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Analysis of diversity and drug susceptibility profiles of yeast isolates and their biofilm production characteristics among HIV and AIDS patients presenting with TB

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ABSTRACT

Although several fungal organisms are known to infect HIV and AIDS patients, the diversity of yeast pathogens that infect individuals in Kenya is not known. In the present study sputum samples were collected from HIV positive patients who presented at the hospital with TB. The yeasts organisms were isolated using Potato Dextrose Agar and identified by germ tube test, CHROMagar and Urease based agar. The production of biofilm was tested using the microtitre plate method. The activity of Amphotericin B, Fluconazole and Ketaconazole was tested using the standard disc diffusion method. Out of the 91 patients, 53 (58%) were females. Thirty seven patients (41%) were aged between the ages 26 and 45 years old. The most common isolates were *Cryptococcus neoformans* (28%), followed by *Cndida parapsilopsis* (19%), *C. krusei* (16%), *C. albicans* (15%) and *C. tropicalis* (13%). The different species showed high levels of drug resistance to fluconazole (74.0%), ketaconazole (74.0%) and amphotericin B (33.7%). Biofilm production was observed in 28% of all the isolates and was not associated with drug resistance ($p>0.01$). This study shows that the lungs of HIV patients with TB are often colonized by opportunistic yeasts such as *Cryptococcus* and *Candida* spp. The most common isolates included *Cryptococcus neoformans*, *Candida parapsiliosis*, *Candida krusei* and *Candida albicans*, and amphotericin B was the most active drug against these organisms. Further studies are needed to determine the potential impact of these organisms on the emergence of multi-drug resistant (MDR) and extensively drug resistant tuberculosis (XDR TB).

KEYWORDS

Candida spp;
Cryptococcus neoformans;
Co-infections;
Fungal infections;
HIV and AIDS;
Tuberculosis.

INTRODUCTION

Fungal infections are a major cause of morbidity and mortality in the immunocompromised host and examples of fungal pathogens responsible for such infections include *Pneumocystis jiroveci*, several *Candida*

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spp., *Cryptococcus* spp. *and Penicillium* spp[26]. In the last two decades the frequency of invasive fungal infections has increased remarkably[2]. Respiratory infections are among the most common causes of morbidity and mortality among HIV and AIDS patients and tuberculosis is one of the most common infections among HIV and AIDS patients[21, 28]. Over the past decade, studies have indicated that TB is the most common opportunistic infection in HIV patients particularly those with CD4 counts less than 200cells/ mm^3[17].

The prevalence of TB-HIV co-infection is high and reaches 65% for example in Uganda while in South Africa; up to 75% of the TB patients are co-infected with HIV[4, 15]. In Kenya, several studies have demonstrated the impact of HIV and TB co-infection with more than 20 times increase in mortality among TB-HIV co-infected cases compared to non-HIV infected individuals[13, 29]. However, very few studies have documented the occurrence of yeast infections among TB infected HIV patients[16].

The factors contributing to the pathogenesis of *Candida* and its progression in HIV positive patients are poorly understood, but may include biofilm production, an interrelationship between HIV and *Candida* and/or a dysfunctioning in the local immunity superimposed on weakened cell-mediated immunity and depletion of CD4 T cells[1, 11]. Clinical diagnosis is often presumptive. Therefore laboratory diagnosis is required for conclusive identification and advice for therapy. However, microbiological examination is rarely conducted for patients attending health care centres in the Nyanza, Kenya and consequently little is known about the different types of yeast organisms occurring in the Nyanza province as well as the antifungal susceptibility profiles of Kenyan yeast isolates[5]. As such it is not certain that treatments being administered to individuals for fungal infections are taking appropriate effect. The isolation and identification of opportunistic yeast pathogens from patients presenting symptoms associated with these agents is important for establishing the appropriate treatment regimen. The present study investigated the occurrence and diversity as well as drug susceptibility profiles of yeast isolates and their biofilm production characteristics among HIV and AIDS patients presenting with TB in treatment centers in the Nyanza Province.

