Spoligotyping Profile of MTBC organisms

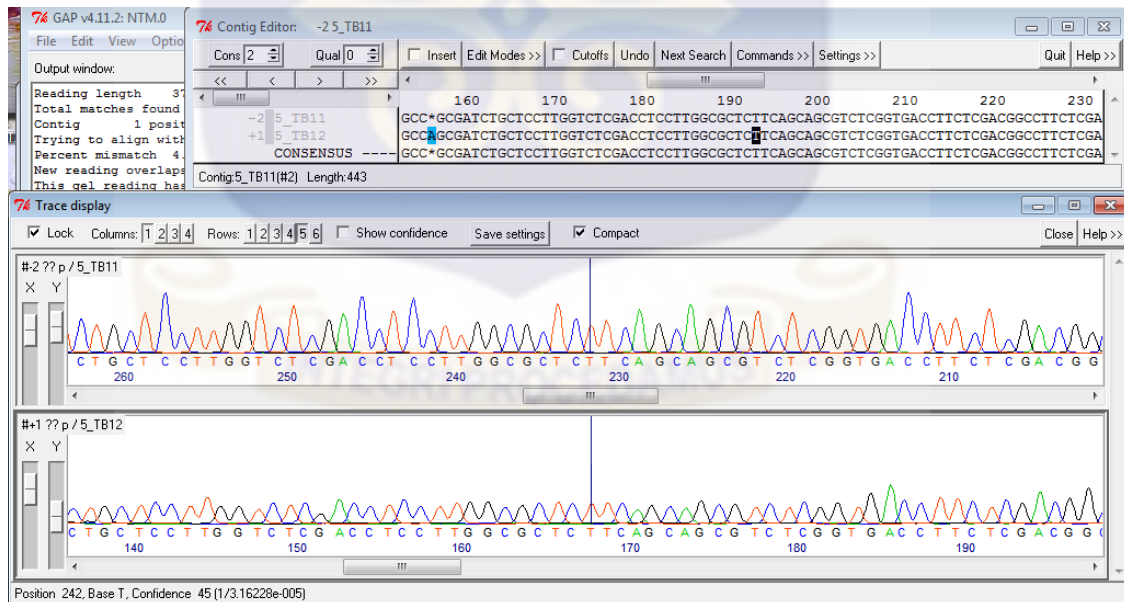

Figure 4.9A: Chromatogram of *Norcadia farcinica* cleaned using Staden Package

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Figure 4.9B: Chromatogram of *M. abscessus* cleaned using Staden Package

4.6 Drug Resistance Profiles of the MTBC

The twenty-five identified MTBC isolates were screened against rifampicin and isoniazid using line probe assay (LPA) (Genotype MTBDR*plus*). Twenty-one (21) of the isolates were susceptible to both isoniazid (INH) and rifampicin (RIF) whilst one isolate was determined as isoniazid mono-resistant and three isolates were found to be rifampicin resistant (Table 4.8). No multi-drug resistance was detected among the isolates. The INH-mono resistant isolate was confirmed based on the absence of the *katG* wild type band and presence of a *katG* mutant-2 band determined from the line probe assay results. For the three RIF-resistant isolates, one had no corresponding band to any of the *rpoB* mutant loci, but the *rpoB* wild type-8 band was absent in one whereas two were absent in the other as shown in appendix 1.

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Table 4.8: Drug Susceptibility Profile of MTBC isolates using Genotype MTBDR*plus*

Anti- TB Agent	Number of Resistant strains	Number of Susceptible strains
Isoniazid (INH) Mono	1	24
Rifampicin (RIF) Mono	3	22
INH+ RIF (MDR)	0	-

4.7 Viral Pathogens Detected

A total of 69 viruses were detected by real-time reverse transcriptase PCR (q-RT-PCR) and nested-PCR methods. *Enterovirus* and *Parainfluenza-2* virus were the two RNA viruses detected in 2 out of 51 samples tested using q-RT-PCR with cycle threshold (Ct) values of 39.73 and 41.98 respectively (Figures 4.10A and B). Data for the amplification curve detection step was extracted and saved using the Biorad software, then analysed using the Seegene viewer. The Seegene viewer completes analysis in a few seconds and the interface shows coloured wells representing wells with the tested samples as indicated in figures 4.10A and 4.10B. The red coloured wells represent wells containing samples, positive for any of the seven viruses detected by the assay. Wells coloured blue contained samples, negative for all the screened viruses and yellow coloured wells are indicative of invalid results. The positive samples produced signals depicted as characteristic curves for the internal control (included in the samples during RNA extraction) and any of the viruses under investigation with specific C (t) values. The Ct is the number of cycles required for the fluorescent signal to cross the threshold and exceed background level. Ct levels are

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inversely proportional to the amount of target nucleic acid in the sample (the lower the Ct level the greater the amount of target nucleic acid in the sample).

Negative samples produced signals for only the internal control whereas the samples that gave invalid results produced no signals at all. The positive control for the q-RT-PCR contained all the seven viruses and produced positive signals for all seven viruses as well as the internal control (Figure 4.10C).

A total of 67 out of the 97 samples tested positive for *Cytomegalovirus,* a DNA virus. A representative agarose gel showing samples that were positive and negative together with the controls is shown in Figure 4.11. Positive samples showed an expected band size of 301bp.

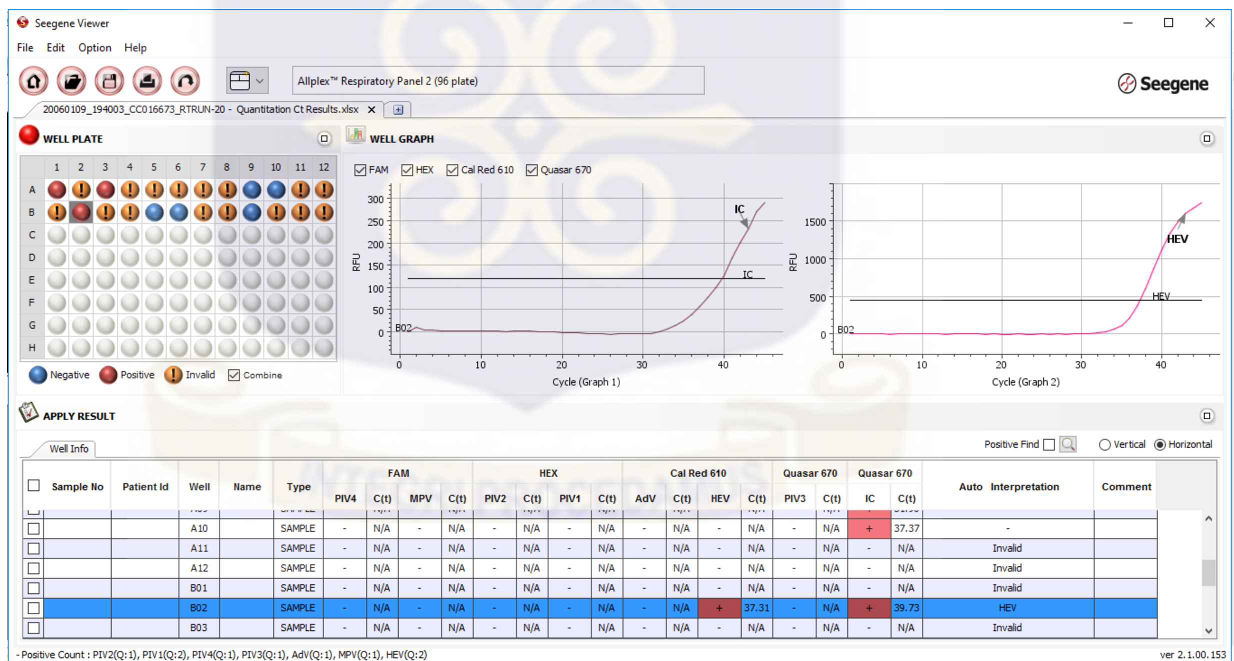

Figure 4.10A: Detection of Enterovirus using Seegene viewer after Real-time RT PCR

