TRIAZOLE RESISTANCE AND SEQUENCE ANALYSIS OF *ASPERGILLUS* SPECIES FROM FUNGICIDE NAÏVE AND EXPERIENCED SOILS IN NAIVASHA SUB-COUNTY AND NAIROBI COUNTY IN KENYA

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Triazole Resistance and Sequence Analysis of *Aspergillus* Species from Fungicide Naïve and Experienced Soils in Naivasha Sub-County and Nairobi County

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

I dedicate this work to my loving mum Ann C. Cheruiyot, Son Ryan Edson Kemoi, Daughter Addison Edson Kemoi and wife Faith Yator for their love, encouragement and financial support.

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LIST OF ABBREVIATIONS AND ACRONYMS

ABC ……..ATP binding sites

ABPA ……Allergic bronchopulmonary aspergillosis

AIDS …….Acquired immunodeficiency syndrome

ARAF…….Azole resistance *aspergillus fumigatus*

ARTEMIS ……. Advanced responsive tactically effective military imaging spectrometer

ARVs……..Antiretroviral

AtrF……..….. ATP binding cassette transported F

BAL……..Broncho alveolar

CCPA……..Chronic cavitary pulmonary aspergillosis

CFU…… Colony forming units

CLSI …….. Clinical Laboratory Standard Institute

CLSI……Clinical laboratory standard institute

CYP51A…….. Cytochrome P450 sub-family 51A

DMO……..... Dimethyl sulfoxide

EUCAST…….…… European Committee for Antibiotic

Susceptibility Testing

HIV……. Human immunodeficiency virus

Hr…….….Hours

IA……..……Invasive pulmonary aspergillosis

IgE……....Immunoglobulin E

IgG……..…..Immunoglobulin G

ITR…… ..Itraconazole

ITS……...… Internal transcribed spacer

KEMRI……Kenya Medical research institute

MALDI TOF …… ……Matrix-Assisted Laser Desorption ionization time of flight

MFS…….….Major facilitators

MLP…….…….Microsatellite length polymorphic

PCR…… Polymerase chain reaction

PSZ……..… Posaconazole

RNA…….…. Ribonucleic acid

RT……. Room temperature

SAFS……..Severe asthma associated with fungal sensitization

SDA……. Sabouraud dextrose agar

TB…….Tuberculosis

TMS …….Transmembrane spanning

TR…….tandem repeats

VCZ…….. Voriconazole

ABSTRACT

New triazole antifungals are recommended for the management of Aspergillosis infections. The emergence of *Aspergillus fumigatus* strains resistant to azole have been identified in some countries ascribed to either previous antifungal treatment, prophylaxis or triazoles use in agriculture. Azole based fungicides use is robust horticulture in Kenya is a significant risk factor for antifungal resistance. The objective of the study was to isolate, characterize and determine triazole resistance amongst *Aspergillus* species from fungicide naïve and experienced soils compared to those from clinical sources. A total of 250 naïve soils, 252 experienced soils, and eleven clinical samples were analyzed. Dry top surface soils were sampled from the study site. Each soil sample was processed and approximately 100µl of the preparations plated onto SDA containing no drug, 1µl/ml itraconazole and 1µl voriconazole. *Aspergillus* species were identified by MALDI TOF MS and ITS genes amplification. Absence or presence of TR34 in the promoter region was determined by *A. fumigatus* DNA amplification using AFCYPPR and AFCYPPF primers. While L98H the mutation was determined by amplification of isolates DNA using AFCYP98R and AFCYP98F primers. Gel electrophoresis was run to detect DNA amplicon *Aspergillus* species isolated were as follow: Nairobi experienced soils 22/45 (48.89%) *A. fumigatus*, 20/45 (44.44%) *A. niger*, 3/45 (6.67%), *A. flavus*, 0/51 (0%) *A. terreus*. Naivasha experienced soils. 23/42 (54.76%) *A. fumigatus*, 12/42 (28.57%) *A. niger*, 3/42 (7.14%) *A. flavus*, 3/42 (7.14%) *A. terreus*. In naïve soils from Nairobi 26/40 (65%) *A. fumigatus*, 12/40 (30%) *A. niger*, 0/40 (0%) *A. flavus*, 2/40 (5%) *A. terreus* while in Naivasha 21/45 (46.67%) *A. fumigatus*, 23/40(51.11%) *A. niger*, 0/45 (0%), *A. flavus*, 0/45 (0%), *A. terreus* and 1/45 (2.2%) *A. ochraceus* were isolated. The resistance of *Aspergillus* species isolated from Nairobi experienced soils were 3/45 (6.7%), 5/45 (11.1) and 27/45 (60%) against itraconazole, voriconazole, and posaconazole respectively. Naivasha experienced soils had a resistance of 3/42 (7.1%) against Itraconazole, 4/42 (9.5%) against voriconazole and 32/42 (76.2%) against posaconazole. Naïve soils form Nairobi had a resistance of 6/40 (15%) against posaconazole compare to 3/45 (6.7%) from Naivasha. *Aspergillus fumigatus* with TR34 in their promoter regions yielded 139bp amplicon and wild type (L98H) yielded DNA fragments of 279bp. Hence the study detected TR34/L98H mutation in all the sequenced isolates. The finding is worrisome and we must strive for more surveillance of environmental and clinical source to detect azole resistance in *Aspergillus* species.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background of the study

Aspergillus species, particularly *A. fumigatus* pose a health risk to susceptible patients suffering from hematological malignancies and Human immune deficiency (HIV). It is a frequent colonizer of cavitary lesions in tuberculosis patients and cause of mortality in post TB treatment cases (Denning *et al*., 2011a). Azole-resistant *A. fumigatus* (ARAF) strains from environments have been reported in azole-naive patients from several European and Asian countries (Alanio *et al*., 2011; Chowdhary *et al*., 2012a; Seyedmousavi *et al.*, 2013). *A. fumigatus* is the most pathogenic species followed by *A. niger, A. flavus*, *A. terreus* and *A. nidulans* (Balajee *et al*., 2006; Hope *et al*., 2005).

Aspergillus species are the most prevalent airborne, filamentous human fungal pathogen. There are approximately 300 *Aspergillus* species, however few cause animal and human illness (Femenia *et al*., 2009). It causes aspergillosis, a condition that can manifest as allergic, invasive, or chronic noninvasive syndromes. Aspergillosis is the second leading cause of death after cryptococcosis in patients suffering from fungal infections (Brown *et al*., 2012). *A. fumigatus* resistance to azole is evolving to be a global health problem (Vermeulen *et al*., 2012).

Aspergillus resistance to azole can occur through long term exposure to azole compounds in agriculture (environmental) or treatment of a patient with an azole (Snelders *et al*., 2008). Due to high humidity in tropical countries, *A. niger, A. flavus and A. fumigatus* cause high rates of infestations and contaminations of agricultural crops. Apart from mycotoxin production and subsequent food contamination, the human is exposed to high levels of fungal spores through inhalation and contact in agricultural practice (Femenia *et al*., 2009).

Itraconazole, posaconazole and voriconazole are first line triazoles recommended for the management of aspergillosis (Lass-Florl, 2011). *Aspergillus fumigatus* resistance to triazoles have been reported in South America, United States, Europe, Japan, India and Iran (Chowdhary *et al*., 2012a; Pfaller *et al*., 2011; Seyedmousavi *et al*., 2013; Van der Linden *et al*., 2011a,). In the United Kingdom and Netherland 17% to 20% and 10% of *A. fumigatus* are resistant to Itraconazole respectively (Bowyer *et al*., 2011; Van der Linden *et al*., 2011b). Global surveillance is done by ARMTEMIS involving sixty-two medical centers reported 5.8% of *A. fumigatus* resistant to at least one triazoles (Pfaller *et al*., 2011).

In 1998 *Aspergillus* with TR34/L98H resistance was reported in the Netherlands (Snelders *et al*., 2008). While TR34/Y121F/T289A resistance was first noted in the Netherlands in 2009 (Van der Linden *et al*., 2013). Unrelated *Aspergillus* strains harboring TR34/L98H and TR46/Y121F/T28 showed genetic relatedness, compared to wild types, hence mutation could have originated from the same ancestor (Van der Linden *et al*., 2013, Chowdhary *et al*., 2012a, Camps, *et al*., 2012b). In the Netherlands and India's resistant isolates harboring TR46/Y121F/T289A were highly similar genetically (Chowdhary *et al*., 2015).

Clinically, Aspergillosis is either managed with azoles, such as itraconazole or amphotericin B. However, resistance to azoles has been increasingly reported especially from high- and middle-income countries (Arendrup, 2010; Bueid, 2010; Burgel *et al*., 2012; Chowdhary *et al*., 2012a; Lockhart *et al*., 2011; Mortensen *et al*., 2010; Mortensen *et al*., 2011; Snelders *et al* 2012; Snelders *et al* 2011; Snelders *et al*., 2008). Studies from a different region of the worlds: African region (Tanzania), European region (Netherlands, Belgium, Denmark and Germany), Asia (Kuwait, India and Iran) and in the USA, have reported multiple sources of Azole-resistant *Aspergillus fumigatus* from soils sample, flower beds, plants, compost and hospitals and its

environs (Ahmad *et al*., 2014; Chowdhary *et al*., 2012a; Snelders *et al*., 2012; Snelders *et al*., 2008).

Azole naïve patients have been reported to harbor *Aspergillus fumigatus* resistant to azole through the following mechanism: Substitution of G432S in CYP51A gene, Substitution of Y121F/T289A coupled with 46bp TR46 in CYP51A genes and L98H substitution in CYR51A gene coupled with 34bp tandem repeat. TR34/L98H was detected in 12/13 of patient resistant to itraconazole in Dutch Centre (Lortholary *et al*., 2011; Snelders *et al*., 2008; Snelders *et al*., 2010).

Natural fungicides were used to control fungal diseases until mid-20th century when synthetic fungicides were introduced (Morton and Staub, 2008). In 1970S azole antifungal were introduced, which was aromatic unsaturated molecules with at least one atom of nitrogen. Azole was preferred because it is broad-spectrum activity and efficiency (Price *et al*., 2015). Inhibition of ergosterol biosynthesis in fungus genes e.g. cyp51A in *Aspergillus fumigatus* and ERG11 (lanosterol 14a-demethylase) (Azevedo *et al*., 2015). Failure of agricultural azoles to control fungal diseases has been addressed by either mixing more than one fungicides or increasing the doses (Russell, 1995). Exposure of *Aspergillus* species to different azole fungicides in the farms can lead to emerging resistance with serious health consequences in human (Snelders *et al*., 2009).

Fungal resistance to triazoles has not been reported in patients undergoing azole treatments (Snelders *et al*., 2009). However, resistance has been noted in ecological niches e.g. aquatic environment and agricultural where soil azole remain active for several months (Chowdhary *et al*., 2013a). Hence hypothesized exposure to fungal azole in the environment leads to cross-resistance to azoles used in medical practice (Chowdhary *et al*., 2013a). Other studies have reported the mutation of *A. fumigatus* in TR46/Y121F/T289A, G448S and TR34/L98H genes isolated from two individuals (one previously exposed to environmental azole and another one to medical azole) (Chowdhary *et al*., (2013a) and Verweji *et al*., (2016). Approximately 5.8%

of *Aspergillus* isolates from 144 soils previously exposed to metconazole, tebuconazole, hexaconazole, epoxiconazole and propiconazole were found to be resistant to medical triazoles (Ren *et al*., 2017).

Azoles used in agriculture have similar chemical structures and mechanism of action as those used in medical practices (Chowdhary *et al*., 2013b). The TR34/L98H resistance mechanism is widespread across the world; African, Australia, North and South America, Asia and Europe. The Cyp51A gene mutations linked to resistance have been found in clinical and environmental samples in the United Kingdom, Denmark, Belgium, Spain, Sweden, Netherlands, France, Portugal, Germany, Poland, Greece, Romania, Italy, Turkey and Austria. The most recent being in Taiwan, Norway, Brazil, Iran, and Kuwait (Garcia-Rubio *et al*., 2017; Rivero-Menedez *et al*., 2016).

High resistance to azole was recently reported in Tanzania (Chowdhary *et al*., 2014a). An ARAF strains with bothTR46/Y121F/T289A and TR34/L98H mutations were isolated in Moshi from agricultural soils used for farming of maize, sugar cane, and beans. However, little information pertaining to the usage of azole fungicides in this area was available. Use of azole-based fungicides in agriculture has been suspected to contribute to azole resistance in clinical practices. The rampant and irrational use of agriculture fungicides (azole) in robust flower and horticultural industry in Kenya is a significant risk factor likely to contribute to a significant level of triazole resistance in Kenya.

1.2 Statement of problem

Opportunistic fungal pathogens such as *Cryptococcus* species, *Candida* species and *Aspergillus* species, are emerging infections in immunosuppressed individuals. Aspergillosis infection mainly affects immunosuppressed individuals such as HIV, cancer and TB. Kenya has been ranked as one of the countries with a high rate of HIV and TB infection burden. Aspergillosis is a fatal disease which has been associated with

high mortality. The disease is common among people in the agricultural sector. Triazoles are recommended antifungal for management of aspergillosis inhuman. In agriculture, there is widespread use of Azoles based fungicides for the control of powdery mildew in tomatoes, vines, cereal and berry; rusts and mildews of vegetables, fruits and ornamentals. *Aspergillus* resistant strains have emerged posing a significant risk to farm workers and the general public. In Kenya, limited data is available on the level of triazole resistance among *aspergillus* species.

1.3 Justification

The widespread use of fungicides in the internationally visible flower and horticulture industry in Kenya and the conducive climatic conditions for fungal infestations and by irrational use of fungicides is a recipe for the emergence of antifungal resistance. The irrational use of azole-based fungicides in the flower and horticulture industry in Kenya, formed the basis of this to find out whether the use of azole-based fungicide in agriculture is a linked to the emergence of triazole resistance in clinical practices through horizontal transfer of resistance genes. *A. fumigatus* resistant to itraconazole have been reported from part of the world. In Kenya, antifungal drug resistance surveillance has not been done to determine whether the use of azole-based fungicides is associated with triazole resistance. Naivasha sub-county study site was selected because it is the site where they area concentration of large scale horticultural farming in the area, while Nairobi County has a concentration of small scale farming and the issue of pollution. Cyp51A gene was sequence to determine the type of mutation involved. Most of the *Aspergillus* species resistance involves cy51A gene. Data generated will serve to inform on the current status of triazoles resistance pattern and to raise concern emerging antifungal resistance in clinical practice and need for more surveillance.

1.4 Null hypothesis.

1. *Aspergillus* species diversity in fungicide naïve and experienced soils from Naivasha sub-county and Nairobi County is low.

2. The rate of azoles resistance is low among fungal isolates from the fungicides experienced and naïve soils from Naivasha sub-county and Nairobi County and clinical sources.

3. They are no mutation associated with Cyp51A gene among triazole resistance *Aspergillus fumigatus* isolates from soils from Naivasha sub-county and Nairobi County.

4. They are no genetic relatedness by sequence analysis of the cyp51A gene of *Aspergillus* species from fungicide naïve soils and experienced soils from Naivasha sub-county and Nairobi County and clinical isolates.

1.5 Research questions

1. What is the diversity in *Aspergillus* species in fungicide naïve and experienced soils from Naivasha Sub-county and Nairobi County?
2. What is the rate of azoles resistance among fungal isolates from the fungicides experienced and naïve soils from Naivasha sub-county and Nairobi County and clinical sources?
3. What is the type(s) of mutations associated with Cyp51A gene among triazole resistance *Aspergillus fumigatus* isolates from soils from Naivasha sub-county and Nairobi County?
4. What is the genetic relatedness by sequence analysis of the cyp51A gene of *Aspergillus* species from fungicide naïve soils and experienced soils from Naivasha sub-county and Nairobi County and clinical isolates?

1.6 General Objectives

To determine triazoles (itraconazole, posaconazole and voriconazole) resistance and sequence analysis of cyp51A gene among *Aspergillus* species from fungicide naïve and experienced soils compared to those from clinical sources.

1.7 Specific objectives

1. To determine *Aspergillus* species diversity in fungicide naïve and experienced soils from Naivasha sub-county and Nairobi County.
2. To estimate the rate of azoles resistance among fungal isolates from the fungicides experienced and naïve soils from Naivasha sub-county and Nairobi County and clinical sources.
3. To determine the type(s) of mutation associated with Cyp51A gene among triazole resistance *Aspergillus fumigatus* isolates from soils from Naivasha sub-county and Nairobi County.
4. To determine the genetic relatedness by sequence analysis of the cyp51A gene of *Aspergillus* species from fungicide naïve soils and experienced soils from Naivasha sub-county and Nairobi County and clinical isolates.

CHAPTER TWO

LITERATURE REVIEW

Aspergillus genus, consist of about 185 species, out of these, 20 species are of medical importance. Among them are; *A. terreus, A. flavus, A. versicolor, A. niger, A. ustus, A. clavatus, A. fumigatus* and *A. ustus*. *Aspergillus* species are known to cause: allergic toxicoses and opportunistic infections in man (Ikeda *et al*., 2000).

Immunosuppression is a predisposing element to opportunistic infections of *Aspergillus* species. *Aspergillus* species are the most isolated filamentous fungi causing invasive infections followed by *Candida* species (Ikenda *et al*., 2000). Aspergillosis can affect almost any organ and system: hepatosplenic aspergillosis, endophthalmitis, cutaneous aspergillosis, otomycosis, osteomyelitis, pulmonary aspergillosis, *Aspergillus* fungemia, and disseminated aspergillosis may develop (Arikan *et al*., 1998; Galimberti *et al*., 1998; Rouby *et al*., 1998). Construction around the hospital may lead to aspergillosis development in patients with neutropenic (Loo *et al*., 1996).

2.1 Triazole antifungal drugs

Antifungals are classified based on mechanism of action and their chemicals structure. Triazoles and azole inhibit biosynthesis pathways of ergosterol by blocking the P450 dependent cytochrome 14 demethylase and 14 methyl lanosterol leading to changes in lipid membrane structure. Azole also inhibit lipid biosynthesis and surface membrane enzymes (Chloe, 2009).

Triazoles include voriconazole, fluconazole, and itraconazole. While azole includes imidazole (miconazole and ketoconazole). Shortly after itraconazole was introduced; a report the first resistant *A. fumigatus* isolates were published and since then, several reports from all over the world, with the exception of Africa, have been published. In Germany for example, 3.2% of clinical and approximately 7% of environmental isolates

have an elevated MIC to itraconazole (Bader *et al*., 2013; Fraczek *et al*., 2013; Snelders *et al*., 2011).

2.1.1 Itraconazole

Itraconazole is a broad spectrum antifungal and synthetic triazole which is a cytochrome P-450 inhibitor. It is commercially available as amorphous nanostructured ITR and oral solution ITR (Alvarez *et al*., 2007). Chronic necrotizing aspergillosis, Aspergilloma, and invasive aspergillosis have been treated successfully with Itraconazole (Dannaoui *et al*., 2001). It has been licensed for short term treatment of vaginal candidiasis since it's safer than ketoconazole and amphotericin B (Watson & Pirotta, 2011). Aerosolized nanostructured ITR is used to prevent pulmonary aspergillosis against *Aspergillus fumigatus* (Alvarez *et al*., 2007).

2.1.2 Posaconazole

Posaconazole is a broad spectrum antifungal agent against *Cryptococcus neoformans, Aspergillus fumigatus and Blastomyces dermatitis* (Felton *et al*., 2010; Simon *et al*., 2015). It was triazole approved for use in the year 2006 http://cpip.gsm.com.ezproxy.samford.edi/. Posaconazole is also available in oral suspension www.crlonline.comezproxy.samford.edu/crlsql/servrlet/crlonline). Posaconazole has activity against *zygomycete*, unlike voriconazole. The drug is fungistatic against *Histoplasma*, *Fusarium* species, *Candida*, *Scedosporium* and *Coccidioides* (Simon *et al*., 2015).

In the USA and Europe, it has been approved for use as prophylaxis in immunosuppressed individuals having candidiasis and invasive aspergillosis for persons aged 13 years or older (Petrikkos and Skiada, 2007, FDA website). Posaconazole mechanism of action is by inhibiting ergosterol biosynthesis. It binds to a cofactor located at lanosterol 14α-demethylase at the target site (Ghannoum and Rice, 1999; Xiao *et al*., 2004).

Posaconazole prevent the ergosterol demethylation at C-4 and C-14 leading to alteration of structure and function of the plasma membrane, causing fungal death. Posaconazole is used in the management of patients with invasive aspergillosis when Voriconazole fails (Heinz *et al*., 2013). Posaconazole has been reported to have a success rate of 72% of which 41% showed complete responses and 30% partial response (Heinz *et al*., 2013).

2.1.3 Voriconazole

Voriconazole is a broad spectrum triazole with good bioavailability (compared to itraconazole and voriconazole) hence the acidic environment is not necessary for its absorption and as a treatment of the choice for aspergillosis (Asma & George, 2011). In 2002 FDA approved the use of voriconazole (www.crlonline.comezproxy.samford.edu/crlsql/servrlet/crlonline). Voriconazole is active against most of invasive mycoses e.g. dimorphic fungi, *Cryptococcus,* Fusarium species, *Candida, Scedosporium apiospermum and Trochosporon* species. *Sporothrix schenckii* and *Zygomycetes* are resistant to Voriconazole (Panackal *et al*., 2006; Sanguinetti *et al*., 2005, www.crlonline.comezproxy. samford.edu/crlsql/servrlet/crlonline).

Voriconazole offered at least 22% survival rate better than amphotericin B (Panackal *et al*., 2006). Voriconazole is metabolized hepatically by CYP450 isoenzymes. The voriconazole drugs are associated with interactions with other drugs, due to CYP3A4, CYP2C19, and CYP2C9 inhibition. (http://cpip.gsm.com.ezproxy.samford.edi/. www.crlonline.comezproxy.samford.edu/crlsql/servrlet/crlonline.)

2.1.4 Echinocandins

Echinocandins constitute the new antifungal approved for the treatment of invasive fungal infection (Denning, 2002; Ostrosky-Zeichner, 2004). The USA and Europe approved three types of echinocandins: Caspofungin in 2001, anidulafungin in 2006 and caspofungin in 2001. It was approved in USA and Europe because they possess low toxicity, can be used in combination with amphotericin, daily injection is sufficient, have broad spectrum against *Aspergillus* and *Candida* and is slowly degraded (Denning, 2002). Echinocandins mechanism of action is targeting the 1-3-β- D glucan synthetase of pathogenic fungus (Kartsonis *et al*., 2003).

Echinocandins have been noted to be active against Amphotericin resistant *Aspergillus terreus* and itraconazole resistant *Aspergillus fumigatus* (Warn *et al*., 2003). Echinocandins are not active against *Fusarium*, *Trichophyton* and *Cryptococcus neoformans* (Feldmesser *et al*., 2000) and intermediate susceptibility to *Scedosporium prolificans*, *Cladophialophora bantiana* and *Scedosporium apiospermum* (Espinel-Ingroff, 1998).

2.1.5 Polyenes

These include nystatin, amphotericin B and natamycin. Amphotericin and echinocandins were used in the treatment of systemic mycoses before the introduction of azoles. Their mechanism of action is by binding to cell membrane causing cellular content leakage and increases permeability (http://cpip.gsm.com.ezproxy.samford.edi; Jenks and Hoenigl, 2018; Patterson *et al*., 2016). Amphotericin B has activity against *Aspergillus* species, *Candida* species and dimorphic fungi (http://cpip.gsm.com.ezproxy.samford.edi/).

Side effects include hematologic toxicity, related infusion reaction and hephrotoxicity (http://cpip.gsm.com.ezproxy.samford.edi/www.crlonline.comezproxy.samford.edu/ crlsql/servrlet/crlonline; Jenks and Hoenigl, 2018; Patterson *et al*., 2016). Nystatin is

related structurally to amphotericin B. Nystatin at high concentration is fungicidal and fungistatic in vivo. Side effects of the drugs include: abdominal pain, vomiting, diarrhea, and nausea. Nystatin is found commercially in topical and oral forms (http://cpip.gsm.com.ezproxy.samford.edi; Jenks and Hoenigl, 2018; Patterson *et al*., 2016).

2.1.6 Echinocandins

Are antifungal drugs which include: caspofungin, anidulafungin, and micafungin. Mechanism of action is inhibiting (1, 3) beta- d-glucan synthase synthesis, thus cell death (Onishi *et al*., 2000). Echinocandins are used to treat invasive aspergillosis (Bennett, 2006). *Cryptococcus neoformans* resistance to echinocandins because in addition to (1, 3) beta d glucan synthase it has (1, 6) glucan (Onishi *et al*., 2000). Echinocandins side effects include: headache, dyspepsia, in case of rapid infusion with histamine-like symptoms and increase hepatic transaminases (Bennett, 2006). The use of capofungin and voriconazole in combination have increased efficacy in the animal? models (Marr *et al*., 2004a).

Caspofungin is used in the treatment of invasive aspergillosis and infection caused by *Candida* species: peritonitis, esophageal candidiasis, pleural infection and abscesses of intra-abdominal (www.crlonline.comezproxy.samford.edu/crlsql/ servlet/crinoline; Jenks and Hoenigl, 2018; Patterson *et al*., 2016). Caspofungin in 2001 was approved for use. Micafungin approved in 2005 for esophageal candidiasis treatment (http://cpip.gsm.com.ezproxy.samford.edi/; Jenks and Hoenigl, 2018; Patterson *et al*., 2016) and in stem cell transplantation patient as prophylaxis (http://cpip.gsm.com.ezproxy.samford.edi/). Anidulafungin was approved in 2006 by FDA for peritonitis, esophageal candidiasis, pleural infection, abscesses of intra-abdominal and candidemia. Product of degradation for anidulafungin passed to feces and urine (Dowell *et al*., 2004).

2.2 Azole resistance mechanism

Most of the fungal resistances associated with azole are mediated by 34 bases tandem repeat in cyp51A promoter region and mutation. Also, efflux pump has been linked to the emergence of triazoles resistance among *Aspergillus* species (Arendrup *et al*., 2010; Bowyer *et al*., 2012; Camps *et al*., 2012a; Camps *et al*., 2012c; Chowdhary *et al*., 2012a; Howard *et al*., 2009; Slaven *et al*., 2002; Snelders *et al*., 2008; Van der Linden *et al*., 2008). Irrational and rational application of agricultural antifungal has been linked to play an important role in the development of resistance against azole and also used of azole in treatment of patients.

Mutation in the TR34/L98H cyp51A has been associated with environmental development of azole resistance. This Azole resistance mechanism is linked to multi triazole resistant isolates from clinical and environmental sources in European countries, and reported in India, Japan, China, and the Middle East (, Badali *et al*., 2013; Tashiro *et al.*, 2012). Cross-resistance involving isolates with TR34/L98H in agricultural azole fungicides with evidence that clinical isolates can acquire resistance from the used environmental azole fungicides (Chowdhary *et al*., 2014b; Chowdhary *et al*., 2012a; Snelders *et al*., 2012).

In Kenya surveillance and studies on antifungal resistance is limited. The only documented is fluconazole emerging resistance in treatment of opportunistic fungal mycoses in HIV individuals, Resistance of 12% among clinical isolates of *Cryptococcus* and *Candid*a species have been reported (Bii *et al*., 2002; Bii *et al*., 2006; Ooga *et al*., 2011). Although antimicrobial resistance has been ascribed to irrational use of antibiotics and poor prescription practices, fungicide use in agriculture is more likely to fuel antifungal resistance particularly the azole based fungicides.

2.2.1 Drug efflux resistance mechanism

This form of resistance is due to activation of associated efflux pumps in the fungal membrane, leading to the recognition of different chemicals resulting in multidrug resistance. There is three drug efflux, associated with azole resistance: superfamily, ATP binding cassette, and major facilitator superfamily. This resistance mechanism caused by overexpression of ATP-binding cassette family efflux pumps and superfamily facilitator (Cannon *et al*., 2009). ATP binding cassette and major facilitator superfamily confer resistance to many drugs in some *Candida* species. MDP pumps in *Aspergillus fumigatus* have been linked with increased resistance to Itraconazole (ITR). Biofilm in *Aspergillus fumigatus* has been linked to resistance. It was revealed *A. fumigatus* has 49 ABC transporters and 278 different Major facilitator's (MFS) (Rajendran *et al*., 2011).

When *Aspergillus* is grown in the presence of Itraconazole it expresses *atrF* gene (an efflux pump) (Albarrag *et al*., 2011). However other reports indicated isolates to be having a substitution of G54E in Cyp51A gene (Albarrag *et al*., 2011; Balashov *et al*., 2005; Diaz-Guerra et *al*., 2003). Which is sufficient for thedevelopment of resistance to Itraconazole (Diaz-Guerra et *al*., 2003). Another case of overexpression of efflux pump is *Aspergillus fumigatus* mutant resistant to itraconazole produced by UV mutagenesis with mutation at codon G54 of Cyp51A (Nascimento *et al*., 2003). However, resistance could be due overexpression of *mdr3* and *mdr4* efflux pump (Nascimento *et al*., 2003).

Some studies have also reported overexpression of *mdr1* and *mdr2* pumps (da Silva *et al*., 2004). Increased cases of overexpression could be a stress response not necessarily associated to resistance (Albarrag *et al*., 2011). *Aspergillus fumigatus* azole resistance transporters are enigmatic and are up-regulated in exposures to triazoles, ABC transporter genes aiding in resistance are ABC-A to ABC-E, AfuMDR2, AfuMDR1 and in clinical resistant strain (da Silva Ferreira *et al*., 2006, Nascimento *et al*., 2003,). CDR1B is involved directly in *Aspergillus fumigatus* resistance against azole (Fraczek *et al*., 2013).

Major facilitator's (MFS) transporters expel drug using electrochemical proton force because it has a lot of TMS. MDR1 has been associated in *Candida dubliniensis* and *Candida albicans* resistance to azole, which enhanced efflux pump to azole. Also, MDR1 in *Saccharomyces cerevisiae* have been linked to confer fluconazole resistance (Lamping *et al*., 2007. In other fungal species, azole resistance have been linked to specific ABC transporters i.e. Afr1 in *Cryptococcus neoformans* and CgSnq2, CgCdr2 and CgCdr1 in *Candida glabrata* and (Coleman and Mylonakis, 2009).

2.2.2 Cyp51A gene mutations mechanism

Most of *Aspergillus fumigatus* resistant to azole have been linked to overexpression or point mutations of cy51A gene (Snelders *et al*., 2010). In cyp51A gene a single mutation at G138C has been detected, the mutant had altered TGC from GGC by deletion of glycine and insertion of cysteine (Howard *et al*., 2006) associated to cross resistance to voriconazole, itraconazole,e and posaconazole (Diaz –Guerra *et al*., 2003; Nascimento *et al*., 2003). Another one is substitution of methionine at M220 linked to reduced susceptibility to azoles (Mellado *et al*., 2004a), resistance to voriconazole and itraconazole due to glycine mutation (Howard *et al*., 2006). Another mutation associated with Cy51A gene are F219I, F219C, P216L, G434C, G432S, Y431C, A284T that have been associated to resistance among *Aspergillus* species (Camps *et al*., 2012b; Prigitano *et al*., 2014).

Another form of *Aspergillus fumigatus* mutation is the alterations in the promoter section resulting in strains resistant to several azoles (Rondriguez-tudela *et al*., 2008; Verweji *et al*., 2007). Resistance is due to modification of cy51A or combinations: A 46bp tandem insertion and substitutions of threonine to alanine, tyrosine to phenylalanine in the promoter region i.e. TR46/Y121F/T289Aoverexpression of cy51A due to the integration of 34bp in the promoter region (TR34/L98H) (Van der Linden *et al*., 2013). A 53bp tandem repeat without substitution acid in cyp51A at the promoter region (Hodiamont *et al*., 2009).

2.2.3 Alteration of ergosterol biosynthesis pathway

Any alteration in the biosynthesis pathway could lead to azole resistance. E.g. alteration through ERG3 gene inactivation will result in resistance to all azole drugs (Sanglard, 2002). Azole relies on 14α methylated sterol synthesis for its fungal activity which is synthesis by ergosterol biosynthesis pathway. In case of a point mutation in ERG3 gene, leading to the stop of 14α methylated sterol synthesis (Martel *et al*., 2010). Amphotericin resistance is liked to the ERG3 mutations and ergosterol absence (Morio *et al*., 2012a). Susceptibility to Amphotericin in *Aspergillus fumigatus* is not affected by egr3 genes deletion (Alcazar-Fuoli, 2006).

Terbinafine is an antifungal class which inhibits squalene epoxidase i.e. Erg 1. In *Aspergillus fumigatus* alterations of erg 1 gene increase resistance to terbinafine (Liu *et al*., 2004). *Aspergillus nidulans* resistance mechanism to terbinafine is linked to naphthalene enzymes degrading, thus resistance could be due to the degradation of this enzyme rings in the terbinafine (Graminha *et al*, 2004).

2.2.4 Azole naïve patients found harbouring *Aspergillus fumigatus* resistant to medical triazole

Azole-resistant *Aspergillus fumigatus* (ARAF) isolated in patients not exposed to azoles before was found to have a least one of the following resistance mechanism: Substitution of G432S in cyp51A gene, two a 46bpTR46 in cyp51A coupled with substitution, three 34bp tandem repeat in cyp51 a gene (Verweji *et al*., 2007). The TR34/L98H genotype found in most of the Dutch Centre were itraconazole resistant (Snelders *et al*., 2010; Snelders *et al*., 2008). The resistances were mediated by 34bp tandem repeat and substitution in cyp51A gene leading to azole-resistant *Aspergillus* phenotype (Snelders *et al*., 2011).

By use of mutagenesis side directed, Snelders *et al*., (2011) found out that *Aspergillus* phenotype which is resistant to a different type of azoles cannot be induced exclusively by induction of L98H mutation or TR34 mutation. This indicates that mutation in L98H is dependent on the promoter region (TR34).

2.3 Emergence of Azole resistance strains in Environment

Many authors agree with the hypothesis, Azole resistance *Aspergillus fumigatus* is environmentally acquired in patients with invasive aspergillosis rather than de novo mutation. The first Azole resistance among *Aspergillus fumigatus* was isolated in an individual previously not exposed to azole drugs (Arendrup *et al*., 2010; Chowdhary *et al*., 2012b; Howard *et al*., 2009; Van der *et al*., 2011).

