ISOLATION AND CHARACTERIZATION OF YEAST FROM *Gallus gallus* DROPPINGS IN KABIGERIET VILLAGE, OLENGURUONE

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I56/12219/09

A research thesis submitted in partial fulfillment of the requirements for the award of the degree of Master of Science (microbiology) in the school of pure and applied sciences, Kenyatta University

October, 2012

DECLARATION

I declare that the work presented in this thesis is my original work and has not been presented for the award of a degree in any other university or any other award.

Kemoi Edson Kipyegon

Signature…….. …….Date……

We confirm that the work reported in this thesis was carried out by the candidate under our supervision.

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DEDICATION

To my loving, mum Ann C. Cheruiyot and sister, Hellen Chepng'etich.

ACKNOWLEDGEMENTS

I express my sincere gratitude to my supervisors: Prof. Paul Okemo and Dr. Christine Bii for their professional guidance offered tirelessly in supervising the project from its inception to its completion. Thanks also go to Dr. Charles Mutai KEMRI for his advices which lead to inception of this project.

Lastly my heartfelt gratitude and thanks are to all my friends and relatives for their patience, support and encouragement during the preparation of this thesis. It is impossible to mention you all by name as I would want, but to everybody else who contributed both materially and morally in one way or the other, towards the achievements made in this study thanks a lot. God bless you all.

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ABBREVIATIONS AND ACRONYMS

AIDS……. Acquired Immunodeficiency syndrome

API…… Analytical profile index

ATCC…… American Type Culture Collection

C…… *Candida*

CDC…….Center for Diseases Control

CHROM agar……Chromogenic agar

DNA……Deoxyribonucleic acid

HIV……... Human Immunodeficiency Virus

KEMRI ……. Kenya Medical Research Institute

LCB…….. ..Lacto phenol Cotton Blue

MLEE……..Multilocus Enzymes Electrophoresis

NCCLS…….. National Control of Clinical Laboratory Standards

NIH…….....National Institute of Health

SDA…… Sabouraud's Dextrose Agar

SDS PAGE……. Sodium dodecyl polyacrylamide gel eletrophoresis

SOPS…….. Standard Operation Procedures

SPP……. Species

SPSS …….. Statistical Package for the Social Sciences

USA…….United States of America

WHO……World Health Organization

ABSTRACT

The Avian family has been a significant source of human epidemic. Over the last three decades, reports on yeast infections in humans have increased especially with respect to immunocompromised individuals. This is associated with increased morbidity and mortality especially in HIV/AIDS immunocompromised individuals. The purpose of this study was to isolate and characterize pathogenic yeasts from domestic Chicken (*Gallus gallus*) dropping. The droppings were collected from Kabigeriet Villages, Olenguruone Division, Kuresoi District and Nakuru County. The samples were collected from cages, houses and roosting sites. The samples (droppings and soil) were collected by swabbing or scooping fresh dropping from Chicken houses, grass, soil and trees using sterile plastic spoons, labeled and inserted in a zip lock safety bag. A total of Eighty four samples (dropping and soil enriched with chicken droppings) were sampled during the study. The samples were transported to the lab using a cool box. Processing of the samples was done at the Mycology laboratory, Centre for Microbiology Research, Kenya medical Research Institute. The samples were plated onto Typan blue agar and incubated at 37°C for two weeks checking was done daily. The isolates were identified according to morphological and biochemical characteristics. The droppings were tested for *Cryptococcus* by direct plating on Niger seed while *Candida* and *Saccharomyces* species by direct plating on Typan blue agar. *Candida* and *Saccharomyces* species were sub cultured on CHROM agar and Corn meal agar for presumptive identification of various *Candida* species. *Cryptococcus neoformans* were sub cultured onto Christensen's urease agar. *Geotrichum* species were presumptively identified by lactophenol cotton blue. Analytical profile index test (API 20C AUX) was used for confirmation. Four types of yeasts were isolates; 35(57.4%) *Candida* species (9 *Candida lusitanie*, 7 *Candida glabrata*, 5 *Candida albicans*, 5 *Candida tropicalis*, 3 *Candida parapsilosis*, 2 *Candida lipolytica* and 2 *Candida krusei*), 23(37.7%) *Geotrichum candidum*, 2(3.3%) *Cryptococcus* species (*Cryptococcus neoformans* and *Cryptococcus laurenti*) and 1(1.6%) *Saccharomyces cerevisiae* were isolated from Chickens dropping sampled. The results of this work demonstrated that domestic chicken (*Gallus gallus*) harbor *Cryptococcus* in their dropping and their close proximity to human habitation poses a risk of cryptococcal infection in HIV/AIDS immunocompromised persons. This could partly explain the high incidence of cryptococcosis in HIV/AIDS patients in Kenya.

CHAPTER ONE

1. INTRODUCTION

1.1 Background

The Chicken (*Gallus gallus*) is a domesticated fowl, which is a subspecies of the Red Jungle fowl. It is one of the most widespread and the most common domesticated birds. In 2003 the total population was more than 24 billion worldwide (Perrins and Christopher, 2003) and out of this population, Chickens were the majority compared to any other species of birds. Chicken is kept by humans primarily as a source of food i.e. meat and eggs. Human beings can acquire diseases from domestic Chickens in two ways. The first is getting in contact with sick Chicken or faeces of the sick Chicken, usually by a veterinarian or a caretaker. The other way is for disease causing pathogens to reside in sick Chicken/eggs. When an individual eats these eggs, then she/he can also get infected. In particular pathogen like fungi, bacteria, protozoa, chlamydial or viral agents are of great concern to human health (htt://www.ehow.com/recipes). This study was designed to identify pathogenic yeasts harbored by Domestic Chicken dropping such as *Candida albicans, Cryptococcus, Blastomyces, Histoplasma* etc.

1.2 *Candida* species

In the genus *Candida,* most species exist as commensals in most healthy individuals (Ramage *et al*., 2001a, Sullivan *et al*., 2004). Pathogenic *Candida* species is a growing problem in medical Science (Calderonne, 2002). *Candida albicans* is the most common

species causing human infections (Vazquez *et al*., 2002), however emergence of non-*albicans* species such as *C. krusei, C. parapsilosis, C. tropicalis* and *C. glabrata* has been reported in the last decade as human pathogens, mainly among immuno-suppressed individuals and hospitalized patients (Brawner *et al.,* 2002, Fleming *et al.,* 2002, Sanchez *et al.,* 2005a, Resende *et al.,* 2004). In United States the proportion of fungal infection in comparisons to all nasocomial infections doubled over a period of ten years (Calderonne, 2002). The increased reporting of non-*albican Candida* species could be due to increased recognition in the laboratory (Sanchez *et al.,* 2005b). Candidiasis presents a growing challenge for patients who are immunocompromised due to HIV/AIDS, organ transplant, cancer and other risk factors. This has increased the emergence of other *Candida* species as opportunistic pathogens (Calderonne, 2002, Gutierrez *et al.,* 2002, Vazquez *et al.,* 2002). *C. dubliniensis* has been associated mainly with oral candidiasis in HIV positive individuals worldwide. However in HIV negative individuals it has been reported to cause systemic and superficial diseases with prevalence rate of less than 5% (Gutierrez *et al*., 2002, Jewtuchowicz *et al*., 2008). Over the last decade *C. dubliniensis* has been misidentified as *C. albicans* due to phenotypic similarity between the two species of *Candida* (Ramage *et al*., 2001b).

Mainly the mode of transmission and the reservoir for *Candida* species are important in development of prevention and control measures of resistant strains (Ptailer *et al.,* 2001). In immunocopromised individuals, large proportions of fungal infections are caused by *Candida* species: *Candida* species are found in 40-65% of feaces from healthy individuals as normal flora, and oropharyngeal colonization in approximately 30-55% of healthy adults (Colombo *et al*., 2006), causing different infections, such as candiduria

(Da Silva *et al*., 2007) candidemia (Colombo *et al*., 2006) and Oropharyngeal candidiasis (Costa *et al*., 2006). Oropharyngeal candidiasis is developed by more than 90% of individual infected with HIV and is not on HAART (highly active antiretroviral therapy) (Repentigny *et al*., 2004). Isolation of *Candida* species in oral cavity does not indicate infection in the absence of clinical correlation. Dorsal tongue and buccal mucosa are the areas heavily colonizer by *Candida* species (Vazquez and Sobel, 2002). Candida carriage occurs in up to 78% of hospitalized elderly, 65% of denture wearers and more than 90% of patients with HIV receiving antibiotic therapy (Vazquez and Sobel, 2002, Akpan and Morgan, 2002).

1.3 *Cryptococcus* species

Cryptococcosis is an opportunistic disease mainly caused by an encapsulated fungus; *Cryptococcus neoformans* (Burker, 2001). *Cryptococcus neoformans* is a basidiomycete. In Sub-Saharan Africa, adult meningitis is the most common complication of cryptococcossis, mainly caused by *Cryptococcus neoformans* due to high prevalence of HIV. *C. neoformans* has been reported to cause infections in persons infected with human immunodeficiency virus worldwide, while *C. gattii* causes infection primarily in HIV-uninfected persons both in tropical and subtropical countries (Dixit *et al*., 2009).

Biochemical and genetic studies have established the existence of the two main varieties of *C. neoformans*. *Cryptococcus neoformans* var. n*eoformans* includes serotypes A and D, while *C. neoformans* var. *gatt*i includes serotypes B and C (Monica, 2004) and the new species *Cryptococcus bacillispora* var. *gatti* (Barreto *et al*., 2004). Due to AIDS

pandemic Cryptococcossis has emerged as the major cause of death in HIV/AIDS infected individual (Grusse *et al.,* 2001). *Cryptococcus neoformans* causes cryptococcal meningitis in patients with AIDS which is lethal. In Thailand the incidence of cryptococcal meningitis has been reported to be at 18.5% in individuals suffering from HIV AIDS (Suwat *et al*., 2001), while in USA the annual incidence is 2-4 cases per 1,000 persons (http://.rightdiagnosis.com/artic/cryptococcossis_dbmd.htm). *C. neoformans* var. *gattii* was first isolated from *Guettarda aereana* tree in Brazilian Amazon rainforest (Fortes *et al.,* 2001). Cryptococcosis is one of the most common lethal infections in patients with AIDS worldwide (Lakshimi *et al*., 2007). The habitats of this yeast remain unknown and the present study is aimed at investigating the occurrence of this yeast in domestic Chicken droppings. Kenya being one of the countries affected by AIDS it's important to investigate the habitat of Cryptococcus.

1.4 *Blastomyces dermatitidis*

Blastomyces dermatitidis is a dimorphic fungus that is pathogenic to humans and other mammals, causing Blastomycosis a chronic granulomatous mycosis. *Blastomyces dermatitis* causes severe infection and substantial mortality in immunosuppresed individual by acquired malignancy and immunodeficiency (Gauthier *et al*., 2007, Bradsher *et al*., 2003). The cumulative incidence of post transplant blastomycosis in USA has been reported to be 0.14% in the year 2000 to 2004 (Gauthier *et al*., 2007). In the environment, *B. dermatitidis* is a mould and produces conidia (microscopic spores). Humans can inhale the conidia into the lungs. When temperatures are high conidia turn

into budding yeasts cells. Blastomycosis is a self-resolving pulmonary disease, but in immunosupressed conditions may disseminate to other organs of the body, which is fatal (*Kurt et al*., 2008). The annual incidence rate for Blastomycosis is approximately 1 in 100,000 of the reported cases in USA (Census Bureau's, 2004, Tracy *et al*., 2002). Ecological niche of *B. dermatitidis* in Wisconsin has been reported to be in association with waterways in northeastern, northern and southeastern regions of the states in urban and rural environments (Reed *et al*., 2008, Baumgardner *et al*., 2006) and also the fungus has been isolated in soil enriched with compost piles and nasal cavities of dogs infected with Blastomycosis (John *et al*., 2011, Varan *et al*., 2009).

1.5 *Histoplasma Capsulatum*

Histoplasma capsulatum causes histoplasmosis which is one of the most common mycoses (endemic) in patients living with HIV/AIDS (Wheat *et al*., 2000). The mode of infection for histoplasmosis is inhalation of conidia and spores (Richardson and Warnock, 2002). In the HIV AIDS era Histoplasmosis has emerged as the most important opportunistic infection among HIV/AIDS patients with CD4 <75 cells/µl. Clinical manifestations of histoplasmosis ranges from asymptomatic mild illness in immunocompetent individuals to disseminated infection in immunocompromised individuals (Richardson and Warnock, 2002). Dissemination of histoplasmosis from pulmonary infection to other organs in the body, occur mainly in advanced stages of HIV/AIDS which is common also in non-immunosupressed individuals. Singhi *et al*., (2003) reported a case of immunocompetent female from North-West Rajastain, India

with disseminated cutaneous histoplasmosis successfully treated with intraconazole. In United States *Histoplasma capsulatum var. capsulatum* have been reported to affect primarily citizens living in Latin America and those living in Ohio valley and Mississippi river while *H. capsulatum var. duboisii* has been reported only in Africa (Wheat *et al*., 2000). Population studies conducted in Ohio Valley and Mississippi valley showed >80% of young adults from the two regions have been infected previously with *H. capsulatum* (Kauffman, 2007). In the USA, almost all citizens living in Midwest are infected with histoplasmosis. Data shows that each year 500,000 people in US are infected with histoplasmosis, however only 5 out of 100 infected individuals developed symptoms of the diseases (http://wwwrightdiagnosis.com/artic.histoplasmosis_dbmd.htm). Endemic distribution of *Histoplasma* variants has be studied and founded out to be due to the difference in environmental conditions from one location to the other such as soil, humidity and climate characteristics (Wheat *et al*., 2000). Activities like farming that disturb soil are associated with *H. capsulatum* exposure. Spores can be carried in the air for several miles, exposing individuals without contact with soil at risk (Wheat *et al*., 2000).

1.6 Problem statement and justification

The study was carried out in Kabigeriet village, Olenguruone because it represents a typical rural setting where domestic Chickens are reared in close proximity with human. In some homesteads human beings share the same house with Chicken hence they are at a risk of contracting infections from Chicken. The Avian family has been a significant source of human epidemics including the recent deadly avian flu. *Gallus gallus* also has been the source or reservoir of some enteric pathogens including drug resistant *E. coli* (Adelaide *et al*., 2009). This raises the question of whether other potentially pathogenic strains are harbored by the birds. The close proximity to human habitation is a significant risk factor for acquisition of potential pathogens. Due to economic hardships and security concerns, humans and Chicken sometimes share the same room. The close contact with humans is a significant risk factor especially in those with debilitating conditions such as HIV/AIDS, cancer. In Western countries pathogenic yeasts such as *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Saccharomyces cerevisiae*, *Geotrichum candidum* and *Candida* species have been isolated in different birds' species droppings such as pigeons. In Thailand, Phayao province, isolation of *Cryptococcus neoformans* was done on Chicken dropping (Kuroki *et al*., 2004) hence there is need to study domestic Chicken droppings in Kenya if they also harbor pathogenic yeasts, because Chicken is largely kept in Kenyan homesteads.

1.7 Hypothesis

Null hypothesis: *Gallus gallus* do not harbor *Cryptococcus* spp. and other potentially pathogenic yeasts in their droppings.

Alternative hypothesis: *Gallus gallus* harbors *Cryptococcus* spp. and other potentially pathogenic yeasts in their droppings.

1.8.1 General objectives:

To isolate, characterize and determine the spectrum of potentially pathogenic yeasts from domestic Chicken droppings in Kabigeriet village, Olenguruone Division, Kuresoi District.

1.8.2 Specific objectives:

1. To isolate, identify and characterize *Cryptococcus* and *Candida* species from domestic Chicken dropping.
2. To isolate and identify the spectrum of other yeasts from domestic Chicken dropping.
3. To determine the relation between the occurrence of yeasts and humidity.
4. To determine the relation between the occurrence of yeasts and temperature.
5. To determine the relation between the occurrence of yeasts and elevation.

CHAPTER TWO

2. LITERATURE REVIEW

2.1 Infections associated with Chicken

Bird-keepers (pet bird and poultry owners) should be aware that some avian diseases can be transmitted to humans (http://www.idph.state.il.us/public/hb/hbb&bdrp.htm). The infectious agents can be protozoal, fungal, bacterial, chlamydial or viral. Individual susceptibility and the seriousness of these various microbial infections vary with age, health status, immune status (immunodeficient or immunosupressed), and whether early therapeutic intervention is sought. The ability of a micro-organism to make a person sick varies with the virulence of the organism, the dose to which the person is exposed, as well as route of infection. Chlamydiosis, Salmonellosis, Arizonosis, Histoplasmosis, Blastomycosis, Candidiasis, Cryptococcosis and Colibacillosis are the most common of these infections (Valerie and Demel, 2010, htt://www.bird-x.com). Avian influenza a viral disease of birds, which is also transmitted to humans, is a highly pathogenic virus whose death rate can be as high as 100% in some birds. Human infections can be acquired through direct or indirect contact with live or dead birds and also from contaminated environments. The H5N1 influenza virus is the most pandemic and is spread widely in birds and humans. Most human beings have no immunity to H5N1 (WHO, 2005, Peiris *et al*., 2004). It has been reported that birds' droppings, the sources of cryptococcosis diseases (Reimao *et al*., 2007) and up to 84% of samples from old roost sites, are a significant source of infection (CDC, 2010). Histoplasmosis is also an infection associated with bats/birds droppings. National Institute of Health (NIH)

reported a blinding eye condition (Ocular histoplasmosis syndrome). It is estimated that up to 4% of histoplasmosis patients develop this serious condition (CDC, 2010).

2.2 *Cryptococcus neoformans*

Cryptococcus species (*Cryptococcus adeliensis* and *Cryptococcus neoformans* var. *neoformans*) has been isolated from various environmental and non environmental sources: hollow of living trees, soil, oral cavity, brain, lung, liver and intestinal of striped grass mouse and its association with avian guano have been reported (Montenegro and Paula, 2000, Filiu *et al*., 2002, Bauwens *et al*., 2004, Kartrin and Heidemarie, 2005, Reimao *et al*., 2007, Kidd *et al*., 2007). High numbers of yeasts have been isolated from pigeon weathered droppings and the environment favors the growth of most bacteria and fungi (micro-organism). There have been isolations of *Cryptococcus* species in pigeon excreta in Western countries where pigeon and pet/captive birds are kept in homesteads (Kielstein *et al*., 2000, Ferreira-Paim *et al*., 2010).

Isolation of *C. neoformans* var. *grubii* and *C. neoformans* var. *gatti* from a number of tree species in Havana, Cuban and Amazon rainforests, Brazil suburban respectively was of great importance, since it confirms the existence of these variants in plants (Illnait *et al*., 2012, Fortes *et al*., 2001, Lazera *et al*., 2000). Restrepo *et al*., (2000) showed that *C. neoformans* has been isolated from 22 different species of trees. The ability of the *Cryptococcus* species to produce enzyme laccase (used to degrade lignin) may be an important feature for yeasts to colonized decayed wood (Fortes *et al*., 2001), and the

ability of *Cryptococcus* to use substrate presence in decaying wood and tree trunk, explains why it grows in trunk hollow (Randawa *et al*., 2000). *C. laurentii* has been isolated from trachea of broiler Chicken (Laubscher *et al*., 2000) and *Cryptococcus neoformans* var. *grubii* have been isolated in honeybee (*Apis mellifera*) colonies (Ergin *et al*., 2004). *Cryptococcus* genus consists of about 19 species, usually characterized as yeast. *Cryptococcus neoformans* is a spherical encapsulated yeast and major pathogenic species in the genus. *Cryptococcus neoformans* var. *neoformans* and *Cryptococcus neoformans* var. *gatti* are two recognized varieties that can be differentiated according to their genomic sequences. The two species have 5 serotypes based on antigenic specificity present on capsular polysaccharides i.e. serotypes A, D and AD of *Cryptococcus neoformans* which have been indentified and serotypes B and C of *Cryptococcus gattii* (Buekhout *et al*., 2001, Sorell, 2001, Kwon-chung and Varma, 2006, WHO, 2009).

Cryptococcus species in culture, after 24h of incubation showed a high pigmentation in 91.3% of isolates in *Pinus halepensis* seed agar medium, 100% in Blackberry agar and 34.8% in Niger agar; however after 48h all strains showed brown colouration in *Pinus halepensis* agar medium and 95.6% of them in Niger seed (Mseddi *et al*., 2010). In Sabouraud glucose agar – Emmon's modification *Cryptococcus* develop creamy-White colonies after 7 days whereas in ground red hot pepper agar and *Guizotia abyssinica* creatinine agar it produces creamy-White colonies in the first 2 days and turns to brown-pigmented colonies after 5 days (Stepanovic *et al*., 2002).

2.2.1 Cryptococcosis

Cryptococcosis is a fungal infection caused by *Cryptococcus neoformans* and *Cryptococcus gattii*, infecting human and animals e.g. dogs and Cats (Syket *et al*., 2010). Cryptococcosis is acquired mainly by inhalation of *Cryptococcus* spores into the lungs, where it will disseminate to other parts of the body e.g. central nervous system, causing pneumonia, meningitis and development of cryptococcomas in lung, muscle and brain (Uicker *et al*., 2005, Sorell, 2001). However the infection caused by the two species (*C. neoformans* and *C. gattii*) differ in that *Cryptococcus gattii* infection is less responsive to antifungal drugs and most likely to cause cryptococcomas, a tumor-like lesion. *Cryptococcus neoformans* infection on the other hand is sensitive to antifungal drugs and rarely causes cryptococcomas (Datta *et al*., 2009, Sorell, 2001). The increasing numbers of immunocompromised individuals due to HIV pandemic have raised populations susceptible to cryptococcosis (Tintelnot et al., 2004). *Cryptococcus neoformans* have been reported to cause meningitis and meningoencephalitis in case of Central nervous involvement and pneumonia in HIV/AIDS patients (Brol *et al*., 2002, Klock *et al*., 2009) and also in patients with systemic lupus erythematous (Ramos *et al*. 2001).