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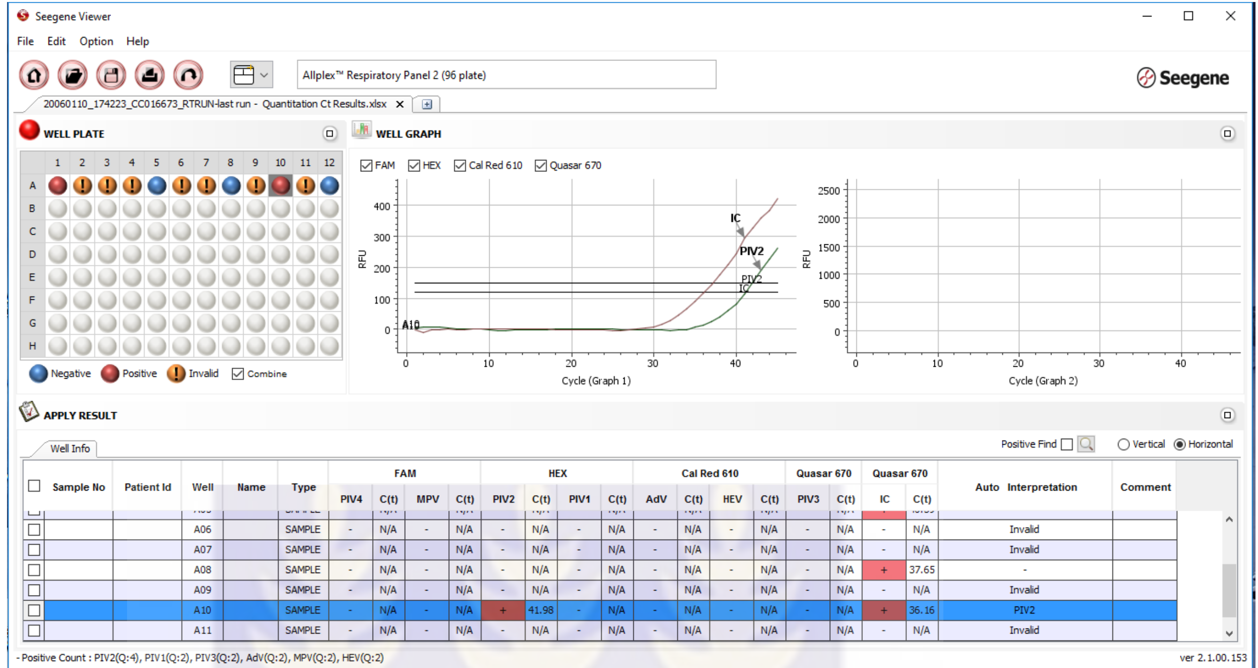

Figure 4.10B: Detection of Parainfluenza-2 using Seegene viewer after Real-time RT PCR

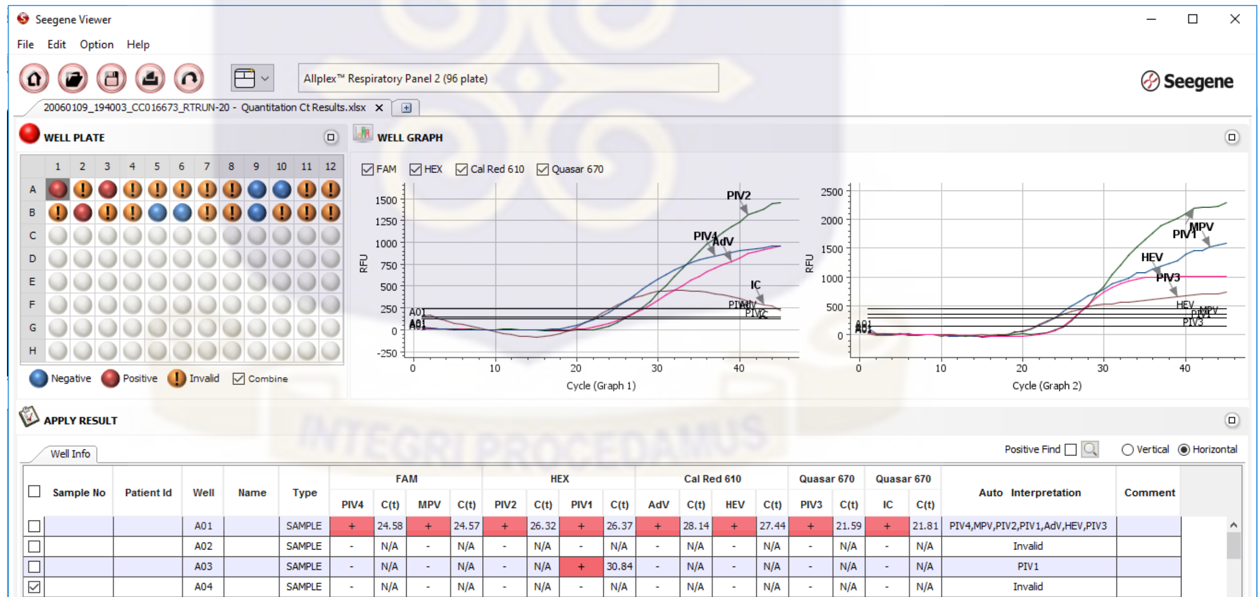

Figure 4.10C: Detection of Allplex Respiratory Assay Positive Control using Seegene viewer after Real-time RT PCR

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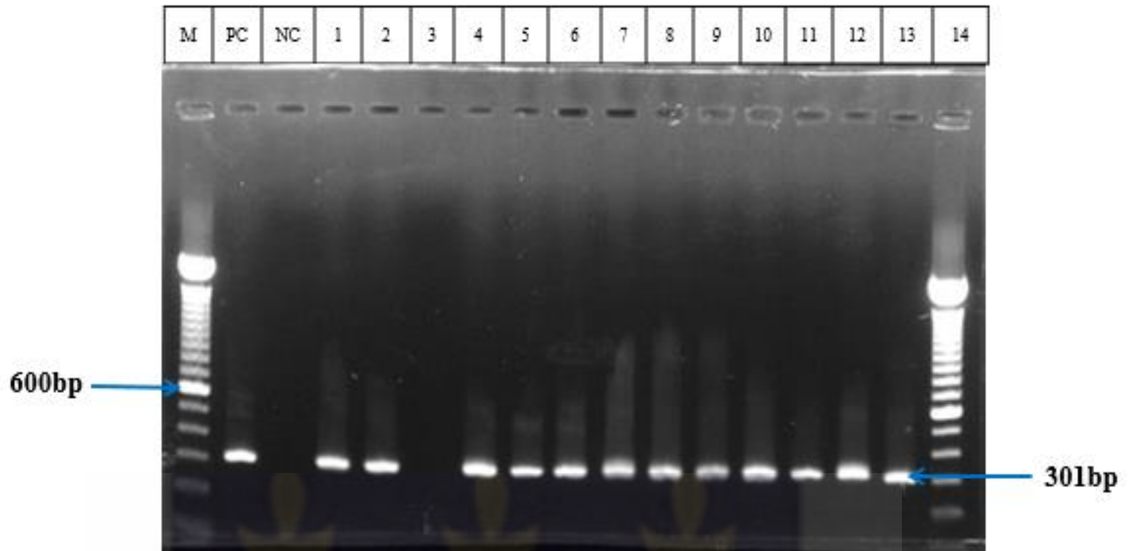

Figure 4.11: Electrophoresis gel showing *Cytomegalovirus* positive samples. *M: 100bp Molecular weight marker, PC: Positive control* (A *known positive Cytomegalovirus DNA sample*) *NC: Negative control, Lanes 1, 2, 4-14: Cytomegalovirus positive samples, Lane 3: Negative sample*

4.8 Rate of Isolating Pathogens from Samples

Out of the total 102 lung biopsy samples investigated, the highest number of isolates identified per sample was 7 and this was detected in 5 samples. Among 7 other samples, 6 different microorganisms were isolated and 5 microorganism were isolated from 18 samples. Four organisms each were isolated from 22 samples, 3 isolated in 28 of the samples, 2 isolated from 15 of the samples and 1 organism each from 7 samples (Figure 4.12).

The most frequently isolated multiple co-infecting pathogens from the biopsy samples examined included fungi, *Mycobacterium tuberculosis complex* (MTBC) species, Non-tuberculous mycobacteria (NTM) and viruses. Majority of the samples (25) had dual presence of general bacteria and viruses whiles, 6 of the samples had evidence of fungi,

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MTBC, other bacteria and virus. Samples with single microorganism type isolated were fifteen (15) of which 14 where general bacteria and 1 virus (Figure 4.13).

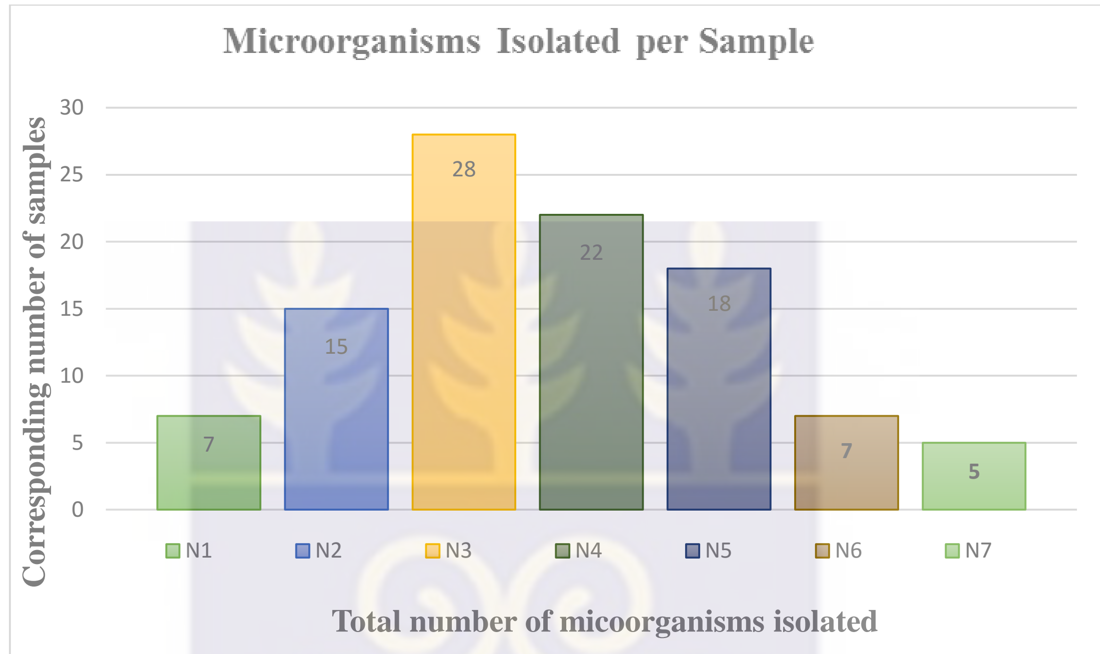

Figure 4.12: Number of Samples with Multiple Microorganisms Identified. *N1: One (1) isolate per sample, N2: Two (2) isolates per sample, N3: Three (3) isolates per sample, N4: Four (4) isolates per sample, N5: Five (5) isolates per sample, N6: Six (6) isolates per sample, N7: Seven*

