THE SPECTRUM AND ANTIFUNGAL DRUG SUSCEPTIBILITY PROFILES OF FUNGI OBTAINED FROM SPUTUM OF TUBERCULOSIS PATIENTS AT SELECTED REFERENCE FACILITIES IN KENYA

BY

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DECLARATION

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DECLARATION

This thesis is my original work and has not been submitted for the award of a degree in any University.

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DEDICATION

I dedicate this work to my nuclear family, my parents, brothers and sisters who offered overwhelming support for this academic achievement.

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I am deeply grateful to the almighty God for giving me the strength and health to carry out this work to completion.

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ABREVIATIONS AND ACRONYMS

ABPA	Allergic bronchopulmonary aspergillosis
AMB	Amphotericin B
AST	Antifungal susceptibility testing
ATCC	American Type Culture Collection
Cyp 51	Cytochrome P450 14-α sterol demethylase gene (gene coding for a demethylase enzyme in the ergosterol biosynthesis pathway)
CDC	United States Center for Disease Control
CMR	Centre for Microbiology Research
Conc	Concentration
COVID-19	Coronavirus disease 2019
CPA	Chronic pulmonary aspergillosis
DMI	Demethylation inhibitors
ECDC	European Centre for Disease Prevention and Control
FCZ	Fluconazole
GM-Ag	*Aspergillus* galactomannan antigen
IA	Invasive aspergillosis
ICZ	Itraconazole
ITS	Internal transcribed spacer
KEMRI	Kenya Medical Research Institute
MIC	Minimum Inhibitory Concentration
NCBI	National Center for Biotechnology Information
NTLDP	National Tuberculosis Leprosy and Lung Disease Program
PSC	Posaconazole
PTB	Pulmonary tuberculosis
RVC	Ravuconazole
TB	Tuberculosis
TR34/L98H	Resistance mechanism based on a 34-base pair tandem repeat in the gene promoter combined with a point mutation in the cyp51A gene at codon 98 leading to an amino acid change
VCZ	Voriconazole

ABSTRACT

Background

Opportunistic fungal infections and antifungal resistance represent a major global health challenge. HIV/AIDS, tuberculosis (TB), diabetes, malnutrition, cancer, and other immunosuppressive conditions are increasing fungal diseases globally. The WHO estimates that more than one million people successfully treated for TB later develop a fatal fungal infection each year. This is often left untreated because it is mistaken for a recurrence of TB and subsequently retreated. Similarly, fungal growth in TB sputum culture is disregarded as contaminants yet they could be the etiological agents in lung disease.

Objective

The study sought to determine the spectrum of pulmonary fungal pathogens and antifungal drug sensitivity among TB retreatment and relapse patients at selected reference facilities in Kenya.

Materials and Methods

The study evaluated 340 expectorated sputa samples from patients who consented to participate in the study. The samples were subjected to mycological analysis, including microscopy and isolation of fungi on Sabouraud Dextrose Agar (SDA). Moulds were identified morphologically by macroscopic and microscopic features while yeasts were inoculated on CROME-agar *Candida* and further identified using analytical profile index **(**API 20C AUX**).** Four antifungal drugs were tested against the isolates, namely itraconazole (ICZ), voriconazole VCZ), fluconazole (FCZ) and amphotericin B (AMB) using broth micro-dilution methods according to CLSI M38 A2 and CLSI M27 recommendations for moulds and yeast, respectively. Minimum inhibitory concentrations (MICs) were determined. Fungi contaminating TB sputum cultures on Lowenstein Jensen (LJ) media were also identified. Molecular characterization of *Aspergillus* spp isolates from sputum was done by sequencing of beta-tubulin (*Btu*) and Calmodulin (*CaM*) genes using specific primers. Sequences were cleaned and examined by BLAST analysis and subsequently deposited in the NCBI database.

Results

Diverse fungal species isolated from the sputa samples were as follows; 16% (n=53) were positive for moulds, with *Aspergillus* being predominant constituting 68 % (n=36) of the moulds. Among the *Aspergilli, A. flavus and A. niger* were the most frequently isolated constituting 23%, (n=12) and 15% (n=8), respectively. Other moulds recovered from the sputa were *Paecillomyces variotii* (9%, n=5), *Scedosporium aspiospermum* (6%, n=3), *Mucor racemosus* (8%, n=4) and *Penicillium* spp (9%, n=5). A total of 14% (n=49) of the samples were positive for yeasts. *Candida albicans* and *C. krusei* were the major yeast species isolated from sputum constituting 50% (n=24) and 20.8% (n=10) of the yeasts, respectively. *C. albicans* (33%, n=22) was also a predominant isolate from LJ tubes. Members of the *Aspergillus* spp with MICs ≥4µg/ml to some antifungal agents were noted, and all the moulds except two (n=2) isolates of *S. aspiospermum* exhibited MICs higher than >4µg/ml for fluconazole. The moulds generally showed greater sensitivity to AMB and VRC, while the yeasts, particularly *C. albicans* exhibited greater sensitivity to the four antifungal agents. There was a statistically significant difference (Chi Square; F=3.7, P=0.004) in the sensitivity pattern of moulds while yeasts exhibited no statistically significant difference (F=1.7, P=0.154>0.05). Genome sequence analysis of *Aspergillus* spp from sputum showed a similarity index of 98% to 100% compared to those in GenBank.

Conclusion

The study demonstrates a wide variety of moulds and yeasts that are potential respiratory pathogens in TB retreatment patients. The fungi could be responsible for persistent TB like symptoms despite treatment and are likely to be misdiagnosed as relapse requiring retreatment. Fungal investigation in presumptive TB relapse cases should be encouraged to reduce unnecessary retreatment, delayed antifungal intervention and poor outcomes associated with misdiagnosis. Fungi from sputum showed varied sensitivity patterns to the antifungals tested hence antifungal sensitivity testing is essential to guide treatment choices in light of possible resistant isolates.

CHAPTER ONE
INTRODUCTION

1.1 Background information

Current evidence shows that the prevalence of fungal infections is on the rise globally. Fungal infections contribute to increased morbidity and mortality especially among immunosuppressed individuals. In Kenya, it is estimated that 7% of the population experiences a severe fungal infection each year (Bowyer *et al*., 2011). The most frequently encountered fungal infections include aspergillosis, penicillosis, histoplasmosis, blastomycosis, candidiasis, and cryptococcosis. The genus *Aspergillus* represents a large group of fungal species of both agricultural and medical importance. *Aspergillus* infections are associated with chronic pulmonary aspergillosis (CPA), aspergilloma, allergic bronchopulmonary aspergillosis (ABPA), as well as, invasive aspergillosis (IA), which presents a severe clinical and pathological form of the infection (Garcia-Effron et al., 2008). *Aspergillus fumigatus* accounts to more than 80–90 % of human infections (Alcazar-Fuoli & Mellado, 2014). Opportunistic fungal infections are now a significant public health concern, and the situation has worsened due to the increased number of immunocompromised patients, majorly those with cancer, HIV/AIDS and diabetes (Loeffler & Stevens, 2003). Majority of patients inhale fungal spores that are abundant in the environment, this exposure is usually severe in patients with asthma who develop severe reactions (Muthu *et al*., 2020). In recent months, fungal secondary infections especially aspergillosis & mucormycosis have been recognized as a significant causes mortality among COVID-19 patients (Alanio *et al.,* 2020; Koehler *et al.,* 2020; Tabarsi *et al.,* 2021).

Pulmonary fungal infections correlate with increased fingal contamination in TB cultures. However, the fungi are usually ignored as contaminants, yet they could be the primary etiologic agents for the patient's condition (David, 2011; Iwata *et al*., 1989; Jabeen, 2016). In a study carried out to determine the prevalence of aspergillosis among patients suspected of tuberculosis, out of 200 sputa samples analyzed, 30 (15%) yielded *Aspergillus* spp, and of these, 10 (5%) had *A. fumigatus* and, on the other hand *A. terreus, A. flavus,* and *A. niger,* were isolated in 5 (2.5%), 6 (3%) and 9 (4.5%) of the patients respectively. The co-infection rate among 27 (13.5%) of the patients with *M. tuberculosis* was 9 (4.5%) (Anna, *et al*., 2012). Pulmonary fungal infections are associated with increased morbidity and mortality and information regarding these diseases in Kenya is scanty.

Azole based antifungal agents are the standard therapy recommended as first-line medication and prophylaxis against aspergillosis and other fungal infections. Azoles target the sterol 14- α demethylase, a vital enzyme in the ergosterol biosynthesis pathway in fungi (Azevedo *et al*., 2015; Bowyer *et al*., 2011; Ghannoum & Rice, 1999). These agents include voriconazole (VRC), itraconazole (ICZ), fluconazole (FCZ) and posaconazole (PSC). Azoles are also used in agriculture to manage plant and animal fungal diseases and resistance is currently emerging in various parts of the world (Azevedo *et al*., 2015; Berger *et al*., 2017). Majority of studies conducted mainly for surveillance in Europe have demonstrated a surge in the level of resistance to azoles in *A. fumigatus* (Mortensen *et al.,* 2010). Resistance to triazole antifungals in *Aspergillus* spp was regarded as a rare occurrence and has only been reported in the past at low incidence, mostly as inpatient case reports. In Spain and the United Kingdom, studies also show an increasing trend of resistance to triazoles where *cyp51* mutations occur due to pressure in patients under azole therapy (Bowyer *et al.,* 2011). Extensive studies conducted in the Netherlands since the year 2002 have reported a substantial number of clinical *A. fumigatus* isolates with multiple resistance to voriconazole, itraconazole, ravuconazole, and posaconazole (Garcia-Effron *et al*., 2008). The strains exhibiting resistance were frequently isolated in azole naive and azole-exposed patients, along with those from the environment. In Kenya, there is a high prevalence of HIV and TB co-infection and in 2018, the HIV positive TB incidence was 79% per 100,000 population (WHO, 2019). Other immune debilitating conditions exist predisposing the population to opportunistic fungal infections. Additionally, little has been done to explore the incidence and prevalence of pulmonary fungal infections in presumptive tuberculosis patients as well as the level of antifungal drug susceptibility. In this regard, this study sought to determine the spectrum of pulmonary fungal pathogens and antifungal drug sensitivity in TB retreatment and relapse patients from selected reference facilities in Kenya.

1.2 Problem statement

Globally, the burden of fungal infections is increasing significantly. Pulmonary fungal infection is a major problem among individuals with suppressed immunity such as the HIV infected, diabetics and TB patients. It is estimated that 1.2 million people live with CPA globally as a sequel of TB. In Kenya, the burden of CPA is estimated at 12, 927 (32 per 100,000 population). Tuberculosis is a chronic disease and the treatment often leads to immunosuppression which predisposes patients to pulmonary fungal infection. On the other hand, coexistence of fungi and TB in the lung has been shown to aggravate and amplify the clinical course of TB patients. Fungal contamination in TB cultures is also a common phenomenon and few studies have been done to determine their etiologic significance in TB smear-negative, relapse and retreatment patients. The assumption that fungi are just contaminants in TB cultures denies the patient appropriate clinical management. The cost implications of wrong treatment, morbidity and mortality associated with missed, delayed or misdiagnosis of TB or pulmonary fungal infection is unwarranted. Similarly, the development of resistance to first line azole-based antifungal drugs could negatively influence the therapeutic use of azoles in the management of fungal diseases, including aspergillosis. The azole antifungal resistance problem has been observed among *Aspergillus* spp particularly *A. fumigatus.* This trend would eventually exclude the usage of orally administered antifungal medicines leaving a limited choice of injectable echinocandins and amphotericin B for management of severe infections. Misdiagnosis or missed diagnosis of respiratory fungal infections among presumptive TB patients coupled with emerging resistance to antifungal and anti-TB drugs could also lead to increased morbidity and mortality, protracted illness and a more significant threat of complications. Genetic diversity of fungal species responsible for pulmonary infections in presumptive TB patients is vital for tretaemnet interventions. However, such data is limited, hence the need for genomic characterization of the fungi.

1.3 Justification

The global incidence and prevalence of respiratory mycoses is not elaborate and information regarding pulmonary fungal infections in Kenya is also limited. With the advent of rising debilitating immune conditions, opportunistic fungal infections are taking center stage. In this context, fungal positive mycobacterial cultures may not be just contaminants but may have an etiological significance in TB smear-negative and retreatment patients. Despite the use of antibiotic supplements and antifungal agents to prevent contamination of TB cultures, the

frequency remained high at NTRL (10%). This was indicative of possible occurrence of resistant fungal and bacterial pathogens among smear-negative and TB retreatment cases. Studies have demonstrated that antifungal resistance in *Aspergillus* spp is rising in many parts of the world. Existence of azole resistant isolates from clinical specimens in Kenya has not been explored comprehensively and there is need to investigate and evaluate the implications of this occurrence. Evidence accumulated in the past has established that fungal strains showing out of range MICs to azole based antifungal agents may not respond to azole treatment. Given the current trend, it is predicted that pulmonary fungal infections could reach epidemic proportions. The present study presents data regarding fungal pathogens and antifungal drug resistance among TB retreatment patients. The findings will inform policy and form a basis for considering possible fungal aetiology in presumptive TB patients for proper management.

1.4 Research Questions

1. What is the spectrum of respiratory fungal pathogens in sputum of tuberculosis relapse and retreatment patients from; Moi Teaching and Referral Hospital (MTRH), National TB reference laboratory (NTRL) and Mbagathi Hospital?
2. What is the level of antifungal drug resistance and sensitivity of fungal isolates from the sputum of tuberculosis relapse and retreatment cases in the selected reference facilities?
3. What are the common fungal TB culture contaminants at the National TB Reference Laboratory?
4. What is the molecular diversity of *Aspergillus* spp isolated from the sputum of tuberculosis relapse and retreatment cases at the selected reference facilities?

1.5 Hypothesis

Drug-resistant pulmonary fungal pathogens represent significant etiologic agents for clinical symptoms among tuberculosis relapse and retreatment cases and threaten therapeutic use of common antifungal and anti-TB medicines.

1.6 General objective

To determine the spectrum and antifungal drug susceptibility of fungal pathogens from the sputum of tuberculosis relapse and retreatment patients at selected reference facilities in Kenya.

1.6.1 Specific objectives

1. To isolate and identify pulmonary fungal pathogens from the sputum of tuberculosis relapse and retreatment patients from Moi Teaching and Referral Hospital (MTRH), the National Tuberculosis Reference Laboratory and Mbagathi Hospital.
2. To determine the antifungal drug sensitivity of fungi isolated from the sputum of tuberculosis relapse and retreatment patients from selected reference facilities in Kenya.
3. To identify fungal Lowenstein Jensen (LJ) sputum culture contaminants in TB relapse and retreatment samples at the National TB Reference Laboratory (NTRL).
4. To characterize *Aspergillus* spp diversity isolated from the sputum of TB relapse and retreatment patients from selected reference facilities in Kenya.

CHAPTER TWO
LITERATURE REVIEW

2.1 Burden of fungal infections

The global incidence of opportunistic fungal infections has increased significantly and has become a significant concern in healthcare delivery particularly among the immunocompromised. It is estimated that over 300 million people are suffering a serious fungal infection globally and 25 milllion are at increased risk of dying or losing sight (Troeger *et al.,* 2017). Fungal diseases range from invasive aspergillosis, chronic pulmonary aspergillosis, severe asthma with fungal sensitization (SAFS), *Cryptococcal meningitis* and invasive candidiasis. These infections are associated with high mortality and are often misdiagnosed, missed or underdiagnosed (Bongomin *et al*., 2017).

The burden is disproportionately more significant in resource limited settings where appropriate diagnostic capabilities and quality drugs are scarce or unavailable (Bongomin *et al*., 2017; Dellière, Rivero-Menendez, *et al*., 2020). New advances in medicine such as the use aggressive and invasive therapeutic technologies has also led to the emergence of a new population of individuals at risk of fungal infections (Richardson & Warnock, 2011). The main portal of entry for respiratory *Aspergillus* infection is inhalation of fungal spores from the environment.

In 2007 the frequency of chronic pulmonary aspergillosis (CPA) following pulmonary tuberculosis (PTB) particularly in patients with cavitation in the lungs was estimated at 372,000 among 7.7 million cases with pulmonary TB. Among these cases, 11, 400 were documented in Europe whereas 145, 372 were reported in South-East Asia. The prevalence range for CPA was < 1 case per 100,000 in Nigeria and the Democratic Republic of Congo in the same year (Denning *et al*., 2011). In Ukraine, out of 29,265 cases of active pulmonary TB in 2012, it was estimated that 2,881 new cases of CPA occurred and that 5 years prevalence was 7,724 cases with a total CPA burden of 10,054 cases (Osmanov & Denning, 2015). Figure 2.1 shows the global burden of CPA across countries with rates per 100,000 populations.

In Cameroon, a study that was carried out to determine the occurrence of respiratory aspergillosis in hypothetical cases of PTB, 30 (15%) of *Aspergillus* spp were isolated from 200 sputum samples and a co-infection rate of 9 (4.5%) with tuberculosis was observed among the cases (Anna, *et al*., 2012). Findings from a similar literature search of all epidemiological papers that reported fungal infections in Kenya revealed that a major fungal infection infects approximately 7% of the population at any one time. Further, the prevalence of CPA after TB stands at 10,848

cases translating to 32/100,000 while the prevalence of asthma in adults was recorded at 3.1%. Regarding the asthma cases, there was an assumption that 2.5% were suffering from allergic bronchopulmonary aspergillosis, implying that 17,696 (44/100,000) are involved (Guto *et al*., 2016). It is important to note that the disease burden estimates are essential in setting national public health priorities for disease surveillance prevention and establishment of targeted interventions for control and management.

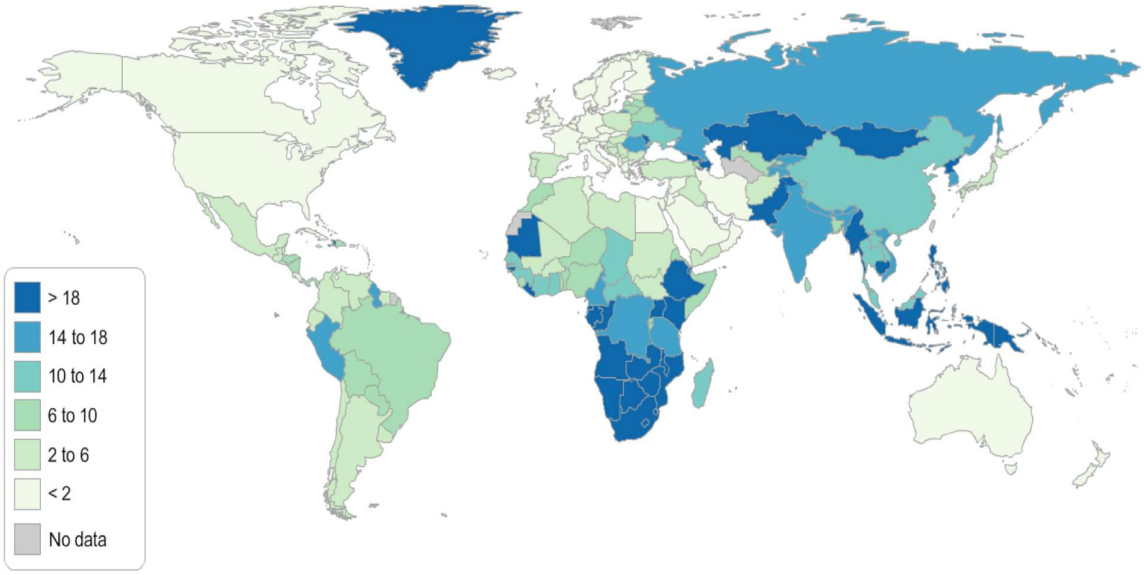


Figure 2.1: Estimated global burden of chronic pulmonary aspergillosis (CPA). Source**:** Global Action Fund for Fungal Infections (GAFFI) (2017), https://www.gaffi.org/why/burden-of-disease-maps/cpa-prevalence

2.2 Burden of tuberculosis in Kenya

According to a national survey carried out in 2016, the prevalence of tuberculosis in Kenya was 558 (455-662) per 100,000 adult population (Ministry of Health, 2016). It was a concern that about 40% of cases were missed in 2015 and a higher prevalence of TB than reported through routine surveillance. The significant number of individuals with TB who were undiagnosed and untreated (missed) continue to transmit the disease in the population, fueling the rate of infection, morbidity, and TB-associated mortality.

In the survey, there was a wide variation in the burden of TB by location, economic status and age category, while a higher male to female ratio in the prevalence of TB was noted. Data from

routine surveillance have previously indicated that the burden of TB was twice as high in males as in females. The high burden among men could be due to biological susceptibility and other common social factors such as cigarette smoking and alcoholism (Sugawara & Nikaido, 2014). Additionally, malnutrition a known predisposing factor to TB is more common among men (Ministry of Health, 2014).

The survey highlighted the need to address fundamental determinants and obstacles to TB control. It demonstrated a higher burden of TB in urban (760 per 100,000 population) compared to rural settings (453 per 100,000 population) consistent with routine TB data which have shown higher notification in the big cities of Nairobi and Mombasa (Ministry of Health, NTLD-Program, 2015). Overcrowding, poor housing and sanitation conditions usually found in the informal settlements are the key drivers for TB disease transmission. The prevalence was highest among individuals in the higher wealth index [741 (507 -975)/100,000 $p< 0.05$], conflicting with the general association of TB with poverty. Tuberculosis and HIV co-infection rate among the prevalent TB cases (16.7%) was lower than those reported among notified TB cases (31%) in Kenya (Ministry of Health, 2016).

2.3 Biotechnological importance of *Aspergillus* spp

The genus *Aspergillus* represents a remarkable genome sequence diversity. The genomes of *Aspergillus* sequenced thus far vary in size from 28 to 40 Mb and the chromosomes are eight in number. They seem to have analogous qualities, even though karyotype analysis shows that naturally existing populations of most of the species harbor variants in the chromosome (Kaphingst *et al*., 2010).

Some species in this genus are extremely harmful, while others are beneficial to humans-many important in industrial and biomedical applications. For example, *Aspergillus nidulans* represents a fundamental model species utilized for genetic and cellular studies. The fermentation industry exploits *A. niger* to produce citric acid, while *A. oryzae* is vital in the fermentation of most Japanese traditional sauces and beverage (Tamang, 2016). *Aspergillus caespitosus* and *A. phoenicis* are known to produce xylanases which have a good potential for pulp bleaching (Guimarães *et al.,* 2006). Contrary to this, *Aspergillus flavus* is pathogenic to humans and animals and it is also responsible for the production of aflatoxin a carcinogenic metabolite, while *A. terreus* and *A. fumigatus* are the main opportunistic pathogens in individuals with impaired immune function (Hedayati *et al*., 2007). Table 2.1 summarises the impact and biotechnological importance of some *Aspergillus* species.

Table 2.1: Biotechnological significance of different *Aspergillus* species.

***Aspergillus* species**	**Impact**	**Biotechnological importance**
A. oryzae	Positive	Fermented food and industrial enzyme.
A. niger	Positive	Citric acid, Glucoamylase and heterologous enzyme.
A. nidulans	Positive	Gene regulation model and cell biology.
A. terreus	Positive	Cholesterol lowering agent and itaconic acid.
A. awamori	Positive	Protein production.
A. fumigatus	Negative	Pathogenic mould in human and animal.
A. clavatus	Negative	Pathogenic mould in human and animal.
A. fischeri	Negative	Pathogenic mould in plant and humans.
A. aculeatus	Negative	Pathogenic mould in plant.
A. flavus	Negative	Pathogenic mould in plant, human and animal and aflatoxin producer.
A. parasiticus	Negative	Aflatoxin producer
A. carbonaris	Negative	Ochratoxin producer caused in food spoilage.
A. rambellii, A. arachidicola	Negative	Aflatoxin B (B1, B2) producer that cause food spoilage.

Source: (Prakash & Jha, 2014)

2.4 Pathogenesis and immunity to fungi

Fungal propagules are ubiquitous in the environment, and this acts as a constant source of infection and colonization. The immune status of the human host is the main determinant of the outcome of exposure (Gogia, 2015). The innate and adaptive immune systems play a key role in the body's defense against fungal infections. Cellular immunity conferred by macrophages, monocytes, neutrophils, dendritic cells (DCs), and epithelial & endothelial cells facilitate the killing and clearance of fungal spores by phagocytosis and complement mediated mechanisms, through the recognition of cell wall structures (Mora-Montes & Lopes-Bezerra, 2017). An infectious process ensues when the delicate balance between the immune system and the invading organism is altered. Neutropenia is the most significant predisposing factor to invasive aspergillosis, candidiasis and mucormycosis (Croft *et al*., 2016; Margalit & Kavanagh, 2015).

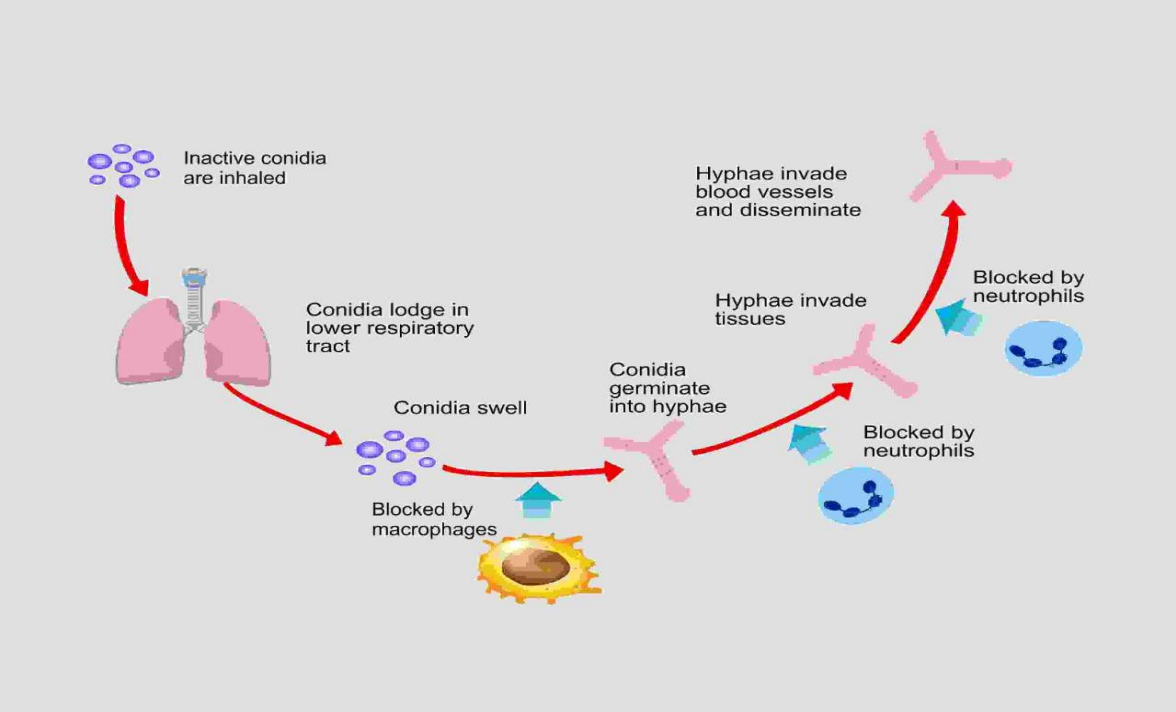


Figure 2.2: Overview of host defence mechanisms against *Aspergillus* spp. Phagocytic uptake and intranuclear killing is the most important line of defence. *Redrawn*; Source: Atlas of infectious diseases-fungal infections (Diamond, 2000).

Pathogen recognition receptors (PRRs) are expressed by host cells and they play a major role in triggering downstream response pathways by specifically recognizing pathogen-associated molecular patterns (PAMs). Toll like receptors (TLRs) are the most vital PRRs in antifungal immunity (Mora-Montes & Lopes-Bezerra, 2017). The innate immune defense mechanisms interact with the fungus at the initial site of infection. These includes the protective barrier of the skin and mucous membranes of the respiratory system, gastrointestinal and the urogenital epithelium (Jiang, 2016). Dendritic cells (DCs) demonstrate high antifungal potency concerning cellular immunity since they initiate innate and adaptive immune responses to *Aspergillus fumigatus*, *Candida albicans* and *Cryptococcus neoformans.* DCs are phagocytic and efficient antigen presenting cells (APCs) (Pathakumari *et al*., 2020). One mechanism through which DCs trigger antifungal immunity, is by capturing and processing of antigens and expression of lymphocyte co-stimulatory molecules. DCs later migrate to lymphoid organs and secrete cytokines including IL-10 and IL-12 that initiate immune response by activating naive T cells to effector Th cells (Kim & Park, 2021). Intensive research on DCs is currently in progress and it

may contribute to development of potent antifungal vaccines in future (Huffnagle & Deepe, 2003).

On the other hand, natural Killer (NKs) have shown great potential against numerous types of fungi such as *C. albicans* and *Aspergillus fumigatus in vitro.* NKs are known to produce interferon gamma (IFN-γ) with further activity against *Cryptococcus neoformans*. Other cells of the innate immunity such as mast cells, basophils and eosinophils contribute immensely to protection against fungal infection (Jiang, 2016).

Regarding adaptive immune responses, T cell immunity is mainly protective and the level of activation of dendritic cells determines the initiation of a range of T-Cell responses such as Th17 and Th2 through signaling pathways involving TLRs. Ultimately a balance between CD4+ effector T cells and regulatory T (Treg) cells is achieved and is possibly exploited by fungi to establish commensalism or infection (Mora-Montes & Lopes-Bezerra, 2017). The role of antibodies in antifungal immunity is increasingly being recognized in medical mycology. Several studies have documented the use of humoral factors as immunotherapeutic options in the treatment of fungal infections (Huffnagle & Deepe, 2003). Majority of antibody related studies have focused on treatment of *Candida albicans* and *Cryptococcus neoformans*. Antibodies generally prevent entry of fungal pathogens and inhibit their replication and suppress polysaccharide release and germ tube growth. Further, antibodies mediate antibody dependent cellular cytotoxicity (ADCC) that results in opsonization which activates complement cascade downstream enhancing phagocytosis. Antibodies can also act direct on fungal pathogens. One example is the action of monoclonal antibodies targeting manoprotein in *C. albicans* preventing adherence ability and growth of the organism (Pathakumari *et al*., 2020).

In the study of humoral immunity against *C. albicans*, one intriguing aspect is that serum antibodies formed due to infection do not confer protection. One explanation to this observation is that there are few protective antibodies in serum that are insufficient to prevent infection (Kim & Park, 2021). Additionally, it is reported that a category of specific immunoglobulins arise, that directly block the activity of protective antibodies in particular anti-glucan antibodies (Dellière, Sze Wah Wong, *et al*., 2020). The role of antibodies in protection against fungal infections is evident in patients with chronic fungal diseases. It is well established that numerous anti-fungal antibodies targeting fungal products such as peptides or glycolipids, polysaccharides, protect the host against such infections. The antibodies essentially interact with extracellular as well as intracellular pathogens. Recent studies have demonstrated the ability of monoclonal antibodies

(mAbs) to promote phagocytosis of *Aspergillus fumigatus* conidia in mice (Dellière, Sze Wah Wong, *et al.*, 2020). The low prevalence of *Cryptococcus neoformans* infections in humans has also been attributed to specific antibodies formed against this organism following natural exposure.

The number of individuals susceptible to fungal infections is increasing significantly due to immunosuppressive agents, invasive medical procedures, organ transplants, and the overuse of broad-spectrum antimicrobial agents. It is also believed that the genetic makeup of some individuals may play a role as a predisposing factor for fungal infection. It has been observed that not all patients at high risk of invasive infection develop the disease, especially candidiasis. Therefore, the risk factors may not fully explain the susceptibility to this infection and thus the suggested role of the host genetic make-up (Gow & Netea, 2016).

The pathogenesis of fungal infections occurs through various mechanisms. Fungi possess a range of virulence factors that enable its survival and capacity to cause severe disease in the human host. The virulence factors in fungi include the ability to adhere to tissues at the site of infection, invasiveness and immune evasion mechanisms (Dagenais & Keller, 2009). Fungal cell wall polysaccharides mainly protect the fungi from harsh environmental conditions while melanin in pigmented fungi facilitate immune evasion by preventing opsonization and subsequent biding of C3 component of the complement (Garcia-Solache & Casadevall, 2010; Garth & Steele, 2017). *Candida albicans* is mainly associated with the formation of true hyphae and pseudo-hyphae which facilitate tissue invasion. Some fungi also secrete extracellular enzymes and proteases which breakdown host tissues. Rapid growth rate, siderophore production, ability to survive in oxygen deprived environments and tolerance to increased temperature conditions is a critical feature in *Aspergillus* and *Mucormycosis*. The major virulence factor for *Cryptococcus neoformans* is the polysaccharide capsule which facilitates immune evasion and melanin synthesis. Infection is acquired through inhalation which establishes in the lungs and later disseminates to the meninges through the blood (Gogia, 2015).

2.5 Clinical significance of *Aspergillus* spp

The *Aspergillus* represents a large group of fungi over 180 species (Rodrigues *et al.,* 2007). It has been demonstrated that over 20 can infect both humans and animals (Yu *et al*., 2005). Members of this genus are ubiquitous and are found in varied environments such air, water, soil and decaying material. *A. flavus* is the most prominent species in the genus and follows *A. fumigatus* the most prevalent cause of invasive aspergillosis in humans and animals. It is known that *A.*

flavus produces several secondary metabolites, including aflatoxins which are highly potent and toxic carcinogens capable of inducing malignancies in mammals (Arapcheska *et al*., 2015). Clinically, diseases resulting from *A. flavus* infection include chronic sinusitis with granuloma formation, wound infection, keratitis and osteomyelitis resulting from traumatic inoculation (Hedayati *et al*., 2007).

The role of *Aspergilli* in human infection and disease was initially described by Virchow (1856) who noticed similarities between *Aspergillus* related diseases described in animals and those he had seen in human infections. *A. fumigatus* is known to survive in a broader range of environmental conditions than other species hence more widespread. This is one of the reasons for the high prevalence of clinical conditions associated with this species. Figure 2.3 illustrates the saprophytic and pathogenic cycles of *A. fumigatus* which begins by inhalation of airborne conidia.

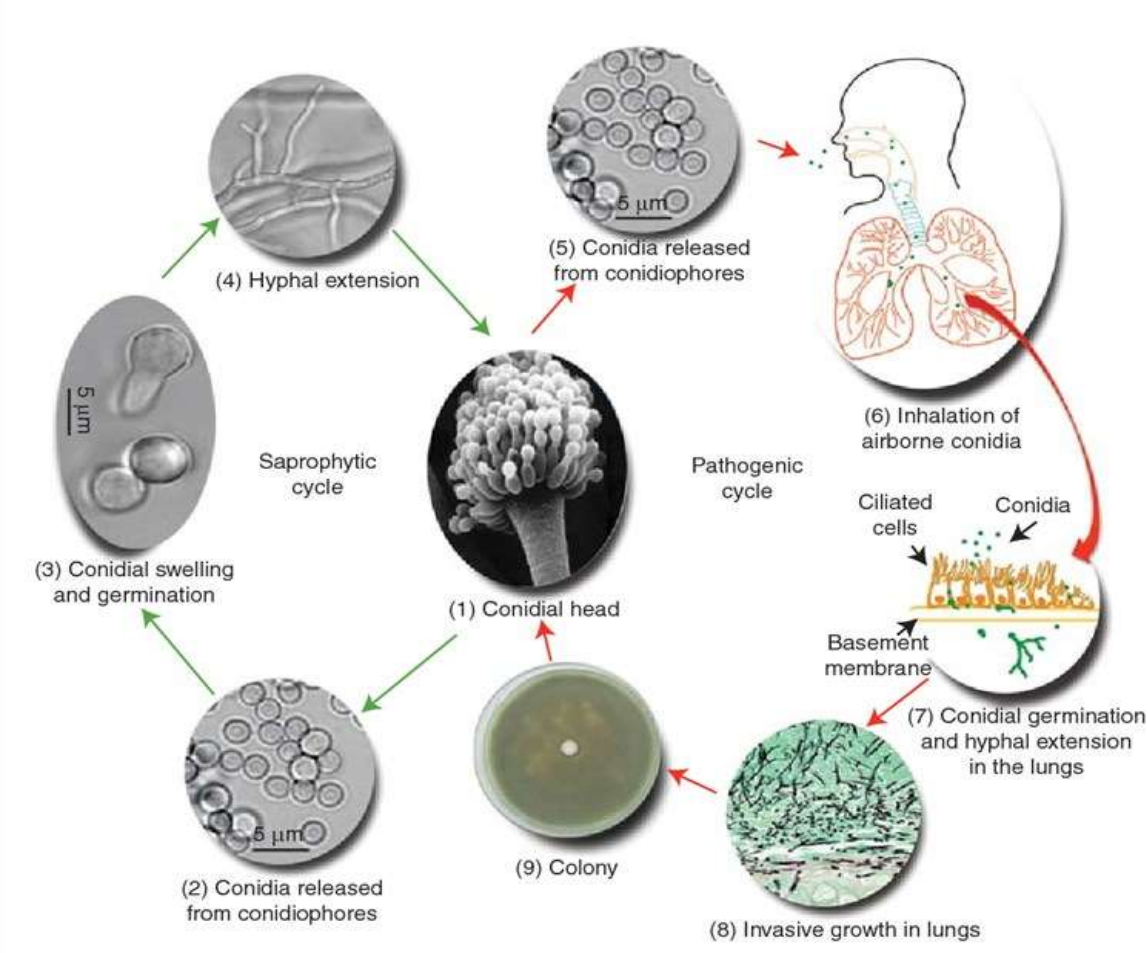


Figure 2.3: Saprophytic and pathogenic cycles of *A. fumigatus*. **Source** (Sugui *et al*., 2015).