The prevalence of cryptococcosis has declined in HIV/AIDS patients who are on active anti-retroviral therapy, however in Southeast Asia and Africa it is still an epidemic affecting approximately 30% of AIDS infected individuals (Steenbergen and Casadevall, 2003). Invasive mycoses have been reported to be the major cause of opportunistic infections in individuals with immunosupressed conditions like HIV/AIDS. Mortality rate remains high despite availability of drugs (antifungal) for treatment of this infection

(Sing, 2003). It has been reported that AIDS is currently a predisposing factor in approximately 90% of all cryptococcal infection (Kachoko *et al*., 2002).

Cryptococcosis is the indication of HIV patients transforming to AIDS, and Cryptococcosis is ranked the second most common fungal infection in AIDS patients. The Centre for diseases control and prevention in US (CDC) reported that out of 274,150 patients with AIDS, 6% develop cryptococcal diseases. In Zimbabwe, 88% of AIDS patients developed cryptococcal infection (Brol *et al*., 2002, John and Meredith, 2010).

Patients' receiving organ transplant are at risk of contracting cryptococcosis (Husain *et al*., 2001) and solid organ recipients contract invasive forms of Cryptococcosis within 30 days (Sun *et al*., 2010). Approximately 55% of organ transplant recipients with Cryptococcosis develop central nervous system infections (Singh *et al*., 2003). Cryptococcosis mortality rate in solid organ transplant patients is higher than in HIV patients, approximately five times higher (Kretschmar *et al*., 2001).

2.3 *Histoplasma* species

Isolation of *Histoplasma* species from the environment has been done mainly in soil enriched with birds and bats guano (Ruchel, 2008) Genus, *Histoplasma* consists of three variants namely; *Histoplasma capsulatum* var. *duboissii* and *Histoplasma capsulatum* var. *capsulatum* and *Histoplasma capsulatum* var. *farciminosum.* Variants *duboissii* and *capsulatum* infect mammals (Human being included) and variant *farciminosum* cause infection in mice and horses (WHO, 2009). Early strain typing techniques of *Histoplasma*

capsulatum were by chemotyping and serotyping (Jessica *et al*., 2010). Currently, strain typing is based on restriction fragment length polymorphism in ribosomal DNA, nuclear genes or in mitochondrial DNA, including ribosomal DNA (Soll, 2000, Jiang *et al*., 2000, Bertha *et al*., 2010, Lindsley *et al*., 2001). Further studies on fragment length polymorphism based on single stranded conformational polymorphism and multi allelic loci have been done (Taylor et al., 2005, Bertha *et al*., 2010)). Such studies show that there some genetic recombination and some degree of clonality among the strain of *Histoplasma*, The studies show that *Histoplasma* can be further classified into at least seven phylogenetic separate species (David *et al*., 2010).

Genetic diversity of *Histoplasma capsulatum* strains can be demonstrated by use of flow cytometric and pulsed field electrophoresis of an intact chromosome classes, with class 1 down strain showing seven chromosomes, class 3 G186B four and Class 2G217B three (Gil-lamaignere *et al*., 2003). *Histoplasma capsulatum* in culture produce conidiophores that are hyphae-like which are at right angles to the mother hyphae. In corn meal agar at 25°C *Histoplasma capsulatum* produce both macro- and micro conidia; however at 37°C they form ovoid, narrow- budding cells. Var. *capsulatum* yeasts are smaller than 2-4µm and those of var. *duboissii* are (12-15µm). On SDA at 25°C *H. capsulatum* produce slow-growing colonies, which are glabrous or verrucose, white to brown and cottony in appearance with transverse folds (pale brown) (WHO, 2009). *Histoplasma* species cause histoplasmosis infection in humans.

2.3.1 Histoplasmosis

Histoplasmosis is classified into different forms based on the organ they infect in the human body:

2.3.2 Acute pulmonary histoplasmosis

Approximately 90% of individuals infected with acute pulmonary histoplasmosis do not show symptoms of the diseases (Hage *et al*., 2008). However if symptoms develop, it occurs within 3-14 days after exposure. Acute pulmonary histoplasmosis patients present themselves with complaints of weight loss, malaise, fever, abdominal pain, chills and some patients may develop dyspnea resulting from diffuse pulmonary involvement. Approximately 5-6% of patients develop skin, joint lesion rheumatologic manifestation of erythema nodusum arthritis and erythema multiforme, while enlarged lymph nodes (mediastinal) and hilar occurs in 5-10% of them (Kauffman, 2007b). Compression of circulation and pulmonary airways can occur, characterized by dyspnea, chest pain, hemoptysis and cough, rarely esophagus compression occur, which causes dysphagia.

2.3.3 Progressive disseminated histoplasmosis

This form of histoplasmosis affects mainly immunocompromised subjects. Major pre-disposing factors include, AIDS patients with CD4 counts <150/µl, hematologic malignancy, solid organ transplantation, cancer patients, use of corticosteroids and

infants exposure to fungus (Kauffman, 2009). Patients suffering from this form of histoplasmosis present the following symptoms, worsening cough, fever, malaise, dyspnea and weight loss. Central nervous system involvement occurs in 5-20% of patients. The patient presents the following symptoms; seizures, confusion, headache, visual and giat disturbances, neck stiffness or pain and altered consciousness (Kauffman, 2007).

In USA skin involvement occurs in 10% of the cases and gastrointestinal involvement occur in 10% of the cases, characterized by diarrhea, fever, abdominal pain and intestinal obstruction and also perforation have been reported. In severe cases lesion occur in colon and small intestine (Karimi *et al*. 2002, Kauffman, 2007), skin infection manifestation range from erythema multiforme to ulcer and vary from location to location. Approximately 66% of HIV positive patients in Brazil have skin infection, compared to those in USA, where skin lesion manifestations are at 1-7% of the reported cases (Karimi *et al*., 2002, Kauffman, 2007). *Histoplasma* isolated from Brazil and USA, show genetic differences based on gene typing and account for the differences in clinical manifestation earlier observed (Karimi *et al*., 2002).

Studies reviewing factors related to severity of histoplasmosis disease and death, found out that neurologic involvement is not a major factor (Wheat *et al*., 2000, Couppie *et al*., 2004). Factors such as serum creatinine >2.1 mg/dl, alkaline phoshatase >2.5 times normal, thrombocytopenia < 100,000 platelets/µl and elevated lactate dehydrogenase are associated with severe disseminated histoplasmosis (Couppie *et al*., 2004, Wheat *et al*., 2000,). AIDS patients who developed immunological improvements during ART treatment, develop histoplasmosis – associated immune reconstitution inflammatory

syndromes, accompanied by arthritis, uveitis, intestinal obstruction and elevation of hepatic enzymes (Breton *et al*., 2006, Shelborne *et al*., 2005).

2.3.4 Ocular histoplasmosis syndrome

Ocular involvements occur in approximately 1-10% of individual living in *histoplasma capsulatum* endemic area. In most cases patients suffering from this form of histoplasmosis are asymptomatic (Hage *et al*., 2008). Macula involvements lead in blindness (CDC, 2010).

2.4 *Candida* species

There are several reports of *Candida* isolates recovered from dorsal tongue and buccal mucosa (Vazquez and Sobel, 2002, Pappas *et al*., 2003). It has also been reported by some authors to have been isolated from human stool (Khatib *et al*., 2001, Repentigny *et al*., 2004). In birds' isolation has been done in Chicken comb, lungs and spleen specimen (Grundet *et al*., 2005, Botello *et al*., 2012) and in turkeys that died after therapeutic treatment of coccidiosis (Moretti *et al*., 2000). This increases the need for more environmental niches to be investigated to be discovered. Characterization of *Candida* species is done using various methods:

2.4.1 *Candida* characteristics on CHROM and Cornmeal media

CHROM agar *Candida*, which contains β-hexosaminidase substrate and other chromogenic substrates, is widely used in differentiation of *Candida* species mainly in mixed samples (Cookie *et al*., 2002, Perry and Freydiere, 2002). Chromogenic *Candida* agar also has the white opaque background; this allows good discrimination among colonies of different species with similar hues (Ghelardi *et al*., 2008). Differentiation of *Candida* species on CHROM agar *Candida* is based on differences in colour produced by different *Candida* species due to presence of species-specific enzymes, incubated at 37°C for 48h. *Candida albicans* and *Candida dubliniensis* colonies appear light green and dark green respectively after incubation at 37°C for 48h, however it has been reported that more prominent colour develop after 72h of incubation (Fotedar and Al-hedaithy, 2003). Some authors have examined the influence of temperature on colour development by some species precisely; *C. albicans* and *C. dubliniensis* and that incubation at 37°C must be adhered to (Odds and Davison, 2000). 30% *C. dubliniensis* isolates produced colonies with different shades of green colour (Aivarez *et al*., 2009) some *C. albicans* also produced the same dark green colonies (Kurzai *et al*., 2000, Manh *et al*., 2005). Other chromogenic media containing substrates like *Candida* ID2 and *Albicans* ID2 are used to differentiate the two *Candida* species (*C. albicans* and *C. dubliniensis*) (Likit *et al*., 2007). However *C. albicans* don't reduce 2, 3, 5- triphenyltetra-zolium chloride and produce pink to white colonies (Giammanco et al., 2002). The sensitivity of Chromogenic *Candida* agar for the identification of *Candida* species; *C. albicans* is at 88% and 100% after incubation for 24 to 48h respectively, *C. krusei and C. tropicalis* at 100% while *C. guilliermondii* is at 99.8% after 48hrs incubation. The sensitivity of

Chromogenic *Candida* agar at 24h is 95.0%, 87.5% and 70.8% for *C. tropicalis, C. krusei,* and *C. guilliermondii* respectively, and reaches 100% after 48h for all species (Ghelardi *et al*., 2008).

In cornmeal agar, *Candida albicans* is known to form single chlamydospores mainly at the tip of suspensor cell and grape-like blastoconidia are formed along the hyphae, while *Candida dubliniensis* is known to form more than one chlamydiospore at the end of each hyphae (Peter and Joachim, 2006). *Candida guilliermondii* produce blastospores in cluster along the pseudohyphae (pseudohyphae are usually few in number) and mainly at septal points (WHO, 2009, Sayyada *et al*., 2010). *Candida glabrata* when incubated at 25°C for 72h forms blastoconidia (Kurtman and Fell, 2000). It has been reported that differentiation of *Candida* species, based on chlamydiospore production is not reliable, since some species of *Candida* like *C. dubliniensis* do not show a consistent pattern in formation of chlamydospores (Ellepola *et al*., 2003). Niger seed agar, which was develop mainly for identification of *Cryptococcus neoformans* has been found to be useful in the differentiation of *Candida dubliniensis* and *Candida albicans* based on absence and presence of chlamydiospore formation in Niger seed (Eileen and Richard, 2003). *Candida albicans* does not produce chlamydospores but produces pseudohyphae after prolonged period of growth while *Candida dubliniensis* produces chlamydospores, pseudohyphae and rough colonies.

2.4.2 Protein fingerprinting

The identification of bacteria strains has been known to be achieved by analysis of a microorganism's cellular protein (Neppelenbroek *et al*., 2006). SDS-PAGE is one of the most used techniques, where microorganism proteins are extracted then denatured and finally separated by use of polyacrylamide gel, however this technique has been found not to be useful for identification of *Candida* species, specifically *Candida albicans* and *C. dubliniensis* (Cunningham and Noble, 1989). Some authors' have disagreed with Cunnigham and Noble's findings and have found out that SDS-PAGE technique is useful in *Candida* species identification (Rosa *et al*., 2000, Sahand *et al*., 2006). Multilocus enzymes electrophoresis (MLEE) is a protein based technique used to identify Isolates based on polymorphism of enzymes i.e. alloenzymes and isoenzymes (Neppelenbroek *et al*., 2006). In MLEE the microorganisms' cellular protein is extracted and then separated by means of polyacrylamide gel, finally enzymes activity is revealed by use of specific staining protocols and enzymes examined, assuming these enzymes are not or are less prone to pressure (Gil-lamalghere *et al*., 2003), and data obtained are considered indirectly representative of organism genome (Gil-Lamalghere et al., 2003). MLEE have been widely used in the study of *Candida* species. Few studies however have been carried out to differentiate *Candida albicans* and *C. dubliniensis* by use of MLEE (Neppelenbroek *et al*., 2006, Rosa *et al*., 2000). *Candida* species has been documented by many authors to cause different forms of candidiasis infection in humans as discussed below.

2.4.1 Candidiasis infection

This infection is caused by *Candida* species in most cases, *Candida albicans*. It's divided in two broad categories: oral candidiasis and systemic candidiasis.

2.4.2 Oral candidiasis

There is increasing fungal infection; cutaneous and mucosal worldwide, mainly due to oral candidiasis due to HIV/AIDS pandemic (Samaranayake, 2002). Oral candidiasis ranges from pseudomembranous and erythematous to gingival erythema and to rhomboid glossitis (Ellepola and Samaranayake, 2000). Increasing incidences of this form of candidiasis is due to presence of predisposing factors, aiding in conversion of *Candida* from a commensal to a parasitic organism. Some authors have reported that 84-100% of HIV/AIDS infected individuals get infected with at least one episode of *Candida* species (Samaranayake, 2002, Ellepola and Samaranayake, 2000).

Primary oral candidiasis is further classified into three variants: erythematous, pseudo-membranous and hyperplastic. Hyperplastic candidiasis present as a chronic lesion while erythematous and pseudomembranous manifest as acute lesions. Apart from these well defined lesions (candidal), a group of *Candida* diseases known as "*Candida* associated lesions" has been reported as their cause is multifactorial with or without *Candida* infection such as median rhomboid glossitis, linear gingival erythema, and denture stomatitis common in denture wearers.

2.4.3 Pseudomembranous and Erythematous candidiasis

Pseudomembranous candidiasis is common in immunocompromised individuals e.g. cancer, newborns, Leukemia patients, HIV infection/AIDS and long term use of broad-spectrum antibiotic therapy (Sherman *et al*., 2002, Akpan and Morgan, 2002). Characterized by White plaque on tongue that "look like milk curd" that can be wiped off with gauze revealing erosive base that bleed easily (Reichat *et al*., 2010, de Almeida and Scully, 2002). The plaque consists of bacteria, food debris, hyphae, yeast cells and necrotic material.

Erythematous candidiasis is one of the most common variant in HIV infection; it is associated with a prolonged use of broad-spectrum antibiotics, corticosteroid and HIV infection. The infection commonly occurs in the palate, dorsum of the tongue and in cheek mucosa and is characterized by red to pink colour, red patches, blisters on the soles of feet, palm of hands and joint pain, itching, fatigue and fever (Fraser and Kimberly, 2011, http://www.softdental.com/diseases/oral_candidiasis_moniliasis_thrush.html).

2.4.4 Atrophic and Hyperplastic candidiasis

Atrophic candidiasis is characterized by marked pebbled patches on dorsal surface of the tongue, buccal mucosa, palate (hard or soft) and erythematous. Diabetes mellitus, vitamin deficiency or poorly fitting dentures are some of the predisposing factors associated with this infection (Miller, 2003).

Up to 15% of reports have associated hyperplastic candidiasis with malignance (Sitheeque and Samaranayake, 2003). This form of candidiasis appears as a chronic discrete raised lesion and varies from small, palpable, translucent, whitish areas to large, dense plaques that are hard and rough areas on palpation (de Ameida and Scully, 2002, Scardina *et al*., 2009, Sitheeque and Samaranayake, 2003). Histology of hyperplastic candidiasis lesion is characterized by *Candida* hyphae. Biopsy is taken when the lesion does not respond to antifungal drugs (Sitheeque and Samaranayake, 2003, Sherman *et al*., 2002). Most patients suffering from Hyperplastic candidiasis are smokers; and this condition has been associated also with malignancy and dysplasia (Muzyka, 2005).

2.4.5 *Candida*- associated lesion

Candida associated lesions are mainly associated with *Candida* species, however yeasts are not the sole causative agents (Golecka *et al*., 2006). These are;

Candida- associated denture stomatitis are characterized by chronic erythema and edema. *Candida* associated denture stomatitis occur when there is overgrowth of *Candida* between palate and denture surface (Golecka *et al*., 2006,). It has been reported by authors that denture stomatitis in HIV individuals is caused by *Candida* in conjunction with other factors like; allergic reaction and bacterial infection (Golecka *et al*., 2006).

Angular cheilitis is a *Candida* infection that forms ulcers and fissures radiating from commissures (oral cavity), accompanied by white plaques and is associated with cutaneous infections of both *Staphylococcus* and *Streptococcus* bacteria, yeasts and deficiencies of iron and vitamin B_{12} are some of the predisposing factors. In young men, angular cheilitis could be the first sign of HIV infection, while in the elderly; it may be due to vitamin deficiency (Reichat *et al*., 2000, Miller, 2003, Akpan and Morgan, 2002, Sauders, 2000,). It is seen in children as it is associated with sucking of the thumb and due to immunosuppression where as in adults it is due to wrinkles or fold of skin at oral commissures (Neville *et al*., 2002, Akpan and Morgan, 2002, Sherman *et al*., 2002).

Median rhomboid glossitis is characterized by an elliptical, erythematous or rhomboid area representing papillary atrophy of filiform on the tongue (Reichat *et al*., 2000). *Candida* linked to median rhomboid glossitis has been under a lot of debate, some authors have reported that it may be an acquired chronic oral candidiasis or a mixture of fungal and bacterial infection (Reichat *et al*., 2000).

Linear gingival erythema candidiasis is commonly referred to as “red band gingivitis” because of its red band appearance (Mark, 2009). However it has been reported that linear gingival erythema occurs also in HIV negative, immunocompromised individuals (Oral health care, 2001). Linear gingival erythema is limited to periodontium and manifests as a red line measuring at least 2mm in width (Oral health care, 2001). Linear gingival erythema can be localized or generalized mainly in gingival margins of teeth. Some authors consider it to be a progressive infection in individuals suffering from HIV/ AIDS leading to necrotizing periodontitis when the CD+4 cells counts is low, However

the disease can be prevented from progressing to necrosis by treating linear gingival erythema (http://www.softdental/com/diseases/oral_candidiasis_moniliasis_thrush.html).

2.4.6 Chronic mucocutaneous candidiasis syndromes (CMC)

This form of candidiasis is characterized by recurrent superficial candidosis of the nails, vagina, skin and mucous membrane of the mouth (de Almeida and Scully, 2002). This infection may be diffuse or chronic, and often associated with endocrine and immune systems disorders, Addison's diseases, myeloperoxidase deficiency, Nezelof sydrome and defect in cell-mediated immunity (Williams *et al*., 2011, Miller, 2003, Saunder's, 2007). Patients suffering from this form of candidiasis do not respond to topical application of antifungal agents such as polyenes but may respond to Azole administered systemically.

2.4.7 Systemic candidiasis

Systemic candidiasis is divided into two categories: disseminated and candidemia candidiasis. When *Candida* species spread to deep organ in the body it is usually referred to as dissemination form of candidiasis.

Candidemia is commonly caused by *Candida* species which is ranked the fourth most common organism recovered in blood cultures and nasocomial infection and are generally considered the causes of most *Candida* infections (Guery *et al*., 2009). The history of the patient's often reveals: Fever not responding to broad-spectrum antibiotic, skin lesion**,** Candidal endophthalmitis and septic shock (Picozo *et al*., 2008). Other causes of candidemia infections: include Endocarditis and it has been reported that *C. albicans* and *C. parapsilosis* account for about 60% of fungal endocarditis cases (Falcone *et al*., 2009). Endocarditis is mainly acquired through direct inoculation during surgery or due to hematogenous dissemination during bloodstream invasion. *Candida* endocarditis has been associated with some risk factors, including chemotherapy, intravenous heroin use, prolonged use of central venous catheters and prosthetic valves. Physical examination of the patient reveal new or changing murmurs, hypotension, and fever not responding to antimicrobials, shock and large septic emboli organs is the characteristic of fungal endocarditis.

Disseminated candidiasis is associated with multiple or single deep organs. However blood cultures from patients suffering from this infection are negative in up to 40-60% of the cases (Shah *et al*., 2008). Risk factors associated with this form of candidiasis include: burns, severe trauma, prolonged hospitalization, bone marrow transplantation, recent chemotherapy or radiation therapy, Foley catheters, premature birth (Pappas, 2006).

Renal candidiasis occur frequently occurs due to disseminated candidiasis or candidemia. Individuals suffering from candidiasis have of fever that doesn't respond to treatment by use of broad spectrum antimicrobials. In most cases, patients are asymptomatic and may lack symptoms showing kidney infection. Diagnosis is mainly done by renal biopsy and urinalysis. Renal candidiasis is diagnosed commonly by autopsy (Malani *et al*., 2007).

Candida peritonitis in most cases tends to be localized and in 15% of the cases it is disseminated into the bloodstream. The individual history reveals the association of the disease with peritoneal dialysis, viscous perforation or gastrointestinal tract surgery. The manifestation of the disease includes vomiting, nausea, fever and chills abdominal pain and at times constipation. Isolation of the etiological agents (*Candida* species) from peritoneal fluid (in surgical cases) needs to be evaluated carefully (Blot *et al*., 2007). Physical examination of the patients may reveal; abdominal pain, absent bowel sounds, fever, localized mass, abdominal distention, and rebound tenderness (Blot *et al*., 2007).