METHODS

The objectives of the study were explained to the patients and the patients were required to sign a consent form if they agreed to participate in the study. Samples used in the present study were de-identified sputum samples submitted for analysis to the hospital laboratory to ensure confidentiality of the patients.

Patients and sample collections

De-identified sputum samples, were collected from HIV positive patients visiting hospitals with respiratory syndromes such as cough or persistent cough, night sweating, fever or weight loss. After collection, the samples were inoculated on potato dextrose agar and incubated over night at 37°C. After incubation the samples were collected in a cooler box and taken to the laboratory for analysis.

HIV and TB diagnosis

The study participants were screened for antibodies to HIV with the OraQuick HIV1/2 (OraSure Technologies, USA), using oral fluids, and with DetermineHIV1/2 (Abbott Laboratories, USA), employing finger prick whole blood according to manufacturer's instruction as described previously[31]. TB diagnosis was performed at the Hospital by the acid fast staining as previously described[6].

Yeast Isolation and identification

The pure colonies from the plates were cultured in Sabouraud dextrose agar (SDA) at 37°C over night. Identification of the yeast isolates was done by noting colonial morphology, color, size, texture, and topography of the colonies. Urea agar based method was used for the identification of *Cryptococcus neoformans*. Chromogenic *Candida* agar (CHROMagar) was used to identify different species of *Candida* by noting the color change as previously described[24]. The germ tube test was done by adding 50µl of broth culture to 450µl of horse blood serum in a microcentrifuge tube and incubating at 37°C for three hours. After incubation the culture was suspended on a glass slide and viewed under a microscope at 40x magnification.

Biofilm production assay

All the microorganisms were tested for the pro-

duction of biofilm using the microtiter plate method as described by[7]. Briefly, Suspension of cultures were prepared in brain heart infusion (BHI) DIFCO™ broth with 1% sucrose and incubated over night. After washing with phosphate buffered saline (PBS), the plates were stained with 1% crystal violet, incubated at room temperature for 15minutes, and the plates were washed again and incubated at 37°C for 30 minutes to dry. Then 200µl of a mixture of 80% ethanol and 20% acetone was added to the plates to solubilise the crystal violet, the plates were incubated at room temperature for 20 minutes and the results were read at 490 nm using the ELISA reader.

Antifungal Susceptibility test by disc diffusion method

The activity of some commercial available drugs (antifungal agents) was tested using the disc diffusion method which was performed according to the Clinical Laboratory Standards Institute (CLSI). The antifungal agents used were: amphotericin B, fluconazole and ketoconazole. Petri dishes with potato dextrose agar (PDA) were inoculated with the culture using sterile swabs and a disc (antifungal drug) of each antifungal agent was placed on the cultured medium and incubated at 37°C over night. After incubation, the zone of inhibition (which is the diameter around the antifungal disc where the growth of the microorganism was inhibited) was measured using a ruler.

Statistical analysis

The data acquired were entered in Microsoft Excel sheet, and analysis was conducted by using statistical package for social sciences (SPSS) program version 17.1. Chi-square was used to determine the correlation between the different tests performed, and p values were used to determine the significance of the findings, considering p values ($p<0.05$) as significant.

RESULTS

Demographic information on the study population

A total of 91 sputum samples were collected from patients of different age groups and different gender. Out of the 91 samples; 53 were from females, 36 from males and 2 were from unknown gender. A total of 26 samples were from individuals aged less than 25 years, 37 samples were from patients aged between 26 – 45years while 18 samples were from patients aged above 45 years. Ten samples were from individuals of unknown age.