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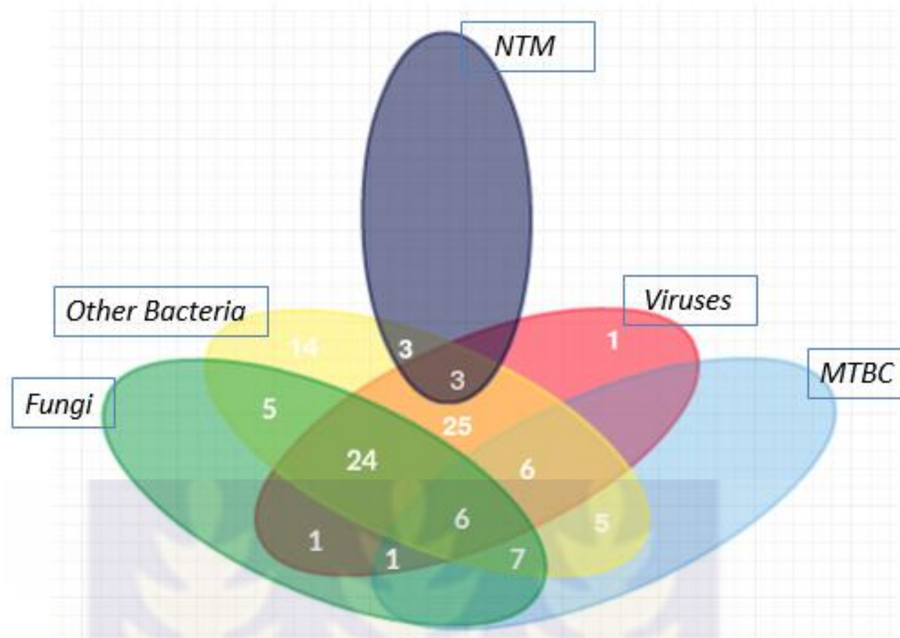

Figure 4.13: Venn Diagram showing the Multiple Microorganisms Identified from Samples

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CHAPTER FIVE

5.0 DISCUSSION

Pulmonary infections often arise from aberrations of the immune responses in HIV infection because, the altered immune homeostasis gives pathogenic microorganisms; otherwise a minute part of the much larger and complex microbial lung ecology (comprising bacteria, fungi and viruses), the competitive advantage for establishing infections. (Chen & Kolls, 2013; Boyton *et al*., 2012). The disarrayed immunity facilitates a switch of commensals to opportunistic pathogens manifesting pathologically as their pathogenesis progresses unhindered (Honda & Littman, 2012). This thesis is the first of its kind in Ghana and sought to characterize the lung microbiome of post-mortem biopsy samples of HIV-positive decedents using conventional culture methods, PCR and MALDI-TOF mass spectrometry. The antibiotic susceptibility profiles of identified bacteria were also determined using the Genotype MTBDR*plus* and Kirby-bauer assays. A better understanding of the lung microbiome is pivotal to the discovery of new drug targets and therapeutic approaches for controlling pulmonary infections in HIV-infected people.

Polymicrobial Interactions within the Lung microbiome of HIV-positive Patients

The lung, which was previously believed to be the most sterile organ has recently been shown to harbour variable number of microbes in both healthy and diseased states (Dickson *et al*., 2013). Emerging studies have partly addressed components of the lung microbiome as the bacteriome, virome and mycobiome (Cui *et al*., 2015; Twiggs *et al*., 2017). In addition, reports from the Lung Human Immunodeficiency Virus (HIV) Microbiome

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Project (LHMP) strongly indicate the presence of general bacteria, mycobacteria, fungi and viruses in the lungs of the HIV/AIDS decedents.

In this study, majority (98%) of the HIV-positive decedents analysed showed evidence of polymicrobial interactions. The observed heterogeneity could be attributed to the micro-ecology of the environment and its influence on the host microbiome. Although all the decedents were randomly recruited at the same tertiary referral hospital, they were of different ecological backgrounds with varied environmental contacts. This may explain the polymicrobial nature observed from analysing the biopsies. In a recent lung microbiome studies on HIV-positive subjects with similar underlying lung diseases, differences in geographical locations were implicated as a source of variable microbial recovery (Stressmann *et al*., 2011). Likewise, Fujimura and colleagues demonstrated that, microbial differences existed between dusts found in homes with and without pets and the hosts microbiota in each setting was differently influenced (Fujimura *et al*., 2010).

Secondly, opportunistic infections are common occurrence in HIV patients and the management of such infections entail the use of antibiotic therapy, which may consequently alter host microbial diversity since they antagonize normal flora in addition to targeted pathogens and could account for the diverse microbes identified in the various samples (Haahtela *et al*., 2013; Etebu & Arikekpar, 2016). This reinforces the need for strict regulations guarding antibiotics usage as well as the inclusion of definitive diagnosis prior to administration of antibiotics as an integral aspect of HIV management in Ghana.

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Furthermore, host immunity has been shown to be a major driver of microbial diversity. Twigg *et al.,* 2017 showed that, the deteriorating effect of HIV infection on lung immunity disturbs the microbial ecology of the lungs especially in the advanced stages of HIV infection. This may impact the dynamics of lung microbiota as well as influences the kind of infections that ensue (Twigg *et al*., 2017). Therefore, the differences in microorganisms identified from this study and other studies on lung biopsy could be due to disparities in immune and anti-retroviral therapy status as well as the stage of HIV infection in the study recruits.

Most lung microbiome studies (Erb-Downward *et al*., 2011; Hilty *et al*., 2010; Huang *et al.,* 2011; Morris *et al*., 2013; Twigg *et al*., 2013) have focused mainly on the constitution of bacterial communities, even though viral and fungal pathogens are known to be major causes of pulmonary infections (Twigg *et al.,* 2017; Nguyen *et al*., 2015). This study employed a broad spectrum of assays aimed at isolating and detecting bacterial, viral and fungal pathogens from autopsy samples. Although a complex mix of these organisms were detected, bacteria were the most abundant microbes isolated with *Enterococcus faecalis*, *Staphylococcus species, E. coli*, *K. pneumoniae, Streptococcus species, Pseudomonas species* and *Neisseria meningitides* being the most pathogenic and clinically relevant.

Enterococcus species are commensals of the gastrointestinal tract (GIT) known to cause endogenous infections such as urinary tract infections (UTI), endocarditis and bacteremia. For instance, *Enterococcus* has been implicated in pneumonia albeit very rare and often

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coincides with immunosuppression in the host (Vanschooneveld *et al.,* 2009). The high isolation rate of *E. faecalis* in the lung biopsies therefore indicates its role as an invasive colonizer of the lungs as well as its high pathogenic potential (Moellering, 1992). *E. coli* is also a known commensal of the GIT that provides some benefits to the host including biosynthesis of vitamins, providing colonization resistance to pathogenic microorganisms and aiding in the extraction of calories from non-digestible polysaccharides (Manson *et al.,* 2008). Nonetheless, *E. coli* has been implicated as opportunistic and evidently associated with urinary and gastrointestinal tract infections (Wang *et al*., 2000) with few reported respiratory infections that arise from hematogenous dissemination from the gastrointestinal or urinary tract as a result of bacteremia or through micro-aspiration from the pharynx. Since bacteremia is a common complication in HIV due to damages in the immune system of the GIT together with destruction of the epithelial micro-ecology, it is possible that *E. coli* translocation and isolation from the lungs of the study decedents was via these routes (Mootsikapun, 2007).

Also, the other pathogenic bacteria identified compares with earlier findings that determined the predominant bacteria phyla in HIV-infected individuals as *Actinobacteria, Bacteroidetes, Firmicutes* and *Prevotella* (Iwai *et al*., 2012; Iwai *et al*., 2014). On the other hand, *Tropheryma whipplei,* which is abundant in asymptomatic HIV infection and significantly declines in abundance following ART was absent in the samples analyzed in this study probably because most of the decedents were symptomatic (Lozupone *et al*., 2013).