In some geographic areas such as South Arabia and Sudan with semi-arid conditions, *A. flavus* is the common cause of invasive aspergillosis (IA) instead of *A. fumigatus* (Yu *et al.,* 2005). Patients acquire the infection by inhaling airborne fungal spores released to the environment by the saprophytic mould. Individuals with underlying conditions are at highest risk of infection. These include hematological malignancies, whole organ transplant recipients, pulmonary diseases (such as chronic obstructive pulmonary disease) and patients receiving immunosuppressive corticosteroids (Verweij *et al*., 2009).

In one study, an assessment of the clinical picture of patients with *Aspergillus flavus* associated keratitis and aflatoxin-producing capacity of the keratitis strains in comparison with environmental strains, it was demonstrated that Aflatoxin B1 is significantly higher in clinical isolates (80%) as relative to environmental isolates (40%) (Leema *et al.,* 2010). Corneal scrapings and biopsy material from patients who had suppurative keratitis yielded *A. flavus* on culture. It is thought that pressure from antifungal therapy and toxic factors released by epithelial cells in the cornea triggered the over-production of aflatoxins in the clinical isolates (Selvam *et al.,* 2014). Nevertheless, the clinical significance of *Aspergilli* is a great concern for clinicians and medical mycologists.

2.6 Emerging antifungal resistance

Fungal pathogens have evolved substantially over the years, exhibiting greater resistance to the few existing antifungal agents (Nosanchuk, 2008). This phenomenon is evident due to antifungal clinical treatment failures and a surge in the prevalence of fungal infections. Epidemiological studies have shown the mortality rate of patients infected with *Aspergillus* spp. crosses 50% (Ravikant, 2015). With the advent of the ever-increasing infections, recent developments indicate a specific change in the fungal pathogen towards the resistant strain among the common genera mainly *Candida* and *Aspergillus* spp. In these instances, there is an occurrence of formerly uncommon species that are particularly difficult to treat (Chen *et al*., 2001). Typical cases include; *Candida krusei* and *C. glabrata* yeasts showing reduced antifungal sensitivity and among filamentous fungi, these include the non-fumigatus *Aspergillus* spp (such as *Aspergillus terreus*), *Zygomycetes* and *Fusarium* spp. Emergence of uncommon mould species has been attributed to selective pressure from over prescription of prophylactic antifungals in patients deemed at high-risk of infection (Bansod & Rai, 2008a).

For a long time, amphotericin B (AMB) and itraconazole (ICZ) have been the only drugs available to treat aspergillosis. Oral and intravenous preparations of itraconazole were available

while posaconazole, caspofungin and voriconazole have recently been accepted to treat *Aspergillus* spp infections (Hedayati *et al.,* 2015).

In Denmark, a study focusing on cystic fibrosis patients infected by *A. fumigatus,* reported azole resistance of 4mg/ml to itraconazole in the isolates on screening agar and EUCAST antifungal susceptibility broth dilution (Yu *et al.,* 2005). Sequencing of *cyp*51A gene was performed on the same isolates showing high MICs and 6 out of 133 patients (4.5%) had azole-resistant *A. fumigatus* strains. Five of the six patients had strains of the mould harboring changes in *cyp*51A gene (Mortensen *et al.,* 2010). Another study carried out in the US reported elevated itraconazole MIC >8 mg/ml in *A. fumigatus isolates* (Ghannoum & Rice, 1999).

Screening methods to determine antifungals' in vitro and in vivo efficacy are still undergoing standardization and micro-dilution techniques are the gold standard (Arikan, 2007). However, significant milestones have been achieved so far for specific fungal pathogens (such as *Candida* spp) and antifungal agents (Sanguinetti & Posteraro, 2018). It is essential to understand the sensitivity of a fungal etiologic agent implicated in an infectious process for the selection of appropriate treatment.

2.7 Mode of action and mechanism of resistance to azole antifungals

There are different antifungal medications with varying clinical spectrum and mode of action. Four main classes of antifungals are currently in clinical use namely azoles, allylamines, polyenes and echinocandins (Perlin *et al*., 2017). Additionally, a diverse group of other compounds exist, such as flucytosine and griseofulvin (Yamauchi, 2018). The development of new antifungal molecules has been hampered by several factors particularly limited selective toxicity of candidate compounds and primary and secondary resistance (A. Espinel-Ingroff, 2009). Fungi are eukaryotic organisms sharing substantial structural features with mammalian cells, making it difficult to target the fungal cells without significant harm to the host. In contrast, prokaryotes mainly bacteria offer a wide variety of metabolic and structural components that can be targeted selectively thus a wide range of antibiotics are available (Xie *et al*., 2014).

Azoles form the main class of synthetic antifungal drugs and roughly 20 compounds are in use. Some of the agents are used superficially to treat dermatophytes and yeast infections. A heterocyclic five member aromatic structure present in these molecules is responsible for the antifungal activity, either an imidazole or a triazole (Ghannoum & Rice, 1999). Triazole antifungal agents are greatly effective against *Aspergillus* spp. and voriconazole, itraconazole,

and posaconazole are approved to treat infections caused by this group of fungi (Denning *et al.,* 2011). Triazoles and imidazoles hinder *cyp*-P450 14 α- demethylase enzyme, preventing the conversion of lanosterol to ergosterol (Espinel-ingroff *et al*., 2009; Xie *et al*., 2014). Inhibiting the enzyme alters the shape of the cells, increasing permeability and malfunctioning of the fungal cell membrane. The molecules have a low affinity on mammalian P450s hence sufficient selective toxicity (Mortensen *et al.,* 2010)

The enzyme 14 α-demethylase is encoded in the gene *cyp51* in filamentous fungi and *erg11* in yeast. Numerous genetic alterations in the region have been linked with resistance to azoles. Over-expression of genes encoding efflux pumps that increase the ability of the fungal cell to expel antifungal agents is believed to be central in resistance seen in *Candida* spp (Arendrup, 2014). Numerous mutations in cyp51A of *Aspergillus spp*. have been connected with the emerging resistance to azoles. Azole-naive patients have also been found with resistant isolates. The isolates are frequently found with a particular genetic change characterized by a 34-base pair tandem repeat in the promoter combined with a point mutation in the *cyp*51A target gene. The mutation brings about substitution of an amino acid at codon 98 (TR34/L98H) resulting in resistance to multiple-azole molecules (ECDC, 2013).

2.8 The role of azole fungicides in antifungal resistance development

Azole fungicides are used to protect crops from fungal diseases. In Europe, approximately 50% of acreage under cereals are protected with fungicides (Segal & Walsh, 2006). *Aspergillus* spp in these environments are therefore exposed to azoles continually. Azoles used in agriculture (epoxiconazole, prothioconazole, tebucanazole, and prochloraz, etc.) differ from those used in clinical practice (Bowyer *et al.,* 2011). It is thought that the application of azoles as antifungal pesticides in Europe led to the rise of *cyp*51A mutations conferring resistance.

Resistance to azole fungicides, also referred to as sterol demethylation inhibitor (DMI), documented in *Blumeriella jaapii* a significant pathogen affecting tart cherry can only be controlled effectively by continuous fungicide application. The resistance is mediated by over-expression of 14 α-demethylase target gene (cyp51) (Ma *et al*., 2006). A rise in multi-triazole-resistant *A. fumigatus*, has been observed mainly in the Netherlands (Segal & Walsh, 2006). The isolates in this region were recovered from patients with history of exposure to azoles, non-exposed, and the environment. It is suggested that the resistant strains were acquired from the environment and might have evolved due to selective pressure of agricultural fungicide use (ECDC, 2013).

In Kenya, a study focusing on the susceptibility of mycotoxigenic fungi to commercial fungicides revealed that isolates of *A. flavus, A. niger* and *Fusarium spp* from maize exhibited phenotypic resistance to four agricultural fungicides (Tonui *et al*., 2014). The study highlighted the influence of fungicide use in agriculture as a possible cause of antifungal drug resistance in clinical settings.

The literature review in the present study demonstrates significant gaps regarding fungal diseases that are increasingly being identified the human population. The escalating burden of fungal infections is further complicated by emerging antifungal resistance involving both filamentous fungi and yeasts. The frequency of infections related to fungal species previously known to be of less clinical relevance is also rising. Antifungal resistant species have the potential to cause severe disease and mortality in infected patients, hence warranting further investigation to understand the pathology and underlying factors. The literature explicitly highlights antifungal resistance seen in *Candida* spp and *Aspergilli*, common human pathogens. A number of studies point out an emerging trend of fungal co-infection in pulmonary TB patients. It is reported that the pathology of *Mycobacterium tuberculosis* is similar to pulmonary mycosis. There exists a knowledge gap on the interaction between *M. tuberculosis* and fungi in the lung. Some studies reported that up to 40% of PTB patients were co-infected with *Candida* spp while 24% had *Aspergillus* spp and PTB co-infection, the majority being multi drug-resistant. This trend calls for further studies to address new therapeutic challenges in presumptive TB patients with potential coinfection by fungi. There is also a need for better diagnostic approaches to identify pulmonary fungal infections mimicking PTB necessitating retreatment.

CHAPTER THREE

MATERIALS AND METHODS

3.0 Flow of investigations

Figure 3.1 illustrates the flow of investigations carried out in the study, including recruitment of eligible participants, collection of sputa samples, mycological investigations, DNA sequence analysis & phylogeny of *Aspergillus* spp and antifungal susceptibility testing of fungal isolates.

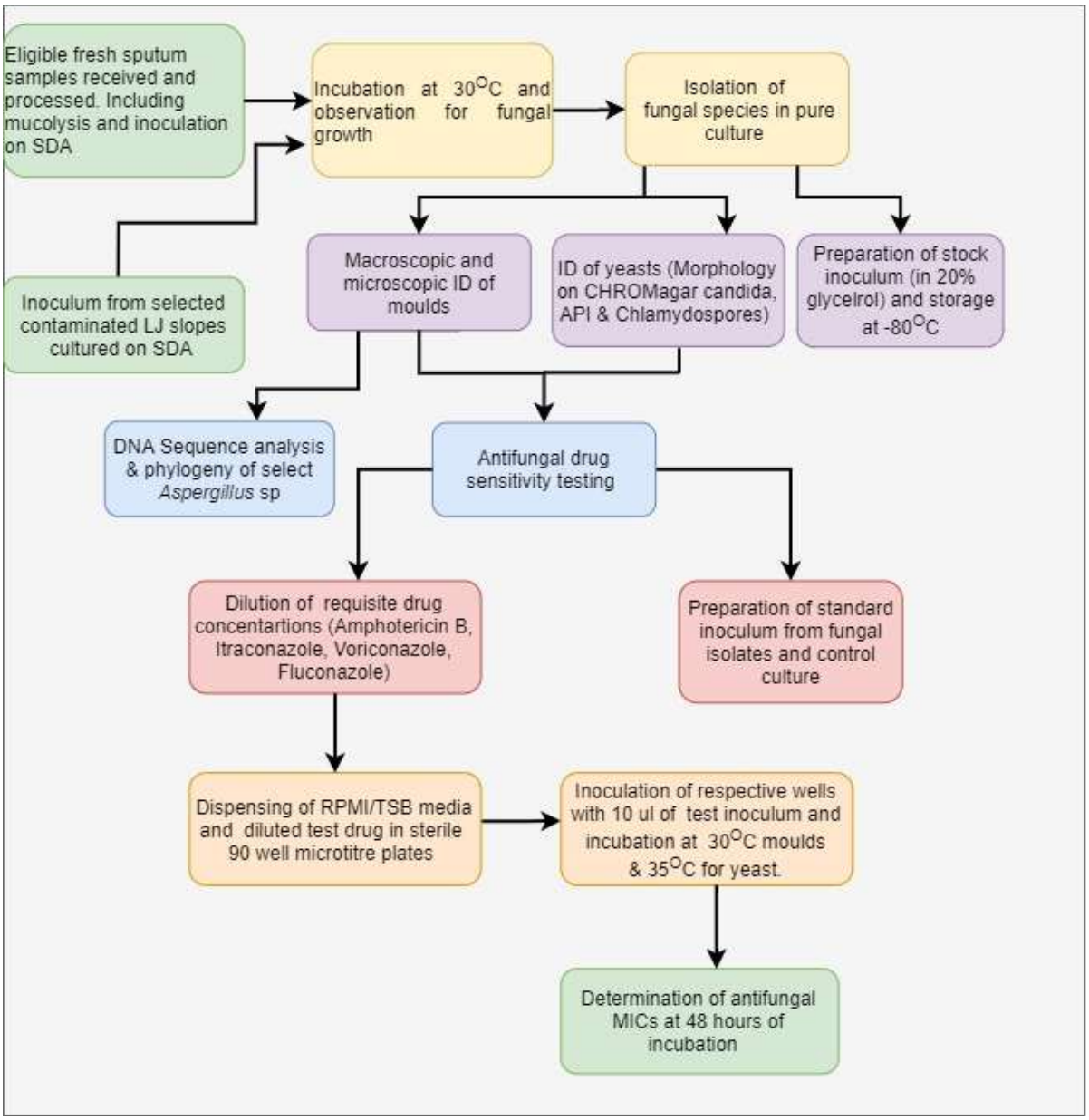


Figure 3.1: Flow diagram of investigations in the study

3.1 Study design

The study employed a cross-sectional laboratory-based design. The research was carried out on sputa samples collected from patients in three reference facilities: Moi Teaching and Referral Hospital (MTRH), National TB Reference Laboratory (NTRL), and Mbagathi Hospital. The study was nested in an-ongoing-research titled "*Mapping antifungal, antibacterial drug resistance and the quality of antimicrobial agents against fungal and bacterial pathogens from smear-negative and retreatment cases in high TB prevalence Counties in Kenya*" (protocol number: KEMRI/SERU/0037/3213) under the KEMRI target area on emerging and reemerging infections/ antibiotic-resistant strains/tuberculosis and respiratory diseases research.

3.2 Study sites

The study sites selected are known high volume facilities serving a large population with a high burden of tuberculosis. Moi Teaching and Referral Hospital serves as a diagnostic and treatment facility for TB patients. Similarly, Mbagathi Hospital serves a large catchment population mainly urban, with an active TB clinic. The National Tuberculosis Reference Laboratory (NTRL) performs culture for TB diagnosis and drug sensitivity testing for relapse and retreatment cases.

3.3 Target population

The study targeted adult participants (>18years) who had been previously treated for tuberculosis and relapse cases who had apparent clinical symptoms presumptive for TB. Minors were excluded from the study due to challenges in obtaining expectorated sputum requiring hospitalization. Participants were provided with adequate information (Appendix 6 & 8) after which they were asked to sign the consent form (Appendix 7 & 9) for participation in the study. Instructions for collecting the quality specimen were explained and samples collected were shipped to the Mycology Laboratory at Kenya Medical Research Institute (KEMRI) for mycological investigations.

3.4 Inclusion and exclusion criteria

3.4.1 Inclusion criteria

1. Tuberculosis relapse or retreatment cases
2. Any patient with recurrent symptoms despite compliance with first-line anti TB therapy
3. HIV-TB co-infected relapse and retreatment cases
4. Only those who consented to participate

3.4.2 Exclusion criteria

1. TB treatment defaulters
2. Patients confirmed to have XDR TB. These patients are usually referred to Kenyatta National Hospital (KNH) for management and require high-level containment
3. Any newly diagnosed smear-positive TB patient
4. Patients with extrapulmonary TB

3.5.1 Sample size determination

The minimum sample size for the study was determined according to Fischer's *et al.,* 1998 with 95% a confidence level. The reported prevalence (67%) of CPA in PTB patients in Kenya was used (Guto, Bii, & Denning, 2016).

$$N = \frac{z_{I-\alpha/2}^2 P (1-P)}{d^2}$$

Where: N= Sample size
$Z_{I-\alpha/2}$= 1.96
P= 67%
d= Absolute precision (0.05)

Therefore: $N = \frac{1.96^2 \times 0.67 (1-0.67)}{0.05^2} = 340$

A minimum of 340 sputa samples were collected for the study.

3.5.2 Sampling technique

The purposive sampling technique was used to collect respective samples from the selected sites based on the proportionate sample size as shown below. The samples were distributed according to the most representative estimated prevalence of TB by proportionate probability.

Facility	Estimated prevalence of TB per 100,000 population for the sites	Proportion calculation	Sample size
NTRL	558	558/1496*340	127
Mbagathi	490	490/1496*340	111
MTRH	448	448/1496*340	102

Source: (MOH, 2016), (Health sector report, 2016), (Nyamogoba *et al.,* 2012)

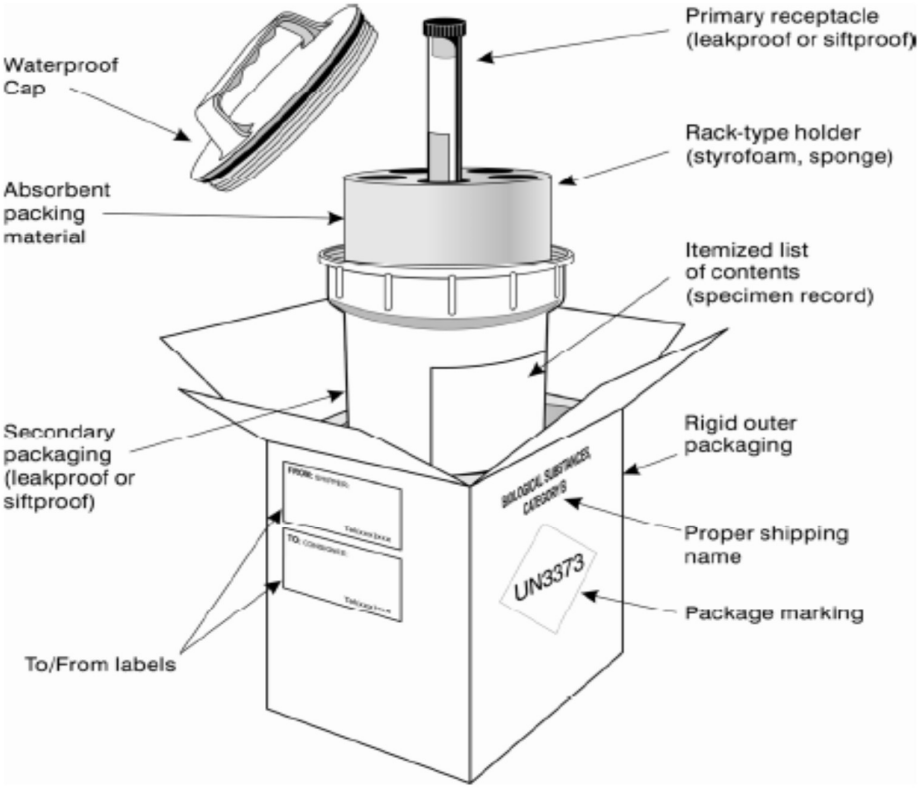
3.6.1 Sputum sample collection

Clinicians examined participants, and those who met the inclusion criteria were provided with clear instructions on collecting good quality sputum specimens. Specimen containing food or other particles were discarded as unsatisfactory. The specimens were collected in clearly labelled biohazard labelled leak proof sample containers in accordance with requisite biosafety protocols. Participants were advised to follow the steps outlined below to produce the best specimen. Only good-quality sputum specimens appearing as purulent and mucoid were accepted for mycological examinations.

1. Patients were asked to rinse their mouth with clean water to remove food and other particles
2. Inhale deeply 2–3 times and breathe out strongly each time
3. Cough deeply from their chest to produce sputum
4. The patient was instructed to place the open container close to the mouth to collect the specimen and to avoid contaminating the outside of the container
5. The patient was thereafter instructed to clean their hands after collecting the sample.

3.6.2 Specimen packaging and transport

Sputum samples collected at the study sites were packaged following triple packaging technique (illustrated below) as per WHO guidelines on transportation of infectious material (Ogbu & Arah, 2016). The specimens were transported to the laboratory in cool boxes. Any delay, leakage or possible contamination was minimized, and the samples were refrigerated at 4^{o}C when there was delay in processing. Tuberculosis reference numbers were used to identify the recruited patients to link the microbiological information with TB management.



Triple packaging technique (WHO, 1997)

3.6.3 Sampling of Lowenstein Jensen (LJ) tubes

Contaminated LJ tubes were collected from the national TB reference laboratory and transported according to guidelines for infectious material to the mycology laboratory at KEMRI to isolate possible fungal contaminants. The sample size (*n=138*) was determined using the formula described below for simple random sampling (Taherdoost, 2017). The calculation was based on 10% culture contamination rate during the sampling period.

$$\boldsymbol{n} = \frac{p\,(100\text{-}p)\,z^2}{E^2}$$

n = required sample size
P = percentage occurrence of contamination (10%)
E = percentage maximum error required (5%)
Z = value corresponding to 95% level of confidence required (1.96)

$$n = \frac{10\ (100\text{-}10)\ 1.96^2}{5^2} = 138$$

3.7 Laboratory procedures

All laboratory tests conducted complied with appropriate biosafety measures at KEMRI mycology research laboratory. The procedures are documented in the standard operating procedure (SOP) for sputum collection and culture; SOP number-KEMRI/CMR/MOI/B007 (controlled document).

3.7.1 Isolation of fungi

Coded sputum samples were first mucolysed with sputasol and inoculated on sabouraud dextrose agar (SDA) in duplicates. The media was supplemented with 10 ml of Chloramphenicol and 0.65 ml gentamicin (40 mg/ml) to inhibit bacterial contamination. Sterility check and ability to support the growth fungi was performed on each batch of media and subsequent negative and positive controls were incubated with every batch of cultures. Inoculated plates were incubated at 30^{o}C for up to 4 weeks and evaluated daily for growth of moulds and 35^{o}C for yeasts. Plates that had no growth at the fourth week were deemed negative and disposed appropriately following laboratory waste disposal procedures by autoclaving and subsequent incineration. Inoculums from LJ tubes were also obtained aseptically and inoculated directly on SDA plates in duplicates with growth monitoring similar to that of sputum cultures.

3.7.2 Identification of fungal isolates

3.7.2.1 Moulds

Filamentous fungi growing on culture were identified by morphologic characteristics using macroscopic and microscopic features. Macroscopic features such as colony growth rate, color on the obverse and reverse, elevation, texture, and colony size were captured. Specimen for microscopy were prepared using tease-mount technique and stained with lacto-phenol cotton blue as described by (Colin Campbell, Elizabeth M. Johnson, 2013). Microscopic features were observed at X40 magnification and features including conidia production, shapes, and arrangement of phialides were noted to aid in identification of the isolates. Confirmation of some *Aspergillus* spp was done by sequencing of calmodulin and beta tubulin genes. The sequencing was carried out courtesy of support from medical mycology research Institute-Chiba University, Japan. Manipulation of all live cultures was done under level II biosafety containment facilities.

3.7.2.2 Yeasts

Yeasts were sub-cultured on CHROMagar™ *Candida* for presumptive identification by morphologic characteristics such as colony color, size and shape of the cells. The isolates were also inoculated on corn meal agar to detect chlamydospores while preliminary identification of *Cryptococcus* spp. was done using urea hydrolysis and India ink. Further identification and confirmation were carried out on analytical profile index (API 20 C AUX-bioMerieux Durham, USA).

3.7.3 Antifungal susceptibility testing

Fungal isolates were screened for susceptibility to itraconazole (ICZ), voriconazole (VCZ), fluconazole (FCZ) and amphotericin B. Antifungal susceptibility testing was performed using broth micro-dilution techniques according to Clinical and Laboratory Standards Institute guidelines (CLSI), M27-A3 and M38-A2 for yeasts and moulds respectively (ClSI, 2009; CLSI, 2008). Voriconazole, itraconazole and amphotericin B were diluted in analytical grade dimethyl sulfoxide (DMSO) while fluconazole was dissolved in sterile distilled water. The dilutions were prepared in 0.03 to 32 µg/ml. Sterile test tubes were used for drug dilutions and sterile, disposable, multi-well microdilution plates (96 U-shaped wells) were utilized for susceptibility assays. The final required drug concentration was achieved with RPMI media. Drugs were diluted in tryptic soy broth (TSB) for mould testing, giving distinct endpoints and RPMI 1640 for yeasts. 200µl of broth containing the drug were dispensed in the test wells and drug free media was dispensed in the positive and negative control wells. A 0.5 McFarland of inoculums were prepared from pure cultures of the test fungi and 10 µl were inoculated from the lowest to highest drug concentration on the micro-titer wells. The micro-titer wells were incubated at 35^{o} C for yeast testing and 30 ^{o} C for moulds and thereafter the MICs were read at 48 hours. *Aspergillus flavus* ATCC®204304 and *Candida parapsilosis*-ATCC® 22019 strains were included for quality control based on their defined minimum inhibitory concentrations (MICs). The end point was determined as the lowest concentration that prevented visible growth for amphotericin B, voriconazole, and itraconazole while the MIC for fluconazole was determined as the lowest concentration corresponding to 50% reduction in turbidity compared to the control well.

3.7.4 Molecular characterization of fungal isolates

3.7.4.1 Fungal DNA isolation

Selected *Aspergillus* spp isolates from the study were sent for sequencing at Chiba University, School of Medicine, Centre for Mycology Research in Japan under the custody of Professor

Gonoi. Genomic DNA extraction from the *Aspergillus* spp isolated from sputum was performed using a standard DNA extraction kit (QIAamp® DNA Mini Kit) (Qiagen, Hilden, Germany) according to manufacturer's protocols. Briefly isolates were grown on sabouraud dextrose agar (SDA) plates at 30°C for 48 hours and thereafter, the plate surface was flooded with 10 ml fungal saline (0.9% w/v NaCl) to harvest conidia. 1 ml saline suspension containing 1–5 x 106 conidia (determined photometrically at A530) was prepared and centrifuged after which the pellet was collected and processed further by lyticase digestion (Sigma Aldrich), centrifugation and elution of DNA by buffer.

3.7.4.2 Sequencing and analysis

Coding sequences and promoter of calmodulin (*CaM*) and beta tubulin (*BtU*) genes were amplified and sequenced in both directions using respective universal primers (Table 3.1) by Sanger sequencing (Samson *et al.,* 2014). The sequences were cleaned and alignment was performed using Jalview version 2.11.1.4 (http://www.jalview.org/). Basic local alignment search tool (BLAST) analysis was performed to confirm identity of isolates in comparison with reference species in the National Centre for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) database.

Table 3.1 Primers and annealing temperatures used for amplification and sequencing

Gene locus	Amplification	Annealing temp (°C)	Cycles	Primer	Direction	Primer sequence (5'-3')
Beta tubulin (*β*-tubulin)	Standard	55 (alt 52)	35	*BtU*	Forward	GACTCGCTACTAGGCCAACGG
				BtU	Reverse	AGTCCGGTGCTGGTAACAAC
Calmodulin (*CaM*)	Standard	55 (alt 52)	35	*CaM*	Forward	CCGAGTACAAGGAGGCCTTC
				CaM	Reverse	CCACGTTATGACCTCCATCGG

Source (Samson *et al.,* 2014)

3.8.1 Data analysis and management

Demographic data such as age and sex of patients, were captured on Ms. Excel. The software was also used to generate comparative graphs of from the data. Statistical analysis was done using STATA version 13. Analysis of variance (ANOVA) was used to compare the frequency of different fungal species and their antifungal susceptibility profiles. Multivariate and univariate analysis was performed to determine the relationship of variables with the positivity of yeasts or moulds as possible predictors. P-values <0.05 were considered statistically significant. Antifungal susceptibility data (MICs) were interpreted according the CLSI reference standards for yeast and moulds (ClSI, 2009; CLSI, 2008). Sequence data analysis and alignment was

performed by BLAST against reference sequences available at home library in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm *Aspergillus* spp by calmodulin (*CaM*) and beta tubulin (*BtU*) genes. Partial coding sequences 15 isolates were cleaned and submitted to the NCBI database through Bankit (https://www.ncbi.nlm.nih.gov/WebSub/) after which they were verified and allocated accession numbers (Tables 4.3.1 & 4.3.2).

Phylogenetic analysis was performed to determine the relationship of *Aspergillus* spp isolates from sputum with isolates in GenBank using one click mode in phylogeny.fr (http://www.phylogeny.fr/simple_phylogeny.cgi) (Dereeper *et al*., 2008). Pairwise identity matches of sequences from the study were also done using clustal omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) in comparison to sequences from GenBank. See Tables 4.3.1 & 4.3.2.

3.8.2 Quality control

Pre-analytical and analytical processes in the study were checked to ensure quality and reliability of results. Sterile sputum mugs were used for colklection of specimen and proper guidance was give to the participants on the appropriate sample collection procedure. Expiry dates of all reagents and the test antifungals were checked and lot to lot testing was performed to ascertain reproducibility of results. Delay of samples before and during transport was monitored and adequate measures were taken to ensure sample integrity. Samples that were not processed immediately were refrigerated at 2-8^{o}C. Aseptic technique was adhered to during sample processing and QC for culture media was performed by inoculation of standard American Type Culture Collection (ATCC) strains. Sterility testing was performed on all prepared media to prevent false positive cultures by incubating un-inoculated culture plates. As part of QC for antifungal broth micro-dilutions testing, ATCC strains of both yeast and mould were included concurrently in each set of test testing on microtitre well plates.

3.9 Ethics consideration

The study was nested in the ongoing research titled "*Mapping antifungal, antibacterial drug resistance and the quality of antimicrobial agents against fungal and bacterial pathogens from smear-negative and retreatment cases in high TB prevalence Counties in Kenya*". (KEMRI/SERU/0037/3213). The protocol was further reviewed and approved by Kenyatta National Hospital-University of Nairobi Ethics Review Committee (KNH-UoN ERC number P108/02/2018). Written informed consent was done for participants and personal information

was handled with the utmost confidentiality. Participants were provided with adequate information (Appendix 6 & 8) after which they were asked to sign the consent form (Appendix 7 & 9) if they agreed to participate in the study. Once a participant consented, a sputum sample was collected appropriately. The samples were recorded with unique numbers to conceal identifying information of patients. However, results were relayed back to the respective facility for further management. Further, data was kept in secure password protected devices to avoid unauthorized access. All analytical processes were carried out at KEMRI- Centre for Microbiology Research in compliance with institutional research policies.

CHAPTER FOUR

The spectrum of filamentous fungi from sputum of tuberculosis relapse and retreatment patients in tuberculosis reference facilities

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4.1 Abstract

Fungal infections represent a significant cause of morbidity and mortality among immunocompromised individuals. Pulmonary fungal infection may be missed or misdiagnosed as tuberculosis (TB) hence complicating the management of these patients. The current study reports the spectrum of filamentous fungi isolated from sputum of TB relapse and retreatment cases at selected reference facilities in Kenya. Methods: A total of 340 sputum samples collected from June 2018 to June 2019 were subjected to mycological investigations. The samples were mucolysed, inoculated on sabouraud dextrose agar (SDA), incubated at 30˚C for 7 days, and checked daily for fungal growth. Macroscopic and microscopic morphological features identified moulds and the species were confirmed by sequencing. The diversity of fungi out of the 340 sputum samples analyzed was as follows; 16% (n = 53) were positive for moulds with Aspergillus species being the predominant constituting 68 % (n = 36). Among the Aspergilli, *A. flavus* and *A. niger* were the most frequently isolated adding up to 23%, (n = 12) and 15% (n = 8) respectively. Additionally, *Paecillomyces variotii* (9%, n = 5), *Scedosporium aspiospermum* (6%, n = 3), *Mucor racemosus* (8%, n = 4) and *Penicillium* spp. (9%, n = 5) were also recovered. The isolated fungi represented potential respiratory pathogens responsible for persistent TB like symptoms despite treatment that could be misdiagnosed as relapse requiring treatment. Fungal investigation of all presumptive TB relapse cases should be advocated before treatment. This will reduce unnecessary retreatment, delayed antifungal intervention and unwarranted morbidity and mortality associated with misdiagnosis.

Keywords: Filamentous Fungi, Tuberculosis, Relapse, Retreatment, Spectrum

4.2 Introduction

Fungi are emerging significant pathogens mainly as an opportunist in individuals with impaired immunity; particularly in people living with HIV, diabetes, cancer, and those treated with toxic chemotherapy (Bongomin *et al*., 2017). Certain species of fungi are also capable of invading and causing disease in immunocompetent individuals. The increase in fungal infections has been exacerbated by the escalation of immune debilitating conditions such as HIV (Bansod & Rai, 2008b). Tuberculosis (TB) remains a significant public health threat in Kenya and the country is listed among the 30 high burden countries (WHO, 2015). A prevalence survey carried out in 2016 confirmed that Kenya's TB burden is higher than previously estimated. The highest burden was recorded in young men of the reproductive age group 25-34 years (Ministry of Health, 2016). Fungal spores being ubiquitous, can colonize pre-existing TB cavities in the lungs. Tuberculosis retreatment cases respond poorly to anti-TB medication and it is thought that there exists a synergistic growth relationship between *Mycobacterium tuberculosis* and fungal respiratory pathogens among other co-morbidities (Marais *et al*., 2013). The most common respiratory fungal diseases include; candidiasis, aspergillosis, cryptococcosis and mucormycosis (Yahaya et al., 2015).

The WHO estimates that more than a million people develop chronic pulmonary aspergillosis after being treated for TB every year (WHO, 2011). Majority of the cases occur in countries with high burden of TB including Bangladesh, China, India, Indonesia, Philippines and sub-Sahara Africa. Due to fungal diagnostic challenges, almost half of those who develop the disease die within five years (WHO, 2011). The symptoms of chronic pulmonary aspergillosis (CPA) i.e. weight loss, severe shortness of breath, fatigue and coughing up blood are similar to those of TB that cannot be differentiated clinically. This often leads to misdiagnosis and subsequent inappropriate treatment. Fungal infection can progress undetected for years until it is too late for antifungal intervention (Limper et al., 2011).

Around one-third of TB patients develop cavities in their lungs, making them vulnerable to infection by air-borne fungi ubiquitous in the environment (Denning *et al*., 2016;David W, 2011). Studies focusing on the global burden of chronic pulmonary aspergillosis have highlighted the importance of following up on TB patients after their initial treatment, and having the right diagnosis available (Suman & Kumar, 2016). Chronic pulmonary aspergillosis (CPA) requires long-term antifungal therapy and surgical intervention. The mycological investigation is required to diagnose the disease early to increase the chance of survival (Kalyani *et al*., 2016).