Nearly 200 *Candida* species exist in nature, but only a few species have been documented to cause humans diseases (Vazquel *et al*., 2003). The medically significant *Candida* species are: *C. albicans* (the most common *Candida* species) accounting for 50-60% of isolated cases, *C. parapsilosis* (10-20%), *C. tropicalis* (6-12%), *C. glabrata* (15-20%), *Candida krusei* (1-3%), *C. guilliermondi* (<5%), *C. kefyr* (<5%), *C. lusitaniae* (<5%) and *Candida dubliniensis*, that is mainly recovered from individuals infected with HIV/AIDS (Vazquel *et al*., 2003).

In individuals suffering from candidemia or invasive candidiasis 70-80%, of *Candida* species isolated are *C. glabrata* and *C. albicans.* It has been reported that incidence of *C.*

glabrata has been increasing worldwide due to its resistance to fluconazole in up to 20% of hospital cases (Vazquel *et al*., 2003). *C. krusei* has been shown to be resistance to fluconazole and ketoconazole; it is less susceptible to amphotericin B and itraconazole (Vazquel *et al*., 2003). *C. lusitaniae* is not as common as other candida species; however it is of medical importance since it is resistant to amphotericin intrinsically and susceptible to echinocandins and azoles (Vazquel et al., 2003). *C. parapsilosis* is also *Candida* species of medical importance mainly in hospitalized patients. It is associated with infections acquired through the use of vascular catheter devices. *In vitro* analysis have shown that echinocandins is more effective on *C. parapsilosis* than in other species of *Candida*. However the relevance of this *in vitro* finding is yet to be determined (Eiland *et. al*., 2008) and *C. tropicalis* is considered to be causing candidemia in cancer patients and those that have undergone bone marrow transplantation (Vazquel *et al*., 2003).

2.4.8 Vaginal candidiasis

This is a Candidal infection of the vulva and vagina. In United States approximately one million women, each year develop this form of candidiasis (Miller, 2003). It is not a serious infection but can be frustrating and uncomfortable to the patients. It is characterized by severe vaginal itching, cottage-cheese like discharge and has bread or sweet-like odor. Vagina can be red, painful and swollen (Sauder's, 2007, Miller, 2003).

2.5 *Blastomyces dermatitidis*

Blastomyces dermatitidis has been isolated in clinical specimen (Tracy *et al*., 2002), culture being the most reliable method for the identification of *Blastomyces dermatitidis*. In Sabouraud dextrose agar at 25°C after 2 - 4weeks period of growth, *Blastomyces dermatitidis* produce white fluffy colonies (Bradsher *et al.,* 2003, Koneman *et al*., 2007). Microscopy also provides an equally firm method to identify budding of *Blastomyces dermatitidis* in sputum, exudates and in tissue following potassium hydroxide preparation (Lemos *et al*., 2002). *Blastomyces dermatitidis* is known to cause Blastomycosis infection.

2.5.1 Blastomycosis

Blastomyces dermatitidis causes blastomycosis infection in humans and is classified into: cutaneous, pulmonary, primary and disseminated blastomycosis (Kauffman, 2007, Tracy *et al*., 2002).

2.5.2 Cutaneous blastomycosis

Cutaneous primary blastomycosis is a manifestation of disseminated pulmonary blastomycosis and has been reported in 80% of infected individuals. The skin becomes infected as the causative agent disseminates to other areas of the body, characterized by

papules, pustules or nodules that are found on the exposed body areas, leading to loss of skin pigment and scarring (Kauffman, 2007, Garvey *et al*., 2006, Kisso *et al*., 2006).

Chest radiograph shows diffuse parenchyma and the patient develops verrous plaque on buttocks, torso and hilar mass (Mason *et al*., 2008). Center of the lesion is crusted and atrophic and fails to show the organisms if biopsy is taken from this region. Biopsy specimen from periphery of the lesion contains micro-abscesses and it is used for diagnosis since it yields better results (Mason *et al*., 2008). Primary cutaneous blastomycosis has been reported in an immunosupressed child, characterized by development of chancriform ulcer at the site of inoculation with lymphadenitis and lymphangitis (Zampogna *et al*., 2003, Garvey *et al*., 2006).

2.5.3 Pulmonary blastomycosis

Pulmonary blastomycosis is the most common form of blastomycosis infection. Acute infection of primary pulmonary blastomycosis has an incubation period of between 3 weeks to 3 months after exposure and resembles pneumonia or could be asymptomatic. In symptomatic patients, they present the following symptoms; chest pain, dyspnea, cough, sputum production and hemoptysis. The most common systemic manifestation; weight loss, fever and night sweats (Kauffman, 2007a, Baumgarder *et al*., 2004).

Solid organ transplant have been reported to transmit blastomycosis and the patients develops blastomycosis approximately 26 months after transplantation (Gauthier *et al*., 2007), with an incidence rate of between 0.14% to 64% of the reported cases between

2000 and 2004 in USA. Tran-placental infection of newborn can occur during pregnancy, from an infected mother (Lemos *et al*., 2002).

2.6 *Saccharomyces* species

Environmental isolation of *Saccharomyces* species has been done from fruits, soil, grain and other materials with high carbohydrates content like in vineyard environments and in clinical specimens (Salomen *et al*., 2000, Smith *et al*., 2002, Sniegowki *et al*., 2002, Erdem *et al*., 2003, Reuter *et al*., 2007, Schulled *et al*., 2007, Valero *et al*., 2007, Murphy and Zeyl, 2011). *S. cerevisiae* is differentiated from other yeasts mainly based on its ability to ferment individual sugars and growth characteristics. Clinical identification of yeasts is done by use of diagnostic kits commercially available and principally classified yeasts according to their ability to utilize distinct types of carbohydrates as sole carbon sources (Torija *et al*., 2001, Amendola and Rees, 2002, Sun *et al*., 2009). More sophisticated techniques for yeasts classification have been developed e.g. DNA sequence, polymorphism (Liti *et al*., 2009, Schacherer *et al*., 2009). *Saccharomyces* species cause infection in humans.

2.6.1 *Saccharomyces* infection

Saccharomyces species has been reported to be an emerging agent causing opportunistic mycoses in immunocompromised individuals (Salonen *et al*., 2000). Prolonged use of

antibiotics a is major risk factor for *Saccharomyces* infection development (Adela and Christopher, 2005). Aortic graft and fungemia infections as well have been observed in immunocompetent individuals (Smith *et al*., 2002). Critically ill patients develop fungemia due to overload during therapeutic treatment of *Saccharomyces boullardii*, approximately 51.3% of fungemia is caused by *Saccharomyces boullardii* (Lherm *et al*., 2002, Adela and Christopher, 2005). Also, *Saccharomyces cerevisiae* causes vaginitis, and periodontal lesion (Erdem *et al*., 2003, Jabra *et al*., 2001).

2.7 *Geotrichum* species

Geotrichum species have been isolated from soil, water, air, cheese and from clinical specimen; sputum, faeces, skin, genital and gastrointestinal tracts of humans and other mammalians (Bouakline *et al*., 2000, Larpin *et al*., 2006, Hojjatollah *et al*., 2010). In Northeastern Thailand *Geotrichum phurueaesis* was isolated from forest soil, while *Geotrichum siamensis* was isolated in water from mangrove forest in southern part of Thailand (Kaewwichian *et al*., 2010). In MEYE agar at 20°C *Geotrichum* strain colonies are 25- 30mm in diameter and form white, dry, centrally powdery colonies with hairy margins. Hyphae are wide approx. 3-5µm, with lateral braches and round apices. Abundant arthroconidia measuring approximately 2.5 - 4.0 × 5.0 - 36.0µm and mycelia are formed (Takahiko *et al*., 2008), while on ME agar, hyphae and arthroconidia (measuring 6-12 × 3-6 µm) produce clusters of blastospores which are globose or subglobose, 4.0 – 6.0µm in diameter. Gematangia are formed on the opposite sides of

hyphae or septa, and asci contain one to four ascospores (Takahiko *et al*., 2008). *Geotrichum* species cause geotrichosis infection in human.

2.7.1 Geotrichosis

In nature *Geotrichum candidum* is saprophytic; however, it causes opportunistic infection in humans (Herich *et al*., 2009). Dissemination of geotrichosis occurs in conditions such as in patients with leukemia diseases. Oral lesions caused by *Geotrichum* species are clinically indistinguishable from candidiasis thrush, due to misdiagnosis and low incidence (0.5% in leukemia patients) of geotrichosis. Some reports indicate its clinical appearance is as erythematous gingivae, ulceration and edematous (Fanci and Pecise, 2003, Corrado *et al*., 2005). Pulmonary involvements have been documented in 9-26% cases of patients with hematological malignancies (Corrado et al., 2005). Diagnosis confirmation of *Geotrichum* species in clinical setting is done by culture (Takahiko *et al*., 2008).

CHAPTER THREE

3. MATERIALS AND METHODS

3.1 Study area

The study area comprised of Kabigeriet Village situated in South Western Kenya in the Rift Valley Province, Nakuru county, Kuresoi District, and Olenguruone Division. Olenguruone is Approximately 282 KM from Nairobi, Kenya. The area lies at about 35° 41'E and 0.1° 35'S. The climate is sub-humid consisting of one rainy season (April to December) and dry season (January – March). The average annual rainfall is 1200 mm and the average temperature is 28^{o}C with small variations (±5^{o}C) throughout the year (Meteorological Kenya, 2009). Here the soil comprises a complex of well drained shallow to moderated deep dark loam soil to clay loam soil. These soils are mostly good for agriculture mainly Maize, Tea, wheat, potatoes as well as animal keeping; Cattle, goats and sheep, it is also good for Chicken rearing. Cypress, Bamboo and Eucalyptus trees dominate the vegetation of the area (Olenguruone village). The study area was chosen since it is atypical rural setting where domestic Chicken are reared in close proximity with people. Unlike in other Divisions of Olenguruone, in Kabigeriet village most farmers rear only domestic chicken. In domestic way (out door rearing) without feeding them with commercial feeds.

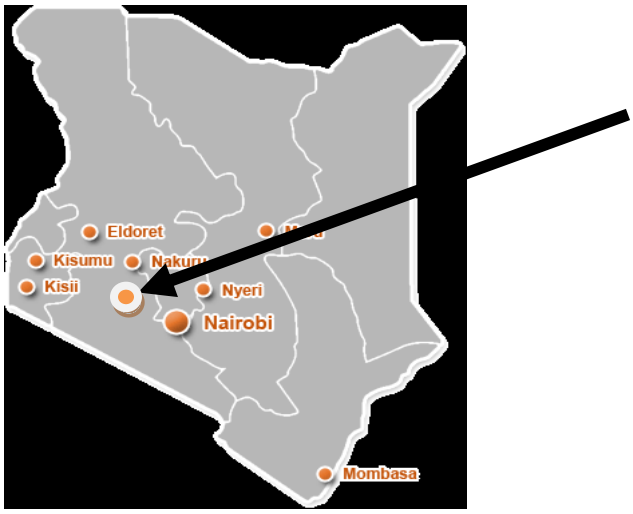

Figure 1: Kenya map showing study area. Courtesy of Copyright©2008-09, www.maps of world.com

Figure 2: The study area (Olenguruone) and the neighboring major towns (Courtesy of map data ©2011, Google, tracks4Africa)

3.2 Research design

The study was a cross sectional laboratory based study carried out for a period of five months, (April 2010 to August 2010). Sampling of the homesteads; complete random design was done. Where the homes were chosen at random and the owner's consent was sort. Stratified sampling was carried out within the selected homesteads for domestic Chicken dropping. These were therefore taken to the laboratory for analysis.

3.3 Sample size

The study was carried out in thirty two homesteads that reared domestic Chicken (*Gallus gallus*) in Kabigeriet village, Olenguruone Division, Kuresoi District. Kabigeriet village has a total of forty five homesteads but only thirty two were sampled because some homesteads had no Chicken while others did not consent to participate in the study. However the study managed to Sample 84 domestic Chickens droppings from different homes/houses which were more than the minimum sample size (64) required for the study. The number of Chicken that were sampled in this study was sufficient according to Mugenda and Mugenda, (1999) formula which states that 13% of the population is sufficient to be sample size, with an estimated population of domestic Chicken in Kabigeriet village being 495. Mugenda and Mugenda formula was used due to the following factors; the research design of the study (it was carried out in a village, with a total of 45 homesteads only) and the size of the accessible population. Farmer's consent was obtained before sampling. Majority consented to the study (by limiting the numbers of Chickens to be sampled) while others didn't.

Therefore 495=100%

?= 13%

$\frac{13\%}{100\%} \times 495 = 64.35$

Number of Chicken droppings sampled = 64

3.4 Sample collection

A semi-structured questionnaire was administered to collect basic household information. Environmental conditions for the area where the samples were collected including time, humidity, temperature, elevation (using Garmin GPSMAP ® 60csx, Garmin international, inc. Kansas USA) (Appendix 1) and groundcover were recorded. The following specimens were collected: Domestic Chicken dropping and soil enriched with domestic Chicken dropping. Environmental collection of domestic Chicken dropping was done by scooping fresh dropping from Chicken houses, grass, soil and trees (contaminated with Chicken dropping in homestead where Chicken sleep on trees) using sterile plastic spoons. Each spoon was used once and discarded into ziplock bags. Droppings which could not be collected using a spoon were swabbed by passing a sterile swab over each sample until it turned “dirty”. A total of 84 specimens were collected and some of the sample collection sites are shown in Plates 1-6


Plate 1 and **2**: Chicken droppings inside human houses.

Plate 3:Chicken droppings on top of timber inside an abandoned house.

Plate 4: Chicken droppings inside a poultry house.

Plate 5: A typical poultry house.

Plate 6: Chicken droppings under maize Store.

3.5 Specimen transportation, storage and bio safety measures

The samples were labeled by giving non random (NR) numbers which were inserted in a zip lock safety bag and transported to the laboratory using a cool box soon after collection and stored at 4°C. All cultures were handled inside a level 2 containment biosafety cabinet.

3.6 Processing of samples, isolation and characterization of isolated yeasts

Two procedures were used in isolation and characterization of yeasts, one for *Candida* species and other yeasts and the other procedures for *Cryptococcus* species.

3.7 *Candida* species isolation and characterization procedure

3.7.1 Specimen preparation

One gram of domestic Chicken dropping was weighed using a scale balance and then transferred to a 10 ml sterile round bottom tube containing 5 ml distilled water then incubated for 1h with agitation (mix every 15 min). Two 1.7ml appendoff tubes were each filled with 900 µl sterile distilled water and 100 µl of the original sample added to the first tube (dilution factor 1:10) then 100 µl of the 1:10 dilution added to the last tube containing 900 µl sterile distilled water, giving a final dilution factor of 1:100. For swabs, 100 µl of sterile distilled water were added to round bottomed tubes and the tip of the swab containing the sample dipped into distilled water with agitation to make a suspension.

3.7.2 Primary isolation

Trypan blue agar (Oxoid) was used for primary isolation and cultivation of yeast (John *et al*., 2009). Media were prepared aseptically according to the manufacturer's instructions (Appendix 2) and dispensed in 25 ml amounts into 90 mm-diameter sterile Petri dishes. The procedure was done inside a Laminar Airflow Biosafety cabinet which was sterilized by wiping with 70% Alcohol to decontaminate. After agar had solidified in Petri dishes, each Petri dish was labeled with the same Non random number given to respective samples. Approximately100 µl of the second dilution and similar amounts for swabs suspension, were inoculated onto the centre of Trypan blue agar and spread using glass rods which were sterilized by autoclaving and incubated at 30°C for 3 days (it was checked daily for any growth before colony purification).

3.7.3 Purification

Yeasts colonies from Trypan blue Agar were sub-cultured onto Sabouraud agar (Oxoid) to purify cultures (Isenberg and Henry, 2004). The procedures were done according to the manufacturer instructions using aseptic techniques then 25 ml amounts were dispensed into 90 mm-diameter Petri dishes (Appendix 2). Each Sabouraud agar plate was equally labeled with the same Non random number which was given to Typan blue agar on samples from the field. Suspected colonies were transferred from Typan blue agar to Sabouraud agar by using a wire loop sterilized by heating over a Bunsen burner until it was red hot then cooled. Using a cooled wire loop, suspected colonies from Typan blue agar were picked and inoculated onto Sabouraud agar and then labeled as earlier

described. The procedure was done inside a level 2 Biosafety cabinet for Biosafety reasons. The sub-cultures were incubated for 48h at 30°C to purify yeast before biochemical tests (yeasts identifications) were done. However, colonies that appeared to be mould were subjected to Lactophenol cotton blue stain.

3.7.4 Lactophenol cotton blue stain test

Lactophenol cotton blue stain was used for staining and microscopic identification of fungi mainly moulds (Forbes *et al*., 2002). Briefly a drop of lactophenol cotton blue stain was placed in the center of a clean slide and a fragment of the moulds picked from colonies that looked like moulds in Sabouraud agar. Colonies 2-3 mm in diameter which were removed from a sporulating culture using an inoculating or teasing needle were emulsified in lactophenol cotton blue stain at the center of a slide and covered using a sterile clean cover slip. The cover slip was sterilized by holding with forceps over a burning Bunsen burner, cooled then put on top of the suspension and then gently pressed. The fragment in the preparation was squashed with the butt of the inoculation needle and excess fluid blotted off. All the procedures were done inside the Biosafety cabinet. The preparation was examined under low (×10) and high (×40) magnifications, for the presence of characteristic mycelia and fruiting structures. Identification of the moulds was done morphologically using macro and micro-morphological features (Forbes *et al*., 2002).

3.7.5 Identification of yeasts

Purified yeast colonies from Sabouraud agar were sub- cultured onto CHROM agar (CHROM agar Company, Paris, France) for preliminary identification of yeasts and to detect mixed cultures as described by Sivakumar *et al*., (2008). Media was prepared according to the manufacturer's instruction under sterile conditions (Appendix 2) and then dispensed in 25 ml amounts into 90 mm-diameters Petri dishes. Suspected colonies were transferred from Sabouraud agar by using a sterile wire loop and inoculated onto CHROM agar. It was incubated at 30°C for 48h for preliminary identification: C. *albicans* appeared green, *C. tropicalis* appeared blue, *C. parapsilosis* appeared pink fizzy and other species appeared white pink (Ghelardi *et al*., 2008). However colonies that could not be identified using CHROM agar were further sub- cultured onto Cornmeal agar (Oxoid) for identification of yeast that produces pseudohyphae, true hyphae, arthrospores and chlamydospores (Peter and Joachim, 2006, WHO, 2009, Kurtman and Fell, 2000).

Colonies from CHROM agar were transferred, inside a lamina airflow Biosafety cabinet using a sterile wire onto cornmeal agar by making a parallel cut of approximately ½ inch into the agar and inoculating by holding the wire at an angle of 45°C and a cover slip put onto the agar, covering a portion of the inoculation streaks. The incubation of the inoculated plates was done at 30°C for 24 to 48 h. The cultures were examined microscopically, through the cover slip to prevent inadvertent contamination of the microscope objective with the agar and dislodging of the conidia. The preparation was examined under low (×10) and high (×40) magnification, for the presence of

characteristic pseudohyphae, true hyphae, arthrospores and chlamydospores. However not all yeasts were identifiable using CHROM agar and cornmeal agar those that gave contradicting result were subjected to confirmation tests (API 20 AUX).

3.7.6 Confirmation test of *Candida* species

Confirmation of yeasts was done using API 20C AUX (Bio Merieux SA) for non-chlamydiospore, pseudohyphae and arthrospores producing *Candida* species and those species that gave contradicting results when subjected to CHROM agar and Cornmeal agar. The basal medium in the ampoules was melted by placing in a boiling water bath for two minutes and allowed to cool. 20 mls of distilled water was dispensed into incubation tray and strip placed with the open ampoules open (2 mm diameter). Suspension of the test yeast was prepared and the density adjusted just below 1+ on a wickerham card. The strip was inoculated (20 cupules; approximately 0.2 ml each) following the manufacturer's instructions, and incubated at 30ºC for 72 h as described in the manufacturer's manual (Appendix 5). The results were read and recorded after 72 h using the API index Chart provided in the KIT.

3.8 *Cryptococcus* species isolation and characterization procedure

3.8.1 Specimen preparation

One gram of domestic Chicken dropping was weighed using a scale balance and then transferred to a 10 ml sterile round bottomed tube containing 5 mls distilled water then incubated for 1h with agitation (mix every 15 min). Two 1.7 ml appendoff tubes were filled with 900 µl distilled water and 100 µl of the original sample added to the first tube, (dilution factor 1:10) then 100 µl of the 1:10 dilution added to the last tube containing 900 µl distilled water, giving a dilution factor of 1:100 in the second dilution. For swabs, 100 µl of distilled water was added into a round bottomed tubes and the tip of each swab containing sample dipped into distilled water with agitation to make a suspension.

3.8.2 Primary isolation

The Niger seed agar and biphenyl media (Teknova, France) were used in detecting, isolation and identification of *Cryptococcus* species (Mseddi *et al*., 2010). The media was prepared according to the manufacturer's instructions under aseptic condition (Appendix 2). After autoclaving, the media was supplemented with 10 mls biphenyl stock solution, 400 µl Chloramphenical stock solutions and 500 µl ampicillin stock solution. The media was cooled to 50°C and dispensed in 25 ml amounts into 90 mm-diameter Petri dishes. The Petri dishes were labeled with Non random sample numbers. Approximately 100 µl of the sample's second dilution and similar amounts for swabs suspension, were inoculated onto the centre of Niger seed agar and spread using glass rods sterilized by

autoclaving and incubated at 30°C for 72h. The inoculums were checked daily for signs of growth.

3.8.3 Purification

Yeasts colonies from Niger seed agar were sub-cultured onto Sabouraud agar to purify cultures (Isenberg and Henry, 2004). The procedures were done according to the manufacturer's instructions using aseptic techniques. Approximately 25 mls of the media was dispensed into 90 mm-diameter Petri dishes (Appendix 2). Each Sabouraud agar plate was labeled with the same non random sample numbers. Suspected colonies were transferred from Niger seed agar to Sabouraud agar by using wire loops and incubated for 24h at 30°C to purify yeast before biochemical tests were done.