Azole resistance *Aspergillus fumigatus* has been isolated in different environmental niches namely soil samples of tea gardens, compost, plant seeds, flowerbeds, hospital surroundings and paddy fields (Badali *et al*., 2013; Chowdhary *et al*., 2012b; Mortensen *et al*., 2010; Snelders *et al*., 2009). Most isolates with mutations at TR34/L98H region of the cyp51A gene were detected in India, Iran, Denmark and the Netherlands from the environment (Badali *et al*., 2013; Chowdhary *et al*., 2012b; Mortensen *et al*., 2010; Snelders *et al*., 2009).

Environmental survey of ARAF done in Europe showed that 9% of Danish and 12% of Dutch soil had TR34/L98H genotype (Mortensen *et al*., 2010; Snelders *et al*., 2009). Similarly, in India 5% of soil samples and 7% of all environmental isolated of *Aspergillus fumigatus* had TR34/L98H mutation (Chowdhary *et al*., 2012b). All strains had developed resistance to the three azoles Itraconazole, Voriconazole, Posaconazole and six other azole-based agriculture fungicides use for the control of fungal infection.

In the Netherlands, another mutation TR46/Y12F/T289A of cyp51A gene in Azole resistance *Aspergillus fumigatus* was noted in fourteen individuals from different

hospitals (Van der *et al*., 2013). The same gene mutation has been identified in Belgium and India from both non-clinical and clinical sources (Chowdhary *et al*., 2013b; Vermeulen *et al*., 2012). As individuals acquire *Aspergillus fumigatus* from the environment, the spread and emergence of azole resistant strains is of great concern especially among HIV/TB co-infected immunocompromised individuals.

2.4 Azole resistance trends in *Aspergillus* species globally

Europe has reported high cases of azole resistance (Table 2.1). In Dutch patients, the first cases of TR34/L98H resistance mechanism was reported in 2007, followed by CYP51A mutations in UK patients in 2009. This raises concerns about the increased cases in triazoles resistance in Europe (Howard *et al*., 2009). Azoles resistance *Aspergillus* isolated from clinical sources have noted in almost all of the European countries (Table 2.1). In the Netherlands the first cases of TR46/Y121F/T289A were reported, followed by the detections of the same mechanism in other countries (Lavergne *et al*., 2015; Montesinos *et al*., 2014; Pelaez *et al*., 2015). Resistance mechanism involving TR34/ L98H and TR46/Y121F/T289A genes mutation has been associated with the use of azole-based fungicides in agriculture (Van der Linden *et al*., 2013).

In Latin American little was known about Azole resistance, until Le Pape *et al*., (2016) isolated *Aspergillus fumigatus* with CYP51A changes with TR34/L98H and TR46/Y121F/ T289A mutation. Recently Leonardelli *et al*., (2017) reported *A. fumigatus* strains resistant to itraconazole carrying G54E substitution at cyp51A in a patient with keratitis in Argentina.

Table 2.1: Summary of cyp51A mutation in *Aspergillus fumigatus*

Cyp51A amino Acid no. or changes	Continents	References
TR34/L98H	African,	Chowdhary *et al*., 2014a
	Asia,	Chowdhary *et al*., 2015; Liu *et al*., 2015;
		Mohammadi *et al*., 2015,
	Europe,	Bader *et al*., 2015; Bader *et al*., 2013; Burgel *et al*., 2012; Howard *et al*., 2009; Jeurissen *et al*., 2012; Mortensen *et al*., 2010; Morio *et al*., 2012b; Prigitano *et al*., 2014; Steinmann *et al*., 2015; Vermeulen *et al*., 2015.
	Oceania	Kidd *et al*., 2015
TR46/Y12F/T289A	America	Le Pape *et al*., 2016; Wiederhold *et al.*, 2016.
	Europe	Arabatzis *et al.*, 2011; Bader *et al*., 2015; Escribano *et al.*, 2011; Lavergne *et al*., 2015; Montesinos *et al*., 2014; Steinmann *et al*., 2015; Vermeulen *et al*., 2015; Pelaez *et al*., 2015.
	Asia	Chen *et al.*, 2015; Hagiwara *et al.*, 2016.
TR53	America	Le Pape *et al*., 2015
	Europe	Hodiamont *et al*., 2009
G448S	America	Wiederhold *et al*., 2016

	Oceania	Kidd *et al*., 2015
	Asia	Toyotome *et al*., 2016
	Europe	Bellete *et al*., 2010; Howard *et al*., 2009; Pelaez *et al.,* 2012.
G54/W/R/E/V/A	America	Wiederhold *et al*., 2016
	Oceania	Kidd *et al*., 2015
	Asia	Chowdhary *et al*., 2015; Tashiro *et al*., 2012; Xu *et al*., 2010,
	Europe	Bader *et al*., 2015; Bader *et al*., 2013; Burgel *et al*., 2012; Howard *et al*., 2009; Morio *et al*., 2012b; Steinmann *et al*., 2015.
M220/T/V/I/K/R/L	America	Pharm *et al*., 2014; Wiederhold *et al*., 2016.
	Europe	Bader *et al*., 2015; Burgel *et al*., 2012; Howard *et al*., 2009.
	Asia	Bader *et al*., 2013; Xu *et al*., 2010

Novel mutation like F332K (Asano *et al*., 2011), G448S&TR46/Y121F/T289A (Toyotome *et al*., 2016) and P216L (Hagiwara *et al*., 2014) has been reported. In India, the prevalence of azole resistance (Table 2.2) (Chowdhary *et al*., 2015; Chowdhary *et al.,* 2012c; Chowdhary *et al.,* 2012d) and Iran has a prevalence of 3.5% (Hagiwara *et al.,* 2016). In the year 2001-2014 a study in the USA involving two hundred and twenty *Aspergillus fumigatus* detected TR46/Y121F/T289A, TR34/L89H, G448S, G138S/C, G54R/W/E, F219S, and M2201/K/V mutation (Wiederhold *et al*., 2016).

Table 2.2: Prevalence summary of azole resistance by country/continent

Isolates sources	Resistance (%)	Country/ Continent	References
Clinical and Environment	1.9%-11.1%	Asia	Ahmad *et al*., 2015; Ahmad *et al*., 2014; Liu *et al*., 2015; Mohammadi *et al*., 2015
Environment	13.9%	Tanzania	Chowdhary *et al*., 2014a
Environment	0.6%-11.8%	U.S.A	Wiederhold *et al*., 2016,
Clinical	2.6%	Oceania	Kidd *et al*., 2015
Clinical	6.6%-28%	United Kingdom	Bueid *et al*., 2010; Howard *et al*., 2009
Clinical	1.8%	Spain	Escribano *et al.*, 2013
Clinical	0.85%-10.6%	France	Alanio *et al*., 2011; Burgel *et al*., 2012; Morio *et al*., 2012b.
Clinical and Environment	1.1%-12%	Germany	Bader *et al*., 2015; Bader *et al*., 2013; Fisher *et al*., 2014.
Clinical	2.25%	Poland	Kurzyk *et al*., 2015
Clinical	5.7%	Belgium	Vermeulen *et al*., 2015
Clinical	10.2%	Turkey	Ozmerdiven *et al*., 2015
Clinical and Environment	21%-20%	Belgium	Vermeulen *et al*., 2015

2.5.1 *Aspergillus* species resistance link to azoles use in agricultural

The discovery of TR34/L98H mutation in clinical and environmental isolates (Mortensen *et al*., 2010; Snelders *et al*., 2008) raised the questions that it might not only result from long term azoles treatment of patients with aspergillosis, resulting in mutants' selection but may be acquired from the environment directly (Snelders *et al*., 2009). The above notion was supported by the subsequent finding that tebuconazole under laboratory conditions can mediate tandem repeats in the cyp51A gene. Five agricultural fungicides (epoxiconazole, tebuconazole, propiconazole, difenoconazole and bromuconazole) have a similar molecular structure to medical triazoles hence target the same enzymes in a similar manner as clinical use azoles (Table 2.3) (Snelders *et al*., 2012; Snelders *et al*., 2009).

Drug resistance is triggered by azole pressure during medications and developes over years. Lateral gene has no role in transfer and antifungal resistance in *Aspergillus* spp. and is not spread from one person to the other (O Gorman *et al*., 2009; Verweji *et al*., 2009). These factors are used in the interpretation of research reports involving cyp51A mutations and to predict the mutation pattern in cohort patients. Hence it is not surprising to find TR34/L98H mutation in patients with aspergillosis in regions or country where mutations occur in the environment. However, Azole-resistant isolates from clinical sources having Cyp51A mutations and TR34/L98H strains depend on the relative fitness and exposure of the mutants competing. TR34/L98H genotypes were recently detected with Polymerase chain reaction positive sputum but culture negative samples from individuals having chronic or allergic pulmonary aspergillosis (Chowdhary *et al*., 2012a).

Clonal expansion in the Netherlands suggested that TR34/L98H genotype fit in the environment since antifungal susceptibility testing depend on cultured isolates (Klaassen *et al*., 2012) and research by use of animal model failed to show virulence loss. However,

G432S isolated in individuals previously not exposed to azole in France (Alanio *et al*., 2011) and TR46/Y121F/T289A from environment and clinical sources in the Netherlands (Kujpers *et al*., 2011) both cyp51A mutants, supported that azole resistance from the environment is often no associated with the up-regulation of CYP51A genes (Leroux and Walker, 2011).

Table 2.3: Commonly used fungicides in agriculture

Common compound's name	**Treatment**	**Year**
Prothioconazole	Broad	2002
Triticonazole	Broad	1992
Fluquinconazole	Foliar	1992
Metconazole	Broad	1992
Epoxiconazole	Broad/cereal	1990
Difenoconazole	Seed/foliar	1988
Tretraconazole	Broad	1988
Hexaconazole	Broad	1986
Tebuconazole	Foliar and seed	1986
Cyproconazole	Broad	1986
Diniconazole	Broad	1983
Triflumizole	Broad	1982
Propiconazole	Broad	1979
Prochioraz (Imidazole)	Seed treatment	1977
Imidazole	Broad	1973

Source: Morton and Staub, 2008, Kleinhauf *et al*., 2013.

2.6 *Aspergillus* species infection clinical manifestations

There are approximately 180 species of medical important *Aspergillus* species causing human disease in genus *Aspergillu*s. Diseases manifestation is determined by the host immune system status and diseases evolution but also governed by individual immune status (Lowes *et al*., 2015). *Aspergillus fumigatus* cause most of the diseases accounting for approximately 90% of the cases (Balajee *et al*., 2005; Li *et al*., 2011). *Aspergillus* species have been noted to causes infections after stem cell transplantation in patients as follows: *Aspergillus terreus* (16%), *A. fumigatus* (56%), *A. niger* (8%), *A. flavus* (18.7%) and *A. versicolor* (1.3%) (Morgan *et al*., 2005).

2.6.1 Allergic Bronchopulmonary Aspergillosis

The diseases affect individuals who are allergic to spores of *Aspergillus*. The inflammation of the lung due to overreactions of the immune system to the fungus leads to mucus accumulation in the airways and bronchospasm. The maladaptive immune reaction can be simple to severe asthma (Agarwal, 2011; Knutsen *et al*., 2012). *Aspergillus* species are found in every habitat, living in the soil. Human infections are usually acquired through spores inhalation, measuring 2-3µm in size, hence can easily be deposited in alveoli (Knutsen *et al*., 2012). Allergic bronchopulmonary aspergillosis (ABPA) pathogenesis has not been fully understood since the intensity of the spore exposure does not correlate with the rate of sensitization (Beaumont *et al*., 1985). Because of the *Aspergillus fumigatus* immune evasive properties and virulence, this factor contributes to invasiveness and allergic properties of the ABPA (Kwon-Chung and Sugui, 2013).

Diagnosis of ABPA is by combinations of radiology, laboratory parameters and clinical manifestations (Denning *et al*., 2006). Laboratory detections of above 500IU/ml of specific IgE serum against *Aspergillus fumigatus* antigens (Denning *et al*., 2006) or performance of skin test and detection of IgG (Denning *et al*., 2006). Treatment of

Allergic bronchopulmonary aspergillosis by a combination of steroid to block reaction and prevent inflammation and use of antifungal drugs such as Itraconazole and voriconazole to kill the fungi (Cheng *et al*., 2010; Harrison *et al*., 2015; Wark *et al*., 2004).

2.6.2 Invasive Aspergillosis

Invasive Aspergillosis (IA) was first reported in 1953 (Rankin, 1953). There has been an increase in IA incidences over the past decades (Chamilos *et al*., 2006). Because of chemotherapy and immunosuppressive agents use (Chamilos *et al*., 2006; Mc Neil *et al*., 2001). *Aspergillus fumigatus* is most virulent causing approximately 90% of invasive aspergillosis (Li *et al*., 2011), IA varies geographically for unknown reasons. In 1978 and 1992 incidence of IA in all the autopsies carried out were 3.1% to 0.4%. While in Invasive pulmonary aspergillosis it rinsed to 60% from 17% (Groll *et al*., 1996). The mortality rate in Invasive pulmonary aspergillosis was approximately 90% in patients who had undergone Hematopoietic transplant and 50% in neutropenic patients (Fukuda *et al*., 2003; Yeghen *et al*., 2000).

The main risk factor of IA is neutropenia, Invasive pulmonary aspergillosis correlates well with neutropenia degree and duration of organ transplantation is also an important risk factor (Kotloff *et al.,* 2004). Other risk factors are: use of multiple anti-rejections drugs, multiple antibiotics use long stay in hospital and multiple immune defects. Invasive pulmonary aspergillosis in not common in HIV patients using ARVs. The incidence has been approximately 3.5 cases in 1000 persons (Holding *et al*. 2000). Majority of HIV/AIDS individuals with IA has been noted as having coexisting low CD^4 counts, neutropenic or undergoing corticosteroid therapy. However, a minority have only AIDS has a predisposing factor (Nathan *et al*., 2000).

Clinical presentation: they present nonspecific symptoms which mimic bronchopneumonia: Production of sputum, cough, fever, not responding to antibiotics, pleuritic chest pain, dyspnoea, and hemoptysis. *Aspergillus* may haematogenously disseminate to other organs namely: brain, skin, heart, Liver, esophagus, liver, and pleura. In the brain, it leads to meningitis, hemorrhage, intracranial lesion, cerebral infarctions and seizures (Wu *et al*., 2010).

A unique form of invasive pulmonary aspergillosis called *Aspergillus* tracheobronchitis, which involve the invasion of the tracheobronchial tree by the fungi. The risk factors of this infection are AIDS, Cancer, and transplantation of the lung (Natha *et al*., 2000, Wu *et al*., 2010). *Aspergillus* tracheobronchitis is characterized by tracheobronchial tree extensive inflammation and presence of *Aspergillus* species in mucosa membrane. Pseudomembranous *Aspergillus* tracheobronchitis is a severe type and the patients will be characterized with dyspnoea and cough, lobar or segmental might be present (Wu *et al*., 2010).

2.6.2.1 Diagnosis of Invasive pulmonary aspergillosis

Invasive pulmonary aspergillosis can be diagnosed using Bronchoalveolar lavage (BAL). Use of BAL fluid have specificity and sensitivity of 97% and 50% respectively (Machmeyer *et al*., 2003). It is also important to send BAL fluid, sputum and lung tissues for histological examination and culture (Panackal and Marr, 2004). The recent diagnosis method is by detecting antigens of *Aspergillus* species i.e. 13-β-d-glucan and galactomannan in fluid (Marr *et al*., 2004b). CT scan and chest X-ray, or microscopy examination of *Aspergillus* species, stain with silver stains can also be used in diagnosis (Table 2.4) (Kradin and Mark, 2008). *Aspergillus* hyphae measure 2.5 to 4.5 µm and they are septate. Treatment is by use of Amphotericin, Itraconazole, and Voriconazole. However, Voriconazole has been found to be the most effective (Herbrecht *et al*., 2002).

2.6.3 Aspergilloma

Aspergilloma presents as a mass of *Aspergillus* fibrin, hyphae, cellular debris and mucus formed in lung cavity or pulmonary scar.In the cavity previously caused by Histoplasmosis, Tuberculosis, Coccidioidomycosis, Lung cancer, Lung abscess, Sarcoidosis or Cystis fibrosis (Denning *et al*., 2016; Horan –Saullo and Alexander, 2016; Kosmidis and Denning 2015; Walsh, 2016).

Aspergilloma can co-exist with other forms of aspergillosis or can occur in isolation. Simple Aspergilloma is defined as a single fungal ball in the lung cavity which is not in conjunction with other forms of aspergillosis and is very slow in progressive (Godet *et al*., 2014; Jhum *et al*., 2013; Patterson and Strek, 2014; Smith and Denning, 2011) reported a patient who had Aspergilloma caused by *Aspergillus flavus* recovered from surgical specimen.

2.6.4 Chronic Cavitary Pulmonary Aspergillosis (CCPA)

The diseases is defined as at least three months symptoms of systemic and respiratory, secondary to *Aspergillus fumigatus* infections with pre-existing lung disease (Denning *et al*., 2016). CCPA is characterized by pleural thickening, the granulomatous, formation of thick-walled cavities and necrosis in the parenchyma. About 50% of the cavities contain Aspergilloma (Godet *et al*., 2014).

2.6.5 Chronic Fibrosing Pulmonary Aspergillosis

The diseases occur from unmanaged cases of chronic cavitary pulmonary aspergillosis (Denning *et al*., 2013). In CFPA there are extensive damages of fibrotic tissue affecting two of the lung lobes (Denning *et al*., 2016).

2.6.6 *Aspergillu*s bronchitis (AB)

Aspergillus bronchitis is caused by *Aspergillus* species. It is a chronic lower airway chronic infection involving mainly bronchi and trachea. In *Aspergillu*s bronchitis parenchyma of the lung is normal unlike in chronic pulmonary *Aspergillu*s where there are nodules, pleural fibrosis and cavitation features in the parenchyma. The occurrence of AB can happen in asthmatics pattern unlike in *Aspergillus* broncho-pulmonary Aspergillosis (Chrdle *et al*., 2012). Case series study in twenty-nine Spanish hospitals, from 2002 to 2010 showed that 38 patients were having *Aspergillus* bronchitis (Barberan *et al*., 2014). Another study reported 17 cases in a population of 400 patients (Chrdle *et al*., 2012).

Cystic fibrosis is one of the risk factors of AB (Armstead *et al*., 2014). Women aged 54 and above are at risk unlike in other forms of aspergillosis where men are predominant (Chrdle *et al*., 2012). Patients produce tenacious sputum in a large amount of any color and productive cough. The second common sign is recurring chest infection (Chrdle *et al*., 2012). Others are shortness of breath, malaise, and weight loss. Diagnosis is through culture and measuring of serum IgG usually above 75mg/l is highly suggestive. The use of PCR in sputum is sensitive compared to culture (Fraczek *et al*., 2013). Treatment is usually by using both voriconazole and itraconazole (Chrdle *et al*., 2012).

2.6.7 Chronic Necrotizing Aspergillosis (CAN)

Also referred to as Subacute or semi-invasive Aspergillosis described in the year 1981 by Gefter *et al*., (1981). The diseases are caused by the infection of parenchyma tissues in lungs by *Aspergillus* species. This disease is rare and literature review is mainly from case studies (Kim *et al*., 2000). The most affected are group are elderly and middle-aged individuals previously affected by pulmonary tuberculosis, cystic fibrosis, sarcoidosis, lung infarction, thoracic surgery and underlying lung diseases (Denning, 2001). Patients with liver diseases, ankylosing spondylitis, rheumatoid arthritis, diabetes mellitus, corticosteroid therapy, and alcoholism are at risk of contracting the infection.

The disease presents itself with the following symptoms: fatigue, malaise, hemoptysis, productive cough, and fever. Chest imaging shows cavitary lesion and thickening of the pleura in the lung. Diagnosis is by the culture of *Aspergillus* species and histological examination (Table 2.4) (Parra *et al*., 2004).

2.6.8 Pulmonary *Aspergillus* overlap syndromes

Syndromes co-infect with other forms of aspergillosis or progress to another entity of aspergillosis (Fig 2.1). This form of aspergillosis occurs due to coincidental, corticosteroid therapy, genetic factors, underlying severe lung diseases or fungal load (Pasqualotto & Soubani, 2010).

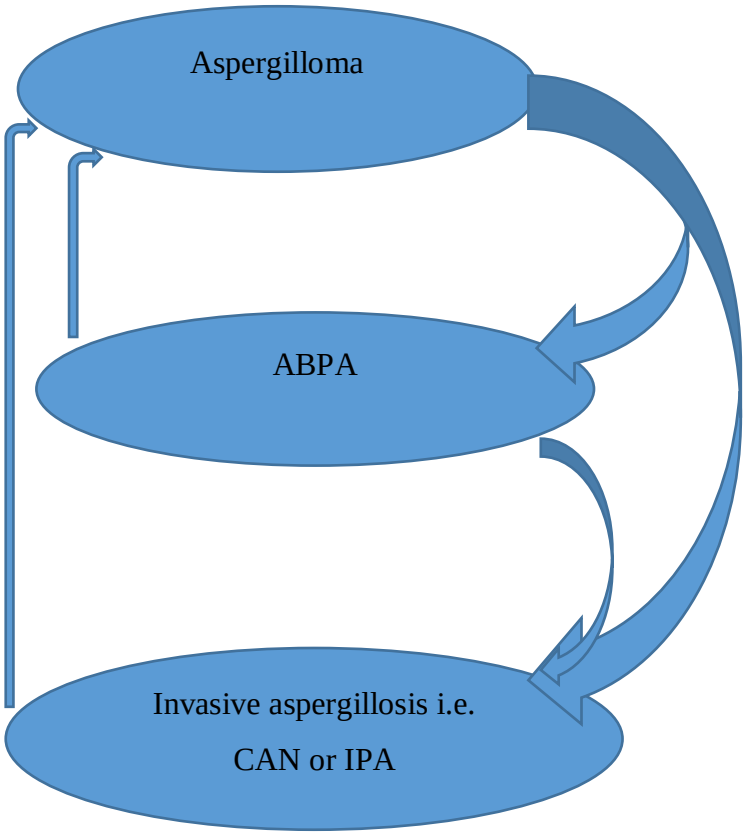


Figure 2.1: Pulmonary *Aspergillus* overlap syndromes, Sources, Pasqualotto & Soubani, 2010.

Table 2.4: Diagnosis of different form of aspergillosis

Features	**Types of aspergillosis**			
	Aspergillus bronchitis	Allergic Bronchopulmonary Aspergillosis	Chronic pulmonary aspergillosis	Invasive aspergillosis
Serum Ig E	Normal	Elevated >1000	Normal or mild elevated	Occasionally positive
Aspergillus IgG and precipitins	Negative or Positive	Negative or Positive	Positive	Negative
Skin prick and fungal specific tests	Negative	Positive	Often low level positive	No data
Systemic symptoms	Uncommon	Uncommon	Common	Common
Onset	Subcute or chronic	Chronic	Chronic	Acute
Host factors	Asthma, Bronchiectasis and other airway disease	Allergies e.g. Asthma	Lung sequelae, Asthma, Bronchiectasis	Transplantation, immunosuppres-sion, neutropenia

Chest Imaging	Bronchiectasis	Obstruction of airway with bronchiectasis and mucus	Aspergilloma, Fibrosis, formation of cavity, nodules	Infarction, consolidation, air crescent and Halo signs
Respiratory symptoms	Dyspnea, productive cough, thick sputum, recurring chest infections	Dyspnea, productive cough and wheezing	Dyspnea, productive cough, hemoptysis	Nonspecific and usually mild

2.6.9 The current trends in *Aspergillus species* resistance and treatment

Treatment of fungal infections is becoming complicated worldwide due to ever-increasing resistance of fungi to antifungal (Chowdhary *et al*., 2017; Perlin *et al*., 2017). In the past resistance was only considered a menace to human health y, currently, it is a multi-sectorial problem requiring experts from environmental, human and animal health (Chowdhary and Meis, 2018). Azole based fungicides with similar chemical structure as clinical triazoles, such as epoxiconazole, propiconazole, tebuconazole, bromuconazole, and difenoconazole are linked to emerging *A. fumigatu*s resistance due to selection pressure (Alvarez-Moreno *et al.,* 2017). The uses of these fungicides in the environment coincide with emerging clinical ARAF (Meis *et al.*, 2016). *A. fumigatus* exhibiting azole-resistance to agricultural azole-based fungicides and cross-resistance to clinical triazoles in Taiwan environment was demonstrated by Wang *et al*., (2018). The study reinforces why one health approach in azoles resistance is needed (Chowdhary and Meis, 2018).

Agriculture is the neglected niche next to Veterinary antimicrobial resistance and Human antimicrobial resistance. A study in the Netherland reported the resistance of *Aspergillus fumigatus* to azoles as an increasing trend (Buil *et al*., 2019).

The recommended treatment for symptomatic chronic pulmonary aspergillosis individuals is six months oral therapy with i.e. oral posaconazole, voriconazole, and itraconazole as first-line treatment (Garcia-Vidal *et al*., 2018). Patients who fail to respond to first-line treatments are given amphotericin B or echinocandins intravenously. Also, surgical resection can be used in patients with hemoptysis, triazole resistance, or localized diseases (Garcia-Vidal *et al*., 2018). The infectious diseases society of America (IDSA) recommend the used of voriconazole as the first treatment for invasive aspergillosis. In patients with neutropenia and hematologic malignancy, a combination of Isavuconazole and voriconazole is recommended (Garcia-Vidal *et al*., 2018; Patterson et al., 2016). Voriconazole is recommended as a first-line treatment for central nervous aspergillosis. Voriconazole compare to posaconazole and itraconazole has good brain tissues penetration (Beardsley *et al*., 2018)

2.7 Phenotypic characterization

The main method used in the identification of *Aspergillus* species is morphological characteristics of conidiophores, conidia, and cultures (http://www.ncbi.nlm.nih.gov/pubmed/21510879).

2.7.1 Morphology

Aspergillus fumigatus grow faster in media compared to *Aspergillus terricola, Aspergillus flavus* and *Aspergillus inccum* then *Aspergillus terreus* (Hina *et al*., 2013; McClenny, 2005). *Aspergillus flavus* var. *Columnaris* have uniseriates head with few biseriates heads while *Aspergillus fumigatus* have uniseriates heads.

Aspergillus flavus produces green to dark green colonies with rough conidiophore wall and radiate heads, while *Aspergillus flavus* var. *columnaris* have columnar heads in Malt Extract agar (Hina *et al*., 2013; Kim *et al*., 2009). *Aspergillus fumigatus* key identifications features are flask to ovate shape vesicle with ampulliform phialides, septate hyphae with abundant dichotomous branching, grey green colonies on SDA measuring 4±1 cm in a week and blue-grey colony on Malt Extract agar. Under the microscopy *A. fumigatus* showed hyaline which is brownish or green at the vesicle tip. *Aspergillus terricola* is identified by globose conidia and green colonies on Malt Extract agar (Diba *et al*., 2007; Hina *et al*., 2013). *Aspergillus niger* show hyphae measuring 2.5-8 mm in wide, branching at an acute angle and are septate conidia are in chains, having a biseriate head (McClenny, 2005). In SDA they appear as black colonies.

2.8 Serological diagnosis of *Aspergillus* infection

2.8.1 (1, 3)-β-D- glucan

(1, 3)-β-D- glucan is found on fungal cell wall (Obayashi *et al*., 2008). They are in high concentration in the cell wall of the following fungi: *Aspergillus, Fusarium, Penicillium, Trichosporon, Saccharomyces, Candida,* and *Fusarium*. *Cryptococcus neoformans* have low (1, 3)-β-D- glucan (Odabasi *et al*., 2006). (1, 3)-β-D- glucan is relatively high in *Aspergillus* (Latge, 2007) and a low level in *Cryptococcus* (Mennink-Kersten *et al*., 2006). (1, 3)-β-D- glucan in *Aspergillus fumigatus* is produced during logarithmic growth (Marty & Koo, 2009).

Scientist have developed laboratory methods for detection of the complex carbohydrates (1, 3)-β-D- glucan) in Cerebrospinal fluid, serum, abscess fluid, and Bronchio lavage fluid (Acosta *et al*., 2011; Lamoth *et al*., 2012). Most of the assays are constructed colorimetric or enzymatic methods depending on (1, 3)-β-D- glucan triggering Amebocyte cells coagulation cascade (Karageorgopoulos *et al.*, 2011). Validation of the assay in hospitalized patients with fungal infection in Japan showed high specificity of 76.8% and sensitivity of 85.3% (De Pauw *et al.*, 2008; Obayashi *et al*., 2008).

2.8.2 Galactomannan

Galactomannan is a type of carbohydrate composed of mannose residues. It is produced by *Aspergillus* species growing in vitro, on their hyphae release and measured in body fluids and serum (Segal, 2009). Galactomannan is a molecule of about 20 kDa or a large glycoproteins molecules (Morelle *et al*., 2005). Galactomannan is composed part of the fungal cell wall with chitin (Segal, 2009). Galactomannan is agglutination test, with detection strength of 5ng/ml. However, the test was replaced with ELISA sandwich with a detection limit of > 1ng/ml.

Platelia immune-enzymatic microplate fluid assay is used to detect galactomannan in broncho-alveolar lavage and serum using monoclonal antibodies refer to as rat EBA directed against polysaccharide (Danpornprasert *et al*., 2010). Galactomannan is released into the bloodstream during angioinvasion (Franquent *et al*., 2001). Invasive aspergillosis is widely diagnosed using galactomannan assay using serum, which specificity reported being 82.4% and sensitivity at 96.8% (Danpornprasert *et al*., 2010; Hadrich *et al*., 2011). However in an individual not undergoing any antifungal therapy, the sensitivity is reported to be 87.5% (Marr *et al*., 2004b). Galactomannan is also found also in the following fungi *Histoplasma* species, *Fusarium* species, *Alternaria* species and *Penicillium* species in varying amounts (Mikulska *et al*., 2012).

2.9 Molecular typing and identification techniques

2.9.1 Microsatellite length polymorphism (MLP)

Microsatellites are DNA segments with repeated sequence e.g. CACACACA (de Valk *et al*., 2007a). MLP relies on microsatellite sequences amplification, which are two to five repetitive stretches of nucleotide. MLP is used to detect different types of alleles in a given locus (Eloy *et al*., 2006; Sampaio *et al*., 2005).

This method is reproducible and rapid (Lair-Fulleringer *et al*., 2003). The microsatellite is widely used in laboratory identification of microorganism. The method showed approximately 100% type-ability and very high discriminatory powers (de valk *et al*., 2007a; Legendre *et al*., 2007). Microsatellites techniques have been successfully used in identifications of *Aspergillus fumigatus* (Bart Delabesse *et al*., 1998).

2.9.2 Restriction fragment length polymorphism

The techniques are used in laboratory identification of several microorganisms' since its rapid, simple and inexpensive compared to other laboratory methods (Aravindhan *et al*., 2007). Organisms are differentiated by cleaving the DNA and analyzing the pattern. The sizes of the DNA fragments produced will differ in length after being digested with a restriction endonuclease. Specific DNA is cut at a specific site using endonuclease (Aravindhan *et al*., 2007). Despite Restriction fragment length polymorphism (RFLP) reproducibility, some authors have reported that the method does not effectively differentiate between *Aspergillus fumigatus* strains (Girardin *et al*., 1993).

2.9.3 Multilocus sequence typing

Multilocus sequence typing (MLST) was originally invested to investigate pathogenic fungi and bacterial (Bain *et al*., 2007; Taylor *et al*., 2003). It is recommended for differentiation of isolates by comparing nucleotide sequence (Taylor *et al*., 2003). MLST is increasingly used in most laboratories for international comparison of isolates. It is used in the identification of isolates by comparing the 5^{th} and the 7^{th} genes of nucleotide polymorphism.

MLST data have been used to estimate mutation rates and recombination among bacteria of the same genus (Urwin *et al*., 2003) and identification of new species e.g. *Aspergillus lentulus* (Balajee *et al*., 2005). The multilocus was mainly used for sequencing *A. fumigatus* seven genes. It was revealed that 93% of the isolates differed with the other by only one sequence (Bain *et al*., 2007). The high discriminative power of multilocus sequence typing was found out to be 0.93 and discover that *Aspergillus fumigatus* species had dissimilarity level in sequence (Bain *et al*., 2007).

2.9.4 Sequence based identification

This method is used in determining nucleotide bases order i.e. thymine, cytosine, adenine and guanine in DNA oligonucleotide. It is an appropriate tool that can be used in future understanding and research of fungi (Ronning *et al*., 2005). Complete DNA sequence has been generated using these methods (Hutchison, 2007). The first complete bacterial genome sequences appeared in 1995 and eukaryotic DNA genomes in 2007 by Hutchison. In 2005 *Aspergillus fumigatus* Af93 were sequenced using random sequencing methods and was found to contain approximately 9,900 genes and 8 chromosomes. The third genes function is still not well understood (Ronning et *al*., 2005).

2.9.5 Random Amplified Polymorphic DNAs (RAPD)

Welsh and McClelland was the first to describe random amplified polymorphic DNAs in 1990. RAPD techniques used ten long bases pairs which is a short synthetic oligonucleotide as primers, under low PCR annealing temperatures to amplify DNA (Williams *et al*., 1990). Products obtained are separated based in size by agarose gels stained in crystal violet or ethidium bromide (Welsh and McClelland, 1990). RAPD techniques are relatively easy to perform compared to other methods using DNA markers (Raclasky *et al*., 2006).

However RAPD has low reproducibility power because of lower intensity case bands or unprepared DNA and most PCR machine are sensitive to changes in reaction temperatures (Bardakci, 1996; Welsh and McClelland, 1994). The method is useful in fingerprinting of fungus especially *Aspergillus fumigatus* (Welsh and McClelland, 1994). In RAPD genotyping *Aspergillus fumigatus* GTATTGCCCT primer is used for giving the best discrimination of isolates (Anderson *et al*., 1996; Raclasky *et al*., 2006).

2.9.6 Multilocus enzyme electrophoresis (MLEE)

MLEE were develop by Bertout *et al*. (2001) for *Candida albicans* identifications. MLEE is used in conjunction with other methods in the study of *Aspergillus fumigatus* diversity, since it has good discriminatory power (Bertout *et al*., 2001, Rodriguez *et al*., 1996).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The study was conducted in Nairobi County and Naivasha Sub County (fig 3.1 and 3.2). These are the counties where horticultural practices and greenhouses are concentrated. Nairobi is the capital city of Kenya. It comprises of Starehe, Dagoretti South, Kibra, Langata, Dagorretti North, Makadaa, Westlands, Ruaraka, Kamukunji, Roysambu, Mathare, Embakasi east, Embakasi central, Embakasi south, Embakasi north, Embakasi west and Kasarani sub-counties. It's lies at about 1°17'S and 36°49'E. Nairobi County was included as one of the study site because of pollution (urban area).