The National Tuberculosis Leprosy and Lung Disease Program (NTLD-P) in Kenya seeks to reduce mortality and morbidity due to chronic lung diseases while promoting lung health in the general population. Currently, there is an increased mortality among tuberculosis patients including those who have completed treatment, due to development of cavitation and bronchiectasis that favors the colonization of filamentous fungi (Dhooria *et al*., 2014). Therefore, undiagnosed or missed diagnosis of respiratory fungal infection significantly increases the morbidity and mortality despite successful TB treatment (Agarwal *et al*., 2014). There is limited empirical evidence on the existence and extent of fungal respiratory diseases and possible co-morbidities with pulmonary TB in Kenya. In light of this inadequate information, the current study sought to isolate and characterize pulmonary fungal pathogens from TB relapse and retreatment patients in Kenya, a sub-Sahara Africa setting.

4.3 Materials and Methods

4.3.1 Sampling sites

Sputum samples were obtained from 340 patients in three reference facilities in the current cross-sectional study: Moi Teaching and Referral Hospital (MTRH), Mbagathi County Referral Hospital, and the National Reference Laboratory. These facilities serve as referral centers for the management of tuberculosis in the country. The study was nested in the protocol approved by KEMRI Scientific and Ethics Review Unit (SERU) in which patients provided written informed consent to participate in the study. Ethics approval for the study was granted by Kenyatta National Hospital/University of Nairobi Ethics Review Committee (KNH/UoN-ERC) (study number P108/02/2018).

4.3.2 Sample size and participant selection criteria

Tuberculosis relapse and retreatment cases with recurrent symptoms consistent with pulmonary TB despite compliance with first-line anti TB therapy were included in the study. Relapse and retreatment cases with known HIV/TB co-infection were also recruited. Tuberculosis treatment defaulters, newly diagnosed smear-positive TB and patients with extensively drug resistant tuberculosis (XDR-TB) and extra-pulmonary TB were excluded from the study. The sample size was determined based on the country estimated burden (67%) of chronic pulmonary aspergillosis (CPA) among pulmonary tuberculosis patients (Guto, Bii, & Denning, 2016). Fischer's *et al*. (1998) formula with a 95% confidence level was used, where a minimum of 340 participants was worked out for the study as indicated below.

$$n = \frac{z^2_{1-\alpha/2}\, P\,(1-P)}{d2}$$

Where: n= Sample size

$Z^2_{1-\alpha/2} = 1.96$

p= 67% (estimated prevalence of CPA in PTB patients)

d= Absolute precision (0.05)

Therefore: $N = \frac{1.96^2 \times 0.67\,(1-0.67)}{0.052} = 340$

4.3.3 Sputum sample processing

Fresh expectorated morning sputum specimen were collected in sterile sputum mugs and packaged using the triple packaging technique following the requisite biosafety guidelines (Heifets, 2014). The samples were transported to Kenya Medical Research Institute (KEMRI), Centre for Microbiology Research-Mycology Laboratory. Coded samples were first mucolysed with sputasol and inoculated on sabouraud dextrose agar (SDA) in duplicates. The media was supplemented with 10 ml of Chloramphenicol and 0.65 ml of Gentamicin (40 mg/ml) to inhibit bacterial contamination. Sterility check and ability to support the growth fungi was performed on each batch of media and subsequent negative and positive controls were incubated with every batch of cultures. Inoculated plates were incubated at 30^{o}C for 4 weeks and evaluated daily for fungal growth. Plates that had no growth at the fourth week were deemed negative and disposed appropriately following the laboratory waste disposal procedures.

4.3.4 Identification of fungal isolates

Fungi growing on culture were identified by morphologic characteristics using macroscopic and microscopic features. Macroscopic features such as colony growth rate, color on the obverse and reverse, elevation, texture and colony size were captured. Specimen for microscopy were prepared using tease-mount technique and stained with lacto-phenol cotton blue as described by (Colin Campbell, Elizabeth M. Johnson, 2013). Microscopic features were observed at X40 magnification and features including conidia production, shapes and arrangement of phialides were noted to aid in identification of the isolates. The isolates were confirmed using the Calmodulin and beta tubulin gene sequence. This was achieved courtesy of support from Medical Mycology Research Institute-Chiba University-Japan. Manipulation of all live cultures was done under level II containment facilities in compliance with the Mycology Laboratory safety standards and KEMRI biosafety guidelines.

4.3.5 Data analysis

The isolation frequency (*Fq*) of individual mould species was recorded and expressed as percentage of the total number of moulds isolated from the sputum samples according to Gonzalez *et al* (1999). Pictures of representative fungal species on culture and microscopy were also captured to demonstrate their morphological diversity.

$$Fq\ (\%) = \frac{\text{No. of colonies of fungal species x 100}}{\text{Total No. of colonies of all species}}$$

Univariate analysis was performed using STATA version 13 by multiple regression to determine the relationship between HIV status and positivity of sputum for mould.

4.4 Results

4.4.1 Characteristics of participants

A total of 340 participants were enrolled into the study. The median age of the participants was 39 years. The patients constituted 206 male and 134 female participants. All the participants had at least one clinical syndrome consistent with presumptive pulmonary TB i.e cough, chest pain and difficulty in breathing with a history of treatment for tuberculosis.

4.4.2 Filamentous fungal isolates

Diverse filamentous fungi were recovered from sputum samples of the study participants. Out of the 340 sputa analyzed, 16% (*n*=53) were positive for pathogenic filamentous fungi. The genus *Aspergillus* were the most frequently isolated accounting for 68 % (*n*=36) of the isolates with *Aspergillus flavus and A. niger* the being most frequently isolated species constituting 23%, (*n*=12) and 15% (*n*=8) respectively. Only 4% (*n*=2) isolates of *Aspergillus candidus* were isolated. Other *Aspergilli* present in the samples were *A. fumigatus*, *A. terreus* and *A. vesicolor.* Additionally*, Paecillomyces variotii* (9%, *n*=5), *Scedosporium aspiospermum* (6%, *n*=3)*, Mucor racemosus* (8%, *n*=4) and *Penicillium* spp (9%, *n*=5) were isolated from the sputum. The frequency, macroscopic and microscopic morphological characteristics of the representative fungi isolated are described in Table 4.1 and subsequently in Plates 4.1 (a) to 4.1 (j).

Name of Isolate and reference plate	Colony morphology (obverse)	Colony morphology (reverse)	Structure of phialides	Frequency *n* (%)
Plate 4.1 (b). *Aspergillus flavus*	Light green powdery colony	Cream-yellow	Biseriate phialides and aerial hyphae with rough, spiny conidiophores	12 (23%)
Plate 4.1 (d). *Aspergillus niger*	Black with white margin	Cream-white	Biseriate phialides covering entire vesicle forming radiate head	8 (15%)
Plate 4.1 (a). *Aspergillus fumigatus*	Velvety, white, dark greenish	Cream white	Uniseriate phialides covering upper two thirds of vesicle.	6 (11%)
Plate 4.1 (j). *Mucor racemosus*	White powdery colony	Cream white	Aerial hyphae bearing conidiophores	4 (8%)
Plate 4.1 (e). *Aspergillus terreus*	Velvety, cinnamon brown	Whitish brown	Compact fan-shaped heads with metulae, phialides, and upright conidiophore	5 (9%)
Plate 4.1 (f). *Aspergillus candidus*	White	Orange yellow	Intact sporing head with metulae and phialides, over the entire vesicle	2 (4%)
Plate 4.1 (g). *Penicillium spp*	Bluish green, white margin, exudates present, raised with radial furrows	Creamish yellow	Branching, flask shaped conidiophores with metulae	5 (9%)
Plate 4.1 (i). *Paecilomyces variotii*	Yellowish brown	Pale brown	Long slender phialides appearing singly along hyphae	5 (9%)
Plate 4.1 (c). *Aspergillus vesicolor*	Greenish yellow	Orange yellow	Biseriate, loosely radiate phialides covering entire vesicle	3 (6%)
Plate 4.1 (h) *Scedosporium aspiospermum*	White cottony aerial mycelia	Greyish black	Septate hyphae with short conidiophores bearing condia singly	3 (6%)
Total number of isolates				**53 (16%)**

Table 4.1: The frequency, macroscopic and microscopic features of moulds from sputum

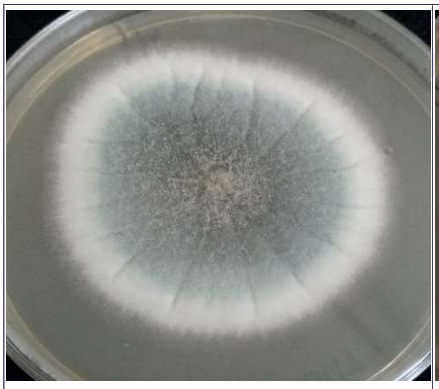
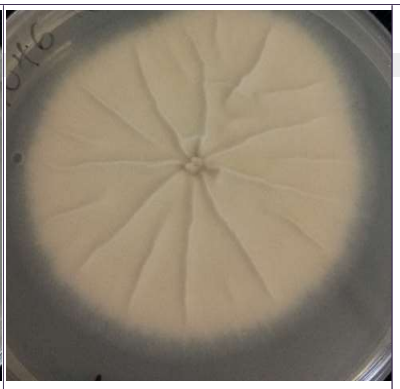


Plate 4.1 (a). *Aspergillus fumigatus* (i) colony surface



(ii). Reverse

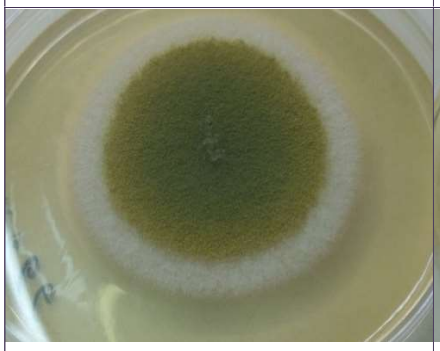
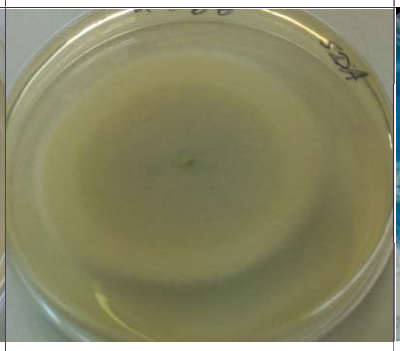
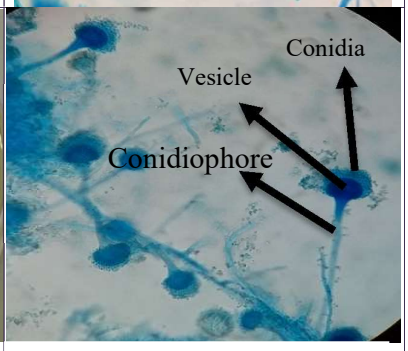


Plate 4.1 (b). *Aspergillus flavus* (i) Colony surface



(ii). Reverse



(iii) Microscopic features

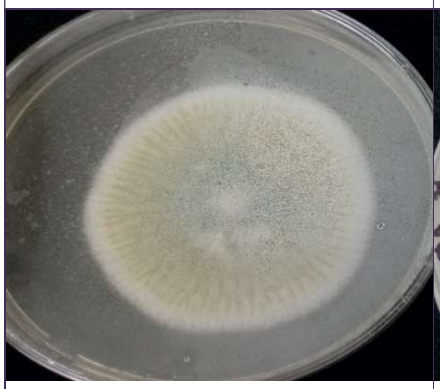
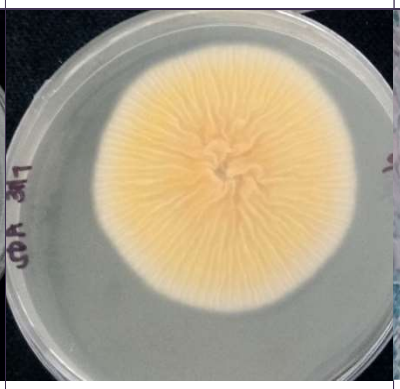
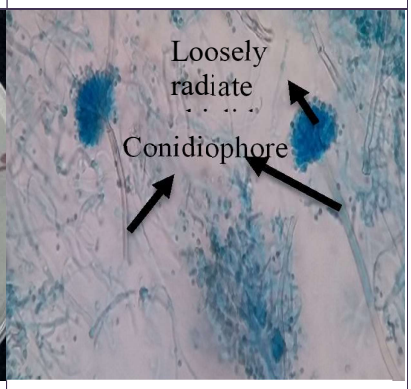


Plate 4.1 (c). *Aspergillus vesicolor* (i) Colony surface



(ii). Reverse



(iii) Microscopic features

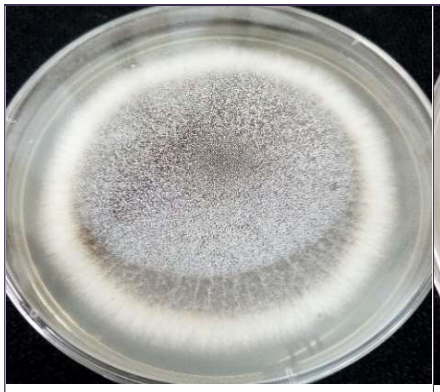
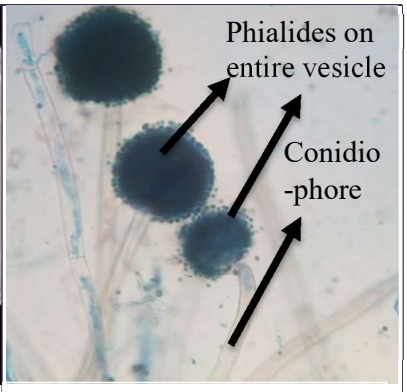


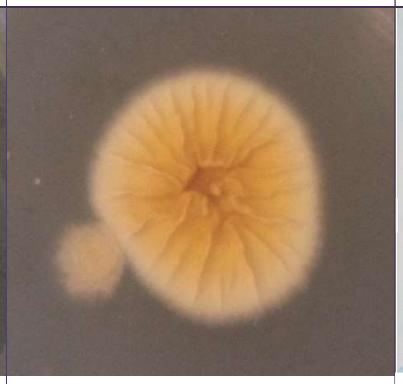
Plate 4.1 (d). *Aspergillus niger* (i) Colony surface

(ii) Colony reverse

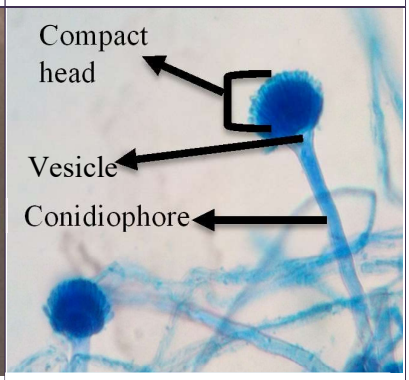


(iii) Microscopic features

Plate 4.1 (e). *Aspergillus terreus* (i) (Colony obverse)

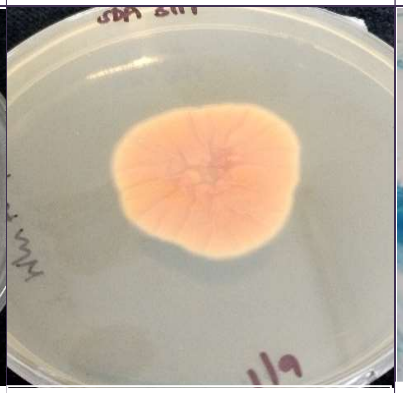


(ii) Reverse

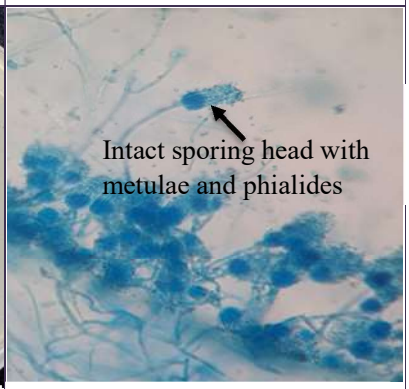


(iii) Microscopic features

Plate 4.1 (f). *Aspergillus candidus* (i) Floccose colony surface



(ii) Reverse



(iii) Microscopic features

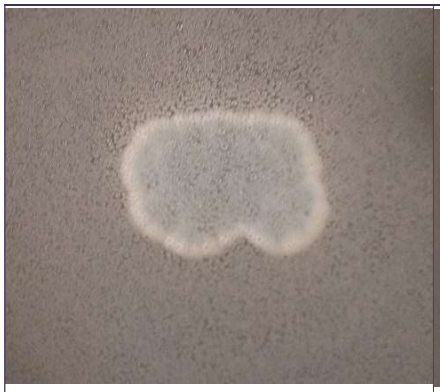
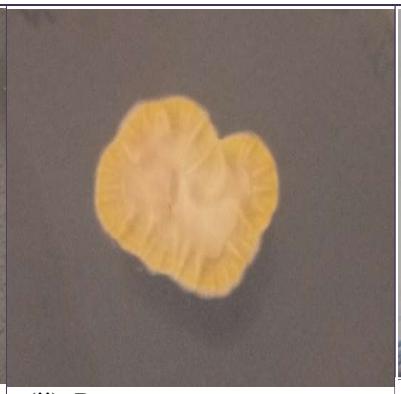
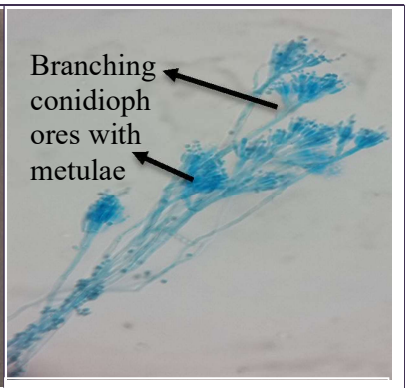


Plate 4.1 (g). *Penicillium spp* (SDA) (i). Colony surface



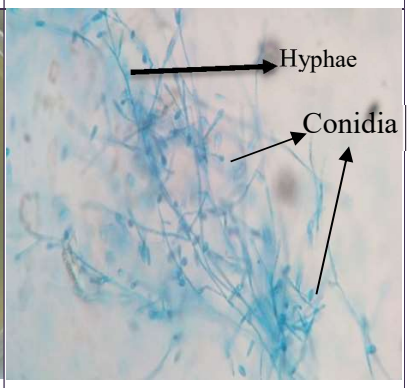
(ii). Reverse



(iii) Microscopic features

Plate 4.1 (h). *Scedosporium aspiospermum* (i) Colony surface

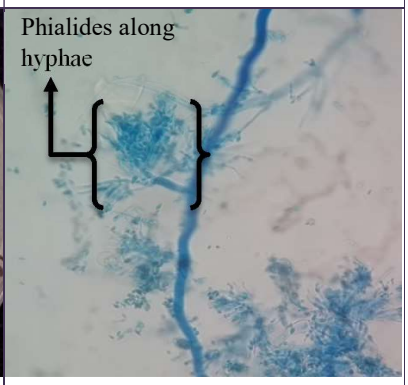
(ii) Reverse



(iii) Microscopic features

Plate 4.1 (i). *Paecilomyces variotii* (i) Colony surface

(ii) Reverse



(iii) Microscopic features

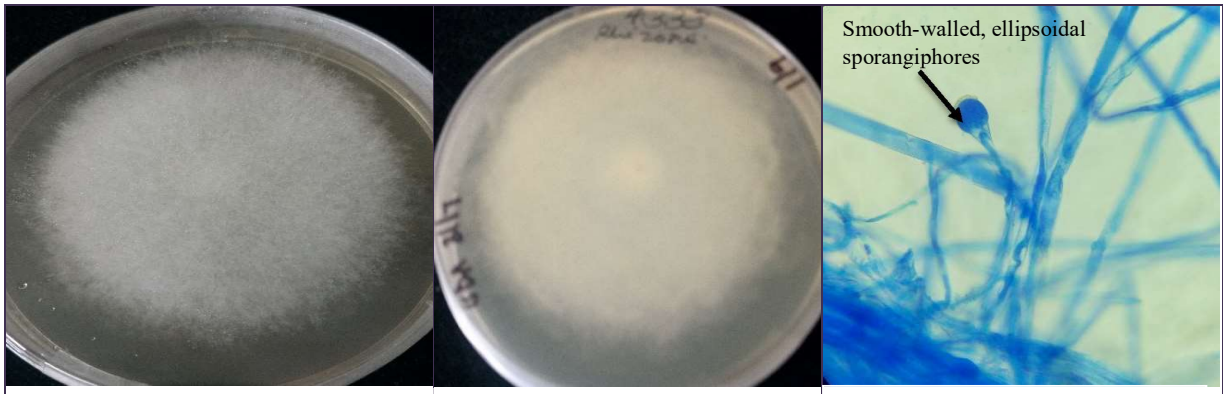


Plate 4.1 (j). *Mucor racemosus* (i) Colony surface-aerial mycelia	(ii) Reverse	(iii) Microscopic features

4.4.3 Distribution of mould isolates by HIV status of patients

About twenty-six percent (26.4%, n=14) of patients positive for moulds were female while majority (73.6%, n=39) were male. Regarding the distribution of mould isolates from sputum by HIV status, majority of some isolates were recovered from HIV positive participants. Approximately thirteen percent (13.2%) of *Aspergillus flavus* were frequently isolated from HIV positive patients compared to 9.4% in HIV negative (Table 4.2.2). Equally, *A. fumigatus* were frequent (9.4%) among the HIV positive compared to 1.9% in HIV negative. *A. candidus* (3.7%) were only isolated from HIV negative patients. Nevertheless, the HIV status of 15.1% of patients positive for moulds were unknown, majority from whom *A. terreus* (5.6%) were isolated (Table 4.2). Based on univariate analysis, there was a statistically significant positive correlation (R=0.4906) between positivity of sputum for moulds and HIV status of the patients (p=0.047).

	HIV Status			
Mould species	**ND**	**Neg**	**Pos**	**Total**
Aspergillus candidus	0	2	0	2
	0.0%	3.7%	0.0%	3.7%
Aspergillus flavus	0	5	7	12
	0.0%	9.4%	13.2%	22.6%
Aspergillus fumigatus	0	1	5	6
	0.0%	1.9%	9.4%	11.3%
Aspergillus niger	2	3	3	8
	3.7%	5.7%	5.7%	15.1%
Aspergillus terreus	3	2	0	5
	5.6%	3.7%	0.0%	9.4%
Aspergillus vesicolor	1	1	1	3
	1.9%	1.9%	1.9%	5.7%
Mucor racemosus	1	0	3	4
	1.9%	0.0%	5.7%	7.6%
Paecilomyces variotii	1	2	2	5
	1.9%	3.7%	3.7%	9.4%

Table 4.2.1: Distribution of mould isolates from sputum by HIV status of patients

Penicillium spp	0 0.0%	2 3.7%	3 5.7%	5 9.4
Scedosporium aspiospermum	0 0.0%	2 3.7%	1 1.9%	3 5.7%
Total	**8** **15.1%**	**20** **37.7%**	**25** **47.2%**	**53** **100.0**
R=0.49, 95% CI {0.7164-1.2836} p=0.047				
Key: ND-not done, Pos-positive, Neg-negative				

Table 4.2.2 shows the distribution of mould isolates obtained sputum by gender of the patients. Majority of the participants with sputum positive for moulds were male (73.58%, n=39), while female participants constituted (26.42%, n=14). The mould isolates were disproportionately prevalent in males ($p<0.05$, $p=0.039$) compared to female patients. *A. terreus* species were exceptionally frequent in females (7.55%) compared to males (1.89%).

	GENDER		
Mould species	**Female**	**Male**	**Total**
Aspergillus candidus	0	2	2
	0.00%	3.77%	3.77 %
Aspergillus flavus	3	9	12
	5.66%	16.98%	22.64 %
Aspergillus fumigatus	1	5	6
	1.89%	9.43%	11.32%
Aspergillus niger	3	5	8
	5.66%	9.43%	15.09 %
Aspergillus terreus	4	1	5
	7.55%	1.89%	9.43 %
Aspergillus vesicolor	1	2	3
	1.89%	3.77%	5.66 %
Mucor racemosus	1	3	4
	1.89%	5.66%	7.55 %
Paecilomyces variotii	1	4	5
	1.89%	7.55%	9.43 %
Penicillium	2	0	2
	0.00%	3.77%	3.77 %
Penicillium spp	0	3	3
	0.00%	5.66%	5.66 %
Scedosporium aspiospermum	0	3	3
	0.00%	5.66%	5.66 %
Total	**14**	**39**	**53**
	26.42%	**73.58**%	**100.00**%
R=0.61, 95% CI {6.8336-8.7335} p=0.039			

Table 4.2.2: Relationship between gender and sputum positivity for moulds

4.5 Discussion

Opportunistic fungi have emerged as significant pathogens in immunocompromised individuals (Ravikant, 2015). The frequency of infections is generally on the rise and appropriate strategies are needed to avert the problem. The current study examined sputa samples from TB relapse and retreatment patients presenting with clinical symptoms typical of pulmonary TB. Of the 340 samples, 16% (n=53) were positive for various moulds implicated in invasive infections and other fungal-related illnesses.

The genus *Aspergillus* were the most frequent filamentous fungi recovered. Members of this group form spores which become airborne and are acquired through inhalation (Ohba *et al*., 2012). There are over 200 species of *Aspergilli,* but a few are associated with clinical disease. *Aspergillus flavus* and *A. niger* were the most dominant species followed by *A. fumigatus, A. terreus*, *A. versicolor* and *A. candidus*. Individuals with a debilitated immune system such as those with leukaemia, severe asthma, cystic fibrosis, HIV/AIDS and people with lung injuries including those who have had tuberculosis are at highest risk of invasive infection and disease (Taccone *et al.*, 2015). The mould isolates were frequently isolated from sputum of male (73.58%) participants compared to females (26.42%). This demonstrates that males are more likely to be affected by pulmonary fungal infection compared to females. These findings are consistent with observations in other studies that have also reported a higher prevalence of pulmonary fungal infection males than females (Bitew & Bati, 2021) .

Pulmonary fungal infections such CPA, ABPA or other fungal etiologies in the lung, can present with clinical symptoms mimicking pulmonary TB hence warranting adequate differential diagnosis (Denning *et al*., 2016). Various diagnostic approaches for invasive aspergillosis have been established and it is mainly suggested that detecting aspergillus galactomannan antigen (GM-Ag) in serum is the most reliable non-invasive technique for detection of active infection (Taghavi *et al*., 2015). The positivity rate of sputum samples is known to be relatively low in patients with CPA despite a high number of *Aspergillus* spp in the lung cavities (Denning *et al.,* 2018). Therefore, the fungal burden of patients in the current study could be higher. However, the findings paint clear picture of the existing situation among individuals presenting with lung conditions clinically thought to be TB. The results depict the significance of unattended pulmonary fungal colonisation and may determine disease progression and outcome of TB management efforts. A study conducted in Uganda revealed significantly elevated serum aspergillus GM antigen in HIV infected patients with TB, suggesting the possible TB complication by *Aspergillus* infection (Kwizera *et al*., 2017).

Other studies have demonstrated the existence of *Aspergillus* spp in respiratory specimen in patients presumed to be infected with TB. In Cameroon, a study carried out to determine the prevalence of respiratory tract aspergillosis in sputum of patients suspected of pulmonary TB, 30 (15%) of *Aspergillus* spp were isolated from 200 sputa samples and a co-infection rate with TB of 9 (4.5%) was observed among the cases (Anna, *et al*., 2012). Literature search of all epidemiology papers that reported fungal infections in Kenya revealed that about 7% of the Kenyan population suffers from a significant fungal infection at any one time. Further, chronic pulmonary aspergillosis (CPA) following TB was reported at a prevalence of 10,848 cases (32/100,000). The reported adult asthma prevalence was 3.1% and it was assumed that 2.5% had ABPA, implying that 17,696 (44/100,000) individuals were affected (Guto *et al*., 2016). The high concentration of fungal spores both in indoor and outdoor environments, particularly the *Aspergillus* spp acts as a source of most infections. Immunosuppression is the significant risk factor for fungal infection, determining the outcome and severity (Richardson *et al*., 2019).

Individuals who are culture positive for diverse *Aspergillus* species have the highest risk of IA, ABPA, as well as bronchitis *(Gago et al*., 2019). Participants in the current study presented with various clinical symptoms such frequent chest pain, tightness, fatigue, weight loss, productive cough and labored breathing among other clinical signs. However, these symptoms are nonspecific and patients with other lung etiologies and tuberculosis may present with the same, making it indistinguishable clinically (Bongomin *et al*., 2017). In Kenya, there is a well-established program for tuberculosis and lung diseases. Nevertheless, considerable efforts and resources have been focused on diagnosing and managing tuberculosis with minimal attention to possible pulmonary fungal diseases. Therefore, such cases may be missed in the patient diagnosis and management phases.

The present study identified *Paecillomyces variotii* from the sputum specimens. This species represents an important emerging opportunistic pathogen among the immunocompromised individuals (Houbraken *et al*., 2010). The fungus has been previously recovered in food products, soil, and indoor home environments (Taekhee *et al,* 2008). Furthermore, *P. variotii* has been implicated in pulmonary mycoses in diabetics and cases of endocarditis, sphenoid infections and other related diseases (Abolghasemi & Tabarsi, 2015; Goldschmidt & Victor, 2016; Robert *et al*., 2015). The presence of *P. variotii* in TB relapse and retreatment cases highlights possible missed diagnosis or co-infection complicating the treatment of the affected individuals.

Penicillium spp represents a large group of ubiquitous genus consisting over 350 recognized species (Visagie *et al.,* 2014). Members of this group are mainly responsible for the contamination of food products and mycotoxin production. Opportunistic infections attributable to *Penicillium* spp have also been reported, mainly entailing prosthetic endocarditis, otomycosis, and pulmonary involvement in immunocompromised individuals (Lyratzopoulos *et al*., 2002). It may be that the genus is a significant pathogen in pulmonary TB. It would be necessary to do further in-depth inquiry to elaborate the clinical relevance and prognosis.

Another mould species isolated from the sputum samples included *Scedosporium aspiospermum*. The fungus *S. aspiospermum* is a ubiquitous filamentous mould found in soil and polluted water (Nucci *et al*., 2009). It has been implicated in a wide variety of severe infections including; lungs, sinuses, bone and disseminated disease involving the central nervous system among immunocompromised individuals and those receiving steroidal medication with consequent mortality (Goldman *et al*., 2016). Infection with *S. aspiospermum* has also been reported in immunocompetent individuals signifying the virulence of this species. In the spectrum of infections associated with *S. aspiospermum*, lung infection is fairly overriding and often progressing in patients with prevailing lung conditions and surgical resection is main intervention since *S. aspiospermum* is intrinsically resistant to various antifungals. A greater survival rate in immunocompetent individuals has been noted compared to the immunocompromised (Agatha *et al*., 2014). Although rare*, S. aspiospermum* and zygomycete infections do occur and are associated with a mortality rate exceeding 60% (Cooley *et al*., 2007). It would be essential to evaluate presumptive TB relapse and retreatment cases for possible fungal infection or co-infection. This should primarily be investigated in HIV/AIDS infected individuals presenting with severe pulmonary symptoms.

Mucor racemosus, also recovered from the sputum is a dimorphic species belonging to a large group of fungi collectively named *Mucormycetes* and are responsible for a group of infections referred to as mucormycosis (Marisa *et al*., 2011). There are frequent human infections caused by *Rhizopus, Absidia* and *Mucor*, and *Chlamydoconidia* species. Mucor is the most common, usually acquired through inhalation (Panigrahi *et al*., 2014). The spectrum of disease associated with mucormycosis includes rhino-cerebral affecting the sinuses and the brain with ensuing fever, sinus congestion and headache. Pulmonary mucormycosis mainly infects the lung resulting in chest pain, breathing problems, cough and fever with disseminated infections (Hassan & Voigt, 2019). Since lung infections with Mucorales present a similar clinical picture as TB or pneumonia, this could be misdiagnosed, and the patient put on anti-TB medication as the

infection progresses. Mucormycosis has also been considered a neglected tropical disease (NTD) (Queiroz-Telles *et al.,* 2017).

Many pulmonary TB patients currently on treatment were solely diagnosed clinically or are considered smear negative. These individuals could likely have other etiologies such as pulmonary fungal etiologies. According to Denning (2018), the WHO reported that approximately 2.2 million out of 5.2 million new cases of pulmonary TB were clinically diagnosed or smear-negative. It has also been observed that there is a low specificity of presumptive TB suggestive X-ray hence the diagnosis is often inconclusive. The utility of computed tomography (CT) has been evaluated for diagnosis of pulmonary infection by filamentous fungi in the immunocompromised. In one of the studies, CT-guided lung biopsies showed high diagnostic accuracy comparable to antigen-based tests and molecular assays (Lass-Flörl *et al.,* 2017).

CHAPTER FIVE

The spectrum of pulmonary yeasts from sputum of tuberculosis relapse and retreatment patients

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5.1 Abstract

The global burden of fungal infections is increasing significantly**.** It is estimated that over 1.5 million people die of fungal diseases and over 1 billion are affected. This study sought to characterize yeast isolates from tuberculosis relapse and retreatment patients from sputum**.** The study utilized a cross-sectional study design. 340 patients were enrolled for the study after obtaining their written informed consent. Participants were instructed to produce sputum samples packaged appropriately and transported to Kenya Medical Research (KEMRI) Mycology Laboratory. The sputa were inoculated on Sabouraud Dextrose Agar (SDA) and incubated at 30^{O}C. Yeasts were preliminarily identified by morphological features on culture and gram stain and subsequently inoculated on CHROMagar^{TM} Candida and cornmeal tween 80 agar. The isolates were further confirmed using API 20C AUX profile. Out of 340 sputa examined, 14% (n=49) were positive for yeast. The most predominant isolate was *Candida albicans* and *C. krusei* constituting 49% (n=24) and 21% (n=10) respectively. Other isolates were *Candida famata* (4%, n=2), *C. zeylanoides* (4%, n=2), *C. lusitaniae* (2%, n=1), C. *guilliermondii* (2%, n=1), *C. parapsilosis* (2%, n=1) and *Cryptococcus neoformans* (2%, n=1). A significant positive correlation (*r=0.5826, p=0.0420*, 95% CI 0.2911-0.7099) between HIV positive cases and positivity of yeast cultures was noted. The study demonstrates possible infection of presumptive TB retreatment patients by yeasts that could be treated clinically as relapse. Non albicans *Candida* represents a major threat in these patients due to possible antifungal resistance and increased virulence associated with these species.

Keywords: Yeast, Tuberculosis, Retreatment, Relapse, Pulmonary

5.2 Introduction

Opportunistic fungal diseases have emerged as significant causes of morbidity and mortality in immunocompromised individuals (Gupte, 2015). It is estimated that there are over 300 million people who are acutely or chronically infected by fungi often resulting to death, prolonged illness, psychological problems, blindness and reduced work capacity, while the estimated collective mortality attributed to fungal diseases stands at 1.6 million annually (LIFE, 2017). The rising burden of fungal infections is primarily linked to the emergence of immune debilitating conditions such as HIV/AIDS and cancer and invasive medical procedures and the use of immunosuppressive chemotherapy (Limper et al., 2011). Tuberculosis (TB) is among the top ten killer diseases and it is a major public health concern globally. It is estimated that 10 million people develop TB disease each year and approximately 1.7 Billion are infected with *Mycobacterium tuberculosis* worldwide, with up to 90% of the cases in 30 high burden countries (WHO 2019b).

There is a rising concern of possible coinfection of patients suspected of pulmonary tuberculosis with fungal pathogens that is often missed or misdiagnosed (Miller & Wilmott, 2018). In 2018, the number of TB case notification in Kenya was 96, 478 and only 48% of these cases were bacteriologically confirmed (WHO 2019a). The clinical presentation (signs and symptoms) of pulmonary fungal infection can be non- specific and therefore not easily distinguishable from other pulmonary conditions such TB. Several filamentous fungi and yeasts have been associated with severe pulmonary infection in individuals with impaired immunity as well as the immunocompetent (Pfaller & Diekema, 2007). The most common and virulent fungal pathogens include *Aspergillus* species, *Candida albicans* and *Cryptococcus neoformans* associated with significant mortality. *Candida albicans* is part of the human microflora but can cause invasive systemic infection in immunocompromised persons with a mortality rate reaching 40% (Xie *et al*., 2014). *Cryptococcus neoformans* is responsible for more than 600,000 deaths and over 1 million cases of meningitis in HIV infected individuals mainly in sub-Saharan Africa (Bongomin *et al*., 2017). *Cryptococcus* infection is one of the AIDS defining illness and it is mainly acquired through the respiratory route by inhalation and subsequent dissemination to the meninges.

