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CHANGING EPIDEMIOLOGY AND ANTIFUNGAL SUSCEPTIBILITY PATTERNS OF *CANDIDA* SPECIES ISOLATED FROM GHANAIAN HIV-POSITIVE PATIENTS

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ABSTRACT

The most common type of fungaemia, candidaemia is caused by Candida spp. There is an alarming emergence of resistant strains of Candida spp. to antifungal treatment patterns. In this study, candidaemia among Ghanaian HIV/AIDS patients was compared to a similar study in 2008 to ascertain the changing prevalence of different Candida spp. and novel susceptibility patterns to the common antifungal drugs; amphotericin B, clotrimazole, ketoconazole, fluconazole itraconazole and nystatin. From 2010 – 2014, a total of 176 Candida spp. (including 15 non-speciated isolates) were obtained from HIV positive individuals. The species were isolated and identified by culture on Sabouraud agar, sugar fermentation, assimilation, urease production tests and confirmed by mini API bioMerieux analyzer. Comparing the two studies over the nine-year period, though not statistically significant (p=0.362), we observed that the incidence of Candida albicans reduced by 9.1%, whereas Candida tropicalis, Candida parapsilosis, Candida glabrata and the non-speciated increased in incidence by 3.6%, 1.7%, 3.3% and 3.1%, respectively. Similarly, amphotericin B (p=0.071) and clotrimazole (p=0.261) despite observable declines in susceptibility of isolates showed no significant decline in drug potency. There was significant increase in resistance of isolates to commonly prescribed antifungal agents; itraconazole (p=0.003), fluconazole (p=0.000), ketoconazole (p=0.008) and nystatin (p=0.049). Most probably, treatment failure of candidaemia among HIV positive individuals on antiretroviral therapy is increasing.

Key words: ***Antifungal drugs – candidaemia – epidemiology – HIV/AIDS***

INTRODUCTION

Candida species were merely regarded as culture contaminants until the early parts of the twenty first century when they evolved into the major causative agents of candidaemia (Klepser, 2001). *Candida* spp. can grow on mucous membranes or elsewhere in the body resulting in symptoms known as candidiasis or thrush. Oropharyngeal candidiasis occurs in the gums, tongue, inner cheek or upper throat while esophagheal candidiasis is more serious since it can make eating painful among HIV/AIDS patients. Genital candidiasis can occur in the vagina in women and under the foreskin of men (David et al., 1997). Patients with advanced HIV/AIDS may also suffer from pulmonary candidiasis. Over time, *Candida* spp. have emerged as the most frequent nosocomial fungal pathogen and most recently, the health risks associated with candidaemia have even been complicated by the emergence of pathogenic non-albicans *Candida* spp. (Amar, et al., 2013 and Chakrabarti et al., 2002).

Candida infection in humans is normally controlled by the immune system (Mulley and Goroll, 2006). This implies that immunocompromised states such as HIV/AIDS and cancer render the host susceptible to a wide spectrum of infections including fungal infections (Kumar et al., 2007). For instance, a high incidence of candidiasis has been shown in individuals with limited neutrophil function as well as people with immunocompromised conditions like HIV/AIDS (Amar, et al., 2013). On the other hand, HIV-negative individuals may experience candidiasis when the immune system is temporally depressed by factors like

stress or alcohol, as well as diabetes (Mårdh et al., 2003).

Between 2003 – 2005, a study we conducted in Ghana indicated that a majority of isolates from HIV/AIDS patients with candidaemia and disseminated candidiasis were *Candida albicans* whereas non-*albicans Candida* constitute the minority (Siakwa et al., 2008). However, a distinct increase has been noted in the proportion of cases resulting from infection with non-*albicans Candida* spp. (Amar et al., 2013; Quindos et al., 1994 and Pfeller et al., 2001).