Naivasha is located about 90KM North West of Nairobi. It is located in Nakuru County. Naivasha sub-county comprising of Mai Mahiu, Maeilla, Olkara, Naivasha East and Viwanda wards. Naivasha lies at about 0°43'S36°26E. Horticulture is the main economic in Naivasha. The majority of Horticulture firm in the area produced cut rose flower which is sold in European markets. They also grew food crops such as kales, cabbages, peas, beans, carrots and tomatoes. Naivasha is one of the Sub-county has two rainy seasons' short rains between October to December and Long rains between April to August. Receiving a mean annual rainfall of 80mm. Temperatures ranging from 10°C (July, August) and 20°C (January, February and March) in cold and hot months respectively. The GPS coordinates were not collected because the farm owners did not consent to it as the coordinates can identify individual farms by name. Also, the generation of a map using GPS coordinates could raise and generate ethical issues as it violated the privacy of the farm owners.

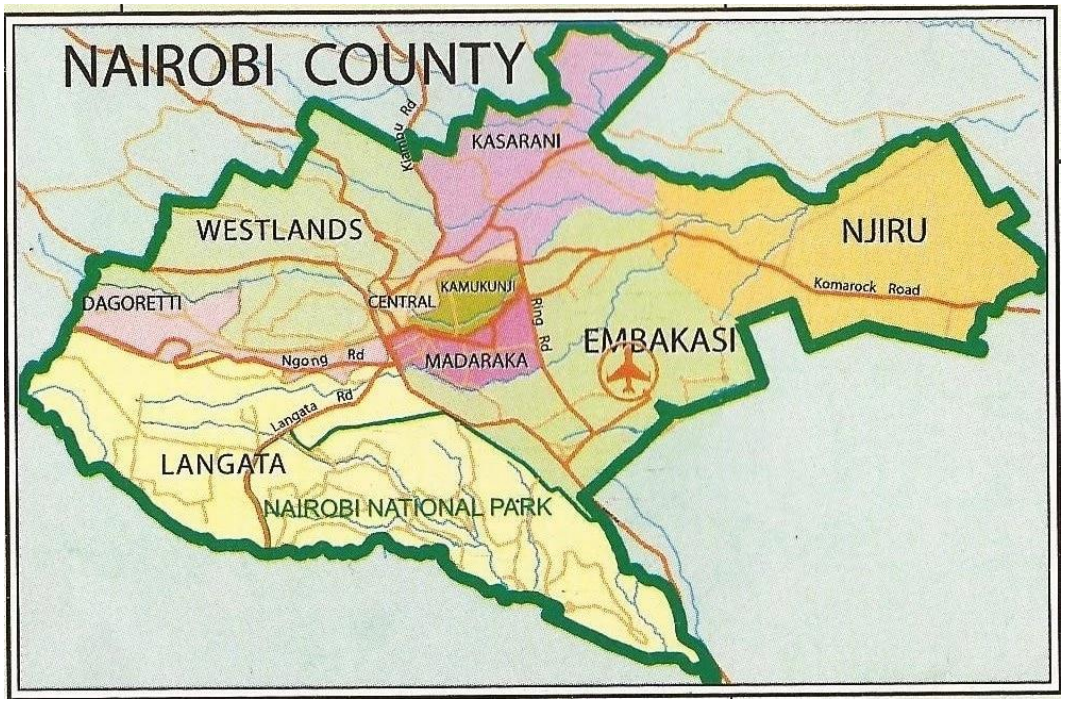


Figure 3.1. Nairobi County study site courtesy of https://netstoragetuko.akamaized.net/images/0fgjhs84r0486fl1a.jpg. Retrieved on April 13, 2019.

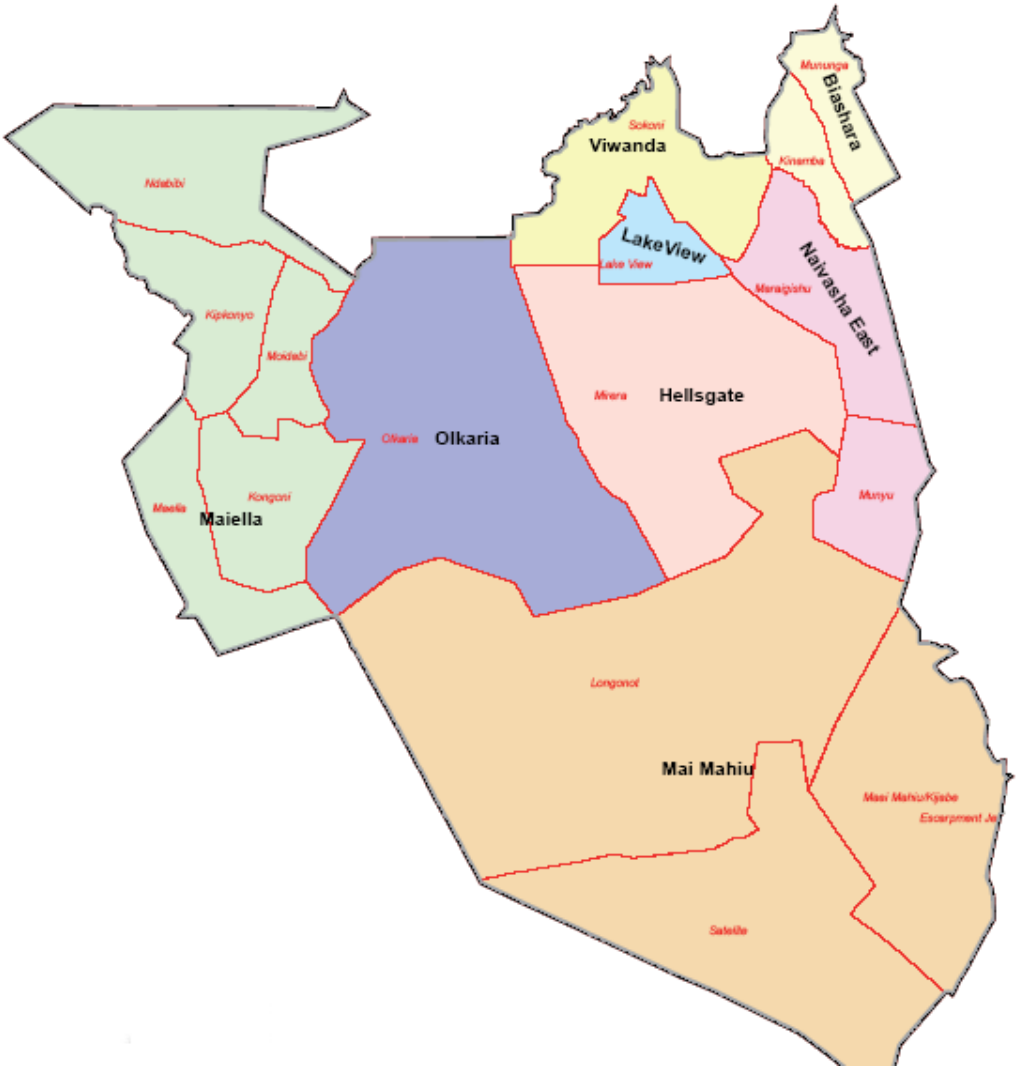


Figure 3.2. Naivasha sub-county study sites courtesy of https://www.ngcdf.go.ke/images/com_constituencies/560/Naivasha%20Constituency. PNG. Retrieved on April 13, 2019.

3.2 Study design

The study involved both laboratory-based and cross-sectional study design involving Nairobi county and Naivasha sub-county. The study was carried out for approximately two years, seven months from May 2015 to December 2017. The soils were collected from random points from the selected site (Each farm/green-houses were sampled in five

points (directions) i.e. east, south, west, north and Centre of the farms and then pooled to make one sample) meeting the inclusion criteria. Simple random sampling was used in the collection of soil samples from the horticulture farm. Farms identification was by use of simple randomization techniques.

3.2.1 Inclusion criteria

Fungicide experienced soil sampled met the following criteria: Soil under cultivation, evidence of fungicide used in at the last one year and permission obtained from the farm owner or organization. While fungicide naïve soils sampled was a virgin land that has never been cultivated, or had no agricultural activities nearby or pollution from nearby human or industrial activities.

3.2.2 Exclusion criteria

Soils were excluded if the owner decline to give consent, no information about fungicides use or where there was evidence of pollution.

3.2.3 Clinical samples inclusion and exclusion criteria

Since clinical isolates were from archived samples, those that did not yield growth or yielded non- *Aspergillus* species were excluded. All the samples that grew *Aspergillus* species were included in the study.

3.3 Sample size determination

3.3.1 Sample size for Environmental samples

A total sample size of 492 for the two study sites was derived using fisher formula (1998). Triazole resistance prevalence rate of 20% for *Aspergillus fumigatus* reported in Tanzania was used according to Chowdhary *et al*., (2014a).

Using fisher formula;

$$n = \frac{t^2 \, x \, P(1-P)}{m^2}$$

Where;

n= required sample size

t= confident level at 95% (standard value of 1.96)

p= Estimated prevalence of resistance at 20%

m= margin of error at 5% (standard value of 0.05)

Hence, n= $\frac{1.96^2 \text{ x } 0.2 \text{ (1-0.2)}}{0.05^2}$ = 245.8 (approximately 246).

The study involved two study sites; Nairobi county and Naivasha sub-county. In each study site, 246 soils samples were collected giving a total of 492 samples. From each study site, an equal number of naïve and experienced soils were collected i.e. for Nairobi County 123 naïve soils and 123 experienced soils were collected. However, during the actual study, a total of 502 samples were collected i.e. 250 fungicides naïve soils and 252 fungicides experienced soils.

3.3.2 Sample size for Clinical samples

Total sample size of 10 archival clinical samples was also derived using fisher formula (1998). Clinical triazole resistance prevalence rate of 0.81% for *Aspergillus fumigatus* was used according to Abdolrasouli *et al*., (2018) reported in the United Kingdom.

Using fisher formula;

$$n = \frac{t^2 \, x \, P(1-P)}{m^2}$$

Where;

n= required sample size

t= confident level at 95% (standard value of 1.96)

p= Estimated prevalence of resistance at 0.81%

m= margin of error at 5% (standard value of 0.05)

Hence, $n = \frac{1.96^2 \text{ x } 0.0018 \text{ (1-0.2)}}{0.05^2} = 9.85$

Approximately 10 clinical samples, however in the study 11 samples were collected. All the clinical samples were archival samples from the sputum of suspected aspergillosis patient referred for fungal investigation at Mycology laboratory-KEMRI.

3.4 Sampling of environmental isolates

Approximately 5g dry top surface soils from the agricultural site were collected into a sterile 15 ml Falcon tube using a sterile plastic spoon (Bader *et al*., 2013). Collected soils were triple packages using paper bags. The soil samples were transported in a cool box as soon as possible for mycological investigations at KEMRI-Center for Microbiology Research. Photo of the study site and information on use and name of fungicide (Appendix XX) and frequency of use were collected from the farm owners using a questionnaire (Appendix XIX). A total of 502 samples were analysed and some of the sample collection sites are shown in plates 3.1 to 3.8. Eleven clinical *Aspergillus* species used for comparisons were from archived isolates from sputum samples of suspected aspergillosis patient referred for fungal investigations at Mycology Laboratory-KEMRI.

Plate 3.1

Plate 3.2

Plate 3.3

Plate 3.4

Plate 3.5

Plate 3.6

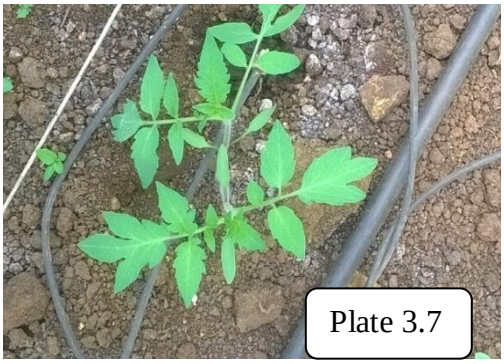


Plate 3.7

Plate 3.8

Legend:

Plate 3.1&Plate 3.2: A forest where fungicides naive soils were collected.

Plate 3.3 & Plate 3.4: Virgin lands where fungicides naive soils were collected

Plate 3.5 & Plate 3.6: Roses flower grown inside greenhouse where experience soils were collected.

Plate 3.7 & Plate 3.8: Traditional vegetables grown a green houses where experienced soils were collected.

3.5 Soil processing in the laboratory

Approximately one gram of the soil samples was suspended and vortexed thoroughly in 5 ml (0.5% w/v) saponin. The debris was allowed to settle and the supernatant transferred to a fresh tube. The resulting suspension centrifuged, and the pellet suspended in a final volume of 500µl sterile Normal saline (Bader *et al*., 2015).

3.6. Culturing of Soil samples

Approximately hundred microliters of the suspension were plated on SDA containing; (a) no drug, (b) 1 µg/ml Itraconazole (c) 1 µg/ml voriconazole. The plates were incubated at 30 ^{o}C for 72 hours. Plates scoring were done daily, for approximately 5 days and picking individual colonies from azole-containing agar plates as they appeared and subculturing onto a fresh medium. Colonies growing after incubation on triazole free media were used as control and to access fungal diversity. Fungal colonies growing Triazole containing agar were used to determine resistant isolates. Identification of fungal colonies was done using standard mycological procedures (Riat *et al*., 2018). The pure isolates were stored in glycerol as stock for molecular analysis and duplicate stocks at-70 °C.

3.6.1 Observation of fungal structures

A drop of lactophenol cotton blue stain was placed at the center of a slide. Fungus fragment was removed using an inoculating needle from the edge of the colony then mixed with stains gently and the coverslips held between the thumb and index finger, one edge of the coverslip was allowed to touch preparations and lower gently to prevent air bubbles forming. Preparation was examined under low magnification power checking for the presence of the fruiting and mycelia structure (Stephen *et al*., 2005).

3.6.2 Species confirmation

All species of fungi isolated in this study were confirmed by MALDI TOF MS (Bruker Germany) using the 'Fungi Library' database at the University of Gottingen. Shipment of the isolates was requested in the proposal and approved by the Director-KEMRI. Briefly, coded samples were grown on SDA slants in a non-breakable culture bottle for one week after which the cultures were double sealed with a parafilm and tripled packaged according to international shipment protocol for biological material. The shipment was by use of international currier company and shipment permit was obtained from the Scientific and Ethical Approval Unit of KEMRI with the permission of the Director KEMRI.

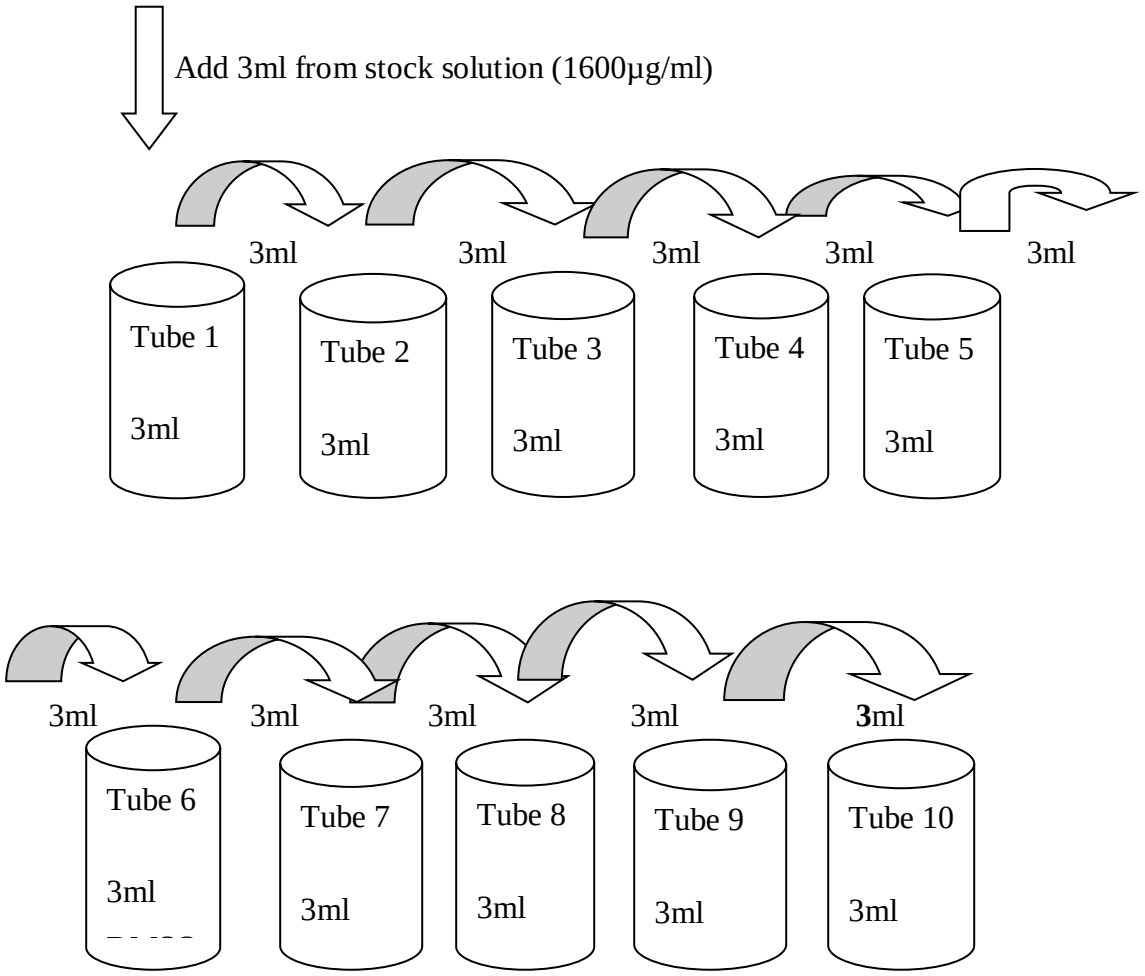
3.6.3 MALDI-TOF MS procedure

MALDI-TOF MS (Bruker Germany) was used for *Aspergillus* species identification. From a sixteen hours old *Aspergillus* culture in SDA incubated at 37°C, one ml of the *Aspergillus* species were centrifuged for 2 min at 20, 800 ×g. The resulting pellets washed twice in deionized water, 1 ml and suspended in 900 µl absolute ethanol

and 300 µl deionized water and centrifuged. The pellet was dry then dissolved in acetonitrile 50 µl and formic acid 50 µl and then centrifuged. Approximately 100 µl supernatant was applied to the MALDI-TOF MS target plate and dry. Dried spot on the target plate was cover with saturated α-cyano-4-hydroxycinnamic acid liquefy in 2.5% trifluoroacetic acid matrix solution and 50% acetonitrile (HCCA, Daltonics). Within a mas range of 2, 000 to 20, 000 Da of 20Hz laser frequency spectra were recorded. Laser short generated were collected and analyzed to create a spectrum according to Masih *et al*., (2016).

3.7 Triazoles susceptibility testing

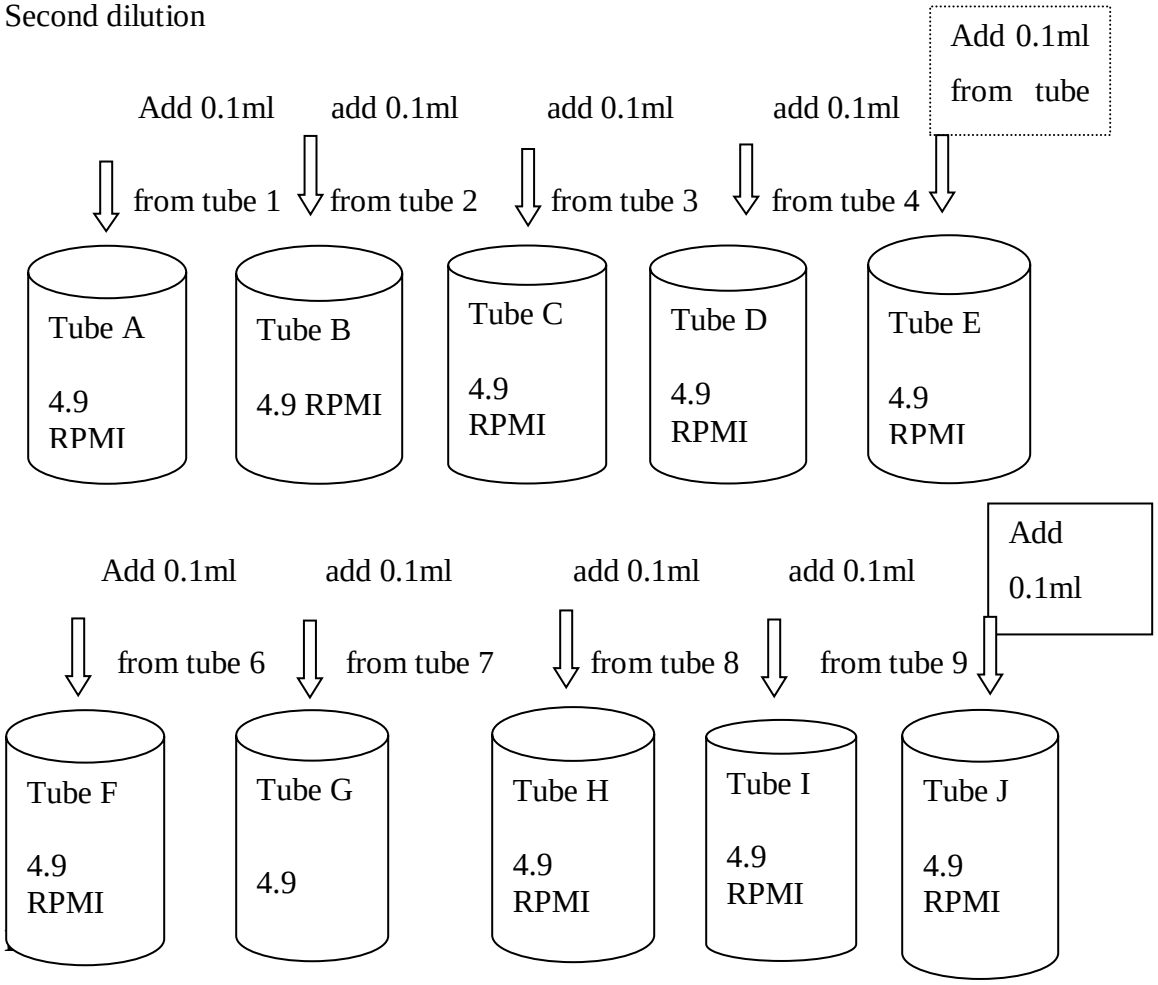
Minimum inhibitory concentrations to Itraconazole (ITZ), Voriconazole (VCZ) and Posaconazole (PSZ) were tested by broth microdilution according to the EUCAST reference method (EUCAST, 2008). Briefly, exactly 9.6 mg of azoles (Itraconazole, voriconazole, posaconazole) powder was weighed and dissolved in 3.0 ml of DMSO to give 1600 µg/ml stock. Ten sterile tubes were used for serial dilution in each tube 3 mls of DMSO were added. Starting with the first tube 3mls of the stock solution was added, mix and 3 mls from the mixed of the first tube was transfer to the second tube mixed and pipette 3 mls from the second and transfer to the third tubes and the process was continued up to the ten tubes. The second serial dilution was done using ten tubes, in each of the tubes 4.9 ml of RPMI were added to each of the ten tubes.



Legend

Dimethyl sulfoxide (DMSO) were added to Tube 1 to tube 10. In tube 1, exactly 3ml stock solution were added, follow by serial dilution to tube 10.

Second dilution



Legend

Second dilution were performed by adding 4.9 RPMI from tube A to tube J, followed by transferred of 0.1 ml of tube I to Tube A, the same were repeated to tube J.

From each tube of the second dilution 200µl were dispensed to microtitre plates and inoculated with 10µl of the inoculums. Plates were then incubated at 30°C for 72 h. The MIC values of all drugs were determined visually as the lowest concentrations with no visible growth. EUCAST drug-susceptible controls strain DSM819 and ATCC46645

were used while azole resistant control isolates (allele TR/L98H) and (allele G54W, ITZR + PSZR86) was used.

3.8 DNA extraction

Resistant *Aspergillus* species to at least one of the triazole with MIC greater than 4µg/ml had their DNA extracted by Phenol chloroform method. Briefly, using a sterile toothpick, a small lump of mycelia from fresh young culture was added to 500ml of lysis buffer (400Mm Tris-HCl [Ph 8.0], 150 mM NaCl, 1% Sodium dodecyl Sulfate, 60 mM ethylene diaminetetra acetic acid [EDTA] [pH 8.0]). The mixture was contained in a 1.5-mL Eppendorf tube. The lump of mycelia was disrupted using the sterile toothpick. The tube containing the mixture is incubated for 10 min at room temperature. The tube is vortexed slightly and centrifuged for 1 min at 1000xg after addition of 150 mL of potassium acetate (pH 4.8; made of 60 mL of 5 M potassium acetate, 28.5 mL of distilled water and 11.5 mL of glacial acetic acid). To another 1.5-mL Eppendorf tube, the supernatant was transferred and spun in a centrifuge as described above. The supernatant was again transferred into yet another 1.5-mL Eppendorf tube to which an equal amount of isopropyl alcohol is added. The tube is centrifuged at 10000 × g for 2 min after mixing by inversion and the supernatant eventually discarded. In 300 mL of 70% ethanol, the resultant DNA pellet was washed, centrifuged for 1 min and the supernatant discarded. After air-drying the final DNA pellet and dissolving in 50 mL of de-ionized water, 1 mL of the purified DNA was used for PCR. (Al-Samarrai, 2000).

3.8.1 Molecular identification

3.8.2 Polymerase Chain reaction amplification of ITS genes

The extracted DNA of the isolates had their ITS region amplified using ITS 4 Forward (5′-TCCT CCGC TTATT GTATG C -3′) and ITS 5 reverse (5′- GGAA GTAA AAGT CGTA ACAA GG -3′) primers(Kham *et al*., 2008). The PCR amplification was done

using ThermocyclerAxiva Sichem Biotech., Japanusing 50µl vol. containing 0.1mM each dNTP, AmpliTaq polymerase, 1µl DNA, 4 pmol each for primers and 1× amplitaq? PCR buffer. The 35 PCR cycles were; denaturation at 95°C for 1 min, Annealing at 60°C for the 30s and extension at 72°C for 1 min. and final extension step at 72°C for 10 min. The amplified PCR products were subjected to 2% electrophoresis. The expected band sizes were 596bp for *A. fumigatus,* 599bp for *A. niger*, 609bp for *A. terreus,* 595bp for *A. flavus*, 565bp for *A. nidulans* and 570bp for *A. ustus* (Hentry *et al*., 2000).

Gel electrophoresis was run using molecular agarose grade 1% w/v (Melford UK) dissolved in 1×TBE buffer (Severn Biotech Ltd, UK), stained with 0.5µg/ml ethidium bromide. To determine the size and quantity of DNA a loading dye 5× (Bioline, UK London), Tris-HCL pH 7.0. 30% glycerol and DNA markers (Appleton, UK) then loaded onto the gel. Gel electrophoresis was performed at 80 V for approximately 20 minutes in TBE buffer then UV transilluminator was used to visualize DNA

3.8.3 TR34 mutations detection

The presence or the absence of TR34 in the promoter region were determined by PCR amplification using: AFCYPPR (5'-TGGTATGCTGGAACTACACCTT-3') and AFCYPPR (5'-AATAATCGCAGCACCACTTC-3') primers (Invitrogen, UK). PCR carried out in 50µl vol. containing, AmpliTaq DNA polymerase, 0.1mM each dNTP, 2µl DNA, 4 pmol each for primers and 1× amplitap PCR buffer. PCR 35 cycles: denaturation at 95°C for 1 min, Annealing at 60°C for the 30s and extension at 72°C for 1 min. initial denaturation at 95°C for 5min and final extension step at 72°C for 10 min. Theamplicons was detected using 2% agarose gels according to Al-Wathiqi *et al*., (2013)

3.8.4 Detection of L98H mutations

The presence or the absence of L98H mutation at cyp51A98 was done using DNA amplification using; AFCYP98F (5'-CAAGTTCTTCTTTGCGTGCAGA-3') and

AFCYP98R (5'-ATAAGTGGCACATGAGACTCT-3') primers. The PCR reaction and cycling condition was as described in above for TR34 mutations. The DNA was purified using Qiagen purification kit according to the manufactured instruction. The purified DNA was digested in AluI (New England Bio-Labs) and electrophoresed for 5 hr at 37°C. The digest was detected by 2% agarose gels to generate PCR-restriction fragment length polymorphism (Ahmad *et al*., 2014). For quality control know *A. fumigatus* containing TR_{34} in the promoter region and wild type were included.

3.8.5 Gel extraction

The DNA fragment of interest was excised using a sterile clean sharp scalpel (According to QlAquick Gel extraction kit protocol). The sliced gel was weighed into a colorless tube. Three (3) volumes of QG buffer was added to 1 volume of gel slice i.e. 100mg ≈ 100 µl. The mixture was then incubated for 10 minutes at 50 °C where tubes were vortexed during incubation every 2 to 3 minutes. After the gel dissolved completely and the color changed to yellow, 1 gel volume and isopropanol were added to the sample and mixed (i.e. 100mg gel slice, 100 µl were added).

In the 2 ml collection tube provided by the manufacturer, QlAquick spin column was placed. The sample was then applied to the QlAquick column for DNA to bind and centrifuged for 1 minute. The flow was then discarded through the QlAquick column to the same collection tube. To ensure that all agarose was removed, 0.5 ml of QG buffer was added to QIAquick column and centrifuged for 1 minute. This was followed by washing by adding 0.75 ml of buffer PE to QlAquick column and spinning in a centrifuge for 1 minute. The flow was discarded through QlAquick column and centrifuged for another 1 minutes at 13,000 rpm. QlAquick column was placed into a clean sterile microcentrifuge where DNA was eluted by adding 50 µl EB buffer to QlAquick membrane center and spinning in the centrifuge at maximum speed for 1 minute.

3.8.6 Sequence analysis

Cyp51A were amplified using the following Primers: CYP51A -5[5'-ATA ATCGCAGCAGCACCACTTCAGA-3'], cyp51A-8 [5' CGGATCGGACGTGGTGT ATG-3'] cyp51A 6 [5'-TGGATGTGTTTTTCGACCGCTT-3'] and cyp51A-7 [5'CCTTGTCACCGTCAAGACGG-3'], and sequencing of each fragment was done from both ends. Sequences from all isolates were assembled using Conting assembly program and nucleotide changes were done by manual inspection of isolates using Conting assembly program. According to Al-Wathiqi *et al*., (2013) known *A. fumigatus* with L98H mutation and wild type were included for quality control.

3.9 Data analysis

Data obtained were analyzed using: Shannon weigner diversity index to determine *Aspergillus* species diversity, two-sample t-test was used to compare itraconazole, voriconazole and posaconazole minimum inhibitory concentrations and CHI square test was used to compare the resistance among *Aspergillus* species against triazoles. Data were presented in tables and bar graphs.

3.9.1 Quality control

All the tests were done in three biologically independent replications. Triazole susceptible and resistant QC isolates were used in each batch of the test. In order to ensure the accuracy and reliability of data, all data collection forms were filled in immediately by the study staff conducting the relevant procedures. All data were double entered into a database and hard copy kept in a lockable cabinet.

3.9.2 Ethical considerations, including safety issues

The study protocol was submitted to the Centre Scientific Committee, Scientific Steering Committee, and Ethical Review Board of KEMRI for approval before implementation.

3.9.3 Study subject/Samples:

There was no direct human contact or human participation in the study but only environmental samples consisting of soils from agricultural fields and non-agricultural fields and coded fungal isolates. Approved permission to access the farms was obtained from the relevant institutions or flower farms owners or individual farm owners. Samples collected did not bear personal or farm identification but were coded immediately after collections to protect the privacy of the farm and the farmers.

3.9.4 Disseminations of information

The data was disseminated through scientific publication (European Scientific Journal and International Journal of Microbiology) and presented at 7rd KEMRI annual scientific & health (KASH) conference (Appendix XVIII).

3.9.5 Potential Benefits

There was no direct benefit to park, farm or field owners. However, the data from this study will contribute to policies on the rational use of fungicides in agriculture and data on the current rate of triazole resistance.

3.9.6 Potential Risks/Protection against Risk

Both privacy and confidentiality in all the interactions with farm and field owners were maintained. Field visits were conducted by an experienced local Kenyan scientist. Soil samples were coded with laboratory number without reverence to the farm or landowner.

Data on azole resistance were compared with that of isolates from clinical sources. The isolates are coded and culture collection at the mycology laboratory.

3.9.7 Biosafety Issues

All environmental samples were transported in unbreakable containers in plastic bags. All procedures of isolation, culture, identification, storage and dispose of fungal cultures were performed by experienced mycologists. All the procedures were done under level II containment facility in the Mycology Laboratory, CMR. The disposal of biohazardous material was done according to KEMRI waste management guidelines.

CHAPTER FOUR

RESULTS

The study analyzed a total of 250 fungicide naïve soils (appendix III), 252 fungicides experienced soil (Appendix II) and a total of 11 clinical samples (appendix 8.I) for comparisons purposes only. The distributions of fungi isolated from fungicides experienced Soils from Nairobi were dominated by *A. fumigatus* and *Rhizopus* species which accounted for 24.5% each. While in Naivasha it was dominated by *Rhizopu*s species at 49.3%. This was followed by *A. niger* in Nairobi at 24.5% and *Fusarium* species in Naivasha at 17.25. In Nairobi *A. terreus* was not isolated whereas in Naivasha soils isolation rate was at 2.9% as per table 4.1.

Table 4.1: Distribution of fungi species isolated from fungicides experienced Soils in Nairobi County and Naivasha sub-county.

Site	*A. fumigatus*	*A. niger*	*A. flavus*	*A. terreus*	*Fusarium* spp.	*Rhizopus* spp.	Total
Nairobi	24 (24.5%)	24 (24.5%)	3 (3.1%)	0 (0.0%)	23 (23.5%)	24 (24.5%)	98 (100%)
Naivasha	25 (18.7%)	13(9.7%)	3 (2.2%)	4 (2.9%)	23 (17.2%)	66 (49.3%)	134 (100%)

Aspergillus species distribution isolated from fungicides experienced soils from Nairobi and Naivasha was dominated by *A. fumigatus* which accounted for 47.1% and 55.6% respectively. This was followed by *A. niger* which accounted for 47.1% prevalence in fungicides experience soils from Nairobi and 28.9% in Naivasha. Soils from Naivasha had 8.9% *A. terreus* and also *A. flavus* were isolated from the two study sites as per table figure 4.1.