Prevalence of yeast organisms in sputum samples from HIV and AIDS patients con-infected with TB

Out of the 91 sputum samples collected from 91 consecutive patients, a total of 104 strains of different yeast species were isolated and identified. Out of the 91 patients, 64 (70.32%) were infected with at least one yeast species. Males were more infected (75%) compared to females (69.8%) although the difference was not significant ($\chi^2=5.124$; $p=0.77$). Generally the prevalence of yeast infections in the respiratory tract was higher among individuals aged above 45years (77.7%). In this study, there were 3 males aged above 45 years and all the 3 were infected while 4 out of the 5 males of unknown age were infected. Among the females, the highest rate of infection was observed among those aged above 45years.

Occurrence of single and multiple respiratory yeast infections among the HIV and AIDS patients co-infected with TB

Of the 91 patients, 27 (30%) were not infected with any yeast organism. Single infection defined as infection by only one yeast species was found in 38 (42%) patients while multiple infections defined as infection with more than 1 yeast species was found in 26 (29%) of the patients. Out of all the single infections, *C. krusei* was the most common representing 14% of all infections while *C. famata* was the least common with only 1 case.

Species distribution among isolates infecting HIV and AIDS patients co-infected with TB

The most commonly isolated yeast was *Cryptococcus neoformans* with 29 (28%) isolates followed by *C. Parapsilosis* with 20 (19%) isolates and *C. Krusei* with 17 (16%). *Candida albicans* was the fourth most commonly isolated yeast from the sputum of these patients with 15 (15%) isolates while the least common was *C. guillermondii* 1 (1%) isolates.

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Distribution of the different yeast species among HIV and AIDS patients co-infected with TB

Of all the yeast isolates *C. albicans* was more common among female patients (18%) compared to males (10%) however the difference was not statistically significant (χ^2=1.159 ; p=0.214). Similar situations were observed with *C. Krusei* and *C. Tropicalis* that were more common among females than males. *Cryptococcus neoformans* and *C. Parapsilosis* were more common among the males. *C. famata* and *C. guillermondii* were not isolated from females. While *Candida albicans* was more common among patients aged between 26 and 45 years, the non-albicans *Candida* showed a progressive distribution among the three different age groups considered in the present study. *C. krusei* and *C. tropicalis* were more common among the individuals aged above 45years and less common among patients less than 26 years old. On the other hand, *C. parapsilosis* was more common among individuals less than 26 years old and less common among individuals aged above 45 years, the same as *Cryptococcus neoformans* which was more common among individuals aged less than 26 years. Although *Candida albicans* was more common among females in general, the prevalence was higher among males aged above 45 years but *C. albicans* was not found among the males individuals aged less than 26 years. *Cryptococcus neoformans* was more common among the males aged more than 25 years. However, it was more common among the females aged less than 26 years.

Antifungal drug resistance of respiratory yeast organisms from HIV and AIDS patients co-infected with TB

Of the 3 antifungal drugs tested, amphotericin B was the most active with only 33.7% resistance among the fungal organisms compared to 74% resistance against fluconazole and 74% against ketoconazole. Non- *Candida albicans* appeared to be significantly more resistant to amphotericin B compared to *C. albicans* (χ^2=6.056; p=0.048) except *C. glabrata* from which none of the 4 isolates was resistant to amphotericin B. All the 2 *C. famata* (100%) and the *C. guillermondii* (100%) isolates were resistant to amphotericin B while *C. albicans* had a resistance rate of (6.7%). *Cryptococcus neoformans* had a resistance rate of 31% to amphotericin B while *C. krusei* had a resistance rate of 17%. Resistance to fluconazole and ketoconazole was generally high among all the fungal isolates with general resistance rates of 74% for both drugs. *C. krusei* showed the least resistance to fluconazole (64.7%) while *C. glabrata* (50%) followed by *C. parapsilosis* (65%) were the least resistant to ketoconazole.