It is also evident that candidiasis affects some people on antibiotics, since the antibiotic temporally kills some of the harmless bacteria that inhabit the body, thus providing an opportunity for *Candida* spp. to replace such microbes (Mardh et al., 2003). There are several forms of antifungal drugs for the treatment of candidiasis: tablet forms (ketoconazole, itraconazole and fluconazole), suspensions for oral candidiasis, creams for skin and nail infections as well as pessaries for vaginal candidiasis. Other antifungal drugs are offered as lozenges and these include clotrimazole, nystatin and amphotericin B. No matter the form, antifungal drugs have pharmacologic side effects such as nausea, vomiting and rashes, while other drugs, like itraconazole and ketoconazole interact with drugs administered to people with HIV/AIDS (Dooley et al., 2008). Dooley et al. (2008) affirmed earlier findings of Albengres et al. (1998) that azoles do present as potent inhibitors of cytochrome P450 3A4 (CYP3A4). Some *Candida* strains become resistant to fluconazole, particularly among HIV/AIDS patients with low CD4 counts or those who have taken the drug for a long time (Ha and White, 1999). Azole antifungal drug resistance has become a major problem in the treatment of oral infections of *Candida albicans* in HIV/AIDS patients (Ha and White, 1999).

Drug resistance is becoming a major source of concern in HIV/AIDS patients. Resistance to 5-fluorocytosine and azoles appears to be increasing in some groups of patients (Juine et al., 2007; Abdel et al., 2007). A periodic surveillance of the antifungal susceptibility pattern of prevailing *Candida* spp. has become important. Earlier, we reported the distribution of *Candida* species isolated from patients with candidaemia as well as the antifungal susceptibility pattern of both *Candida albicans* and non-*albicans Candida* spp. from the Cape Coast Teaching Hospital of Ghana over a 3-year period (Siakwa et al., 2008). The present study thus aimed at determining novel characteristics in the incidence and distribution of *Candida* spp. isolated from HIV/AIDS patients from 2010 to 2014 to enable us assess the changes in epidemiology and antifungal susceptibility patterns of the isolated *Candida* spp..

MATERIALS AND METHODS

Isolation, identification and antifungal susceptibility testing

The study was carried out between January, 2010 and June, 2014 at the Cape Coast Teaching Hospital, a referral hospital in the Central Region of Ghana. *Candida* spp. were isolated from samples of blood, oral, sputum, cerebrospinal fluids, vaginal swabs, urine and stool specimen from HIV positive patients at the Central Regional Hospital, Cape Coast. The growth pattern in Sabouraud agar, germ tube formation and chlamydospore formation on corn meal agar were examined. Also sugar fermentation, assimilation and urease production tests were performed on these isolates for identification and speciation (Huppert et al., 1975 and Clinical and Laboratory Standards Institute (CLSI), 2009). Candidaemia was defined when *Candida* spp. were isolated from one or multiple blood cultures in association with clinical symptoms of septicaemia or sepsis. For fungal blood cultures, 5-10 ml blood was drawn aseptically from patients and inoculated immediately in two sets of biphasic media containing brain-heart infusion agar and broth. One set was incubated at 37°C and the other at 25°C. Subculture was done by tilting the culture bottle to cover the solid phase by liquid phase for 1h after 2, 7 and 14 days from the day of inoculation. Bottles were checked every day for the first week, and twice weekly for the next 2 weeks for growth of yeast, if any. Yeast colonies were identified by germ-tube test, urease test, reduction of tetrazolium media, different conidial arrangements on corn meal agar, sugar assimilation and fermentation tests (Taschedjian et al., 1960; Hasenelever, 1971; Huppert et al., 1975; Tierno and Milstoc 1977; Martin, 1979).

Pure cultures of Candida spp. were confirmed using API ID 32C test kit strips

(BioMerieux, Marcyl`Etoile, France). The mini API VITEK is a semi-automated system, which has features for full interpretation of results. It has several benefits, including assistance for validation of all results, identification of resistance phenotypes and more refined prediction of therapeutic results (Angus et al., 2001; Vincent et al., 2006). During this process, a minimum of five colonies were suspended in 0.9% saline and adjusted to a 0.5 McFarland standard (corresponds to 1 x 10^6 to 5 x10^6 CFU/ml) by using a Vitek colorimeter (BioMerieux, Vitek Inc). The stock solution was then diluted 1:50 in RPMI 1640 medium and then 1:20 to obtain a 2X test concentration. Following this, 100 µl of the 2X inoculum was pipetted to prepare antifungal dilutions in microwells to achieve a final concentration of 0.5 x 10^3 to 2.5 x 10^3 CFU/ml in a final test volume of 200 µl. The microwell plates were incubated at 35°C for 48 ± 2 h. (mean ± SE). The minimum inhibition concentrations (MICs) were calculated as the lowest concentration with no growth. All tests and controls were performed in duplicates while the final inoculum size was confirmed by subculture and colony count.