Aspergillus species are widely distributed in the environment with *A. fumigatus* being the most frequent cause of invasive aspergillosis (IA) and the mortality can reach 94%. *Aspergillus* spores are small and when inhaled, they are deposited in the lungs and can reach the alveolus where they germinate if unchecked by the local immune defenses in the lung epithelium (Gago et al., 2019). Studies have demonstrated that synergistic growth of pulmonary tuberculosis and fungi such as

Aspergillus spp, *Cryptococcus* spp and *Candida* spp is fatal contributing to the overall mortality of infected patients (Fang *et al*., 2017). The current study sought to isolate and characterize yeasts from sputum of tuberculosis relapse and retreatment patients in Kenya a sub Sahara Africa setting.

5.3 Materials and methods

5.3.1 Study design and participant recruitment

The study employed a cross-sectional study design. A total of 340 participants from three selected facilities namely Moi Teaching and Referral Hospital, the National Reference Laboratory and Mbagathi Hospital who were above 18 years of age were recruited into the study. The patients were recruited through a consenting process. All the patients had a history of treatment for tuberculosis and they were presenting with clinical symptoms such as cough, chest pain, and extreme fatigue.

5.3.2 Ethical considerations

The study protocol was reviewed and approved by Kenyatta Hospital -University of Nairobi Ethics Review Committee (KNH-UoN-ERC) (study number: P108/02/2018) and Kenya Medical Research Medical Research Institute Scientific and Ethics Review Unit (SERU) (study number: KEMRI/SERU/0037/3213). The study was carried out according to the approved protocol and participants were informed on the purpose of the study and consent was sought before recruitment. Patient information was kept confidential and samples were assigned unique numbers to safeguard identifying information. The results were communicated to the respective clinicians for further patient management.

5.3.3 Sample collection

The participants were instructed to collect the sample onto a well labelled sputum mug. Each participant was asked to produce a single sputum sample (at least >2ml) which were then transported promptly to Kenya Medical Research Institute, Center for Microbiology Research-Mycology Laboratory for mycological investigations.

5.3.4 Sample processing and isolation of yeasts

The details of each expectorated sputum sample received at the laboratory were recorded accordingly and the quality and volume were assessed. The sample was subjected to sputasol (Thermo-Scientific™) for 10 minutes to homogenize. Primary isolation was done by inoculation of 100 µl on sabourauds dextrose agar (SDA) supplemented with chloramphenicol (16µg/ml) and gentamicin (4µg/ml) in duplicates. The inoculated plates were sealed with parafilm and incubated

at 35^{O}C. The cultures were examined after 48 hours and incubation was continued for a maximum of 4 days for cultures with no growth.

5.3.5 Identification of yeast isolates

Yeasts were preliminarily identified based on morphological characteristics on primary SDA culture and confirmed by gram stain. The isolates were subsequently subcultured on CHROMagar^{TM} Candida (bioMérieux®, Durham) medium prepared according to the manufacturer's instructions. Isolates were also subjected to API 20C AUX^{TM} (bioMérieux®) identification profile based on carbohydrate utilization. Chlamydospore formation was also used to differentiate *Candida albicans* and other *Candida* species further. The isolates were inoculated onto corn meal agar plates (Dalmau inoculation technique) containing 1% Tween 80 and theater incubated at 30^{O}C at high humidity for 24 to 72 hours. Thereafter, the cultures were examined under the microscope through the cover slip. *Cryptococcus* species was identified based on India Ink used to demonstrate the prominent polysaccharide capsule.

5.3.6 Data analysis

Data was captured and analyzed using excel spreadsheets and STATA version 13. Summaries of the frequency of yeast isolates were generated along with demographic information. Univariate analysis was performed by regression analysis to determine the relationship between HIV positivity of the retreatment patients with the positivity of sputum culture for yeasts.

5.4 Results

5.4.1: Distribution of yeast isolates from sputum

Out of the 340 sputa samples tested, 14% (*n*=49) were positive for yeasts. *Candida albicans* and *C. krusei* were the most predominant species constituting 49% (*n*=24) and 21% (*n*=10) of the total yeast isolates respectively. Other species included *Candida tropicalis* 14.5% (*n*=7), *C. famata* (*n*=2), *C. zeylanoids* (*n*=2), *C. lusitaniae* (*n*=1), *C. guilliermondii* (*n*=1), *C. parapsilosis* (*n*=1) and *Cryptococcus neoformans* (*n*=1). The isolates exhibited various morphological features on Cornmeal agar-tween 80 and characteristic colony pigmentation on CHROMagar^{TM} Candida used as the basis for identification. The identities of isolates were also confirmed by API 20C AUX (bioMérieux®). Descriptions of different characteristics of the yeast isolates are shown in Table 5.1 with representative culture plates in Plates 5.1 (a), 5.1 (b) and 5.1 (c) and microscopic features in Figure 5.1.

Table 5.1: Characteristics of yeast isolates on Corn meal-tween 80 agar, CHROMagarTM

Yeast species	Morphological features on Cornmeal agar-tween 80	Colony features on CHROMagar^{TM} Candida	Identification by API 20C AUX	No. of isolates *n* (%)
Candida albicans	Presence of elongated pseudohyphae with blastoconidia appearing in grape-like clusters at the septa. Chlamydospores are produced at the terminal end of the hyphae.	Green	Identified as *C. albicans*	24 (49%)
Candida krusei	Presence of long, straight cells exhibiting tree-like branching appearance and blastoconidia in chains resembling a "crossed matchsticks" arrangement.	Pink	Identified as *C. krusei*	10 (21%)
Candida tropicalis	Presence of abundant pseudohyphae composed of elongated cells. Blastoconidia appear in small groups along mycelia showing a typical "pine forest arrangement"	Dark blue	Identified as *C. tropicalis*	7 (14%)
Candida famata	Pseudohyphae are absent.	White to pink	Identified as *C. famata*	2 (4%)
Candida zeylanoides	Presence of long curved pseudohyphae. Blastoconidia in clusters or single are seen.	Bluish-green	Identified as *C. zeylanoides*	2 (4%)
Candida lusitaniae	Presence of ovoid (egg-shaped) yeast cells appearing in chains and pairs and production branching pseudohyphae.	White to ivory	Identified as *C. lusitaniae*	1 (2%)
Candida guilliermondii	Presence of sparse short pseudohyphae and round blastoconidia in clusters.	Purple	Identified as *C. guilliermondii*	1 (2%)
Candida parapsilosis	Presence of abundant thin long branching pseudohyphae. Blastospores appear along the large pseudomycelia.	Pale pink	Identified as *C. parapsilosis*	1 (%)
Cryptococcus neoformans	Not done	Gray	Identified as *C. neoformans*	1 (%)
Total number of isolates				**N=49 (14%)**

Candida and identification by API 20C AUX

Plate 5.1 (a) (SDA). Primary sputum culture with mucoid yeast typical of *Cryptococcus* spp. after 72-hour incubation at 35ºC yeast colonies.

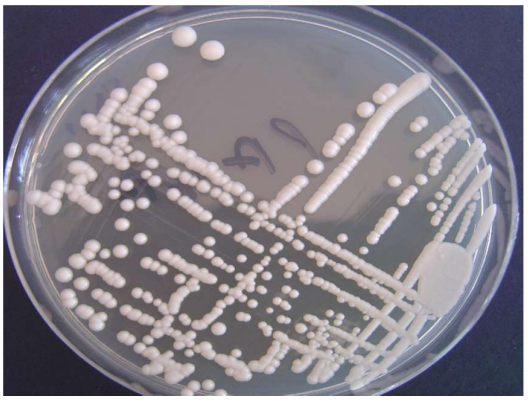

Plate 5.1 (b) (SDA). Primary sputum culture with single, white cream, dry yeast colonies presumptively identified as *Candida albicans*.

Plates 5.1 (c) (A&B). CHROM™ agar Candida for selective and presumptive identification of Candida species. *C. albicans* (green) *C. tropicalis*- blue, *C. krusei*- dark pink rough colonies. Other *Candida* spp (white)

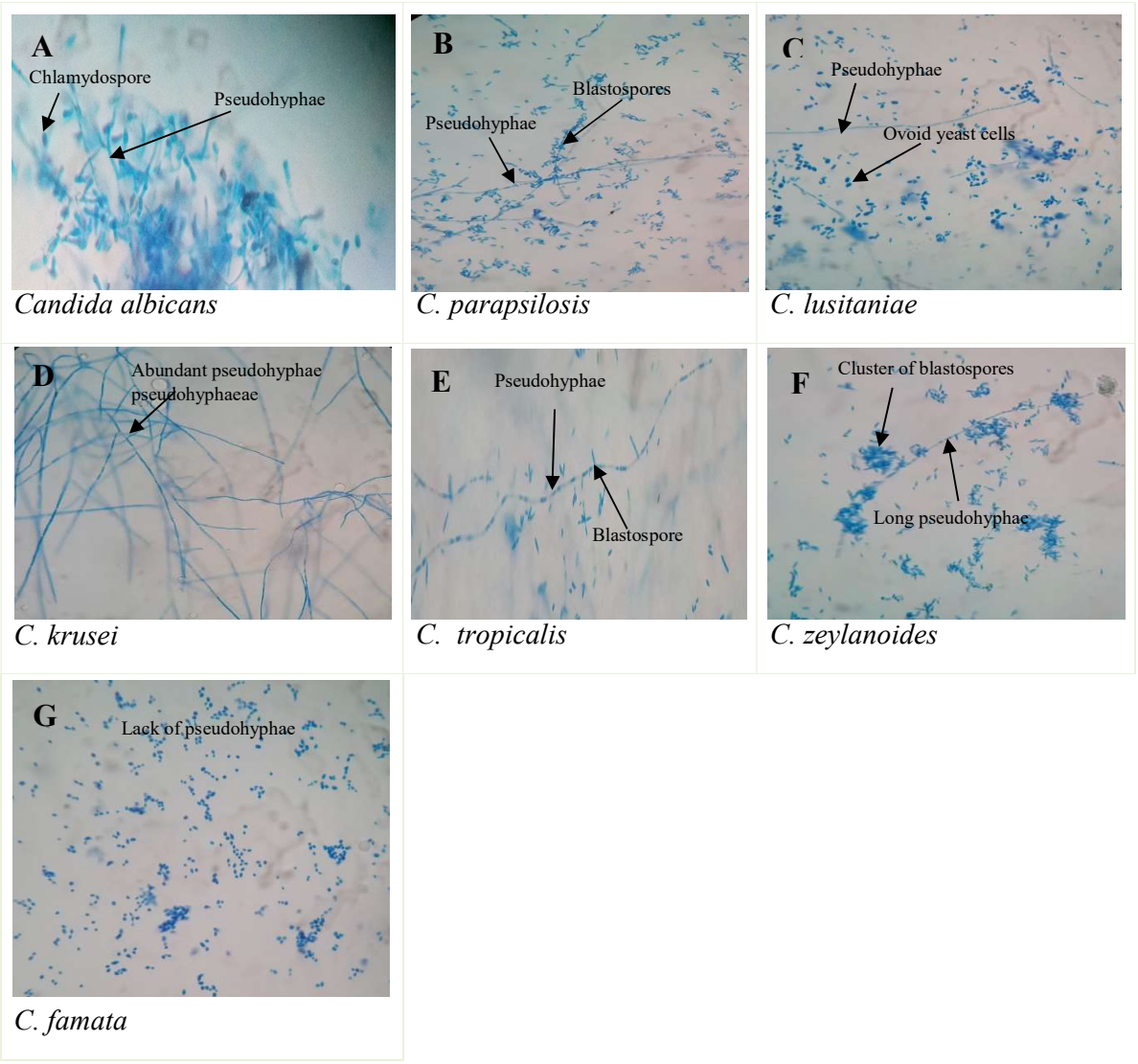

Figure 5.1: Morphological features of representative yeast isolates. A to G shows the microscopic features on cornmeal agar tween 80.

5.4.2 Distribution of yeast isolates from sputum by HIV status of patients

Thirty-eight percent 38.8% (n=19) of patients that were positive for yeasts, were female while 61.2% (n=30) were male. The distribution of yeast isolates from sputum according to HIV status showed that majority were from HIV positive patients (table 4.2.4). Twenty-six percent of *Candida albicans* (26.5%) were from HIV positive participants compared to 18.4% from HIV negative. Similarly, *C. krusei* isolates were frequent (10.20%) in HIV positive patients with 8.16% among the HIV negative. *Cryptococcus neoformans* (2.04%) was isolated from a HIV positive patient. However, the HIV status of 12.24% of participants positive for yeasts were unknown (Table 5.4.2). According to univariate analysis, a positive correlation (R=0.336)

between HIV status of the patient and positivity for yeast in sputum was observed. However, this was not statistically significant (p=0.1817>0.05).

Table 5.2: Distribution of yeast isolates by HIV status of patients

	HIV Status			
Yeast species	**ND**	**Neg**	**Pos**	
Candida lusitiniae	0 0.00%	1 2.04%	0 0.00%	1 2.04%
C. famata	1 2.04%	0 0.0%	1 2.04%	2 4.08%
C. parapsilosis	1 2.04%	0 0.00%	0 0.00%	1 2.04%
C. albicans	2 4.08%	9 18.4%	13 26.53%	24 48.98%
C. guilliermondii	0 0.00%	0 0.00%	1 2.04%	1 2.04%
C. krusei	1 2.04%	4 8.16%	5 10.20%	10 20.41
C. tropicalis	1 2.04%	3 6.12%	3 6.12%	7 14.29%
Cryptococcus neoformans	0 0.00%	0 0.00	1 2.04%	1 2.04%
Candida zeylanoides	0 0.00%	0 0.00	2 4.08%	2 4.08%
Total	**6** **12.24%**	**17** **34.69%**	**26** **53.06%**	**49** **100.00%**
R=0.336, 95% CI {0.2332-0.3536} p=0.1817				
Key: ND-not done, Pos-positive, Neg-negative				

Majority of patients whose sputum samples were positive for yeasts were male (61.22%, n=30), while female participants constituted (38.78%, n=19). *Candida albicans* were more frequent in males (28.57%, n=14) compared to female (20.14%, n=10) patients. A positive correlation between gender and positivity for yeasts was observed. However this was not significant (p=0.074 >0.05,

	Gender		
Yeast species	**Female**	**Male**	**Total**
C. Famata	1	0	1
	2.04%	0.00%	2.04%
C. Lusitiniae	0	1	1
	0.00%	2.04%	2.04%
C. famata	0	1	1
	0.00%	2.04%	2.04%
C. parapsilosis	0	1	1
	0.00%	2.04%	2.04%
C .albicans	10	14	24
	20.41%	28.57%	48.98%
C. guilliermondii	1	0	1
	2.04%	0.00%	2.04%
C .krusei	3	7	10
	6.12%	14.29%	20.41%

Table 5.3: Frequency of yeasts obtained from sputum based on gender of participants

C .tropicalis	3	4	7
	6.12%	8.16%	14.29%
C. neoformans	0	1	1
	0.00%	2.04%	2.04%
C. zeylanoides	1	1	2
	2.04%	2.04%	4.08%
Total	**19**	**30**	**49**
	38.78%	**61.22**%	**100.00**%
R=0.48, p= 0.074; 95% CI {4.8333-6.7335}			

5.5 Discussion

A variety of yeast species were isolated from sputum samples in the current study. *Candida albicans*, *C. krusei* and *C. tropicalis* were the most predominant consisting 49%, 21% and 14%, respectively. Other studies have also highlighted *C. albicans* as the most common yeast isolated in patients with pulmonary tuberculosis. In Nigeria, a study reported a high prevalence of *C. krusei* (9.3%) and *C. albicans* (7.3%) in 300 patients suspected of pulmonary tuberculosis (Yahaya *et al*., 2014). Another study reported *Candida* co-infection rate of 50% in TB smear positive patients with *C. albicans* co-infection of 40%, C. *tropicalis* (20%) and *C. glabrata* (20%). The mean age of patients with *Candida* co-infection and those without co-infection had no significant difference (Kali *et al*., 2013). One systematic review reported *Candida* co-infection with PTB of 25.7% with *C. albicans* being attributed to a pooled prevalence of 65.8% (Hadadi *et al*., 2020). A similar trend was observed in this study with *C. albicans* beign the most frequent. Literature has also shown that *C. tropicalis* in particular has a greater potential to cause invasive and more severe disease (Astekar *et al*., 2016). Other non albicans *Candida* recovered were *C. zeylanoides* (4%), *C. lusitaniae* (2%), *C. guilliermondii (2%)* and *C. parapsilosis* (2%). These species are seen as emerging and new pathogens in patients with other lung pathologies and immunosuppression and are associated with significant mortality and antifungal drug resistance (Ziesing *et al*., 2016).

Candida albicans is known to exist as a normal human commensal in 10% of healthy individuals but it has been recovered in over 50% of patients with PTB (Astekar *et al*., 2016). Evidence suggests that microbial interactions occur in disease often promoting colonization and increasing virulence of pathogenic organisms. For instance, in patients with cystic fibrosis, *Pseudomonas aeruginosa* is consistently detected in individuals with *Aspergillus fumigatus* infection or frequent *C. albicans* colonization than in cases without these fungi in brochoalveolar lavage cultures (Van Dijck & Jabra-Rizk, 2019). A synergistic interaction between *Mycobacterium tuberculosis* and *C. albicans* has been observed where *M. tuberculosis* is seen to facilitate the growth of *C. albicans* hence aggravating TB and the associated symptoms (Astekar *et al.*, 2016). Sequencing techniques have demonstrated that the lung mycobiome contributes immensely to the impact of chronic respiratory diseases (Nguyen *et al*., 2015). However the interplay with the immune system and other existing pulmonary conditions plays a major role (Nguyen *et al*., 2015; Richardson *et al*., 2019).

Cryptococcus neoformans was recovered from one sputum sample in the present study. The organism is widely distributed in the environment mainly in soils and areas contaminated with avian droppings. It is usually acquired through inhalation of propagules into the lungs from where it disseminates to the brain via the blood stream leading to severe cryptococcal meningitis (Li et al., 2019). The disease affects more than 1 million individuals living with HIV globally and is often the cause of death. *C. neoformans* has been isolated previously in patients with PTB and it is mainly considered an emerging pathogen in this category of patients (Bansod & Rai, 2008a; Li et al., 2019; Sethi et al., 2015; Shouyong Tan, Haobin Kuang, 2017). Its presence from sputum suggests possible active infection or colonization.

Yeasts recovered from sputum in the study could complicate management the retreatment patients. *C. albicans*, the most predominant (49%) species could be attributed to majority of complications in pulmonary infections and may need special consideration during clinical interventions in such pastients. However other non albicans *Candida* spp were isolated and perhaps they may be of great clinical significance in the patient's condition. Findings suggest that male are more frequenty affected compared to females. However, this was not statistically significant $p>0.05$. Evidence shows that yeasts could complicate the diagnsosis treatment and management TB patients. It is therefore essential to carryout sufficient clninical and laboratory evaluation of presumptive TB patients for proper management and to increase the number of bacteriologically confirmed TB cases before treatement initiation.

CHAPTER SIX

In vitro antifungal susceptibility of yeasts and moulds isolated from sputum of tuberculosis relapse and retreatment patients

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6.1 Abstract

Opportunistic fungal infections due to immunosuppression coupled with antifungal drug resistance is an emerging challenge globally. The present study examined the antifungal susceptibility of yeasts and molds from sputum of tuberculosis retreatment and relapse patients at selected reference facilities in Kenya. 340 sputa samples from patients who gave written informed consent were examined. Fungal culture was done on sabouraud dextrose agar (SDA). Macroscopic and microscopic features identified moulds while yeasts were inoculated on CHROM^{TM} agar *Candida* and confirmed using API 20C AUX^{TM}. Itraconazole (ICZ), voriconazole (VCZ), fluconazole (FCZ) and amphotericin B (AMB) were tested using broth micro-dilution methods according to Clinical and Laboratory Standards Institute (CLSI). Out of the 340 samples, 14.4% (n=49) and 15.6% (n=53) were positive for yeasts and molds respectively. *Candida albicans* and *C. krusei* were the most predominant isolates constituting 49.0% (n=24) and 20.4% (n=10) of the total yeasts respectively. *Aspergillus* spp were the most frequent (22.6%) molds and isolates with MICs ≥4µg/ml on the antifungal agents were noted. All the molds except two (n=2) isolates of *Scedosporium aspiospermum* exhibited MICs >4µg/ml for fluconazole. Overall, molds were more sensitive to AMB and VCZ. *C. albicans* had MIC_{50} <0.06µg/ml, and MIC_{90}<4µg/ml. There was a statistically significant difference (F=3.7, P=0.004<0.05) in the overall sensitivity pattern of moulds for the four antifungal agents while there was no significant difference (F=1.7, P=0.154>0.05) in sensitivity exhibited by the yeasts. The study demonstrates the significance of fungal colonization in presumptive tuberculosis retreatments or relapse with evidence of triazole resistance. There is need to strengthen fungal diagnostic and antifungal sensitivity testing for proper clinical management of susceptible populations.

Keywords: Yeast, molds, antifungals, susceptibility, tuberculosis, retreatment

6.2 Introduction

Fungal diseases constitute a substantial cause of morbidity and mortality in humans and animals (Bongomin et al., 2017). Recently, cases of superficial and systemic fungal infections have escalated with a possibility of reaching epidemic magnitude (Denning, 2011). The most affected members of society are the immunocompromised and those undergoing invasive medical procedures and infants (Miller & Wilmott, 2018). Current knowledge depicts an increase in pulmonary mycoses as a co-morbidity or a sequelae of TB that frequently leads to mortality (Jabbari *et al.,* 2015). Lung infection with fungi mainly occurs following inhalation of airborne fungal propagules ubiquitous in the environment (Chew *et al*., 2003). Numerous yeasts and moulds have been implicated in pulmonary pathologies and the clinical features and severity of disease varies from asymptomatic in otherwise healthy individuals to overt symptoms such as cough, dyspnea, chest pain, and bronchiectasis (Ohba *et al*., 2012). The symptoms exhibited are usually non-specific and consistent with other pulmonary conditions and infections. Fungal species of great importance that have been found clinically in pulmonary cases include moulds of the *Aspergillus* spp such as *A. fumigatus A. flavus* and *A. niger* while yeasts comprise *Cryptococcus* spp mainly *C. neoformans*, *Candida* spp majorly *C. albicans and C. tropicalis.* Other significant pathogens include *Mucor* spp *and Histoplasma capsulatum* (K. Chen *et al*., 2001).

Fungal pathogens have evolved substantially over the years exhibiting greater and increased resistance to the few existing antifungal agents (Sanguinetti & Posteraro, 2018). The trend is worrisome to clinicians and patients as well as microbiologists and other researchers alike in an effort to understand this phenomenon (Laxminarayan *et al*., 2013). A greater challenge in diagnosing and managing pulmonary fungal infections exists in developing countries with resource constraints, scarcity of quality medicine, and poor health care systems (Denning *et al*., 2018). There are different classes of antifungal medications with varying clinical spectrum and mode of action. Four main antifungals are currently in clinical use namely azoles, allylamines, polyenes and echinocandins.

Additionally, a diverse group of other compounds exist, such as flucytosine and griseofulvin (Yamauchi, 2018). The development of a new antifungal regimen has been hampered by several factors, particularly limited selective toxicity of candidate compounds and primary and secondary resistance (A. Espinel-Ingroff, 2009). Fungi are eukaryotic organisms sharing substantial structural features with mammalian cells, making it difficult to target the fungal cells without significant harm to the host. In contrast, prokaryotes mainly bacteria offer a wide variety of

metabolic and structural components that can be targeted selectively thus a wide range of antibiotics are available (Xie *et al*., 2014). Antifungal drugs exert their activity on various biosynthetic processes within the cell or interfere with ergosterol a vital component of the fungal cell membrane leading to loss of integrity and consequential death. Mechanisms of resistance that have been described encompass alteration of drug targets, altered drug uptake or efflux, and lack of microbial enzymes to metabolize the drug to an active form (Sanglard, 2016). Screening methods to determine antifungals' in vitro and in vivo efficacy are still undergoing standardization and micro-dilution techniques are the gold standard. However significant progress has been achieved for specific fungal pathogens such *Candida* spp and drugs (Sanguinetti & Posteraro, 2018). It is essential to understand the sensitivity of a fungal etiological agent implicated in an infectious process to select appropriate treatment. In view of the impending antifungal resistance, the current study reports the response of pulmonary yeasts and moulds from sputum of tuberculosis retreatment and relapse patients to three azole antifungals, precisely fluconazole (FCZ), voriconazole (VCZ) and itraconazole (ICZ), and the polyene-amphotericin B (AMB).

6.3 Materials and Methods

6.3.1 Study samples

The current study was cross-sectional and laboratory investigations were carried out at Kenya Medical Research Institute (KEMRI), Center for Microbiology Research-Mycology Unit. Expectorated sputum samples were obtained from tuberculosis retreatment and relapse cases presenting with clinical symptoms typical of pulmonary tuberculosis and a history of tuberculosis treatment. The participants were recruited from three reference facilities in Kenya namely Moi Teaching and Referral Hospital, Mbagathi Hospital and the National TB Reference Laboratory and all were above 18 years of age. The samples were transported promptly to the mycology laboratory according to the standard operating procedures for collecting, biosafety, and shipment infectious material. The study was reviewed and approved by Kenyatta National Hospital/University of Nairobi Ethics Review Committee (KNH/UoN-ERC) (study number P108/02/2018). Written consent was obtained for all patients, and personal information was handled with the utmost confidentiality.

6.3.2 Isolation and identification of fungi

Sputum samples were digested with sputasol and subsequently inoculated in duplicate on sabouraud dextrose agar (SDA) supplemented with 0.65 ml gentamicin (40 mg/ml) and 10 ml of chloramphenicol. The cultures were incubated at 30^{o}C and 35^{o}C and monitored for fungal growth

for up to four weeks. Mould isolates were identified by morphological colony growth characteristics such as topography, color on the reverse and obverse, and microscopic features on lactophenol cotton blue mount. Yeasts were subcultured on CHROMagar™ *Candida* for presumptive identification by morphologic characteristics such as color, size of colonies and shape of the cells. India Ink was performed to differentiate *Cryptococcus neoformans* based on the presence of a capsule as per the available standard operating procedures. Confirmation of the identity of yeasts was carried out using Analytical Profile Index (API 20 C AUX-bioMerieux Durham, USA).

6.3.3 Antifungal susceptibility testing

Antifungal susceptibility was performed using broth micro-dilution techniques according to Clinical and Laboratory Standards Institute guidelines (CLSI), M27-A3 and M38-A2 respectively. The antifungal drugs tested were; voriconazole, itraconazole, fluconazole and amphotericin B. Voriconazole, itraconazole and amphotericin B were diluted in analytical grade dimethyl sulfoxide (DMSO) while fluconazole was dissolved in sterile distilled water. The dilutions were prepared in 0.03 to 32 µg/ml. Sterile test tubes were used for drug dilutions and sterile disposable multi-well microdilution plates (96 U-shaped wells) were utilized for susceptibility assays. The final required drug concentrations were achieved with RPMI media. Drugs were diluted in tryptic soy broth (TSB) for mold testing, giving distinct endpoints and RPMI 1640 for yeasts. 200µl of broth containing the drug were dispensed in the test wells and drug free media was dispensed in the positive and negative control wells. A 0.5 McFarland of inoculums were prepared from pure cultures of the test fungi and 10 µl were inoculated from the lowest to highest drug concentration on the micro-titer wells. The micro-titer wells were incubated at 35^{o} C for yeast testing and 30 ^{o} C for molds and after that the MICs were read at 48 hours. *Aspergillus flavus* ATCC®204304 and *Candida parapsilosis*-ATCC® 22019 strains were included for quality control based on their defined minimum inhibitory concentrations (MICs). The end point was determined as the lowest concentration that prevented visible growth for amphotericin B, voriconazole, and itraconazole while the MIC for fluconazole was determined as the lowest concentration corresponding to 50% reduction in turbidity compared to the control well.

6.4 Results

6.4.1 Antifungal susceptibility results for filamentous fungi

The *Aspergillus flavus* and *A. niger* were the most frequent moulds recovered from the samples making up 23%, (n=12) and 15% (n=8) respectively. Some moulds exhibited MICs ≥4µg/ml considered resistant for azoles. See Table 6.1. For Amphotericin B (AMB), 41.6% (n=5) of *A. flavus* had MICs ≥4µg/ml, while 12.5% (n=1) of *A. niger,* 100% (n=3) *of A. versicolor* and 40% (n=2) of *Penicilium spp* showed similar MIC results for AMB (Table 6.1). Similarly, mould isolates exhibiting high MICs for Itraconazole were identified and *A. flavus* (25%, n=3), *A. versicolor* (33%, n=1), *A. candidus* (50%, n=1), and *Penicillium* spp (20%, n=1) had MIC ≥4µg/ml. Likewise, isolates with high MICs to voriconazole were noted mainly *A. terreus* (20%, n=1), *Mucor racemosus* (75%, n=3), *Paecillomyces variotii* (40%, n=2) and *Penicillium* spp (60%, n=3). All the moulds except two (n=2) isolates of *Scedosporium aspiospermum* exhibited MICs higher than >4µg/ml for fluconazole (Table 6.1). Overall, the moulds showed greater sensitivity to Voriconazole and Amphotericin B (Figure 6.1). Most of the moulds had MIC_{50} >32 µg/ml for Fluconazole. The MIC_{50} and MIC_{90} are also shown in Table 6.1. Varying sensitivity of the mould isolates to the four antifungal agents at high and low drug concentrations on micro-titer well plates are shown in Figure 6.1 and Figure 6.2.

Table 6.1: Distribution of MICs for filamentous fungal isolates from sputum

Antifungal agent and isolate	No. of isolates with respective MICs (µg/ml) n=53 (48 hours)												
Amphotericin B	**<0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**>32**	**Total**	**MIC_{50}**	**MIC_{90}**
Aspergillus flavus	1	-	2	-	2	2	1	1	2	1	12	1	8
Aspergillus niger	1	-	0	-	2	4	1	-	-		8	2	2
Aspergillus vesicolor	-	-	-	-	-	-		1	-	2	3	8	>32
Aspergillus terreus	-	-	-	-	-	-	1	1	1	2	5	16	>32
Aspergillus candidus	-	-	-	-	-	-	1	1	-	-	2	4	8
Aspergillus fumigatus	1	1	1	-	-	1	1	-	-	-	5	0.25	2
Mucor racemosus	2	-	-	-	-	2	-	-	-	-	4	2	2
Paecilomyces variotii	-	-	1	3	1	-	-	-	-	-	5	0.5	0.5
Scedosporium aspiospermum	3	-	-	-	-	-	-	-	-	-	3	<0.06	<0.06
Penicillium spp	1	-	1	1	0	-	-	-	1	1	5	0.5	16
Aspergillus flavus (ATCC® 204304)	-	-	-	-	1	-	-	-	-	-	1	1	1
Itraconazole	**<0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**>32**	**Total**	**MIC_{50}**	**MIC_{90}**
Aspergillus flavus	3	2	-	1	3	-	2	-	1	-	12	0.5	1
Aspergillus niger	1	1	1	1	2	2	-	-	-	-	8	0.5	2
Aspergillus vesicolor	-	-	-	-	2	-	-	1	-	-	3	1	8
Aspergillus terreus	-	-	-	2	3	-	-	-	-	-	5	0.5	1
Aspergillus candidus	1	-	-	-	-	-	-	1	-	-	2	<0.06	8
Aspergillus fumigatus	1	-	-	1	1	1	1	-	-	-	5	1	2
Mucor racemosus	-	-	-	2	-	2	-	-	-	-	4	0.5	2
Paecilomyces variotii	4	-	-	-	1	-	-	-	-	-	5	<0.06	<0.06
Scedosporium aspiospermum	3	-	-	-	-	-	-	-	-	-	3	<0.06	<0.06
Penicillium spp	-	1	-	-	1	2	1	-	-	-	5	2	2
Aspergillus flavus (ATCC® 204304)	-	-	1	-	-	-	-	-	-	-	1	0.25	0.25
Voriconazole	**<0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**>32**	**Total**	**MIC_{50}**	**MIC_{90}**
Aspergillus flavus	4	2	-	-	2	4	-	-	-	-	12	<0.12	2
Aspergillus niger	-	-	-	2	5	1	-	-	-	-	8	1	1
Aspergillus vesicolor	-	-	-	-	2	1	-	-	-	-	3	1	2
Aspergillus terreus	-	-	-	-	2	2	1	-	-	-	5	2	2
Aspergillus candidus	-	-	-	1	-	1	-	-	-	-	2	0.5	2
Aspergillus fumigatus	1	-	1	1	2	-	-	-	-	-	5	0.5	1
Mucor racemosus	-	-	-	-	-	1	-	1	2	-	4	8	16
Paecilomyces variotii	1	-	-	1	-	1	1	-	-	1	5	2	4
Scedosporium aspiospermum	3	-	-	-	-	-	-	-	-	-	3	<0.06	<0.06
Penicillium spp.	-	-	1	-	1	-	1	1	-	1	5	4	8
Aspergillus flavus (ATCC® 204304)	-	-	-	-	-	1	-	-	-	-	1	2	2
Fluconazole	**<0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**>32**	**Total**	**MIC_{50}**	**MIC_{90}**
Aspergillus flavus	-		-	-	-	-		2	1	9	12	>32	>32
Aspergillus niger	-	-	-	-	-	-	-	-	1	7	8	>32	>32
Aspergillus vesicolor	-	-	-	-	-	-	-	-	-	3	3	>32	>32
Aspergillus terreus	-	-	-	-	-	-	-	1	-	4	5	>32	>32
Aspergillus candidus	-	-	-	-	-	-	-	-	-	2	2	>32	>32
Aspergillus fumigatus	-	-	-	-	-	-	-	-	-	5	5	>32	>32
Mucor racemosus	-	-	-	-	-	-	-	-	-	4	4	>32	>32
Paecilomyces variotii	-	-	-	-	-	-	-	-	-	5	5	>32	>32
Scedosporium aspiospermum	-	-	-	2	-	-	-	1	-	-	3	0.5	8
Penicillium spp	-	-	-	-	-	-	-	-	-	5	5	>32	>32
Aspergillus flavus (ATCC®204304)	-	-	-	-	-	-	-	-	1	-	1	16	16

Quality control strain *Aspergillus flavus* ATCC®204304 MIC range AMB (0.5-4), ICZ (0.25-0.5), VCZ (0.5-4), MIC_{50}-drug concentration that inhibited 50% of isolates, MIC_{90}- drug concentration that inhibited 90% of isolates.