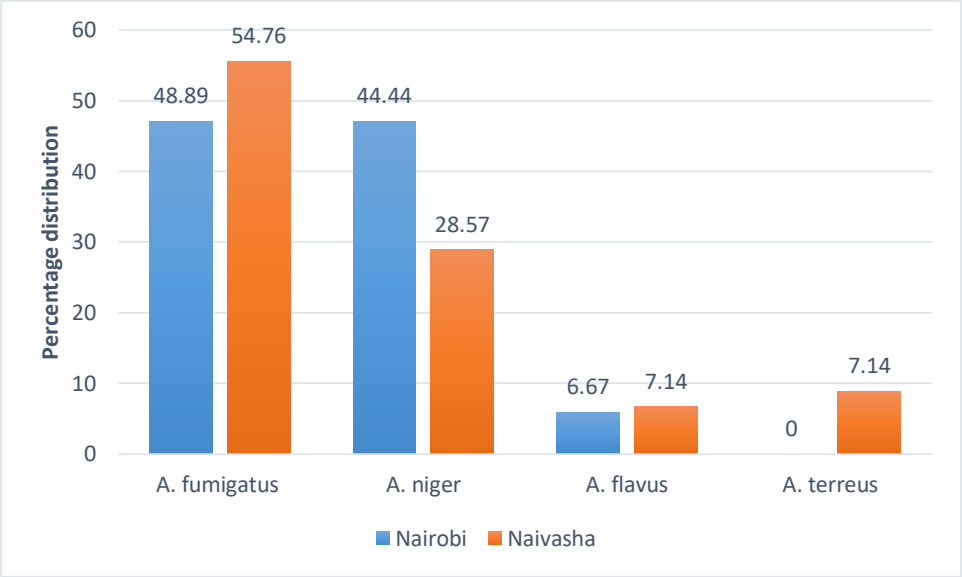


Figure 4.1: *Aspergillus* species distribution in fungicides experienced soils in Nairobi County and Naivasha sub-county.

Fungi distributions in naïve soils were dominated by *Rhizopus* species and follow by *Fusarium* species from the two study sites. Then follow by *A. fumigatus* in Nairobi at 23.6% and *A. niger* in Naivasha at 16.8%. Also *A. flavus*, *A. terreus* and *A. ochraceus* were isolated as shown in table 4.2.

Table 4.2: Distribution of fungi species isolated from fungicides naive soils in Nairobi County and Naivasha sub-county.

Site	*A. fumigatus*	*A. niger*	*A. flavus*	*A. terreus*	*Fusarium* spp.	*Rhizopus* spp.	*A. ochraceus*	Total
Nairobi	26 (23.6%)	12 (10.9%)	0 (0.0%)	1 (0.91%)	25 (22.7%)	46 (41.8%)	-	110 (100%)
Naivasha	18 (14.4%)	21 (16.8%)	0 (0.0%)	0 (0.0%)	26 (20.8%)	59 (47.6%)	1 (0.8%)	125 (100%)

The distributions of *Aspergillus* species isolated from Nairobi county fungicide naïve soils were as follows: *A. fumigatus* (66.7%), *A. niger* (30.8%), *A. terreus* (2.6%) and no *A. ochraceus*. While in Naivasha naïve soils it was dominated by *A. niger* at 52.5% follows *A. fumigatus* (45%) and 2.5% *A. ochaceus*. *A. flavus* were not isolated from Nairobi and Naivasha fungicides naïve soils as per figure 4.2.

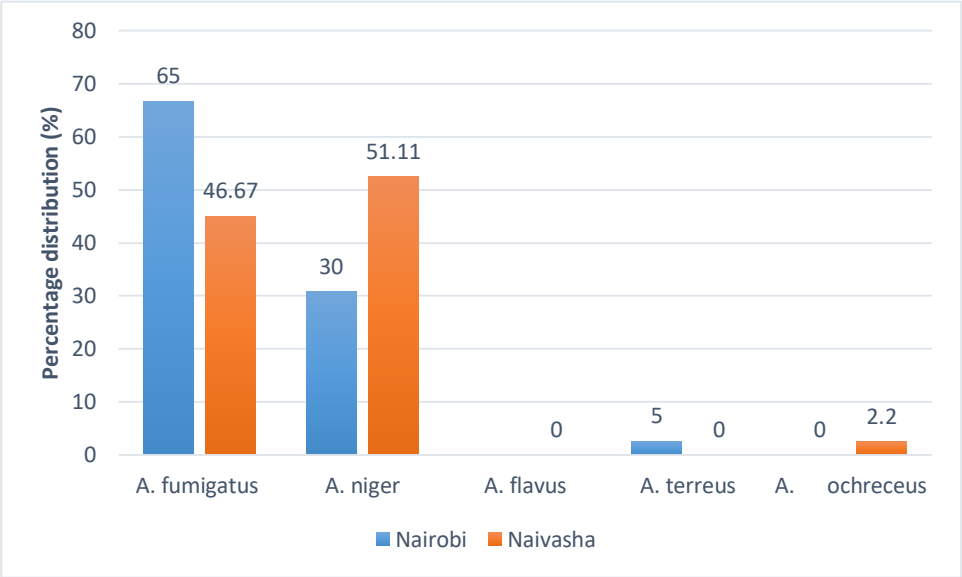


Figure 4.2: *Aspergillus* species distribution in fungicides naive Soils in Nairobi County and Naivasha sub-county.

4.1 Phenotypic characteristics of *Aspergillus* species isolated from fungicide naïve and experienced soils

4.1.1 Soil samples inoculation on SDA agar

Four species of *Aspergillus* were isolated namely: *A. fumigatus*, *A. niger*, *A. flavus*, *A. terreus* and *A. ochraceus* has shown on Plate 4.1 to plate 4.11 below. Also *Fusarium* species, *Penicillium* species, and *Rhizopus* species were also, isolated from the soils.

Plate 4.1: *Aspergillus fumigatus* in azole free SDA media

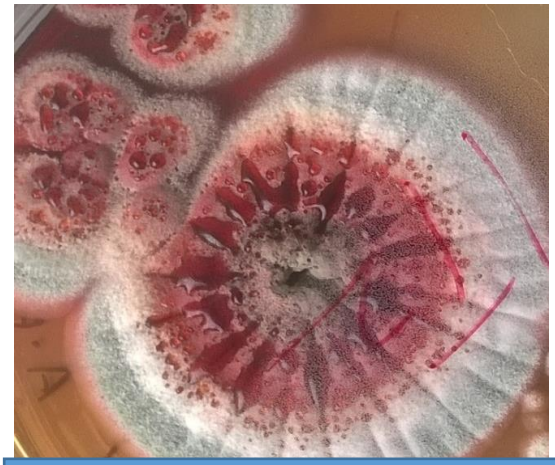


Plate4.2: *Aspergillus fumigatus* in ITZ+VE SDA plate

Plate 4.1 Plate 4.2: Colonies of *Aspergillus fumigatus* growth surface is powdery, Grey-green to a blue-Green with thin white border and reddish center. Read after 24hours incubation at 40°C. The change of color in plate 4.2 may be due to azole added to the media.

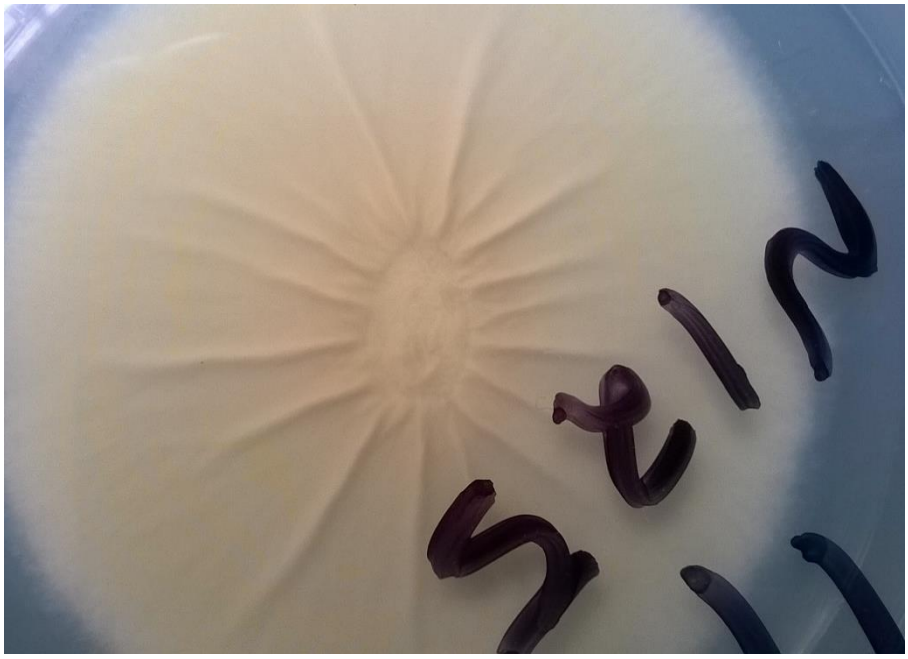


Plate 4.3: *Aspergillus fumigatus* reverse side showing pale yellowish coloration, read after 72hours incubation at 35°C.

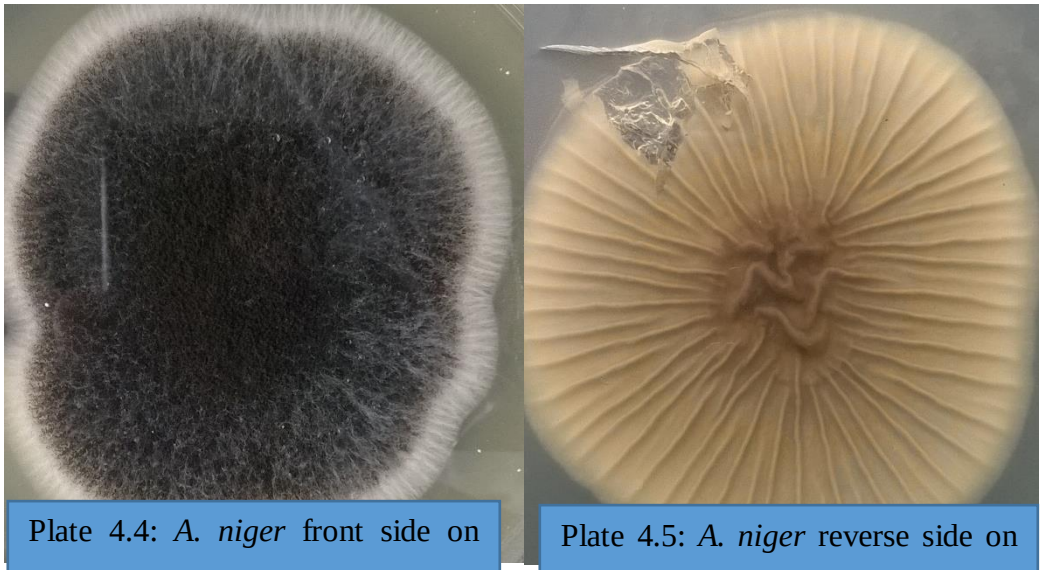


Plate 4.4: *A. niger* front side on SDA

Plate 4.5: *A. niger* reverse side on SDA

Plate 4.4 & Plate 4.5: Colonies of *Aspergillus niger* from fungicides experienced soils with characteristic black colony with white on SDA after 72hours incubation at 35°C.

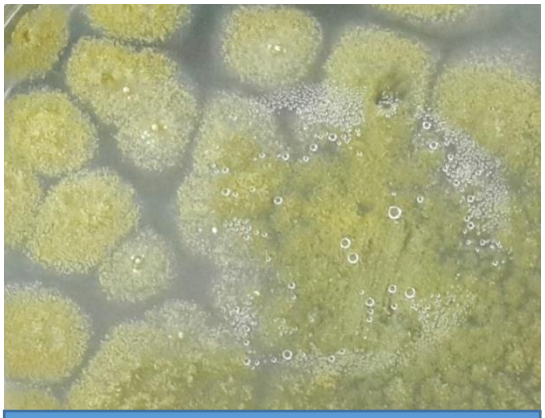


Plate 4.6: *A. flavus* front side on SDA

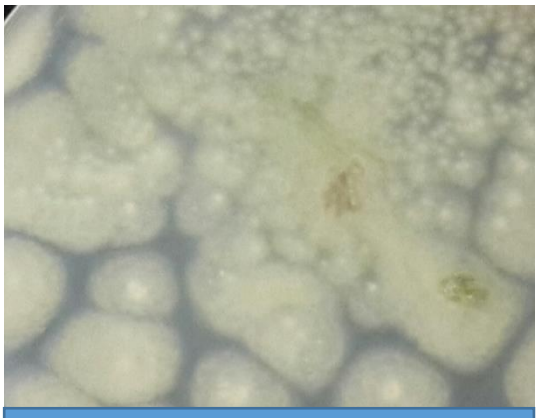


Plate 4.7: *A. flavus* reverse side on

Plate 4.6 & Plate 4.7: Colonies of *Aspergillus flavus* from fungicide experienced soils on Itraconazole negative SDA plate showing greenish colonies after 72hours incubation at 35°C.

Plate 4.8: *A. terreus* front side on SDA

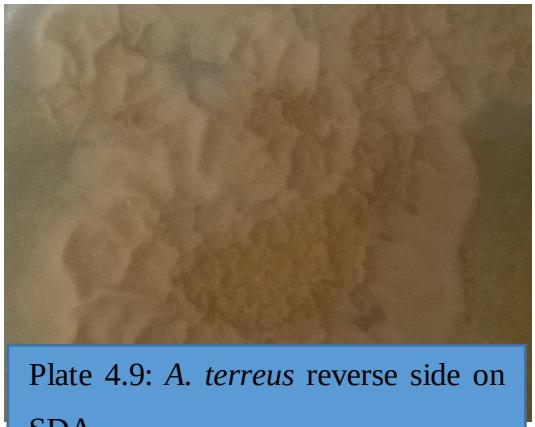


Plate 4.9: *A. terreus* reverse side on SDA

Plate 4.8 & Plate 4.9: Colonies of *Aspergillus terreus* from fungicide experienced soils in Voriconazole positive plate after 72hours incubation at 35°C.

Plate 4.10: *A. ochraceus* front side on SDA

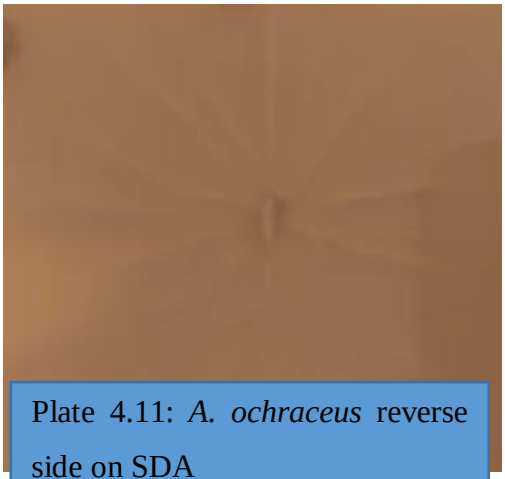


Plate 4.11: *A. ochraceus* reverse side on SDA

Plate 4.10 & Plate 4.11: Colonies of *Aspergillus ochraceus* from fungicide experienced soils after 72 hours incubation at 35°C.

4.1.2 Lactophenol cotton blue stain

Isolated *Aspergillus* species were stain with lactophenol cotton blue to characterize by their structures (sterigmata, vesicle, conidia). Lactophenol cotton blue composed of phenol which kills the pathogenic organism, in the process prevents cell lysing, lactic acids and cotton blue preservers and stain fungal structures respectively.

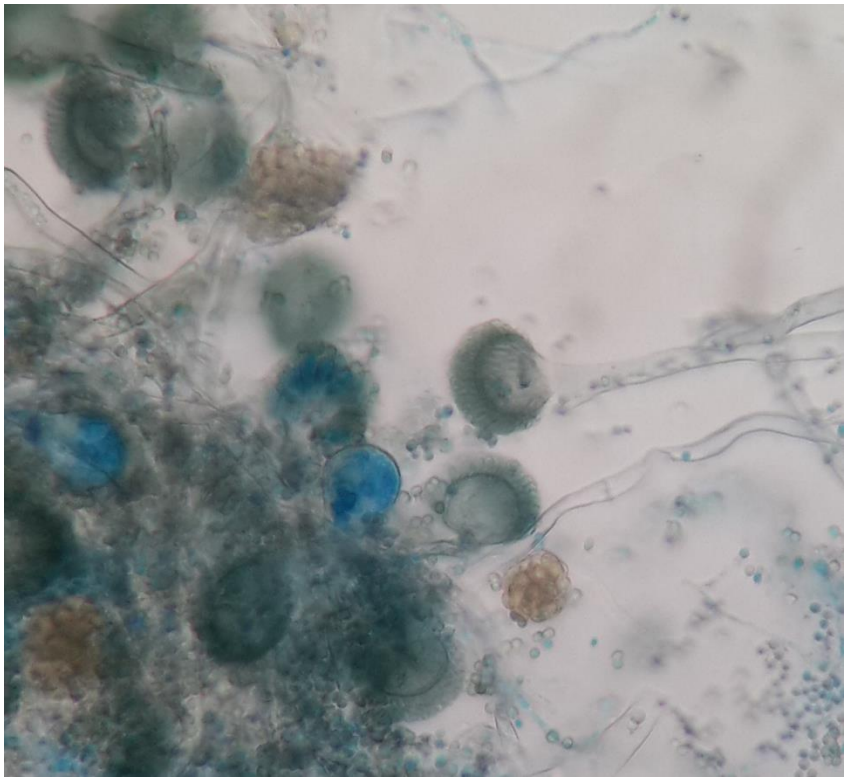


Plate 4.12: *Aspergillus fumigatus* microscopic morphology lactophenol cotton blue stain (×40 magnification).

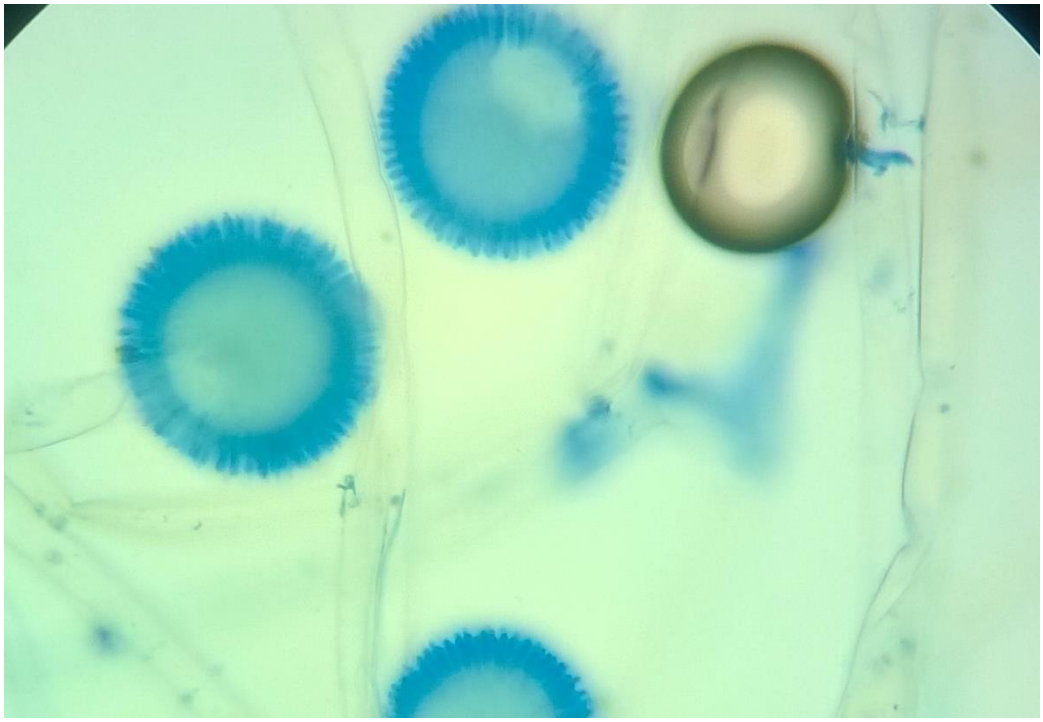


Plate 4.13: *Aspergillus niger* microscopic morphology lactophenol cotton blue stain (×40 magnification).

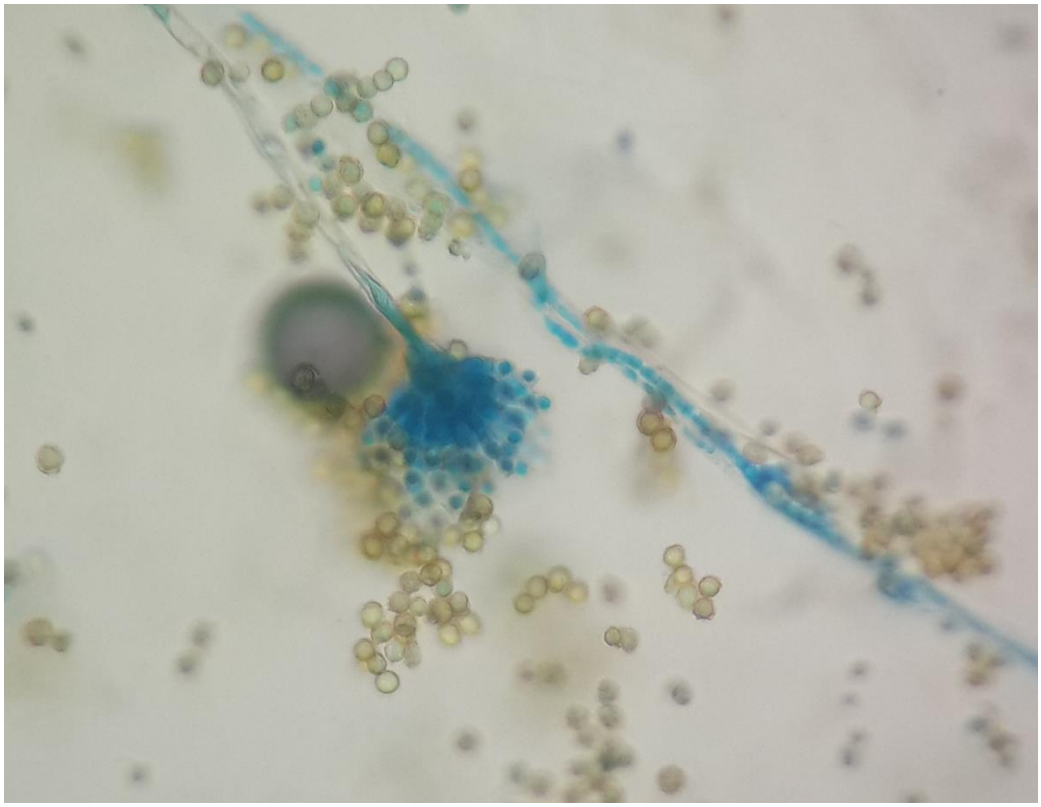


Plate 4.14: *Aspergillus flavus* microscopic morphology lactophenol cotton blue stain (×40 magnification).

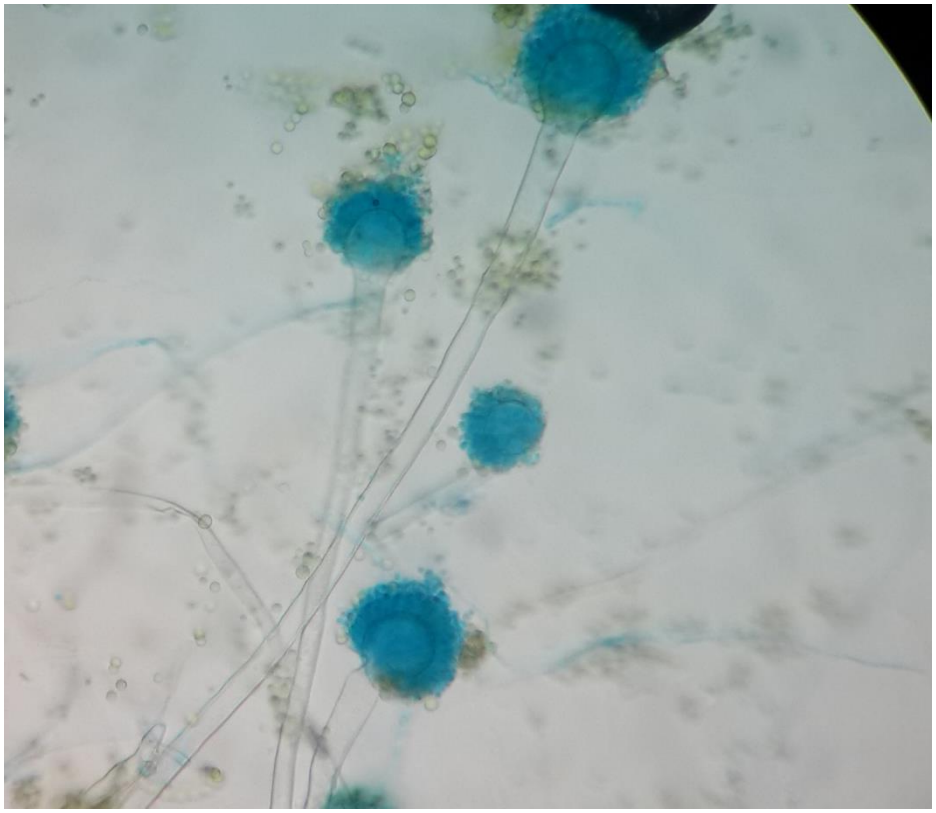


Plate 4.15: *Aspergillus terreus* microscopic morphology lactophenol cotton blue stain (×40 magnification).

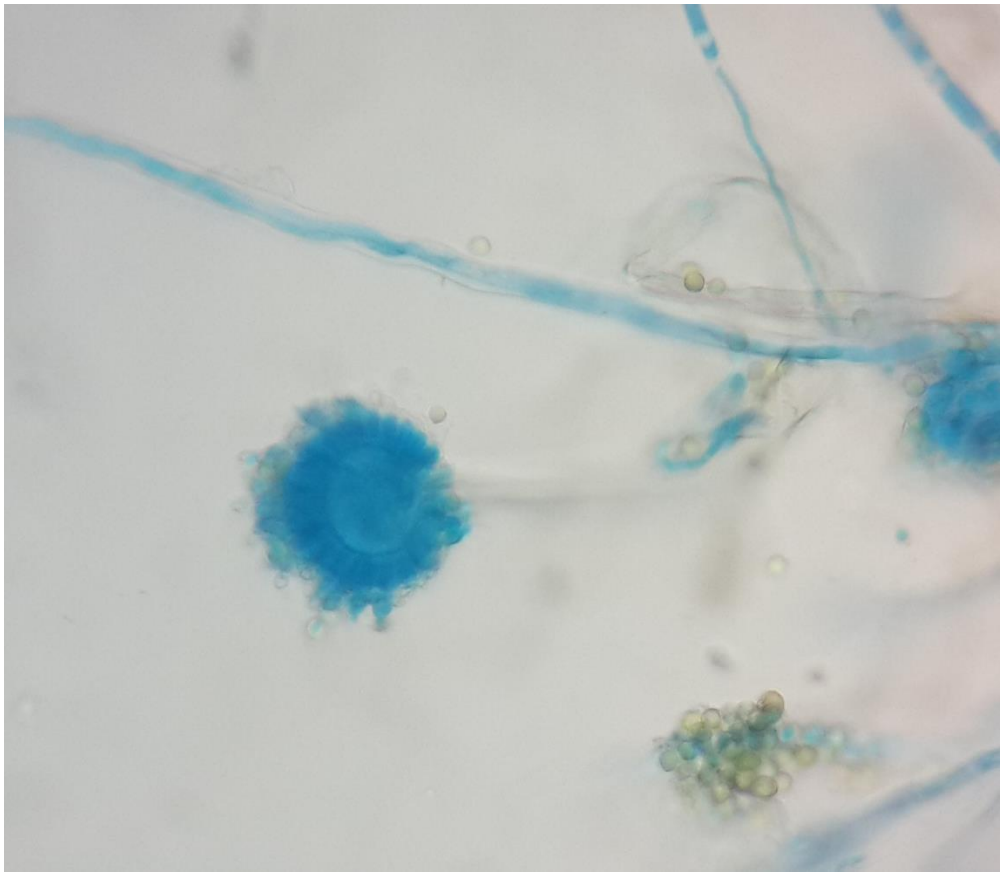


Plate 4.16: *Aspergillus ochraceus* microscopic morphology lactophenol cotton blue stain (×40 magnification).

4.2 *Aspergillus* species diversity in Nairobi experienced Soils

Using the Shannon Weigner diversity index as; Shannon Index = $\sum_i \left(\frac{n_i}{N} \cdot \log_{10}\left(\frac{n_i}{N}\right)\right)$.

The diversity of *Aspergillus* species in Nairobi experienced soils was 1.507. The Berger –Parker Dominance index ($\frac{n_{\max}}{N} = 0.4717$) showed that in this area, *Aspergillus* species was 47.17% dominated by *Aspergillus* fumigatus in the soils. Therefore there was high diversity of *Aspergillus* species in this type of soils (Appendix IV).

4.2.1 *Aspergillus* species diversity in Naivasha experienced Soils.

In Naivasha experienced soils, diversity of *Aspergillus* species was 1.368. The Berger – Parker Dominance index ($\frac{n_{\max}}{N} = 0.625$), indicating that in this area, *Aspergillus* species was high, (62.5%), dominated by a specific *Aspergillus fumigatus*. The result showed that there was less fungal diversity in the soils (Appendix V). Colony forming units (CFU) of the *Aspergillus* species were established in the two collection sites (Nairobi County and Naivasha Sub-County). Comparison of the CFU in the two sites was done using two-sample T-test and the colony forming units in Nairobi mean 33.8 ± 5.5 was higher than in Naivasha (mean 23.0 ± 3.8).

4.2.2 *Aspergillus* species diversity in Nairobi County fungicide naïve Soils

Using Berger –Parker Dominance index ($\frac{n_{\max}}{N} = 0.60$), the diversity of *Aspergillus* species in Nairobi fungicide naïve soils was 0.60, *Aspergillus* species accounted for 60.0% of fungi isolated with higher species diversity dominated by *Aspergillus fumigatus* in the soils. Therefore there was high diversity of *Aspergillus* species in this soils (Appendix VI).

4.2.3 *Aspergillus* species diversity in Naivasha sub-county fungicides naïve Soils

In Naivasha sub-County, fungicide naïve soils, the diversity of *Aspergillus* species was 0.3966. Using Berger –Parker Dominance index ($\frac{n_{\max}}{N} = 0.3966$). In this area, *Aspergillus* species prevalence was, 39.66%, dominated by A. *fumigatus*. This result showed that there was less diversity in Naivasha naïve soils compared to Nairobi naïve soils (Appendix VII).

4.2.4 MALDI-TOF MS *Aspergillus* results

Identification of closely related *Aspergillus* species were done using MALDI-TOF MS, which enabled the differentiation of species that were indistinguishable using phenotypic methods. Mass spectra generated by each *Aspergillus* species was used to run against the Bruker Daltonic, Bremen database. Most of the Species mass spectra agreed with similar species in the database (Appendix XXI).

4.3 Antifungal sensitivity testing

The Minimum inhibitory concentration of Posaconazole, Voriconazole and Itraconazole were determined by broth micro dilution according to the EUCAST reference method. Data obtained were analyzed as showed in the tables 4.3 & 4.5.

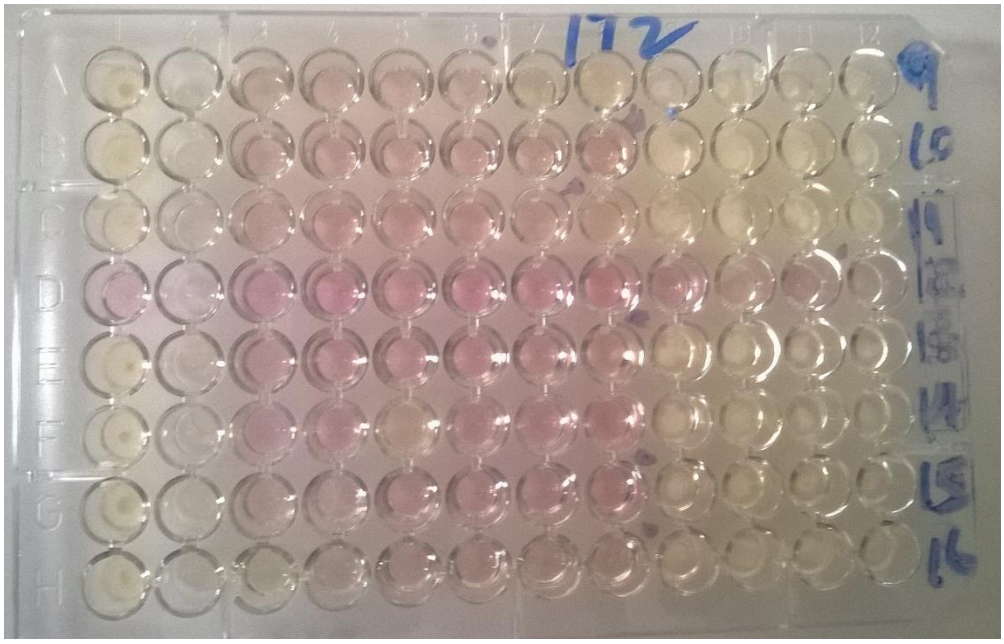


Plate 4.17: Microtiter plate with RPMI media inoculated with the test fungi at different Itraconazole concentrations

4.3.1 Comparison of Itraconazole, Voriconazole and Posaconazole Minimum inhibitory concentration among *Aspergillus* species from fungicides experienced soils from Naivasha Sub-county and Nairobi County.

Comparing the MIC ranges to Itraconazole in the two sites carried out using two-sample t-test. MIC ranges in Nairobi mean 1.21 ± 0.25 was lower than in Naivasha (mean 1.32 ± 0.21). Comparing the MIC ranges to Voriconazole in the two sites when carried out using two-sample t-test at 95% confidence interval using SPSS packages, MIC ranges in Nairobi mean 2.02 ± 0.80 was higher than in Naivasha (mean 1.47 ± 0.42). After treatment with Posaconazole, comparing the MIC ranges to PSZ in the two sites using two-sample t-test, MIC zones in Nairobi mean 2.23 ± 0.84 was lower than in Naivasha (mean 2.81 ± 0.92) (Table 4.3).

Table 4.3: Minimum inhibitory concentration (MIC) of triazoles on *Aspergillus* from fungicide experienced soils in Nairobi and Naivasha.