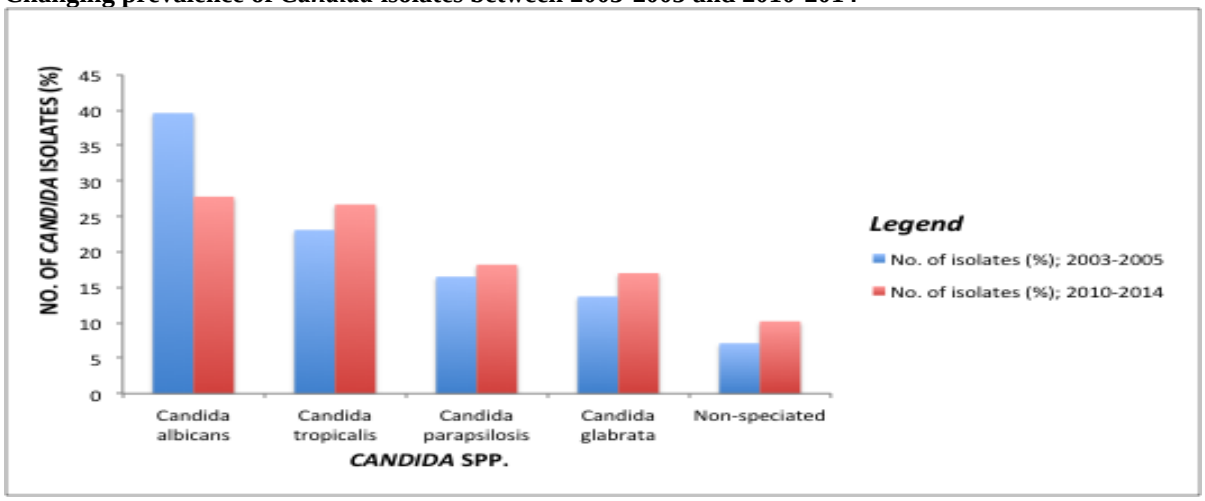
Following this, the antifungal susceptibility patterns of isolates were evaluated against amphotericin B, itraconazole, fluconazole, clotrimazole, ketoconazole and nystatin. The susceptibility tests were performed by standard broth macro-dilution and micro-dilution antifungal susceptibility testing of yeasts, approved by the Clinical and Laboratory Standards Institute (CLSI) (2009) and Dota et al. (2011).

Statistical analysis

Descriptive statistics such as mean, standard deviation, percentages and graphs were used to describe the data. The paired t-test was used to compute the differences between the means of the two study groups for 2003-2005 and 2010-2014. The paired t-test was also used to evaluate the differences in performance of the individual antifungal drugs on the *Candida* spp. or isolates for the two study periods. Finally, the One-way Analysis of Variance Test (ANOVA) was then used to analyze unique differences in efficacies of the drugs (pooled together) against every individual isolate of *Candida* spp. A p-value < 0.05 was considered a statistically significant difference between any compared mean groups.

RESULTS AND DISCUSSION

Changing prevalence of *Candida* isolates between 2003-2005 and 2010-2014



t-value=1.02; p-value=0.364; No significant difference

Figure 1: A clustered bar chart showing the changing prevalence of *Candida* spp. isolated from the HIV-positive patients over the two study periods 2003-2005 and 2010-2014

Despite an observable change in prevalence of *Candida* spp. between the study periods as seen descriptively from figure 1, the p-value of 0.364 indicates that the observed change in prevalence is not statistically significant to allow us draw solid conclusions