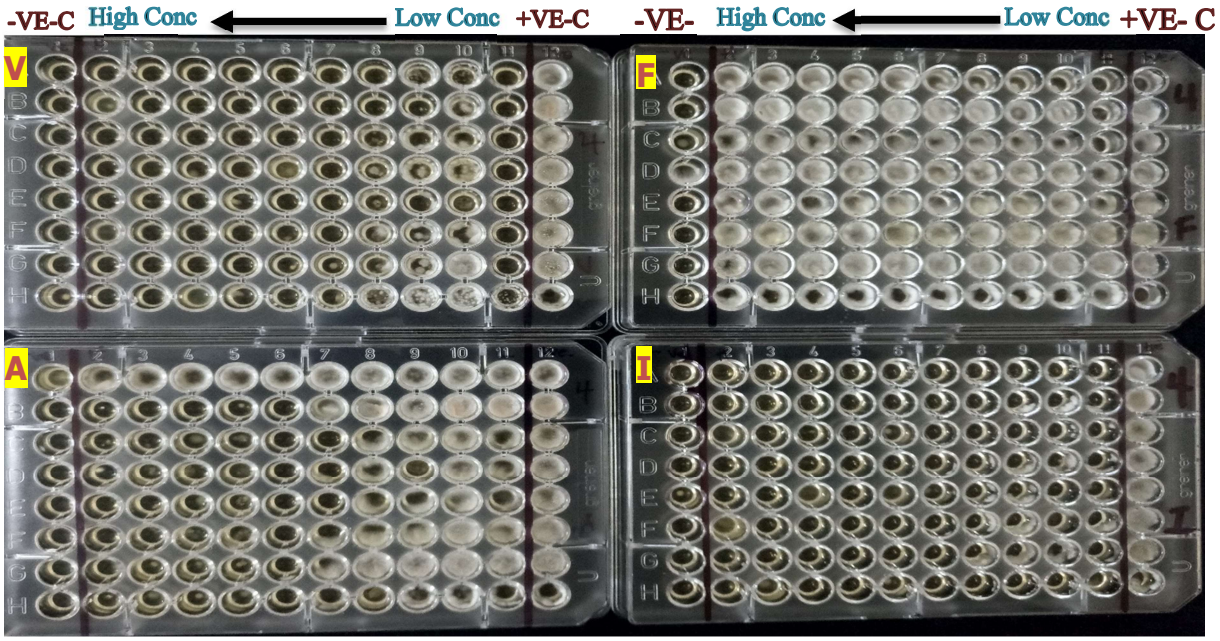

Figure 6.1: Micro-titer well plates showing varied sensitivity of the same mould isolates to voriconazole (V-top left), fluconazole (F-top right), amphotericin B (A-bottom left) and itraconazole (I-bottom right) with diminishing growth at higher concentration compared to the positive and negative control wells (-VE-C & +VE-C respectively). The moulds are typically extensively resistant to fluconazole (F) while V&I exhibit greater activity at low concentrations (MICs).

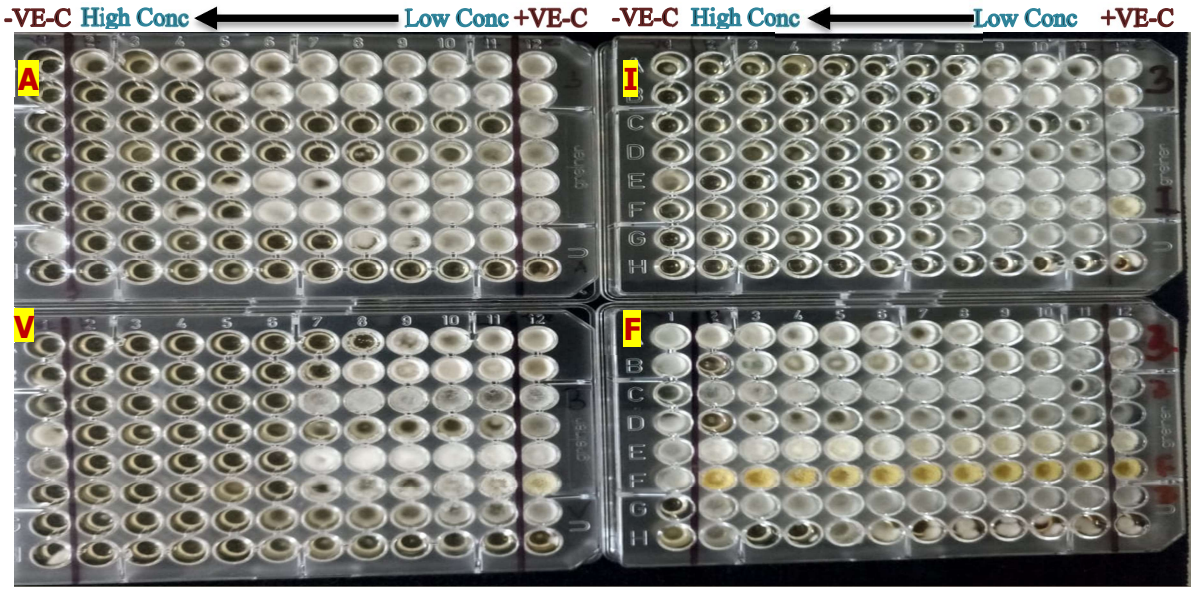

Figure 6.2: Micro-titer well plates showing a few moulds with relatively high MICs on amphotericin B (A-top left), itraconazole (I-top right) and voriconazole (V-bottom left) with resistance to fluconazole (F-bottom right) typical of filamentous fungi.

6.4.2 Antifungal susceptibility results for yeast isolates

Yeast isolates showed relatively low MICs to the antifungal agents (Table 6.2). A few isolates exhibited high MICs to the four drugs hence considered resistant. Twenty-one percent of *Candida albicans* (21%, n=5) had MICs≥4µg/ml for Amphotericin B, 4% (n=1) for Itraconazole, 8% (n=2) for Voriconazole and none of the *C. albicans* isolates had MIC ≥4µg/ml for Fluconazole. MIC ≥4µg/ml was also recorded for 40% (n=4) of *Candida krusei* isolates for both Voriconazole and Fluconazole, 30% (n=3) for Amphotericin B and 20% (n=2) for Itraconazole. One isolate of *Candida tropicalis* had >32µg/ml for both Fluconazole and Voriconazole. Other isolates with MICs ≥4µg/ml for AMB were *C. famata* (n=2) and *C. parapsilosis* (n=1) (Table 6.2). The MIC_{50} and MIC_{90} for the yeasts are shown in Table 6.1. *C. albicans* exhibited the greatest sensitivity to the four antifungals with MIC_{50} and MIC_{90} of <0.06µg/ml and <4µg/ml respectively. The antifungal sensitivity pattern for some yeasts on microtiter well plates are shown in Figure 6.3.

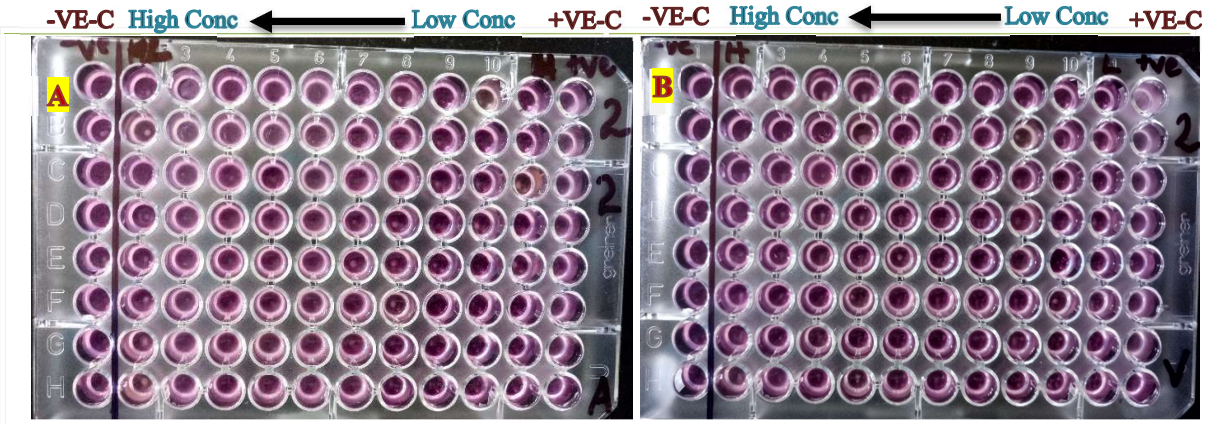

Figure 6.3: Micro-titer plates showing significant sensitivity of yeast isolates to the antifungal agents tested, example here amphotericin B (A) and voriconazole (B) depicted by the clarity of inoculated wells at low drug concentrations compared to the positive control (+VE-C) on RPMI-1640

Table 6.2: MICs of yeast isolates from sputum for the four antifungal agents

Antifungal agent and yeast species	Number of isolates with respective MICs (µg/ml) *n*=49 (48 hours reading)												
Amphotericin B	**<0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**>32**	**Total**	**MIC_{50}**	**MIC_{90}**
Candida albicans	13	-	2	1	-	3	4	1	-	-	24	<0.06	4
Candida krusei	2	1	1	1	-	2	1	1	-	1	10	0.5	8
Candida tropicalis	2	-	-	2	2	1	-	-	-	-	7	0.5	2
Candida famata	-	-	-	-	-	-	2	-	-	-	2	4	4
Candida zeylanoids	1	-	-	1	-	-	-	-	-	-	2	<0.06	0.5
Candida lusitaniae	-	-	-	-	-	1	-	-	-	-	1	2	2
Crypto coccus neoformans	1	-	-	-	-	-	-	-	-	-	1	<0.06	<0.06
Candida guilliermondii	-	-	-	-	-	1	-	-	-	-	1	2	2
Candida parapsilosis	-	-	-	-	-	-	1	-	-	-	1	4	4
Candida parapsilosis-ATCC® 22019	-	-	-	-	-	1	-	-	-	-	1	2	2
Itraconazole	**<0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**>32**	**Total**	**MIC_{50}**	**MIC_{90}**
Candida albicans	22S	-	-	-	-	1R	-	-	-	1R	24	<0.06	<0.06
Candida krusei	4S	2S	-	-	2R	-	-	-	-	2R	10	0.12	>32
Candida tropicalis	6S	-	1S	-	-	-	-	-	-	-	7	<0.06	<0.06
Candida famata	2	-	-	-	-	-	-	-	-	-	2	<0.06	<0.06
Candida zeylanoids	1	-	1	-	-	-	-	-	-	-	2	<0.06	0.25
Candida lusitaniae	1	-	-	-	-	-	-	-	-	-	1	<0.06	<0.06
Cryptococcus neoformans	1	-	-	-	-	-	-	-	-	-	1	<0.06	<0.06
Candida guilliermondii	-	-	-	1	-	-	-	-	-	-	1	0.5	0.5
Candida parapsilosis	1S	-	-	-	-	-	-	-	-	-	1	<0.06	<0.06
Candida parapsilosis-ATCC® 22019	-	-	1S	-	-	-	-	-	-	-	1	0.25	0.25
Voriconazole	**<0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**>32**	**Total**	**MIC_{50}**	**MIC_{90}**
Candida albicans	15S	-	2S	4S	1R	-	1R	-	-	1R	24	<0.06	0.5
Candida krusei	2S	-	3S	1S	-	-	-	1R	-	3R	10	0.25	>32
Candida tropicalis	4S	-	-	1R	1R	-	-	-	-	1R	7	<0.06	
Candida famata	-	-	-	2	-	-	-	-	-	-	2	0.5	0.5
Candida zeylanoides	1	-	-	-	1	-	-	-	-	-	2	<0.06	1
Candida lusitaniae	1	-	-	-	-	-	-	-	-	-	1	<0.06	<0.06
Cryptococcus neoformans	-	-	1	-	-	-	-	-	-	-	1	0.25	<0.25
Candida guilliermondii	-	-	1	-	-	-	-	-	-	-	1	0.25	0.25
Candida parapsilosis	-	-	-	1R	-	-	-	-	-	-	1	0.5	0.5
Candida parapsilosis-ATCC® 22019	-	1S	-	-	-	-	-	-	-	-	1	0.12	0.12
Fluconazole	**<0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**>32**	**Total**	**MIC_{50}**	**MIC_{90}**
Candida albicans	22S	2S	-	-	-	-	-	-	-	-	24	<0.06	0.12
Candida krusei	3S	-	1S	-	1R	1R	-	-	2R	2R	10	1	>32
Candida tropicalis	3S	-	-	2S	1S	-	-	-	-	1R	7	0.5	1
Candida famata	-	-	-	-	1	1	-	-	-	-	2	1	2
Candida zeylanoids	1	-	-	-	1	-	-	-	-	-	2	<0.06	1
Candida lusitaniae	1	-	-	-	-	-	-	-	-	-	1	<0.06	<0.06
Cryptococcus neoformans	1	-	-	-	-	-	-	-	-	-	1	<0.06	<0.06
Candida guilliermondii	-	1	-	-	-	-	-	-	-	-	1	0.12	0.12
Candida parapsilosis	1S	-	-	-	-	-	-	-	-	-	1	<0.06	<0.06
Candida parapsilosis-ATCC® 22019	-	-	-	1S	-	-	-	-	-	-	1	0.5	0.5

Quality control (QC) strain *Candida parapsilosis*-ATCC® 22019 MIC range AMB (0.5-4), ICZ (0.12-0.5), VCZ (0.03-0.25), FCZ (1.0-4.0), R-resistant, S-susceptible, I-Susceptible doze dependent, MIC_{50}-drug concentration that inhibited 50% of isolates, MIC_{90}- drug concentration that inhibited 90% of isolates.

6.4.3 Comparative sensitivity of yeasts and moulds from sputum

Mould isolates from sputum showed greater sensitivity to Voriconazole (85.5%) and Amphotericin B (64.7%) while the yeasts showed relatively higher sensitivity to the four antifungal agents (Figure 6.3). There was a statistically significant difference (F=3.7, p=0.004) in the sensitivity pattern of the moulds while the yeasts exhibited no statistically significant difference (F=1.7, p=0.154). Figure 6.3 summarises the susceptibility pattern for both moulds and yeasts isolated from sputum.

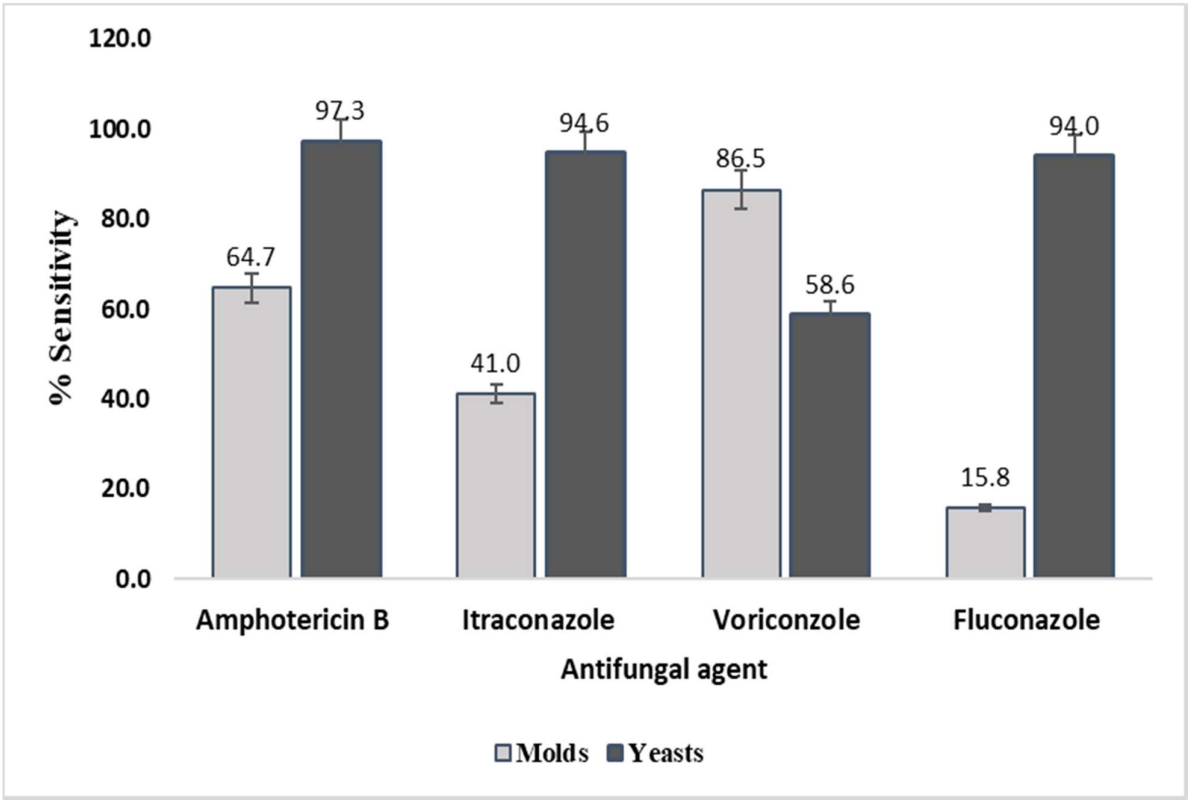

Figure 6.4: Comparative sensitivity of mould and yeast isolates from sputum on four antifungal agents.

6.5 Discussion

The study reports the antifungal susceptibility patterns of yeast and moulds isolated from the sputum samples. Multi-drug resistance in *Mycobacterium tuberculosis* is a major challenge in TB management that often leads to treatment failure and increased morbidity and mortality (Corbett *et al*., 2003). Coinfection in patients with pulmonary TB by potentially resistant fungal pathogens constitutes a key threat to successful treatment of these individuals (Dhooria *et al*., 2014). In that regard, clinicians encounter presumptive PTB cases whose clinical symptoms persist despite compliance with prescribed anti-TB regimen and often with inadequate bacteriological confirmation (Bansod & Rai, 2008a).

Four antifungal agents namely Amphotericin B, Itraconazole, Voriconazole and Fluconazole were tested in this study. Treatment of fungal infections with an antifungal agent depends on the clinical condition of a patient but the drug may also be used as prophylaxis in high risk conditions (Yamauchi, 2018). Similar to antibacterial resistance, antifungal resistance is an emerging challenge complicating the antimicrobial resistance control agenda (Wiederhold, 2017). Emerging triazole resistance in *Aspergillus fumigatus* is well recognized (Azevedo *et al*., 2015; Dunne *et al*., 2017; Xie *et al*., 2014). The current study identified fungal isolates exhibiting resistance and high MICs to the four drugs tested. *A. flavus* (41.6%), and *C. albicans* (21%) exhibited MICs ≥4µg/ml to *Amphotericin B* while all the *A. versicolor* isolates were resistant to Amphotericin B. All the filamentous fungi except *Paecillomyces variotii* and *Penicillium* spp were susceptible to triazole (Voriconazole and Itraconazole) suggesting favorable therapeutic options for management of mould infections. *P. variotii* and *Penicillium* spp were resistant to triazoles tested exhibiting MIC >32 µg/ml. Triazole resistance especially among the *A. fumigatus* is an emerging challenge in clinical practice *(Guillermo Garcia-Effron et al*., 2008). Cultures that turn positive for *Aspergilli* during the course of treatment with voriconazole have been associated with treatment failure, resistance and subsequent mortality (Jenks *et al*., 2019).

Based on the available CLSI interpretive criteria for the most predominant yeast isolates in the study, 92%, 71%, and 100% of *C. albicans* were susceptible to itraconazole, Voriconazole and fluconazole respectively (table 4.2.6). Only 60% of *C. krusei* were susceptible to both itraconazole and voriconazole with a decreased sensitivity to fluconazole (40%) (CLSI, 2008). Other studies have reported fluconazole resistance in *C. albicans* ascribed to prophylactic use of fluconazole in HIV patients (Badiee *et al*., 2010). There is also evidence of fluconazole resistance among clinical *Candida* species in Kenya and Tanzania (Mushi *et al*., 2016; Ooga *et al*., 2015). All isolates of *C. tropicalis* were susceptible to Itraconazole (100%) and 57% were susceptible to

Voriconazole. It is good to note that availability of triazole antifungals is limited and the cost is prohibitive hence this could explain the relatively high sensitivity observed. Nevertheless, increasing azole resistance is being encountered clinically ascribed to prophylactic use of azoles in immunocompromised patients and use of azole-based fungicides in agriculture.

The need for routine *in vitro* antifungal resistance testing is becoming essential. This is meant to facilitate the correlation of *in vivo* efficacy, track the occurrence of resistance in fungal etiological agents, and assess the therapeutic potential of new molecules (Rex *et al*., 2001). A major consideration in antifungal susceptibility testing is the clinical relevance of *in vitro* MICs. The correlation of increased MICs with molecular resistance to triazole antifungals and treatment failure has been recognized notably for *Aspergillus* spp. (Espinel *et al*., 2012). Itraconazole is mainly used to treat CPA and Voriconazole has been used as a first-line regimen for invasive aspergillosis (IA). New molecules introduced in the recent years include Isavuconazole, accepted for treatment of IA and Posaconazole administered as a prophylaxis in patients with increased risk of infection such as those with acute myeloid leukemia (Meis *et al*., 2016). The cost and availability are the greatest challenge for newer triazoles in resource constrained settings who need it the most. Therefore, resistance to these agents is unlikely to arise due to clinical use but as a result of irrational use of triazole based fungicides in agriculture.

Antifungal susceptibility testing (AST) using broth microdilution techniques such as reference standards by the CLSI and EUCAST is a challenge in our setting. First, the procedures for performing AST with microdilution methods are laborious and time consuming hence the turn around time for results is signficantly slow. Secondly, other impediments include the unpredictable correlation between treatment outcome and *in vitro* MIC results. Capabilities for diagnosis, treatment, and monitoring antifungal resistance are still limited in Kenya, hence requiring investment in these technologies. The study demonstrated varied antifungal sensitivity patterns for both yeast and moulds, suggesting greater efficacy of Amphotericin B and Itraconazole for moulds which could be vital for patient management decisions.

With the advent of antimicrobial resistance (AMR), it has been argued that it would be impossible to alleviate the increasing menace without sufficient attention to diagnosis and management of fungal diseases (Denning *et al*., 2017). Inaccurate diagnosis of fungal sepsis in hospital settings, administration of broad spectrum antibiotics, inability to diagnose pulmonary aspergillosis in presumptive TB patients and misdiagnosis of fungal related asthma constitute the vital drivers of AMR (Denning *et al*., 2017). Antifungal resistance varies significantly depending on the fungal

agent and the geographical region, a great variability in this phenomenon has been observed in *Candida glabrata* following the introduction of new antifungals particularly Echinocandins (Sanglard, 2016). *C. glabrata* was however not isolated in the study. One key strategy to address the problem of antifungal resistance is the establishment of antifungal stewardship in clinical settings to keep track and reduce the overall use of antifungal drugs and the adoption of proper infection prevention and control strategies (Ha *et al*., 2019). Successfully implemnted antifungal stewardship programmes have been acompanied by substancial benefits in minimizing healthcare costs, improving patient outcomes and mitigation of toxicities resulting from unnecessary antifungal prescriptions (Micallef *et al*., 2017). Considerable emphasis has been placed on antibiotic resitance disregarding antifungal stewardship. Antifungal drugs are relatively expensive medicines and a well implimented stewardship program would be beneficial in all facets (Ananda-Rajah et al., 2012). Resource contrains and lack of adequate incentives for clinical personnel to carry out satisfactory antimicrobial stewardship activities are among the greatest challenges to successful implementation of such programs (Safdar et al., 2014). Further, the role of agricultural fungicides in the emergence of clinical antifungal resistance should be adressed adequately as there is evidence suggesting azole use in agriculture as the main driver (Azevedo *et al*., 2015; Berger *et al*., 2017; Chowdhary *et al*., 2014; Dunne *et al*., 2017; Meis *et al*., 2016; Ren *et al*., 2017; Verweij *et al*., 2016; Xie *et al*., 2014).

The present study underscores the need to address the emerging clinical challenge of fungal pulmonary infections and antifungal resistance. These infections are often missed or misdiagnosed as tuberculosis and disregarded as contaminants in tuberculosis culture laboratories. Fungi exhibiting high MICs to the antifungals tested, represents an imminent threat to antifungal therapy hence resistance testing is vital for the selection of appropriate antifungal treatment. However, correlation of microbiological resistance and clinical outcome as well as pulmonary fungal colonization of retreatment and relapse patients is essential. Overall, a greater sensitivity to amphotericin B and voriconazole was observed in moulds while yeasts, mainly *C. albicans*, showed nearly uniform sensitivity to the four drugs tested.

CHAPTER SEVEN

Diversity of fungi from Lowenstein Jensen TB sputum cultures at the National TB Reference Laboratory, Kenya

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7.1 Abstract

Tuberculosis remains a global public threat. Various technologies have been developed over the years for diagnosis of *Mycobacterium tuberculosis*. However, culture is the gold standard for diagnosis and drug susceptibility testing for TB. Often, cultures get contaminated by a range bacteria and fungi that are usually disregarded as contaminants. The study sought to isolate and characterize fungi from contaminated Lowenstein Jensen solid media. One hundred and thirty-eight contaminated slopes (138) were sampled from the National TB Reference Laboratory. Visual examination of the slopes was performed and an inoculum was cultured of Sabouraud Dextrose Agar (SDA) plates. The cultures were incubated at 30^{o}C and examined subsequently for fungal growth. Diverse yeasts and moulds were recovered. Out of the 138 LJ slopes, 48% (*n*=66) yielded diverse fungal species. The most frequent isolates were *Candida albicans* 33% (*n*=22) *C. krusei* 21% (*n*=14) and *Rhizopus* spp 14% (*n*=9). Other fungi recovered included *Aspergillus niger* 2% (*n*=1), *Penicillium* spp 3% (n=2) and *Acremonium* spp 5% (*n*=3). Majority of the slopes, (44%, n=61) had various discoloration, while 33% (n=45) had visible growth of mould along with discoloration. The study highlights the challenge of culture contamination by fungi that hampers the diagnosis of TB. However, higher frequency of TB culture contamination suggests that the fungi could be of clinical significance in the patient's condition. Therefore, there is need for further clinical workup to ascertain, in light of emerging fungal complications in patients with pulmonary tuberculosis.

Key words: Fungi, Tuberculosis, Sputum, Lowenstein Jensen, Contamination

7.2 Introduction

Tuberculosis (TB) is still a major global health problem particularly in low- and middle-income countries. In 2019, the estimated global incidence of active TB was 10.0 million with TB associated mortality reaching 1.4 million deaths (WHO, 2021). A number of new TB diagnostic platforms such as GeneXpertMTBRif assay and MTBDRplus have been approved for use by WHO. However, culture remains the gold standard for diagnosis and drug susceptibility testing (DST) for *Mycobacterium tuberculosis* (WHO, 2009). Lowenstein Jensen (LJ) solid media and Mycobacterium Growth Indicator Tube (MGIT) (liquid media) are routinely used for culture of TB from a variety of clinical specimen. Contamination of these cultures is a common and expected occurrence in TB culture laboratories. Nevertheless, contamination rate for both solid and liquid media are monitored as part of quality assurance in TB diagnostics. Contamination rate above 5-8% is generally considered high for MGIT while contamination rate above 3-5% is unacceptable for LJ solid media (Caulfield & Wengenack, 2016).

Contamination of TB cultures represents a significant problem to TB diagnosis and patient management. Contaminated cultures cannot be interpreted and often have to be repeated (Kassaza *et al*., 2014). This generally increases the cost of diagnosis and delay in obtaining critical results needed for patient management (Muzanyi *et al*., 2021). Historically, fungi and bacteria have been recognized as the most common contaminants in TB cultures. On the contrary, recent evidence suggests the possible etiological role of fungi in presumptive pulmonary TB patients (Amiri *et al*., 2018; Fang *et al*., 2017; Hadadi-Fishani *et al*., 2020a, 2020b; Hosseini *et al*., 2020). In this regard, fungal contaminants in TB sputum cultures may no longer be disregarded as contaminants and hence further evaluation may be needed to ascertain the clinical relevance. The fungal infection may exist as coinfection with TB or as a primary infection due to the affected patient's underlying immunosuppression.

Diverse fungal species have previously been isolated from LJ cultures meant for isolation of *Mycobacteria*. These include members of the genus *Aspergillus* spp and *Mucor* spp *Candida albicans* (Murahari *et al*., 2019). It is vital for TB referernce laboratories to understand the profile of contaminants in TB cultures in their laboratory. The understanding facilitates implementation of targeted interventions to address the challenge. TB culture contaminants have not been previously profiled in Kenya. At the time when the current study was carried out at the National TB Reference Laboratory (NTRL), contamination rate had reached 10%. Therefore, the study

sought to characterize common fungal contaminants in LJ tubes previously inoculated with sputum from TB relapse and retreatment patients at the reference labratory.

7.3 Materials and Methods

7.3.1 Study design

The study utilized a cross-sectional study design. Lowenstein Jensen solid media tubes that had likely contamination by moulds were randomly selected at the National TB Reference laboratory. The tubes were transported according to guidelines for handling and transportation of infectious material with triple packaging to the Mycology Laboratory at KEMRI for isolation of possible fungal contaminants.

7.3.2 Sampling and sample size determination

The sample size (*n=138*) for LJ tubes was determined using the formula described below for simple random sampling (Taherdoost, 2017). The calculation was based on 10% culture contamination rate during the sampling period of the study.

$$\boldsymbol{n} = \frac{p\,(100\text{-}p)\,z^2}{E^2}$$

n = required sample size
P = percentage occurrence of contamination (10%)
E = percentage maximum error required (5%)
Z = value corresponding to 95% level of confidence required (1.96)

$$\boldsymbol{n} = \frac{10\,(100\text{-}10)\,1.96^2}{5^2} = 138$$

The required minimum sample size was 138 LJ tubes.

7.3.3 Ethical considerations

The study protocol was reviewed and approved by Kenyatta Hospital Ethics Review Committee (KNH-ERC) (study number: P108/02/2018) and Kenya Medical Research Medical Research Institute Scientific and Ethics Review Unit (SERU) (study number: KEMRI/SERU/0037/3213). Approval to carry out the study was also granted by the management of NTRL. The study was carried out according to the approved protocol. Patient information on samples were kept confidential by assigning unique numbers to safeguard identifying information. Data was secured in password protected computers restricting unauthorized access.

7.3.4 Sample processing and isolation of fungi

At the mycology laboratory, LJ tubes were examined physically to ascertain their condition and record the contamination pattern. The tubes were allowed to settle for at least 30 minutes before processing. Procedures involving manipulation the LJ tubes and fungal cultures were carried in a Class IIA biosafety cabinet. An inoculum was obtained from the slants using a sterile swab and inoculated on Sabouraud Dextrose Agar (SDA) supplemented with 0.65 ml gentamicin (40 mg/ml) and 10 ml of chloramphenicol in duplicates. One set of cultures were incubated at 30ºC for up to 7 days for isolation of moulds while another set was incubated at 35ºC examined at 48 and 72 hours for possible isolation of yeasts.

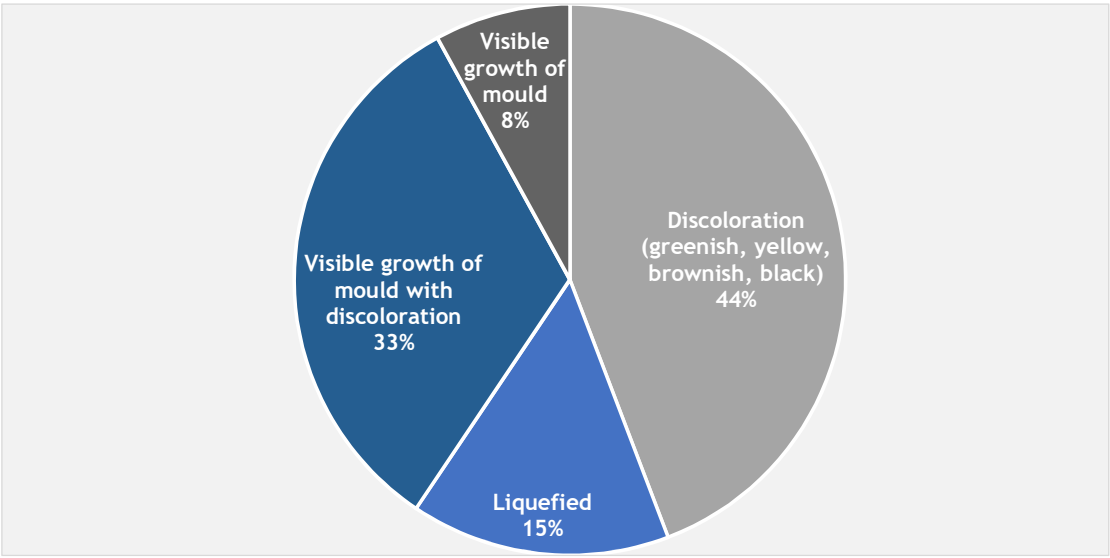
7.3.5 Identification of fungal isolates

Yeast isolates were initially identified by morphological characteristics on primary SDA culture and subsequently confirmed by gram stain. The isolates were then subcultured on CHROMagar™ *Candida* (bioMérieux®, Durham) medium prepared according to manufacturer's instructions. API 20C AUX™ (bioMérieux®) identification profile based on carbohydrate utilization was also used to further characterize yeasts. Chlamydospore formation was also used to differentiate *Candida albicans* from non albicans *Candida* species. Briefly isolates were inoculated onto corn meal agar plates (Dalmau inoculation technique) containing 1% Tween 80 and thereafter incubated at 30^{O}C at high humidity for 24 to 72 hours and examined microscope through the cover slip for chlamydospores. Mould isolates were identified by colony growth characteristics such as topography, color on the reverse and obverse, and microscopic features of conidial arrangement and phialides on lactophenol cotton blue mount at X40 magnification.

7.4 Results

7.4.1 Physical characteristics of contaminated LJ tubes

A visual examination of the LJ slopes revealed varied patterns of contamination. Majority, (44%,



n=61) had various discoloration, while 33% (n=45) had visible growth of mould along with discoloration (Figure 7.1 & 7.2). Eight percent (8%, n=11) of the slants exhibited mould growth without discolouration in the entire tube (Figure 7.1). Some LJ slopes were liquefied (15%, n=21%).

Figure 7.1: Pattern of LJ slope contamination on physical examination

Figure 7.2: LJ slopes with visible growth of fungal contaminants

7.4.2 Fungal species distribution from contaminated LJ slopes

Out of 138 contaminated LJ slopes inoculated on SDA, 48% (*n*=66) yielded diverse fungal species. The most frequent isolates were *Candida albicans* 33% (*n*=22) *C. krusei* 21% (*n*=14) and *Rhizopus* spp 14% (*n*=9). Other fungi recovered included *Aspergillus niger* 2% (*n*=1), *Penicillium* spp 3% (n=2) and *Acremonium* spp 5% (*n*=3). Some LJ tubes had visible contamination by filamentous fungi (Figure 7.3) while others had mixed growth on SDA. Fifty-two percent (52%) of the LJ tubes were fungal culture negative.

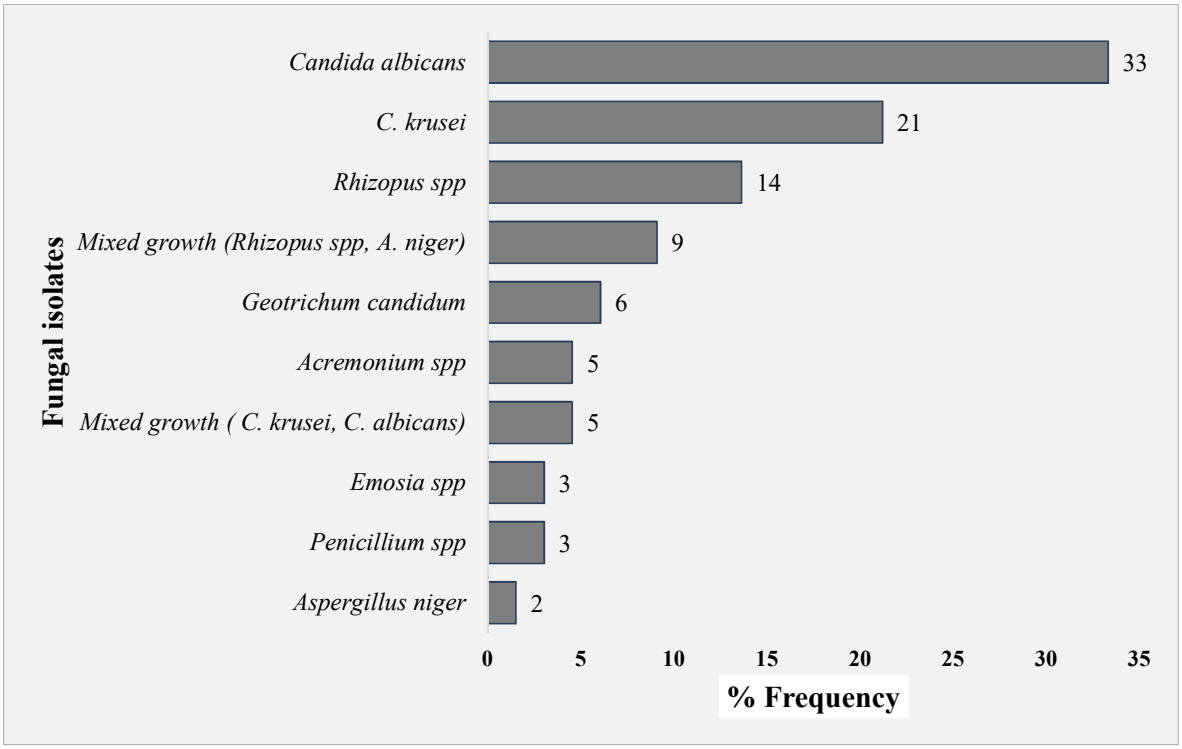


Figure 7.3: Frequency of fungal isolates from contaminated LJ tubes

7.5 Discussion

Contamination of cultures remains a major challenge in TB diagnosis. This occurrence causes delay in TB case detection and management, increased disease transmission in the community, and severity and mortality in affected patients (Murahari *et al*., 2019). In the current study, mycological findings from 138 LJ slopes (solid media) examined revealed that a variety of both yeasts and moulds are responsible. Overall, 48% were positive for fungi with *Candida albicans* and *Rhizopus* spp being the most abundant. *Aspergillus niger* and *Penicillium* spp were also identified with mixed growth in some SDA cultures, albeit at a low prevalence, while some tubes had visible contamination by moulds. In previous studies, a variety of bacteria and fungi (such as *C. albicans*, *A. fumigatus*) have been recovered from contaminated LJ slopes consistent with results from the current study (McClean *et al*., 2011; Vianney *et al*., 2017). Fungi identified in this study grew despite aseptic and routine decontamination processes in the laboratory. The isolates may represent exogenous contaminants or actual etiological agents of pulmonary disease in the retreatment patients from whom the sputum samples were obtained. However, this requires further investigation to correlate with the clinical condition of patients.