3.8.4 Urease test

Yeasts colonies from Sabouraud agar were further sub-cultured onto Christensen's urea agar to detect their ability to hydrolyze urea (urease positive) and incubated at 30°C for 24h. Urease positive yeasts turned the colour of media to pinkish. *Cryptococcus* suspected colonies were subjected to API 20C AUX (Bio Merieux, SA) for confirmation purposes.

3.8.5 Confirmation test of *Cryptococcus* species

Confirmation of *Cryptococcus* species was done using API 20C AUX (Bio Merieux SA). The basal medium in the ampoules was melted by placing in a boiling water bath for two minutes and allowed to cool. 20 mls of distilled water was dispensed into incubation trays and strip placed with the ampoules open (2 mm diameter). Suspension of the yeast was prepared and the density adjusted just below 1+ on a wickerham card, the strip was inoculated (20 cupules; approximately 0.2 ml each) following the manufacturer's instruction, and incubated at 30ºC for 72h (Appendix 5). The results were read and recorded after 72h using the API index Chart provided in the KIT.

CHAPTER FOUR

4. RESULTS

4.1 Yeasts isolated from Chicken droppings

The occurrence of yeasts in Kabigeriet village, were either as single or multiple infestations in all the samples analyzed. Out of all the samples analyzed 72.62% were positive for yeasts while 27.38% were negative (Fig. 3).

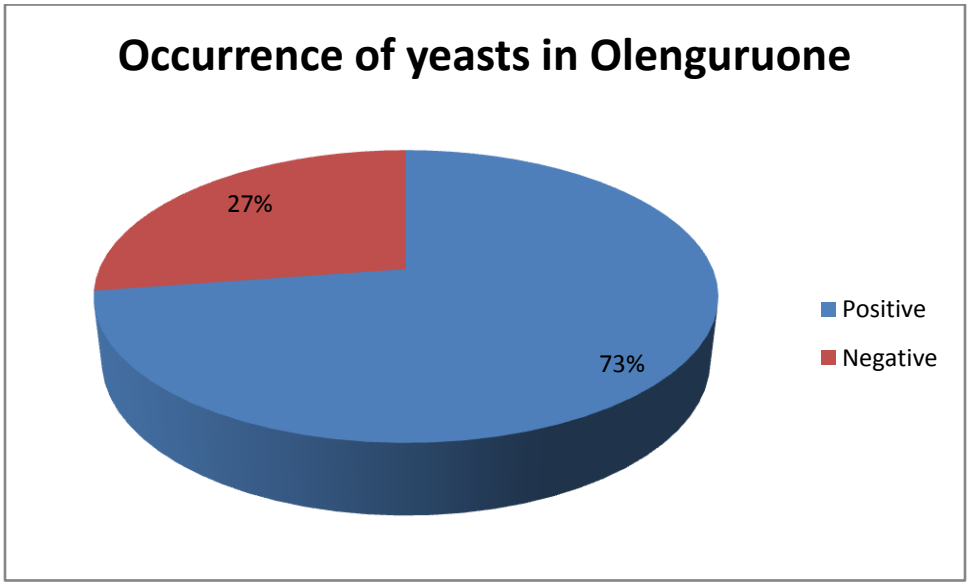

Figure 3: Occurrence of yeasts in Chicken droppings in Kabigeriet village, Olenguruone

4.2 Prevalence of yeasts in Kabigeriet village, Olenguruone

Four types of yeasts species were isolated in the study; *Candida* species 35/61(57.38%), *Geotrichum candidum* 23/61(37.70%), *Cryptococcus* species 2/61(3.28%) and *Saccharomyces cerevisiae* 1/61(1.64%) (Fig.4).

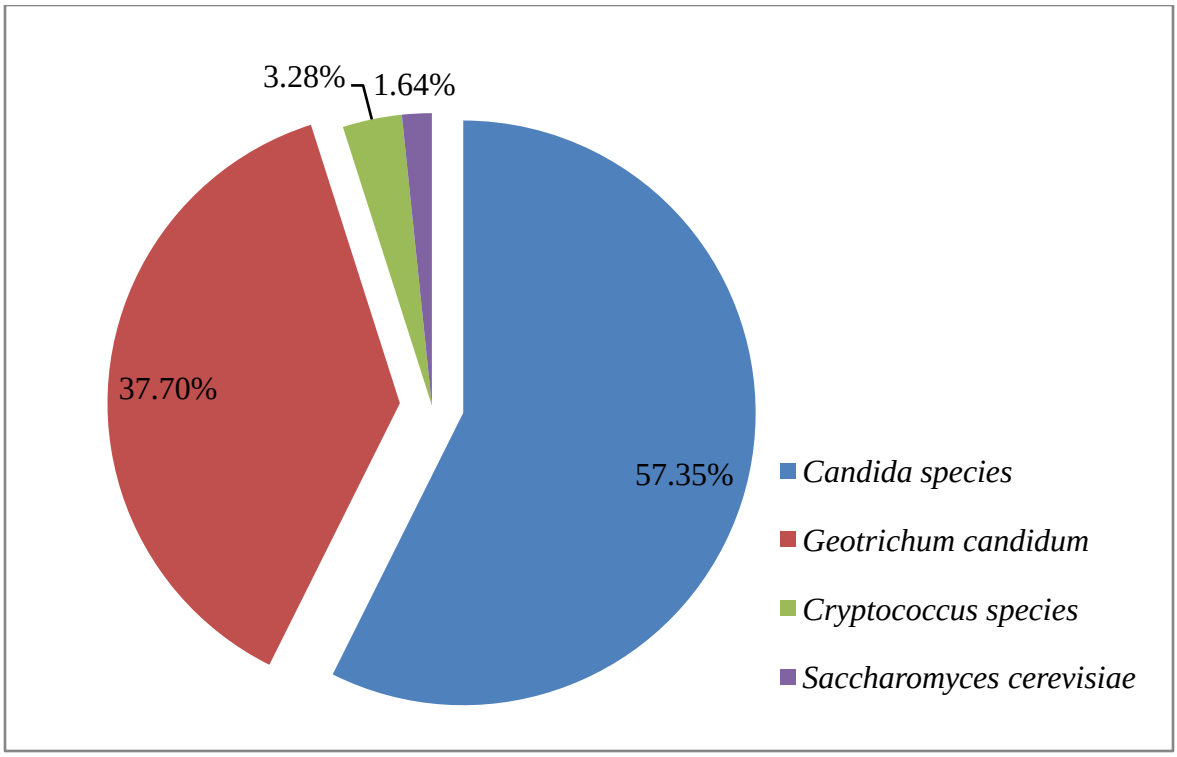

Figure 4: Percentage distribution of yeasts from samples collected from *Gallus gallus* droppings**.**

4.2.1 Yeasts species isolated from domestic Chicken droppings

During the study a total of 84 samples (58 guanos and 26 soils enriched with Chicken dropping), were collected from 32 homesteads out of which a total of 61 different yeasts species were isolated and identified (Table 1). The dominant yeast species was found to be *Geotrichum candidum* which was 37.70% of the total isolated species of yeast. This was followed by *Candida lusitanie (*14.75%); other species were *Saccharomyces cerevisiae, Candida ciferri, Candida guilliermondii, Cryptococcus neoformans,* and *Cryptococcus laurenti* which were 1.63% each.

Table 1: Species of yeasts isolated from the samples

Yeasts	n	%	Dominants
Geotrichum candidum	23	37.70	1
Candida lusitanie	9	14.75	2
Candida glabrata	7	11.47	3
Candida tropicalis	5	8.19	4
Candida albicans	5	8.19	4
Candida parapsilosis	3	4.91	5
Candida lipolytica	2	3.27	6
Candida krusei	2	3.27	6
Cryptococcus laurenti	1	1.63	7
Cryptococcus neoformans	1	1.63	7
Candida guilliermondii	1	1.63	7
Candida ciferri	1	1.63	7
Saccharomyces cerevisiae	1	1.63	7

4.3 Characterization and identification of the isolated *Candida* species

4.3.1Trypan blue agar

All samples collected were plated onto Trypan blue agar for primary isolation and purification purposes (Plate 7).

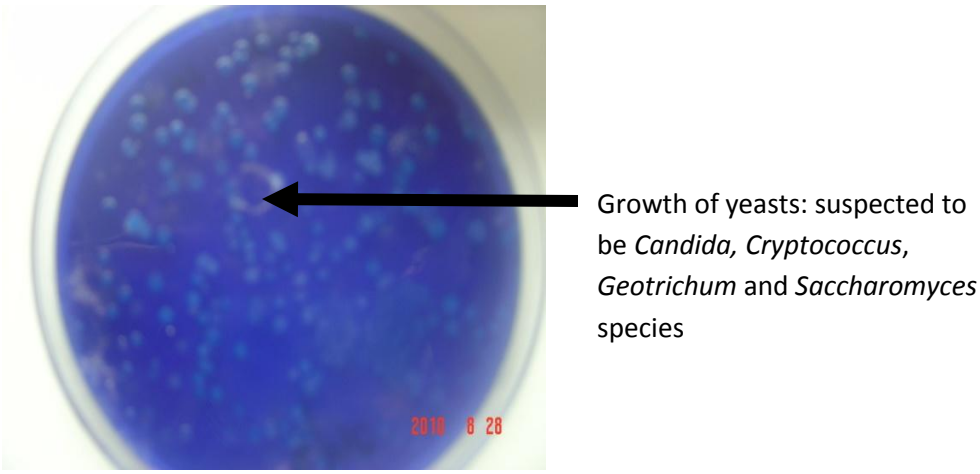

Plate 7: Trypan Blue agar, read after 3days incubation at 30°C. Only whitish coloured colonies with smooth edged were seen. It was not possible to determine whether there was a pure or mixed culture of yeasts based on the appearance.

4.3.2 CHROM agar

The CHROM agar supported the growth of all environmental yeasts isolates and its opaque to white background allows good discrimination among of colonies of different species with almost similar hues. A wide variety of colony colours were seen some which were species specific. Three *Candida* species, *Candida tropicalis* (Plate 8), *Candida krusei* (Plate 9) and *Candida albicans* (Plate 11), were presumptively identified. For conformations purposes the above *Candida* species were sub cultured to cornmeal agar.

The identification of 12 isolates based on CHROM agar was specific due to the presence of β-hexosaminidase substrate and other chromogenic substrates in the media. Differentiation of *Candida* species is due to presence of species-specific enzymes. *Candida tropicalis* colonies appeared smooth, mucoid, wide and blue in colour. These were differentiated from *Candida albicans* and *Candida krusei* which presented with greenish, smooth convex colonies and dry flat, rough pink colonies with pale borders with dark centre respectively and *Cryptococcus* species colonies appeared white, mucoid and raised (Plate 10).

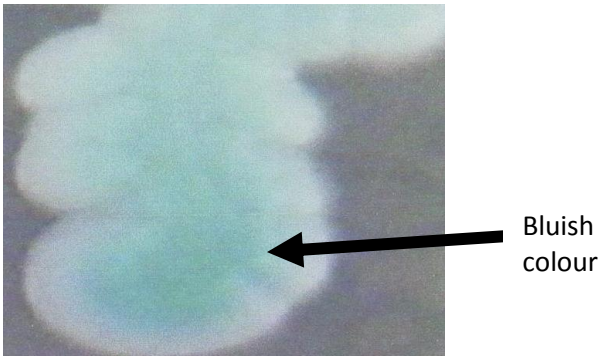

Plate 8: *C. tropicalis* showing bluish colouration of colonies on CHROM agar read after 48h at 30°C.

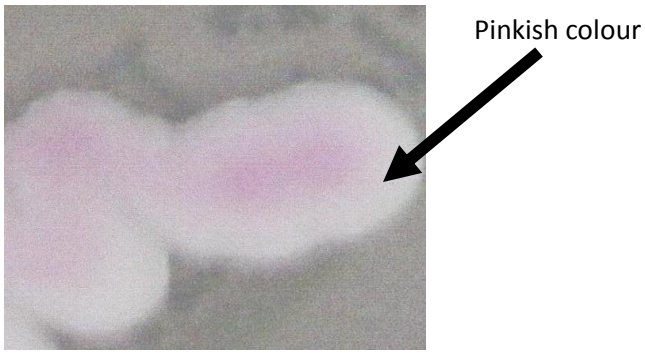

Plate 9: *C. krusei* showing pinkish colouration of colonies on CHROM agar read after 48h at 30°C.

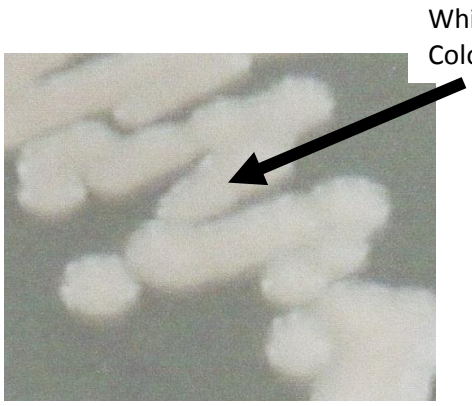

Plate 10: *Cryptococcus species* note whitish Colouration of colonies on CHROM agar read after 48h at 30°C.

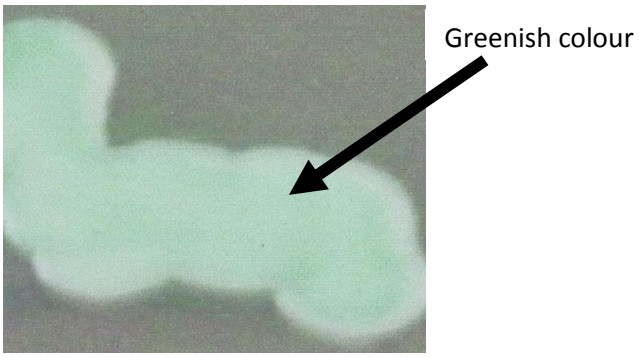

Plate 11: *Candida albican* note greenish colouration of colonies on CHROM agar read after 48h at 30°C.

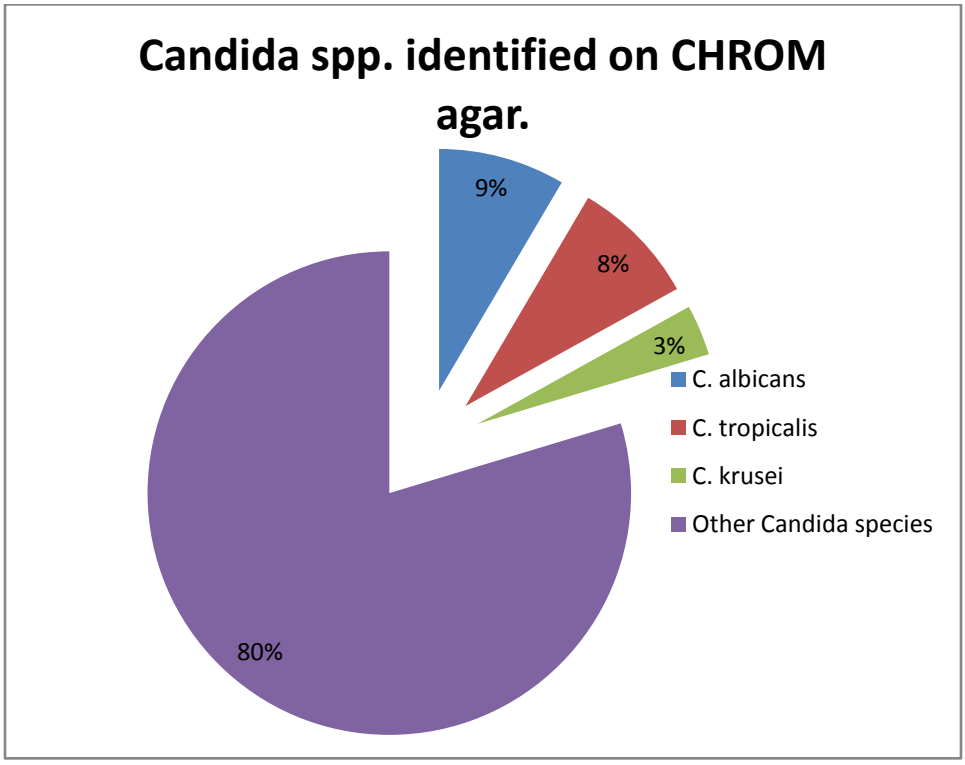

Figure 5: Types of *Candida* species Identified using CHROM agar.

The following types of yeasts after plating in CHROM agar were not presumptively indentified, since the colonies colour were not species specific (Table 2). They were subjected to further supplementary tests i.e. plated on cornmeal agar and API 20 AUX.

Table 2: Appearances of yeasts on CHROM agar after incubation at 37°C for 48h

Yeasts	n	Colour
Geotrichum candidum	23	Dark pink
Candida lusitanie	9	Light to dark brown
Candida glabrata	7	Light to dark brown
Candida parapsilosis	3	Light brown
Candida lipolytica	2	Light brown
Cryptococcus species	1	White
Candida guilliermondii	1	Blackberry wine
Candida ciferri	1	Light brown
Saccharomyces cerevisiae	1	Violet to brown

4.3.3 Cornmeal agar

All the *Candida* species which were not identified using CHROM agar were plated on cornmeal agar to study the formation of pseudohyphae, true hyphae, arthrospores and chlamydospores. 47 *Candida* species were identified in cornmeal agar at 37°C (WH0, 2009).

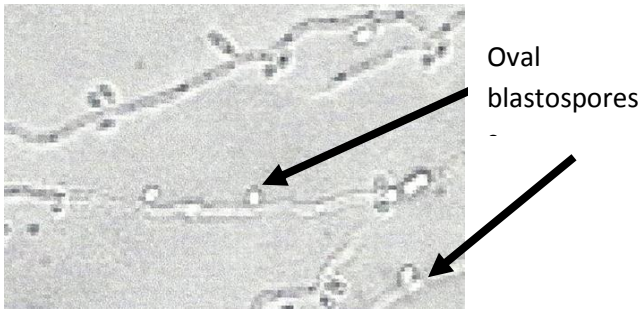

Plate 12: *C. tropicalis* in cornmeal agar showing oval blastospores read after 48h at 30°C.

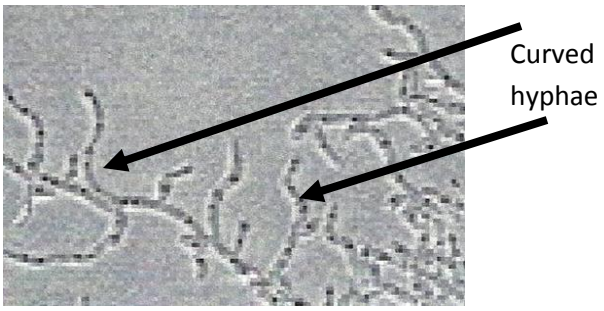

Plate 13: *C. parapsilosis* in cornmeal agar showing curved and large hyphae read after 48h at 30°C.

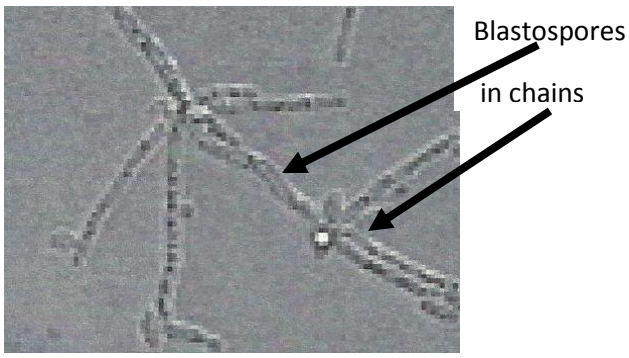

Plate 14: *C. krusei* in cornmeal agar showing pseudohyphae with moderate branching read after 48h at 30°C.

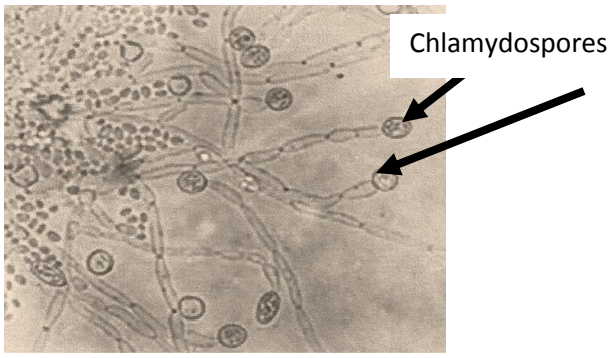

Plate 15: *C. albicans* in cornmeal agar showing chlamydospores read after 48h at 30°C.

On cornmeal tween 80 agar *Candida tropicalis* (Plate 12) at 30°C after 72h, along the long pseudohyphae produces blastospores which are oval in shape and blastospores may occur in clusters or singly. These is differentiated from *Candida parapsilosis* which on cornmeal tween 80 agar at 30°C after 72h (Plate 13), produces blastospores usually located along pseudohyphae and pseudohyphae are curved and 'giant cells' (large hyphal) are observed, while *Candida krusei* (Plate 14) on corn meal tween agar produces branched pseudohyphae usually with chains blastospores. Along the hyphae and at the septa points *Candida albicans* produces round blastoconidia (Plate 15) and also asexual

spores (typical) know as chlamydospores which are large, round and thick walled usually terminal.