Effect of gender and age of the HIV and AIDS patients co-infected with TB on drug resistance among the fungal isolates

Generally isolates from males were more resistant to all antibiotics compared to those isolated from females, however, the difference was not significant (p>0.05). Isolates obtained from individuals aged less than 25 years were generally more resistant to amphotericin B compared to those isolated from individuals aged 26 and above. Resistance to fluconazole and ketoconazole were more common among isolates obtained from patients aged above 45 years. A more detailed analysis of the drug resistance rate indicated that males aged between 26 and 45 were the least resistant to amphotericin B while isolates obtained from females aged above 45 years, were the most resistant to ketoconazole. None of the *C. albicans* isolated from males was resistant to amphotericin B while 10% of the isolates from females were resistant to amphotericin B. Similarly, *C. lambica* isolated from females were resistant to amphotericin B as compared to those isolated from males which showed 0.0% resistance. *C. albicans*, *C. parapsilosis* and *C. tropicalis* isolated from males showed 100% resistance to fluconazole where as *C. lambica* and *C. glabrata* isolated from females showed 100% resistance to the same drug. All the species isolated from males showed high resistance to ketaconazole with an exception of *C. lambica* which was the only species that showed resistance in isolates from females and 0.0% resistance in isolates from males.

Multiple drug resistance among the isolates obtained from HIV and AIDS patients co-infected with TB

Multiple drug resistance (MDR) defined as the simultaneous resistance of an isolate to more than one drug was common and 75 (72.8%) were resistant to

more than 1 drug at a time. Of all the isolates tested, 19 (18.4%) were susceptible to all the 3 drug tested, 9 (8.7%) were resistant to only one drug. Forty six isolates (44.7%) were resistant to 2 drugs while 30 (28.8%) were resistant to all the three drugs at a time. Of all the yeast organisms, *C. tropicalis* showed 46.2% resistance to all the three drugs while all the 2 *C. famata* strains and the only *C. guilliermondii* strain were resistant to all the 3 drugs investigated. Of all the isolates, those obtained from males had the highest MDR rate (81.3%) compared to those isolated from females (61.5%) (χ^2=3.231; p=0.057). The rate of MDR was similar among the age groups, however, the rate of resistance to 3 drugs was higher among the isolates obtained from individuals aged less than 26 years (41.8%) compared to individuals aged 26 – 45 (21.1%) and those from individuals aged more than 45 years (26.3%). Of all the species, *C. famata* and *C. guillermondii* were all resistant to more than 1 drug at a time. *C. glabrata* was the least resistant species with MDR while 73.3% of *C. albicans* isolates were resistant to more than 1 drug.

Biofilm production among the respiratory yeast isolates obtained from HIV and AIDS patients co-infected with TB

Biofilm production was tested among the yeast organisms and 29 (27.9%) of all the isolates were biofilm producers. Of all the organisms identified, the only *C. guillermondii* isolate was positive for biofilm while 1 of the 2 *C. famata* and 2 of the 4 *C. glabrata* were biofilm producers. One third of all the 15 *C. albicans* strains were biofilm producers. When all the other *Candida* spp. were considered together, biofilm production appeared only in 25% of non-albicans *Candida*. The sex and the age of the patients from whom the isolates were obtained did not have any impact on biofilm production by the isolates (p=0.661 for sex and 0.158 for age). In the present study, biofilm production did not seem to have any effect on drug resistance.