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The main pathogenic bacteria detected in this study have been implicated as respiratory pathogens that are often diagnosed empirically as pneumonia or tuberculosis and treated as such at various health facilities (Crothers *et al*., 2011; Benito *et al*., 2012; Klatt, 2016). Under or misdiagnosis of these pathogens are common, mostly due to the similar and overlapping signs and symptoms of infections they cause (Chakaya, 2011). For instance, the pre-mortem diagnosis of one of the cases was cerebral toxoplasmosis but autopsy detected pneumonia as the cause of death whereas *M. abscessus, K. pneumoniae* and *E. faecalis* were the main pathogens detected by laboratory investigations. Clearly, there was a disparity between the clinical and autopsy diagnosis but since autopsy is the gold standard for ascertaining cause of death (Castelnuovo *et al*., 2009; Etard *et al*., 2006), it may suffice as the true diagnosis in this instance. Even if pneumonia is considered as the cause of death, questions such as 1) the exact pathogens causing the pneumonia 2) the etiology of infection; mono- or polymicrobial and 3) what should the treatment target may arise. For this reason, it is imperative to incorporate definitive diagnosis that involves improved molecular characterization techniques in the detection of the exact pathogens causing various infections. This will inform on appropriate treatment and management of HIV patients, rather than just adopting the “treat all, kill all” strategy which is the common practice in health facilities. This current treatment strategy could have adverse effect on the patient, possibly drive the emergence of resistance especially if the microbial communities do not comprise solely of superficial isogenic pathogens and thus, exhibit ranging sensitivity patterns (El-halfawy & Valvano, 2015).

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From the laboratory investigation, mycobacterial species (31.4%) were also predominantly isolated as expected. They included members of the *Mycobacterium tuberculosis complex* (MTBC) (24.5%) and *M. abscessus* (6.9%). One of the acid-fast isolate was identified as *Norcardia farcinica.* All these pathogens cause severe life-threatening pulmonary infections which are potentiated by the immune-suppressed state in HIV infection (Pawlowski *et al*., 2012; Marquez-Diaz *et al*., 1998; Griffith *et al*., 2007). *M. abscessus* is a non-tuberculous mycobacterium (NTM) that causes pulmonary infections that resemble tuberculosis especially in vulnerable individuals. These fast growers pose great challenges to therapy since their infections are usually not differentiated from true TB disease (Brown-Elloit *et al*., 2003; Nessar *et al*., 2012).

Based on the current tuberculosis (TB) diagnostic algorithm in Ghana, peripheral health facilities use sputum smear microscopy as the main diagnostic tool to define TB cases. This is less sensitive, non-specific for differentiating between mycobacterial species and highly subjective especially in HIV co-infected persons (Getahun *et al.,* 2007). As a result patients infected with MTBC and those with NTM are both categorized as tuberculosis patients and are put on the same treatment regimen that targets MTBC. Unfortunately, these NTMs such as the *M. abscessus* identified in this study, are often difficult to treat because of their inherent resistance to anti-tuberculosis therapy (Brown *et al*., 2003; Nessar *et al*., 2012). This results in treatment failure, classification of the infection as a multi-drug resistant type and the subsequent administration of second-line anti-TB drugs to patients who do not need

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this therapy (Maiga *et al*., 2012). Apart from the serious side effects these patients have to endure, it also complicates the existing deteriorating effects that HIV presents.

To address this problem, the National Tuberculosis Control Programme (NTP) is rolling out Xpert MTB/RIF to peripheral health facilities following endorsement of this method by the WHO for the simultaneous detection of MTBC and rifampicin (RIF) resistance (Reider *et al*., 2007). This will help in accurate diagnosis of etiological causes of pulmonary infections and improve treatment. With the existing heterogeneity of the lung microbiome demonstrated by several studies including this current study, host-directed therapy that focuses on immunity and diet in addition to specific pathogens responsible for infection could be exploited rather than the use of broad spectrum antibiotics after empirical diagnosis.

A number of fungal species including *Candida species*, *Cryptococcus neoformans*, *Pneumocystis jiroveci, Yarrowia lipolytica* and *Pichia occidentalis* were also isolated form the lung biopsies. This is not surprising as colonization of mucosal surfaces (for example, the oral cavity, gastrointestinal tract and urogenital tract) is a very common phenomenon with fungi. The genus *Candida* (about 160 species) is adapted to live in mammalian hosts and cause infections in both immune-competent and immune-compromised individuals (Cui *et al*., 2015). *Cryptococcus neoformans* can also exist at low levels within the lungs for several years without causing any infection but then bloom to cause life-threatening infections when the immune system is suppressed as in the case of HIV (Shirley & Baddley,

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2009). This study just like findings from recent mycobiome studies in HIV-infected persons, detected *Pneumocystis jiroveci* (Cui *et al.,* 2015; Benito *et al*., 2012) in 42% of the autopsy biopsies. This far exceeds the projected low prevalence that ranged from 0%-11% by earlier studies (Elvin *et al*., 1989; Abouya *et al*., 1992; Ansari *et al*., 2002; Batungwanayo *et al*., 1994; Kamanfu *et al*., 1993) in HIV-infected patients with respiratory manifestations (Fisk *et al*., 2003) and gives compelling evidence that incidence of *Pneumocystis jiroveci* pneumonia (PJP) in Africa is on the rise in contrast to the observed decline in most developed countries (Palella *et al*., 1998). This is probably due to the use of definitive and more sensitive diagnostic techniques (Wasserman *et al*., 2016). For the other isolated fungal species (*P. occidentials* and *Yarrowia lipolytica),* their clinical relevance is still unknown although *Yarrowia lipolytica* is a known commensal of respiratory tract.

Aside bacterial and fungal species, quite a substantial number of viruses are able to cause pulmonary infections either alone or as co-infection with other infectious agents. In this study, the main viruses detected were *Cytomegalovirus* (CMV) together with *Enterovirus* and *Parainfluenza virus-2*. *Cytomegalovirus* usually establishes as latent infections in healthy individuals but causes active disease in immune-compromised states (Drew *et al*., 2006). This finding confirms other microbiome studies on HIV infected individuals in which CMV were identified to be associated with persistent infections (Naeger *et al*., 2010; Springer *et al*., 2004). Although some microbiome studies identified viruses such as lymphocryptovirus*;* the genus for Epstein-Barr virus, bacteriophages, anelloviruses and

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parvoviridae (Young *et al*., 2015; Twiggs *et al*., 2017), no such detection were made due to the use of primers specific PCRs for detection of targeted viruses, which did not include the above pathogens.

Limited Response to Selected Antimicrobials

Bacterial tolerance to commonly prescribed antibiotics poses a greater challenge for the treatment and management of infectious diseases as these pathogens subsequently evolve some adaptation mechanisms and become resistant to these antibiotics. The antibacterial profiles of the identified bacteria were determined by screening against selected antimicrobial agents on the basis of their Gram status. This is because differences in cell wall composition between Gram negative and positive bacteria confer on them different cell wall properties and sensitivities to different antibiotics (Mai-Prochnow *et al*., 2016).

Generally, the Gram-positive bacteria were highly resistant to the beta-lactams. This could be driven by the increased inappropriate use of these antimicrobials especially in developing regions where they are easily accessible and are fairly cheap (Levy, 2002). The *Enterococcus* and *Lactobacillus species* also exhibited such high resistance pattern. According to Munita *et al.,* (2015), enterococcal infections pose considerable challenges to antimicrobial therapy due to the decreased expression of peptidoglycan binding proteins in these microorganisms, which are the targets of beta-lactam drugs (Hollenbeck & Rice, 2012). This could account for the *in vitro* antimicrobial tolerance exhibited by the enterococcus species observed in this study. Although some studies have shown sensitivity of lactobacillus species to the beta-lactams, the contrary is reported in this study

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(Gueimonde *et al*., 2013). The high inhibitory effect to vancomycin and the aminoglycosides such as gentamicin is in agreement with other studies (Gueimonde *et al*., 2013). The fair susceptibility recorded for levofloxacin on the other hand for all the Gram-positives could due to their limited inappropriate use in the populace since they are mainly through the intravenous route (Turnidge, 1999; Hooper, 2000).

Among the Gram-negative bacteria tested, high levels of resistance to cefuroxime; a cephalosporin commonly called Zinnat was recorded across different bacteria species (*E. coli, K. pneumoniae, Enterobacter* and *Pseudomonas)*. This drug has been overly abused in the country because of its easy accessibility. In addition, the spread of resistant bacteria strains could be aided by poor sanitary conditions and this is a major challenge in Ghana (Levy, 2002); thus the high resistance is not surprising. The varied resistance profiles observed for the different antimicrobials could be due to differences in drug pressure experienced by the individual bacterial species (Kolar *et al*., 2001). The fact that these isolates were recovered from biopsies of HIV-positive decedents is also worth considering in trying to explain the observed antimicrobial resistance of these isolates. This is because there is often the challenge of poor adherence to treatment due to high pill burden which could highly fuel resistance (Krentz, 2012).

Anti-mycobacterial profiles for the 25 *Mycobacterial tuberculosis complex* (MTBC) isolates against two of the first line anti-TB drugs were also determined. One (1) isoniazid mono-resistance and three (3) rifampicin mono-resistances were detected whiles the

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remaining isolates were susceptible to both drugs. These two drugs are very essential in the treatment of tuberculosis (Zumla *et al.,* 2015). In the general population, isoniazid mono-resistance is usually about 5 folds higher than rifampicin mono-resistance (Asante-Poku *et al.*, 2015; WHO, 2012; Jenkins *et al*., 2011). However, the reversed of a 3 fold higher rifampicin mono-resistance in comparison to isoniazid mono-resistance was observed in this study. This interesting turn could be due to the drug-drug interactions that occur between Anti-retroviral drugs (such as Efavirenz) and rifampicin, which alters the metabolism and absorption of these drugs leading to the emergence of drug resistance (Karanja *et al*., 2016).