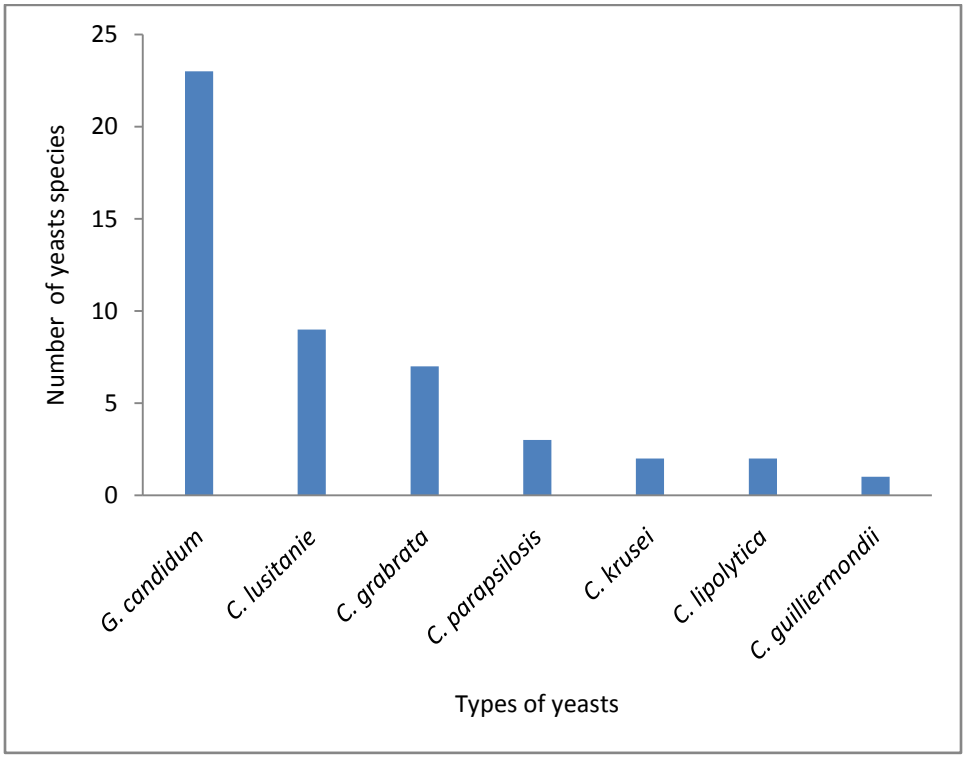

Figure 6: Number of yeasts species identified using Corn meal agar**.**

4.3.4 Confirmation of *Candida* species using API 20C AUX

API 20C AUX was able to confirm 5 *Candida* species and *S. cerevisiae* which could not be indentified using cornmeal agar, which analyses each species based on carbohydrates assimilation profile. The identification of each species was possible by referring to the manufacturers analytical profile index provided by Bio Merieux SA. The most common types of *Candida* species were *Candida lusitanie*, *Candida ciferri*, *Candida parapsilosis* and *Candida albicans* respectively in the ration of 2:1:1:1:1 respectively (Table 3).

Table 3: *Candida* species identified using API 20 C AUX

Candida species	n (+ve)	% (+ve)
Candida lusitanie	2	33.33
Candida ciferri	1	16.66
Saccharomyces cerevisae	1	16.66
Candida parasilosis	1	16.66
Candida albican	1	16.66

4.4 Characterization and identification of *Cryptococcus* species

4.4.1 Niger seed agar

All the 84 suspected samples were plated onto Niger seed agar to study the colony morphology and the existence of black colouration on agar which is suggestive of *Cryptococcus* species (Plate 16). Only 8 isolates showed *Cryptococcus* characteristics: brownish-black colouration due to uptake of diphenolic substrates in presence of phenoloxidase. Phenoloxidase of *Cryptococcus* oxidizes diphenolic via labile dopachrome intermediates, to melanin polymers.

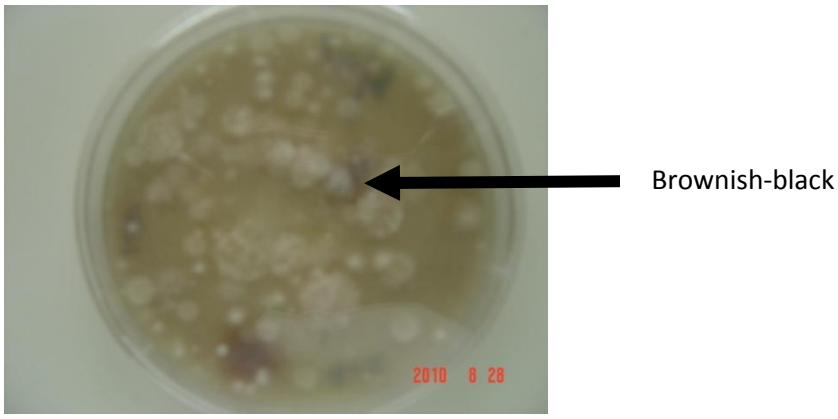

Plate16: Yeasts on Niger seed agar showing brownish-black colouration typical of *Cryptococcus*, read after 72h incubation at 30°C.

4.4.2 Urease test

All the 8 isolates tested were positive for urease (Plate 17). Hydrolysis of urea in Christensen's urea agar by *Cryptococcus* species was indicated by change of colour of the indicator from yellow-orange to pinkish red.

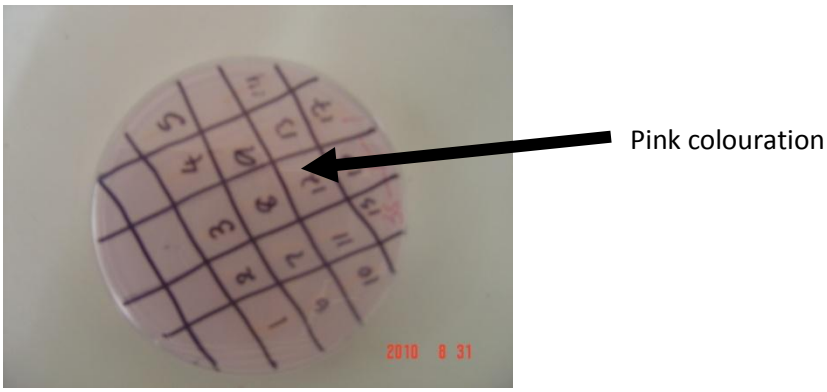

Plate17: Urease positive yeasts in Christensen's urea media, read after 24h at 30°C.

4.4.3 Confirmation of *Cryptococcu*s using API 20 C AUX

All the 8 urease positive isolates were subjected to API 20 C AUX and only 2 were confirmed as *Cryptococcus* species (Table 4).

Table 4: *Cryptococcus* species identified using API 20 C AUX

Types of *Cryptococcus*	n(+ve)	%(+ve)
Cryptococcus laurentii	1	12.5%
Cryptococcus neoformans	1	12.5%
None	6	75%

4.5 Identification of *Geotrichum candidum* using lactophenol cotton blue

Geotrichum candidum was mostly indentified in Lactophenol cotton blue stain characterized by chains of hyaline, one-celled, oval to rectangular arthroconidia. Arthroconidia give a bud appearance when they germinate at one end and they usually vary in size (Plate 18). Out of 35 colonies suspected to be mould, only *Ochroconic gallopana* which is known to be of medical importance was indentified. Also Hazard level 3 pathogens: *Histoplasma capsulatum* and *Blactomyces dermatidis* were suspected, however further characterization was not done because of bio-safety issues.

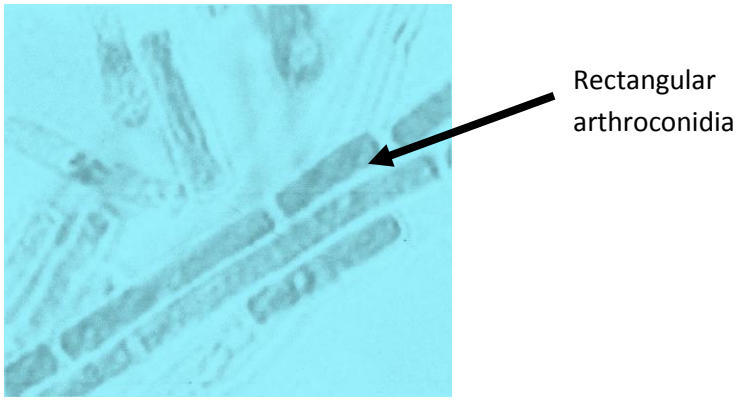

Plate 16: *Geotrichum candidum* in wet preparation of lactophenol cotton blue stain showing: chains of hyaline, smooth and one-celled, rectangular arthroconidia.

4.6 Student t- test

The student t-test was used to compare the means of various types of yeasts isolated from soils and Chicken dropping at 95% confidence interval using SPSS packages (Table 5).

Table 5: Yeasts isolated in Chicken droppings and soil

Yeasts types	Droppings isolated	Soil isolated
Geotrichum candidum	17	6
Candida species	26	9
Cryptococcus species	2	0
Saccharomyces cerevisae	1	0
Means ($\bar{x}$)	11.25	3.75

The finding showed that there was no significant difference in the yeasts isolated in the dropping to those isolated from the soil ($t = 2.07$, $df = 3$, $P= 0.13$). However, yeasts from droppings (mean 11.25 ± 5.86) were higher than those from soil (3.75 ± 2.25).

4.7 Correlation between yeasts occurrence and humidity

Yeasts grows well in environment where they is high humidity, however the study found out that when the humidity was high, few types of yeasts were isolated.

The interval and width were arrived at by the help of Stunge's rule

Interval: $K= 1+3.22 (\log_{10} n)$ i.e. K= no. of intervals, n= no. of value in a data set

$K= 1+ 3.22 (1.924) = 7.1824 \approx 7$

Width: $(W) = \text{Range } (R) / \text{No. of intervals } (K)$

$R= X \text{ largest} – X \text{ smallest} = 100\%-32\% = 68\%$

$W=68/7.1824 = 9.4 \approx 9$

Table 6: Correlation of yeasts isolated and humidity

Humidity range	***Geotrichum candidum***	***Candida species***	***Cryptococcus Species***	***Saccharomyces cerevisiae***	**Total isolated**
0 – 10	0% (0)	0% (0)	0% (0)	0% (0)	0
11 – 20	40% (2)	60% (3)	0% (0)	0% (0)	5
21 – 30	20% (2)	70%(7)	0% (0)	10% (1)	10
31 – 40	37.5%(3)	62.5% (5)	0% (0)	0% (0)	8
41 – 50	50% (2)	25% (1)	25% (1)	0% (0)	4
51 – 60	13.3% (2)	80% (12)	6.7% (1)	0% (0)	15
61 – 70	0% (0)	0% (0)	0% (0)	0% (0)	0
71 – 80	37.5% (3)	62.5% (5)	0% (0)	0% (0)	8
81 – 90	50% (2)	50% (2)	0% (0)	0% (0)	4
91 – 100	50% (2)	50% (2)	0% (0)	0% (0)	4
Mean % isolated	29.83a	46.0a	3.17b	1.0b	

NB: Mean percentages isolated denoted by similar letters are not significantly different at 95% CI.

No significant relationship in number of yeasts and humidity (r = 0.93). The numbers of yeasts were found to decrease slightly with the increase in the humidity. When the humidity was high, few types of yeasts were isolated. However, the mean number (29.83a) of *Geotrichum candidum* and *Candida* species was higher than that of *Cryptococcus* Species and *Saccharomyces cerevisae.*

4.8 Relation between yeasts occurrence and temperature

At low temperature (0-10°C) yeasts will not grow, but not die either. At temperatures 10-37°C yeasts will grow and multiply faster. At higher temperature the yeasts cell become stressed.

Interval and width were arrived at by the help of Stunge's rule

Interval same as describe correlation between yeasts occurrence and humidity.

Width (W) = Range (R)/No. of intervals (K), R= 41°C – 11.4°C = 29°C, K = 7.1824

W = 29.6/7.2 = 4.11

Table 7: Correlation of yeasts isolated and temperature

Temperature range	***Geotrichum candidum***	***Candida species***	***Cryptococcus Species***	***Saccharomyces cerevisae***	**Total isolated**
11 – 15	50% (5)	50% (5)	0% (0)	0%(0)	10
16 – 20	43.8% (7)	56.3% (9)	0% (0)	0% (0)	16
21 – 25	8.3% (1)	83.3%(10)	0% (0)	8.3% (1)	12
26 – 30	22.2% (2)	66.7% (6)	11.1% (1)	0% (0)	9
31 – 35	36.4% (4)	54.5% (6)	9.1% (1)	0% (0)	11
36 – 40	33.3% (1)	66.7% (3)	0% (0)	0% (0)	3
Mean %	32.33a	62.92a	3.37b	1.38b	

NB: Mean percentages isolated denoted by similar letters are not significantly different at 95% CI.

No significant relationship in number of yeasts and temperature (r = -0.67). The numbers of yeasts were found to decrease with the increase in temperature. When the temperature was high, few types of yeast were isolated. Using ANOVA to establish the effect of temperature on the occurrence of the fungi species, it was established that there

was a significant difference ($P<0.05$) in the temperature effects. Growth of *G. candidum* and *Candida* species were significantly affected particularly by the temperature ranges of 11–15 and 16 – 20^{o}C.

4.9 Relation between yeasts occurrence and elevation

In this study it was found out that the numbers of yeasts were found to decrease with the increase in elevation.

Intervals and width were arrived at by help of Stunge's rule

Interval same as described in 3.5.2

Width (W) = Range (R)/No. of interval (K)

R = X largest – X smallest = 9747 – 7839 = 1908

W = 1908/7.2 =265

Table 8: Correlation of yeasts isolated and elevation

Elevations range	***Geotrichum candidum***	***Candida species***	***Cryptococcus species***	***Saccharomyces cerevisae***	**Total isolated**
7839 – 8139	42.1% (8)	57.9% (11)	0%(0)	0%(0)	19
8140 – 8439	22.5% (9)	70% (28)	5% (2)	2.5% (1)	40
8440 – 8739	0%(0)	0%(0)	0%(0)	0%(0)	0
8740 – 9039	0%(0)	0%(0)	0%(0)	0%(0)	0
9040 – 9339	100% (2)	%(0)	0%(0)	0%(0)	2
Mean %	32.92a	25.58a	1.00b	0.00b	

NB: Mean percentages isolated denoted by similar letters are not significantly different at 95% CI. There were no significant differences in the means.

There was no significant relationship in number of yeasts and Elevation ($r = 0.99$). The numbers of yeasts were found to decrease with the increase in elevation. When the elevation was high, few types of yeasts were isolated.

CHAPTER FIVE

5. DISCUSSION

The aim of this study was to isolate and characterize yeasts that are found on domestic Chicken dropping and soil enriched with dropping as a possible source of infections to humans particularly immunosupressed individuals. Faeces from birds such as pigeon or soil contaminated from birds' faeces provide good nutrients for growth of pathogenic yeasts like *Cryptococcus* species, are therefore possible source of infection and reservoir (Reimao *et al*., 2007). In Kabigeriet village, Olenguruone, most people breed Chickens in their yards, faeces were cultured. The kind of yeasts isolated from Chicken samples in this study, such as: *G. candidum* (37.70%), *Candida* species (54.05%), *Cryptococcus* species (3.27%) and *S. cerevisae* (1.63%) (Table 1), agree with what has been isolated in the past (Cafarcha *et al*., 2008) *Candida albicans*, *C. tropicalis, C. glabrata, C. lusitanie*, *C. guilliermondii*, *Cryptococcus neoformans*, *C. lauretii*, *Trichosporon beigelii* and *Saccharomyces cerevisiae* have been isolated in wild birds dropping before. There was higher isolation rates of yeasts in Chicken dropping samples (72.13%) compared to soil (27.87 %) (Table 5). This could be attributed to soil exposure to harsh environmental condition like availability of nutrient in the soil, dilution of soil especially during raining seasons and sunlight that could not be conducive for yeast survival for a long time.

Candida species identification was performed using three mycological methods applied sequentially to avoid false results and ensure accuracy. This is because misidentification of yeasts species has been previously reported in results from the use of single identification methods (Lo *et al*., 2001). Culture CHROM agar contains a chromogenic, 5-bromo-6-chloro -3- indolyl phosphate P-toluidine (chromogenic substrates) and 5-

bromo-4-chloro-3-indolyl/ N-acetyl-β-D-glucosaminide, which reacts with species specific enzymes to give yeasts colonies which are different in colour (Plate 8, 9, 10, 11) (Ghelardi *et al*., 2007), and Corn meal, which identifies *Candida* species based on formation of chlamydospores, pseudohyphae, true hyphae, and arthrospores (Plate 12, 13, 14, 15), were performed initially as these are the recommended methods (WHO, 2009). However, API 20C AUX system was then applied as a third step to identify non-chlamydospore forming species based on their carbohydrate assimilation profiles. While *Cryptococcus* species identification was performed using three mycological methods to avoid false results; culture on Niger seed agar which identifies *Cryptococcu*s species by existence of brownish-black colouration (Mseddi *et al*., 2010 Cheikn *et al*., 2008). Formation of black colouration by *Cryptococcus* is due to uptake of diphenolic substrates in presence of phenoloxidase (Fatma *et al*., 2011, Mseddi *et al*., 2010, Cheikn *et al*., 2008, Cheikn *et al*., 2006, Nasri and Triki, 2004, Fortes *et al*., 2001). Phenoloxidase of *Cryptococcus* oxidizes diphenolic via labile and dopachrome intermediates, to melanin polymers. Urease test which was performed by sub-culturing on Christensen's urea media and API 20C AUX system was then applied as a final step for confirmation purposes.

Culture results indicated high yeasts prevalence (98.36%) than moulds (1.64%). Of the samples investigated 10.71% had mixed yeasts infestations. Yeast multi-species co-existence has been noted in previous studies which indicated that *Candida* species especially *C. albicans* coexist in synergy with other species (Patricia *et al*., 2012). Culturing is regarded as the gold standard for confirming diagnosis of fungal infection with a specificity of 100% but variable sensitivity (Lebowitz *et al*., 2002, Stepanovic *et al*., 2008, Cheik-rouhou *et al*., 2008, Cheik-rouhou *et al*, 2006, Bradsher *et al.*, 2003,

Koneman *et al*., 2007). However the sensitivity of the test is determined by sample collection techniques, storage and transport conditions. *Candida* species were the most common yeasts isolated in domestic Chicken droppings and soils enriched with dropping as follows: *C. lusitanie* (14.75%), *C. glabrata* (11.47%), *C. tropicalis* (8.19%), *C. albicans* (8.19%), *C. parasilosis* (4.19), *C. lipolytica* (3.27%), *C. krusei* (3.27%), *C. guilliermondii* (1.63%) and *C. ciferri* (1.63%). The present results show that *C. lusitanie* is the most common *Candida* species isolated from the droppings in contrast with previously reported studies which indicated that *Candida albicans* was the most common species (Brilhante *et al*., 2010). This difference could be due to regional variations associated with geographical location and environmental condition such as humidity, soil type and temperature and the species of birds.

*Cryptococc*i found in soils or decaying wood contaminated with bird faeces e.g. pigeon faeces (Lazera *et al*., 2000, Reimao *et al*., 2007) and desiccated yeast from Chicken dropping can transmit the fungal infection to humans primarily through inhalation of spores. *C. laurentii* species occur worldwide but its natural habitat is yet to be discovered and properly identified. However infections caused by this species have been reported in severe immunosupression cases (Esaki *et al*., 2006). There were minimal cases of Cryptococcosis before the AIDS pandemic. Due to HIV/AIDS pandemic the disease has emerged as the major cause of infirmity and death in infected HIV victims (Esaki *et al*., 2006). Also pneumonia cases caused by *C. laurentii* in HIV patients have been reported. *C. neoformans* has been mostly isolated in dropping (faeces) from avian species (Granados and Castaneda, 2005, Rosario *et al*., 2005, Abegy *et al*., 2006, Fili u *et al*., 2002, Kobayashi *et al*., 2005).

The isolation rate of 1.81% of *C. neoformans* from cloaca specimen of birds e.g. pigeons has been reported, similar results were obtained from the present study with isolation of 3.26% of *Cryptococcus* species (*C. laurentii* (1.63%) *and C. neoformans* (1.63)) from domestic Chicken dropping. This is in agreement with a study done by Laubscher *et al*., (2000) which isolated the same species of *Cryptococcus* (*C. laurentii*) in the trachea of broiler Chicken. It is possible that such low isolation rate is mainly due to the high temperature of birds leading to poor/low survival of the fungi in the Chicken body. The high concentration of ammonia in fresh birds dropping (alkaline pH) is unfavorable for yeasts growth. Also the fast growth of fungus such as Zygomycetes found in soil and faeces (contaminant fungi) can lead to false negative results due to competitive inhibition (Kobayashi *et al*., 2005). Isolation of *Cryptococcus* from Chicken dropping is difficult since it can be affected by some biotic factors such as mites, amoebae and soil bacteria when grown on media by inhibiting or killing the yeasts. It has been reported that the number of *Cryptococcus* species colonies is inversely proportional to the number of other pathogenic yeasts colonies in a culture plate (Akram *et al*., 2005, Springer *et al*., 2010, Misuzu *et al*., 2004).

Several studies have been done to examine *Cryptococcus* presence in birds' and birds dropping, including aviaries in which multiple species of birds are housed together at close range (Nosanchuk *et al*., 2000, Gugnanii *et al*., 2004, Malik *et al*., 2003, Gokulshankar *et al*., 2004, Bauwens *et al*., 2004, Kielstein *et al*., 2000). All these studies found out that avian species harbors' *Cryptococcus* but no reasons for this specificity has been determined. The data available comparing the level of growth of *Cryptococcus* in avian species is mainly due to the composition of nutrient in droppings. This finding has

showed the role birds play, particularly domestic Chicken in the transmission of *Cryptococcus*. The growth of *Cryptococcus* and other pathogenic organism on Chicken and birds dropping explains the mechanism through which birds e.g. pigeon and Chicken play in harboring *Cryptococcus* and other organism, in their body parts i.e. internal organs or on external tissues e.g. feathers or feet that come in contact with their droppings (Fisher *et al*., 2002, Malik *et al*., 2003, Nosanchuk *et al*., 2000, Kao *et al*., 1957).