DISCUSSION

Respiratory infections are commonly found among HIV and AIDS patients and the etiology varies between bacteria, viruses and fungi. Several studies have investigated the involvement of fungal pathogens in respiratory infections and most reports so far have implicated *C. albicans* as the most common agent of *Candida* pneumonia[8]. However, recent studies have reinforced the growing importance of non- albicans *Candida* such as *C. parapsilosis* in fungal diseases of the respiratory tract. The present study indicates that non-albicans *Candida* co-infect the lungs of HIV and AIDS patients with TB. Data on the diversity of *Candida* species affecting human is increasing steadily, and several studies have highlighted the involvement of different species in human diseases in relation to pneumonia[30]. Recent studies in 41 different countries have indicated increased rates of isolation of the common non-albicans species *C. glabrata* (10.2% to 11.7%), *C. tropicalis* (5.4% to 8.0%), and *C. parapsilosis* (4.8% to 5.6%) when the time periods 1997 to 2000 and 2005 to 2007 were compared[27]. In the present study non-albicans *Candida* spp. appeared to be important opportunistic pathogens causing respiratory infections in HIV patients in Vhembe district with *C. parapsilosis* (19%) being the most common followed by *C. krusei* (16%) while *C. albicans* was only the third most common species isolated from sputum samples. Studying yeast isolates from different types of samples from 24 hospitals in Taiwan, Yang et al.,[35] found that *Candida albicans* (69.1%) was the most common species, followed by *Candida tropicalis* (12.9%), *Candida glabrata* (8.3%), *Candida parapsilosis* (2.7%), *Candida krusei* (0.6%), and others (6.4%), whereas[3] also found that *C. albicans* (45%) was the predominant species isolated followed by *C. tropicalis* (24.7%) and *C. parapsilosis* (10.5%) from all clinical specimens (Sputum, blood, urine and others), except blood from which *C. krusei* was most frequently (38.4%) recovered. In a study on oropharyngial candidiasis among HIV patients in Nigeria, *Candida albicans* (40.5%) was the most frequently isolated species, while the non-albicans species included *C. tropicalis, C. Krusei,* C. *glabrata* and *C. neoformans* for species having up to 4 isolates[12]. The contribution of these organisms to the pathogenicity of respiratory disease could not be established from present study since these were secondary to TB infections. Therefore further studies need to be conducted with emphasis on clinical presentation of the disease in order to determine the pathogenic impact

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of the fungal isolates. The management of fungal infections among HIV and AIDS patients as well as immunocompetent patients includes the use of various antifungal agents including azoles and polyenes[25]. Fluconazole (FLC) belongs to the azoles and interferes with ergosterol biosynthesis by binding to the *ERG11* gene product, lanosterol 14-α- demethylase. In a study in Nigeria, resistance to fluconazole was 22%[12]. This prevalence is far much lower compared to the prevalence of resistance observed in the present study which was 74%. Although fluconazole had been shown to be very active against most clinical yeast organisms mostly *Candida* spp, with susceptibility levels of 90%, an increase in fluconazole resistance over time was seen with *C. parapsilosis, C. guilliermondii, C. lusitaniae, C. sake*, and *C. pelliculosa*[27]. Among the emerging fluconazole-resistant species were *C. guilliermondii* (11.4% R), *C. inconspicua* (53.2% R), *C. rugosa* (41.8% R), and *C. norvegenesis* (40.7% R). In another study on the susceptibility of *Candida* spp. to amphotericin B and fluconazole, Yang et al.,[36] reported that the prevalence of non-albicans *Candida* species had increased as compared to previous studies, and this was due to the frequent exposure of non-*albicans* to fluconazole which induces the exposed species to develop resistance. Resistance among fungal organisms to antifungal drug is often the result of multiple factors including the expression of efflux pumps in the fungal cell membrane, biofilm matrix permeability, and a stress response in the fungal cell[23]. Although several factors might be responsible for the high resistance of yeast isolates to floconazole in the present study, frequent use of fluconazole in the treatment of fungal related infections among HIV and AIDS patients in the region might be an important cause of this high resistance. The present study did not obtain clinical information such as the CD4 counts of the patients or the viral load. However, the potential effects of fungal infections in the lungs need to be clarified. In fact, many patients treated for TB in the hospitals are "smear negative"[18]. This raises the possibility of other pathogens been involved in the symptoms experienced by the patients. Recent studies by Jarvis and colleague have demonstrated the disastrous consequences of pulmonary cryptococcosis misdiagnosed as smear-negative pulmonary tuberculosis with fatal consequences[16]. The present study also demonstrates the high prevalence of Cryptococcus neoformans in sputum samples of HIV patients co-infected with TB and therefore supports the possibility of TB patients to be susceptible to other fungal infections as well or the possibility of these fungal infections being misdiagnosed in a limited number of cases as "smear negative TB". Further studies are needed to confirm the role of pulmonary fungal infections in "smear negative" patients. The formation of biofilm is a major virulence factor in *C. albicans* pathogenesis and is related to drug resistance among these organisms. Studies by[22] indicated that sessile *C. albicans* cells show controlled regulation of gene expression, as they quickly mount a drug specific transcriptional response in the presence of high doses of antifungal agents. Biofilm production depends on the different species of *Candida*. In the present study we found that 33% of *C. albicans* produced biofilm and appeared to be the most biofilm producing species when all the other *Candida* species were considered. In a study conducted by[9], (2007) the biofilm production rate of non-albicans (41%) was found higher than albicans (23%), and the difference was statistically significant ($p < 0.05$). We did not find any association between biofilm production and drug resistance in the present study as previously stipulated [32]. This difference could be due to factors that could be experimental or strain related. Studies on larger samples with different conditions might give more insight into the role of biofilm on drug resistance among yeast isolates in the Kisumu Town district. *Cryptococcus neoformans* is responsible for several syndromes of which the most important is cryptoccoccal meningitis followed by cryptococcal pneumonia[32]. Deok-Jong Yoo et al.,[10] in a study done in Mulago Hospital (Uganda) reported that pulmonary cryptococcosis was common in HIV patients suspected with tuberculosis and the median CD4 count among those with pulmonary cryptococcosis was 23 cells per microliter. In the present study *C. neoformans* was the most commonly isolated organism in sputum samples (28%) among patients with tuberculosis. Previous studies have indicated that the isolation of *C. neoformans* from bronchial secretion of HIV positive patients is a signal of disseminated cryptococcosis[14] (Other types of *Cryptococcus* have also been reported in the sputum of AIDS patients. In Tamilnadu, India, a case of pulmonary cryptococcosis