that there has been a rapid change in prevalence between the two study periods. Figure 1 shows a decrease in the incidence of *Candida albicans* from 39.6% in the 2003-2005-study period to 27.8% in the 2010-2014-study period. The non-*albican Candida* spp. however, were observed to have increased from the 2003-2005 period to the 2010-2014-study period. For instance, in *C. tropicalis*, the incidence increased from 23.1% in the 2003-2005 period (Siakwa et al., 2008) to 26.7% in the 2010-2014 period. Similarly, the incidence increased from 16.5% to 18.2% for *C. parapsilosis*, 13.7% to 17.0% for *C. glabrata* and 7.1% to 10.2% for the non-speciated *Candida* isolates. The increase in incidence could be due to the development of resistance to the drugs used. In fact the development of resistance to antifungal drugs in *Candida* spp. following prolonged exposure to azole antifungals have been reported (Cowen et al., 2000; Mishra et al., 2007). Even though the incidence of *C. albicans* was reduced, it is still the most common. Strikingly, the increased incidence of non-*albicans Candida* indicates that susceptibility testing should be an important tool in the management of patients with invasve candidiasis since *in vitro* resistance and toxicity issues should be considered when selecting an antifungal agent (Hoffman and Pfaller, 2001; Pfaller and Yu, 2001; Rex and Pfaller, 2002).

Changing susceptibility patterns of *Candida* isolates against antifungal drugs

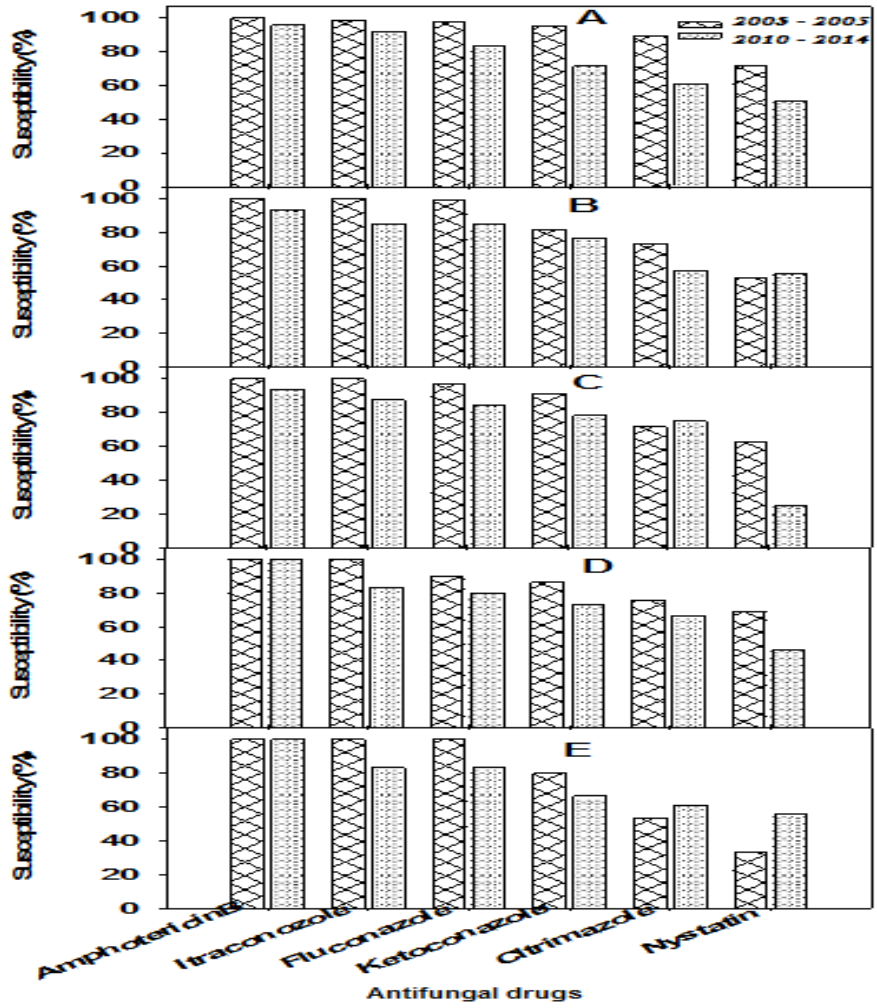


Figure 2. A chart showing the change in susceptibility patterns of *Candida* spp. against the antifungal drugs for the two study periods, 2003-2005 and 2010-2014. (A= *Candida albicans*; B= *C. tropicalis*; C= *C. parapsilosis*; D= *C. glabrata*; E= Non-speciated).

a. Paired t-test of individual drug-efficacy on pooled *Candida* isolates

Antifungal drug	t-value	p-value (CI=95%)
Amphotericin B	2.45	0.071
Itraconazole	6.47	0.003
Fluconazole	16.10	0.000
Ketoconazole	4.88	0.008
Clotrimazole	1.31	0.261
Nystatin	2.79	0.049