The mechanism by which the birds' excreta get infected with pathogenic yeasts is still not known (Filiu *et al*., 2002). However, this can be attributed to availability of fungus in air, soil or foods consumed by Chicken and spores/fungi cell can be dispersed by blowing wind settling on birds' excreta. It has been found out that non-infected birds (pigeon) dropping get infected after they get exposed to air containing aerosolubilized cells of pathogenic yeasts (*C. neoformans*) (Camile *et al*., 2008). The results obtained in the present study, together with the claims made by authors (cited), confirm the environmental contamination of the guano.

Student t-test at 95% confidential intervals were used to compare the means of various types of yeast obtained from the soil with those obtained from the Chicken droppings and it showed that there were no significant differences in occurrence of yeasts in soil and dropping (P=0.13). However, yeasts from droppings (mean 11.25±5.86) were higher than those from soil (3.75±2.25) (Table 5). This could be due to the harsh environmental condition in the soil. In this study, there was no significant difference in relationship of occurrence of yeasts and humidity (r = 0.93), however, most yeast were isolated between humidity of 51 % to 60% (Table 6). This agreed with a study done by Misuzu *et al*.,

(2004) where the majority of yeasts were isolated during dry season compared to rainy season. This showed there were no relationships between humidity and the occurrence of yeasts in the environment. Also there were no relationships in occurrence of yeasts with temperature (r = -0.67) (Table 7) and elevation (r = 0.99) (Table 8). Attention now is focused on the origin and epidemiology of the yeasts, due to the rise in yeasts infections and involvements of yeasts in different types of human diseases.

5.1 Conclusions

The results of this work demonstrated that domestic chicken (*Gallus gallus*) harbor *Cryptococcus, Candida species, Geotrichum candidum* and *Saccharomyces cerevisae* in their dropping and their close proximity to human habitation poses a risk of cryptococcal, *Candida, Geotrichum* and *Saccharomyces* infections in HIV/AIDS immunocompromised persons. This could partly explain the high incidence of cryptococcosis and candidiasis in HIV/AIDS patients in Kenya. The study signifies the need to discover more environmental niches for yeasts especially of *Cryptococcus* species. The results obtained in the present investigation suggest that humans cohabiting with Chicken are at a risk of contracting yeasts infections. The isolated yeasts species are not endosaprobiotic in the avian digestive tract and that their primary source might be the environment.

5.2 Recommendations

More research should be done to identify and understand more about the habitat of the pathogenic yeasts specially *Cryptococcus* species and their transmission from environment to humans. Farmers rearing Chicken should observe the following measures to reduce transmission of yeasts from Chicken to people; this will help to significantly reduce the risk:

1. Proper hygiene should be observed when handling Chicken e.g. wear protective clothing
2. Have regular vet checks and keep detailed records of any testing done on Chicken.

3. High standards of hygiene should be maintained in poultry house i.e. regularly cleaning to avoid accumulation of dropping.
4. The disturbance of soil and other materials associated with a high risk of exposure to pathogenic yeast should be avoided by all persons with HIV infection. In particular, avoidance of accumulations of bird droppings is prudent. However, certain risk groups such as the very young, the elderly and the immune compromised should take extra care.

6. REFERENCES

Abegy M. A., Cella F.L., Faganello J., Valente P., Schrank A., Vainstein M.H. (2006). *Cryptococcus neoformans e Cryptococcus gattii* isolated from excreta of psittaciformes in a Southern Brazilian Zoological garden. Mycopathologia. 161 (2):83-91.

Adela E.A., Christopher H. (2005). Invasive *Saccharomyces* infection: A comprehensive review. Clinical Infectious Diseases. 41:1559-1565.

Adelaide O.A, Bii C, Okemo P, (2008). Antibiotic resistance and virulence factors in *Escherichia Coli* from broiler Chicken slaughtered at Togoni processing Plant in Limuru. East African Medical Journal. 85(12):597-606.

Akram M.S.H., Sinasi T.Y., Ahmet G., Mehmet A.S. (2005). *Cryptococcus neoformans* varieties from material under the canopy of eucalyptus trees and pigeon dropping samples from four major cities in *Jordan*. Mycothologia. 158:195-199.

Akpan A., Morgan R., (2002). Oral candidiasis. Postgraduate Medical Journal. 78: 455-459.

Ailvarez M.L., Suarez B.L., Calcedo L.D. (2009). Isolation of *Candida dubliniensis* for the first time in Cali, Colombia and its identification with phenotypic methods. Mycopathologia, 167:19-44.

Amendola J., Rees N. (2002). Understanding baking: The art and science of baking. John Wiley and Sons. P. 36. ISBN9780471405467.

Barreto de Oliveira M.T., Boekhout T., Theelen B. (2004). *Cryptococcus neoformans* shoos'remarkable gemotypic diversity in Brazil. Journal Clinical Microbiology. 42:1356-1359.

Baumgardner DJ., Knavel E.M., Steber D., Swain G.R. (2006). Geographic distribution of human Blastomycosis case in Milwaukee, Wisconsin, USA: association of urban watershed. Mycopathologia. 161:275-282.

Baumgarder D.J., Halsmer S.E., Egan G. (2004). Symptoms of pulmonary Blastomycosis: northern Wisconsin, United States. Wilderness Environmental Medicine. 15:250-256.

Bauwens L., Veicamment F., Wuytack C., Van looveren K., Swinne D. (2004). Isolation of *Cryptococcus* in Antwerp zoo's nocturnal house. Mycoses. 47:292-296.

Bertha M., Maria A.M., Gabriel P., Amado R. (2010). Molecular characterization of *Histoplasma capsulatum* isolated from an outbreak in treasure hunters. Infectious Diseases. 10:264.

Blot S.I., Vandewoude K.H., De waele J.J., (2007). *Candida* peritonitis. Curriculum Opinion of Critical Care. 13:195-1999.

Buekhout T., Theelsen B., Diaz M. (2001). Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans*. Microbiolology. (Reading England) 147:155-168.

Bouakline A., Lacrox C., Roux N., Gangneux J.P., Derovin F. (2000). Fungal contamination of food in hematology units. Journal Clinical Microbiology. 38:4272-4273.

Botello N.S., Depaila S.B., Panagio L.A., Pinge-Filho P. (2012). *Candida* species isolated from urban Bats of Londrina-Parang Brazil and their potential virulence. Zoonoses. 15:16-22.

Bradsher R.W., Chapman S.W., Pappas P.G. (2003). Blastomycosis. Infectious Diseases Clinical North America. 17:21-40.

Brawner D.L and Cutler J, (2002). Oral albicans isolates from non hospitalized normal carriers, immunocompetent hospitalized patients and immucompromised patients with or without acquired immunodeficiency syndrome. Journal Clinical Microbiology. 27:1335-1340.

Breton G., Adle-Biassette H., Therby A., Ramanoelina J., Chaudat L., Bissuel F., Huerre M., Drumer F., Dupent B., Lortholary O., (2006). Immune reconstitution inflammantory syndrome in HIV-Infected patients with disseminated histoplasmosis AIDS.20:118-1121.

Brilhante R.S.N., Debora S.C.M., Casteto B., Duarte P.S.G., Palva M.A.N., (2010). Yeast microbiota of raptors: a possible tool for environmental monitoring. Blackwell LTD. 4:189-193.

Brol C.G., Cotton L., Samayoa B., Paz L., Samayoa C.A., Quattrini E., Gonzalez M., Velasquez-porta T., Arathan E. (July7-12,2002). *Cryptococcus neoformans* infection in person living with HIV/AIDS that attend to urban hospitality clinic in: proceeding of the international conference.14 (Abstract no. B10259).

Burker K., Mycotic diseases. In: Willians E.S., Barker L.K. (Eds) (2001). Infectious diseases of wild mammals. Indian: Manson publishing. The veterinary press. P520-522.

Camile L., Larissa A.Z., Graziella C.G.S., Renata C.F.S., Marisol D.M. (2008). Screening of antigenemia and isolation of *Cryptococcus neoforman* and *C. gattii* from cloaca and crop of bird in the State of Parana Brazil. Veterinary press. 28:7.

Cafarcha C., Romito D., Iatta R., Carmarda A., Montagna M.T., Otronto D. (2006). Role of birds of prey as carries and spreaders of *Cryptococcus neoformans* and other zoonotic yeast. Medical Mycology. 44 (6):484-492.

Calderonne R. (2002). *Candida* and Candidiasis. Washington: America Society of Microbiology press.

Centre for Diseases Control and Prevention National centre for emerging and zoonotic infectious diseases (NCEZID) July 28, 2010. Division of Foodborne, waterbone and environmental diseases (DFWED).

Cheikn-Rouhou S., Besbes S., Lugnay G., Bleckerc C., Deroamec C., Attiaa H., (2008). Sterol composition of black cumin (Nigella sativa L.) and Aleppo pine (Pinus halepensis mill). Seed oils. Journal food compost anal. 21:162-168.

Cheikn –Rouhou S., Hentati B., Besbes S., (2006). Chemical composition and lipid fraction characteristics of Aleppo pine (Pinus halepensis mill) seed cultivated in Tunisia. Food Science Techniques international. 12:407-415.

Colombo A.L., Niccs M., Park B.J., Nover S.A., Da Matta D. A. (2006). Epidemiology of candidemia in Brazil: a nationawide sentinel surveillance of candidemia in eleven medical centers. Journal Clinical Microbiology 44:2816-2828.

Cookie V.M., Miles R.J., Price R.G., Midley G., Khamri W., Richardson A.C. (2002). New chromogenic agar medium for the identification of *Candida* species. Environmental Microbiology. 68:3622-3627.

Corrado G., Livio P., Bruno M., Dumenico D., Rosa F. (2005). Invasive infection caused by Trichosporon species and *Geotrichum capitatum* in patients with hematological malignancies. Journal Clinical Microbiology. 43:1818-1828.

Costa C.R., de lemos J.A., Passes X.S., de Araudo C.R. (2006). Species distribution and antifugal susceptibility profile of oral *Candida* isolates from HIV-infected patients in the antiretroviral therapy era. Mycopathology 162:45-50.

Couppie P., Sobesky M., Aznar C., Bichart S., Clyti E., Bussel F., Elguedj M., Alvarev F., Demar M., Louvel D., Pradinaid R., Carme B. (2004). Histoplasmosis and acquired immunodeficiency syndrome a study of prognostic factors. Clinical Infectious Diseases. 38:134-138.

Cunningham M.J., Noble W.C., (1989). SDS-PAGE protein patterns of yeasts from human sources. Mycoses. 32:344-348.

Da silva E.H., Ruiz L.S., Matsumoto F.E., Auler M.E., Giudice M.C. (2007). Candiduria in a public hospital of Sao Paulo (1999-2004): Characteristics of the yeast isolates. Institute Medicine Tropical Sao Paulo. 49:349-353.

Datta K., Barlett K.H., Baer R. (2009). *Cryptococcus gattii* working group of the Pacific Northwest. Sread of *Cryptococcus gattii* into Pacific Northwest region of the United States. Emerging Infectious Diseases. 15:1185-1191.

David R.A., Robert G.R., Patricia A.C., Sulanne M. (2010). Detection of Latin America strains of *Histoplasma* in a murine model by use of a commercially available antigen test. Clinical Vaccine. Immunology. 17:802-806.

De Almeida O.P., Scully C. (2002). Fungal infection of the mouth. Brazil Journal oral Science. 1:19-26.

Dixit A., Carroll S.F., Qureshi S.T. (2009). *Cryptococcus gattii*: an emerging cause of fungal diseases in North America. Interdisciplinary Perpect infectious Diseases 840452, Dmid, 9503836, published online May 25, 2009.

Eileen L., Richard C.B. (2003). The use of Niger seed agar to screen for *Candida dublieniensis* in the clinical microbiology laboratory. Diagnostic micro-infectious. Diseases. 46:13-17.

Eiland E.H., Hassoun A., English T., (2008). Points of concern related to the micafungin versus caspofungin trial. Clinical Infectious Diseases. 46:640-641.

Ellepola A.N.B., Hust S.F., Elie C.M., Morrison C.J., (2003). Rapid and unequivocal differentiation of *Candida dubliniensis* from other *Candida* species using species-Specific DNA probes Comparison with phenotypic identification methods. Oral microbiology Immunology 18:379-388.

Ellepola A.N.B., Samaranayake L.P. (2000). Antimycotic agents in oral candidosis: an overview: 1. clinical variants. Dent Update 27: 111–116.

Erdem H., Cetin M., Timuruglo T., Citin A. (2003). Identification of yeasts in public hospital primary care patients with or without clinical vaginitis. Aust. Z.Nzl. Obstetrics Gynaecalogy.43:312-316.

Esaki M.S., Nagaligeswatan K., Devaleenol B., Srinivasan R. (2006). Pneumonia and pleural effusion due to *Cryptococcus laurenti* in clinical proven case of AIDS. Clinical Respiratory Journal, 13 (5):275-278.

Falcone M., Barzaghi N., Carosi P., Minoli L., Ravasio V., (2009). *Candida* infective endocarditis: report of 15 cases from a prospective multicenter study. Medicine (Baitimore) 88:160-168.

Fanci R., Pecile P. (2003). *Geotrichum capitatum* fungemia in a patient with acute myeloid leukemia: case report. Journal chemotherapy. 13:412-413.

Fatma M., Amira S., Hayet S., Fatma C., Fattouma M. (2011). Two new media Pinus halepensis seed agar and blackberry agar for rapid identification of *Cryptococcus neoformans*. Mycoses.54:350-353.

Ferreira-Paim K., Andrade-Silva L., Mora D.J., Pedrosa A.L., Rodrigues V., and Silva-Vergata M.L., (2010). Genotyping of *Cryptococcus neoformans* isolated from captive birds in Uberaba, Minas Gerais, Brazil. Mycoses. 10:1439-0507.

Filiu W.F., Wanke B., Anguena S.M., Vilela V.O., Macedo R.C., Lazera M. (2002). Avian habitats as a source of *Cryptococcus neoformans* in the city of Campo Grande, Mato Grussu do Sul, Brazil. Rev. Sol. Brazil Medicine Tropical. 35:591-595.

Fisher M.C., Koenig G.L., White T.J., Taylor J.W. (2002). Molecular and phenotypic description of *coccidiodes posadasii* sp. Nov. previously recognized as the non-California population of *coccidiodies immitis*. Mycologia. 94:74-84.

Fotedar R., Al-hedaithy S.A., (2003). *Chlamydospores*-negative *Candida albicans* using CHROM agar Candida medium. Mycoses. 46:96-103.

Forbes B.A, Anaissie M.J., Taylor B.H. (2002). Bailey and Scott's Diagnostic microbiology, 10th edition. Pg 995. Elservier health sciences.

Fortes S.T., Lazera M.S., Nishikawa M.M., Macedo R.C.L., Wanke B. (2001). First isolation of *Cryptococcus neoformans* var. *gatii* from a native jungle tree in Branzilian Amazon forest rainforest. Mycoses. 10:1439-0509.

Fleming R.V, Waist T.J and Anaissie E.J, (2002). Emerging and less common fungal pathogen. Infectious Diseases Clinical North America. 16:915-933.

Fraser M., Kimberly S. (2011). Erythema multiforme. http://northshoreji.staywellsolutiononline.com/library.Enctclopedia/85,p00282.

Gauthier G., Safdar N., Klein B., Andes D. (2007). Blastomycosis in solid organ transplant recipients. Transplant infectious disease. 9: 310-317.

Ghelardi, E., Pichierri, G., Castagna, B., Barnini, S., Tavanti, A., Campa, M. (2008). Efficacy of Chromogenic *Candida* Agar for isolation and presumptive identification of pathogenic yeast species. Clinical Microbiology Infectious. 14: 141–147.

Giammanco G.M., Pizzo G., Pecorella S., Pecoraro V., Micici M.E., (2002). Identification of *Candida dubliniensis* among oral yeast Isolates from an Italian population of human immunodeficiency virus- infected (HIV+) subject. Oral Microbiology Immunology 17:89-94.

Gil-Lamaignere C., Roilides E., Hacker J., Müller F-MC.(2003). Molecular typing for fungi – a critical review of the possibilities and limitations of currently and future methods. Clinical Microbiology Infectious. 9: 172–185.

Gokulshankar S., Ranganathan S., Ranjith M.S., Ranjithsign A.J. (2004). Prevalence serotypes and mating patterns of *Cryptococcus neoformans* in pellents of different avifauna in Madras, India. Mycoses. 47:310-314.

Golecka M., Oldakowska –jedynak U., Mierzwinskia-Nastalska E., Adamczyk-Sosinska (2006). *Candida*-associates denture stomatitis in patients after immunosupression therapy. Transplant procedure. 38:155-156.

Gugnani H.C., Mitchell T.G., Litvintseva A.P. (2004). Isolation of *Cryptococcus gattii* and *Cryptococcus neoformans* var. *grubii* from the flowers and bark of Eucalyptus trees in India. Medical Mycology. 43:565-569.

Gutierrez J., Morales P., Gunzalez M.A., Quindos G. (2002). *Candida dubliniensis*, a new fungal pathogen. Journal Basic Microbiology. 42:207-227.

Grundet S., Mayser P., Redmann T., Kaleta E.F., (2005). Mycological examination on the fungal flora of the Chicken comb. Mycoses, 48:114-119.

Grusse P., Tinteinot K., Sullver O., and Schmitz B. (2001). Encephalomyelitis due to *Cryptococcus neoformans* var. *gattii* presenting as spinal tumor: Case report and review of the literature. Journal of Neurology, Neurosurgery and psychiatry. 70:113-116.

Garvey K., Hinshaw M., Vannes E. (2006). Chronic disseminated cutaneous Blastomycosis in an 11-year old, with a brief review of the literature. Pediatric Dermatology. 23:541-545.

Granados D.P., Castaneda E., (2005). Isolation and characterization of *Cryptococcus neoformans* varieties recovered from natural sources in Bogota, Colombia and study of ecological conditions in the area. Microbiology Ecology. 49:282-290.

Guery B.P., Arendrop M.C., Auzinger G., Azoulay E., Borges M., Johnson E.M., (2009). Management of invasive candidiasis and candidemia in adult non-neutropenic intensive care unit patients: Part I. Epidemiology and diagnosis. Intensive care medicine. 35:155-162.

Gutierrez J, Morales P, Gunz Lez M.A., (2002). *Candida dubliniensis*, a new fungal pathogen (Review). Journal Basic Microbiology. 42:207-227.

Hage C.A., Wheat L.J., Loyd J., Allen S.D., Blue D., Knox K.S. (2008). Pulmonary histoplasmosis. Semir Respiratory. Critical Care medicine. 29:151-165.

Herich L.T., Marty F., Milner Jr. D., Thickner A. (2009). Disseminated Geotrichum candidum infection in a patient with relapsed acute myelogenous leukemia following allogeneic stem cell transplantation and review of the literature. Transplant. Infectious Diseases. 11; 458-462.

Hojjatollah S., Alireza K., Aghil S., Zanra T., (2010). Isolation and identification of yeast flora genital tract in healthy female camels (Camels drumedarius). Veterinary Microbiology. 144:183-186.

http://www.bird-x.com (25, January 2007). Bird-x-iac 3000N. Elizabeth st. Chicaco. Il 60607 USA, 800-662-5021 or 312-226-2473. Fax: 312-226-2480. Sales@Birds-x.com.

http:// www.ehow.com/recipes by Lydan altman.

http://www.idph.state.il.us/public/hb/hbb&bdrp.htm

http://www.rightdiagnosis.com/artic/cryptococcosis-dbmd.html.

http://www.rightdiagnosis.com/artic/histoplasmosis.dhmd.htm.march 2012.

http://www.softdental/com/diseases/oral_candidiasis_moniliasis_thrush.html).

Husain S., Wagener M.M., Singh N. (2001). *Cryptococcus neoformans* in organ transport recipients: Correlates of variability in clinical characteristics and outcome. Emerging infectious Diseases. 7:1-7.

Illnait-Zaragozi M.T., Martinez-Machin G.F., Fernandez-Andreu C.M. (2012). Environmental isolation and characterization of *Cryptococcus* species from living trees in Havana city, Cuba. Mycoses. 10:1439-1507.

Isenbery W.D., Henry S.C. (2004). Manual of clinical microbiology. 8th ed. Society of microbiology.

Jabra-Rizk M.A., Ferreira S.M.S, Sabet M., Falker W.A., Merz W.G., Meiller T.F. (2001). Recovery of *Candida dubliniensis* and other yeasts from human immunodeficiency virus associated periodontal lesion. Journal Clinical Microbiology. 39:4520-4522.

Jessica A.E., Elizabeth A.A., Chad A.R. (2010). The yeast-phase virulence requirement for α-glucan synthase differs among *Histoplasma capsulatum* chemo-types. Eurkaryotic cell.10:87-97.

Jewtuchowicz V.M., Mujica M.T., Brusca M.J., (2008). Phenotypic and genotypic identification of *Candida dubliniensis*, from subgingival sites in immunicompetent subjects in Argentina. Oral Microbiology Immunology. 23:505-509.

Jiang B., Bartlett M.S., Allen S.D., Smith J.W., Wheat L.J., Connolly P.A. (2000). Typing *Histoplasma capsulatum* isolates based on nucleotide sequence variation in the internal transcribed spacer regions of rRNA genes. Journal Clinical Microbiology. 38:241-245.

John I., Pitt, Ailsan D. (2009). Methods for isolation, enumeration and identification. Fungi and food spoilage. 19-52, Doi: 10.1007/978-0-387-92207-2_4.