Treatment	Site	Mean MIC ± SE	T value	P value
ITZ	Nairobi	1.21 ± 0.25	0.33	0.744
	Naivasha	1.32 ± 0.21		
VRZ	Nairobi	2.02 ± 0.80	0.61	0.545
	Naivasha	1.47 ± 0.42		
PSZ	Nairobi	2.23 ± 0.84	0.47	0.638
	Naivasha	2.81 ± 0.92		

Key:

ITZ: Itraconazole

VRZ: Voriconazole

PSZ: Posaconazole

4.3.2 Triazole resistance of *Aspergillus* species from fungicides experienced soils from Nairobi and Naivasha

Resistance of fungal isolates from Nairobi experienced soils were compared to the isolates from Naivasha using chi-square. To compare resistance to ITZ, VRZ and PSZ, isolates from Nairobi were compared to those from Naivasha using Chi-square test at 95% confidence interval using SPSS packages. The results showed that there was no significant differences in the number of resistant isolates from Nairobi to the number of resistant isolates from Naivasha ($P > 0.05$).

Isolates from Nairobi soils resistant to Itraconazole 3/45(6.7%) were slightly lower than isolates from Naivasha soils 3/42(7.1%).Fungi isolates from Nairobi soils resistant to Voriconazole 5/45(11.1%) was slightly higher than isolates from Naivasha 4/42(9.5%). Higher resistance to Posaconazole was recorded in the soils. The result showed that fungi isolates from Nairobi soils resistant to Posaconazole 27//45(60.0%) were slightly higher than isolates from Naivasha soils resistant to Posaconazole 32/42(76.2%) (Table 4.4).

Table 4.4: Isolates sensitivity to Itraconazole, Voriconazole and posaconazole from Nairobi and those from Naivasha experienced soils.

Site	Azole	Resistant	Intermediate	Susceptible	Total	χ^2-Value	P value
Nairobi	ITZ	3(6.7%)	20(44.4%)	22(48.9%)	45(100%)	1.586	0.452
Naivasha		3(7.1%)	24(57.1%)	15(35.7%)	42(100%)		
Nairobi	VRZ	5(11.1%)	15(33.3%)	22(55.6%)	45(100%)	1.298	0.523
Naivasha		4(9.5%)	19(45.2%)	19(45.2%)	42(100%)		
Nairobi	PSZ	27(60.0%)	13(28.9%)	5(11.1%)	45(100%)	2.800	0.247
Naivasha		32(76.2%)	8(19.0%)	2(4.8%)	42(100%)		

Key:

ITZ - Itraconazole

VRZ - Voriconazole

PSZ – Posaconazole

4.3.3 Comparison of Itraconazole, Voriconazole and Posaconazole Minimum inhibitory concentration among *Aspergillus* species from fungicides naïve soils from Naivasha Sub-county and Nairobi County.

Aspergillus species colony forming units (CFU) were established in naïve soils in the two collection sites (Nairobi and Naivasha) using two-sample t-test at 95% confidence interval using SPSS packages. Colony forming units in Nairobi soils mean 24.7 was lower than that in Naivasha soils (mean 27.1. However, the CFU in the two sites was no significantly different ($t = 0.38$, $P = 0.707$). The minimum inhibition concentration (MIC) to Itraconazole in the two sites were compared using two-sample t-test. The MIC in Nairobi (mean 1.85) was higher than in Naivasha (mean 1.71 ± 0.72). Isolates from fungicides naïve soils were generally susceptible to Itraconazole with 77.6%, intermediate, 14.1% and 8.2% were resistant.

The MIC range to Voriconazole in the two sites was compared using two-sample t-test. The MIC ranges in Nairobi mean (1.97 ± 0.59) was higher than that in Naivasha (mean 1.15 ± 0.30).To Voriconazole, 81.2% were susceptible, 5.9% were intermediate and 12.9% were resistant. The MIC ranges to Posaconazole in the two sites were compared using two-sample t-test. The MIC ranges in Nairobi mean (3.57 ± 1.2) was higher than that in Naivasha (mean 1.21 ± 0.42).Posaconazole, recorded 11.8% susceptible, 77.6% intermediate and 10.6% resistant (Table 4.5).

Table 4.5: Minimum inhibitory concentration (MIC) of triazole on Aspergillus in naïve soils in Nairobi and Naivasha.

Treatment	Site	Mean MIC ± SE	T value	P value
ITZ	Nairobi	1.85± 0.80	0.13	0.896
	Naivasha	1.71 ± 0.72		
VRZ	Nairobi	1.97 ± 0.59	1.23	0.223
	Naivasha	1.15 ± 0.30		
PSZ	Nairobi	3.57 ± 1.2	1.85	0.070
	Naivasha	1.21 ± 0.42		

Key:

ITZ- Itraconazole

VRZ- Voriconazole

PSZ- Posaconazole

4.3.4 Triazole resistance of *Aspergillus* isolates from Nairobi fungicides naïve soils to those isolates from Naivasha fungicides naïve soils

Resistance to ITZ, VRZ and PSZ isolates from Nairobi were compared to those from Naivasha using Chi-square test at 95% confidence interval using SPSS packages. The results showed that there were no significant differences in the number of resistant isolates from Nairobi to the number of resistant isolates from Naivasha ($P > 0.05$). It was established that Isolates from naïve soils in Nairobi resistant to Itraconazole 2/40(5.0%) were slightly lower than isolates from Naivasha soils resistant to Itraconazole 5/45(11.1%) (Table 4.6).

Number of Isolates from Nairobi naïve soils resistant to Voriconazole 7/40(17.5%) were slightly higher than isolates from Naivasha naïve soils were resistant to Voriconazole 4/45(8.9%). Higher resistance to Posaconazole was recorded in naïve soils from Nairobi. The result showed that, fungi isolates from Nairobi soils resistant to Posaconazole

6/40(15.0%) were slightly higher than isolates from naïve soils from Naivasha soils resistant to Posaconazole 3/45(6.7%) (Table 4.6).

Table 4.6: Isolates sensitivity to Itraconazole, Voriconazole and posaconazole from Nairobi and those from Naivasha fungicides naïve soils.

Site	Azole	Resistant	Intermediate	Susceptible	Total	χ^2-Value	P value
Nairobi		2 (5.0%)	9 (22.5%)	29 (72.5%)	40 (100%)		
Naivasha	ITZ	5 (11.1%)	3 (6.7%)	37 (82.2%)	45 (100%)		
						4.979	0.083
Nairobi	VRZ	7 (17.5%)	2 (5.0%)	31 (77.5%)	40 (100%)		
Naivasha		4 (8.9%)	3 (6.7%)	38 (84.4%)	45 (100%)		
						1.439	0.487
Nairobi	PSZ	6 (15.0%)	27 (67.5%)	7 (17.5%)	40 (100%)		
Naivasha		3 (6.7%)	39 (86.7%)	3 (6.7%)	45 (100%)		
						4.305	0.105

Key:

ITZ- Itraconazole

VRZ- Voriconazole

PSZ- Posaconazole

4.4 Molecular analysis of the isolated *Aspergillus fumigatus* with triazole resistance

Aspergillus fumigatus with high MIC were subjected to PCR and its amplified bands were subjected to gel electrophoresis to identify the conferring resistance. The expected band size for the presence of TR_{34} was139bp for isolates containing tandem repeats and wild type with no tandem repeats yielded 105bp. For L98H mutation detection or wild type sequence. The expected band sizes were 71bp, 90bp, and 189bp for wild type, 189bp been a diagnostic fragment. For L98H mutation it yielded two DNA fragments i.e. 71bp and 279bp. Diagnostic fragment has been 279bp.

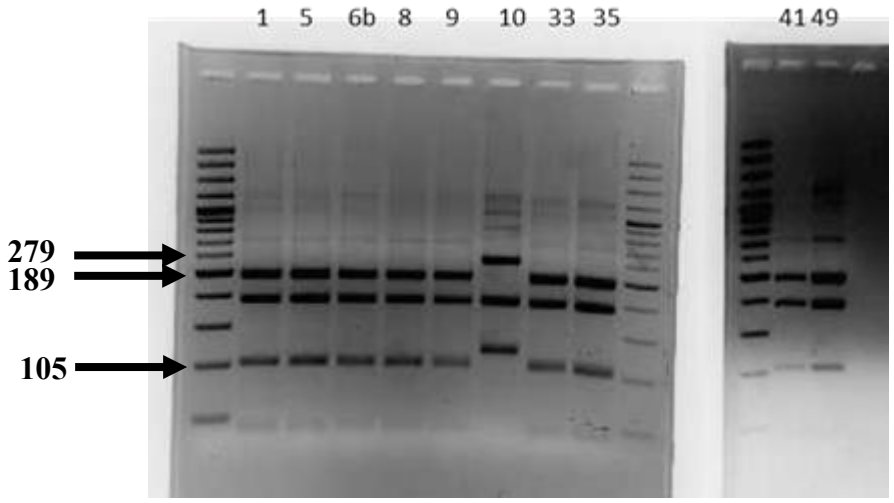


Figure 6.1: Gel picture of the PCR-RFLP expected from Alu1 digestion 1kbDNA marker, 1, 5, 6b, 8, 9, 33, 35, 41, 49 a wild type and 10 TR34/L98H resistant strains

Legend Numerical numbers 1, 5, 6b, 8, 9, 10, 33, 35, 41 and 49 are *Aspergillus fumigatus* isolates. Wild type are *Aspergillus* species with no mutation.

4.4.1 Short tandem repeat *Aspergillus fumigatus* (STRAf) microsatellite typing

The nine samples with high MIC were subjected to STRAf assay. *Aspergillus fumigatus* DNA was extracted as in section 3.8.0 and analyzed using nine loci STRAf panel made up of three nucleotide repeats. Polymarase chain reaction, Amplification condition and fluorescent labels as used (de Valk *et al*., 2005). Fragment profiler software (Diegem) was used to assign repeat number to each marker and checked visually by electropherogram. STRAf data below shown low discriminatory microsatellite panel among *Aspergillus fumigatus* isolates from fungicides experience and naïve soils and also clinical sources. Most of the markers are similar indicating these isolates could be from the same source.

Table 4.7: STRAf microsatellite typing triazole resistant *Aspergillus fumigatus*

Strain	CYP51A	2A	2B	2C	3A	3B	3C	4A	4B	4C	Sources
E10	TR34/L98H	15	22	8	33	9	6	8	10	18	Environmental
N047	TR34/L98H	15	22	8	33	9	6	8	10	18	Environmental
N064	TR34/L98H	14	21	8	32	9	38	8	10	18	Environmental
E7	TR34/L98H	14	21	8	32	9	38	8	10	18	Environmental
N127	TR34/L98H	14	21	8	28	9	6	8	10	18	Environmental
C07	TR34/L98H	14	21	8	28	9	6	8	10	18	Clinical
E208	TR34/L98H	10	20	8	32	9	7	8	9	19	Environmental
E230	TR34/L98H	14	20	8	32	9	6	8	10	20	Environmental
C10	TR34/L98H	14	23	8	30	9	6	8	10	20	Clinical

Legend: 2A, 2B, 2C, 3A, 3B, 3C, 4A, 4B and 4C are short tandem repeat *Aspergillus fumigatus* (STRAf) markers.

CHAPTER FIVE

DISCUSSION

The study report for the first time triazole resistance among *Aspergillus* species isolated from fungicides naïve soils, experienced soils and clinical sources in Kenya. Among the isolated *Aspergill*us species 5% of the isolates were resistant to at least one of the three azoles tested in fungicides naïve soils in Nairobi and 11.1% in Naivasha. In fungicides experienced soils in Nairobi and Naivasha the study report the resistance level of 6.7% and 7.1% respectively against itraconazole. The finding of the study is within the range on level of resistance reported in several Eurasian countries of between 6%-14% (Badali *et al.,* 2013; Bader *et al*., 2015; Hsuan-Chen *et al*., 2018; Mortensen *et al.,* 2010; Tangwattanachuleeporn *et al.,* 2017). However, data from this research showed a high resistance rate compared to a study in China which reported 1.4% (Chen *et al*., 2016) and low compared to a study in the UK, where about 70% of the isolates had itraconazole MIC $\geq$ 8 µg/mL (Denning *et al*., 2011b). The study report 67.8% *Aspergillus* species resistance to posaconazole.

ARAF against azole therapy has been reported to be increasing over the last decades (Verweij *et al*., 2016) both in the environmental and clinical (Fraczek *et al*., 2013; Meneau *et al*., 2016). The study supported the hypothesis that the used of fungicides in the environment could induce resistance in *Aspergillu*s species since more of resistance species were isolated from fungicides experienced soils compared to naïve soils.

Sabouraud dextrose agar containing azoles at low concertation was used to select-Itraconazole, Voriconazole, and posaconazole resistant isolates. Although this may induce resistance due to exposure to medical triazoles, this is unlikely to occur due to a short exposure time of 24hrs to 48hrs (Mellado *et al*., 2007). Some studies have shown that azoles at sub-inhibitory concentration have an effect on pigmentation and conidiation (Varanasi *et al*., 2004). When *A. fumigatus, A. nidulans, A. flavus* and *A. niger* is cultured

in a media containing voriconazole concentration of 0.125-0.5mg/l. At this concentration, it will inhibit conidition leading to producing colonies which are whitish. However, the effect is reversible by growing the fungus on azoles free medium (Varanasi *et al*., 2004). Exposure to Posaconazole leads to changes in *Aspergillus fumigatus* colonies pigmentation from a green color to white (Faria-Ramos *et al*., 2014).

Azole-resistant *A. fumigatus* associated mortality is estimated as 88% inpatient with aspergillosis with a high rate of treatment failure (van der Linden *et al*., 2015; Van Der Linden *et al*., 2011; Verweji *et al*., 2012). The true prevalence azole resistance in *Aspergillu*s species is still largely unknown, in clinical practice but has been estimated at, 0%-6% (Howard & Arendrup, 2011). Azole-resistant *Aspergillus fumigatus* has been isolated from azole exposed and naïve patients (Chowdhary *et al*., 2013a; Snelders *et al*., 2012; Verweij *et al*., 2009) and Previously studies from a different region of the worlds: African region (Tanzania), European region (Netherlands, Belgium, Denmark, and Germany), Asia (Kuwait, India and Iran) and in the USA) have reported multiple sources of Azole resistant *Aspergillus fumigatus* from soil sample, flower beds, plants, compost and hospitals and its environs (Ahmad *et al*., 2015; Ahmad *et al*., 2014; Bader *et al*., 2015; Bader *et al*., 2013; Chowdhary *et al*., 2014a; Chowdhary *et al*., 2012a; Liu *et al*., 2015; Mohammadi *et al*., 2015; Snelders *et al*., 2008; Snelders *et al*., 2012; Vermeulen *et al*., 2015; Wiederhold *et al*. 2016).

This study reports for the first time Azole resistance in Kenya from fungicides naive and experienced soils in Nairobi county and Naivasha sub-county. Triazoles resistant *Aspergillu*s species have been noted in all the continents except Antarctica (Chowdhary *et al*., 2013a). Triazole resistance the Netherlands was 20% (Fuhren *et al*., 2015) and between 17% and 20% in the United Kingdom (Verweij *et al*., 2015). In this study the prevalence range of between 5% - 11.1% in fungicides naïve soils and 6.7% - 7.1% in experienced soils from Nairobi and Naivasha respectively. However, comparing

the colony forming units in the two sites (Nairobi County and Naivasha sub-county) using a two-sample t-test showed that there was no significant difference (t = 1.60, P = 0.114).

Most research has shown that *Aspergillus fumigatus* are of different genetic diversity, despite the use of different typing methods (de Valk *et al*., 2005b; Lasker, 2002; Menotti *et al*., 2005; Warris *et al*., 2003). It is extremely rare to find strains with similar genetic composition, even when molecular methods with high-resolution power is used (de Valk *et al*., 2005b; de Valk *et al*., 2007b; Warris *et al*., 2003). However in the present study, the sequenced A. *fumigatus* were similar to genetically (Table 4.7). These results did not agree with the finding of the following authors (Bain *et al*., 2007; Lasker, 2002; Menotti *et al*., 2005; Warris *et al*., 2003; de Valk *et al*., 2005b) who reported high diversity in *Aspergillus fumigatus*.

In this study the diversity of *Aspergillus* species isolated from fungicides naïve and experienced soils from Nairobi County and Naivasha sub-county was done using Shannon Weigner diversity index was compared from Nairobi experienced soils, fungal diversity index was 1.507, compared to fungicides Naïve soils at 0.6 in Nairobi. The fungal diversity in fungicide experienced soils was 1.368 compared to fungicides naïve soils of 0.3966. In all the study sites irrespective of fungicides exposure *Aspergillus* species were the majority of the fungi species that were isolated. This is an indication that fungi are cosmopolitan and ubiquitous as reported by (Klich, 2002; Rousk *et al*., 2010; Wheeler *et al*., 1991).

Fungi have high adaptability to different environmental condition and can grow in a wide range of pH (Rousk *et al*., 2010), however some have a preference for a certain pH range for optimum growth (Domsch *et al*., 2007; Rousk *et al*., 2010; Wheeler *et al*., 1991). Differentiation of *Aspergillus* species based on physiological and morphological characteristics is the key identification although molecular methods have shed more light into fungal taxonomy. In this study A. *niger*, A. *flavus, A. terreus*, and *A. fumigatus* were

isolated from both fungicide naïve and experienced soils from the Nairobi county and Naivasha sub-county study sites.

Aspergillus fumigatus is the most virulent species, but also *A. flavus* has be reported to been causing infection both in human and avian (Hadrich *et al*., 2010, Okoye and Aba-Adulugba *et al*., 1998). This study shows a high diversity of *Aspergillus* species from experience and naïve soils, and agreed with work done by, Hadrich *et al*., (2013) who reported the isolation of *A. fumigatus*, *A. flavus*, *A. niger*, *A. ochraceus*, *A. terreus* and also we isolated *Penicillium, Cladosporium* and *Rhizopus* from environmental samples.

Aspergillus terreus is restricted geographically to certain areas compared to *Aspergillus fumigatus* (Lass-Florl *et al*., 2007). According to Neal *et al*., (2011), *A. terreus* population sub-clustering from European countries and America are identical whereas the previous study reported dissimilarity of *A. terreus* isolates due to recombination (Kathuria *et al*., 2015). This may be attributed to different environments which support the existence of different genotypes. There is a high level of invasive aspergillosis associated with *A. terreus* reported in Innsbruck and Houston (Lass-Florl *et al.,* 2007; Lass-Florl *et al*., 2005). Although the two do not have the same climatic conditions, it could be due to several factors and the fact that *A. terreus* adapt to different climatic condition (Blum *et al*., 2008).

Aspergillus fumigatus resistance in clinical isolates have been linked to the use of azole based fungicides in crop farming to prevent fungal diseases (Snelders *et al*., 2009). Approximately 90% of ARAF with resistant genotype TR_{34}/L98H isolates have been isolated from a patient previously not exposed to azole (Snelders *et al*., 2008) and most of the environment isolates have been reported to harbor this form of mutation (Badali *et al*., 2013; Chowdhary *et al*., 2012c; Mortensen *et al*., 2010; Snelders *et al*., 2009). This study reported the same azole-resistant *Aspergillus* with TR_{34}/L98H resistance from naïve soils, experienced soils and clinical sources. However, in the present study, it was not possible to verify whether the clinical sample was from azole naïve patients or patient's

previously exposed to azole as this information was missing from the clinical information of the archived.

Aspergillus fumigatus from both environmental and clinical isolates with TR_{34}/L98H mutation showed resistance against the three test medical azoles (Itraconazole, posaconazole, and Voriconazole. Snelders *et al*., (2012) and Van Ingen *et al*., (2015), detected isolates with TR34/L98H mutation, which was resistant to Posaconazole and itraconazole. Fungicides exposure in the environment through fungicide use in agriculture inhibit the growth of susceptible strains and promotes the multiplication of the resistant type though antifungal pressure.

Fungal spores are dispersed by wind over a long distance, this supports the hypothesis that ARAF adapts to different environmental habitat as witnessed in Asia countries (Badali *et al*., 2013; Chowdhary *et al.*, 2012c). This could explain the isolation of resistant *Aspergillus* species in fungicides Naïve soils. Although fungicide naïve soil was collected from uncultivated soils known for non-fungicides use, fungal spores are also known to be wind dispersed over a long distance. This was mitigated by sampling soils that have never been cultivated away from cultivated farms outside urban influence and away from main roads. Cyp51A sequence analysis showed that the isolates have similar resistant mutation from the different study counties and sources (Table 4.7). Widespread use of triazole in agriculture and its environmental persistence in the soils may lead to a reduction in azole-susceptible strains and results in the emergence of the resistant genotypes (Verweji *et al*., 2013).

The isolation of azole-resistant *Aspergillus* from naïve soils suggest that soil harboring resistant strains are dispersed by wind, material or human being as they traveled. Fungi have been noted to acquire resistance through azole exposure as reported in patient and plant fungi exposed to triazole before (Chen *et al*., 2005; Howard *et al*., 2006). Exposure to azole over a long duration and the ability of the fungi's to reproduce in high numbers are some of the risk factors in resistance selections (Anderson, 2005). A survey done by

Hollomon (2017) suggested that resistance involvement in clinical practice is higher compared to that in the environment due to high selections pressure in patients receiving azoles medication compared to crops in the fields.

Fungicides in the environment have been there for decades but recently there has been an increase in Azole-resistant *Aspergillus* species (Verweij et *al*., 2009). Some researchers have linked this emergence to the use of newer triazoles fungicides in agriculture which may easily induce TR/L98H mutation (Faria-Ramos *et al*., 2014; Snelders *et al*., 2012; Verweij *et al*., 2009). A study by Snelders *et al.* (2009) has shown cross-resistance to agricultural fungicides (metconazole and tebuconazole) by wild type and TR_{34}/L98H isolates from environment and clinical. This supports the hypothesis of fungicides role in the development of azole-resistant *Aspergillus* species in environmental fungi. Previous studies have linked Cyp51A expression to change of amino acid leucine and at 34bpTR at the promoter (Bueid *et al*., 2010).

Resistance involving the substitution of G54 in the gene, in the past, have linked to prolonged exposure of patients with azoles (Chen *et al*., 2005; Tashiro *et al*., 2012; Xu *et al*., 2010). However several studies have proved otherwise by isolation of isolates with the same resistance from the environment e.g. in Romania, Tanzania, Germany, and India (Bader *et al*., 2015; Sharma *et al*., 2015). Most azole mechanisms of resistance in *A. fumigatus* has been characterized a lot (Fraczek *et al*., 2013; Meneau *et al*., 2016) and usually attributed to cyp51A gene mutations. Isolates resistant to azole have point's mutations in the cyp51A genes such as G138, G54, and M220 which alter the sequence of the amino acids (Chowdhary *et al*., 2014c).

This is commonly found in patients who have been exposure to azoles for long (Verweji *et al.,* 2016). Several research reports have discovered 54 codons from clinical isolates as the main region associated with resistance in gene cyp51A (Balashov *et al*., 2005; Chen *et al*., 2004; Diaz-Guerra *et al*., 2003, Mann *et al*., 2003; Nascimento *et al*., 2003) also codon 220 (Chen *et al*., 2004; da Silva *et al*., 2004; Mellado *et al*., 2004b; Rodriguez *et*

al., 2008; Snelder et al., 2008) and codon 98 (Mellado *et al*., 2007; Rodriguez *et al*., 2008; van der Linden *et al*., 2009; Verweji *et al*., 2007).

In this study all the sequence azole-resistant *Aspergillus fumigatus* had TR34/L98H mutation having similar nine microsatellite loci genotype i.e. 2A, 2B, 2C, 3A, 3B, 3C, 4A, 4B and 4C. The same finding was reported in *A. fumigatus* isolates in India (Chowdhary *et al*., 2012c). Because of the similarity in microsatellite loci, it was not possible to draw organogram for genetic relatedness comparison among isolated *Aspergillus* species between the two study sites (Nairobi County and Naivasha sub-county). STR analysis of *Aspergillus fumigatus* is recommended for epidemiological studies (de Valk *et al*., 2005; Guinea *et al*., 2011). Several authors have studied deletions or the insertions of tandem repeats, Vigouroux *et al*., (2002) and Noller *et al*., (2006) found out that they is high chances of the tandem repeats to increase. While Vogler et al., (2006) reported the opposite. However, some studies found out that the longer the repeats the higher chances of deletions or insertions (Huang et al., 2002; Lai and Sun, 2003). The study report single repeat unit insertion, where as other study found out most of mutations involving several repeat (Bustamante *et al*., 2013). This could be due to the used of larger repeat in the assay hence would most likely involved more repeat (Bustamante *et al*., 2013). The study report low diversity of *A. fumigatus* due to closing similar in Short tandem repeat *Aspergillus fumigatus*.

Even though there was a shortcoming in PCR fragment sizing, however, STR typing is the most recommended method because of its reproducibility in the laboratory and good discriminatory power (de Valk *et al*., 2007b; Vanhee *et al*., 2009). *Aspergillus fumigatus* can be identified correctly using the following genes ITS (Internal transcribed spacer), actin, β tubulin, calmodulin and rodlet A by sequencing (Samsom *et al*., 2007; Yoguchi *et al*., 2007). Other molecular methods are restriction fragment length polymorphism, multilocus sequence typing) (Balajee *et al*., 2006; Staab *et al*., 2009), microsphere based

luminex assay (Etienne *et al*., 2009) and random amplified polymorphic (Brandt *et al*., 1998).

In a phylogenetic analysis of ITS regions have been used since it showed variation between microorganism of the same species and minor variation within the strains (Sugita *et al*., 1999). Identification of *Aspergillus* species using ITS 1 and ITS 2 sequence analysis is an efficient and reliable method compared to culture methods (Henry *et al*., 2000). *Aspergillus* species identification to species level is not possible using the 18S rRNA gene. In this study *Aspergillus fumigatus, A. niger*, *A. terreus,* and *A. flavus* were using ITS sequencing accurately. ITS region has been noted as the most reliable target for differentiation of *Aspergillus* species. The assay is cost-effective when ITS region is target using specific primers (Zhao *et al*., 2011). MALDI TOF MS is a diagnostic tool used for accurate and rapid identification of microorganism (Barker *et al*., 2014; Chao *et al*., 2014; Putignami *et al*., 2014; Triest, *et al*., 2015) and also to identify fungi (Alshawa *et al*., 2012; De Carolis *et al*., 2012; Kolecka *et al*., 2013; Posteraro *et al*., 2012; Sanguinetti & Posteraro, 2017). MALDI TOF MS also used to differentiate closely related species.

Variation among *A. fumigatus* resistant to multiples azole in India have posed a challenge in patient's management (Chang *et al*., 2016) and also recent isolation of TR34/L98H *Aspergillus fumigatus* genotype in Tanzania (Chowdhary *et al*., 2014a). In developing countries like Kenya, Tanzania and most of the Africa countries, treatment and prompt diagnosis is a big challenge because of a limited resource. This is complicated by the high population of tuberculosis patients at risk of a chronic form of aspergillosis post TB treatment.

Overexpression of cyp51A is caused by tandem repeats insertion to the cyp51A gene. TR34 (34 base pair) tandem repeat always occur together with nucleotide substitution of lysine to histidine at codon 98 (Lelievre et al., 2013). This study reports the same findings. Price et al., (2015) hypothesized an increase in mRNA levels decrease the sensitivity

of *Aspergillus* to azole. In clinical and environmental samples TR34/L98H is the most common type of mutation (Rivero-Menedez *et al*., 2016; Verweij *et al*., 2016). Among all the resistant *Aspergillus fumigatus* showed TR34/L98H mutation and had cross-resistance to both agricultural and clinical azoles. The study detected TR34 mutation with 130bp and 105bp DNA amplicon for wild type. While for L98H mutation 189bp for wild type and 279bp for resistance strain was detected. The results agreed with Al-Wathiqi *et al*., (2013) finding.

Point mutations associated resistance have been shown to change the shapes channels thus inhibit the binding of the triazoles molecules to these channels (Monk *et al*., 2014; Snelders *et al*., 2010). Different forms of cyp51A points mutation have been linked to triazoles used e.g. TR34/L98H have linked to the used of Itraconazole, Isavuconazole, Posaconazole and Voriconazole (Chowdhary *et al*., 2014a; Van der Linden *et al*., 2015; Verweji *et al*., 2016; Wiederhold *et al*., 2016) similarly mutation which were found to be TR34/L98H. M220 and G54 have been linked to Posaconazole and Itraconazole (Verweji *et al*., 2016) and TR53 and TR46/Y121F/T289A to Itraconazole, Isavuconazole, Posaconazole (Chowdhary *et al*., 2014a; Jensen *et al*., 2016; Le pape *et al*., 2016; Van Der Linden *et al*., 2015; Vermeulen *et al*., 2015; Verweji *et al*., 2016) and Isavuconazole, itraconazole, posaconazole, voriconazole (Le pape *et al*., 2016; Verweji *et al*., 2016) respectively.

The TR34/L98H has been reported worldwide and it is the most common form of mutation (Arendrup *et al*., 2010; Bignell, 2014; Lockhart *et al*., 2011; Morio *et al*., 2012b; Mortensen *et al*., 2011; Wiederhold *et al*., 2015). However since 2009 when TR46/Y121F/T289A resistance was identified where it has migrated rapidly across the continents (van der Linden *et al*., 2013), even though in this study it was identified this type of mutation. Environmental hotspot for development of resistance among *Aspergillus* species is still unknown. However it has been suggested that resistance can development via sexual reproduction, but no data to support this notion.

The relatedness of *Aspergillus* isolates from the environment and clinical genetically and its single mutation (TR34) dominance is an indication of resistance being acquired from the environment is the most common route. Questions have risen on how resistant to clinical uses azoles emerge from the environment, but it has been suggested to due application of azole based fungicides in the preservation of materials, crops protection, post-harvest application to reduce spoilage (Verweji *et al*., 2007, Snelders *et al*., 2008, Hof, 2008).

The triazoles used in both agriculture and medical practice have similar chemical structures (3-dimensional) but a different mode of action against *Aspergillus fumigatus* (Snelders *et al*., 2012, Warrilow *et al*., 2010). However, demethylation inhibitor (DMI) like bromuconazole and propiconazole have the same structures as itraconazole and voriconazole have a similar structure to epoxiconazole and tebuconazole (Hollomon, 2017). Relatedness of *Aspergillus fumigatus* genetically has been done using two to four tandem markers using microsatellite fingerprinting (de Valk *et al*., 2005a).

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

Aspergillus species distribution isolated from fungicides experienced and fungicides naïve soils from Nairobi and Naivasha was dominated by *A. fumigatus* except naïve soils from Naivasha which were dominated by *A. niger*. Five species of *Aspergillus* were isolated namely: *A. fumigatus, A. niger, A. terreus, A. flavus* and *A. ochraceus*. The study report for the first time in Kenya the presence of triazole resistance among *Aspergillus* species: *Aspergillus fumigatus, A. niger,* A. *flavus, A. ochraceus* and *A. terreus* from clinical and environmental sources. Most of the isolated *Aspergillus* species resistance to triazoles were from Naivasha soils. Indiscriminate and use of common azole-based fungicide in both clinical and agriculture could be a significant risk factor for emerging triazole resistance. TR34/L98H mutation was detected in all the sequenced isolates from both naïve and experienced soils.

6.2 Recommendation

- There is a need for ethical and rational use of fungicide in agriculture. New, effective and safe should be developed in clinic and agriculture that do not induce the development of azole resistance.
- There is a need for triazole surveillance, collection of precise information and monitoring resistance both in agriculture and clinical practice to investigate the magnitude of the triazoles resistance.
- There is a need for government policies and regulation for use of clinical and agricultural based fungicide.
- The adoption of good field practice such as restricted and prudent use of azole-based fungicides in terms of period and applications doses and also the rotation of the product. When resistance is detected a different azoles based fungicides should be used.

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APPENDICES

Appendix I: Clinical Isolates:

*The isolates were archived at Mycology laboratory, Kenya Medical Research Institute from sputum of patients suspected of aspergillosis.