Further, in other studies a broader range of unique fungal species have been recovered from sputa than LJ tubes. Fifty-two percent (52%) of LJ tubes in the current study were negative for fungi despite various physical changes observed in the media. Contamination in these tubes could have been caused by bacteria and other factors such as chemical degradation, which were not examined in the present study. Various approaches have been explored in an attempt to reduce Mycobacterial culture contamination. In one study, presumptive TB patients were asked to rinse their mouth with 10 ml of 0.1% chlorhexidine digluconate before sputum production. The study

did not find significant reduction in culture contamination for patients who rinsed their mouth and those who had not (Kabore *et al*., 2019). In another study, suspected TB patients were asked to perform an oral rinse for 60 seconds with chlorhexidine and subsequently for an additional 60 seconds with nystatin before sputum collection. The study concluded that there was a reduction in culture contamination downstream but however the rinse interfered with sample integrity, hampering the isolation of *M. tuberculosis* and reduction in smear sensitivity (Kalema *et al*., 2012).

The current study highlights the likely etiology of the fungal isolates from LJ tubes in the patient's pulmonary lung condition. Fungi such as *C. albicans* and *Aspergillus* spp are known respiratory pathogens and a number of studies have identified these species as co-infection in patients with *Mycobacterium tuberculosis* (Amiri *et al*., 2018; Fontalvo *et al*., 2016; Hadadi-Fishani *et al*., 2020b). The ignored fungi could exacerbate TB symptoms, severity and overall mortality since it is presumed to be a contaminant and hence remains unattended in the patient management cycle.

CHAPTER EIGHT

Genomic characterization of *Aspergillus* species from sputum of tuberculosis retreatment patients

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8.1 Summary of the chapter

Tuberculosis (TB) is an ancient disease that remains a major global public health threat. Opportunistic fungal infections are also on the rise due to immunocompromised individuals' growing population. *Aspergilli* are frequently being recognized as major etiological agents in pulmonary TB co-infection and post TB associated mortality. The aim of the study was to characterize *Aspergillus* spp isolates from sputum of TB retreatment patients from three reference facilities in Kenya. The study was carried out at Kenya Medical Research Institute, Mycology Laboratory. Sputum samples were collected from presumptive TB retreatment patients who gave written informed consent to participate. The samples were mucolysed and processed for isolation of *Aspergillus* on Sabouraud Dextrose Agar (SDA). Genomic DNA from a total of 15 pure *Aspergillus* spp recovered from sputum was extracted and sequencing was performed on *β-tubulin* and *Calmodulin* genes using universal primers. Gene sequences were cleaned and alignment was performed using Jalview. Basic local alignment search tool (BLAST) analysis was performed to confirm identity of isolates in comparison with reference species in NCBI database. Phylogenetic analysis was also done and the sequences were deposited in NCBI database through Bankit. Partial coding sequences of the *Aspergillus* spp were accepted and given NCBI accession numbers. The analysis revealed intriguing relationship of *Aspergillus* spp from sputum and those in GenBank. In the current study, clustal analysis & pairwise identity matches for *β*-tubulin and *calmodulin* gene sequences showed similarity index ranging from 98.2% to 100%. Genomic characterization of *Aspergillus* spp is essential in identification of clinical isolates to facilitate targeted antifungal therapy, prognosis of infected patients and overall management.

Key words: *Aspergillus*, Genomic, Sputum, Tuberculosis, Retreatment

8.2 Introduction

Aspergillus genus comprises a diverse group of saprophytic filamentous fungi that are widespread in the environment. They can be found in soils, air, indoor environments, water and plant material (Paulussen *et al*., 2017). *Aspergilli* are responsible for a range of human diseases with majority of severe infections occurring in immunocompromised individuals. *Aspergilli* produce spores that are suspended in the air and human infection occurs through inhalation. The clinical spectrum of pulmonary aspergillosis varies significantly, including allergic bronchopulmonary aspergillosis (ABPA), chronic pulmonary aspergillosis (CPA) as well as invasive pulmonary aspergillosis (IPA) (Kanj *et al*., 2018). In the recent years, a variety of fungal pathogens have emerged in patients infected with *Mycobacterium tuberculosis. Aspergillus niger, A. fumigatus, A. flavus, Histoplasma capsulatum* and *Cryptococcus neoformans* are some fungi isolated from sputum of TB patients (Bansod & Rai, 2008a). The fungi are known to aggravate the condition of the affected patients complicating diagnosis, treatment and management of TB with resultant mortality.

The genomes of several *Aspergillus* species have been sequenced with an aim of understanding the biology of these organisms. Some important features of this genus revealed by genomic sequencing include metabolic characteristics, ecology, clinical aspects of related infections, and molecular basis of antifungal resistance (Paulussen *et al*., 2017). Traditionally, identification of *Aspergillus* species relies heavily on colony morphological characteristics and conidia & conidiophore arrangement. However, there is an increasing need for more definitive taxonomic characterization of the respective species due to the rising clinical relevance of *Aspergilli* (Arastehfar *et al*., 2021).

DNA based genomic characterization is reliable in distinguishing members of the *Aspergillus* spp. One helpful approach has been the analysis of introns and sequencing of entire or segments of unique genes such as *β*-tubulin and hydrophobin genes (Latgé & Chamilos, 2020). The *β*-tubulin gene was the first to be isolated and sequenced in *A. flavus* and was found to be highly conserved (Seip *et al*., 1990). Tubulin proteins are vital in eukaryotic cellular function and constitutes a major structural componenent of microtubules. Microtubles associate with other proteins such as kinesins and dyneins which facilitate chromosome segregation, cell division, intracellular transport among other functions (Einax & Voigt, 2003). Due to their conserved nature, bet tubulin genes re the hallmarks to condtruct phylogenetic tree of fungi. *Calmodulin* (*CaM*) is an important protein found in eukaryotes and it reversibly binds to Ca2+ as a key receptor. The calmodulin gene is highly conserved and has been reliably sequenced to characterise *Aspergillus niger* (S.

Chen *et al*., 2010; Liao *et al*., 2017). One study identified 25 well defined species clades based on *calmodulin* gene along with 70 different haplotypes among representative strains (Samson *et al*., 2014).

Molecular characterization approaches provide the greatest number of distinguishing characters for fungal taxonomy. Several efficient bioinformatics tools are now available to facilitate phylogenetic analysis and comparison of various species in Genebank databases. The objective of the present study was to characterize *Aspergillus* spp isolates from sputum of tuberculosis retreatment patients based on *β*-tubulin and *Calmodulin* gene sequences.

8.3 Material and Methods

8.3.1 Study design and sample collection

The study utilized a cross-sectional study design. Initial isolation of fungi from sputum was carried out at Kenya Medical Research Institute (KEMRI), Centre for Microbiology Research (CMR)–Mycology Laboratory. Fresh sputum samples were collected from relapse and retreatment patients recruited from three reference facilities namely Moi Teaching and Referral Hospital, Mbagathi Hospital and the National TB Reference Laboratory. The samples collected in sterile sputum mugs were packaged using triple packaging technique in accordance with requisite biosafety guidelines (Heifets, 2014). The samples were transported to the laboratory for mycological investigations.

8.3.2 Ethical considerations

The study protocol was reviewed and approved by Kenyatta Hospital-University of Nairobi Ethics Review Committee (KNH-UoN ERC) (study number: P108/02/2018). The study was nested in a parent study approved by Kenya Medical Research Medical Research Institute Scientific and Ethics Review Unit (SERU) (study number: KEMRI/SERU/0037/3213). The study was carried out according to the approved protocol and participants gave written informed consent. Patient information was kept confidential by assigning unique numbers to samples to safeguard identifying information. Data was secured in password protected computers lockable cabinets restricting unauthorized access.

8.3.3 Isolation of *Aspergillus* species from sputum

Sputum samples were mucolysed with sputasol and inoculated on sabouraud dextrose agar (SDA) in duplicates. To inhibit bacterial contamination, the media was supplemented with 10 ml of Chloramphenicol and 0.65 ml of Gentamicin (40 mg/ml). Sterility check and ability to support

growth fungi were performed on each culture media batch. Inoculated plates were incubated at 30ºC for up to 4 weeks and evaluated daily for fungal growth. Fungi growing on the plates were subcultured on fresh SDA to obtain pure cultures. *Aspergilli* were initially identified based on phenotypic morphological characteristics on culture such as colony colour, size, presence exudates, topography, and microscopic features of conidia and phialides. Plates that had no growth at the fourth week were believed to be negative and therefore disposed appropriately as per the laboratory waste disposal procedures.

8.3.4 *Aspergillus* spp DNA extraction

Fifteen pure isolates of *Aspergillus* spp selected from the study were sent for sequencing at Chiba University, School of Medicine, Center for Mycology Research in Japan under the custody of Professor Gonoi. Genomic DNA extraction from the *Aspergillus* spp isolates was performed using a standard DNA extraction kit (QIAamp® DNA Mini Kit) (Qiagen, Hilden, Germany) according to manufacturer's protocols. Briefly isolates were grown on SDA agar plates at 30°C for 48 hours. The plate surface was then flooded with 10 ml fungal saline (0.9% w/v NaCl) to harvest conidia. 1 ml saline suspension containing 1-5 x 106 conidia (determined photometrically at A530) was prepared and centrifuged after which the pellet was collected and processed further by lyticase digestion (Sigma Aldrich), centrifugation and elution of DNA by buffer.

8.3.5 *Aspergillus* spp DNA sequencing and alignment

Coding sequences and promoter of calmodulin (*CaM*) and *β* tubulin (*BtU*) genes were amplified and sequenced in both directions using respective universal primers (Table 8.1) by Sanger sequencing (Samson *et al.,* 2014). The sequences were cleaned and alignment was performed using Jalview version 2.11.1.4 (http://www.jalview.org/). Basic local alignment search tool (BLAST) analysis was performed to confirm identity of isolates in comparison with reference species in the National Centre for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) database.

Table 8.1 Primers and annealing temperatures used for amplification and sequencing

Gene locus	Amplification	Annealing temp (ºC)	Cycles	Primer	Direction	Primer sequence (5'-3')
Beta tubulin (*β*-tubulin)	Standard	55 (alt 52)	35	*BtU*	Forward	GACTCGCTACTAGGCCAACGG
				BtU	Reverse	AGTCCGGTGCTGGTAACAAC
Calmodulin (*CaM*)	Standard	55 (alt 52)	35	*CaM*	Forward	CCGAGTACAAGGAGGCCTTC
				CaM	Reverse	CCACGTTATGACCTCCATCGG

Source (Samson *et al.,* 2014)

8.3.6 *Aspergilllus* spp DNA sequence data analysis

Sequence data analysis and alignment was performed by BLAST against reference sequences available at home library in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm *Aspergillus* spp by calmodulin (*CaM*) and beta tubulin (*BtU*) genes. Partial coding sequences of 15 isolates were cleaned and submitted to the NCBI database through Bankit (https://www.ncbi.nlm.nih.gov/WebSub/) after which they were verified and allocated accession numbers (Tables 8.3 & 8.4). Phylogenetic analysis was performed to determine the relationship of *Aspergillus* spp isolates from sputum with isolates in Genebank using one click mode in phylogeny.fr (http://www.phylogeny.fr/simple_phylogeny.cgi) (Dereeper *et al*., 2008). Pairwise identity matches of sequences from the study were also done using clustal omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) in comparison to sequences from Genebank.

8.4 Results

8.4.1 *Aspergillus* spp DNA sequences from sputum

A total of 15 sequences of *Aspergillus* spp isolates comprising of *A. fumigatus*, *A. flavus*, *A. terreus* and *A. niger* obtained from the study are shown in table 8.2 and 8.3. Assigned Genebank (GB) accession numbers, partial coding sequences (CDS) and corresponding protein sequences for *β*-tubulin and *Calmodulin (Cam)* are included in table 8.2 and 8.3 respectively. An illustration of BLAST analysis of the sequences is shown in figure 8.3.

Table 8.2: *Aspergillus* spp sequences based on *β*-tubulin gene sequencing

Isolate name & number	Assigned GB accession number	***β*-tubulin gene sequences with partial coding sequence (CDS) & corresponding protein sequences**	
68-5278 *Aspergillus fumigatus*	**MZ044668**	**Gene sequence**	TGGTATGTCTTGACCTCAAAGCTTGGATGACGGGTGATTGGGATCTCTCATCTTAGCAGGCTACCTCCATGGGTTCAGCC TCACTGTCATGGGTATCAGCTAACAAATCTACAGGCAGACCATCTCTGGTGAGCATGGCCTTGACGGCTCTGGCCAGTAA GTTCGACCTATATCCTCCCAATTGAGAAAGCGGCGGAAACACGGAAAACAAGGAAGAAGCGGACGCGTGTCTGATGGGAA ATAATAGCTACAATGGCTCCTCCGATCTCCAGCTGGAGCGTATGAACGTCTATTTCAACGAGGTGTGTGGATGAAACTCT TGATTTATACTATTTCGGCAACATCTCACGATCTGACTCGCTACTAGGCCAACGGTGACAAATATGTTCCTCGTGCCGTT CTGGTCGATCTCGAGCCTGGTACCATGGACGCTGTCCGTGCCGGTCCCTTCGGCGAGCTATTCCGTCCCGACAACTTCGT CTTCGGCCAGTCCGGTGCTGGTAACAACTGGGCCAAGGGTCACTACACCGAGGGCGCCGAGTTGGTCGACCAGGTTATCG ATGTCGTCCGTCGTGAGGCTGAAGGCTGTGACTGCCTCCAGGGCTTCCAGGTCACCCACTCTCTCGGTGGTGGTACCGGT GCCGGTATGGGTACTCTTCTGATCTCCAAGATCCGTGAGGAGTTCCCCGACCGTATGATGGCGACCTTCTCCGTTGTTCC CTCCCCCAAGGTTTCCGACACTGTCGTTGAGCCTTACAACGCTACCCTCTCCGTTCACCAGCTCGTTGAGCACTCCGATG AGA
		Coding region	ATGGACGCTGTCCGTGCCGGTCCCTTCGGCGAGCTATTCCGTCCCGACAACTTCGTCTTCGGCCAGTCCGGT GCTGGTAACAACTGGGCCAAGGGTCACTACACCGAGGGCGCCGAGTTGGTCGACCAGGTTATCGATGTCGTC CGTCGTGAGGCTGAAGGCTGTGACTGCCTCCAGGGCTTCCAGGTCACCCACTCTCTCGGTGGTGGTACCGGT GCCGGTATGGGTACTCTTCTGATCTCCAAGATCCGTGAGGAGTTCCCCGACCGTATGATGGCGACCTTCTCC GTTGTTCCCTCCCCCAAGGTTTCCGACACTGTCGTTGAGCCTTACAACGCTACCCTCTCCGTTCACCAGCTC GTTGAGCACTCCGATGAGA
		Protein sequence	MDAVRAGPFGELFRPDNFVFGQSGAGNNWAKGHYTEGAELVDQVIDVVRREAEGCDCLQGFQVTHSLGGGTGAGMG TLLISKIREEFPDRMMATFSVVPSPKVSDTVVEPYNATLSVHQLVEHSDE
69-7046 *Aspergillus fumigatus*	Too short to bank	**Gene sequence**	TGGTATGTCTTGACCTCAAAGCTTGGATGACGGGTGATTGGGATCTCTCATCTTAGCAGGCTACCTCCATGGGTTCAGCC TCACTGTCATGGGTATCAGCTAACAAATCTACAGGCAGACCATCTCTGGTGAGCATGGCCTTGACGGCTCTGGCCAGTAA GTTCGACCTATATCCTCCCAATTGAGAAAGCGGCGGAAACACGGAAAACAAGGAAGAAGCGGACGCGTGTCTGATGGGAA ATAATAGCTACAATGGCTCCTCCGATCTCCAGCTGGAGCGTATGAACGTCTATTTCAACGAGGTGTGTGGATGAAACTCT TGATTTATACTATTTCGGCAACATCTCACGATCTGACTCGCTACTAGGCCAACGGTGACAAATATGTTCCTCGTGCCGTT CTGGTCGATCTCGAGCCTGGTACCATGGACGCTGTCCGTGCCGGTCCCTTCGGCGAGCTATTCCGTCCCGACAACTTCGT CTTCGGCCAGTCCGGTGCTGGTAACAACTGGGCCAAGGGTCA
		Coding region	ATGGACGCTGTCCGTGCCGGTCCCTTCGGCGAGCTATTCCGTCCCGACAACTTCGTCTTCGGCCAGTCCGGT GCTGGTAACAACTGGGCCAAGGGT
		Protein sequence	MDAVRAGPFGELFRPDNFVFGQSGAGNNWAKG

74-3993 *Aspergillus flavus*	MZ044672	**Gene sequence**	TGGTATGTCTCAATGCCTTCGAGTTAGTATGCTTTGGACCAAGGAACTCCTCAAAAGCATGATCTCGGATGTGTCCTGTTA TATCTGCCACATGTTTGCTAACAACTTTGCAGGCAAACCATCTCTGGCGAGCACGGCCTTGACGGCTCCGGTGTGTAAGTA CAGCCTGTATACACCTCGAACGAACGACGACCATATGGCATTAGAAGTTGGAATGGATCTGACGGCAAGGATAGTTACAAT GGCTCCTCCGATCTCCAGCTGGAGCGTATGAACGTCTACTTCAACGAGGTGCGTACCTCAAAATTTAAGCATCTATGAAAA CGCTTTGCAACTCCTGACCGCTTCTCCAGGCCAGCGGAAACAAGTATGTCCCTCGTGCCGTCCTCGTTGATCTTGAGCCTG GTACCATGGACGCCGTCCGTGCCGGTCCCTTCGGTCAGCTCTTCCGTCCCGACAACTTCGTTTTCGGCCAGTCCGGTGCTG GTAACAACTGG
		Coding region	ATGGACGCCGTCCGTGCCGGTCCCTTCGGTCAGCTCTTCCGTCCCGACAACTTCGTTTTCGGCCAGTCCGGT GCTGGTAACAACTGGGCCAAG
		Protein sequence	MDAVRAGPFGQLFRPDNFVFGQSGAGNNWAK
75-4291 *Aspergillus flavus*	MZ044673		TGGTATGTCTCAATGCCTTCGAGTTAGTATGCTTTGGACCAAGGAACTCCTCAAAAGCATGATCTCGGATGTGTCCTGTTA TATCTGCCACATGTTTGCTAACAACTTTGCAGGCAAACCATCTCTGGCGAGCACGGCCTTGACGGCTCCGGTGTGTAAGTA CAGCCTGTATACACCTCGAACGAACGACGACCATATGGCATTAGAAGTTGGAATGGATCTGACGGCAAGGATAGTTACAAT GGCTCCTCCGATCTCCAGCTGGAGCGTATGAACGTCTACTTCAACGAGGTGCGTACCTCAAAATTTCAGCATCTATGAAAA CGCTTTGCAACTCCTGACCGCTTCTCCAGGCCAGCGGAAACAAGTATGTCCCTCGTGCCGTCCTCGTTGATCTTGAGCCTG GTACCATGGACGCCGTCCGTGCCGGTCCCTTCGGTCAGCTCTTCCGTCCCGACAACTTCGTTTTCGGCCAGTCCGGTGCTG GTAACAACTGG
		Coding region	>75 [Organism=Aspergillus flavus] beta tubulin partial coding sequence ATGGACGCCGTCCGTGCCGGTCCCTTCGGTCAGCTCTTCCGTCCCGACAACTTCGTTTTCGGCCAGTCCGGT GCTGGTAACAACTGGGCCAAG
		Protein sequence	MDAVRAGPFGQLFRPDNFVFGQSGAGNNWAK
76-6830 *Aspergillus terreus*	MZ044674	**Gene sequence**	TGGTACGTCTGGAATCAACCTGGGGAATACTGGCTCTCGTGGGATGCAGAGTCCTACGGACATGCGTCCTCGGGCTAAAAA GGGTTCCGTGGTGGCATGATGCTGACAACTGTACAGGCAAACCATCTCTGGCGAGCACGGCCTTGATGGCTCCGGTGTGTA AGTGTCACCGACGCCCGCTCAATGGGCTCCCATAATGGAGAATTACACGACGATGGACGATTCTGATGGAAAACAGCTTCA ATGGCTCCTCCGACCTCCAGCTCGAGCGCATGAACGTCTACTTCAACGAGGTACGTTCCCTTCCTACACCATCCTGTGATA GATTCTCCACGCCTCCAAAGACCTCGACACTAATTTCCGACCCCCTTTAGGCCAGCGGAAACAAGTATGTTCCTCGTGCCG TCCTCGTTGACCTTGAGCCCGGTACCATGGACGCCGTCCGTGCCGGTCCCTTCGGTCAGCTCTTCCGTCCCGACAACTTCG TCTTCGGCCAGTCTGGTGCCGGTAACAACTGGGCAAGGGGTCAT
		Coding region	ATGGAAAACAGCTTCAATGGCTCCTCCGACCTCCAGCTCGAGCGCATGAACGTCTACTTCAACGAGGTACGT TCCCTTCCTACACCATCCTGTGATAGATTCTCCACGCCTCCAAAGACCTCGACACTAATTTCCGACCCCCTT TAG
		Protein sequence	MENSFNGSSDLQLERMNVYFNEVRSLPTPSCDRFSTPPKTSTLISDPL

77-4645 *Aspergillus terreus*	MZ044675	**Gene sequence**	TGGTACGTCTGGAATCAACCTGGGGAATACTGGCTCTCGTGGGATGCAGAGTCCTACGGACATGCGTCCTCGGGCTAAAAA GGGTTCCGTGGTGGCATGATGCTGACAACTGTACAGGCAAACCATCTCTGGCGAGCACGGCCTTGATGGCTCCGGTGTGTA AGTGTCACCGACGCCCGCTCAATGGGCTCCCATAATGGAGAATTACACGACGATGGACGATTCTGATGGAAAACAGCTTCA ATGGCTCCTCCGACCTCCAGCTCGAGCGCATGAACGTCTACTTCAACGAGGTACGTTCCCTTCCTACACCATCCTGTGATA GATTCTCCACGCCTCCAAAGACCTCGACACTAATTTCCGACCCCCTTTAGGCCAGCGGAAACAAGTATGTTCCTCGTGCCG TCCTCGTTGACCTTGAGCCCGGTACCATGGACGCCGTCCGTGCCGGTCCCTTCGGTCAGCTCTTCCGTCCCGACAACTTCG TCTTCGGCCAGTCTGGTGCCGGTAACAACTGGGCAAGGGGTCATC
		Coding region	ATGGAAAACAGCTTCAATGGCTCCTCCGACCTCCAGCTCGAGCGCATGAACGTCTACTTCAACGAGGTACGT TCCCTTCCTACACCATCCTGTGATAGATTCTCCACGCCTCCAAAGACCTCGACACTAATTTCCGACCCCCTT TAG
		Protein sequence	MENSFNGSSDLQLERMNVYFNEVRSLPTPSCDRFSTPPKTSTLISDPL
78-3328 *Aspergillus terreus*	MZ044676		TGGTACGTCTGGAATCAACCTGGGGAATACTGGCTCTCGTGGGATGCAGAGTCCTACGGACATGCGTCCTCGGGCTAAAAAG GGTTCCGTGGTGGCATGATGCTGACAACTGTACAGGCAAACCATCTCTGGCGAGCACGGCCTTGATGGCTCCGGTGTGTAAG TGTCACCGACGCCCGCTCAATGGGCTCCCATAATGGAGAATTACACGACGATGGACGATTCTGATGGAAAACAGCTTCAATG GCTCCTCCGACCTCCAGCTCGAGCGCATGAACGTCTACTTCAACGAGGTACGTTCCCTTCCTACACCATCCTGTGATATATT CTCCACGCCTCCAAAGACCTCGACACTAATTTCCGACCCCCTTTAGGCCAGCGGAAACAAGTATGTTCCTCGTGCCGTCCTC GTTGACCTTGAGCCCGGTACCATGGACGCCGTCCGTGCCGGTCCCTTCGGTCAGCTCTTCCGTCCCGACAACTTCGTCTTCG GCCAGTCTGGTGCCGGTAACAACTGG
		Coding region	ATGGAAAACAGCTTCAATGGCTCCTCCGACCTCCAGCTCGAGCGCATGAACGTCTACTTCAACGAGGTACGT TCCCTTCCTACACCATCCTGTGATAGATTCTCCACGCCTCCAAAGACCTCGACACTAATTTCCGACCCCCTT TAG
		Protein sequence	MENSFNGSSDLQLERMNVYFNEVRSLPTPSCDRFSTPPKTSTLISDPL
79-045 *Aspergillus fumigatus*	Too short to bank	**Gene sequence**	ATGGTGTTTGCATTGAGCAGAAGCTAAACTTGATTCTTGGTGACAGGGTAACCAAATTGGTGCCGCTTTCTGGTATGTCTTG ACCTCAAAGCTTGGATGACGGGTGATTGGGATCTCTCATCTTAGCAGGCTACCTCCATGGGTTCAGCCTCACTGTCATGGGT ATCAGCTAACAAATCTACAGGCAGACCATCTCTGGTGAGCATGGCCTTGACGGCTCTGGCCAGTAAGTTCGACCTATATCCT CCCAATTGAGAAAGCGGCGGAAACACGGAAAACAAGGAAGAAGCGGACGCGTGTCTGATGGGAAATAATAGCTACAATGGCT CCTCCGATCTCCAGCTGGAGCGTATGAACGTCTATTTCAACGAGGTGTGTGGATGAAACTCTTGATTTATACTATTTCGGCA ACATCTCACGATCTGACTCGCTACTAGGCCAACGGTGACAAATATGTTCCTCGTGCCGTTCTGGTCGATCTCGAGCCTGGTA CCATGGACGCTGTCCGTGCCGGTCCCTTCGGCGAGCTATTCCGTCCCGACAACTTCGTCTTCGGCCAGTCCGGTGCTGGTAA CAACTGGGCCAAGGGTCACTACACCGAGGGCGCCGAGTTGGTCGACCAGGTTATCGATGTCGTCCGTCGTGAGGCTGAAGGC TGTGACTGCCTCCAGGGCTTCCAGGTCACCCACTCTCTCGGTGGTGGTACCGGTGCCGGTATGG GTACTCTTCTGATCTCCAAGATCCGTG
		Coding region	ATGGACGCTGTCCGTGCCGGTCCCTTCGGCGAGCTATTCCGTCCCGACAACTTCGTCTTCGGCCAGTCCGGT GCTGGTAACAACTGGGCCAAGGGTCACTACACCGAGGGCGCCGAGTTGGTCGACCAGGTTATCGATGTCGTC CGTCGTGAGGCTGAAGGCTGTGACTGCCTCCAGGGCTTCCAGGTCACCCACTCTCTCGGTGGTGGTACCGGT GCCGGTATGGGTACTCTTCTGATCTCCAAGATCCGT

		Protein sequence	MDAVRAGPFGELFRPDNFVFGQSGAGNNWAKGHYTEGAELVDQVIDVVRREAEGCDCLQGFQVTHSLGGGTGAGMGTLLISKIR
99-078 *Aspergillus fumigatus*	MZ044669	**Gene sequence**	CGGTGCCGCTTTCTGGTATGTCTTGACCTCAAAGCTTGGATGACGGGTGATTGGGATCTCTCATCTTAGCAGGCTACCTCCA TGGGTTCAGCCTCACTGTCATGGGTATCAGCTAACAAATCTACAGGCAGACCATCTCTGGTGAGCATGGCCTTGACGGCTCT GGCCAGTAAGTTCGACCTATATCCTCCCAATTGAGAAAGCGGCGGAAACACGGAAAACAAGGAAGAAGCGGACGCGTGTCTG ATGGGAAATAATAGCTACAATGGCTCCTCCGATCTCCAGCTGGAGCGTATGAACGTCTATTTCAACGAGGTGTGTGGATGAA ACTCTTGATTTATACTATTTCGGCAACATCTCACGATCTGACTCGCTACTAGGCCAACGGTGACAAATATGTTCCTCGTGCC GTTCTGGTCGATCTCGAGCCTGGTACCATGGACGCTGTCCGTGCCGGTCCCTTCGGCGAGCTATTCCGTCCCGACAACTTCG TCTTCGGCCAGTCCGGTGCTGGTAACAACTGGGCCAAGGGTCACTACACCGAGGGCGCCGAGTTGGTCGACCAGGTTATCGA TGTCGTCCGTCGTGAGGCTGAAGGCTGTGACTGCCTCCAGGGCTTCCAGGTCACCCACTCTCTCGGTGGTGGTACCGGTGCC GGTATGGGTACTCTTCTGATCTCCAAGATCCGTGAGGAGTTCCCCGACCGTATGATGGCGACCTTCTCCGTTGTTCCCTCCC CCAAGGTTTCCGACACTGTCGTTGA
		Coding region	ATGGACGCTGTCCGTGCCGGTCCCTTCGGCGAGCTATTCCGTCCCGACAACTTCGTCTTCGGCCAGTCCGGT GCTGGTAACAACTGGGCCAAGGGTCACTACACCGAGGGCGCCGAGTTGGTCGACCAGGTTATCGATGTCGTC CGTCGTGAGGCTGAAGGCTGTGACTGCCTCCAGGGCTTCCAGGTCACCCACTCTCTCGGTGGTGGTACCGGT GCCGGTATGGGTACTCTTCTGATCTCCAAGATCCGTGAGGAGTTCCCCGACCGTATGATGGCGACCTTCTCC GTTGTTCCCTCCCCCAAGGTTTCCGACACTGTCGTTGAGCCTTACAACGCTACCCTCTCCGTTCACCAGCTC GTTGAGCACTCCGATGAG
		Protein sequence	MDAVRAGPFGELFRPDNFVFGQSGAGNNWAKGHYTEGAELVDQVIDVVRREAEGCDCLQGFQVTHSLG GGTGAGMGTLLISKIREEFPDRMMATFSVVPSPKVSDTVVEPYNATLSVHQLVEHSDE
102-015 *Aspergillus fumigatus*	MZ044670	**Gene sequence**	GAAGCTAAACTTGATTCTTGGTGACAGGGTAACCAAATTGGTGCCGCTTTCTGGTATGTCTTGACCTCAAAGCTTGGATGA CGGGTGATTGGGATCTCTCATCTTAGCAGGCTACCTCCATGGGTTCAGCCTCACTGTCATGGGTATCAGCTAACAAATCTA CAGGCAGACCATCTCTGGTGAGCATGGCCTTGACGGCTCTGGCCAGTAAGTTCGACCTATATCCTCCCAATTGAGAAAGCG GCGGAAACACGGAAAACAAGGAAGAAGCGGACGCGTGTCTGATGGGAAATAATAGCTACAATGGCTCCTCCGATCTCCAGC TGGAGCGTATGAACGTCTATTTCAACGAGGTGTGTGGATGAAACTCTTGATTTATACTATTTCGGCAACATCTCACGATCT GACTCGCTACTAGGCCAACGGTGACAAATATGTTCCTCGTGCCGTTCTGGTCGATCTCGAGCCTGGTACCATGGACGCTGT CCGTGCCGGTCCCTTCGGCGAGCTATTCCGTCCCGACAACTTCGTCTTCGGCCAGTCCGGTGCTGGTAACAACTGGGCCAA GGGTCACTACACCGAGGGCGCCGAGTTGGTCGACCAGGTTATCGATGTCGTCCGTCGTGAGGCTGAAGGCTGTGACTGCCT CCAGGGCTTCCAGGTCACCCACTCTCTCGGTGGTGGTACCGGTGCCGGTATGGGTACTCTTCTGATCTCCAAGATCCGTGA GGAGTTCCCCGACCGTATGATGGCGACCTTCTCCGTTGTTCCCTCCCCCAAGGTTTCCGACACTGTCGTTGAGCCTTACAA CGCTAC
		Coding region	ATGGACGCTGTCCGTGCCGGTCCCTTCGGCGAGCTATTCCGTCCCGACAACTTCGTCTTCGGCCAGTCCGGT GCTGGTAACAACTGGGCCAAGGGTCACTACACCGAGGGCGCCGAGTTGGTCGACCAGGTTATCGATGTCGTC CGTCGTGAGGCTGAAGGCTGTGACTGCCTCCAGGGCTTCCAGGTCACCCACTCTCTCGGTGGTGGTACCGGT GCCGGTATGGGTACTCTTCTGATCTCCAAGATCCGTGAGGAGTTCCCCGACCGTATGATGGCGACCTTCTCC GTTGTTCCCTCCCCCAAGGTTTCCGACACTGTCGTTGAGCCTTACAACGCTACCCTCTCCGTTCACCAGCTC GTTGAGCACTCCGATGAG
		Protein sequence	MDAVRAGPFGELFRPDNFVFGQSGAGNNWAKGHYTEGAELVDQVIDVVRREAEGCDCLQGFQVTHSLG GGTGAGMGTLLISKIREEFPDRMMATFSVVPSPKVSDTVVEPYNATLSVHQLVEHSDE

103-057 *Aspergillus fumigatus*	MZ044671	**Gene sequence**	TGGTGCCGCTTTCTGGTATGTCTTGACCTCAAAGCTTGGATGACGGGTGATTGGGATCTCTCATCTTAGCAGGCTATCCTC CATGGGTTCTGCGTCCGTGTCATGGGTATCAGCTAACAAATCTACAGGCAGACCATCTCTGGTGAGCATGGCCTTGACGGC TCTGGCCAGTAAGTTCGACCTATATCCTCCCAATTGAGAAAGCGGCGGAAACACGGATCGGCAAGAAGGAAGCGGACGCGT GTCTGATGGGAAATAATAGCTACAATGGCTCCTCCGATCTCCAGCTGGAGCGTATGAACGTCTATTTCAACGAGGTGTGTG GATGAAACTCTTGATTTATACTATTTCGGCAACATCTCACGATCTGACTCGCTACTAGGCCAACGGTGACAAATATGTTCC TCGTGCCGTTCTGGTCGATCTCGAGCCTGGTACCATGGACGCTGTCCGTGCCGGCTCCCTTCGGCGAGCTATTCCGTCCCG ACAACTTCGTCTTCGGCCAGTCCGGTGCTGGTAACAACTGGGCCAAGGGTCACTACACCGAGGGCGCCGAGTTGGTCGACC AGGTTATCGATGTCGTCCGTCGTGAGGCTGAAGGCTGTGACTGCCTCCAGGGCTTCCAGGTCACCCACTCTCTCGGTGGTG GTACCGGTGCCGGTATGGGTACTCTTCTGATCTCCAAGATCCGTGAGGAGTTCCCCGACCGTATGATGGCGACCTTCTCCG TTGTTCCCTCCCCCAAGGTTTCCGACACTGTCGTTGAGCCTTACAAC
		Coding region	ATGGACGCTGTCCGTGCCGGTCCCTTCGGCGAGCTATTCCGTCCCGACAACTTCGTCTTCGGCCAGTCCGGT GCTGGTAACAACTGGGCCAAGGGTCACTACACCGAGGGCGCCGAGTTGGTCGACCAGGTTATCGATGTCGTC CGTCGTGAGGCTGAAGGCTGTGACTGCCTCCAGGGCTTCCAGGTCACCCACTCTCTCGGTGGTGGTACCGGT GCCGGTATGGGTACTCTTCTGATCTCCAAGATCCGTGAGGAGTTCCCCGACCGTATGATGGCGACCTTCTCC GTTGTTCCCTCCCCCAAGGTTTCCGACACTGTCGTTGAGCCTTACAACGCTACCCTCTCCGTTCACCAGCTC GTTGAGCACTCCGATGAG
		Protein sequence	MDAVRAGPFGELFRPDNFVFGQSGAGNNWAKGHYTEGAELVDQVIDVVRREAEGCDCLQGFQVTHSLG GGTGAGMGTLLISKIREEFPDRMMATFSVVPSPKVSDTVVEPYNATLSVHQLVEHSDE