Co-existence of Multiple Microorganisms in the Lungs: Impact on management of HIV-associated Pulmonary Infections in Ghana

The lung microbiome even in healthy individuals has been shown to be polymicrobial in nature (Dickson e*t al.,* 2013). The findings from this study furthers shows that majority of the samples had more than one microorganism isolated or detected with the highest being 7 microbes per sample as observed in five different samples. The microbes included general bacteria, mycobacteria, fungi and viruses, signifying the presence of multiple microbial communities within the lungs of HIV-infected persons. The clinically relevant organisms identified such as *Pneumocystic jiroveci*, *Mycobacteria species*, *Cytomegalovirus* and *K. pneumoniae* are known to possess high opportunistic and pathogenic potential with a number of them being implicated in pulmonary infections (Crothers *et al*., 2011; Benito *et al*., 2012). This clearly indicates that immune-suppression, either alters lung microbiome by facilitating translocation of non-lung resident microbes to

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the lungs where they establish infections or decreases/increases the distribution of specific microbes while opportunistic infections are established or both scenarios are true. Since all these organisms potentially cause pulmonary infections with overlapping clinical presentations (Huang & Crothers, 2009), determining an underlying etiology of the infection somehow becomes very complicated. There could be occasions where the infection is as a result of synergistic pathogenesis of multiple organisms and in such case, underdiagnoses could lead to treatment failure. For example, from the laboratory findings of this study, 27 samples had evidence of *Mycobacteria species* and 16 out of these had pre-mortem clinical diagnosis of tuberculosis infection whereas, 11 had not been diagnosed of TB although post-mortem revealed the presence of pneumonia in 3 cases and tuberculosis in 6 cases out of these 11. In addition to *Mycobacteria species*, other microorganisms with pathogenic potential and similar clinical presentations as mycobacteria infection were also detected in the 11 samples. In these cases, two inferences can be drawn, the possibility of the respiratory infection occurring as a result of the synergistic effect of all the pathogens present or only a single pathogen. Deciphering such underlying etiological agent is crucial in informing on the appropriate therapeutic regimen. To circumvent the problem of mis- or under-diagnosis, laboratory investigations and antibiotic testing should be integrated in the management of HIV patients.

This study, first to characterize the lung microbiome in Ghana therefore gives a prognostic insight to multiple organisms occurring in HIV/AIDS patients and their antimicrobial sensitivity profile to commonly prescribed antibiotics. This calls for the implementation of

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effective diagnostic interventions and treatment guidelines by the Ghana AIDS Commission and all other stakeholders.

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CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

1. The main organisms identified that are potential respiratory pathogens included *E. faecalis, Stapylococcus species, K. pneumoniae, Pseudomonas species, Streptococcus species,* MTBC*, M. abscessus, Candida species, Pneumocystis jiroveci* and *Cytomegalovirus*.

2. Majority of the identified bacterial species were highly resistant to commonly prescribed antibiotics such as flucloxacillin and cefuroxime sodium.

3. High mono-resistance to rifampicin was recorded among the MTBC in this study. Therefore, administration of rifampicin to TB/HIV co-infected patients should be critically investigated to fully understand the drug-drug interactions with anti-HIV drugs and their impact on resistance.

6.2 RECOMMENDATIONS

1. This study identified the existence polymicrobial respiratory pathogens that could cause opportunistic infection, in the lung biopsies of HIV decedents. Therefore, I recommend the introduction of comprehensive laboratory investigations by Health Services that go beyond the existing empirical clinical diagnosis carried out in our

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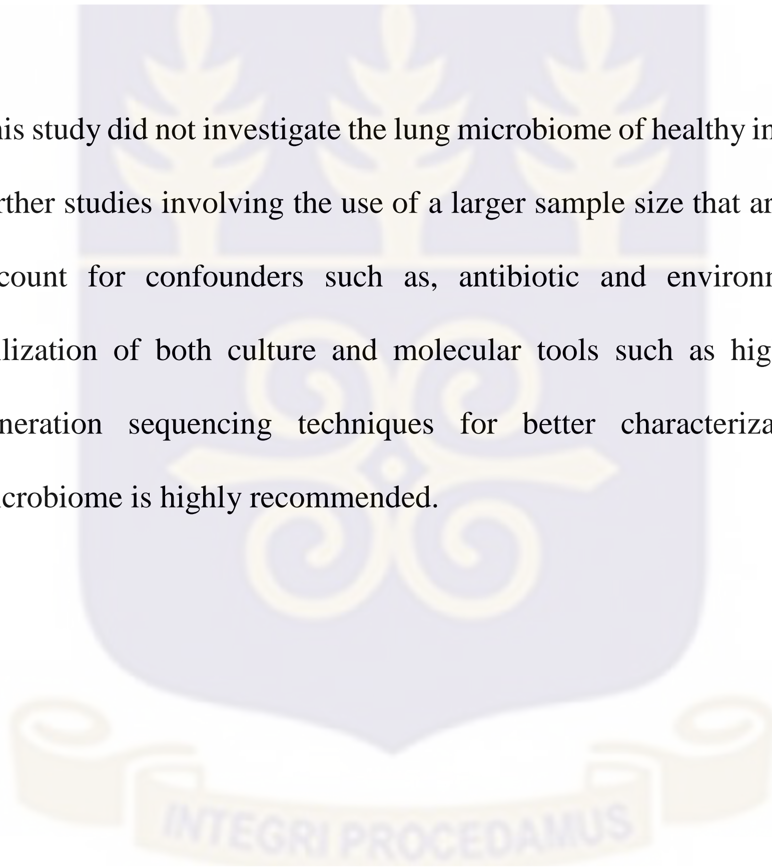
health facilities to identify the exact underlying etiological agents in the management of HIV patients in Ghana.

2. This thesis also identified high level of resistance among bacterial pathogens to commonly prescribed antibiotics, I therefore recommend strict regulations guiding antibiotic patronage by the Ghana Health Service. In addition, all chemical shops and pharmacies should be mandated to routinely provide guidelines on the usage of antibiotics to clients in order to ensure adherence to antibiotic regimen.



3. This study did not investigate the lung microbiome of healthy individuals therefore, further studies involving the use of a larger sample size that are well controlled to account for confounders such as, antibiotic and environmental effects and utilization of both culture and molecular tools such as high through-put next generation sequencing techniques for better characterization of the lung microbiome is highly recommended.

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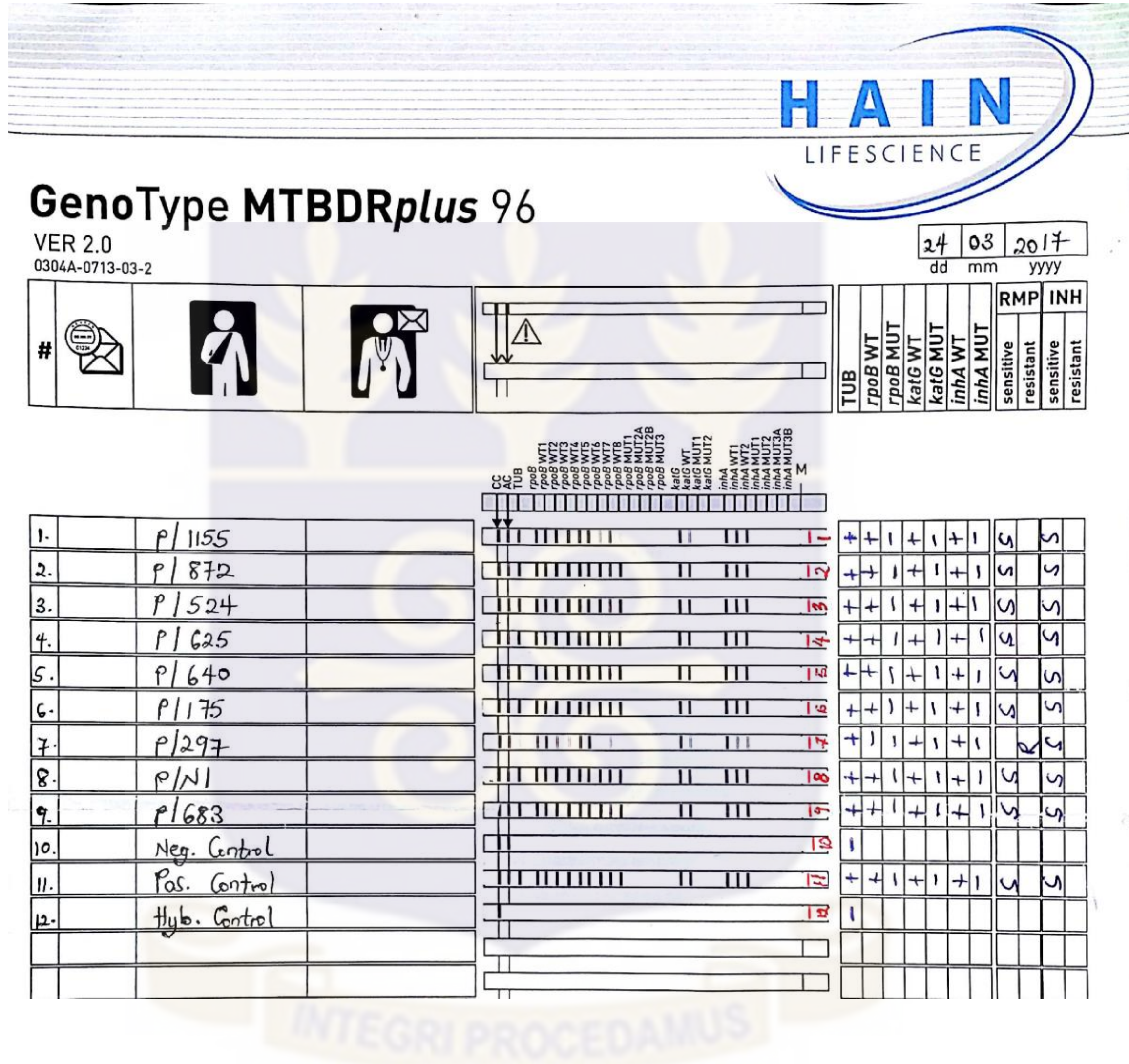
https://www.surveysystem.com/sscalc.htm