caused by *C. laurentii* in a diabetic AIDS patient who was on anti-tuberculosis and antiretroviral treatments was reported[32]. In the present study, the only species isolated was *C. neoformans*, however, no effort was made to further characterize the *Cryptococcus* spp. isolated. *Cryptococcus neoformans* biofilm producing strains have been shown to be more resistant to antifungal drugs than the non biofilm producing strains. Martinez et al.,[20] reported that the exposure of *C. neoformans* cells or preformed cryptococcal biofilms to fluconazole or voriconazole did not result in yeast growth inhibition and did not affect the metabolic activities of the biofilms, respectively. In the present study *Cryptococcus neoformans* was resistant to all the drugs tested with the lowest resistance being observed to amphotericin B (31% resistance). Increased resistance could be associated with exposure to the drug as previously described by[33] who observed in their study that, upon exposure to stepwise increases in the concentration of FLC, subpopulations that could grow at higher concentrations emerged.

CONCLUSIONS

In conclusion, this study shows that HIV patients infected with TB are often colonized with fungal organisms. These organisms might contribute to the exacerbation of the respiratory syndromes although further studies are needed to confirm this hypothesis. The most common opportunistic yeasts found in the respiratory tract of HIV patients co-infected with TB attending Hospital centers in Kisumu Town District, Kenya, are *Cryptococcus neoformans*, *Candida parapsiliosis*, *Candida krusei* and *Candida albicans*. Amphotericin B was the most active drug against these opportunistic pathogens. This study further highlights the need for routine speciation and antifungal susceptibility testing on HIV patients. It also indicates the emergence of non-*albicans Candida* as potential important respiratory pathogens in HIV patients in Kisumu Town District and more studies are needed to clarify the role of these fungal organisms in the clinical presentation of pneumonia in the target population. The identification of the infecting organisms and antifungal susceptibility testing will enhance the ability of clinicians to prescribe appropriate antifungal therapy. Further studies are needed to confirm the role of pulmonary fungal infections in "smear negative" patients.

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