Serial no.	**Isolates**	**MIC against azoles**		
		ITZ	**VRZ**	**PSZ**
C01	*Aspergillus fumigatus*	1	1	0.5
C02	*Aspergillus flavus*	0.5	0.25	0.5
C03	*Aspergillus flavus*	0.5	0.03	0.06
C04	*Aspergillus fumigatus*	0.5	1	0.13
C05	*A. fumigatus*	1	0.25	0.5
C06	*A. fumigatus*	0.5	0.5	0.5
C07	*A. fumigatus**	1	16	1
C08	*A. fumigatus*	0.5	2	0.13
C09	*A. fumigatus*	1	1	0.125
C10	*A. fumigatus**	4	0.5	0.25
C11	*A. fumigatus*	2	2	1

Appendix II: Isolates isolated from experienced soils

Serial No.	Collection site	No Azoles medium	CFU	ITZ MIC	VRZ MIC	PSZ MIC
E001	Nairobi	Fusarium/Rhizopus		-	-	
E002	Nairobi	Fusarium		-	-	
E003	Nairobi	Rhizopus		-	-	
E004	Nairobi	Fusarium		-	-	
E005	Nairobi	Yeasts		-	-	
E006	Nairobi	*Aspergillus fumigatus*	6	0.5	0.06	0.06
E007	Nairobi	*Aspergillus fumigatus*	3	8	8	16
E008	Nairobi	*Aspergillus fumigatus* and *Aspergillus niger*	8	1	8	0.5
E009	Nairobi	*Fusarium/A. terreus*	2	0.25	0.0065	0.25
E10	Nairobi	*Aspergillus fumigatus*	*2*	8	>32	>32
E011	Nairobi	*Aspergillus fumigatus*	9	0.06	0.25	0.25
E012	Nairobi	*Aspergillus fumigatus*	4	0.5	0.13	0.5
E013	Nairobi	*Aspergillus fumigatus* and *Aspergillus niger*	10	0.5	0.5	2
E014	Nairobi	*Aspergillus fumigatus*	4	1	0.5	16
E015	Nairobi	Yeasts		-	-	
E016	Nairobi	Rhizopus		-	-	
E017	Nairobi	Bacteria		-	-	
E018	Nairobi	*Aspergillus fumigatus*	21	4	1	1
E019	Nairobi	*Aspergillus fumigatus*	3	0.5	2	0.5
E020	Nairobi	*Aspergillus niger*	30	1	0.5	2

E021	Nairobi	Rhizopus		-	-	
E022	Nairobi	*Aspergillus fumigatus* and *Aspergillus niger*	6	1	1	1
E023	Nairobi	Yeasts, Bacteria		-	-	
E024	Nairobi	*Aspergillus niger*	4	2	0.25	0.5
E025	Nairobi	*Rhizopus* species		-------	-------	
E026	Nairobi	*Aspergillus fumigatus*	11	1	1	0.25
E027	Nairobi	*Aspergillus niger*	9	2	0.0065	2
E028	Nairobi	*Aspergillus flavus*	13	1	0.5	0.25
E029	Nairobi	*Fusarium*		------	------	
E030	Nairobi	*Fusarium*		------	------	
E031	Nairobi	*Aspergillus fumigatus*	6	0.5	1	1
E032	Nairobi	*Aspergillus niger*	2	0.125	0.065	1
E033	Nairobi	*Fusarium/Rhizopus*		-	-	
E034	Nairobi	*Aspergillus niger*	4	2	1	4
E035	Nairobi	*Rhizopus*		-	-	
E036	Nairobi	*Aspergillus niger*	71	0.25	0.125	2
E037	Nairobi	*Fusarium*		-	-	
E038	Nairobi	Yeasts		-	-	
E039	Nairobi	Yeast		-	-	
E040	Nairobi	*Aspergillus fumigatus*	21	1	0.25	0.125
E041	Nairobi	*Aspergillus flavus*	7	0.5	0.06	0.13
E042	Nairobi	Rhizopus				
E043	Nairobi	*Aspergillus fumigatus*	19	2	4	1
E044	Nairobi	*Aspergillus niger*	<100	0.125	0.065	0.25
E045	Nairobi	*Aspergillus niger*	17	1	0.25	0.125

E046	Nairobi	*Aspergillus niger*	8	0.5	1	0.5
E047	Nairobi	*Aspergillus niger*	54	0.5	1	0.5
E048	Nairobi	*Aspergillus niger*	87	1	0.25	1
E049	Nairobi	*A. fumigatus*	67	2	16	0.065
E050	Nairobi	Yeasts		-	-	
E051	Nairobi	*Aspergillus niger*	17	0.065	1	0.5
E052	Nairobi	*Aspergillus niger*	9	0.5	0.125	0.065
E053	Nairobi	*Aspergillus flavus*	51	0.125	1	2
E054	Nairobi	*Aspergillus niger*	33	1	0.5	0.125
E055	Nairobi	*Rhizopus*		-	-	
E056	Nairobi	*Rhizopus*		-	-	
E057	Nairobi	*Aspergillus fumigatus*	9	1	0.25	0.25
E058	Nairobi	Yeasts		-	-	
E059	Nairobi	*Aspergillus niger and Fusarium species*	79	0.065	0.5	0.5
E060	Nairobi	*Aspergillus fumigatus*	89	0.5	0.13	0.06
E061	Nairobi	*Fusarium*		-	-	
E062	Nairobi	*Fusarium*		-	-	
E063	Nairobi	Bacteria, Yeasts		-	-	
E064	Nairobi	*Penicillium*		-	-	
E065	Nairobi	*Rhizopus*		-	-	
E066	Nairobi	*Fusarium*		-	-	
E067	Nairobi	*Aspergillus fumigatus*	12	0.25	1	2
E068	Nairobi	*Aspergillus fumigatus*	*9*	2	0.25	0.06
E069	Nairobi	*Rhizopus*		-	-	
E070	Nairobi	*Rhizopus*		-	-	
E071	Nairobi	*Penicillium*		-	-	
E072	Nairobi	*Rhizopus*		-	-	

E073	Nairobi	*Rhizopus*		-	-	
E074	Nairobi	*Fusarium*		-	-	
E075	Nairobi	*Rhizopus*		-	-	
E076	Nairobi	Yeasts		-	-	
E077	Nairobi	Bacteria		-	-	
E078	Nairobi	*Fusarium*		-	-	
E079	Nairobi	*Fusarium*		-	-	
E080	Nairobi	*Rhizopus*		-	-	
E081	Nairobi	*Fusarium*		-	-	
E082	Nairobi	*Rhizopus*		-	-	
E083	Nairobi	*Rhizopus*		-	-	
E084	Nairobi	*Rhizopus*		-	-	
E085	Nairobi	*Aspergillus niger*	67	0.25	0.06	0.15
E086	Nairobi	*Fusarium*		-	-	
E087	Nairobi	*Fusarium*		-	-	
E088	Nairobi	*Aspergillus fumigatus*	17	0.5	1	4
E089	Nairobi	*Fusarium*		-	-	
E090	Nairobi	*Rhizopus*		-	-	
E091	Nairobi	Yeasts		-	-	
E092	Nairobi	*Aspergillus fumigatus*	*25*	1	0.25	0.25
E093	Nairobi	*Rhizopus*		-	-	
E094	Nairobi	*Rhizopus*		-	-	
E095	Nairobi	*Fusarium*		-	-	
E096	Nairobi	*Aspergillus fumigatus* and *Aspergillus niger*	<100	1	2	1
E097	Nairobi	Yeasts		-	-	
E098	Nairobi	Yeasts		-	-	

E099	Nairobi	*Aspergillus fumigatus*, *Aspergillus niger* and *Fusarium*	<100	0.5	1	2
E100	Nairobi	*Rhizopus*		------	------	
E101	Nairobi	*Fusarium*		------	------	
E102	Nairobi	*Fusarium*		------	------	
E103	Nairobi	Bacteria		-	-	
E104	Nairobi	*Aspergillus fumigatus* and *Aspergillus niger*	57	2	1	0.5
E105	Naivasha	*Rhizopus*		------	------	
E106	Naivasha	*Fusarium*		------	------	
E107	Naivasha	*Fusarium*		------	------	
E108	Naivasha	*Fusarium*		------	------	
E109	Naivasha	*Fusarium*		------	------	
E110	Naivasha	*Aspergillus niger* and *Fusarium*	37	1	0.5	8
E111	Naivasha	*Aspergillus terreus*	6	0.065	1	0.5
E112	Naivasha	*Rhizopus*		-	-	
E113	Naivasha	*Rhizopus*		-	-	
E114	Naivasha	*Aspergillus terreus*	9	1	0.25	0.25
E115	Naivasha	*Fusarium*		------	------	
E116	Naivasha	*Fusarium*		------	------	
E117	Naivasha	*Bacteria*		------	------	
E118	Naivasha	*Aspergillus niger*	7	1	1	8
E119	Naivasha	*Rhizopus*		------	------	
E120	Naivasha	*Aspergillus fumigatus*	1	2	2	0.065
E121	Naivasha	Yeasts		-	-	
E122	Naivasha	Fusarium		-	-	

E123	Naivasha	Bacteria		-	-	
E124	Naivasha	*Aspergillus flavus*	*6*	2	1	0.5
E125	Naivasha	Rhizopus		-	-	
E126	Naivasha	Fusarium		-	-	
E127	Naivasha	Rhizopus		-	-	
E128	Naivasha	Rhizopus		-	-	
E129	Naivasha	Yeasts, Bacteria		-	-	
E130	Naivasha	*Aspergillus niger and Aspergillus flavus*	32	0.5	0.125	0.25
E131	Naivasha	*Aspergillus fumigatus*	2	0.5	1	1
E132	Naivasha	*Aspergillus niger*	5	0.125	0.065	0.065
E133	Naivasha	*Aspergillus niger*	19	1	0.25	0.5
E134	Naivasha	*Fusarium*		------	-------	
E135	Naivasha	Rhizopus		-	-	
E136	Naivasha	Rhizopus		-	-	
E137	Naivasha	Bacteria		-	-	
E138	Naivasha	*Aspergillus flavus*	35	1	0.5	1
E139	Naivasha	*Aspergillus niger*	2	0.5	1	2
E140	Naivasha	*Aspergillus niger*	8	0.5	0.125	0.25
E141	Naivasha	*Aspergillus niger*	12	2	2	1
E142	Naivasha	*Aspergillus niger*	81	2	8	32
E143	Naivasha	*Fusarium*		-	-	
E144	Naivasha	Rhizopus		-	-	
E145	Naivasha	*Aspergillus niger and Fusarium*	23	1	0.125	0.5
E146	Naivasha	*Aspergillus niger*	18	0.5	2	1
E147	Naivasha	*Aspergillus fumigatus*	4	1	0.5	1
E148	Naivasha	Yeasts		-	-	

E149	Naivasha	Fusarium		-	-	
E150	Naivasha	*Aspergillus niger*	5	2	0.125	0.5
E151	Naivasha	Fusarium		-	-	
E152	Naivasha	Fusarium		-	-	
E153	Naivasha	Bacteria		-	-	
E154	Naivasha	Yeasts		-	-	
E155	Naivasha	Yeasts		-	-	
E156	Naivasha	*Penicillium*		-	-	
E157	Naivasha	*Rhizopus*		-----	-----	
E158	Naivasha	*Fusarium*		-----	-----	
E159	Naivasha	*Rhizopus*		-----	------	
E160	Naivasha	*Rhizopus*		------	------	
E161	Naivasha	Rhizopus		------	-----	
E162	Naivasha	*Rhizopus*		-----	-----	
E163	Naivasha	*Rhizopus* and *Aspergillus fumigatus*	7	0.06	0.5	0.125
E164	Naivasha	*Aspergillus fumigatus*	9	1	1	0.5
E165	Naivasha	*Rhizopus*		------	------	
E166	Naivasha	*Rhizopus*		------	------	
E167	Naivasha	Rhizopus		------	------	
E168	Naivasha	*Aspergillus fumigatus*	*35*	4	1	2
E169	Naivasha	*Rhizopus*		------	------	
E170	Naivasha	*Rhizopus*		------	------	
E171	Naivasha	*Rhizopus*		------	------	
E172	Naivasha	Rhizopus		------	------	
E173	Naivasha	*Aspergillus fumigatus*	57	2	4	16
E174	Naivasha	*Rhizopus*		------	------	
E175	Naivasha	*Rhizopus*		------	------	

E176	Naivasha	Rhizopus		-----	-----	
E177	Naivasha	Yeasts		-	-	
E178	Naivasha	Fusarium		-	-	
E179	Naivasha	*Rhizopus*		-----	-----	
E180	Naivasha	Rhizopus		-----	-----	
E181	Naivasha	*Aspergillus fumigatus*	23	0.5	0.5	0.5
E182	Naivasha	*Aspergillus fumigatus*	7	1	1	4
E183	Naivasha	*Aspergillus fumigatus*	17	2	1	0.125
E184	Naivasha	*Rhizopus*		-----	-----	
E185	Naivasha	Rhizopus		-----	-----	
E186	Naivasha	*Aspergillus fumigatus*	67	1	1	0.5
E187	Naivasha	*Rhizopus*		-----	-----	
E188	Naivasha	Rhizopus		-----	------	
E189	Naivasha	*Aspergillus fumigatus* and *Aspergillus terreus*	89	0.5, 0.25	0.5, 1	4, 1
E190	Naivasha	*Aspergillus fumigatus*	5	0.5	0.25	1
E191	Naivasha	*Rhizopus*		------	------	
E192	Naivasha	*Aspergillus fumigatus*	29	*1*	2	0.5
E193	Naivasha	*Rhizopus*		------	------	
E194	Naivasha	*Aspergillus fumigatus*	3	2	0.5	1
E195	Naivasha	Yeasts		-	-	
E196	Naivasha	*Aspergillus fumigatus*	7	0.125	0.25	1
E197	Naivasha	*Rhizopus*		------	------	
E198	Naivasha	*Rhizopus*		------	------	
E199	Naivasha	*Rhizopus*		------	------	
E200	Naivasha	*Aspergillus fumigatus*	57	1	0.5	2
E201	Naivasha	*Aspergillus fumigatus*	39	1	2	0.125

E202	Naivasha	*Rhizopus*		-----	-----	
E203	Naivasha	*Rhizopus*		------	------	
E204	Naivasha	*Rhizopus*		------	------	
E205	Naivasha	*Rhizopus*		------	-----	
E206	Naivasha	*Rhizopus*		-----	------	
E207	Naivasha	*Rhizopus*		------	------	
E208	Naivasha	*Aspergillus fumigatus*	16	0.065	16	8
E209	Naivasha	*Rhizopus*		------	------	
E210	Naivasha	*Aspergillus fumigatus*	6	0.5	0.065	0.125
E211	Naivasha	*Rhizopus*		------	------	
E212	Naivasha	*Rhizopus*		------	------	
E213	Naivasha	*Rhizopus*		------	------	
E214	Naivasha	*Aspergillus fumigatus*	*3*	2	1	0.5
E215	Naivasha	*Rhizopus*		------	------	
E216	Naivasha	*Rhizopus*		-----	-----	
E217	Naivasha	*Rhizopus*		-----	-----	
E218	Naivasha	*Rhizopus*		-----	------	
E219	Naivasha	*Aspergillus fumigatus*	*9*	4	1	2
E220	Naivasha	*Rhizopus*		-----	-----	
E221	Naivasha	Fusarium		-----	-----	
E222	Naivasha	*Rhizopus*		-----	-----	
E223	Naivasha	*Rhizopus*		-----	-----	
E224	Naivasha	*Rhizopus*		-----	-----	
E225	Naivasha	*Aspergillus fumigatus*	11	0.5	0.125	0.25
E226	Naivasha	*Rhizopus*		-----	------	
E227	Naivasha	*Rhizopus*		-----	-----	
E228	Naivasha	*Rhizopus*		-----	-----	
E229	Naivasha	*Rhizopus*		-----	-----	

E230	Naivasha	*Aspergillus fumigatus*	31	8	4	16
E231	Naivasha	*Rhizopus*		-----	----	
E232	Naivasha	*Rhizopus*		----	----	
E233	Naivasha	*Rhizopus*		-----	-----	
E234	Naivasha	*Fusarium*		-----	-----	
E235	Naivasha	*Fusarium*		-----	-----	
E236	Naivasha	Yeasts		-----	-----	
E237	Naivasha	*Rhizopus*		-----	----	
E238	Naivasha	*Rhizopus*		-----	-----	
E239	Naivasha	Bacteria		-----	-----	
E240	Naivasha	*Rhizopus*		----	-----	
E241	Naivasha	*Yeasts/Bacteria*		-----	----	
E242	Naivasha	*Penicillium*		----	----	
E243	Naivasha	*Fusarium*		----	----	
E244	Naivasha	*Fusarium*		----	-----	
E245	Naivasha	*Rhizopus*		----	-----	
E246	Naivasha	*Fusarium*		-----	-----	
E247	Naivasha	*Aspergillus fumigatus* and *Aspergillus niger*	<100	2	1	2
E248	Naivasha	*Yeasts*		------	-----	
E249	Naivasha	Yeasts		-----	-----	
E250	Naivasha	*Aspergillus terreus*	28	1	0.5	0.5

Appendix III: Isolates isolated from naïve soils

Serial no.	Site	No Azoles medium	C.F.U	ITZ MIC	VRZ MIC	PSZ MIC
N001	Nairobi	*Aspergillus fumigatus*	*7*	0.5	0.5	0.125
N002	Nairobi	*Aspergillus fumigatus*	5	0.25	0.25	0.065
N003	Nairobi	*Rhizopus*		-------	-------	
N004	Nairobi	*Aspergillus fumigatus*	4	0.5	0.125	0.5
N005	Nairobi	*Fusarium*		--------	--------	
N006	Nairobi	*Fusarium*		--------	--------	
N007	Nairobi	*Rhizopus*		--------	--------	
N008	Nairobi	*Fusarium*		--------	--------	
N009	Nairobi	*Aspergillus niger*	67	0.25	0.25	0.25
N010	Nairobi	Yeasts		--------	--------	
N011	Nairobi	*Rhizopus, Fusarium*		--------	--------	
N012	Nairobi	*Aspergillus fumigatus*	11	2	0.5	0.5
N013	Nairobi	*Aspergillus fumigatus*	5	0.5	8	8
N014	Nairobi	*Rhizopus*		--------	--------	
N015	Nairobi	*Aspergillus terreus*	13	0.125	0.25	0.065
N016	Nairobi	*Rhizopus*		--------	--------	
N017	Nairobi	Bacteria		--------	--------	
N018	Nairobi	*Fusarium*		--------	--------	
N019	Nairobi	Bacteria		--------	--------	
N020	Nairobi	*Aspergillus niger*	35	1	0.125	0.5
N021	Nairobi	*Aspergillus niger, Cladosporon* and *Fusarium*	67	0.25	0.5	1
N022	Nairobi	*Fusarium*		--------	--------	

N023	Nairobi	*Rhizopus, Fusarium, A. niger*	6	1	0.5	4
N024	Nairobi	Yeasts, Bacteria		--------	--------	
N025	Nairobi	*Rhizopus*		--------	--------	
N026	Nairobi	*Rhizopus*		--------	--------	
N027	Nairobi	*Fusarium*		--------	--------	
N028	Nairobi	*Rhizopus*		--------	--------	
N029	Nairobi	*Fusarium*		--------	--------	
N030	Nairobi	*Aspergillus niger*	21	2	1	1
N031	Nairobi	*Rhizopus*		--------	--------	
N032	Nairobi	*Aspergillus niger*	9	1	0.5	0.5
N033	Nairobi	*Rhizopus*		--------	--------	
N034	Nairobi	*Rhizopus*		--------	--------	
N035	Nairobi	*Rhizopus*		--------	--------	
N036	Nairobi	*Penicillium*		--------	--------	
N037	Nairobi	*Rhizopus*		--------	--------	
N038	Nairobi	*Fusarium*		--------	--------	
N039	Nairobi	*Fusarium* and *Aspergillus fumigatus*	2	0.5	0.25	1
N040	Nairobi	*Aspergillus fumigatus*	18	1	0.125	0.25
N041	Nairobi	*Rhizopus*		------	--------	
N042	Nairobi	*Rhizopus*		-------	------	
N043	Nairobi	*Fusarium*		------	-------	
N044	Nairobi	*Aspergillus niger* and *Penicillium*	30	2	0.5	0.125
N045	Nairobi	*Rhizopus*		------	------	
N046	Nairobi	*Rhizopus*		------	------	

N047	Nairobi	*A. Fumigatus, Rhizopus*	11	2	8	16
N048	Nairobi	*Fusarium*		--------	--------	
N049	Nairobi	*Rhizopus*		--------	--------	
N050	Nairobi	*Fusarium* , Yeasts		--------	--------	
N051	Nairobi	Yeasts		--------	--------	
N052	Nairobi	Bacteria, Yeasts		-	-	
N053	Nairobi	*Rhizopus*		-	-	
N054	Nairobi	Yeasts		-	-	
N055	Nairobi	*Fusarium* species		--------	--------	
N056	Nairobi	*Aspergillus fumigatus*	19	0.5	1	2
N057	Nairobi	*Aspergillus fumigatus* and *Aspergillus niger*	<100	0.25	0.065	1
N058	Nairobi	*Fusarium*		--------	--------	
N059	Nairobi	*Penicillium*		--------	--------	
N60	Nairobi	*Rhizopus*		--------	--------	
N061	Nairobi	*Rhizopus*		--------	--------	
N062	Nairobi	*Aspergillus niger*	67	0.125	0.5	0.125
N063	Nairobi	*Fusarium* species				
N064	Nairobi	*Rhizopus* and *Aspergillus fumigatus*	2	>32	16	>32
N065	Nairobi	Yeasts		-	-	
N066	Nairobi	*Rhizopus*		--------	--------	
N067	Nairobi	*Rhizopus*		--------	--------	
N068	Nairobi	*Rhizopus*		--------	--------	
N069	Nairobi	*Rhizopus*		--------	--------	
N070	Nairobi	*Aspergillus niger*	17	0.125	0.5	0.125
N071	Nairobi	*Rhizopus*		--------	--------	

N072	Nairobi	*Aspergillus fumigatus*	3	2	1	0.25
N073	Nairobi	*Rhizopus*		--------	--------	
N074	Nairobi	*Rhizopus*		--------	--------	
N075	Nairobi	Bacteria		-	-	
N076	Nairobi	*Fusarium*		--------	--------	
N077	Nairobi	Bacteria		-	-	
N078	Nairobi	Yeasts, Bacteria		-	-	
N079	Nairobi	*Rhizopus*		--------	--------	
N080	Nairobi	*Rhizopus*		--------	--------	
N081	Nairobi	*Rhizopus*		--------	--------	
N082	Nairobi	*Aspergillus fumigatus*	4	0.25	0.125	0.125
N083	Nairobi	*Fusarium* species		--------	--------	
N084	Nairobi	*Aspergillus fumigatus*	11	0.25	0.5	0.5
N085	Nairobi	*Aspergillus fumigatus*	5	0.5	0.125	0.5
N086	Nairobi	*Aspergillus fumigatus*	13	1	2	1
N088	Nairobi	*Aspergillus fumigatus*	2	0.5	0.5	2
N087	Nairobi	*Aspergillus fumigatus*	11	8	16	32
N089	Nairobi	*Fusarium*		--------	--------	
N090	Nairobi	*Rhizopus*		--------	--------	
N091	Nairobi	Yeasts		------	------	
N092	Nairobi	*Rhizopus*		--------	--------	
N093	Nairobi	*Aspergillus niger*	39	1	0.5	0.5
N094	Nairobi	Bacteria		-----	------	
N095	Nairobi	*Fusarium*		--------	--------	
N096	Nairobi	*Aspergillus niger*	87	2	0.5	.5
N097	Nairobi	*Rhizopus*		--------	--------	
N098	Nairobi	*Rhizopus*		------	------	
N099	Nairobi	*Fusarium*		--------	-------	

N100	Nairobi	*Aspergillus niger*	<100	2	4	4
N101	Nairobi	*Rhizopus*		--------	--------	
N102	Nairobi	*Rhizopus*		--------	--------	
N103	Nairobi	Yeast, Bacteria				
N104	Nairobi	*Penicillium*		--------	--------	
N105	Nairobi	Bacteria		------	------	
N106	Nairobi	Bacteria		------	------	
N107	Nairobi	Yeasts		------	------	
N108	Nairobi	*Rhizopus*		-------	--------	
N109	Nairobi	*Fusarium*		-------	--------	
N110	Nairobi	*Fusarium*		-------	--------	
N111	Nairobi	*Rhizopus*		-------	--------	
N112	Nairobi	*Penicillium* and *Aspergillus fumigatus*	97	0.5	0.5	0.5
N113	Nairobi	*Aspergillus fumigatus*	13	0.25	1	.05
N114	Nairobi	*Rhizopus*		-------	-------	
N115	Nairobi	*Aspergillus fumigatus*	3	0.5	0.5	2
N116	Nairobi	*Rhizopus*				
N117	Nairobi	*Aspergillus fumigatus*	9	1	1	0.5
N118	Nairobi	*Aspergillus fumigatus*		2	0.125	0.065
N119	Nairobi	*Rhizopus*		-------	--------	
N120	Nairobi	*Aspergillus fumigatus*	8	1	4	8
N121	Nairobi	*Rhizopus*		-------	--------	
N122	Nairobi	Yeasts		-------	--------	
N123	Nairobi	*Aspergillus fumigatus* and *Rhizopus*	3	1	1	16
N124	Nairobi	*Aspergillus fumigatus*	23	2	2	1
N125	Nairobi	*Aspergillus fumigatus*	15	0.5	4	4

N126	Naivasha	*Aspergillus niger*	45	0.5	0.25	0.25
N127	Naivasha	*Aspergillus fumigatus*	21	8	8	16
N128	Naivasha	*Fusarium*		-------	--------	
N129	Naivasha	*Rhizopus*		-------	--------	
N130	Naivasha	*Rhizopus*		-------	--------	
N131	Naivasha	*Rhizopus*		-------	--------	
N132	Naivasha	*Aspergillus niger*	7	4	2	8
N133	Naivasha	Yeasts		-------	--------	
N134	Naivasha	*Aspergillus niger*	9	1	0.5	8
N135	Naivasha	Yeasts				
N136	Naivasha	*Rhizopus*		-------	--------	
N137	Naivasha	*Rhizopus*		-------	--------	
N138	Naivasha	*Fusarium*		-------	--------	
N139	Naivasha	*Rhizopus*		-------	--------	
N140	Naivasha	*Rhizopus*		-------	--------	
N141	Naivasha	*A. Fumigatus*	5	1	4	1
N142	Naivasha	*Aspergillus fumigatus*	7	0.25	0.25	0.25
N143	Naivasha	*Rhizopus*		-------	--------	
N144	Naivasha	*Aspergillus niger*	9	2	2	1
N145	Naivasha	*Rhizopus*		-------	--------	
N146	Naivasha	*Aspergillus fumigatus*	10	0.125	0.5	0.5
N147	Naivasha	*Rhizopus*		-------	--------	
N148	Naivasha	*Aspergillus fumigatus*	4	0.5	1	0.25
N149	Naivasha	*Fusarium*		-------	--------	
N150	Naivasha	*Rhizopus*		-------	--------	
N151	Naivasha	*A.fumigatus, Fusarium*	7	1	1	1

N152	Naivasha	*Fusarium*		32	8	4
N153	Naivasha	*Aspergillus fumigatus*	21	4	1	0.25
N154	Naivasha	*Rhizopus*		-------	------	
N156	Naivasha	*Rhizopus*		------	------	
N157	Naivasha	*Aspergillus fumigatus*	19	2	8	2
N158	Naivasha	*Rhizopus*		-----	-------	
N159	Naivasha	*Aspergillus fumigatus*	6	0.5	1	0.5
N160	Naivasha	*Rhizopus*		-------	--------	
N161	Naivasha	*Rhizopus*		-------	--------	
N162	Naivasha	*Rhizopus*		-------	--------	
N163	Naivasha	*Aspergillus fumigatus*	7	0.5	1	0.125
N164	Naivasha	*Rhizopus*		-------	--------	
N165	Naivasha	*Rhizopus*		-------	--------	
N166	Naivasha	*Rhizopus*		-------	--------	
N167	Naivasha	*Rhizopus*		-------	--------	
N168	Naivasha	*Rhizopus*		-------	--------	
N169	Naivasha	*Fusarium*		-------	--------	
N170	Naivasha	*Rhizopus*		-------	--------	
N171	Naivasha	*Rhizopus*		-------	--------	
N172	Naivasha	*Rhizopus*		-------	--------	
N173	Naivasha	*Aspergillus niger*	23	4	2	0.125
N174	Naivasha	*Rhizopus*		-------	--------	
N175	Naivasha	*Rhizopus*		-------	--------	
N176	Naivasha	*Rhizopus*		-------	--------	
N177	Naivasha	*Rhizopus*		-------	--------	
N178	Naivasha	*Rhizopus*		-------	--------	
N179	Naivasha	*Rhizopus*		-------	--------	
N180	Naivasha	*Aspergillus niger*	16	2	1	1

N181	Naivasha	*Fusarium*		-------	--------	
N182	Naivasha	Yeasts				
N183	Naivasha	*Aspergillus niger*	19	0.5	0.25	0.25
N184	Naivasha	*Aspergillus fumigatus*	14	0.5	0.125	0.125
N185	Naivasha	*Rhizopus*		-------	--------	
N186	Naivasha	*Rhizopus*		-------	--------	
N187	Naivasha	*Rhizopus*		-------	--------	
N188	Naivasha	*Rhizopus*		-------	--------	
N189	Naivasha	*Fusarium*		-------	--------	
N190	Naivasha	*Aspergillus fumigatus*	6	0.125	0.5	0.125
N191	Naivasha	*Fusarium*		-------	--------	
N192	Naivasha	*Fusarium*		-------	--------	
N193	Naivasha	*Rhizopus*		-------	--------	
N194	Naivasha	*Aspergillus fumigatus*	7	-------	--------	
N195	Naivasha	*Aspergillus fumigatus*	17	*0.5*	0.25	0.125
N196	Naivasha	*Rhizopus*		-------	--------	
N197	Naivasha	*Aspergillus niger*	33	1	0.13	0.125
N198	Naivasha	*Rhizopus*		-------	--------	
N199	Naivasha	*Rhizopus*		-------	--------	
N200	Naivasha	*Rhizopus*		-------	--------	
N201	Naivasha	*Aspergillus niger*	47	0.25	0.065	0.5
N202	Naivasha	*Aspergillus niger*	3	1	0.25	0.5
N203	Naivasha	*A. fumigatus, A. ochraceus*	10	1	0.25	1
N204	Naivasha	Rhizopus				
N205	Naivasha	*Aspergillus fumigatus and A. niger*	38	1	0.125	1
N206	Naivasha	Bacteria		------	------	

N207	Naivasha	*Aspergillus niger*	12	0.5	0.125	0.065
N208	Naivasha	*Fusarium, Rhizopus*		-----	-----	
N209	Naivasha	*Aspergillus fumigatus, Fusarium*	13	0.5	0.065	1
N210	Naivasha	*Aspergillus niger, A. flavus*	55	0.25	1	0.125
N211	Naivasha	*Rhizopus*		------	------	
N212	Naivasha	*Rhizopus, Fusarium, A. niger, Penicillium*	<100	0.5	0.5	1
N213	Naivasha	*Fusarium, Rhizopus, A. niger, A. ochraceus*	87	0.25	1	0.5
N214	Naivasha	Bacteria		------	-----	
N215	Naivasha	*Aspergillus niger, Fusarium*	72	1	0.125	0.25
N216	Naivasha	*Aspergillus fumigatus, Fusarium*	7	1	1	0.25
N217	Naivasha	*Rhizopus, Fusarium*		------	-------	
N218	Naivasha	Bacteria		------	------	
N219	Naivasha	*Aspergillus fumigatus and Aspergillus niger*	75	0.5	1	0.125
N220	Naivasha	Yeasts, Bacteria		-------	------	
N221	Naivasha	*Aspergillus fumigatus and Aspergillus niger*	93	0.065	0.065	0.125
N222	Naivasha	*Fusarium*		-------	------	

N223	Naivasha	*Fusarium, A. fumigatus, A. flavus, A. niger*	85	0.5	1	1
N224	Naivasha	*Aspergillus niger, Fusarium*	27	0.125	0.5	0.25
N225	Naivasha	*Rhizopus*		------	-------	
N226	Naivasha	*Rhizopus*		------	------	
N227	Naivasha	*Fusarium, A. niger*	41	1	0.25	0.065
N228	Naivasha	*Aspergillus niger*	2	0.125	0.5	0.125
N229	Naivasha	Yeasts		------	------	
N230	Naivasha	*Rhizopus, A. niger, A. flavus, A. fumigatus*	21	0.065	0.25	0.125
N231	Naivasha	*Aspergillus niger, Cladosporon*	7	0.25	0.5	0.5
N232	Naivasha	*Rhizopus*		------	------	
N233	Naivasha	*Penicillium*		-----	------	
N234	Naivasha	*Aspergillus niger, Fusarium*	6	0.25	0.125	0.5
N235	Naivasha	Yeasts, *Fusarium*		-----	-----	
N236	Naivasha	*Aspergillus niger, Rhizopus*	15	0.25	0.25	0.125
N237	Naivasha	*Rhizopus*		-----	-----	
N238	Naivasha	*Rhizopus*		-----	-----	
N239	Naivasha	*Rhizopus*		-----	-----	
N240	Naivasha	*Rhizopus*		-----	-----	
N241	Naivasha	Yeasts		-----	------	
N242	Naivasha	*Rhizopus*		-----	-----	

N243	Naivasha	*Aspergillus niger, Fusarium*	18	0.125	0.065	0.065
N244	Naivasha	*Rhizopus*		-----	-----	
N245	Naivasha	*Rhizopus*		-----	-----	
N246	Naivasha	*Fusarium*		-----	-----	
N247	Naivasha	Bacteria		-----	-----	
N248	Naivasha	Yeasts		----	-----	
N249	Naivasha	*Fusarium*		------	------	
N250	Naivasha	*Rhizopus*		-----	-----	
N251	Kajiando	Bacteria		-----	-----	
N252	Kajiando	*Rhizopus*		-----	-----	

Appendix IV: *Aspergillus* species diversity in Nairobi experienced soils

Number of Organisms:	53	Total Number of Species:	4
Average population size:	13.25	Decimal Accuracy:	4
Total Number of Regions:	1	Total Number of Region Sets:	1

Alpha Biodiversity [α]

Simpson Index $\frac{\sum_i n_i(n_i-1)}{N(N-1)}$	0.3897	Simpson Index Approximation $\frac{\sum_i n_i^2}{N^2}$	0.4012
Dominance Index $1-\left(\frac{\sum_i n_i(n_i-1)}{N(N-1)}\right)$	0.6103	Dominance Index Approximation $1-\left(\frac{\sum_i n_i^2}{N^2}\right)$	0.5988
Reciprocal Simpson Index $\frac{1}{\left(\frac{\sum_i n_i^2}{N^2}\right)}$	2.566	Alternate Reciprocal Simpson Index $\frac{1}{\left(\frac{\sum_i n_i(n_i-1)}{N(N-1)}\right)}$	2.492
Shannon Index $-\sum_i\left(\frac{n_i}{N}\cdot\log_2\left(\frac{n_i}{N}\right)\right)$	1.507	Berger-Parker Dominance Index $\frac{n_{max}}{N}$	0.4717
Shannon Index $-\sum_i\left(\frac{n_i}{N}\cdot\ln\left(\frac{n_i}{N}\right)\right)$	1.045	Inverted Berger-Parker Dominance Index $\frac{N}{n_{max}}$	2.12
Shannon Index $\sum_i\left(\frac{n_i}{N}\cdot\log_{10}\left(\frac{n_i}{N}\right)\right)$	-0.4536	Margalef Richness Index $\frac{S-1}{\ln N}$	0.7556
Menhinick Index $\frac{S}{\sqrt{\sum_i n_i}}$	0.5494	Rényi Entropy/Hill Numbers (r=0,1,2,∞) $\frac{1}{1-r}\cdot\ln\left(\sum_i p_i^r\right)$	4, 2.846, 2.492, ≈∞
Buzas and Gibson's Index $\frac{e^{-\sum_i\left(\frac{n_i}{N}\cdot\ln\left(\frac{n_i}{N}\right)\right)}}{S}$	0.7105	Gini Coeffificient $\frac{2\Sigma_i\, in_i}{n\Sigma_i\, n_i}-\frac{N+1}{N}$	0.4009

Equitability Index $-\frac{\sum_i \left(\frac{n_i}{N} \cdot \ln\left(\frac{n_i}{N}\right)\right)}{\ln N}$	0.7535	ln() of Hill Numbers (0,1,2,∞):	1.386, 1.046, 0.9133, ≈∞

Beta Biodiversity [β]