http://www.ghanaids.gov.gh/gac1/aids_info.php

APPENDICES

Appendix 1

Pictures of the results of LPA (Genotype MTBDR*plus*) assay for Samples, Positive, Negative and Hybridization Controls



HAIN LIFESCIENCE

GenoType MTBDR*plus* 96

VER 2.0

0304A-0713-03-2

Date: 24 (dd) 03 (mm) 2017 (yyyy)

#	Sample	TUB	*rpoB* WT	*rpoB* MUT	*katG* WT	*katG* MUT	*inhA* WT	*inhA* MUT	RMP sensitive	RMP resistant	INH sensitive	INH resistant
1.	P/1155	+	+	−	+	−	+	−	S		S	
2.	P/872	+	+	−	+	−	+	−	S		S	
3.	P/524	+	+	−	+	−	+	−	S		S	
4.	P/625	+	+	−	+	−	+	−	S		S	
5.	P/640	+	+	−	+	−	+	−	S		S	
6.	P/175	+	+	−	+	−	+	−	S		S	
7.	P/297	+	−	−	+	−	+	−		R	S	
8.	P/N1	+	+	−	+	−	+	−	S		S	
9.	P/683	+	+	−	+	−	+	−	S		S	
10.	Neg. Control	−										
11.	Pos. Control	+	+	−	+	−	+	−	S		S	
12.	Hyb. Control	−										

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HAIN LIFESCIENCE

Pteonah

GenoType MTBDR*plus* 96

VER 2.0
0304A-0311-03-1

dd	mm	yyyy
12	04	2017

#	Sample	TUB	rpoB-WT	rpoB-MUT	katG-WT	katG-MUT	inhA-WT	inhA-MUT	RMP sensitive	RMP resistant	INH sensitive	INH resistant
1.	P/588	+	+	-	+	-	+	-	S		S	
2.	P/570	-										
3.	P/478	+	+	-	+	-	+	-	S		S	
4.	P/466	+	+	-	+	-	+	-	S		S	
5.	P/357	+	+	-	+	-	+	-	S		S	
6.	P/194	+	+	-	+	-	+	-	S		S	
7.	P/814	+	+	-	+	-	+	-	S		S	
8.	P/894	+	+	-	+	-	+	-	S		S	
9.	P/1123	+	-	-	+	-	+	-		R	S	
10.	P/244	+	+	-	+	-	+	-	S		S	
11.	P/317	+	+	-	+	-	+	-	S		S	
12.	P/318	+	+	-	+	-	+	-	S		S	
13.	P/172	+	+	-	+	-	+	-	S		S	
14.	P/149	+	+	-	+	-	+	-	S		S	
15.	P/122A	+	+	-	+	+	+	-	S			R
16.	P/244	+	+	-	+	-	+	-	S		S	

CC AC TUB rpoB rpoB WT1 rpoB WT2 rpoB WT3 rpoB WT4 rpoB WT5 rpoB WT6 rpoB WT7 rpoB WT8 rpoB MUT1 rpoB MUT2A rpoB MUT2B rpoB MUT3 katG katG WT katG MUT1 katG MUT2 inhA inhA WT1 inhA WT2 inhA MUT1 inhA MUT2 inhA MUT3A inhA MUT3B M

LOT ______ HYB ____ min STR ____ min SUB ____ min ________

Pearah

GenoType MTBDR*plus* 96

VER 2.0
0304A-0311-03-1

Date: 12 / 04 / 2017 (dd mm yyyy)

#	Sample		TUB	rpoB-WT	rpoB-MUT	katG-WT	katG-MUT	inhA-WT	inhA-MUT	RMP sensitive	RMP resistant	INH sensitive	INH resistant
1	P/588		+	+	-	+	-	+	-	S		S	
2.	P/570		-										
3.	P/478		+	+	-	+	-	+	-	S		S	
4.	P/466		+	+	-	+	-	+	-	S		S	
5.	P/357		+	+	-	+	-	+	-	S		S	
6.	P/194		+	+	-	+	-	+	-	S		S	
7.	P/814		+	+	-	+	-	+	-	S		S	
8.	P/894		+	+	-	+	-	+	-	S		S	
9.	P/1123		+	-	-	+	-	+	-		R	S	
10.	P/244		+	+	-	+	-	+	-	S		S	
11.	P/317		+	+	-	+	-	+	-	S		S	
12.	P/318		+	+	-	+	-	+	-	S		S	
13.	P/172		+	+	-	+	-	+	-	S		S	
14.	P/149		+	+	-	+	-	+	-	S		S	
15.	P/122A		+	+	-	+	+	+	-	S			R
16.	P/244		+	+	-	+	-	+	-	S		S	
05-05-17													
17.	P/1198		+	+	-	+	-	+	-	S		S	
18.	P/112/17		+	-	-	+	-	+	-		R	S	
19	P/570		-										
20	P/150		+	+	-	+	-	+	-	S		S	
21	P/40A		-										
22	P/40A		+	-	-	+	-	+	-		R	S	

LOT ______ HYB ____ min STR ____ min SUB ____ min

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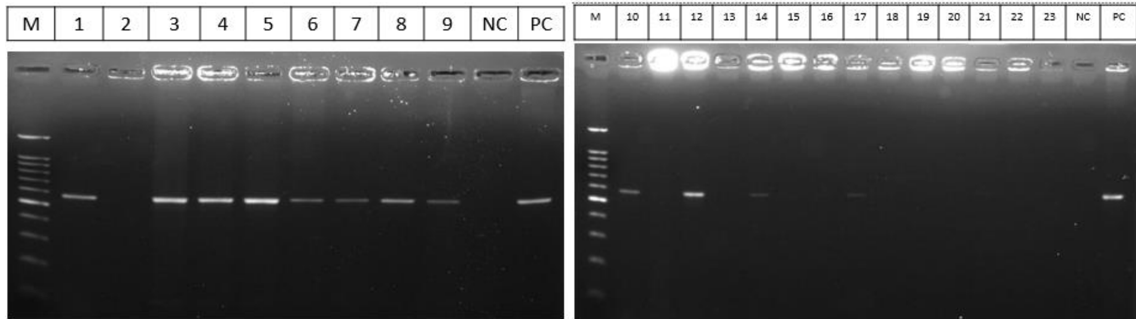
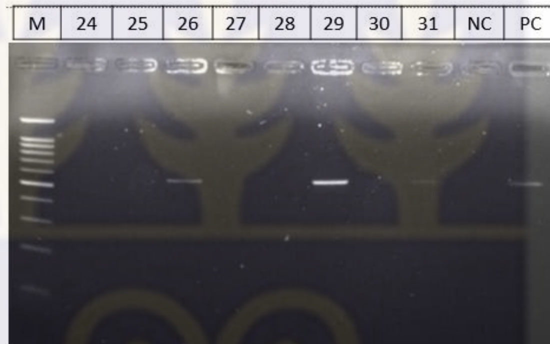
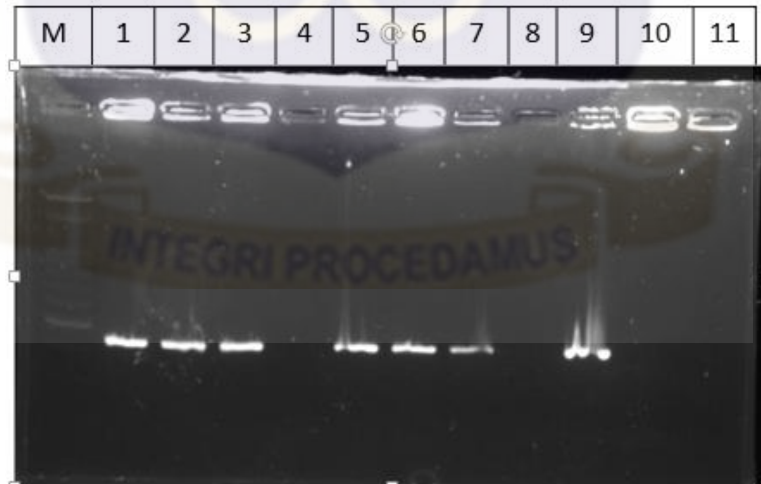
Appendix 2