Table 1. The paired t-test of mean differences on the efficacy of the individual antifungal drugs against pooled *Candida* isolates over the two study periods in comparison, 2003-2005 and 2010-2014.

b. One-way ANOVA of the susceptibility of individual *Candida* isolates to the pooled drug-efficacy

Candida isolate	F-value	p-value (CI=95%)
Candida albicans	2.25	0.176
Candida tropicalis	9.25	0.009
Candida parapsilosis	4.66	0.044
Candida glabrata	4.10	0.058
Non-specified	7.30	0.016

Table 2. The one-way ANOVA-test of mean differences on the changing susceptibility patterns of individual *Candida* isolates upon exposure to the antifungal drugs pooled together with respect to the two study periods in comparison, 2003-2005 and 2010-2014.

As can be observed descriptively from the clustered bar chart above (figure 2), our study findings indicate a change in susceptibility of isolates between the two study periods, 2003-2005 and 2010-2014. All *Candida* spp. showed a decline in susceptibility from the 2003-2005 to 2010-2014 study periods. Despite this decline in susceptibility for all *Candida* spp., only *C. tropicalis*, (p=0.009), C. *parapsilosis* (p=0.044) and the non-speciated *Candida* isolates (p=0.016) produced a significant change in susceptibility pattern between the two study periods. *C. albicans* and *C. glabrata* with respective p-values of 0.176 and 0.058 did not produce any significant change in susceptibility patterns. In fact *Candida albicans* (p=0.176) seemed the most susceptible isolates that have low resistance potential to the drugs and this trend indicates the need for identifying more potent therapeutic interventions to treat the emerging non-albican *Candida* isolates, which possess a significantly resistant potential against the drugs that are currently under usage. These observations are congruent with the findings of Amar et al. (2013).

An assessment of the potency and efficacy of the individual drugs over the two study periods showed that itraconazole (p=0.003), fluconazole (p=0.000), ketoconazole (p=0.008) and nystatin (p=0.049) significantly declined in efficacy against pooled isolates over the two study periods. There arises a remarkable consequence from these observations as scientific evidence has shown that azole antifungal drugs are some of the active inhibiting factors of cytochrome P450 3A4 (CYP3A4) and ultimately significant interactions between these antifungal drugs and antiretroviral drugs (Dooley et al., 2008). Without assessing the cumulative drug-drug interactions, we find reason to be cautious in suggesting a decline in efficacy of these antifungal drugs in HIV-positive

individuals who are on antiretroviral therapy. This also suggests that drugs with fewer interactions are probably the best way to go even though they could prove to be rather expensive to access especially in resource-poor areas of the world (UNAIDS, 1998). On the other hand, amphotericin B (p=0.071) and clotrimazole (p=0.261) despite observable declines in susceptibility of isolates, do not produce a significant decline in potency, thus an indication that these drugs are still highly favourable compared with their counterparts. We must be circumspect in our conclusions however because another study with a similar research design would be beneficial in helping us monitor the possible emergence of highly resistant strains of *Candida* spp. to these rather popular antifungal drugs.

These findings are also consistent with the findings of Amar et al. (2013) where they detected that amphotericin B was the most sensitive drug to the *Candida* spp. They also noted that fluconazole and nystatin faced significant resistance from the *Candida* spp. and this is also consistent with our findings thus, fluconazole, nystatin, itraconazole, ketoconazole had a significant decline in efficacy against the *Candida* spp. between the two study periods. The case for fluconazole is however inconsistent with the findings of Mokaddas et al. (2007) who noted that fluconazole , widely used in Kuwait, showed no evidence of enhanced resistance.

CONCLUSION

The proportion of *Candida albicans* reduced between the two periods of study while the non-*albicans Candida* increased. There has been an increase in resistance to the antifungal drugs assessed in this study, but the susceptibility to the newer drugs such as amphotericin B, itraconazole and fluconazole, was higher than that for the usually prescribed drugs, like nystatin. The resistance to the antifungal drugs may be due to the development of new strains with prolonged single drug usage or few drugs` application in the treatment of infections. Furthermore, there is the need to use advanced molecular/genetic techniques to identify if new strains have evolved, and if so, establish the extent of antifungal drug resistance

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