John R., Archer R., Shelly H., Kurt D. (2011). Non-Rural point sources Blastomycosis outbreak near a yard waste collection site. Clinical Medicine Research 9:57-65.

John W., Meredith L.M., De Witt (2010). Cryptococcossis. http://emedicine medscape.com/article/215354.

Kachoko K.K., Swift P.J., Parrish A.G. (2002). Fine needle aspiration of vesicles in AIDS patients with cutaneous cryptococcosis. South Africa Medical Journal. 92:203.

Kaewwichian R., Yongmanitchai W., Srisuk N., Fujiyama K., Limtung S. (2010). *Geotrichum siamensis* sp. Nov. and *Geotrichum phoruearsis* sp. Nov. two a sexual arthroconidial yeasts species isolated in Thailand. FEMS yeast Research, 10:214-220.

Kauffman C.A. (2007a). In Goldman L., Ausiello D. eds. Cecil medicine 23rd ed. Philadelphia P.A., Sauders Elsevier; Chapter 355.

Kauffman C.A. (2007b). Histoplasmosis: a clinical and laboratory update. Clinical Microbiology Rev. 20:115-132.

Kauffman C.A. (2009). Histoplasmosis. Clinical Chest Medicine. 30:217-225.

Kao L.k., Litman M.L., Stanley S.S. (1957). *Cryptococcus neoformans* in pigeon excrete in New York City. America Journal. 79:49-59.

Karimi K., Wheat L.J., Connolly P., Cloud G., Hajjeh R., Wheat E., Alves K., Lacaz C.D., Keath E., (2002). Differences in histoplasmosis in patients with acquired immunodeficiency syndrome in the United States and Brazil. Journal Infectious Diseases. 186:1655-1660.

Kartrin T., Heidemarie L. (2005). Isolation of *Cryptococcus adeliensis* from clinical samples and the environment in Germany. 43:1007.

Kidd S.E., Chow Y., Mark S. (2007). Characterization of environmental sources of the human and animal pathogen *Cryptococcus gattii* in British Colombia, Canada and the Pacific Northwest of the United States. Appl. Environment Microbiology. 73:1433-1443.

Kielstein, Hotzel, Schmareck, Khaschabi and Glawischinig (2000). Occurrence of *Cryptococcus* species. in excreta of pigeons and pet birds. Mycoses. 43:7-15.

Klock C., Cerski M., Goldan L.Z. (2009). Histopathological aspects of neurocryptococcosis in HIV-infected patients: Autopsy report of 45 patients. International Journal Surgery Pathology. 17:444-448.

Kisso B., Mahmoud F., Thakkar J.R. (2006). Blastomycosis presetting as recurrent tender Cutaneous nodules. SD Journal medical 59:255-259.

Khatib R., Riederer K.M., Ramanathan J., Baran J., (2001). Faecal fungal in healthy volunteers and inpatients. Mycoses. 44:151-156.

Kobayashi C.C., Hasimoto e Souza L.K., Fernandos O.F., Brito S.C., Silva A.C. (2005). Characterization of *Cryptococcus neoformans* isolated from urban environmental sources in Goiana, Goivas Brazil. Revta institute Medicine Tropical 47 (4): 203-207.

Koneman E., Ellen S., Janda W. (2007). Atlas and textbook of diagnostic microbiology. Philadelphia: Lippincott-Raven.

Kuroki M., Phlchalchumpon C., Yasuoka A., Chiranairadul P., Chosa T., Sirinirand P., Miyazaki T., Kakeya H., Higashiyama Y., Miyazaki Y., Ishinda Y., Kuhno S. (2004). Enviromental isolation of cryptococcus neoformans from an edemic region of HIV-associated *Cryptococcus* in Thailand. Yeasts. 21:809-812.

Kurt D., Reed H., Jennifer K., John R., Archer A. (2008). Ecological niche modeling of Blastomyces dermatitidis in Wisconsin. Plos one 3:02034.

Kurtman C.P., Fell J.W (Ed) (2000). The yeasts. A Taxonomic study Elsevier scientific B.V., Amsterdam, Netherlands.

Kurzai O., Korting H-C., Harmson D., (2000). Molecular and phenotypic identification of the yeast pathogen *Candida dubliniesis*. Journal Molecular Medicine. 78:521-529.

Kretschmar M., Buhheidt D., Hor H., Nichterlein T. (2001). Galastomannan enzyme immuassay for monitoring systemic infection with *Aspergillus fumigates* in mice. Mycology. 41:107-112.

Krockenberger M.B., Canfield P.J., Malik R. (2003). *Cryptococcus neoformans* in the Koala (Phascolartos cinereus): Colonization by *Cryptococcus neoformans* var. *gattii* and investigation of environmental sources. Medical Mycology. 40:263-272.

Kwon-Chung K.J. Verma A. (2006). Do major species concepts support one, two or more species within *Cryptococcus neoforman*. FEMS yeast Research. 6:574-897.

Lakshimi V, Sodha T, Jeja V.D, Umabala P, (2007). Prevalence of central nervous system cryptococcosis in AIDS patients. Indian Journal Medical Microbiology. 23:146-9.

Larpin S., Mondoloni C., Georges S., Vernoux J.P., Gueguen M., Desmasures N. (2006). *Geotrichum candidum* dominates in yeast population dynamics in Livaroti, a French red-smear cheese. FEMS yeast research, 6:1243-1253.

Laubscher W.D.F., Viljoen B.C., Albertyn J., (2000). The yeasts flora occurring in the trachea of Broiler Chicken. Food techno. Biotechnol. 38:77-80.

Lazera M.S., Salmito Cavalcanti M.A., Londero A.T., Trilles L., Nishikawa M.M., Wanke B. (2000). Possible primary ecological niche of *Cryptococcus neoformans*. Medical Mycology. 38: 379-383.

Lemos L.B., Soofi M., Amir E. (2002). Blastomycosis and pregnancy. Ann. Diagn. Pathology. 6:211-215.

Lebowitz R.A., Waitzman M.N., Jacob J.B., Pearlman A., Tieno P.m. (2002). Isolation of fungi by standard laboratory methods in patients with chronic rhinosinusitis. The laryngoscope. 112:2189-2191.

Lherm T., Monet C., Noogiere B. (2002). Seven cases of fungemia with *Saccharomyces boulardii* in critically ill patients. Intensive care medicine. 28:797-801

Liti G., Carter D.M., Moses A.M., Warringer J., Parts L. (2009). Population genomics of domestic and wild yeasts. Nature. 458:337-341.

Likit M., Hilmioglu S., Tasbakan M., Aydemir S., (2007). Evaluation of *Albicans* ID2 and Biggy agar for the isolation and direct identification of vaginal yeast isolates. Journal Medical Microbiology. 56: 762–765.

Lindsley M.D., Horst S.F., Lqbal N.J., Morriso C.J. (2001). Rapid identification of dimorphic and yeast-like fungal pathogens using specific DNA probes. Journal Clinical Microbiology. 39:3505-3511.

Lo H.J., Ho Y.A., Ho M., (2001). Factors accounting for misidentification of *Candida* species. Journal Microbiology Immunology Infections. 34:171-177.

Malani A.N., Kauffman C.A., (2007). *Candida* urinary tract infection: treatment options expert. Rev. Antimicrobial Infections Therapy 5:277-284.

Malik R., Krockenberger M.B., Criss G., Doneley R., Madill D.N. (2003). Avian Cryptococcosis. Medical Mycology 41: 115:24.

Mahn B., Stehr F., Scäfer W., Neuber K., (2005). Comparison of standard phenotypic assays with a PCR method to discriminate *Candida albicans* and *C. dubliniensis*. Mycoses. 48: 55–61.

Mark Cichoki (2009). HIV and your oral health (Condition of the mouth caused by HIV). www.esnandental.com

Miller-Keane (2003). Encyclopedia and dictionary of medicine, nursing and allied health, seventh edition by Saunders, an imprint of Elsevier inc.

Mason A.R., Cortes G.Y., Cook J., Maze J.C., Thiers B.H., (2008). Cutaneous Blastomycosis: a diagnostic challenge. International Journal Dermatology 47:824-830.

Misuzu K., Chesenda P., Akra Y., Piphat C., Toru C., Petchsri S., Taiga M., hiroshi K., Yasuhito H., Yushitsuru M., Yutaka I., Shungeru K., (2004). Environmental isolation of *Cryptococcus neoformans* from an endemic region of HIV-associated cryptococcosis in Thailand. Yeast, 21:809-812.

Monica Cheesbrough, (2004), District laboratory practice in tropical countries, Cambridge university press, Cambridge UK, 244-245.

Montenegro H., Paula C.R. (2000). Environmental isolation of *Cryptococcus neoformans* var. *gatti* and *C. neoformans* var. *neoformans* in the city of Sao Pauo, Brazil Medical Mycology. 38:385-390.

Moretti A., Piergili Fioretti D., Buncio L., Pasquali P., Del Russi E., (2000). Isolation of *Candida rugosa* from Turkeys. Journal Veterinary Medicine, series B, 47:433-439.

Murphy H.A., Zeyl C.W. (2011). Prozygotic isolation between *Saccharamyces cerevisiae* and *Sacchraromyces paradoxis* through differences in mating speed and germination timing. Evolution 10:1558.

Muzyka B.C. (2005). Oral fungal infections. Dent. Clinical North America. 49:49-65.

Mseddi F., Sellami A., Sellami H., Cheikhrouhou F., Makni F., Ayadi A. (2010). Two new media *pinus halepensis* seed agar and blachberry agar for rapid identification of *Cryptococcus neoformans*. Mycoses. 10:1439-0509.

Nasri L., Triki N. (2004). Lipids analysis of Tunisia pine seeds: *Pinus halepensis* mill and *Pinus pinea*. Risg 81:244-247.

Neville B.W., Damm D.D., Allen C.M., Bouquot J.E. (2002). Fungal and protozoal diseases. Oral & maxillofacial pathology. 2nd ed. Philadephia. Sauders p. 189-197.

Neppelenbroek K.A., Campanha N.H., Spolidorio D.M.P., Spolidorio L.C., Seo R.S., Pavarina A.C., (2006). Molecular fingerprinting methods for the discrimination between *C. albicans* and *C.dubliniensis*. Oral Diseases 12:242-253.

Nosanchuk J.D., Shoham S., Fries B.C., Shapero D.S., Lebitz S.M., Casadevall A. (2000). Evidence of zoonotic transmission of *Cryptococcus neoformans* from pet cockatoo to an immunocompromised. Annals of internal Medicine. 132:205-208.

Odds F.C., Davidson A. (2000). "Room temperature" use of CHROM agar *Candida*. Diagn. Microbiology Infectious Dis.38:147-150.

Oral health care for people with HIV infection (2001). AIDS institute, NY department of health, page 31-32.

Pappas P.G. (2006). Invasive candidiasis. Infect. Disease Clinical North America. 20:485-506.

Pappas P.G., Rex R.H., Leo J. (2003). A prospective observational study of candidemia: epidemiology, therapy and influences on mortality in hospitalized adult and pediatric patients. Clinical Infectious Disease. 37:634-643.

Patricia I.D., Zhihing Y., Takanori S., Angela T. (2012). Synergistics interaction between *Candida albicans* and commensal oral streptococci in Novel vitro mucosa model. Infectious Immunology. 80:620-632.

Peter S., Joachim M. (2006). Chlamydospores formation in *Candida albicans* and *Candida dubliniensis*-an enigmatic development programmed. 50:1-12.

Peiris J.S., Yu W.C., Leung C.W. Cheung C.Y., (February 2, 2004). "Re-emergence of fatal human influenza A subtype H5H1 disease. Lacet.363:617-619.

Perrins E.D., Christopher, Buffalo (2003). Firetly Encyclopedia of Birds, Firetly books, Ltd.

Perry J.D., Freydiere A.M. (2002). The application of chromogenic media in clinical microbiology. Journal Appl. Microbiology. 103:2046-2055.

Picozo J.J., Gunzalez-Romo F., Cander F.J., (2008). Candidemia in the critically ill patient. International Journal Antimicrobials Agents: 32 suppl 2:s83-s85.

Ptailer M.A and Xu W.L (2001). Antifungal susceptibility testing new technology and clinical application. Infectious. Diseases Clinical North America. 15:1227-1261.

Ramos L., Lopoz C., Gumez C., Mathorin S., Mateo A., (2001). Case Report. Cutaneous cryptococcosis in a patient with systemic erythematous lupus. Mycoses. 44:419-421.

Ramage G., Vande W.K., Wickes B.L., Lopez-Ribot J.L. (2001). Standardized method for invitro susceptibility testing of *Candida albicans* biofilms. Antimicrobials Agents Chemotherapy. 45:2475-2479.

Randawa H.S., Mussa A.Y., Khan Z.U (2000). Decaying wood in tree trunk hollows as a natural substrate for *Cryptococcus neoformans* and other yeast-like fungi of clinical interest. Mycopathologia. 151:63-69.

Reed K.D., Meece J.K., Archer J.R., Peterson A.T. (2008). Ecological niche modeling of Blastomyces dermatitidis in Wisconsin. Plos one 3:02034.

Reichat P.A., Samaranayake L.P., Philipsen H.P. (2000). Pathology and clinical correlates in oral candidiasis and its variants: A review oral diseases. 6:85-91.

Reimao J.Q., Drummund E.D., Tercett M.D.S., Lyon J.P., Franco M.C., De Siqueria A.M. (2007). Isolation of *Cryptococcus neofmans* from hollows of living trees in the city of Alfenas, MG, Brazil. Mycoses. 50:261-264.

Repentighny L., Lewandosky D., Jolicoeur P, (2004). Immunopathogenesis of oropharyngeal candidiasis in human immunodeficiency virus infection. Clinical Microbiology. 17 (4): 729-759.

Resende J.L, France G.R, Rosa C.A, et al., (2004). Phenotypic and genotypic identification of *Candida* species isolated from hospitalized patients. Rev Iberoam mycology. 21:24-28.

Restrepo A., Baumgardner D.J., Bagagli E. (2000). Clues to the presence of pathogenic fungi in certain environments. Medical Mycology. 38:67-677.

Reuter M., Bell G., Greig D. (2007). Increased out breeding in yeast in response to dispersal by an insect vector. Curriculum Biology. 17:R81-R83.

Richardson M.D., Warnock N.W. (2003). Histoplasmosis in: Fungal infection diagnosis and management. 3rd Ed. Oxford: Blackwell: P. 264-278.

Rosa E.A.R., Rosa R.T., Pereira C.V., Boriollo M.F.G., Hutling S.F. (2000). Analysis of parity between protein-based electrophoretic methods for the characterization of oral *Candida* species. Mem Inst Oswaldo Cruz 95: 801–6.

Rosario I. Hermosa de Mendonza M., Deniz S., Soro G., Alamo. (2005). Isolation of *Cryptococcus* species including *C. neoformans* from cloaca of pigeons. Mycoses. 48:421-424.

Ruchel K.L. (2008). Outbreak of histoplasmosis among travelers returning from El salvader-Pennsylvania and Virginia. MMWR Mortal. Wkly. Rep. Dec. 19, 2008: 57:1349-1353.

Sanchez-Vargas LO, Ortiz-Lopez N.G, Villarm M., (2005a). Oral *Candida* isolates colonizing or infecting human immunodeficiency virus-infected and healthy person in Mexico. Journal Clinical Microbiology. 43:4159-4162.

Sanchez- Vargas L.O, Ortiz-Lopez N.G, Villar M., (2005b). Point prevalence, microbiology and antifungal susceptibility patterns of oral *Candida* isolates colonizing or infecting Mexican HIV/AIDS patients and healthy person. Rev. Ibroam. Mycology. 22:83-92.

Sahand L.H., Moragues M.D., Robert R., Quindos G., Pantos S., (2006). Evaluation of Bichro-Dubli Fumouze to distinguish *Candida dubliniensis* from *Candida albican*. Diagn. Microbiology Infectious Diseases. 55:165-167.

Salonen J.H., Richardson M.D., Gallacher K., Issakaiinen J., Helenius A., Lehtunen O.P., Nikoskelainin J., (2000). Fungal colonization of haematological patients receiving cytotoxic chemotherapy, Emergence of a zole-resistant *Saccharomyces cerevisiae*. Journal Hospital Infections 45:293-301.

Samaranayake L.P., (2002). Essential microbiology for dentistry, 2nd ed. London: Churchill Livingstone.

Sauder's (2007). Dorland's medical dictionary for health consumers. An imprint of Elsevier inc.

Sayyada G.N., Shazia T.H., Shahana U.K. (2010). Use of CHROM agar *Candida* for the presumptive identification of Candida species directly from clinical specimen in resource-limited setting. Libyan Journal Medicine. 5.2144.

Scardina G.A., Ruggieri A., Messina (2009). Chronic hyperplastic candidiasis: a pilot study of the efficacy of 0.18% isotretion. Journal oral Science. 51:407-410.

Schacherer J., Shapiro J.A., Ridertet D.M., Kruglyall L. (2009). Comprehensive polymorphism survey elucidates population structure of *Saccharomyces cerevisiae*. Nature 458:321-345.

Schulle D., Pereira L., Alves H., Cambon B., Desavin S. (2007). Characterization of commercial *Saccharomyces cerevisiae* isolates recovered from vineyard environments. Yeasts. 24:625-636.

Shah C.P., Mckey J., Spirn M.J., (2008). Ocular candidiasis: a review. Brazil Journal Ophthaimology. 92:466-468.

Shelborne J.A., 3rd, Visnegarwala F., Adams C., Krause K.L., Hamil R.j., White A.C., Jr. (2005). Unusual manifestation of disseminated Histoplasmosis in patients responding to antiretroviral therapy. America Journal medicine. 118:1038-1041.

Sherman R.G., Prusinsiki L., Ravenel M.C., Joralmon R.A. (2002). Oral candidosis quintessence int. 33:521-532.

Smith D., Metzgar D., Wills C., Fierer J. (2002). Fatal *Saccharomyces cerevisiae* aortic graft infection. Journal Clinical Microbiology. 40:2691-2692.

Sniegowki P.D., Dombrowki P.G., Fingerman E. (2002). *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* coexist in a natural woodland site in North America and display different levelsor reproductive isolation from conspecific. FEMS yeast Research. 1:299-306.

Singh N. (2003). Treatment of opportunistic mycoses: how long is long enough. Infectious Diseases. 3:703-708.

Singhi M.K., Gupta L., Kacchawa D., Gupta D. (2003). Disseminated primary cutaneous histoplasmosis successfully treated with itraconazole. Indian Journal Dermatology Leprosy. 69:405-407.

Sitheeque M.A., Samaranayake L.P. (2003). Chronic hyperplastic candidiasis / candidiasis (*Candidal* leukoplakia). Critical Rev. Oral Biological Medicines. 14:253-267.

Sivakumar V.G., Shankar P., Natina K., Menon T. (2008). Use of CHROM agar in the differentiation of common species of *Candida*. Mycopathologia 67:47-49.

Soll D.R. (2000). The ins and outs of DNA fingerprinting the infections fungi. Clinical Microbiology Rev. 13:332-370.

Sorell T.C. (2001). *Cryptococcus neoformans* variety *gattii*. Medical Mycology. 39:155-168.

Springer D.J., Ren P., Raina R., Dony Y., Behr M.J. (2010). Extracellular fibrils of pathogenic yeast *Cryptococcus gattii* are important for ecological niche, murine virulence and Human neutrophil interactions. Springer Plos one 5(6):e10978. Doi:10:101371.

Steenbergen J.N., Casadevall A. (2003). The origin and maintenance of virulence for the human pathogenic fungus *Cryptoccoccus neoformans*. Microbes infections. 5:667.

Stepanovic S., Vukovic D., Radonjic I., Dinitrijevic V., Svabic- Vlahovic M. (2002). Ground red hot pepper agar in the isolation and presumptive identification of *Cryptococcus*. Mycoses. 45:384-388.

Sullivan D.J., Moran G.P., Pinjon E. (2004). Comparison of the epidemiology, drug resistance mechanisms and virulence of *Candida albicans*. FEMS yeast Research. 4:369-376.

Sun H.H., Ma Hg., Hao M.L., Pretorius I.S., Chen S.W., (2009). Identification of yeast population dynamics of spontaneous fermentation in Beijing wine region, China, Annals of microbiology. 59:69-76.

Sun H.V., Alexander B.D., Lortholary (2010). Unrecognized pretransplant and donor-derived Cryptococcal diseases in organ transplant recipients. Clinical Infectious Diseases. 51:1062-1069.

Suwat C., Thira S., Orapan S., Kenrad E.N. (2001). Clinical presentation and risk behaviors of patients with acquired immune-deficiency syndrome in Thailand, 1994-1998: regional variation and temporal trends. Clinical Infectious Diseases. 32:958-962.

Syket J., Storges B., Cannon M., Gericota B. (2010). Clinical signs, Imaging features, neuropathology, and outcome in Cats and Dogs with Central nervous system Cryptococcosis from California. Journal veterinary Internal Medicine. 24:1427-1443.

Takahiko N., Mohamed A.A., Yuichi N., Masayuki M., Katsuyuki U. (2008). Dipudasas tetraspores SPP. Nov., an ascoporogenus yeast isolated from deep-sea sediments in the Japan. Trench. IJSEM. 58:1040-1046.

Taylor M.L., Chavez-Tapia C.B., Rojac-Martiniz A., Reyes-Montes M.R. (2005). Geographical distribution of genetic polymorphism of the pathogen *Histoplasma capsulatum* isolated from infected bats, captured in a central zone of mexico. FEMS immune. Medical Microbiology. 45:451.