Comparing two sample areas

Absolute beta Value ($(S_0$-c)-(S_1-c)...):	3	Whittaker's Index (S/alpha):	1
Sørensen's similarity index:	1	Alternate Whittaker's Index (S/alpha-1):	0
Sørensen's similarity index (%):	100%	Jaccard Index:	-1
Routledge beta-R Index:	1.333	Jaccard Index (%):	-100%
Mountford Index:	-1	Number of Common Species:	4
Mountford Index (%):	-100%	Bray Curtis dissimilarity	0

Appendix V: *Aspergillus* species diversity in Naivasha sub-county experienced soils

Total Number of Organisms:	40	Total Number of Species:	4
Average population size:	10	Decimal Accuracy:	4
Total Number of Regions:	1	Total Number of Region Sets:	1

Alpha Biodiversity [α]

Simpson Index $\frac{\sum_i n_i(n_i-1)}{N(N-1)}$	0.4577	Simpson Index Approximation $\frac{\sum_i n_i^2}{N^2}$	0.4713
Dominance Index $1-\left(\frac{\sum_i n_i(n_i-1)}{N(N-1)}\right)$	0.5423	Dominance Index Approximation $1-\left(\frac{\sum_i n_i^2}{N^2}\right)$	0.5288
Reciprocal Simpson Index $\frac{1}{\left(\frac{\sum_i n_i^2}{N^2}\right)}$	2.185	Alternate Reciprocal Simpson Index $\frac{1}{\left(\frac{\sum_i n_i(n_i-1)}{N(N-1)}\right)}$	2.122
Shannon Index $-\sum_i\left(\frac{n_i}{N}\cdot\log_2\left(\frac{n_i}{N}\right)\right)$	1.368	Berger-Parker Dominance Index $\frac{n_{max}}{N}$	0.625
Shannon Index $-\sum_i\left(\frac{n_i}{N}\cdot\ln\left(\frac{n_i}{N}\right)\right)$	0.9483	Inverted Berger-Parker Dominance Index $\frac{N}{n_{max}}$	1.6
Shannon Index $\sum_i\left(\frac{n_i}{N}\cdot\log_{10}\left(\frac{n_i}{N}\right)\right)$	-0.4119	Margalef Richness Index $\frac{S-1}{\ln N}$	0.8133
Menhinick Index $\frac{S}{\sqrt{\sum_i n_i}}$	0.6325	Rényi Entropy/Hill Numbers (r=0,1,2,∞) $\frac{1}{1-r}\cdot\ln\left(\sum_i p_i^r\right)$	4, 2.586, 2.122, ≈∞
Buzas and Gibson's Index $\frac{e^{-\sum_i\left(\frac{n_i}{N}\cdot\ln\left(\frac{n_i}{N}\right)\right)}}{S}$	0.6454	Gini Coeffificient $\frac{2\Sigma_i\, in_i}{n\Sigma_i\, n_i}-\frac{N+1}{N}$	0.4875

Equitability Index $-\frac{\sum_i \left(\frac{n_i}{N} \cdot \ln\left(\frac{n_i}{N}\right)\right)}{\ln N}$	0.6841	ln() of Hill Numbers (0,1,2,∞):	1.386, 0.9501, 0.7524, ≈0.4705

Beta Biodiversity [β]

Comparing two sample areas

Absolute beta Value ($(S_0$-c)-(S_1-c)...):	3	Whittaker's Index (S/alpha):	1
Sørensen's similarity index:	1	Alternate Whittaker's Index (S/alpha-1):	0
Sørensen's similarity index (%):	100%	Jaccard Index:	-1
Routledge beta-R Index:	1.333	Jaccard Index (%):	-100%
Mountford Index:	-1	Number of Common Species:	4
Mountford Index (%):	-100%	Bray Curtis dissimilarity	0

Appendix VI: *Aspergillus* species diversity in Nairobi county naïve soils

Dataset Totals

Total Number of Organisms:	45	Total Number of Species:	5
Average population size:	9	Decimal Accuracy:	4
Total Number of Regions:	1	Total Number of Region Sets:	1

Alpha Biodiversity [*α*]

Simpson Index	0.4253	Simpson Index Approximation $\frac{\sum_i n_i^2}{N^2}$	0.438
Dominance Index $1-\left(\frac{\sum_i n_i(n_i-1)}{N(N-1)}\right)$	0.5747	Dominance Index Approximation $1-\left(\frac{\sum_i n_i^2}{N^2}\right)$	0.562
Reciprocal Simpson Index $\frac{1}{\left(\frac{\sum_i n_i^2}{N^2}\right)}$	2.352	Alternate Reciprocal Simpson Index $\frac{1}{\left(\frac{\sum_i n_i(n_i-1)}{N(N-1)}\right)}$	2.283
Shannon Index $-\sum_i\left(\frac{n_i}{N}\cdot\log_2\left(\frac{n_i}{N}\right)\right)$	1.533	Berger-Parker Dominance Index $\frac{n_{max}}{N}$	0.6
Shannon Index $-\sum_i\left(\frac{n_i}{N}\cdot\ln\left(\frac{n_i}{N}\right)\right)$	1.062	Inverted Berger-Parker Dominance Index $\frac{N}{n_{max}}$	1.667
Shannon Index $\sum_i\left(\frac{n_i}{N}\cdot\log_{10}\left(\frac{n_i}{N}\right)\right)$	-0.4614	Margalef Richness Index $\frac{S-1}{\ln N}$	1.051
Menhinick Index $\frac{S}{\sqrt{\sum_i n_i}}$	0.7454	Rényi Entropy/Hill Numbers (r=0,1,2,∞) $\frac{1}{1-r}\cdot\ln\left(\sum_i p_i^r\right)$	5, 2.901, 2.283, ≈∞

Buzas and Gibson's Index $\frac{e^{-\sum_i \left(\frac{n_i}{N} \cdot \ln\left(\frac{n_i}{N}\right)\right)}}{S}$	0.5787	Gini Coeffificient $\frac{2\sum_i i n_i}{n\sum_i n_i} - \frac{N+1}{N}$	0.5511
Equitability Index $-\frac{\sum_i \left(\frac{n_i}{N} \cdot \ln\left(\frac{n_i}{N}\right)\right)}{\ln N}$	0.6602	ln() of Hill Numbers (0,1,2,∞):	1.609, 1.065, 0.8255, ≈0.5113

Beta Biodiversity [β]

Comparing two sample areas

Absolute beta Value ((S_0-c)-(S_1-c)...):	4	Whittaker's Index (S/alpha):	1
Sørensen's similarity index:	1	Alternate Whittaker's Index (S/alpha-1):	0
Sørensen's similarity index (%):	100%	Jaccard Index:	-1
Routledge beta-R Index:	1.667	Jaccard Index (%):	-100%
Mountford Index:	-0.6667	Number of Common Species:	5
Mountford Index (%):	-66.67%	Bray Curtis dissimilarity	0

Appendix VII: *Aspergillus* species diversity in Naivasha sub-county naïve soils

Total Number of Organisms:	58	Total Number of Species:	6
Average population size:	9.667	Decimal Accuracy:	4
Total Number of Regions:	1	Total Number of Region Sets:	1

Alpha Biodiversity [α]

Simpson Index $\frac{\sum_i n_i(n_i-1)}{N(N-1)}$	0.2934	Simpson Index Approximation $\frac{\sum_i n_i^2}{N^2}$	0.3056
Dominance Index $1-\left(\frac{\sum_i n_i(n_i-1)}{N(N-1)}\right)$	0.7066	Dominance Index Approximation $1-\left(\frac{\sum_i n_i^2}{N^2}\right)$	0.6944
Reciprocal Simpson Index $\frac{1}{\left(\frac{\sum_i n_i^2}{N^2}\right)}$	3.408	Alternate Reciprocal Simpson Index $\frac{1}{\left(\frac{\sum_i n_i(n_i-1)}{N(N-1)}\right)}$	3.272
Shannon Index $-\sum_i\left(\frac{n_i}{N}\cdot\log_2\left(\frac{n_i}{N}\right)\right)$	1.944	Berger-Parker Dominance Index $\frac{n_{max}}{N}$	0.3966
Shannon Index $-\sum_i\left(\frac{n_i}{N}\cdot\ln\left(\frac{n_i}{N}\right)\right)$	1.347	Inverted Berger-Parker Dominance Index $\frac{N}{n_{max}}$	2.522
Shannon Index $\sum_i\left(\frac{n_i}{N}\cdot\log_{10}\left(\frac{n_i}{N}\right)\right)$	-0.5852	Margalef Richness Index $\frac{S-1}{\ln N}$	1.231
Menhinick Index $\frac{S}{\sqrt{\sum_i n_i}}$	0.7878	Rényi Entropy/Hill Numbers (r=0,1,2,∞) $\frac{1}{1-r}\cdot\ln\left(\sum_i p_i^r\right)$	6, 3.854, 3.272, ≈∞
Buzas and Gibson's Index $\frac{e^{-\sum_i\left(\frac{n_i}{N}\cdot\ln\left(\frac{n_i}{N}\right)\right)}}{S}$	0.6413	Gini Coeffificient $\frac{2\Sigma_i\, in_i}{n\Sigma_i\, n_i}-\frac{N+1}{N}$	0.4943

Equitability Index $\frac{\sum_i \left(\frac{n_i}{N} \cdot \ln\left(\frac{n_i}{N}\right)\right)}{\ln N}$	0.752	ln() of Hill Numbers (0,1,2,∞):	1.792, 1.349, 1.186, ≈∞

Beta Biodiversity [β]

Comparing two sample areas

Absolute beta Value ((S_0-c)-(S_1-c)...):	5	Whittaker's Index (S/alpha):	1
Sørensen's similarity index:	1	Alternate Whittaker's Index (S/alpha-1):	0
Sørensen's similarity index (%):	100%	Jaccard Index:	1
Routledge beta-R Index:	2	Jaccard Index (%):	100%
Mountford Index:	0.5	Number of Common Species:	6
Mountford Index (%):	50%	Bray Curtis dissimilarity	0

Appendix VIII: Publications and conference presentation

1. **Edson K. Kemoi**, Andrew Nyerere, Uwe Gross, Oliver Bader, Tohru Gonoi, Christine C. Bii (2017). Diversity of Azoles Resistant *Aspergillus* Species Isolated from Experience and Naïve Soils in Nairobi County and Naivasha Sub-County Kenya. European Scientific Journal December 2017 edition Vol.13, No.36 ISSN: 1857 – 7881 (Print) e - ISSN 1857-7431. URL:http://dx.doi.org/10.19044/esj.2017.v13n36p30.

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Diversity of Azoles Resistant *Aspergillus* Species Isolated from Experience and Naïve Soils in Nairobi County and Naivasha Sub-County Kenya

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Abstract

New triazole antifungals voriconazole, itraconazole and posaconazole are recommended for prophylaxis and treatment of both invasive and chronic fungal infections such as aspergillosis and aspergilloma. Emergence of azole-resistant among *A. fumigatus* isolates have been reported in other countries including Tanzania ascribed to either previous antifungal treatment, prophylaxis or triazoles use in agriculture. The use of azole based fungicides in the robust horticulture in Kenya is a significant risk factor for antifungal resistance. The study proposes to analyze environmental isolates of *Aspergillus fumigatus, Aspergillus flavus* and *Aspergillus niger* for the presence of resistance against the triazoles antifungals. Fungicide naïve soils were obtained from uncultivated virgin fields while fungicide experience soils were collected from flower, agricultural and horticultural fields and greenhouses within Naivasha sub-county and Nairobi County. The fungal isolates were subjected to antifungal susceptibility to triazoles using broth micro dilution method. A total of 492 samples were analyzed in Nairobi, 52 isolates were identified and they resistance were as follow: *A. fumigatus* (32%), *A. niger* (26.09%), *A. flavus* (33.33%) and *A .terreus* (0%) and in Naivasha 44 isolates were isolated out of which 25 were *A. fumigatus* and its resistance was at 36%. Data were analyses using student T test and showed they no different between resistant and susceptible isolates from the two location. Data generated will serve to inform on the current status of triazoles resistance pattern and to raise concern emerging antifungal resistance in clinical practice.

Keywords: Azoles resistant *Aspergillus, Environmental isolates, Aspergillus fumigatus*

INTRODUCTION

Aspergillus species are found worldwide, it has isolated in organic matter, soil and decaying organic matter, Most of the *Aspergillus* species sporulated highly (Balajee, 2009, Mann *et al*., 2003). The conidia hence is carried by wind and inhaled causing infection in individual whose immune system is compromised (Perfect *et al*., 2001). Azoles namely posaconazole, Voriconazole and itraconazole are drugs used as first line in treatment of aspergillosis (Walsh *et al*., 2008). Other drugs with sensitive against *Aspergillus* species are Anidulafungin, micafungin, caspofungin and amphotericin B. *Aspergillus fumigatus* which causes most of the chronic and invasive aspergillosis (Denning *et al*., 2003, Walsh *et al*., 2008) is normally sensitive to the three classes of antigungal. However the recent past cases of patient with aspergillosis caused by *A. fumigatus* resistant to azoles has been reported (33). *Aspergillus terreus* is resistant to amphotericin B while *Aspergillus pseudofischeri* and *A. lentulus* is resistant to azoles intrinsically (Alcazar *et al*., 2008).

Azole resistance in *Aspergillus* infection due to azole treatment failure is an emerging health problem (Van der Linden *et al*., 2013. Van der Linden *et al*., 2011, Vermeulen *et al*., 2013). Surveillance reports have showed geographical spread of azole resistant species in clinical and environment in Africa, Europe, Asia, North and South America and Middle East (Vermeulen *et al*., 2013, Chowdhary *et al*., 2014). In contrast Netherland surveillance studies showed that azole resistance is endemic, TR46/Y121F/T289A and TR34/L98H cyp51A gene mediated resistance been the most common (Van der Linden *et al*., 2011).Multicenter international surveillance network study reported 3.2% triazoles resistance. Majority of resistant isolates were *Aspergillus* species at 78% and 22% of the other sibling species (*A. lentulus, A.udagawae and A. thermomutatus*) (Lamoth, 2016, Van der Linden *et al*., 2015).

The common reported mechanism of azoles resistance is alteration of the azoles drugs target i.e. lanosterol 14-α-demethylase and mutation in gene *cyp51A* (Chen *et al*., 2005, Mellado *et al*., 2005, Mellado *et al*., 2004, Diaz-Guerra *et al*., 2003, Mann *et al*., 2003). Substitution usually occurs in codon 98 by usually by substitution of histidine with leucine usually in the promoter region i.e. TR34/L98H. Some authors have attribute widespread uses of azoles in agricultural may have led to selection of strain carrying the resistant gene (Snelders *et al*., 2012, Verweiji *et al*., 2009). The use of azole based fungicides in the robust horticulture in Kenya is a significant risk factor for antifungal resistance. Recently several authors have reported a novel CYP51A mediated resistance with high resistance level to Voriconazole, TR46/Y121F/T289A also has be isolated from clinical sources and environmental in Europe, Africa, Latin America and Indian (Verweij *et al*., 2015,Abdolrasouli *et al*., 2016, Le Pape *et al*., 2016, Fuhren *et al*., 2015)

The aims of this study were to investigate the diversity and prevalence of azoles resistant *Aspergillus* species in Nairobi County and Naivasha sub-County.

MATERIALS AND METHODS

Sampling for environmental isolates

Approximately 5g dry top surface soils from agricultural site were collected into a sterile 15 ml Falcon tube using a sterile plastic spoon. Soil samples were transported in a leak proof packaging in a cool box to KEMRI-Center for Microbiology Research for mycological investigations.

Fungal culture and identification

Approximately a gram of the soil sample were suspended and vortexed thoroughly in 5 ml (0.5% w/v) saponin. The debris allowed to settle and the supernatant transferred to a fresh tube. The resulting suspension were then centrifuged, and the pellet suspended in a final volume of 500 µl sterile NaCl. Hundred µicrolitres of the suspension were plated

on Sabouraud dextrose agar containing; (a) no drug, (b) 1 µg/ml Itraconazole (c) 1 µg/ml voriconazole. All the Plates were incubated 30ºC for 72 hours, checking daily for any growth. Colonies growing after incubation on triazole free media were used as control and to access fungal diversity. Fungal while triazole containing agar were used to determine resistant isolates.

Triazoles susceptibility testing

Minimum inhibitory concentrations to Itraconazole (ITZ), Voriconazole (VCZ) and Posaconzole (PSZ) were tested by broth micro dilution according to the EUCAST reference method (EUCAST, 2008) with minor modification.

Exactly 9.6mg of azoles (Itraconazole, Voriconazole, posaconazole) powder were weighed and dissolved in 3.0ml of DMSO to get 1600µg/ml stock. Ten sterile tubes were used for serial dilution in each tube 3mls of DMSO were added. Starting with the first tube 3mls of the stock solution were added, mix and 3mls form the mixed of the first tube was transfer to second tube mixed and pipette 3mls from the second and transfer to the third tubes and the process were continued up to the ten tubes. The second serial dilution were done using ten tubes, in each of the tubes (From first serial dilution tubes) 4.9ml of RPMI were added to each of the ten tubes

From each tubes of the second dilution 200µl were dispensed to microtitre plates and inoculated with 10µl of the inoculums. Plates were then incubated at 30°C for 72 h. The MIC values of all drugs were determined visually as the lowest concentrations with no visible growth. EUCAST drug susceptible controls strains DSM819 and ATCC46645 were used while azole resistant control isolates allele TR/L98H) and (allele G54W, ITZR + PSZR86) will be used.

RESULTS

A total of 492 of both Naïve and experience soils (246 Naïve soils and 246 experienced soils) were analyzed. At total 52 isolates were isolated from experience soil in Nairobi out which proprtions of azoles resistance were as follows: *A. fumigatus* (32%), *A. niger* (26.09%), *A. flavus* (33.33%) and *A .terreus* (0%) as showed in table I. In Naivasha a total of 44 isolates were isolated, out of which 25 *A. fumigatus* were isolated and 36% were azole resistant as showed in Table 1.

Table 1: Showing isolates from experience soils both in Nairobi and Naivasha

Nairobi Experience soils isolates				**Naivasha experienced soils Isolates**		
Isolates	**Total no.**	**Susceptible**	**Resistant's**	**Total no.**	**Susceptible**	**Resistant's**
A. Fumigatus	25	17 (68.0%)	8 (32.0%)	25	14 (56.0%)	9 (36.0%)
A. Niger	23	17(73.91%)	6(26.09%)	13	10(76.92%)	3(23.08%)
A. Flavus	3	2(66.67%)	1(33.33%)	3	2(66.67%)	1(33.33%)
A. Terreus	1	1(100%)	0 (0.0%)	3	3 (100%)	0(0.0%)
Total	**52**	**37**	**15**	**44**	**29**	**13**

Out of 246 naïve soils analyzed In Nairobi a total of 40 isolates were isolated, of which resistance were as follows: *A. fumigatus* (19.23%), *A niger* (23.08%), *A. flavus* (0%), and *A. terreus* (0%) as showed in table 2. In Naivasha 50 *Aspergillus* species were isolated of which resistance were as follows: *A. fumigatus* (19.05%), *A niger* (15.38%), *A. flavus* (0%), and *A. terreus* (0%) as showed in the table 2 below.

Table 2: Showing isolates from experience soils both in Nairobi and Naivasha

Nairobi Naïve soils isolates				Naivasha Naïve soils Isolates		
Isolates	**Total no.**	**Susceptible**	**Resistant's**	**Total no.**	**Susceptible**	**Resistant's**
A. Fumigatus	26	21(80.77%)	5(19.23%)	21	17(80.95%)	4(19.05%)
A. Niger	13	10(76.92%)	3(23.08%)	26	22(84.62%)	4(15.38%)
A. flavus	0	0(0.0%)	0(0.0%)	3	3 (100%)	0(0.0%)
A. terreus	1	1 (100%)	0(0.0%)	0(0.0%)	0(0.0%)	0 (0.0%)
Total	**40**	**32(80.0%)**	**8(20.0%)**	**50**	**42(84.0%)**	**8(16.0%)**

T-test comparison of the resistant samples of Nairobi Naïve soils isolates to the Nairobi Experience soils isolates

The data was first normalized by getting the percentage resistance of the fungi. These were therefore used in testing for the variations. Using two-sample t-test, there was no significant difference in percentage resistant isolates in Nairobi naïve samples (mean 6.2%) to the percentage resistance isolates in Nairobi experience isolates (mean 7.8%), (T = 1.24, P = 0.271).

Two-Sample T-Test and CI: Nairobi Naive, Nairobi experience

Two-sample T for Nairobi Naive vs Nairobiexperience

	N	Mean	StDev	SE Mean
Nairobi Naive	4	10.6	12.3	6.2
Nairobi exper	4	22.9	15.6	7.8

Difference = mu N. Naive - mu N. experience

Estimate for difference: -12.28

95% CI for difference: (-37.78, 13.23)

T-Test of difference = 0 (vs not =): T-Value = -1.24 P-Value = 0.271 DF = 5

T-test comparison of the resistant samples of Nairobi Naïve soils isolates to the Naivasha naïve soil isolates

The result showed that there was no significant difference in percentage resistant isolates in Nairobi naïve samples (mean 6.2%) to the percentage resistant isolates in Naivasha naive isolates (mean 5.0%), (T = 0.25, P = 0.814).

Two-Sample T-Test and CI: Nairobi Naive, Naivasha naive

Two-sample T for Nairobi Naive vs Naivasha naive

	N	Mean	StDev	SE Mean
Nairobi Naive	4	10.6	12.3	6.2
Naivasha Naïve	4	8.6	10.1	5.0

Difference = mu N. Naive - mu Naivasha naive

Estimate for difference: 1.97

95% CI for difference: (-18.46, 22.40)

T-Test of difference = 0 (vs not =): T-Value = 0.25 P-Value = 0.814 DF = 5

T-test comparison of the resistant samples of Naivasha Naïve soil isolates to the Naivasha experience soil isolates

The result showed that there was no significant difference in percentage resistant isolates in Naivasha naïve samples (mean 5.0%) to the percentage resistant isolates in Naivasha experience isolates (mean 8.2%), (T = 1.51, P = 0.206).

Two-Sample T-Test and CI: Naivasha experience, Naivasha naive

Two-sample T for Naivas experience vs Naivasha naïve

	N	Mean	StDev	SE Mean
Naivas e	4	23.1	16.4	8.2
Naivasha	4	8.6	10.1	5.0

Difference = mu Naivas experience - mu Naivasha naive

Estimate for difference: 14.50

95% CI for difference: (-12.18, 41.17)

T-Test of difference = 0 (vs not =): T-Value = 1.51 P-Value = 0.206 DF = 4

T-test comparison of the resistant samples of Naivasha Naïve soil isolates to the Nairobi experience soil isolates

The result showed that there was no significant difference in percentage resistant isolates in Naivasha naïve samples (mean 5.0%) to the percentage resistant isolates in Nairobi experience isolates (mean 7.8%), (T = 1.54, P = 0.185).

Two-Sample T-Test and CI: Nairobi experience, Naivasha naive

Two-sample T for N. experience vs Naivasha naive

	N	Mean	StDev	SE Mean
Nairobi exper	4	22.9	15.6	7.8
Naivasha Naive	4	8.6	10.1	5.0

Difference = mu N. experience - mu Naivasha naive

Estimate for difference: 14.25

95% CI for difference: (-9.56, 38.06)

T-Test of difference = 0 (vs not =): T-Value = 1.54 P-Value = 0.185 DF = 5

DISCUSSION

We report the detection of environmental azole resistance *Aspergillus* species from both Naïve and experience soil in Nairobi County and Naivasha sub-County, Kenya. In experience they were high percentages in resistance from Naivasha isolates this may be due to irrational used of fungicides in large scale horticultural farming as compare to Nairobi which was mostly scale farming

In the recent decades, there has an increase in opportunities infection one of them is aspergillosis (Walsh *et al*., 2008). Invasive fungal infection disease are associated by high mortality and morbidity, partially because of failure to diagnosis early resulting in delay treatment. Most of the cross resistance in Aspergillus species by several authors have be reported infrequently, which mean they are infrequent to date. Low prevalence is due to variation in laboratory testing between different laboratories.

The detection of azole resistance of *Aspergillus* species was done based on medium containing azoles and micro dilution susceptibility testing. The uses of azole containing in screening environmental samples for the presence of resistance were first described using Itraconazole at a concentration of 4mg/liter (Snelders et al., 2009). Since then breakpoint have been proposed to be 1mg/liter for Itraconazole (Pfaller *et al*., 2009, Verweji *et al*., 2009). Which raises question that resistance isolates may go undetected. However it has been reported by several authors the MIC of most isolates against Itraconazole is 8mg/liter or more (Howard et al., 2009,). In the study we further included

Voriconazole (1mg/liter) and posaconazole (1mg/liter) SDA agar. Student T test showed that they were no significant difference in percentage resistance (P= 0.271) of isolates from Nairobi naïve and experience soils isolates as well as in Naivasha from naïve and experience soils (P= 0.206).

The used of azole containing agar, hypothetically may results in laboratory generation of resistance in the recover isolates though expose. However it is believe that since de novo short azole exposure (Almost 72hr exposure) is for short time it may not cause resistance and detection of cyp51A alterations which cause necessary (Mellado *et al*., 2007). Acquire azole resistance usually develops due to response of fungi to azole exposure in patients or in agricultural (Chen *et al*., 2005, Howard *et al*., 2006, Verweij *et al*., 2009). Drug expose over a long periods and high numbers of reproducing fungi are some of the factors that played a role in the selection of resistance (Anderson, 2005). This condition is met in soil when agricultural azole applied remained in soils over a year and in patients with Cavitary *Aspergillus* (Hof, 2001).

Demethylation inhibitors and Azoles fungicides are recommend for used in plant protection (Hof, 2001). In Netherlands the used for fungicides in agricultural has double since mid1990s (Verweji *et al*., 2009). Similar trends has been observed in Denmark. Resistant *Aspergillus fumigatus* is selected by used of azole fungicides or it can be introduced via importation of commercial compost to the environment, are one of the key factors in development of resistance (Verweji *et al*., 2009). In our study most of the sites with resistant Aspergillus were previously exposed to azoles fungicides before. This is always hard to management aspergillosis because most laboratories do not perform sensitivity test and due to negative cultures. Hence the prevalence of azoles resistant *Aspergillus* species may be underestimated with risk of inappropriate therapy. We believe in our study and emerging reports on azole related resistance, we suggest that susceptibility testing for *Aspergillus* species should be performed routinely.

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Research Article

Triazole-Resistant *Aspergillus fumigatus* from Fungicide-Experienced Soils in Naivasha Sub-county and Nairobi County, Kenya

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The mainstay in prevention and treatment of aspergillosis is the use triazole drugs. In Kenya, the use of agricultural azole is one of the predisposing factors in development of resistance. One hundred fifty-six (156) experienced soils were collected from agricultural farms and cultured on Sabouraud Dextrose Aagar. +e study isolated 48 yielded *Aspergillus fumigatus* and 2 *A. flavus*. All the isolates were subjected to antifungal susceptibility testing against three triazoles: posaconazole, voriconazole, and itraconazole. Out of the isolates, 3 had MIC of 32 and 1 had MIC of 16 against itraconazole, and 1 isolate had MIC of 32 against posaconazole. CYP51A gene was sequenced, and TR34/L98H mutation was identified. Triazole resistance existing in Kenya calls for rational use of azole-based fungicides in agriculture over concerns of emerging antifungal resistance in clinical practice.

Introduction

Aspergillus species and especially *Aspergillus fumigatus* is the most common cause of aspergillosis, which is the second leading cause of death after cryptococcosis in patients suffering from fungal infections [1]. Azole resistant *Aspergillus fumigatus*) is an

evolving global health challenge [2]. It is a frequent colonizer of cavitary lesions in tuberculosis patients and cause of mortality in post TB treatment cases [3].

Aspergillosis treatment is by used of amphotericin B or azoles. However, resistances against azoles have been increasingly reported especially from high- and middle-income countries [4-12]. Previously studies from different region of the worlds: African region (Tanzania), European region (Netherlands, Belgium, Denmark and Germany), Asia (Kuwait, India and Iran) and in USA, have reported multiple sources of Azole resistant *Aspergillus fumigatus* from soil sample, flower beds, plants, compost and hospitals and its environs [4, 10,12-23].

In contrast, limited data is available from low-income countries, especially from sub-Saharan Africa. However, a recent disturbing report of high resistance to azole was reported in Moshi Tanzania associated with *A. fumigatus* with TR34/L98H and TR46/Y12F/T289A mutations [20]. The widespread irrational use of azole based agricultural fungicides in the flower and horticultural industry in Kenya is a significant risk factor for azole resistance. The study aimed to determine the prevalence of triazole resistance among *Aspergillus fumigatus* from fungicide experienced soils.

Methodology

Study area

The study was conducted in Nairobi and Naivasha Sub County where horticultural practices and green houses are concentrated. Nairobi is the Kenya Capital City and lies at about 1°17'S and 36°49'E while Naivasha is located approximately 90KM North West of Nairobi. It is located in Nakuru County at 0°43'S36°26E and horticulture is the main economy. The trade names of commonly used fungicides includes; Milraz, Antracol, Mistress and Victory which are broad spectrum fungicides of ornamental, vegetable and fruit plants.

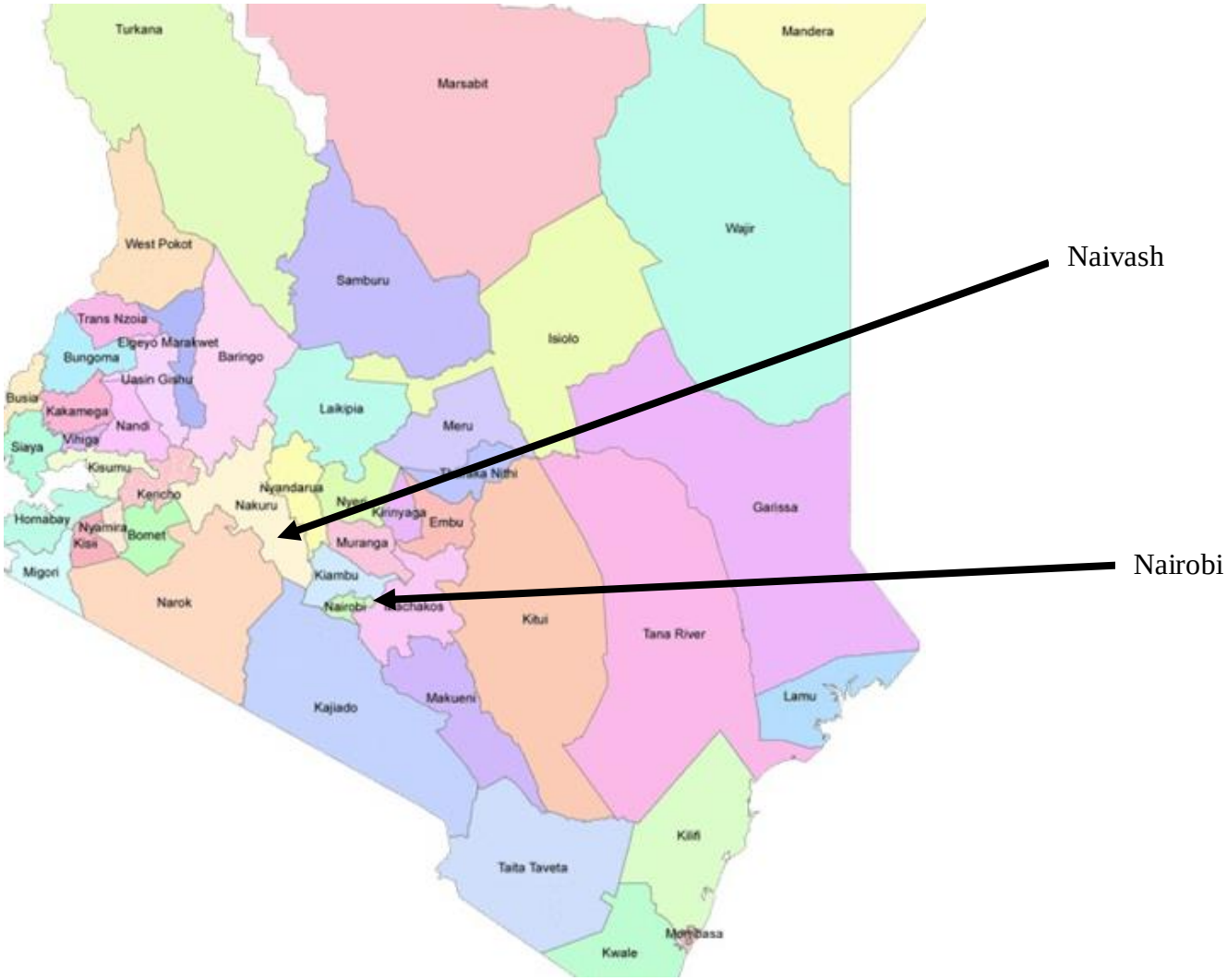

Fig: 1. A Map showing the study areas courtesty of www.mapsofworld.com. Accessed on 12th January, 2015.

Sampling for environmental isolates

A total of 156 samples were collected and analyzed. Approximately 5g dry top surface soils from agricultural site were collected into a sterile 15 ml Falcon tube using a sterile plastic spoon [23]. Samples were transported in a leak proof packaging in a cool box to Mycology laboratory-KEMRI-Center for Microbiology Research for investigation.

Clinical isolates were isolated from sputum samples of suspected aspergillosis patients and were archived isolates at Kenya Medical Research Institute, Mycology Laboratory.