Gel Pictures of IS*6110* PCR Amplicons





Gel Picture of *hsp65* PCR Amplicons



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Gel Pictures of CMV Nested PCR- Round 2 Amplicons

Gel Pictures of PJP Nested PCR- Round 2 Amplicons

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Appendix 3

Table 1: Mineral salts for Lowenstein-Jensen media preparation

Ingredients	**L-J with Glycerol**		**L-J with Pyruvate**
Potassium phosphate (KH_2PO_4)	0.4g		0.4g
Magnesium sulphate ($MgSO_4 7H_2O$)	0.04g		0.04g
Magnesium citrate	0.1g		0.1g
Asparagin	0.6g		0.6g
Glycerol/Pyruvate	2mL		1.3g
Distilled water	100mL		100mL
Egg homogenate	167mL		167mL
Malachite green (2%)	3.3mL		3.3mL
pH	7.0		7.0

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Appendix 4

Aligned Sequences of *hsp65* positive isolates

1. *Nocardia farcinica* strain HN11062 60 kDa chaperonin 2 GroEL2 (groEL2) gene, partial cds

Sequence ID: KF432771.1 Length: 444 Number of Matches: 1
Related Information
Range 1: 1 to 442 GenBank GraphicsNext Match Previous Match

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
811 bits(439)	0.0	441/442(99%)	0/442(0%)	Plus/Plus

Query 1
TACCACCGATGGTGTGTCCATCGCCAAGGAGATCGAGCTGGAGGACCCGTACGAGA
AGAT 60
||||| |||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1
TACCAACGATGGTGTGTCCATCGCCAAGGAGATCGAGCTGGAGGACCCGTACGAGA
AGAT 60

Query 61
CGGCGCTGAGCTGGTCAAGGAAGTTGCCAAGAAGACCGACGACGTCGCGGGTGACG
GCAC 120
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 61
CGGCGCTGAGCTGGTCAAGGAAGTTGCCAAGAAGACCGACGACGTCGCGGGTGACG
GCAC 120

Query 121
CACCACCGCCACCGTTCTTGCCCAGGCCCTGGTCAAGGAAGGTCTGCGTAACGTCGC
CGC 180
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 121
CACCACCGCCACCGTTCTTGCCCAGGCCCTGGTCAAGGAAGGTCTGCGTAACGTCGC
CGC 180

Query 181
CGGCGCCAACCCGCTCGGCCTGAAGCGCGGCATCGAGAAGGCCGTCGAGAAGGTCA
CCGA 240
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 181
CGGCGCCAACCCGCTCGGCCTGAAGCGCGGCATCGAGAAGGCCGTCGAGAAGGTCA
CCGA 240

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Query 241
GACGCTGCTGAAGAGCGCCAAGGAGGTCGAGACCAAGGAGCAGATCGCGGCCACGG
CCGG 300
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 241
GACGCTGCTGAAGAGCGCCAAGGAGGTCGAGACCAAGGAGCAGATCGCGGCCACGG
CCGG 300

Query 301
TATCTCCGCGGGCGACCAGTCCATCGGCGACCTGATCGCCGAGGCCATGGACAAGGT
TGG 360
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 301
TATCTCCGCGGGCGACCAGTCCATCGGCGACCTGATCGCCGAGGCCATGGACAAGGT
TGG 360

Query 361
CAACGAGGGTGTCATCACCGTCGAGGAGTCCAACACCTTCGGCCTGCAGCTGGAGCT
CAC 420
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 361
CAACGAGGGTGTCATCACCGTCGAGGAGTCCAACACCTTCGGCCTGCAGCTGGAGCT
CAC 420

Query 421 CGAGGGTATGCGGTTTCGACAA 442
||||||||||||||||||||||
Sbjct 421 CGAGGGTATGCGGTTTCGACAA 442

2. *Mycobacterium abscessus subsp. bolletii* strain 14I016 heat shock protein 65kDa (hsp65) gene, partial cds

Sequence ID: KT185533.1 Length: 444 Number of Matches: 1
Related Information
Range 1: 4 to 444 GenBank Graphics Next Match Previous Match

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
809 bits(438)	0.0	440/441(99%)	0/441(0%)	Plus/Plus

Query 1
CACCGATGGTGTGTCCATCGCCAAGGAGATCGAGCTGGAGGACCCGTACGAGAAGA
TCGG 60
|| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

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Sbjct 4
CAACGATGGTGTGTCCATCGCCAAGGAGATCGAGCTGGAGGACCCGTACGAGAAGA
TCGG 63

Query 61
CGCTGAGCTGGTCAAGGAAGTTGCCAAGAAGACCGACGACGTCGCGGGTGACGGCA
CCAC 120
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 64
CGCTGAGCTGGTCAAGGAAGTTGCCAAGAAGACCGACGACGTCGCGGGTGACGGCA
CCAC 123

Query 121
CACCGCCACCGTTCTTGCCCAGGCCCTGGTCAAGGAAGGTCTGCGTAACGTCGCCGC
CGG 180
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 124
CACCGCCACCGTTCTTGCCCAGGCCCTGGTCAAGGAAGGTCTGCGTAACGTCGCCGC
CGG 183

Query 181
CGCCAACCCGCTCGGCCTGAAGCGCGGCATCGAGAAGGCCGTCGAGAAGGTCACCG
AGAC 240
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 184
CGCCAACCCGCTCGGCCTGAAGCGCGGCATCGAGAAGGCCGTCGAGAAGGTCACCG
AGAC 243

Query 241
GCTGCTGAAGAGCGCCAAGGAGGTCGAGACCAAGGAGCAGATCGCGGCCACGGCCG
GTAT 300
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 244
GCTGCTGAAGAGCGCCAAGGAGGTCGAGACCAAGGAGCAGATCGCGGCCACGGCCG
GTAT 303

Query 301
CTCCGCGGGCGACCAGTCCATCGGCGACCTGATCGCCGAGGCCATGGACAAGGTTGG
CAA 360
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 304
CTCCGCGGGCGACCAGTCCATCGGCGACCTGATCGCCGAGGCCATGGACAAGGTTGG
CAA 363

Query 361
CGAGGGTGTCATCACCGTCGAGGAGTCCAACACCTTCGGCCTGCAGCTGGAGCTCAC
CGA 420
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

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Sbjct 364
CGAGGGTGTCATCACCGTCGAGGAGTCCAACACCTTCGGCCTGCAGCTGGAGCTCAC
CGA 423

Query 421 GGGTATGCGGTTCGACAAGAA 441
|||||||||||||||||||||
Sbjct 424 GGGTATGCGGTTCGACAAGAA 444

3. *Mycobacterium abscessus* strain FJ06130 60 kDa chaperonin 2 GroEL2 (groEL2) gene, partial cds
Sequence ID: KF432464.1 Length: 445 Number of Matches: 1
Related Information
Range 1: 1 to 437 GenBank Graphics Next Match Previous Match First Match

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand	Frame
802 bits(434)	0.0()	436/437(99%)	0/437(0%)	Plus/Plus	

Features:
Query 1
TTACCACCGATGGTGTGTCCATCGCCAAGGAGATCGAGCTGGAGGACCCGTACGAGA
AGA 60
|||||| |||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1
TTACCAACGATGGTGTGTCCATCGCCAAGGAGATCGAGCTGGAGGACCCGTACGAG
AAGA 60

Query 61
TCGGCGCTGAGCTGGTCAAGGAAGTTGCCAAGAAGACCGACGACGTCGCGGGTGAC
GGCA 120
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 61
TCGGCGCTGAGCTGGTCAAGGAAGTTGCCAAGAAGACCGACGACGTCGCGGGTGAC
GGCA 120

Query 121
CCACCACCGCCACCGTTCTTGCCCAGGCCCTGGTCAAGGAAGGTCTGCGTAACGTCG
CCG 180
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 121
CCACCACCGCCACCGTTCTTGCCCAGGCCCTGGTCAAGGAAGGTCTGCGTAACGTCG
CCG 180

Query 181
CCGGCGCCAACCCGCTCGGCCTGAAGCGCGGCATCGAGAAGGCCGTCGAGAAGGTC
ACCG 240
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

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Sbjct 181
CCGGCGCCAACCCGCTCGGCCTGAAGCGCGGCATCGAGAAGGCCGTCGAGAAGGTC
ACCG 240

Query 241
AGACGCTGCTGAAGAGCGCCAAGGAGGTCGAGACCAAGGAGCAGATCGCGGCCACG
GCCG 300
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 241
AGACGCTGCTGAAGAGCGCCAAGGAGGTCGAGACCAAGGAGCAGATCGCGGCCACG
GCCG 300

Query 301
GTATCTCCGCGGGCGACCAGTCCATCGGCGACCTGATCGCCGAGGCCATGGACAAGG
TTG 360
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 301
GTATCTCCGCGGGCGACCAGTCCATCGGCGACCTGATCGCCGAGGCCATGGACAAGG
TTG 360