Tracy C., Bruce L.R., Greg M.B., Michael P.M., Garx C.S. (2002). Epidemiology and clinical spectrum of Blastomycosis diagnosed at Manitoba hospital. Clinical Infectious Diseases. 34:1310-1316:

Tintelnot K., Lemmer K., Losert H., Schar G., Polak A., (2004). Follows-up of epidemiological data of cryptococcisis in Austria, Germany and Switzerland with special focus on the characterization of clinical isolates. Mycoses. 47:455-464.

Torija M.J., Rozes N., Publet M., Guillamon J.M., Mas A. (2001). Yeast population dynamics in spontaneous fermentation: Comparison between two different wine-producing areas over a period of tree yeast. Antonie Van Leeuwenhoek, 79:345-352.

Uicker W.C., Doyle H.A., James P.M., Langlois M., Bichanans K.L.(2005). Cytokine and chemokine expression in the central nervous system associated with protective cell-mediated immunity against *Cryptococcus neoformans*. Medical Mycology. 43:27-35.

US Census Bureau's, population Estimates, 2004.

Valerie B., Demil B. (March 23, 2010). Diseases caused by Chickens. http://www.livestrong.com/article/91095-diseases-caused-Chickens/#ixzz10espt31k

Valero E., Cambon B., Schuller D., Casn M., Dequin (2007). Biodiversity of *Saccharomyces* yeasts strains from grape berries of wine-producing areas using started commercial yeasts. FEMS yeast research. 7:317-329.

Varan N., Baumgardner D.J., Czuprynski C.J., Paretsy D.P. (2009). Isolation of *Blastomyces dermatitidis* from the nares of dogs, Northern Wisconsin USA. 7:780-782.

Vazquez J.A., Sobel J.D. (2002). Mucosal candidiasis. Infectious Diseases Clinical North America. 16:793-820.

Vazquel J.A., Sobel J.D., (2003). Candidiasis in: Clinical mycology, Dismukes W.E., Pappas P.G., Sober J.D., Eds Oxford University. 143-187.

Wheat L.J., Chetchotisakd P., Williams B., Connolly P., Shuff K., Hajjer R. (2000). Factors associated with severe manifestation of histoplasmosis in AIDS. Clin. Infect. Dis. 30:877-881.

Wheat J., Sarosi G., McKinsey D., Hamil R., Bradsher R., Johnson P., Loyd J., Kauffman, (2000). Practise guidelines for the management of patients with histoplasmosis Infection Diseases society of America. Clinical Infectious Diseases. 30:688-695.

Williams D.W., Kuriyama T., Silva S., Malic S., Lewis M.A. (2011). *Candida* biofilms and oral candidosis: treatment and prevention. Periondotology. 55:250-265.

World Health Organization writing committee consultation on human influenza A/H5N1 (2005). Avian influenza A (H5N1) infection in human. New England Journal Medicine. 353: 1374-1385.

World Health Organization (2009). Laboratory manual for diagnosis of fungal opportunistic infection in HIV/AIDS Patients. Regional office for South-East Asia, Indraprastaha Estate, Mahatha Gandhi Marg, New Delhi.

Zampogna J.C., Hoy M.J., Ramos- Cara F.A. (2003). Primary cutaneous North America Blastomycosis in an immunosuppressed child. Pediatric Dermatology. 20:128-130.

7. APPENDICES

8.1 APPENDIX 1: SAMPLES COLLECTION AND ENVIRONMENTAL CONDITIONS

Sample ID.	Elevation	Temperature	Humidity	Sample type	Isolates
001D1	9747ft	13.9°C	27%	guano	*G. candidum*
001D2	9747ft	13.9°C	27%	guano	Bacteria
001S	9747ft	13.9°C	27%	Soil	*G. candidum*
002D1	8073ft	32.5°C	25%	guano	*C. tropicali*
002D2	8073ft	32.5°C	25%	guano	Bacteria
002S	8073ft	32.5°C	25%	Soil	Bacteria
003D	8210ft	24.3°C	25%	guano	*S. cerevisan*
003SW	8210ft	24.3°C	25%	guano	*C. tropicali*
003S	8210ft	24.3°C	25%	Soil	Bacteria
004D	8177ft	26.3°C	28%	guano	*C. krusei* and *C. glabrata*
004SW	8177ft	26.3°C	28%	guano	*C. lusitaniae*
004S	8177ft	29.3°C	60%	Soil	*C. lusitaniae*
005D	8194ft	29.4°C	47%	guano	Bacteria
005SW	8194ft	29.4°C	47%	guano	*Cry. Laurentii*
005S	8194ft	29.4°C	47%	Soil	Bacteria
006D	8195ft	13.6°C	39%	guano	Bacteria
006SW	8195ft	13.6°C	39%	guano	*C. albican* and *C. tropicali*
006S	8195ft	13.6°C	39%	Soil	*C. lusitanie*
007D1	8132ft	16.3°C	30%	guano	*G. candidum*
007D2	8132ft	16.3°C	30%	guano	Bacteria
007S	8132ft	16.3°C	30%	Soil	*C. albican*

008D	8148ft	16.6°C	83%	guano	Bacteria
008S	8148ft	16.6°C	83%	Soil	Bacteria
009D	8154ft	17.1°C	40%	guano	*G. candidum*
009S	8154ft	17.1°C	40%	Soil	*G. candidum*
010D	8184ft	19.1°C	29%	guano	*C. krusei*
010S	8184ft	19.1°C	29%	Soil	Bacteria
011D	8181FT	23.5°C	75%	guano	Bacteria
011S	8181FT	23.5°C	75%	Soil	Bacteria
012D	8077ft	24.4°C	58%	guano	*C. parapsilosis*
012S	8077ft	24.4°C	58%	Soil	*C. glabrata*
013D	8115ft	31.3°C	42%	guano	*C. lusitanie* and *G. candidum*
013S	8115ft	31.3°C	42%	Soil	*G. candidum*
014D	8140ft	32.6°C	45%	guano	*G. candidum*
014S	8140ft	32.6°C	45%	Soil	*C. albican*
015D	8133ft	33.9°C	54%	guano	Bacteria
015S	8133ft	33.9°C	54%	Soil	*G. candidum*
016D	8152ft	41.0°C	37%	guano	Bacteria
016S	8152ft	41.0°C	37%	Soil	Bacteria
017D1	8177ft	36.0°C	32%	guano	*C. tropicalis* and *G. candidum*
017D2	8177ft	36.0°C	32%	guano	*C. lusitanie*
018D	8162ft	11.4°C	78%	guano	Bacteria
018SW	8162ft	11.4°C	78%	guano	*C. lusitanie*
018S	8162ft	11.4°C	78%	guano	*G. candidum*
019D	8192ft	12.6°C	100%	guano	Bacteria
019SW	8192ft	12.6°C	100%	guano	Bacteria

019S	8192ft	12.6°C	100%	Soil	Bacteria
020D	8189ft	15.6°C	80%	guano	*C. lipolytica*
020SW	8189ft	15.6°C	80%	guano	Bacteria
020S	8189ft	15.6°C	80%	Soil	Bacteria
021D	8157ft	18.9°C	78%	guano	*C. lusitaniae*
021SW	8157ft	18.9°C	78%	guano	Bacteria
021S	8157ft	18.9°C	78%	Soil	*G. candidum* and *C.lusitanie*
022D	8093ft	21.9°C	59%	guano	*G. candidum*
022SW	8093ft	21.9°C	59%	guano	*C. guilliermondii*
022S	8093ft	21.9°C	59%	guano	*C. lipolytica*
023D	8189ft	25.5°C	18%	guano	*C. glabrata*
023SW	8189ft	25.5°C	18%	guano	*G. candidum*
023S	8189ft	25.5°C	18%	guano	*G. candidum*
024D	8200ft	25°C	15%	guano	*C. parapsilosis*
024SW	8200ft	23.6°C	52%	guano	*C. parapsilosis* and *C. glabrata*
024S	8200ft	23.6°C	52%	Soil	*C. albican*
025D	8159ft	30.9°C	52%	guano	*Cry. neoforman* and C. *glabrata*
025SW	8159ft	30.9°C	52%	guano	*C. glabrata*
025S	8159ft	35.4°C	20%	Soil	*C. albican*
026D1	7839ft	15.6°C	85%	guano	Bacteria
026D2	7839ft	15.6°C	85%	guano	Bacteria
026S	7839ft	15.6°C	85%	Soil	*C. lusitaniae*

027D1	7993ft	15.4°C	100%	guano	*G. candidum*
027D2	7993ft	15.4°C	100%	guano	*G. candidum*
027SW	7993ft	15.4°C	100%	Soil	*C. albican*
028D	8163ft	18.8°C	88%	guano	Bacteria
028D2	8163ft	18.8°C	88%	guano	*G. candidum*
028S	8163ft	18.8°C	88%	guano	Bacteria
029D1	8176ft	19.5°C	85%	guano	Bacteria
029D2	8176ft	19.5°C	85%	guano	*C. lusitaniae*
029D3	8176ft	19.5°C	85%	guano	*G. candidum*
030D1	8200ft	29.6°C	51%	guano	Bacteria
030D2	8200ft	29.6°C	51%	guano	*C. glabrata*
030D2	8200ft	29.6°C	51%	guano	*C. tropicali*
031SW1	8128ft	17.1°C	100%	guano	*C. tropcali*
031SW2	8128ft	17.1°C	100%	guano	*O. gallopava*
032SW1	7840ft	18°C	75%	guano	Bacteria
032SW1	7840ft	18°C	75%	guano	*C. glabrata* and *G. candidum*

8.2 APPENDIX 2: 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 → Fiiter 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.

Trypan blue stock (0.1g/ml)

1ml Trypan blue stock makes 1litre Trypan blue media.

To make 10ml stock

1g Trypan blue powder → 10ml distilled water and if possible store in the fridge.

Phenol red (0.012g/ml)

1ml → 1litre Christensen's urea media

To make 20ml stock

0.240g → Dissolve in 20ml distilled water and if possible store in fridge.

Biphenyl stock (0.05g/10ml of 95% ETOH)

10ml stock → 1litre Niger seed agar

To make 100ml stock

0.5g Biphenyl → 100ml of 95% ETOH

8.3 APPENDIX 3: QUESTIONNAIRE FORM

ENVIROMENTAL COLLECTION:

Supplies:

Digital camera

GPS unit

Temperature/ humidity gauge

Landscape characterization sheets

Sterile plastic spoons

Sharpie

Pencil

Tree swabs

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 and excreta

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 classification

Urban: Grassland: Forest: Agricultural: Residential:

Other:

Groundcover classification

Bare soil: Forest: Sand: Vegetative debris: Gravel:

Natural grassland: Maintained grass: Impervious (cement/asphalt): other:

Canopy classification

No canapy: Partial canapy: Full canapy: other:

Distance to water

_____ Meters to water Waters visible at a distance No visible water

Water classification

Creek (running water, < 1ml across) Steam (running water, < 1m but>10ml across)

River (running water,>10ml across) Drainage ditch (describe)

Drainage ditch (describe)

Small puddle (standing shallow water, < 1ml across)

Large puddle (standing shallow water,> 1ml across)

Pond (small body of standing water)

Animal classification

Bird

Bird/ bat Exposure classification

No visible birds

Roosting birds at sample site

Roosting birds in the vicinity

Roosting birds at a distance

Lots of birds flying overhead but not roosting

Sample type

Guano Soil

Additional sample description:

8.4 APPENDIX 4: SOPS FOR MEDIA PREPARATION

Sabourand's dextrose agar.

Sabourand's Dextrose agar supplemented with Chloramphenicol and Gentamicin for the primary isolation and cultivation of yeast and moulds.

Reagents	Measurements

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

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 10 minutes, remove and slopes or pour to plates as required.

Trypan blue agar for yeast and moulds.

Trypan blue media with chloramphenicol and ampicilin for primary isolation and cultivation of yeast and moulds.

Reagents	Measurements
Trypan blue solution	1ml
Peptone	20g
Glucose	1g
Disodium phosphate	2.5g
Chloramphenicol	200µl
Ampicillin	200µl
Distiller water	1L

To 2 litres add the following distilled water, glucose, peptone, agar, disodium phosphate using above measurements.

Autoclave at 121ºC for 25 minutes.

Immediately add Trypan blue stock solution.

5 minutes to cool add Chlorampenical and Ampicillin.

CHROMO agar

Composition:

Reagents	Measurements
Agar	15g/l
Peptone	10.2g/l
Chromogenic mix	22g/l
Chloramphenicol	0.5µl
PH	6.IP

*(Classical formula adjusted and/ or supplemented as required meeting performance criteria)

Preparation: According to quantities desired, weigh out power and use in the proportion 44.7g/l of purified water ,or use full pre-weighed dose with corresponding volume of water. Dispense powder slowly in water by rotating until swelling of the agar. Bring water to boil at 100ºC by repeated heating, swirling or stirring regularly. If using an autoclave, do so without pressure. Do not heats to more than 100ºC mixture may also be brought to boil in microwave oven to stir gently, return to oven for short repeater heating. Continue until complete fusion of agar grains (Large bubbles replacing foam: about 2 minutes).Cool to 45ºC, swirling or stirring gently to homogenize before pouring into sterile Petri dish or tubes. Let dry (Store in dark). Medium may be kept for one day at

room temperature or for at least a month in a refrigerator if properly prepared and properly stored. Streak and incubate for 48 hours at 30ºC to 37ºC.

Interpretation:

Candida albicans------green

Candida tropicalis-----Blue Grey

Candida krusei---------Pink, Fizzy

Other species ---------White pink

Cornmeal agar

This medium is useful for stimulating the formation of pseudohyphae true hyphae, arthrospores and chlamydospores in those species able to produce them.

Reagents	Measurements
Agar	2g
Distiller water	1l
Cornmeal extract	2g

Heat to dissolve.

Autoclave at 121ºC for 15 minutes

Niger seed-biphenyl media 1l

This medium is used in identifications of *Cryptococcus* species.

Reagents	Measurements

Ground Niger seed	70g
Distilled water	1l
Biphenyl solution	10ml
Glucose	1g
Agar	20g
Chloramphenicol solution	400µl
Ampicillin solution	500µl

70g ground Niger seed boil for 15 minutes in 350ml distiller water and filter through cheecloth into a 2l flask.

Bring up to 1 litre distilled water and add glucose and agar.

Autoclave for 25minutes at 121ºC.

After autoclaving add immediately Biphenyl solution.

5 minutes to cool add Chloramphenicol and Ampicillin stock solution.

8.5 APPENDIX 5: USER MANUAL FOR API 20C AUX YEAST IDENTIFICATION SYTEM.

API 20C AUX is a system for the precise identification of the most frequently encountered yeast in clinical microbiology. The complete list of those yeasts that it is possible to identity with this system is given in the identification Table at the end of this package insert.

Principle:

The API 20C AUX strip consists of 20 cupules containing dehydrated substrate which enables the performances of 19 assimilation test. The cupules are inoculated with a semi-solid minimal medium and the yeast will only grow if they are capable of utilizing each substrate as sole carbon source. The reactions are read by comparing them to growth control and identification is obtained by referring to the analytical profile index or using the identification software.

Reagents:

Kit contents (25 tests)

25 API 20C AUX strips.

25 incubation boxes.

25 ampoules of C medium.

25 results sheets.

1 package inserts.

Additional product (not included in the kit):

Suspension medium, 2ml (ref.70 700) or Nacl 0.85% medium, 2ml (ref. 20070).

Rice or corn meal agar Tween (morphology agar).

Sabouraud medium.

Pipettes (ref. 70 250).

Mc Farland standard #2 (ref. 70 900).

API 20C AUX Analytical profile index (ref. 20 290) or identification software (consult bio merieux).

Ampoule rack (ref. 70 200).

Required laboratory equipment:

30ºC incubator

Refrigerator

Bunsen burner

Marker pen

Test names:

GLU-GLUcose

GLY-GLYcerol

2KG-2-Keto-D-Gluconate

ARAL-ARAbinose

XYLD-XYLose

ADO-ADOnitol

XLT-XyLiTol

GAL-GALactose

TRE-TREhalose

RAF-RAFFinose

INO-INOsitol

SOR-SORbitol

MDG-Methyl-D-Glucoside

NAG-N-Acetyl-D-Glucoside

CEL-CELiobise

LAC-LACtose

MAL-MALtose

SAC-SACcharose/sucrose

MLZ-MeLeZitose

Composition of the media

Suspension medium 2ml- Demineralized water.

NACL 0.85%- Sodium chloride 8.5g

Medium 2ml- Dermineralized water 1000ml

C Medium 7ml

Magnesium suphate 0.2g

Histidine 0.005g

Tryptophane 0.02g

Methionine 0.02g

Agar 0.5g

Vitamin solution 1ml

Ammonium suphate 5g

Monopotassium phosphate 0.31g

Dipotassium phosphate 0.4g

Sodium chloride 0.1g

Calcium chloride 0.05gs

Trace elements 10ml

Water qsq 1000ml

Final PH: 6.4-6.8.

Although C medium contain agar, it requires no prior heating and may be as easily pipette as a liquid medium it is preferable to warm it at room temperature a few hours before use. Do not shake.

Storage of the strips and media

For in vitro diagnostic use only

Qualified laboratory personnel should use a septic, technique and established precaution for infection agents.

Do not pipette specimens or reagents by mouth.

Do not use reagents past the expiration date.

Upon removal from refrigerator, allow reagents to come to room temperature (20ºC - 30ºc).

Open ampoules carefully as follows:

Hold the ampoules in one hand in a vertical position (white plastic cap uppermost).

Press the cap down as far as possible.

Cover the flattened part of the cap with the upper part of the thumb.

Apply thumb pressure in an outward motion to the flattened part of the cap to snap off the top of the ampoules inside the cap.

*For ampoules with no dropper-cap:-carefully remove the cap

*For ampoules with the dropper –cap-turn the ampoules upside down and maintain it in a vertical position.

Squeeze on the cap to transfer the entire reagent into the dropper-bottle.

All inoculated product should be considered infectious and handled appropriately.

All specimen and microbial cultures are potentially infectious and should be treated with universal precautions.

NCCLS MZGA: protection of laboratory workers from instrument Biohazards and infectious disease transmitted by blood, body, fluids and tissue: Approved guideline (1997).

After completing test, receding and interpretation, all specimens, spills and inoculated products must be autoclaved, incinerated or immersed in a germicide prior to disposal.

Interpretation of the results should be made by a competent microbiologist who should also take into consideration the sources of the specimen, colonial and microscopic morphology.

Instruction for use

Specimens and yeast cultures should be considered infection and handled appropriately by trained and competent technicians. Aseptic technique and usual handling precaution for yeast should be observed throughout this procedure; refer to universal.

Precaution MZGA: protection of laboratory workers from instrument Biohazards and infectious diseases transmitted by blood, body fluids and tissue: Approved guideline (1997). For additional handling precaution, refer to biosafety in microbiological and biomedical laboratories, HHS publication No.(CDC), 93-8395, 3rd Edition (May 1993) or to the regulation of each country.

Preparation of the strip

Prepare the incubation box (tray and lid) and distribute about 5ML of distilled water or demineralized water (or any water without additives or chemicals which may release gases e.g CL2, CO2 e.t.c) into the honey-combed wells of the tray to create a humid atmosphere.

Record the strain reference on the elongated flap of the tray.

Remove the strip from its individual packaging and place it in the incubation tray.

Preparation of the inoculums

Open an ampoule of suspension medium or NACL 0.85%.

Medium as indicated in the paragraph "warning and precaution" (ampoule with no dropper-cap) or use, any tube containing 2ML of the same solution without additives.

Using a pipette, pick up a portion of a yeast colony either by suction or by successive touches and make a suspension with turbidity equal to 2 Mc Farland.

Dispense one drop of the yeast suspension onto the rice or corn meal agar Tween (or appropriate morphology medium).

Open an ampoule of C medium as indicated in the paragraph "warning and precautions" (ampoule with no dropper-cap) and transfer 100µl (2-4 drops) of the previous suspension into it.

Gently homogenize with the pipette, avoiding the formation of bubbles.

Inoculation of the strip

Fill the cupules with the suspension obtained in the ampoule of C medium. Avoid the formation of bubbles by placing the top of the pipette against the side of the cupules. Care should be taken not to overfill or under fill the cupules (the surface should be flat or slightly, convex, but never concave), otherwise incorrect results may be obtained.

Place the lid on the tray and incubate at 30ºC for 48-72hours.

Reading the strip

After 48 or 72 hours of incubation if the test, in particular glucose, are not clear-cut after 48hours, check for growth (the 0 cupules serves as a negative control). Cupules more turbid than the control indicates a positives reaction to be recorded on the results re incubation is necessary: remove the lid only when reading the strip and replace immediately.

Identification

Identification using analytical profile index: the pattern can obtained: reactions obtained must be coded into a numerical profile. On the results sheet, the tests are separated into groups of 3 and a number 1, 2, or 4 is indicated for each. By adding the numbers

corresponding to positive reaction within each group, a 7- digit number is obtained which constitutes the numerical profile.

NOTE: The presence of hyphae (mycelium) or pseudohyphae (pseudomycelium) constitutes the 21st test and has a value of 4 when positive. Using the identification software by mainly entering the 7- digit numerical profile via the keybord i.e. 2764774 Trichoporon asahii.

Quality control

The strips and media are systematically quality controlled at various stages of their manufacture. For those who wish to perform their own quality control test with the strips, it is recommended that the following stock cultures be used, to obtain the results below: profiles obtained after 72hours of incubation after culture on Sabouraud agar/ Trypan blue.

Disposal of used material

After use, all ampoules, pipettes, strips and incubation boxes should be autoclaved, incinerated or immersed in a disinfectant for decontamination prior to disposal.

Limitations

The API 20C AUX system is intended uniquely for the identification of yeast included in the database. It cannot be used to identify any other microorganism or exclude their presence.