Table 8.3: *Aspergillus* spp sequences based on Calmodulin (*CaM*) gene sequencing

Isolate name & number	Assigned GB accession no.	Calmodulin (*CaM*) gene sequences with partial coding sequence (CDS) & corresponding protein sequence	
70-086 *Aspergillus niger*	MZ044677	**Gene sequence**	CTGCCTGGCATTCTCAACAGGATTGCTGACATCGCATCGTCTAGGCCGACTCTCTGACCGAAGAGCAAGTTT CTGAGTACAAGGAGGCCTTCTCCCTCTTTGTGAGTGCTCCCTGAATGACCCTCCGATCATCCTGATCGATGA GCTATCTTTACCGGAGCATAATGCTAATGTGTTTTCGGACTTAATAGGACAAGGATGGCGATGGTGGGTGGA ATTCTATCCCCTTCACATTTTACCTGTAGCGCTCGATCCGACCGCGGGATTTCGACAGCATTTCTCAGAATT ATTTGGATCATAATACTAATTTAATCGGTGAATCAGGCCAGATCACCACCAAGGAGCTCGGCACTGTGATGC GCTCCCTTGGCCAGAACCCCTCCGAGTCTGAGCTTCAGGACATGATCAACGAGGTTGACGCTGACAACAACG GAACGATCGACTTCCCCGGTATGTGTTAGATTTACGCCTGTAAGGCGGAAATGCGGGCTGGATTGTGATTGA CTTTTGCCGCCAGAATTCCTTACCATGATGGCTCGTAAGATGAAGGACACCGACTCCGAGGAGGAAATCCGC GAGGCTTTCAAGGTCTTTGACCGCGACAACAATGGTTTTATCTCCGCCGCGGAGCTGCGCCACGTCATGACC TCCATTGGCGAGAAGCTCACCGACGACGAAGTCGATGAGATGATCCGTGAGGCGGACCAGGACGGTGATGGC CGCATCGACTGTATGTTTACCATGCCCGATTATACTCATATCATAACATACTGA
		Coding region	ATGATGGCTCGTAAGATGAAGGACACCGACTCCGAGGAGGAAATCCGCGAGGCTTTCAAGGTCTTTGACCGC GACAACAATGGTTTTATCTCCGCCGCGGAGCTGCGCCACGTCATGACCTCCATTGGCGAGAAGCTCACCGAC GACGAAGTCGATGAGATGATCCGTGAGGCGGACCAGGACGGTGATGGCCGCATCGACTGTATGTTTACCATG CCCGATTATACTCATATCATAACATACTGA
		Protein sequence	MMARKMKDTDSEEEIREAFKVFDRDNNGFISAAELRHVMTSIGEKLTDDEVDEMIREADQDGDGRIDCMFTMPDYTHIITY
71-003 *Aspergillus niger*	MZ044678	**Gene sequence**	CTGCCTGGCATTCTCAACAGGATTGCTGACATCGCATCGTCTAGGCCGACTCTCTGACCGAAGAGCAAGTTT CTGAGTACAAGGAGGCCTTCTCCCTCTTTGTGAGTGCTCCCTGAATGACCCTCCGATCATCCTGATCGATGA GCTATCTTTACCGGAGCATAATGCTAATGTGTTTTCGGACTTAATAGGACAAGGATGGCGATGGTGGGTGGA ATTCTATCCCCTTCACATTTTACCTGTAGCGCTCGATCCGACCGCGGGATTTCGACAGCATTTCTCAGAATT ATTTGGATCATAATACTAATTTAATCGGTGAATCAGGCCAGATCACCACCAAGGAGCTCGGCACTGTGATGC GCTCCCTTGGCCAGAACCCCTCCGAGTCTGAGCTTCAGGACATGATCAACGAGGTTGACGCTGACAACAACG GAACGATCGACTTCCCCGGTATGTGTTAGATTTACGCCTGTAAGGCGGAAATGCGGGCTGGATTGTGATTGA CTTTTGCCGCCAGAATTCCTTACCATGATGGCTCGTAAGATGAAGGACACCGACTCCGAGGAGGAAATCCGC GAGGCTTTCAAGGTCTTTGACCGCGACAACAATGGTTTTATCTCCGCCGCGGAGCTGCGCCACGTCATGACC TCCATTGGCGAGAAGCTCACCGACGACGAAGTCGATGAGATGATCCGTGAGGCGGACCAGGACGGTGATGGC CGCATCGACTGTATGTTTACCATGCCCGATTATACTCATATCATAACATACTGA
		Coding region	ATGATGGCTCGTAAGATGAAGGACACCGACTCCGAGGAGGAAATCCGCGAGGCTTTCAAGGTCTTTGACCGC GACAACAATGGTTTTATCTCCGCCGCGGAGCTGCGCCACGTCATGACCTCCATTGGCGAGAAGCTCACCGAC GACGAAGTCGATGAGATGATCCGTGAGGCGGACCAGGACGGTGATGGCCGCATCGACTGTATGTTTACCATG CCCGATTATACTCATATCATAACATACTGA
		Protein sequence	MMARKMKDTDSEEEIREAFKVFDRDNNGFISAAELRHVMTSIGEKLTDDEVDEMIREADQDGDGRIDCMFTMPDYTHIITY
72-3889 *Aspergillus niger*	MZ044679	**Gene sequence**	CTGCCTGGCATTCTCAACAGGATTGCTGACATCGCATCGTCTAGGCCGACTCTCTGACCGAAGAGCAAGTTT CTGAGTACAAGGAGGCCTTCTCCCTCTTTGTGAGTGCTCCCTGAATGACCCTCCGATCATCCTGATCGATGA

			GCTATCTTTACCGGAGCATAATGCTAATGTGTTTTCGGACTTAATAGGACAAGGATGGCGATGGTGGGTGGA ATTCTATCCCCTTCACATTTTACCTGTAGCGCTCGATCCGACCGCGGGATTTCGACAGCATTTCTCAGAATT ATTTGGATCATAATACTAATTTAATCGGTGAATCAGGCCAGATCACCACCAAGGAGCTCGGCACTGTGATGC GCTCCCTTGGCCAGAACCCCTCCGAGTCTGAGCTTCAGGACATGATCAACGAGGTTGACGCTGACAACAACG GAACGATCGACTTCCCCGGTATGTGTTAGATTTACGCCTGTAAGGCGGAAATGCGGGCTGGATTGTGATTGA CTTTTGCCGCCAGAATTCCTTACCATGATGGCTCGTAAGATGAAGGACACCGACTCCGAGGAGGAAATCCGC GAGGCTTTCAAGGTCTTTGACCGCGACAACAATGGTTTTATCTCCGCCGCGGAGCTGCGCCACGTCATGACC TCCATTGGCGAGAAGCTCACCGACGACGAAGTCGATGAGATGATCCGTGAGGCGGACCAGGACGGTGATGGC CGCATCGACTGTATGTTTACCATGCCCGATTATACTCATATCATAACATACTGA
		Coding region	ATGATGGCTCGTAAGATGAAGGACACCGACTCCGAGGAGGAAATCCGCGAGGCTTTCAAGGTCTTTGACCGC GACAACAATGGTTTTATCTCCGCCGCGGAGCTGCGCCACGTCATGACCTCCATTGGCGAGAAGCTCACCGAC GACGAAGTCGATGAGATGATCCGTGAGGCGGACCAGGACGGTGATGGCCGCATCGACTGTATGTTTACCATG CCCGATTATACTCATATCATAACATACTGA
		Protein sequence	MMARKMKDTDSEEEIREAFKVFDRDNNGFISAAELRHVMTSIGEKLTDDEVDEMIREADQDGDGRIDCMFTMPDYTHIITY
100-086 *Aspergillus niger*	MZ044680	**Gene sequence**	CTGCCTGGCATTCTCAACAGGATTGCTGACATCGCATCGTCTAGGCCGACTCTCTGACCGAAGAGCAAGTTT CTGAGTACAAGGAGGCCTTCTCCCTCTTTGTGAGTGCTCCCTGAATGACCCTCCGATCATCCTGATCGATGA GCTATCTTTACCGGAGCATAATGCTAATGTGTTTTCGGACTTAATAGGACAAGGATGGCGATGGTGGGTGGA ATTCTATCCCCTTCACATTTTACCTGTAGCGCTCGATCCGACCGCGGGATTTCGACAGCATTTCTCAGAATT ATTTGGATCATAATACTAATTTAATCGGTGAATCAGGCCAGATCACCACCAAGGAGCTCGGCACTGTGATGC GCTCCCTTGGCCAGAACCCCTCCGAGTCTGAGCTTCAGGACATGATCAACGAGGTTGACGCTGACAACAACG GAACGATCGACTTCCCCGGTATGTGTTAGATTTACGCCTGTAAGGCGGAAATGCGGGCTGGATTGTGATTGA CTTTTGCCGCCAGAATTCCTTACCATGATGGCTCGTAAGATGAAGGACACCGACTCCGAGGAGGAAATCCGC GAGGCTTTCAAGGTCTTTGACCGCGACAACAATGGTTTTATCTCCGCCGCGGAGCTGCGCCACGTCATGACC TCCATTGGCGAGAAGCTCACCGACGACGAAGTCGATGAGATGATCCGTGAGGCGGACCAGGACGGTGATGGC CGCATCGACTGTATGTTTACCATGCCCGATTATACTCATATCATAACATACTGA
		Coding region	ATGATGGCTCGTAAGATGAAGGACACCGACTCCGAGGAGGAAATCCGCGAGGCTTTCAAGGTCTTTGACCGC GACAACAATGGTTTTATCTCCGCCGCGGAGCTGCGCCACGTCATGACCTCCATTGGCGAGAAGCTCACCGAC GACGAAGTCGATGAGATGATCCGTGAGGCGGACCAGGACGGTGATGGCCGCATCGACTGTATGTTTACCATG CCCGATTATACTCATATCATAACATACTGA
		Protein sequence	MMARKMKDTDSEEEIREAFKVFDRDNNGFISAAELRHVMTSIGEKLTDDEVDEMIREADQDGDGRIDCMFTMPDYTHIITY

8.4.2 Phylogenetic relationship of *Aspergillus* spp based on *β*-tubulin gene

Phylogenetic analysis of 11 *β*-tubulin gene sequence revealed significant relatedness of *Aspergillus* spp. from sputum to isolates in NCBI database comprising of *A. terreus*, *A. fumigatus*, *A. hortai, A. oryzae* and *A. flavus* (Figure 8.1). *Aspergillus* spp from the study and reference species from Genebank clustered along three phylogenetic clades. See figure 8.1.

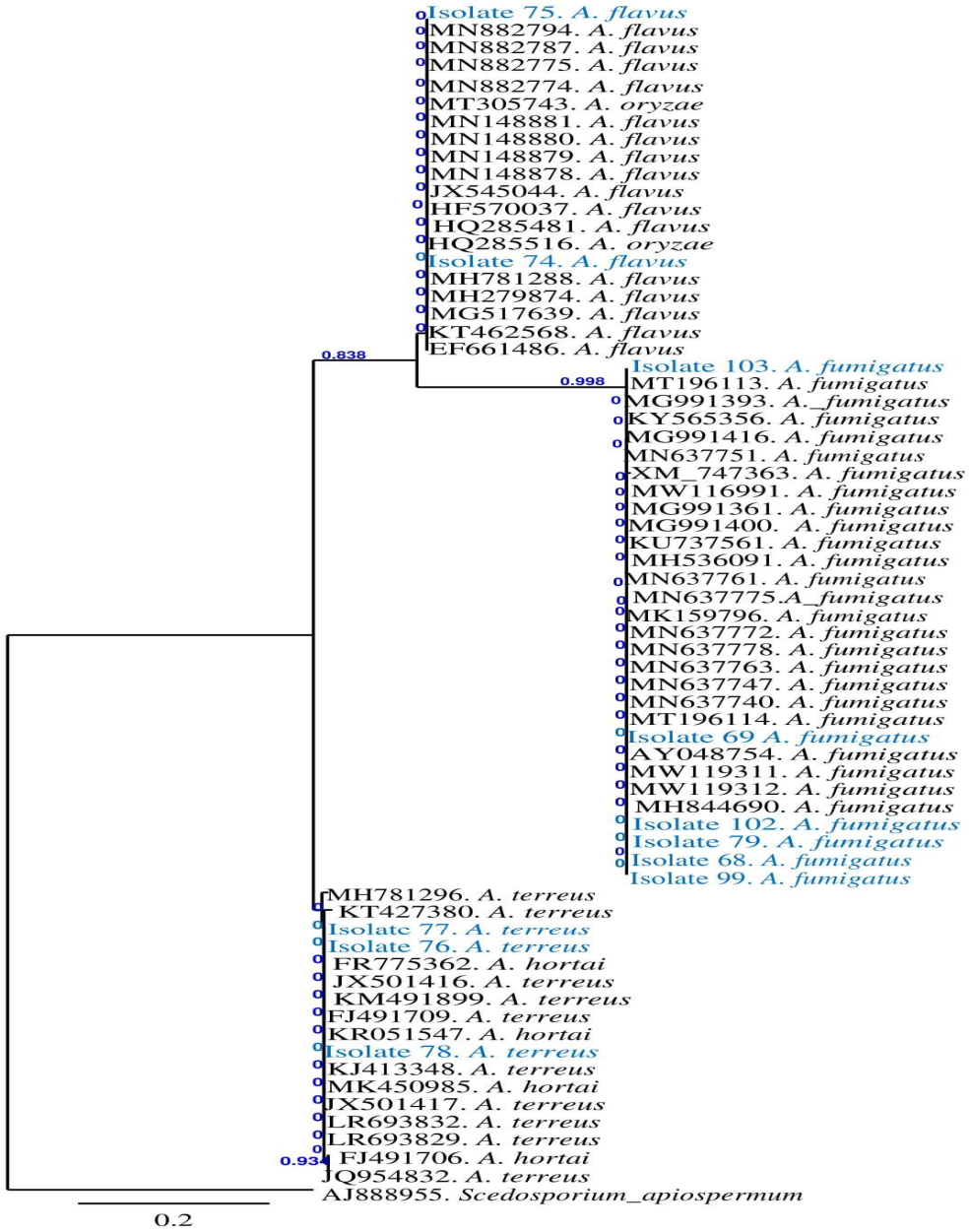

Figure 8.1: Phylogenetic tree of *Aspergillus* spp isolates constructed using partial sequences of *Beta tubulin* gene. The analysis involved 11 nucleotide sequences from the study indicated in blue and 56 others obtained from Genebank. Genebank accession numbers for reference isolates are indicated on the tree respectively. The tree is drawn to scale, with branch lengths measured according to number of substitutions per site. *Scedosporium aspiospermum* was used as the outgroup.

8.4.3 *Aspergillus* spp pairwise identity matches based of based on *β-tubulin* gene

Pairwise identity matches for *Aspergillus* spp sequences performed using clustal omega in the study, revealed a similarity index of 99% to 100% compared to references in NCBI database. *A. terreus* (isolate no. 70) showed similarity of 99.8% *to A. terreus* accession number JX501416 (Table 8.4). On the other hand, *A. flavus* isolate number 74 exhibited 100% similarity to *A. flavus* with accession number MH 701200. Majority of *A. fumigatus* sequences from the study showed 100% similarity to reference *A. fumigatus* species in the database. Three of the isolates (numbers. 69, 79 & 102) had 100% similarity to *A. fumigatus* with accession number MH844690 (Table 8.4).

Table 8.4: Pairwise identity matches of *Aspergillus* spp isolates from sputum based on *β*-tubulin gene sequencing

	Isolate number and GB accesion reference																
	KM491899 *A.terreus*	Isolate 76. *A.terreus*	Isolate 77. *A.terreus*	JX501416 *A.terreus*	Isolate 78. *A.terreus*	Isolate 74. *A.flavus*	MH781288 *A.flavus*	MH279874. *A.flavus*	Isolate 75. *A.flavus*	Isoalte 103. A. *fumigatus*	MT196113. A. *fumigatus*	Isoalte 99. A. *fumigatus*	Isolate 68. *A. fumigatus*	Isolate 69. *A. fumigatus*	Isolate 79. *A. fumigatus*	Isolate 102. *A. fumigatus*	MH844690. *A. fumigatus*
KM491899 *A.terreus*	100.0	98.9	98.7	98.3	99.2	63.8	65.0	65.5	64.0	63.9	64.0	63.8	63.2	63.1	63.8	63.8	63.8
Isolate 76. *A.terreus*	98.9	100.0	100.0	100.0	99.8	64.2	64.2	64.4	64.4	62.9	62.9	62.9	62.9	63.0	62.9	62.9	62.9
Isolate 77. *A.terreus*	98.7	100.0	100.0	100.0	99.8	64.2	64.2	64.3	64.4	62.8	62.8	62.8	62.8	63.0	62.8	62.8	62.8
JX501416 *A.terreus*	98.3	100.0	100.0	100.0	99.8	64.2	64.3	64.4	64.4	62.7	62.9	62.7	62.8	63.0	62.7	62.7	62.7
Isolate 78. *A.terreus*	99.2	99.8	99.8	99.8	100.0	64.2	64.2	64.2	64.4	62.7	62.6	62.6	62.6	62.6	62.6	62.6	62.6
Isolate 74. *A.flavus*	63.8	64.2	64.2	64.2	64.2	100.0	100.0	100.0	99.8	66.6	66.3	66.3	66.3	66.3	66.3	66.3	66.3
MH781288 *A.flavus*	65.0	64.2	64.2	64.3	64.2	100.0	100.0	100.0	99.8	67.5	67.4	67.2	66.7	66.7	67.2	67.2	67.2
MH279874. *A.flavus*	65.5	64.4	64.3	64.4	64.2	100.0	100.0	100.0	99.8	68.0	68.0	67.8	67.2	67.1	67.8	67.8	67.8
Isolate 75. *A.flavus*	64.0	64.4	64.4	64.4	64.4	99.8	99.8	99.8	100.0	66.6	66.3	66.3	66.3	66.3	66.3	66.3	66.3
Isoalte 103. *A. fumigatus*	63.9	62.9	62.8	62.7	62.7	66.6	67.5	68.0	66.6	100.0	97.8	98.0	98.1	98.1	98.2	98.2	98.2
MT196113. *A. fumigatus*	64.0	62.9	62.8	62.9	62.6	66.3	67.4	68.0	66.3	97.8	100.0	99.8	100.0	100.0	99.6	99.6	99.6
Isoalte 99. *A. fumigatus*	63.8	62.9	62.8	62.7	62.6	66.3	67.2	67.8	66.3	98.0	99.8	100.0	100.0	100.0	99.8	99.8	99.8
Isolate 68. *A. fumigatus*	63.2	62.9	62.8	62.8	62.6	66.3	66.7	67.2	66.3	98.1	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Isolate 69. *A. fumigatus*	63.1	63.0	63.0	63.0	62.6	66.3	66.7	67.1	66.3	98.1	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Isolate 79. *A. fumigatus*	63.8	62.9	62.8	62.7	62.6	66.3	67.2	67.8	66.3	98.2	99.6	99.8	100.0	100.0	100.0	100.0	100.0
Isolate 102. *A. fumigatus*	63.8	62.9	62.8	62.7	62.6	66.3	67.2	67.8	66.3	98.2	99.6	99.8	100.0	100.0	100.0	100.0	100.0
MH844690. *A. fumigatus*	63.8	62.9	62.8	62.7	62.6	66.3	67.2	67.8	66.3	98.2	99.6	99.8	100.0	100.0	100.0	100.0	100.0

NB: Percentage similarity matrix based on β-tubulin gene sequence created by clustal Omega 2.1, GB- Gene Bank

8.4.4 Phylogenetic relationship of *Aspergillus* spp based *Calmodulin* gene

Comparison of four *Aspergillus niger* nucleotide sequences from the study and 17 others obtained from Genebank showed substantial relatedness evidenced by clustering on same clades (Figure 8.2). The analysis revealed a unique relationship between *A. niger* and *A. welwitschiae.* See figure 8.2.

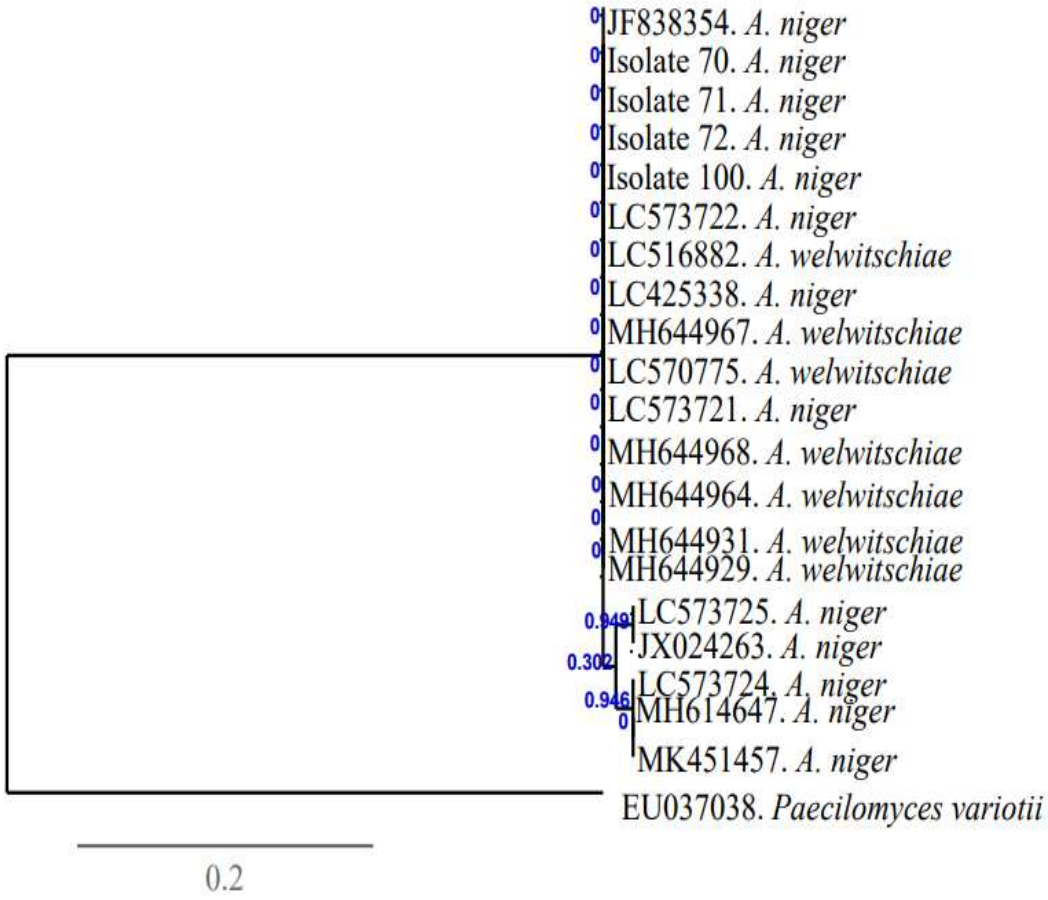

Figure 8.2: Phylogenetic tree of *Aspergillus* spp isolates constructed using partial sequences of calmodulin gene**.** The tree is drawn to scale, with branch lengths measured according to number of substitutions per site. *Paecillomyces variotii* was used as the outgroup. The respective Genebank accession numbers for reference strains are indicated in the tree along with isolate numbers of *A. niger* from the study.

8.4.5 *Aspergillus* spp pairwise identity matches based of based on *Calmodulin* gene

Pairwise identity matches for *A. niger calmodulin* gene sequencing exhibited 100% similarity to references species in NCBI database based clustal omega. *A. niger* isolate number 70 & 71 showed 100% similarity to *A. niger* with NCBI accession number LC573721 while the analysis also revealed 100% similarity for *A. niger* isolate number 72 & 100 to reference *A. niger* with designated accession number LC573722 (Table 8.5). Figure 8.3 shows some multiple sequence alignment performed on sequences from the study compared with reference sequences from the NCBI database.

Table 8.5: Pairwise identity matches of *Aspergillus niger* isolates from sputum based on Calmodulin *(CaM)* gene sequencing

	Isolate number and GB accesion reference											
	MK451457. *A. niger*	LC573724. *A. niger*	LC573725. *A. niger*	JX024263. *A. niger*	JF838354. *A. niger*	Isolate 70 *A. niger*	Islolate 71 *A. niger*	Isolate 72 *A. niger*	Isolate 100 *A. niger*	LC573722 *A. niger*	LC425338 *.A. niger*	LC573721. *A. niger*
MK451457. *A. niger*	100.0	100.0	98.7	98.7	98.9	98.9	98.9	98.9	98.9	98.9	98.9	98.7
LC573724. *A. niger*	100.0	100.0	98.8	98.8	98.9	99.0	99.0	99.0	99.0	99.0	99.0	98.8
LC573725. *A. niger*	98.7	98.8	100.0	100.0	98.8	99.0	99.0	99.0	99.0	99.0	99.0	98.9
JX024263. *A. niger*	98.7	98.8	100.0	100.0	98.8	99.0	99.0	99.0	99.0	99.0	99.0	98.9
JF838354. *A. niger*	98.9	98.9	98.8	98.8	100.0	99.9	99.9	99.9	99.9	99.9	99.9	99.7
Isolate 70 *A. niger*	98.9	99.0	99.0	99.0	99.9	100.0	100.0	100.0	100.0	100.0	100.0	99.9
Islolate 71 *A. niger*	98.9	99.0	99.0	99.0	99.9	100.0	100.0	100.0	100.0	100.0	100.0	99.9
Isolate 72 *A. niger*	98.9	99.0	99.0	99.0	99.9	100.0	100.0	100.0	100.0	100.0	100.0	99.9
Isolate 100 *A. niger*	98.9	99.0	99.0	99.0	99.9	100.0	100.0	100.0	100.0	100.0	100.0	99.9
LC573722. *A. niger*	98.9	99.0	99.0	99.0	99.9	100.0	100.0	100.0	100.0	100.0	100.0	99.9
LC425338. *A. niger*	98.9	99.0	99.0	99.0	99.9	100.0	100.0	100.0	100.0	100.0	100.0	99.9
LC573721.*A. niger*	98.7	98.8	98.9	98.9	99.7	99.9	99.9	99.9	99.9	99.9	99.9	100.0

NB: Percentage similarity matrix based on Calmodulin gene sequence created by clustal Omega 2.1, GB- Gene Bank

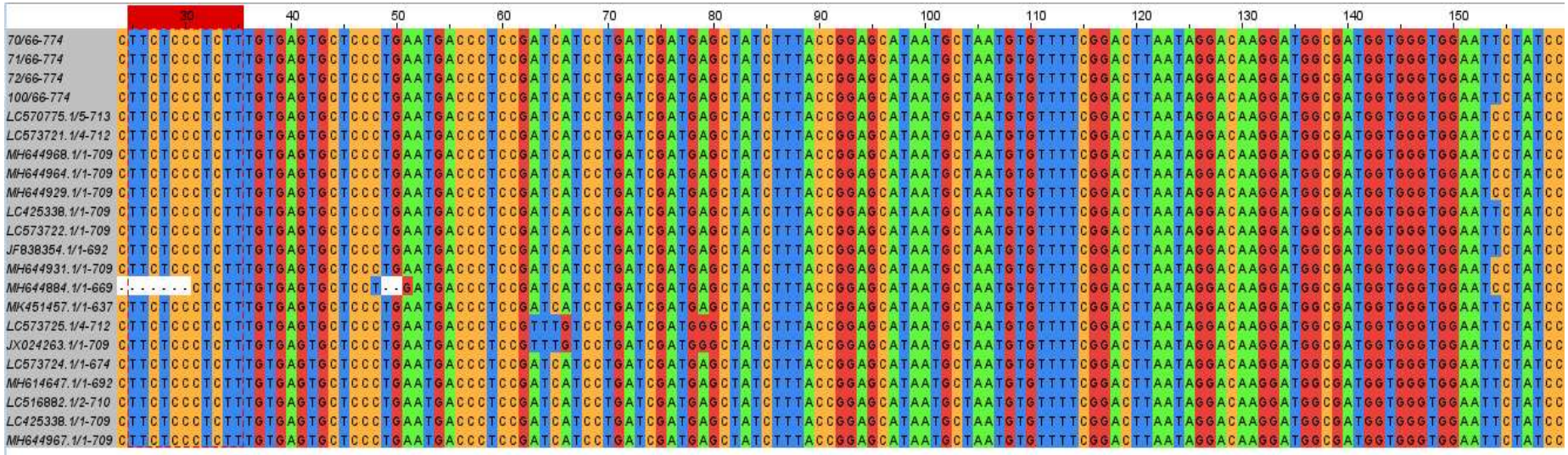

Figure 8.3: Multiple sequence alignment of some *Aspergillus* spp isolates from the study with reference sequences in Genebank. Isolate identities are indicated on the left part of the figure.

8.5 Discussion

Phylogenetic analysis of sequences from *Aspergillus* spp revealed substantial relationship with isolates in Genebank designated by respective accession numbers. Based on *β*-tubulin gene sequences, isolates from the study were clustered with related species in three phylogenetic clades representing *A. flavus*, *A. fumigatus* and *A. terreus*, respectively. Previous studies have reported a variety of distinct lineages for species previously thought to be *A. terreus* (Samson *et al*., 2011). In addition, the present study demonstrated clustering of *A. terreus* with *A. hortai* in the same clade. Phylogenetic analysis using *calmodulin* gene sequences revealed clustering in three phylogenetic clades. Distinctively, the analysis showed clustering of *A. niger* and *A. welwitchiae* on the same clade, demonstrating significant genetic relatedness. Clustal analysis & pairwise identity matches for *β*-tubulin and *calmodulin* gene sequences of *Aspergillus* spp from the study and those in Genebank showed similarity index ranging from 98.2% to 100%.

Molecular based PCR identification and sequencing of fungal pathogens are valuable as adjunct to phenotypic methods (culture based) of species identification. The turnaround time to identify agents of infection is greatly reduced with application of PCR methods. Some studies have demonstrated sensitivity ranging from 72 to 88% and of 75 to 98.7% of *Aspergillus* PCR assays (Imbert *et al*., 2018). However, it is essential to consider existing clinical information of a patient when interpreting PCR results. One study that examined the usefulness of various specimen reported that sputum and bronchial aspirates had a higher culture yield of *Aspergillus* and stronger quantitative PCR (qPCR) signals compared to broncho-alveolar lavage (BAL) (Fraczek *et al*., 2014). This suggests an important consideration on the choice of appropriate specimen for diagnosing aspergillosis.

Fungal genome sequencing and molecular epidemiological studies have contributed significantly to the investigation and analysis of emerging antifungal resistant *Aspergillus* spp and their association with human infection (van der Torre *et al*., 2021). Further, precise identification of pathogenic species facilitates epidemiological investigations and guides clinical management and control of pathogens (Alshehri *et al*., 2020). Findings from the current study demonstrate the genomic diversity of *Aspergillus* spp from sputum that could be of clinical relevance in TB retreatment patients. Previous studies have shown that genome sequencing can be valuable in distinguishing cryptic species of closely related *Aspergilli* that can be phenotypically similar (Arastehfar *et al*., 2021). It is also evident that such closely related species can have extremely different patterns of antifungal sensitivity particularly to amphotericin B and triazoles while some

exhibit intrinsic resistance. This can significantly influence antifungal treatment response and choice of therapy for infected patients.

Overall, *Aspergilli* identified in the study did show significant genetic variations as depicted by phylogenetic trees generated from *β*-tubulin and *calmodulin* gene sequencing. The reliability of molecular and sequencing technologies in diagnosis and characterization of fungal infections warrants adoption and increased utilization in idenfying clinical isolates of pathogens for beter management of patients.

CHAPTER NINE
GENERAL CONCLUSIONS AND RECOMMENDATIONS

9.1 Conclusions

The overall objective of the study was to determine the spectrum of potential fungal pathogens in TB relapse and retreatment patients and antifungal drug sensitivity. Chapter one introduces the background of the study highlighting previous key studies that have focused on the subject providing a basis for the current research. Chapter two, the literature review summarises various aspects regarding fungal diseases including: the burden of fungal infections and tuberculosis, clinical significance of *Aspergilli* and trends in emerging antifungal resistance. Chapter three explains the methods for data collection, participant recruitment, ethics approval, laboratory procedures and general flow of investigations in the study.

Chapter four describes the spectrum of filamentous fungi isolated from sputum of the participants. Mycological investigations revealed *Aspergillus* spp, *Penicillium* spp, *Paecillomyces* spp, *Mucor* spp and *Scedosporium* spp were the most predominant moulds. Similarly, chapter five captures diverse yeast isolates from sputum where *Candida albicans* was the most frequent yeast species. Other non albicans species such as *C. parapsilosis* & *C. lusitaniae* were also recovered. The moulds and yeasts identified in the study have been associated with severe fungal infections and could be of clinically importance in presumptive TB relapse and retreatment patients.

Chapter six focuses on the *in vitro* antifungal susceptibility of yeasts and moulds isolated from sputum samples of the relapse & retreatment patients. Fungi exhibiting high MICs to the antifungals tested were observed and they represent an imminent threat of antifungal resistance. However, the correlation of microbiological resistance and clinical outcome of patients is essential. Overall, a greater sensitivity to itraconazole and voriconazole was observed for moulds while the yeasts particularly *C. albicans* exhibited greater sensitivity to the four antifungal agents tested. Some species of fungi are intrinsically resistant to certain antifungals while some acquire resistance due to irrational use of these agents. Therefore, AST should be performed for all fungal isolates to guide appropriate therapy.

Chapter seven highlights the challenge of TB culture contamination that often hampers diagnsosis of TB and drug susceptibility testing. Diverse mould and yeast species were recovered from contaminated solid LJ cultures majority being *C. albicans*, *C. krusei* and *Rhizopus* spp. The study underscores the need for further investigation to ascertain the clinical importance of these presumed contaminants.

Chapter eight describes the genomic characterization of *Aspergillus* species from sputum of patients in the study. Beta tubulin & Calmodulin genes were sequenced using specific primers. Clustal analysis & pairwise identity matches for *β*-tubulin and *calmodulin* gene sequences of isolates revealed similarity index ranging from 98.2% to 100% with those Genebank suggesting close genetic relatedness.

Opportunistic fungal infections represent a significatnt threat to global health. The burden is on the rise due to an increasing population of immunocomprised individuals. COVID-19 is compounding the problem furher and urgent interventons are needed to mitigate the impact of fungal diseases that cause complications in patients. Generally, the study underscores the need to address the emerging problem of fungal pulmonary infections that are often missed or misdiagnosed as tuberculosis or disregarded as TB culture contaminants. A great deal of information remains to be discovered regarding fungal diseases and fundamentally the correlation of TB and other pulmonary disorders. As new strains of fungal pathogens emerge in the clinical environment, there is a need to embrace PCR and sequencing technology to identify and characterize unique species to guide patient management.

9.2 Limitations of the study

1. The study was confined to three reference facilities that were conveniently selected and not randomly sampled. Therefore the findings may not be representative of what is seen in other facilities.
2. The study was cross-sectional and therefore it does not allow for follow-up of these patients to see the effect of treatment on morbidity and mortality.
3. Exclusion of children in the study does not allow observations that can be seen in that age group.