Query 361
GCAACGAGGGTGTCATCACCGTCGAGGAGTCCAACACCTTCGGCCTGCAGCTGGAGC
TCA 420
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 361
GCAACGAGGGTGTCATCACCGTCGAGGAGTCCAACACCTTCGGCCTGCAGCTGGAGC
TCA 420

Query 421 CCGAGGGTATGCGGTTC 437
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Sbjct 421 CCGAGGGTATGCGGTTC 437

4. *Mycobacterium abscessus* strain RGTB350 65 kDa heat shock protein (hsp65) gene, partial cds
Sequence ID: HM454215.1 Length: 441 Number of Matches: 1
Related Information
Range 1: 1 to 441 GenBank Graphics Next Match Previous Match First Match
Alignment statistics for match #1

Score	**Expect**	**Identities**	**Gaps**	**Strand**	**Frame**
804 bits(435)	0.0()	439/441(99%)	0/441(0%)	Plus/Plus	

Features:
Query 25
ACCACCGATGGTGTGTCCATCGCCAAGGAGATCGAGCTGGAGGACCCGTACG
AGAAGATC 84
|||| |||||||||||||||||||||||||||||||||||||||||||||||||||||

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Sbjct 1
ACCAACGATGGTGTGTCCATCGCCAAGGAGATCGAGCTGGAGGACCCGTACG
AGAAGATC 60

Query 85
GGCGCTGAGCTGGTCAAGGAAGTTGCCAAGAAGACCGACGACGTCGCGGGT
GACGGCACC 144
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 61
GGCGCTGAGCTGGTCAAGGAAGTTGCCAAGAAGACCGACGACGTCGCGGGT
GACGGCACC 120

Query 145
ACCACCGCCACCGTTCTTGCCCAGGCCCTGGTCAAGGAAGGTCTGCGTAACG
TCGCCGCC 204
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 121
ACCACCGCCACCGTTCTTGCCCAGGCCCTGGTCAAGGAAGGTCTGCGTAACG
TCGCCGCC 180

Query 205
GGCGCCAACCCGCTCGGCCTGAAGCGCGGCATCGAGAAGGCCGTCGAGAAG
GTCACCGAG 264
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 181
GGCGCCAACCCGCTCGGCCTGAAGCGCGGCATCGAGAAGGCCGTCGAGAAG
GTCACCGAG 240

Query 265
ACGCTGCTGAAGAGCGCCAAGGAGGTCGAGACCAAGGAGCAGATCGCGGCC
ACGGCCGGT 324
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 241
ACGCTGCTGAAGAGCGCCAAGGAGGTCGAGACCAAGGAGCAGATCGCGGCC
ACGGCCGGT 300

Query 325
ATCTCCGCGGGCGACCAGTCCATCGGCGACCTGATCGCCGAGGCCATGGACA
AGGTTGGT 384
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 301
ATCTCCGCGGGCGACCAGTCCATCGGCGACCTGATCGCCGAGGCCATGGACA
AGGTTGGT 360

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Query 385
AACGAGGGTGTCATCACCGTCGAGGAGTCCAACACCTTCGGCCTGCAGCTGG
AGCTCACC 444
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 361
AACGAGGGTGTCATCACCGTCGAGGAGTCCAACACCTTCGGCCTGCAGCTGG
AGCTCACC 420

Query 445 GAGGGTATGCGGTTCTACAAG 465
||||||||||||||| |||||
Sbjct 421 GAGGGTATGCGGTTCGACAAG 441

5. *Mycobacterium abscessus subsp. bolletii* strain 14I016 heat shock protein 65kDa (hsp65) gene, partial cds
Sequence ID: KT185533.1 Length: 444 Number of Matches: 1
Related Information
Range 1: 4 to 442 GenBank Graphics Next Match Previous Match First Match
Alignment statistics for match #1

Score	**Expect**	**Identities**	**Gaps**	**Strand**	**Frame**
806 bits(436)	0.0()	438/439(99%)	0/439(0%)	Plus/Plus	

Features:
Query 1
CACCGATGGTGTGTCCATCGCCAAGGAGATCGAGCTGGAGGACCCGTACGAG
AAGATCGG 60
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Sbjct 4
CAACGATGGTGTGTCCATCGCCAAGGAGATCGAGCTGGAGGACCCGTACGAG
AAGATCGG 63

Query 61
CGCTGAGCTGGTCAAGGAAGTTGCCAAGAAGACCGACGACGTCGCGGGTGA
CGGCACCAC 120
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Sbjct 64
CGCTGAGCTGGTCAAGGAAGTTGCCAAGAAGACCGACGACGTCGCGGGTGA
CGGCACCAC 123

Query 121
CACCGCCACCGTTCTTGCCCAGGCCCTGGTCAAGGAAGGTCTGCGTAACGTC
GCCGCCGG 180
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Sbjct 124
CACCGCCACCGTTCTTGCCCAGGCCCTGGTCAAGGAAGGTCTGCGTAACGTC
GCCGCCGG 183

Query 181
CGCCAACCCGCTCGGCCTGAAGCGCGGCATCGAGAAGGCCGTCGAGAAGGT
CACCGAGAC 240
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 184
CGCCAACCCGCTCGGCCTGAAGCGCGGCATCGAGAAGGCCGTCGAGAAGGT
CACCGAGAC 243

Query 241
GCTGCTGAAGAGCGCCAAGGAGGTCGAGACCAAGGAGCAGATCGCGGCCAC
GGCCGGTAT 300
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 244
GCTGCTGAAGAGCGCCAAGGAGGTCGAGACCAAGGAGCAGATCGCGGCCAC
GGCCGGTAT 303

Query 301
CTCCGCGGGCGACCAGTCCATCGGCGACCTGATCGCCGAGGCCATGGACAAG
GTTGGCAA 360
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 304
CTCCGCGGGCGACCAGTCCATCGGCGACCTGATCGCCGAGGCCATGGACAAG
GTTGGCAA 363

Query 361
CGAGGGTGTCATCACCGTCGAGGAGTCCAACACCTTCGGCCTGCAGCTGGAG
CTCACCGA 420
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 364
CGAGGGTGTCATCACCGTCGAGGAGTCCAACACCTTCGGCCTGCAGCTGGAG
CTCACCGA 423

Query 421 GGGTATGCGGTTCGACAAG 439
|||||||||||||||||||
Sbjct 424 GGGTATGCGGTTCGACAAG 442

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6. *Mycobacterium abscessus* strain FJ06130 60 kDa chaperonin 2 GroEL2 (groEL2) gene, partial cds
Sequence ID: KF432464.1 Length: 445 Number of Matches: 1
Related Information
Range 1: 1 to 443 GenBank Graphics Next Match Previous Match First Match

Alignment statistics for match #1

Score	**Expect**	**Identities**	**Gaps**	**Strand**	**Frame**
813 bits(440)	0.0()	442/443(99%)	0/443(0%)	Plus/Plus	

Features:
Query 3
TTACCACCGATGGTGTGTCCATCGCCAAGGAGATCGAGCTGGAGGACCCGTA
CGAGAAGA 62
|||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1
TTACCAACGATGGTGTGTCCATCGCCAAGGAGATCGAGCTGGAGGACCCGTA
CGAGAAGA 60

Query 63
TCGGCGCTGAGCTGGTCAAGGAAGTTGCCAAGAAGACCGACGACGTCGCGG
GTGACGGCA 122
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 61
TCGGCGCTGAGCTGGTCAAGGAAGTTGCCAAGAAGACCGACGACGTCGCGG
GTGACGGCA 120

Query 123
CCACCACCGCCACCGTTCTTGCCCAGGCCCTGGTCAAGGAAGGTCTGCGTAA
CGTCGCCG 182
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 121
CCACCACCGCCACCGTTCTTGCCCAGGCCCTGGTCAAGGAAGGTCTGCGTAA
CGTCGCCG 180

Query 183
CCGGCGCCAACCCGCTCGGCCTGAAGCGCGGCATCGAGAAGGCCGTCGAGA
AGGTCACCG 242
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 181
CCGGCGCCAACCCGCTCGGCCTGAAGCGCGGCATCGAGAAGGCCGTCGAGA
AGGTCACCG 240

Query 243
AGACGCTGCTGAAGAGCGCCAAGGAGGTCGAGACCAAGGAGCAGATCGCGG
CCACGGCCG 302

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Sbjct 241
AGACGCTGCTGAAGAGCGCCAAGGAGGTCGAGACCAAGGAGCAGATCGCGG
CCACGGCCG 300

Query 303
GTATCTCCGCGGGCGACCAGTCCATCGGCGACCTGATCGCCGAGGCCATGGA
CAAGGTTG 362
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 301
GTATCTCCGCGGGCGACCAGTCCATCGGCGACCTGATCGCCGAGGCCATGGA
CAAGGTTG 360

Query 363
GCAACGAGGGTGTCATCACCGTCGAGGAGTCCAACACCTTCGGCCTGCAGCT
GGAGCTCA 422
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 361
GCAACGAGGGTGTCATCACCGTCGAGGAGTCCAACACCTTCGGCCTGCAGCT
GGAGCTCA 420

Query 423 CCGAGGGTATGCGGTTCGACAAG 445
|||||||||||||||||||||||
Sbjct 421 CCGAGGGTATGCGGTTCGACAAG 443