No.	Sources	Isolates	MIC itraconazole	MIC Voriconazole	MIC posaconazole
F1	Experienced soils	*Aspergillus fumigatus*	1	0.25	0.25
F2	Experienced soils	*Aspergillus fumigatus*	1	0.15	0.25
F3	Experienced soils	*Aspergillus fumigatus*	0.5	0.5	0.06
F4	Experienced soils	*Aspergillus fumigatus*	1	0.25	0.25
F5	Experienced soils	*Aspergillus fumigatus*	1	0.15	0.25
F6	Experienced soils	*Aspergillus fumigatus*	4	1	1
F7	Experienced soils	*Aspergillus fumigatus*	4	1	1
F8	Experienced soils	*Aspergillus fumigatus*	0.5	0.15	0.25
F9	Experienced soils	*Aspergillus fumigatus*	0.5	0.06	0.15
F10	Experienced soils	*Aspergillus fumigatus*	1	0.5	0.25
F11	Experienced soils	*Aspergillus fumigatus*	0.5	0.13	0.06
F12	Experienced soils	*Aspergillus fumigatus*	0.5	0.06	0.06
F13	Experienced soils	*Aspergillus fumigatus*	0.5	0.03	0.25

F14	Experienced soils	*Aspergillus fumigatus*	0.25	0.15	0.06
F15	Experienced soils	*Aspergillus fumigatus*	0.5	0.15	0.25
F16	Experienced soils	*Aspergillus fumigatus*	0.5	0.15	0.15
F17	Experienced soils	*Aspergillus fumigatus*	0.13	0.5	0.25
F18	Experienced soils	*Aspergillus fumigatus*	1	1	1
F19	Experienced soils	*Aspergillus fumigatus*	0.15	0.06	0.25
F20	Experienced soils	*Aspergillus fumigatus*	0.5	0.15	0.25
F21	Experienced soils	*Aspergillus fumigatus*	1	1	1
F22	Experienced soils	*Aspergillus fumigatus*	0.5	0.15	0.5
F23	Experienced soils	*Aspergillus fumigatus*	32	8	0.5
F24	Experienced soils	*Aspergillus fumigatus*	1	0.25	0.25
F25	Experienced soils	*Aspergillus fumigatus*	1	8	0.5
F26	Experienced soils	*Aspergillus fumigatus*	0.5	0.15	0.15
F27	Experienced soils	*Aspergillus fumigatus*	0.5	0.03	0.06

F28	Experienced soils	*Aspergillus fumigatus*	0.5	1	0.15
F29	Experienced soils	*Aspergillus fumigatus*	1	0.15	0.25
F30	Experienced soils	*Aspergillus fumigatus*	0.5	0.05	0.15
F31	Experienced soils	*Aspergillus fumigatus*	1	0.15	0.25
F32	Experienced soils	*Aspergillus fumigatus*	1	0.15	0.15
F33	Experienced soils	*Aspergillus fumigatus*	0.5	0.25	0.5
F34	Experienced soils	*Aspergillus fumigatus*	1	0.15	0.25
F35	Experienced soils	*Aspergillus fumigatus*	1	2	1
F36	Experienced soils	*Aspergillus fumigatus*	32	16	1
F37	Experienced soils	*Aspergillus fumigatus*	0.5	0.15	0.25
F38	Experienced soils	*Aspergillus fumigatus*	0.5	0.25	0.15
F39	Experienced soils	*Aspergillus fumigatus*	1	0.25	0.25
F40	Experienced soils	*Aspergillus fumigatus*	32	8	32
F41	Experienced soils	*Aspergillus fumigatus*	1	0.15	0.15

F42	Experienced soils	*Aspergillus fumigatus*	0.5	0.15	0.15
F43	Experienced soils	*Aspergillus fumigatus*	0.5	0.06	0.06
F44	Experienced soils	*Aspergillus fumigatus*	8	8	16
F45	Experienced soils	*Aspergillus fumigatus*	1	8	0.5
F46	Experienced soils	*Aspergillus fumigatus*	0.25	0.006	0.25
F47	Experienced soils	*Aspergillus fumigatus*	0.06	0.25	0.25
F48	Experienced soils	*Aspergillus fumigatus*	0.5	0.13	0.5
C21	Clinical	*Aspergillus fumigatus*	4	4	2
C40	Clinical	*Aspergillus fumigatus*	2	1	0.13

Fungal culture and identification

One gram of soil sample was mixed with 5 ml saponin, vortexed and the debris were allowed to settle. One hundred microliters of the supernatant was transferred to 500µl of sterile normal saline and vortexed. Approximately 100µl of the suspension was cultured onto Sabouraud dextrose agar containing: (a). 0.001mg/l of itraconazole (b) 0.001mg/l of Voriconazole (c) no drug control. All the inoculated plates were incubated for 5 days at 30°C (24)

Broth dilution sensitivity testing

Aspergillus fumigatus growing on azole supplemented media were subjected to antifungal susceptibility testing against three triazoles; Posaconazole (PCZ), Voriconazole (VCZ) and Itraconazole (ITZ) using CLSI M38-A2 broth micro dilution method [25].

Sequencing of CYP 51A Gene

Aspergillus fumigatus showing high MIC to Itraconazole, Voriconazole and Posaconazole had their CYP 51A gene sequenced for detection of mutation as previously described (26).

RESULTS

A total 156 fungicide exposed soil samples were analyses, out of which 48 yielded *Aspergillus fumigatus* and 2 *A. flavus*. Antifungal susceptibility testing against three triazoles; Posaconazole, Voriconazole and Itraconazole indicates that, 3 isolates had MIC =32 µg/ml and other 2 had MIC= 4 µg/ml against Itraconazole, against Voriconazole 5 isolates had MIC =8 µg/ml, 1 isolates had MIC =16 µg/ml and against Posaconazole 1 isolates had MIC of 32 µg/ml and one had MIC = 16 µg/ml (Table 1). Two achieved clinical *Aspergillus fumigatus* from KEMRI-Mycology was used for comparison (Table 1).

Table 1: Sources of isolates and MIC against the three triazoles

Triazole Resistance level of all of the *Aspergillus fumigatus* isolates are summarized in table 2. According to Arendrup [27] and Hope [28] breakpoints, the percentage of resistance against both Itraconazole and voriconazole was 12.5% and Susceptible cases at 87.5% and 85.4% against Itraconazole and voriconazole respectively. Against Posaconazole 27.1% were resistant, 60.4% intermediates and 12.5% susceptible.

Table 2: Triazole resistance levels of the isolated *Aspergillus fumigatus*

Resistance	Itraconazole	Voriconazole	Posaconazole
Resistant	6 (12.5%)	6 (12.5%)	13 (27.1%)
Intermediates	0	1(2.08%)	29 (60.4%)
Susceptible	42 (87.5%)	41(85.4%)	6 (12.5%)
Total	48 (100%)	48(100%)	48(100%)

Three samples with high MIC were subjected to sequence analysis for the detected of mutation in CYP51A in which TR34/L98H was confirmed [Table 3].

Table 3: Sequencing analysis of CyP51A of selected *Aspergillus fumigatus*

N0.		Mutation	STRS									Sources
F36	*A. fumigatus*	TR34/L98H	14	21	8	32	9	38	8	10	18	Environmental
F40	*A. fumigatus*	TR34/L98H	14	21	8	28	9	6	8	10	18	Environmental
C21	*A. fumigatus*	TR34/L98H	14	21	8	28	9	6	8	10	18	Clinical

Discussion

In our study, we report the presence of triazole resistant *Aspergillus fumigatus* from clinical and fungicide experienced soils collected from Naivasha and Nairobi Kenya. Azole resistant *Aspergillus* spp. have be detected worldwide including; Asia, Europe, Middle East, Tanzania [20] and in Kenya, Kemoi *et al* reported prevalence of azole resistance *Aspergillus fumigatus* of between 19.23% and 36% from both naïve and experience soils. However the study didn't sequence cyp51A gene to determine the type of mutations involved [21]. The finding of this study is of great medical implications especially in African environments where resources are limited, and effective treatment and early diagnosis is a challenge. Posaconazole, Itraconazole and Voriconazole are the first line drugs used in management and prevention of aspergillosis, hence detections of

environmental isolates resistant to these triazoles poses great challenge in the medical field [28, 27].

In Kenya, Naivasha sub-County is known for extensive flower farming with extensive use of fungicides. The use of azole based fungicide in agriculture introduces antifungal pressure resulting in reduced susceptible fungi and increases azole resistant strains [32]. Detection of *A. fumigatus* with TR34/L98H mutation is the first report in Kenya. It has been reported by several authors that isolates with mutations in TR34/L98H region have cross resistance to both medical azole and azole based fungicides e.g. Propiconazole, tebuconazole, difenoconazole, bromuconazole and epoxiconazole [4, 12]. The TR34/L98H mutation in gene CYP51A involves the substitution of leucine 98 for histidine L98H and two 34-bp tandem copies in CYP51A gene at promoter region [10, 33-36].

Azole based fungicides in the environment has been linked to TR/L98H mutation in *Aspergillus fumigatus*. This form of resistance has been linked to point alteration in 220, 138 and 54 codons from patient on azole treatment [37]. Plant pathogenic molds having tandem repeat which aid in resistance against sterol demethylation inhibitor fungicides has been reported [38]. Because

Fungi including *A. fumigatus* and other plant fungal pathogens share the same habitat and they are constantly exposed to fungicide pressure. Therefore, if *Aspergillus* species harboring TR/L98H resistance is present in the environment, the conidia can be widely dispersed by wind and may cause infection in susceptible individual [9].

In Moshi, Tanzania Azole resistant *Aspergillus fumigatus* was isolated from soil known for extensive farming. It was reported that 20% of the environmental samples harbor azoles resistant *Aspergillus fumigatus*, of which 5.5% was associated with TR46/Y122FT289A mutation and 20% TR34/L98H mutation isolated from woody debris and soil samples [20]. The isolation of *Aspergillus fumigatus* with G54E mutation in

Tanzania, Romania and Indian from environmental samples is considered to occur in patients with prolonged exposure to azole [39]. The G54E mechanisms is responsible for 20.0% from India, 30.4% from Romania and 46.4% of resistant isolates from Tanzania [39].

Conclusion

There is a significant triazole resistance among environmental isolates of *Aspergillus* probably ascribed to use of irrational use of fungicide in agriculture and calls for legislative mechanism for control of fungicide use. This is a grave public health concerns given the limited resources and the limited antifungal options available for the susceptible patients.

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3. 7rd KEMRI annual scientific & health (KASH) conference (8th to 10th February 2017). Triazole resistance and sequence analysis of *Aspergillu*s species from

experienced and naïve soils in Naivasha Sub-County and Nairobi County (Abstract no. 037). (**Conference presentation**).

Appendix IX: SOPs for microscopy techniques and stains

Lactophenol cotton Blue.

For the staining and microscopic identification of fungi

Reagents	Measurements
Cotton Blue (Aniline Blue)	0.05g
Phenol crystals (C6H5O40)	20g
Glycerol	40ML
Lactic acid (CH3CHOHCOOH)	20ML
Distilled water	20ML

This stain was prepared over two days.

On the first day, dissolve the cotton Blue in the distilled water and leave overnight to eliminate insoluble dye.

On the second day, wearing gloves add the phenol crystals to the lactic acid in a glass beaker. Placed on magnetic stirred until phenol is dissolved.

Add the glycerol.

Filter the cotton blue and distilled water solution into the phenol/glycerol/lactic acid solution mix and store at room temperature.

STOCK PREPARATION

Ampicilin 100mg/ml stock

500µL ⟶ 1 L Niger seed agar

200µl ⟶ IL Trypan blue

To make 10ml stock

1g Ampicillin powder ⟶ add 5ml distilled water ⟶ Fill to 10ml with distilled water
Filter sterilize and Store in fridge.

Chloramphenicol (100mg/ml stock)

400µL ⟶ 1L Niger seed agar

200µl ⟶ 1L Trypan blue

To make 10ml stock

1g Chloramphenicol ⟶ Fill to 10ml with 100% ETOH and store in fridge if possible.

Appendix X: SOPs for media preparation

All the media used in the study were prepared in Distilled water in 1L or 500ML capped bottles, then were autoclaved for 15 minutes at 121° and 15 psi. After autoclaving is done the media is allowed to cool to approximately 55°C before specific antifungal (Itraconazole, Voriconazole and posaconazole) and 25µg/ml chloramphenicol (to inhibit bacteria) were added, then the media once ready were poured into petri dishes in a sterile hood to prevent contamination.

Sabourand's dextrose agar

Sabourand's Dextrose agar supplemented with Chloramphenicol and Gentamicin for the primary isolation and cultivation of yeast and moulds and azoles (Itraconazole, voriconazole and posaconzole) to detect the resistance of the *Aspergillus* species.

Reagents	**Measurements**
Sabouraud's Dextrose agar (oxoid cm41)	65g
Chloramphenicol	1×250mg capsule
Getamicin (40mg/ml)	0.65ml
Distilled water	1000ml
Azole	1ml/l

Soak all ingredients, except Gentamicin in 100ml water.

Boil remaining water, add to soaking ingredients, and bring to boil to dissolve stirring well to prevent from charring.

Add the Gentamicin and mix well.

Dispense for slopes it required.

Autoclave at 121ºC for 15 minutes, remove, add appropriate azoles and slopes or pour to plates as required.

RPMI 1640 PREPARATION

Weigh 10.43g of RPMI (Biochrom GMBH, Leonorenstr 2-6, D-12247, Berlin Germany) powder into of 500ml of sterile distiller water. The solution was then stirred to completely dissolve the powder. PH was adjusted by the used of sodium hydroxide to 7.0 and then stored at 4°C.

2% Gel preparation

Weigh 0.25g agarose powder in 125 ml TBE.

PCR reagents preparation

1. **Tri-acetate**

Working solution: 0.04m Tris-acetate

0.002m EDTA

Concentrated stock solution (per liters): 50×: 242g. This base 57.1ml glacial acetic acid 100ml 0.5 M EDTA (PH 8.0).

1ml Tri-acetate Dissolve 121.1g in 800ml H_2O adjust the PH by adding HCL

Desired PH

7.4---------- 70ml HCL

7.6---------- 60ml HCL

8.0---------- 42ml HCL

NB: If the 1m solution is yellow discard it.

2. **0.5M EDTA (PH 8.0)**

   Add 186.1g of disodium ethylene diamine tetraacetate.$2H_2O$ to 800ml of H_2O. Stir vigorously on a magnetic stirrer. Adjust to PH 8.0 with NaOH (220g of NaOH pellets). Dispense into aliquots and sterile by autoclaving.

   NB: Disodium salt of EDTA will not go into solution unless the PH is 8.0.

3. **10% sodium doceyl sulphate (SDS) also called sodium lauryl sulphate).**

   Preparation: Dissolve 100g of electrophoresis grade SDS in 900ml of H_2O, heat to 68°C to dissolve. Adjust the PH to 7.2 by adding HCL and adjust the volume to 1 litre and dispense into aliquots.

4. **Ethidium bromide (10mg/ml)**

   Preparation: Add 1g of ethidium bromide to 100ml of H_2O. Stir on a magnetic stirred for several hours. Wrap the container in alluminium foil or dark bottle and store at 4°C.

5. **Phosphate buffered saline (PBS)**

NaCl	4.0g
KH_2PO4	0.17g
K_2HPO2	0.6g

All of the above reagents were mixed and dissolved in half a litre distilled water. The PH of the solution was adjusted to the required 7.3 USING HCL or NaOH then, autoclave for 20 minutes at 121℃ and 15psi.

6. **Phospahate buffered saline (PBS) (0.05% tween 80.**

Exactly 500ml of PBS buffer were measured and warm 0.25 ml pf tween 80 added to PBS. The PH was then adjusted using HCL or NaOH to 7.3. Phosphate buffered saline tween 80 was then autoclaves for 20 minutes at 121℃ and 15 psi.

7. **TBE buffer**

EDTA	7.4g
Boric acid	55g
Tris-base	108g

All the above ingredients were added onto 1L of sterile distilled water

8. **DNA extraction buffer**

mMTris-HCL	1.20g
1.4M NaCL	8.12g
10mM EDTA	0.37g
2% CTAB	2.00g

The above reagents were dissolve in 100ml of DH2O, PH adjusted to 8.0 and then autoclaved at 121℃ for 20 minutes.

Appendix XI: Informed consent form

Title of the study: **TRIAZOLE RESISTANCE AND SEQUENCE ANALYSIS OF *ASPERGILLUS* SPECIES FROM FUNGICIDE NAÏVE AND EXPERIENCED SOIL FROM NAIVASHA SUB COUNTY AND NAIROBI COUNTY.**

Institutions and Investigators: The study will be led by Edson K. Kemoi at KEMRI. His contacts are mobile 0721759385. The rest of the study team include

NAME	INSTITUTION	UNIT
Dr. Christine Bii	KEMRI	CMR
Dr. Andrew Nyerere	JKUAT	Department of Medical Microbiology

Introduction: Before you decide if you wish to be in this study, you need to know about any things that could happen if you decide to join; good or bad. This form tells you about the study. You can ask any questions you have at any time.

Being in the study is your choice: This consent form gives you information about the study and the risks. These will be explained to you. Once you understand the study, and if you agree to take part, you will be asked to sign your name on this form. Before you learn about the study, it is important that you know the following:

- Your participation in this study is entirely voluntary
- You may decide not to answer questions or withdraw from the study at any time

Human Participants Involvement: There will be no direct human or human participation in the study but only environmental samples consisting of soils from agricultural fields and non-agricultural fields. Approved permission to access the fields, park and farms will obtained from the relevant institutions such as Kenya Wildlife Services or flower farms owners or individual farm owners.

Potential Benefits: There is no direct benefit to park, farm or field owners. However, the data from this study will contribute to policies on rational use of fungicides in agriculture and data on the current rate of triazole resistance in clinical practice.

Potential Risks/Protection against Risk: Privacy and Confidentiality:We will endeavor to maintain both privacy and confidentiality in all our interactions with farm and field owners. Field visits will be conducted by an experienced local Kenyan scientist. Soil samples will be coded with laboratory number without reverence to the farm or land owner.

Data on azole resistance will be compared with that of isolates from clinical sources. The isolates are coded and culture collection at the mycology laboratory from previous studies.

Biosafety Issues: All environmental samples will be transported in unbreakable containers in plastic bags. All procedures of isolation, culture, identification, storage and dispose of fungal cultures will be performed by experienced mycologists. All the procedures will be done under level II containment facility in the Mycology Laboratory, CMR. The disposal of bio hazardous material will be done according to KEMRI waste management guidelines.

Potential biases and mitigation measures in the study: Although soil from virgin land will form fungicide naïve site, although human activities in this area may transport fungal spores in their shoes from fungicide experienced farms. Fungal spores are also known to be wind dispersed over long distance.

Outputs/ Outcomes and expected application of research results: The data generated will contribute to the current knowledge of status of triazole resistance and establish whether fungicide use in agriculture contributes to antifungal resistance in clinical practices. This will serve to educate on the rationale use of fungicides in agricultural

practices to contain emerging resistance in clinical practice. It is also expected that the study will be useful for training of graduate and post graduate students in mycological procedures. The data generated will be available for used by a student at ITROMID for the post graduate thesis

The data will be disseminated through scientific presentation in national, regional and international. Preference will be given to local meetings such as KASH where the findings are expected to have a wider impact.

Costs to you: There is no cost to you for participating in this study.

Consent: I hereby seek your permission as a farmer to collect soils samples from your farms and to ship isolates to Germany.

Problems and questions: If you ever have questions about this study, you should contact the Principal Researcher, Dr. Christine Bii at Mobile 0721759385.

Your rights as a study participant: This research has been reviewed and approved by the Ethical Review Committee of the Kenya Medical Research Institute.

If you have any questions about your rights as a research participant you may contact the secretary of the Kenya Medical Research Institute Ethical review committee (a team of professionals who review the research to protect your rights) at 020-2722541, 020-2726781, 0722 205 901 or 0733 400 003.

Your statement of consent and signature: If you have read the informed consent, or had it read and explained to you, and you understand the information and voluntarily agree to join this study, please carefully read the statements below and think about your choice before signing your name or making your mark below. No matter what you decide, it will not affect anything:

- I have been given the chance to ask any questions I may have and I am content with the answers to all of my questions.
- I know that my records will be kept confidential and that I may leave his study at any time.
- I have been told the name, phone number and address of the person to contact in case of an emergency, and this information has also been given to me in writing.
- I agree to take part in this study as a volunteer, and will be give a copy of this informed consent form to keep.

_____

Participant name Participant signature and date

_____ _____

Name of Researcher Signature and date

_____

Name of witness as appropriate Witness's signature and date

NOTE: You are not giving up any legal rights by signing this informed consent document

Appendix XII: Fomu ya Ridhaa

Jina la uchunguzi: **UPINZANI WA AINA YA *ASPERGILLUS* KWA DAWA YATRIAZOLENA UCHAMBUZI WA VIINI VYAKE KUTOKA KWA UDONGO USIOZOEA DAWA YA FUNGICIDE NA UDONGO ULIOZOEA DAWA HII KUTOKA JIMBO NDOGO LA NAIVASHA NA JIMBO LA NAIROBI .**

Taasisi naWakaguzi: Utafiti utakuwa ikiongozwa na Edson K. Kemoi katika idara ya KEMRI. Mawasiliano yake ya simu ya rununu 0721759385.

Wengine katika timu ya utafiti ni pamoja na:

JINA	**SHULE**	**IDARA**
DR. Christine Bii	KEMRI	CMR
DR Andrew Nyerere	JKUAT	Kitivo cha mikrobiologia

Utangulizi: Kabla ya kuamua kama unataka kuwa katika utafiti huu, unahita jikujua mambo yoyote ambayo yanaweza kutokea kama umeamua kujiunga; mema au mabaya. Fomu hii atakwambia kuhusu utafiti huo. Unaweza kuuliza maswali yoyote unayo wakati wowote. Kuwa katika utafiti niuchaguzi wako: Fomu hii yaridhaa inatoa taarifa kuhusu utafiti na hatari. Utaelezwa mambo haya. Mara tu utakapoelewa somo hili, na kama wewe utakubali kushiriki, utatakiwa kutia sahihi na jina lako katika fomu hii. Kabla yakujifunza kuhusu utafiti huo, ni muhimu kwamba unajua yafuatayo:

• ushiriki wako katika utafiti huu nihiari kabisa.

• Unaweza kuamua kutojibu maswali au kuondoka kutoka katika utafiti wakati wowote.

Binadamu Washiriki Ushirikishwaji: Hakutakuwa n ushirikiano wowote wa moja kwa moja na binadamu lakini sampuli za mazingira tu za udongo kutoka Nyanja za kilimo na mashamba yasiyo ya kilimo. Kibali cha kuzuruviwanja, mbuga za wanyama na

mashamba kitapatika na kutoka taasisi husika kama vile Idara ya wanyama pori ya Kenya, wakulima wa maua, au wamiliki binafsi wa mashamba.

Faida za moja kwa moja kwa washiriki: Hakuna faida moja kwa moja kwa wenyembuga, shamba au wamiliki wa mashamba. Hata hivyo, takwimu kutoka utafiti huu itachangia sera za matumizi bora ya madawa (fungicides) katika kilimo na takwimu kuhusu kiwango cha sasa cha wadudu kuzuia makali ya dawa ya triazole katika utatifiti wa kliniki.

Hatari iloyoko Kinga dhidi ya Hatari: Faragha/Usiri na uaminifu: Tutajitahidiili kudumisha faragha/usiri na uaminifu katika uhusiano wetu wote na wamiliki wa vilimo na shamba.Ziara ya kuzuru Nyanja itafanywa na wana sayansiwa Kenya waliobombea. Sampuli za udongo zitapewa nambari ya maabara bila kuzigatia mmiliki wa shamba au ardhi. Takwimu za wadudu kuzuia makali ya azoleita kuwa ikilinganishwa na ileya chembechembe kutoka vlini kihusika. Chembechembe zitapewa nambari na kutole wakati ka maabara ya mikologia ambapoutafiki mwingine wa haina hii ulifanyiwa.

Masuala ya usalama wa viumbe: Sampuli zote za mazingira zitasafirishwa katika vyombo madhubuti katika mifuko ya plastiki. Taratibu zote za kupima, kupanda, kutambua, kuhifadhi na kuondoa vimeleautafanywana mwanamikologia mwenye uzoefu. Taratibu zote zita fanyika chini ya kiwango chapili (II) cha maabara ya Maikolojia, CMR. Utupajiwataka namadhara nyenzo utafanyika kwa mujibu wa miongozo ya usimamizi wa taka wa KEMRI.

Uwezo wa kutokuwa na usawanahatuaza kukabiliana katika utafiti: Ingawa udongo kutoka shamba lisilotumika utakuwa kama tovuti ya dawa isiyokuwa na madhara yoyote, ingawa shughuli za binadamu katika eneo hili zinaweza kusafirisha chepechepe na vimelea katika viatu yao kutoka mashamba yenye uzoefu wa kutumia madawa (fungicide). Chepechepe na vimelea pia vinajulikana kwa kutawanywa na upepo zaidi ya umbali mrefu.

Matokeo na manufaa yanayotarajiwa kutokana na utafiti: Takwimu itakayopatikana itachangia ujuzi wa kisasa wa hali ya wadudu kuzuia makali ya dawa (triazole) na kuthibitisha kama matumizi ya dawa (fungicide) katika kilimo inachangia katika jambo hili kulingana na takwaimu zingine zilizoko.Hii itatumika kuelimisha wakulima juu ya matumizi mantiki wa dawa (fungicides) katika mbinu za kilimo na kuzuia kujitokeza kwa wadudu kupingana na madawa haya. Pia inatarajiwa kuwa utafiti utakuwa muhimu kwa ajili ya mafunzo ya kuhitimuna viwango zaidi ya masomo katika mikologia. Deta itakayotokana na utafiti huu itakuwa inapatikana kutumiwa na mwanafunzi katika ITROMID kwa ajili waliohitimu masosmo yao Deta itasambazwa kupitia kuwasilisha katika mikutano mikuu ya kisayans ikatika nchi, kikanda na kimataifa. Matokeo ya Utafiti yatapewa kipaombele kwa mikutano ya ndani kama vile KASH ambapo matokeo wanatarajiwa kuwa na athari kuu.

Gharama kwako: Hakuna gharama yoyote kwa wataosajili ya kushiriki katika utafiti huu.

Ridhaa: Kwa hivyo nina tafuta idhini yako kama mkulima kukusanya sampuli ya udongo kutoka mashamba yako na kuisafirisha na meli Ujerumani.

Matatizo na maswali: Kama utakuwa ana maswali kuhusu utafiti huu, unapaswa kuwasiliana na Mtafiti Mkuu, Daktari Christine Bii kupitia nambari ya rununu 0721759385.

Haki zako kama mshirika wa utafiti: Utafiti huu umechambuli wanakupitishwa na kamati ya Uchunguzi wakimaadili Taasisi ya Utafiti ya KEMRI. Kama una maswali yoyote kuhusu haki zako kama mshiriki utafiti unaweza kuwasiliana na katibu waTaasisi yaUtafitiya KEMRI kamati ya kimaadili (timu ya wataalamu ambao kupitia utafiti ilikulinda haki yako) katika nambari ya simu 020-2722541, 020-2726781, 0722 205 901 au 0733 400 003.

Kauli yako yaridhaa na saini: Kama umesoma ridhaa hii, au ulikuwa umesomewa na kuelezwa habari na hiari kukubali kujiunga na utafiti huu, tafadhali soma kwa uangalifu maelezo hapa chini na kufikiri juu ya uchaguzi wako kabla y akutia sahihi jina lako au kutia alama yako hapa chini. Uchaguzi wako hautakuathiri kwa njia yoyote ile.•

Nimepewa nafasi ya kuuliza maswali yoyote ambayo nahitaji majibu nanimeridhika na majibu ya maswali yote yangu.

• Najua kwambare kodi yangu itakuwasiri na ninaweza kuondoka katika utafiti huu wakati wowote.

• Nimeambiwa majina na, namba ya simu naanuani yakuwasiliana katika jambo la dharura, na habari hii pia nilipewa kwa maandishi.

•Nakubaliana kushiriki katika utafiti huu kama kujitolea, nanitapewaa nakala ya fomu hii ya idhini kuweka.

_____	_____
Jina la mshiriki	Sahihi na tarehe
_____	_____
Jina la Mtafiti	Sahihi na tarehe
_____	_____
Jina la shahidi	Sahihi na tarehe

NOTE: Wewe sikutoa juu ya haki za kisheria kwa kusaini hii ridhaahati

Appendix XII: Cheti cha tafsiri

CHETI CHA TAFSIRI

Mimi.....nimetafsiri fomu hii ya ridhaa kutoka lugha ya Kiingereza hadi lugha ya Kiswahili na baadaye kuitafsiri kwa lugha ya Kiingereza, bila kutumia nguvu yoyote na kwa kadri ya ufahamu wangu.

_____ _____

Jina la mtafsiri Tarehe na sahihi

_____ _____

Jina la shahidi Tarehe na sahihi

Appendix XIV: Translation certificate

TRANSLATION CERTIFICATE

I ……. Have translated the consent form from English to Kiswahili and back translated to English, without any coercion and to the best of my knowledge.

_____	_____
Translator name	Date and sign
_____	_____
Witness Name	Date and sign

Appendix XV: Ethical approval I

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 **May 28, 2015**

TO: **KEMOI EDSON KIPYEGON,**
PRINCIPAL INVESTIGATOR

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

Dear Sir,

RE: PROTOCOL NO. KEMRI/SERU/CMR/P000602/3031(*RESUBMITTED INITIAL SUBMISSION*): TRIAZOLE RESISTANCE AND SEQUENCE ANALYSIS OF ASPERGILLUS SPECIES FROM FUNGICIDE NAÏVE AND EXPERIENCED SOILS IN NAIVASHA SUB-COUNTY AND NAIROBI COUNTY

Reference is made to your letter dated 21st May, 2015. KEMRI/Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study documents on May 26, 2015.

This is to inform you that the Committee notes that the issues raised during the 238th SERU meeting of the SERU held on 22nd April, 2015 have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day, **28th May, 2015** for a period of one year. Please note that authorization to conduct this study will automatically expire on **May 27, 2016.** If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **April 15, 2016.**

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,

PROF. ELIZABETH BUKUSI,
ACTING HEAD,
KEMRI/SCIENTIFIC AND ETHICS REVIEW UNIT

Appendix XVI: Ethical approval II (Renewal)

KENYA MEDICAL RESEARCH INSTITUTE

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

KEMRI/RES/7/3/1 **May 11, 2016**

TO: **KEMOI EDSON KIPYEGON,**
PRINCIPAL INVESTIGATOR

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

forwarded 16/5/2016

Dear Sir,

RE: **KEMRI/SERU/CMR/P000602/3031 - (*REQUEST FOR ANNUAL RENEWAL*): TRIAZOLE RESISTANCE AND SEQUENCE ANALYSIS OF ASPERGILLUS SPECIES FROM FUNGICIDE NAÏVE AND EXPERIENCED SOILS IN NAIVASHA SUB-COUNTY AND NAIROBI COUNTY**

Thank you for the continuing review report for the period **28th May 2015** to **1st April 2016.**

This is to inform that during the 251st Committee A meeting of the KEMRI/Scientific and Ethics Review Unit (SERU) held on the **10th May 2016**, the Committee **conducted the annual review and approved** the above referenced application for another year.

This approval is valid from **May 27, 2016** through to **May 26, 2017**. Please note that authorization to conduct this study will automatically expire on **May 26, 2017**. If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to SERU by **April 14, 2017.**

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

You may continue with the study.

Yours faithfully,

pp

DR. EVANS AMUKOYE,
ACTING HEAD,
KEMRI/SCIENTIFIC AND ETHICS REVIEW UNIT

Appendix XVII: Proposal approval

JOMO KENYATTA UNIVERSITY
OF
AGRICULTURE AND TECHNOLOGY
DIRECTOR, BOARD OF POSTGRADUATE STUDIES

P.O. BOX 62000
NAIROBI – 00200
KENYA
Email: director@bps.jkuat.ac.ke

TEL: 254-067-52711/52181-4
FAX: 254-067-52164/52030

REF: JKU/2/11/TM416-2144/2014

02ND AUGUST 2016

Kemoi, Edson Kipyegon
C/o SOBMS
JKUAT

Dear Mr. Kipyegon,

RE: APPROVAL OF Ph.D. RESEARCH PROPOSAL AND OF SUPERVISORS

Kindly note that your Ph.D. research proposal entitled: **"TRIAZOLE RESISTANCE AND SEQUENCE ANALYSIS OF *ASPERGILLUS* SPECIES FROM FUNGICIDE NAÏVE AND EXPERIENCED SOILS IN NAVASHA SUB COOUNTY AND NAIROBI COUNTY"** has been approved. The following are your approved supervisors:-

1. Dr. Christine Bii
2. Dr. Andrew Nyerere

PROF. MATHEW KINYANJUI
DIRECTOR, BOARD OF POSTGRADUATE STUDIES
Copy to: Dean, SOBMS

/cm

Appendix XVIII: Intent to submit (thesis) approval

JOMO KENYATTA UNIVERSITY
OF
AGRICULTURE AND TECHNOLOGY
DIRECTOR, BOARD OF POSTGRADUATE STUDIES

P.O. BOX 62000
NAIROBI – 00200
KENYA
Email: director@bps.jkuat.ac.ke
TEL: 254-067-52711/52181-4
FAX: 254-067-52164/52030

REF: BPS/ AG422-1405/2015 **6TH July 2018**

EDSON KIPYEGON KEMOI
C/o SOBMS
JKUAT

Dear , Kemoi

RE: APPROVAL OF YOUR INTENT TO SUBMIT PhD. THESIS FOR EXAMINATION

We are in receipt of your letter of intent to submit your PhD. thesis for examination.

This is to inform you that your request has been approved. It is a requirement that you clear with all the relevant departments/sections of the University and forward the duly completed Clearance Form to the BPS office to enable us processes your thesis for examination.

The Clearance Form is obtainable from the Office of the Director, Board of Postgraduate Studies.

Yours sincerely

PROF. MATHEW KINYANJUI
DIRECTOR, BOARD OF POSTGRADUATE STUDIES

Copy to: Dean, **SOBMS**

Appendix XIX: Environmental collection of soil questionnaire

Supplies: Digital camera, GPS unit, Temperature/ humidity gauge, Landscape characterization sheets, sterile plastic spoons, Sharpie, Pencil, Tree swabs and Ziplock bags

Procedure:

Record the GPS coordinates on landscape characterization sheet: ……..…..

Take digital photographs of the in general and the sampled material and / or area: ……

Fill out landscape characterization: ……..

Sample the material of interest i.e. soil: ……

After daily sample collection is complete store samples in cool box: …….

Ship samples to Nielsen laboratory on cool box for subsequent culture: ……

LANDSCAPE CHARACTERIZATION SHEET

Sample ID: …… Date: …….. Time: ……

GPS coordinates: …….. Latitude: ……. Longitude: ……..

Waypoint ID: ……. Temperature: ……. Humidity: …….

Picture description:

……

……

…….

Site of soil collections

Agricultural soil: …… Virgin land soils: …… Other: ……

History of fungicide uses

Yes: …….. No: ……

Name of the fungicides commonly used: ……

How often the farmer used the fungicides: …….

Additional sample description:

……

……

……

Appendix XX: Common azole based fungicides used in Agriculture

Sn no.	Chemical compound
1.	Propiconazole
2.	Mancozeb
3.	Chlorothalonil
4.	Hexaconazole
5.	Tebuconazole
6.	Difenoconazole
7.	Tricyclazole
8.	Probenazole
9.	Fluopicolide
10.	Trihydroxide
11.	Propine B

Appendix XXI: *Aspergillus flavus* MALDI TOF MS spectrum