9.3 Recommendations and future work

1. Fungal diseases remain a significant threat to global health. Based on findings, the study recommends screening of smear negative patients presenting with clinical symptoms suggestive of TB relapse for fungal infection or coinfection before retreatment.
2. Diagnosis of fungal infections is still a challenge in many settings particularly in developing countries such as Kenya. Therefore, there is need for investment in training and education of experts and to increase access to affordable fungal diagnostic platforms including molecular tools.
3. Antifungal drug resistance is emerging globally particularly to azoles. More studies should be carried out to further characterize resistant fungal pathogens and to identify related mechanisms and key drivers to prevent further spread and to maintain potency of antifungals.
4. The prevalence and burden of fungal infections in various populations is not elaborate. Further studies should be carried to establish the burden of fungal diseases to facilitate targeted interventions.
5. High frequency of fungal contamination in TB cultures could be a reflection of etiology in presumptive TB patients' pulmonary condition. There is therefore need for further investigations to ascertain their clinical role in lung disease.
6. The study carried out genomic characterization *Aspergillus* species that showed high correlation with reference strains in genebank. There is need for further genetic mapping of pulmonary fungal infections from presumptive TB patients targeting other gene regions including those responsible for antifungal drug resistance.

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Appendix 1: Research acknowledgement letter from the National TB Laboratory.

MINISTRY OF HEALTH
NATIONAL TUBERCULOSIS REFERENCE LABORATORY
P.O.BOX 20750-00202, NAIROBI.
KENYATTA NATIONAL HOSPITAL GROUNDS off NGONG ROAD

26/1/2018

To,
University of Nairobi (UoN),
College of Health Sciences,
Department of Medical Microbiology,
Kenyatta National Hospital Campus,
PO. Box 19676-00202,
Nairobi, Kenya.

Dear sir/madam,

RE: PhD research acknowledgement letter for Mr. Josephat Tonui

The National Tuberculosis Reference Laboratory (NTRL) has been experiencing an increased rate of TB sputum culture contamination. In light of this challenge, the Laboratory has sought to collaborate with the Kenya Medical Research Institute (KEMRI)-Mycology laboratory to investigate the source and identify pulmonary fungal pathogens which could be significant etiological agents among TB retreatment and relapse cases in Kenya.

I am therefore writing in support of the proposed study tittle: ***"Spectrum of pulmonary fungal pathogens and antifungal drug susceptibility among relapse and TB retreatment samples at selected health facilities in Kenya"***. Findings from the research will provide information that will help to mitigate these challenges and facilitate proper management of TB cases in the country.

Yours Faithfully

Nellie Njambi Mukiri

NATIONAL TUBERCULOSIS REFERENCE LABORATORY
TB FREE I SEE
26 JAN 2018
P O Box 20781-00202 NAIROBI

MANAGER-NATIONAL TUBERCULOSIS REFERENCE LABORATORY

ISO 9001:2008 Certified

Appendix 2: Letter of authorization to nest the study (P108/02/2018) under the parent study (KEMRI/SERU/0037/3213).

KENYA MEDICAL RESEARCH INSTITUTE

Centre for Microbiology Research, P.O. Box 19464 - 00202, NAIROBI - Kenya,
Tel: (254) (020) 2720794, 2720038, Nairobi E-mail: cmr@kemri.org, Website. www.kemri.org

15th October, 2018

The Secretary

Ethics KNH/UON ERC

RE: LETTER OF AUTHORIZATION TO NEST THE STUDY AND USE THE ISOLATES FOR PHD

This is to confirm that I have authorized Mr. Josephart Tonui to nest his study and use the isolates from our research project entitled; *Mapping antifungal, antibacterial drug resistance and the quality of antimicrobial agents against fungal and bacterial pathogens from smear negative and retreatment cases in high TB prevalence Counties (KEMRI/SERU/CMR/0037/3213)*

The project has been fully approved by KEMRI Scientific and Ethical Approval Unit and all the consenting and assenting was conducted as per the approved protocol. The work will be undertaken in Mycology Laboratory-KEMRI under my supervision. The study is being funded by KEMRI and Mr. Tonui has signed the KEMRI IP policy document.

Thank You

CENTRE FOR MICROBIOLOGY RESEARCH
P. O. Box 19464-00202,
NAIROBI

Dr. Christine Bii

Head: Mycology Oi Unit,

Center for Microbiology Research, KEMRI

Appendix 3: Ethics approval for the parent study (KEMRI/SERU/0037/3213)

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel: (254) (020) 2722541, 2713349, 0722-205901, 0733-400003, Fax: (254) (020) 2720030
E-mail: director@kemri.org, info@kemri.org, Website. www.kemri.org

KEMRI/RES/7/3/1 April 19, 2018

TO: **DR. CHRISTINE BII,**
PRINCIPAL INVESTIGATOR

THROUGH: **THE DIRECTOR, CMR,** Forwarded SK
NAIROBI

Dear Madam,

RE: KEMRI/SERU/CMR/0037/3213 (***RESUBMISSION-REQUEST FOR ANNUAL RENEWAL)*** **MAPPING ANTIFUNGAL ANTIBACTERIAL DRUG RESISTANCE AND THE QUALITY OF ANTIMICROBIAL AGENTS AGAINST FUNGAL AND BACTERIAL PATHOGENS FROM SMEAR NEGATIVE AND RETREATMENT CASES IN HIGH TB PREVALENCE COUNTIES IN KENYA**

Reference is made to your letter dated 16th April 2018. The KEMRI Scientific and Ethics Review Unit (SERU), acknowledges receipt of the revised application on 17th April 2018.

This is to inform you that the resubmitted request for annual renewal was reviewed, and it was determined that the issues raised have been adequately addressed.

This study is granted approval for continuation effective this day, **April 19, 2018.** Please note that authorization to conduct this study will automatically expire on **April 18, 2019**. If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the SERU secretariat by **March 14, 2019.**

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the SERU for review prior to initiation.

You may continue with the study.

Yours faithfully,

THE HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

Appendix 4: Approval of modifications to nest the present study under the parent study protocol.

UNIVERSITY OF NAIROBI
COLLEGE OF HEALTH SCIENCES
P O BOX 19676 Code 00202
Telegrams: varsity
(254-020) 2726300 Ext 44355

KNH-UoN ERC
Email: uonknh_erc@uonbi.ac.ke
Website: http://www.erc.uonbi.ac.ke
Facebook: https://www.facebook.com/uonknh.erc
Twitter: @UONKNH_ERC https://twitter.com/UONKNH_ERC

KENYATTA NATIONAL HOSPITAL
P O BOX 20723 Code 00202
Tel: 726300-9
Fax: 725272
Telegrams: MEDSUP, Nairobi

Ref: KNH-ERC/ Mod&SAE/434

December 18, 2018

Josephat Kipyegon Tonui
Reg. No.H80/52222/2017(PhD Candidate)
Dept.of Medical Microbiology
School of Medicine
College of Health Sciences
University of Nairobi

Dear Josephat

Re: Approval of modifications – study titled "Spectrum of pulmonary fungal pathogens and antifungal drug susceptibility among TB relapse and retreatment samples at selected health facilities in Kenya" (P108/02/2018)

Your communication received at the KNH-UoN ERC on 21st October 2018 refers.

The KNH-UoN ERC has reviewed your request to have your study nested in a KEMRI SERU approved study(KEMRI SERU/0037/ 3213). It has been noted that the principal Investigator of the main study also happens to be a supervisor of current study and has given permission for the nesting. All appropriate documents have been provided.

The following observations were made -

1. The suggested changes do not alter the study objectives or study populations.
2. The changes will not substantially affect the benefit/risk ratio in the earlier approved study. The well being of study participants will not be altered.

These changes are reflected in the amended protocol and are therefore **approved.**

Yours sincerely,

PROF. M.L. CHINDIA
SECRETARY, KNH-UON ERC

c.c. The Principal, College of Health Sciences, UoN
The Director CS, KNH
The Chairperson, KNH-UoN ERC

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Appendix 5: Ethics approval letter granted by KNH-UoN Ethics Review Committee for the study.

UNIVERSITY OF NAIROBI
COLLEGE OF HEALTH SCIENCES
P O BOX 19676 Code 00202
Telegrams: varsity
Tel:(254-020) 2726300 Ext 44355

KNH-UON ERC
Email: uonknh_erc@uonbi.ac.ke
Website: http://www.erc.uonbi.ac.ke
Facebook: https://www.facebook.com/uonknh.erc
Twitter: @UONKNH_ERC https://twitter.com/UONKNH_ERC

KENYATTA NATIONAL HOSPITAL
P O BOX 20723 Code 00202
Tel: 726300-9
Fax: 725272
Telegrams: MEDSUP, Nairobi

Ref: KNH-ERC/A/215

June 13, 2018

Josephat Kipyegon Tonui
Reg. No.H80/52222/2017
Dept.of Medical Microbiology
School of Medicine
College of Health Sciences
University of Nairobi

KENYATTA NATIONAL HOSPITAL APPROVED 13 JUN 2018 KNH/UoN-ERC P.O. Box 20723-00202 NAIROBI

Dear Josephat

RESEARCH PROPOSAL – SPECTRUM OF PULMONARY FUNGAL PATHOGENS AND ANTIFUNGAL DRUG SUSCEPTIBILITY AMONG TB RELAPSE AND RETREATMENT SAMPLES AT SELECTED HEALTH FACILITIES IN KENYA (P108/02/2018)

This is to inform you that the KNH- UoN Ethics & Research Committee (KNH- UoN ERC) has reviewed and **approved** your above research proposal. The approval period is from 13th June 2018 – 12th June 2019.

This approval is subject to compliance with the following requirements:

a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b) All changes (amendments, deviations, violations etc) are submitted for review and approval by KNH-UoN ERC before implementation.
c) Death and life threatening problems and serious adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH-UoN ERC within 72 hours of notification.
d) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH- UoN ERC within 72 hours.
e) Clearance for export of biological specimens must be obtained from KNH- UoN ERC for each batch of shipment.
f) Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
g) Submission of an *executive summary* report within 90 days upon completion of the study. This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/ or plagiarism.

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For more details consult the KNH- UoN ERC website **http://www.erc.uonbi.ac.ke**

Yours sincerely,

PROF. M. L. CHINDIA
SECRETARY, KNH-UoN ERC

c.c. The Principal, College of Health Sciences, UoN
The Deputy Director, CS, KNH
The Chairperson, KNH-UON ERC
The Assistant Director, Health Information, KNH
The Dean, School of Medicine,UoN
The Chair, Dept.of Medical Microbiology, UON
Supervisors: Prof. Walter Jaoko(UON), Dr.Christine Bii(KEMRI)

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Appendix 6: Consent to participate in the study (English version)-excerpt from the parent study.

Kenya Medical Research Institute

CONSENT TO PARTICIPATE IN A RESEARCH STUDY

Study Title: Mapping antifungal, antibacterial drug resistance and the quality of antimicrobial agents against fungal and bacterial pathogens from smear negative and retreatment cases in high TB prevalence Counties in Kenya.

Name of study site:…….……Site Code……

Investigators	**Institution**	**Study role**
Dr. Bii Christine	KEMRI, Centre for Microbiology Research (Kenya)	Principal Investigator
Dr. Lucia Keter	Centre for Traditional Medicine and Drug Research	Co- Investigator
Dr. Beatrice Irungu	Centre for Traditional Medicine and Drug Research	Co- Investigator
Olga Mashedi	KEMRI, Centre for Respiratory Diseases Research	Co- Investigator
Eva Gatumwa	KEMRI, Centre for Respiratory Diseases Research	Co- Investigator
Tom Ouko	KEMRI, Centre for Microbiology Research (Kenya)	Co-Investigator
Abdi Mohammed	KEMRI, Centre for Microbiology Research (Kenya)	Co- Investigator
Richard Korir	KEMRI, Centre for Microbiology Research (Kenya)	Co- Investigator
Obanda Apollo	KEMRI, Centre for Microbiology Research (Kenya)	Co- Investigator

Purpose of the study

This is a research study. Research studies only include people who choose to take part. Scientists from the Kenya Medical Research Institute (KEMRI), Kenya are conducting a research study to determine the type of germs that are not TB but with signs and symptoms of possible TB infections but test negative for TB, and those who come back for TB treatment because they are not improving. In addition, the study will also look at the quality of the medicines used for treating these germs whether they are working. This is to understand 1. What are the germs that are not TB that cause pneumonia in TB smear negative patients? 2. What are the drugs that treat these germs that cause pneumonia in TB smear negative patients? 3. Is TB repeat treatment due to failure in part from substandard TB drugs? The study will also obtain blood samples from

patients for use in new methods of diagnosis of these germs. Poor quality drugs and drug resistance in non-TB germs may be the cause of significant deaths in TB infected patients. It is therefore important to understand in order to address the health challenge in these patients. You are being asked to participate in this study because you are suspected to have TB/pneumonia and a clinician/ doctor will obtain your sputum and blood for laboratory investigations. The scientists will seek to obtain with your consent, the blood and sputum sample left over after routine laboratory investigations for further investigations at KEMRI/CMR Laboratories in Nairobi. The isolates obtained will be tested for presence of resistance to drugs used for treatment of this infection. Information obtained from this study will be shared with the hospital to be used to guide treatment and management of patients with this infection.

This study is funded by the KEMRI Internal Research Grants.

Who can participate in this study?

Any person can participate who is hospitalized or not hospitalized and suspected of TB and have his/her sputum and blood sample obtained and have agreed to participate and signed the consent form or consent has been given through a relative/guardian.

How many people will take part in this study?

About 100 people from this hospital, with suspected TB infection have their sputum and blood sample taken will participate in this study.

Sputum and blood sample collection

A qualified clinician/medical doctor of the hospital has examined you and collected sputum and blood specimens for investigation at the hospital laboratory that will help in administration of effective treatment for your infections. These are routine procedures done in the hospital to investigate patients with TB/pneumonia infections. This study will not seek for additional sputum and blood sample or increase in sample volume from you. Normally, a small amount of sputum and blood samples remains after routine investigations.

Potential risks

There is no risk for participation in this study. Blood and sputum sample is routinely obtained for laboratory analysis in patients suspected with TB and pneumonia. The blood and sputum samples are taken by qualified hospital medical/clinical doctor and not the study staff. Findings obtained from this study will be shared with the hospital for management of this infection.

Potential benefits

There is no any benefit that a participant will get from participating in the study. However, findings obtained from this study will be shared with the hospital for management of these infections in future.

Payment for participation

There is no payment or compensation to volunteers for their participation in this study.

Confidentiality

All information and records relating to you /relative's participation in the study will remain confidential. You/your child's name will not be used in any report resulting from this study. All information will not be used for any other purpose other than for this study and will only be accessible to study investigators and authorized person. All computerized records and laboratory specimens for this study will contain only unique study number and not your name. All data entered in the computer will be password protected and only accessible to authorized persons in the study.

Protection of research subjects

KEMRI Scientific and Ethical Review Unit (SERU) is a mandated body concerned with protection of persons participating in medical research; it is also eligible to review research records and information as part of their responsibility to protect human subjects.

Consent to participate

This study is seeking for you/relative/guardian consent/assent for participation. Participation will involve giving consent for use of blood and sputum sample, medical information but not name. There is no follow up or further information needed from you or your relative/guardian. If you consent to participate or act as a legal representative for an adult to participate in the study, we will ask you to sign or append your thumbprint on this form. In case you do not give consent for participation, there will be no penalty and the clinician/medical doctor attending will proceed to treat you as required. You will receive a signed copy of this consent form to keep.

If you have understood the information in this consent form and what I have explained to you, I ask you to confirm and sign up for participation of you or your relative.

If there is any portion of this consent form that you do not understand, please ask me before signing it.

Declaration

The purpose of the study has been explained to me. I understand that the study is investigating on fungi and non-TB bacteria that cause infections in TB suspected patients that would lead to better understanding for appropriate treatment, management and control strategies of these infections. I am or my relative is being asked to voluntary participate in the study. I understand the possible risks and benefits for participating in this study. I further understand that

PARTICIPATION IS VOLUNTARY and I may decline to participate at any time without any explanation and without suffering any consequences such as denial of clinical/medical attention.

I have read or have been read to me and understood the information given and willingly consent for me/relative to participate in this study. I have been given the opportunity to ask questions concerning this investigation and any such question has been answered to my full and complete satisfaction.

In case of need for further information about this study please contact**.** Dr. Christine C. Bii Tel No. 020 2722451 or 0721-224351 or KEMRI Centre for Microbiology Research P.O. Box 19462 - 00202 Nairobi or **The Secretary Scientific and Ethics Review Unit P.O. Box 54840-00200 Nairobi. Mobile; +254717719477; Email; seru@kemri.org**

Participants sign…….. Date……..Age…… Sex……

IP NO……

Legal representative Name …….. Sign…….. Date…….

Thumbprint……

Witness Name……. Sign…….. Date…….

Interview /Investigator's Name …… date…….

Appendix 7: Assent to participate in a research study (English version); excerpt from the parent study.

Kenya Medical Research Institute

ASSENT TO PARTICIPATE IN A RESEARCH STUDY

Study Title: Mapping antifungal, antibacterial drug resistance and the quality of antimicrobial agents against fungal and bacterial pathogens from smear negative and retreatment cases in high TB prevalence Counties in Kenya.

Name of study sit……

Investigators	**Institution**	**Study role**
Dr. Bii Christine	KEMRI, Centre for microbiology Research (Kenya)	Principal Investigator
Dr. Lucia Keter	KEMRI, Centre for Traditional Medicine and Drug Research	Co-Investigator
Dr. Beatrice Irungu	KEMRI, Centre for Traditional Medicine and Drug Research	Co-Investigator
Ms. Olga Mashedi	Kenya Medical Research Institute, (Kenya)	Co-Investigator
Eva Gatumwa	KEMRI, Centre for Respiratory Diseases Research	Co-Investigator
Tom Ouko	KEMRI, Centre for microbiology Research (Kenya)	Co-Investigator
Abdi Mohammed	KEMRI, Centre for microbiology Research (Kenya)	Co-Investigator
Richard Korir	KEMRI, Centre for Microbiology Research (Kenya)	Co-Investigator
Obanda Apollo	KEMRI, Centre for Microbiology Research (Kenya)	Co-Investigator

Declaration

This is a research study. The purpose of this study has been explained to me satisfactorily. I understand the study is investigating the fungi and bacteria that cause diseases in TB negative patients that would lead to better understanding for appropriate management and control strategies of these infections. My **PARTICIPATION IS VOLUNTARY** and I am being asked to voluntarily participate in the study. My participation will involve giving assent for use of my blood and sputum samples, medical information but not name. The germs that may be obtained

for this study will be stored at KEMRI/CMR Laboratories, Nairobi and may be exported for further analysis and confirmation where necessary.

I further understand that my participation is voluntary and I may withdraw from the study at any time and without any explanation or loss of right to care and treatment of my condition.

I further understand that there is no compensation to volunteers for their participation.

I have read and/ or have been read to me and understood the information given and willingly consent to participate in the study. I have been given the opportunity to ask questions concerning this investigation and any such question has been answered to my full and complete satisfaction. I will receive a signed copy of this consent form to keep.

In case of need for further information about this study please contact**. Dr. Christine C. Bii Tel No. 020 2722451 or 0721-224351 or KEMRI Centre for Microbiology Research P.O. Box 19464 -00202 Nairobi or The Secretary Scientific and Ethics Review Unit P.O. Box 54840-00200 Nairobi. Mobile; +254717719477; Email; seru@kemri.org.**

Participant sign……. Date…….

Thumbprint…… Age……. Sex……..

Witness Name …… sign……. Date…….

Interviewer /Investigator Name…… Sign……. date…….

IP NO……

Appendix 8: Consent to participate in a research study (Kiswahili version); excerpt from the parent study.

Kenya Medical Research Institute

IDHINI YA KUSHIRIKI KATIKA UTAFITI

Jina la Utafiti: Mapping antifungal, antibacterial drug resistance and the quality of antimicrobial agents against fungal and bacterial pathogens from smear negative and retreatment cases in high TB prevalence Counties in Kenya.

Jina la hospitali ya utafiti huu_____ **Nambari ya hospitali _____**

Mtafiti	**Taasisi Husika**	**Jukumu la Mtafiti**
Dr. Bii Christine	KEMRI, Centre for microbiology Research (Kenya)	Principal Investigator
Dr.Lucia Keter	KEMRI, Centre for Traditional Medicine and Drug Research	Co-Investigator
Dr.Beatrice Irungu	KEMRI, Centre for Traditional Medicine and Drug Research	Co-Investigator
Ms. Olga Mashedi	Kenya Medical Research Institute, (Kenya)	Co-Investigator
Eva Gatumwa	KEMRI, Centre for Respiratory Diseases Research	Co-Investigator
Tom Ouko	KEMRI, Centre for microbiology Research (Kenya)	Co-Investigator
Abdi Mohammed	KEMRI, Centre for microbiology Research (Kenya)	Co-Investigator
Obanda Apollo	KEMRI, Centre for microbiology Research (Kenya)	Co-Investigator

Madhumuni ya Utafiti

Huu ni utafiti. Utafiti huusisha tu watu ambao huchagua kushiriki kwa hiari. Watafiti kutoka shirika la utafiti wa kimatibabu la Kenya Medical Research Institute (KEMRI humu nchini Kenya wanafanya utafiti juu ya viini vinavyo sababisha maradhi ya kifua yaani nimonia kwa wagonjwa ambao vipimo vyao vya maradhi ya kifua kikuu ni negative na walee wagonjwa wa kifua kikuu amabo wamerudi hospitali kwa kukosa kupona katika matibabu ya mara ya kwanza. Hii ni kutaka kujua ni vimelea gani vya maradhi vinasababisha maradhi haya katika wagonjwa hawa. Vimelea vinavyo sababisha maradhi ya kifua yaani pneumonia ni hatari na huathiri sana watu ambao wana upungufu wa kinga mwilini. Vimelea hivi visipotambuliwa mapema kwa mgonjwa na kutibiwa kwa haraka vinaweza kusababisha kifo. Unahimizwa kushirika katika utafi huu kwa sababu unatuhumiwa kuwa na ugonjwa wa pneumonia na daktari anayekutibu. Daktari alichukua sampuli

ya mate yako na kupeleka katika maabara ya hospitali kwa uchunguzi ya maradhi yako. Watafiti wanakuomba idhini yako ya kuweza kuchukua sampuli ya mate na damu yako uliotowa itakayobaki baada ya kumalizika kwa uchunguzi katika maabara ya hospitali ili kufanyia utafiti wa kuwepo kwa viini vingine. Sampuli inayobaki huwa kwa kawaida hutupwa na hospitali. Watafiti watapeleka sampuli yako katika maabara maalum ya utafiti wa vimelea vya fungi na bacteria ya idara ya KEMRI iliyoko jijini Nairobi. Vimelea ambavyo vitawezapatikana kwa sampuli yako vitachunguzwa kuweko kwa upinzani kwa madawa yanayotumiwa kutibu maradhi ya pneumonia yanayo sababishwa na maambukizi ya vimelea hivi. Vimelea hivi vinaweza kusafirishwa katika maabara ya vimelea vya viini kwa maabara ya utafiti ambayo KEMRI iko na uhusianao wa karibu nayo itakapohitajika kufanyiwa uchunguzi zaidi ya kibiologia. Matokeo ya utafiti huu utapatiwa hospitali hii ili kutumika kuongoza matibabu na usimamizi kwa wagonjwa wenye maradhi ya pneumonia ambayo sio yenye kuletwa na vimelea vy TB. Utafiti huu unafadhiliwa na ruzuku kutoka kwa shirika la utafiti wa kimatibabu la KEMRI.

Ambae anaweza kushirikia katika utafiti

Mtu yeyote ambaye amehudumia katika hospitali hii na daktari anaemtibu anatuhumu kuwa ana maradhi ya pneumonia isio ya TB na ameshashukua mate yake na kupeleka kwa maabara ya hospitali kwa uchunguzi anaweza kushirika katika utafiti huu. Pia aweze kutoa idhini kwa kuweka sahihi katika fomu hii ya idhini au idhini kupatikana kupitia kwa mzazi, jamaa au mlezi. Mtu ambaye haitapatikana kwake idhini hatoweza kushiriki.

Ni watu wangapi watashiriki katika utafiti

Watu 100, ambao wametoa sampuli ya mate yao kwa uchunguzi wa maradhi ya kifua kikuu na kupelekwa katika maabara ya hospitali kwa uchunguzi, watashiriki katika utafiti huu.

Kutolewa kwa sampuli ya mate na damu

Dakitari wa hospitali amekupima au kupima jamaa yako na kuchukua sampuli ya mate na damu yako kwa uchunguzi ya maradhi yako katika maabara ya hospitali. Kuchukuliwa kwa sampuli ya mate yako na damu ni taratibu za kawaida katika hospitali wanapo chunguza mambukizi ya maradhi ya TB, Pneumonia na HIV. Utafiti huu hautachukua nyongeza ya sampuli ya mate au damu yako au kuongeza kiasi cha mate au damu yako yanayotolewa na dakitari kwa kawaida. Kwa kawaida kiasi kidogo cha sampuli ya mate na damu hubaki baada ya uchunguzi katika maabara ya hospitali kufanywa. Utafiti huu utachukua tu sampuli ya kikohozi na damu utakaobaki pekee. Sampuli inayobaki huwa kwa kawaida hutupwa na hospitali.

Faida na uwezo wa hatari kwa mshiriki

Hakuna hatari yeyote itakayopatikana kwa mwenye kushiriki katika utafiti huu. Sampuli ya mate au damu hutolewa kwa kawaida katika hospitali kwa mgonjwa yeyote anayetuhumiwa kuwa na maradhi ya TB, Pneumonia na HIV. Sampuli ya damu hutolewa na dakitari wa hospitali aliyefezu kufanya hivyo, na sio watafiti husika katika utafiti huu. Matokeo yatakayo patikana kutoka kwa utafiti huu yatapatiwa hospitali hii ili kutumika kuongoza matibabu na usimamizi kwa wagonjwa wenye maradhi ya Pneumonia isoyo ya TB yanayo sababishwa na vimelea vyitakavyo patikana.

Malipo kwa ajili ya kushiriki

Hakuna malipo au fidia yeyote itakayotolewa au kupatikana kwa mwenye kushiriki. Kushiriki katika utafiti huu ni kwa hiari.

Kuwekwa siri kwa taarifa na rekodi za mshiriki

Itabakia siri taarifa na rekodi zote zinazohusiana na wewe / jamaa yako kuhusu kushiriki katika utafiti huu. Jina lako/ jamaa yako halitatumika katika ripoti yeyote ya utafiti huu Taarifa zote

zitatumika tu kwa ajili ya utafiti huu na sio kwa matumizi mengine yeyote. Taarifa na rekodi zote za utafiti huu zinawezakutizamwa tu na watafiti husika au kuangaliwa na mwenye atapewa ruhusa pekee. Taarifa zote zitanukuliwa katika tarakilishi na sampuli zote zitakuwa tu na nambari maalum ya utafiti, yaani code na sio jina la mshiriki. Taarifa zote zitawekwa katika tarikilishi iliyo na visawe na kuweza kufunguliwa tu na watafiti katika utafiti huu au mwenye kupewa ruhusa.

Utetezi wa haki kwa mshiriki

Kamati ya kitaifa ya maadili ya KEMRI, ina mamlaka husika kutetea na kulinda haki za washiriki katika utafiti wa kimatibabu. Pia kamati hii ina mamlaka ya kukagua taarifa na rekodi zote za utafiti kama sehemu ya majukumu yake. Anwani ya kamati hii inapatikana hapo chini katika fomu hii.

Idhini ya Kushiriki

Utafiti huu unakuhimiza kupata kwa idhini yako /jamaa wako ili kushiriki. Kushiriki kwako kutahusisha kutoa idhini kwa matumizi ya sampuli ya damu na mate yako na taarifa ya afya ya kidakitari kuhusu maradhi ya ukimwi, umri and jinsia. Hakutakua na kufwatilia au nyongeza zaidi za habari kuitishwa. Ukikubali kushiriki au kutenda kama mwakilishi wa kisheria kwa mtu mzima kushiriki katika utafiti, tutakuhitaji uweke sahihi au ishara ya kidolo chako cha gumba kwenye fomu hii. Iwapo utakosa kutoa idhini ya kushiriki kwako/ jamaa yako, hakutakua na adhabu yeyote na dakitari wa hospitali anayekutibu ataendelea kama kawaida. Ukikubali kushiriki utapata nakala ya fomu hii ya idhini kujiwekea. Kama umeelewa maelzeo katika fomu hii ya idhini na niliyokueleza, ninakuomba uthibitishe kwa ishara kwa kuweka sahihi au alama ya kidole chako/jamaa wako kwa kushiriki. Kama kuna sehemu yeyote ya fomu hii ya idhini huelewi, tafadhali niulize mimi kabla ya kuweka sahihi au kidole chako.

Azimio la Kushiriki

Nimefahamishwa kusudi na lengo la utafiti huu. Naelewa kuwa utafiti huu unachunguza kuwepo kwa viini visivyo vya TB vinavyo sababisha maradhi ya pneumonia. Naelewa kuwa matokeo ya utafiti huu yatatumiwa kwa ajili ya matibabu, usimamizi na udhibiti ya mikakati ya mambukizi ya maradhi ya vimelea vya pneumonia. Naelewa pia kuwa mimi/mtoto/jamaa yangu, twahimizwa kushiriki kwa hiari katika utafiti huu. Naelewa hatari na faida inayoweza kupatikana kutokana na kushiriki kwangu, mtoto au jamaa yangu katika utafiti huu. Naelewa pia kuwa kushiriki kwangu, mtoto wangu au jamaa yangu ni kwa hiari. Na kuwa mimi/mtoto wangu au jamaa yangu anaweza kukataa kushiriki bila kutowa maelezo yeyote. Na kutoshiriki kwangu au mtoto au jamaa yangu hakutaathiri haki ya kutibiwa na kupata matibabu katika hospitali ipasavyo. Nimesoma au nimesomewa fomu hii ya idhini na nimeelewa vizuri maelezo yote. Ninatoa idhini yangu au jamaa yangu kushiriki katika utafiti huu kwa hiari. Nimepewa fursa ya kuuliza maswali kuhusu utafiti huu na maswali yangu yote yamejibiwa kwa ukamilifu na nimeridhika na majibu niliopewa. Nitapewa nakala iliyowekwa sahihi ya fomu hii ya idhini kujiwekea.

Iwapo utahitaji maelezo zaidi kuhusu utafiti huu, tafadhali wasiliana na mtafiti mkuu au katibu wa kamati ya maadili ya KEMRI kupitia anwani zifuatazo;

Dr. Christine C. Bii Tel No. 020 2722451 or 0721-224351 or KEMRI Centre for Microbiology Research P.O. Box 19462 -00202 Nairobi au The Secretary Scientific and Ethics Review Unit P.O.Box 54840-00200 Nairobi. Mobile; +254717719477; Email; seru@kemri.org

Sahihi ya Mshiriki…….. Tarehe……..…..Umri……
Jinsia……

IP NO……Hali ya virusi vya HIV vya mshiriki ……
Jina la mwakilishi …….. ……..……..
Sahihi……Tarehe…….……
….
Alama ya kidole cha gumba……..……
Jina la mshahidi ……. Sahihi ……..…….. Tarehe …….…….
Jina la mtafiti…… …… Tarehe…….

Appendix 9: Assent to participate in a research study (Kiswahili version); excerpt from the parent study.

Kenya Medical Research Institute

IDHINI YA KUSHIRIKI KATIKA UTAFITI

Jina la Utafiti: Mapping antifungal, antibacterial drug resistance and the quality of antimicrobial agents against fungal and bacterial pathogens from smear negative and retreatment cases in high TB prevalence Counties in Kenya.

Jina la hospitali ya utafiti huu__

Mtafiti	**Taasisi Husika**	**Jukumu la Mtafiti**
Dr. Bii Christine	KEMRI, Centre for microbiology Research (Kenya)	Principal Investigator
Dr.Lucia Keter	KEMRI, Centre for Traditional Medicine and Drug Research	Co-Investigator
Dr.Beatrice Irungu	KEMRI, Centre for Traditional Medicine and Drug Research	Co-Investigator
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Abdi Mohammed	KEMRI, Centre for microbiology Research (Kenya)	Co-Investigator
Richard Korir	KEMRI, Centre for microbiology Research (Kenya)	Co-Investigator
Obanda Apollo	KEMRI, Centre for microbiology Research (Kenya)	Co-Investigator

Azimio la kushiriki

Huu ni utafiti. Nimeelezwa lengo na kusudi la utafiti huu na nimeridhika. Naelewa kuwa utafiti huu unachunguza kuwepo kwa vimelea vya fungi na bacteria vinavyosababisha maradhi ya Pneumonia isio ya TB. Naelewa kuwa matokeo ya utafiti huu yatatumiwa kwa ajili ya matibabu, usimamizi na udhibiti ya mikakati ya mambukizi ya maradhi ya pneumonia iso ya TB na vimelea hivyo. Ninahimizwa kushiriki katika utafiti huu na naelewa kuwa kushiriki kwangu ni kwa hiari. Naelewa kuwa kushiriki kwangu kutahusisha kutoa idhini kwa matumizi ya sampuli ya mate na damu itakayobaki baada ya uchunguzi katika maabara ya hospitali kukamilika na kutoa taarifa ya afya ya kidaktari kuhusu maradhi ya ukimwi, umri na jinsia. Nafahamu kuwa vimelea vya fungi na bacteria vitakavyo patikana kutokana na utafiti huu vitahifadhiwa katika maabara maalum ya utafiti wa vimelea vya fungi na bacteria ya idara ya kimatibabu ya KEMRI iliyoko jijini Nairobi. Nafahamu pia kuwa vimelea hivi vinaweza kusafirishwa katika maabara ya vimelea vya fungi na

bacteria vya vyuo vilivyo na uhusiano na shirikila la kimatibabu la KEMRI, itakapohitajika kufanyiwa uchunguzi zaidi ya kibiologia.

Naelewa kuwa kushiriki kwangu ni kwa hiari na naweza kukataa kushiriki wakati wowote bila kutowa maelezo yeyote. Na kutoshiriki kwangu hakutaathiri haki yangu ya kutibiwa na kupata matibabu katika hospitali. Nimesoma au nimesomewa fomu hii ya idhini na nimelewa vyema maelezo yote. Ninatoa idhini yangu ya kushiriki katika utafiti huu kwa hiari. Nimepewa fursa ya kuuliza maswali kuhusu utafiti huu na maswali yangu yote yamejibiwa kwa ukamilifu na nimeridhika na majibu niliopewa. Nitapewa nakala iliyotiwa sahihi ya fomu hii ya idhini kujiwekea.

Iwapo utahitaji maelezo zaidi kuhusu utafiti huu, tafadhali wasiliana na mtafiti mkuu au katibu wa kamati ya maadili ya KEMRI kupitia anwani zifuatazo; **Dr. Christine C. Bii Tel No. 020 2722451 or 0721-224351 or KEMRI Centre for Microbiology Research P.O.Box 19462 - 00202 Nairobi au The Secretary Scientific and Ethics Review Unit P.O.Box 54840-00200 Nairobi. Mobile; +254717719477; Email; seru@kemri.org**

Sahihi ya Mshiriki…….. Tarehe……..…..Umri…… Jinsia……

Jina la mshahidi ……. Sahihi ……..…… Tarehe ……..…….

Alama ya kidole cha gumba……..……

IP NO……

Appendix 10: Published manuscripts from the study

1. Josephat Tonui et al. In vitro antifungal susceptibility of yeasts and molds isolated from sputum of tuberculosis relapse and retreatment patients. *Pan African Medical Journal. 2021;38:227. [*doi: 10.11604/pamj.2021.38.227.26485*]*

2. Tonui, J., Mureithi, M., Jaoko, W. and Bii, C. (2020). Spectrum of Filamentous Fungi from Sputum of Tuberculosis Relapse and Retreatment Patients in Tuberculosis Reference Facilities. *Open Journal of Medical Microbiology*, **10**, 190-203. doi: 10.4236/ojmm.2020.104017.