Characterization and antifungal drug susceptibility of clinical isolates of *Candida* species.

V. B. Ooga[1*], J. K. Gikunju[2] , C. C. Bii[3]

1. MSc,School of Health Sciences, Medical Laboratory Science, Kenyatta University. Email:violetooga@yahoo.com
2. PhD,Institute of Tropical Medicine and Infectious Diseases (ITROMID),Jomo Kenyatta University of Agriculture and Technology (JKUAT).E-mail : joekangangi2@yahoo.com
3. PhD,Centre for Microbiology Research, Kenya Medical Research Institute (KEMRI).
E-mail: kiptobi@yahoo.com

[*] ***Corresponding author:*** *V. B. Ooga. Email:violetooga@yahoo.com*

SUMMARY

***Candida* species are responsible for a wide spectrum of infections in man. They can be isolated from most sites of a human body. These mycoses are most common in immunocompromised patients as opportunistic infections. Azoles have been used in treatment, prophylaxis and currently as maintenance therapy for candidiasis in these patients. The aim of the study was to characterize *Candida* species from clinical sources and determine their susceptibilities to azoles in Kenya. The study was conducted in 2009 in a mycology laboratory at Kenya Medical Research Institute (KEMRI). A total of 50 isolates of *Candida* were characterized and correctly identified to species level by germ tube test, Pal's agar, Chromogenic agar Candida, corn meal agar and Analytical Profile Index (API 20C AUX). 45 isolates were identified as *Candida albicans,* 1 as *Candida glabrata,* 1 as *Candida famata* and 3 as *Candida parapsilosis*. Susceptibilities of *Candida* species to fluconazole, posaconazole, itraconazole and clotrimazole were determined using Epsilometer-test and disc diffusion method. Their Minimum Inhibitory Concentrations (MIC's) were correlated. In Epsilometer test, 78% of *Candida* species were susceptible to clotrimazole and posaconazole, 60% to fluconazole and 50% to itraconazole. In disc diffusion method, 92% *Candida* species were susceptible to clotrimazole, 74% to itraconazole, 78% to posaconazole and 46% to fluconazole. There were no significant differences in susceptibility between E-test and disc diffusion methods for clotrimazole, itraconazole and posaconazole which had low significance levels ($p<0.002$). Fluconazole had the greatest difference between the two methods ($p=0.002$) and a kappa value of 0.329. There is emerging fungal resistance to fluconazole and itraconazole therefore further investigations on fungal resistance and rational use of antifungal drugs is necessary.**

[Afr J Health Sci. 2011; 19:76-83]

Introduction

With the rising frequency and spectrum of fungal infections, as well as increasing reports of resistance to antifungal agents, it calls for constant antifungal susceptibility testing in clinical set ups (Barry *et al*., 2000). This will help the clinician in decision making in terms of choice of antifungal treatment (Barry *et al*., 2000).

Candida is now the fourth leading cause of nosocomial infections. The rise may be attributed to advances in medical practice such as intravascular catheters, total parenteral nutrition (TPN), broad spectrum antimicrobials, and dialysis. Patients like those of solid organ and bone marrow transplants, acquired immune deficiency syndrome (AIDS), and intensive chemotherapy regimens are at high risk (Vendettuoli *et al*., 2009). It is therefore imperative that the armamentarium of antifungals expands to treat these infections. Irrational use of antibiotics and prophylactic use on the other hand is fuelling antimicrobial resistance including resistance to antifungal drugs (Maenza *et al.,* 1996). Evidence of emergence azole resistance exists especially in patients empirically treated with fluconazole (Bii *et al.,* 2002).

New generation triazoles have therefore been developed to meet the increasing need for new antifungals, and address the rising incidence of invasive fungal infections and the emergence of fungal resistance (M cGinnis and Rinaldi, 1996). Therefore susceptibility testing of new generation triazoles need to be determined to serve as baseline data for future studies.

The study was aimed at characterizing and comparing antifungal drug susceptibilities of *Candida spp.* from clinical sources.

Materials and Methods

The study was laboratory based carried on clinical isolates of *Candida* species. The study was conducted between May and December, 2009. Fifty isolates of *Candida* species were recovered from archived samples. Specimen from which archived samples were made include: urine, High Vaginal Swabs, necrotic swabs, pus swabs, sputum and throat swabs. Identification, confirmation of the species and susceptibility testing were conducted at a mycology laboratory, Center for Microbiology Research (CMR), KEMRI. The research protocol was reviewed and approved by the Scientific Centre Committee, Scientific Steering Committee and Ethical Review Committee of KEMRI. All *Candida* isolates were subcultured on Sabouraud's Dextrose Agar. Germ Tube Test was used for presumptive identification of *Candida albicans*. Yeasts that were germ tube-negative were cultured on Chromagar Candida and Pal's Agar to differentiate between *Candida dubliniensis* and *Candida albicans*. This was done on the basis of production of a hyphal fringe on Pal's Agar and green color on CHROMagar Candida. Germ tube-negative yeasts were further identified using corn meal agar on a slide culture basing on chlamydospores production. Analytical Profile Index (Biomeriux) was used as a confirmatory test for all the *Candida spp.* The susceptibility tests were done using disc diffusion and E-test methods and recorded as per CLSI guidelines and manufactures' instructions. E-test and Disc diffusion results were compared using Kappa test.

Results

Identification and confirmation

Identification tests of the fungal yeasts yielded the following results as shown in the figures (Fig.3, Fig. 4, Fig.5, and Fig.6 & Fig.7) in Germ tube test, CHROMagar-Candida, Pal's agar and Corn meal agar respectively. Basically colonial morphology was use [d] to classify various species of *Candida.* In germ tube test, *Candida albicans* produced short one piece germ tubes which discriminated them from *C.tropicalis, C.famata* and *C. parapsilosis*. *Candida albicans* were characterized by their green color in CHROMagar-Candida; *Candida parapsilosis* were orange-pink whereas *Candida tropicalis* were noted by their atypical green color (Fig. 4). In corn meal gar, *C .albicans* produced good growth white colonies with chlamydospores in singles whereas *C.tropicalis* and *C. parapsilosis* produced good growth white / cream colonies with chlamydospores in clusters (Fig.7). Colonies of *Candida albicans* produced smooth colonies without a hyphal fringe on Pals' agar (Fig 5) whereas those of *C.tropicalis, C.famata* and *C. parapsilosis* were rough with hyphal fringes (Fig. 6). Confirmatory test Analytical Profile Index (API 20C AUX-Biomeriux) yielded the 4 species that were investigated in the study; *C. albicans C.tropicalis, C.famata* and *C. parapsilosis.* It is based on fermentation and utilization of different substrates.

Figure 3; Appearance of *Candida albicans* showing germ tubes in serum

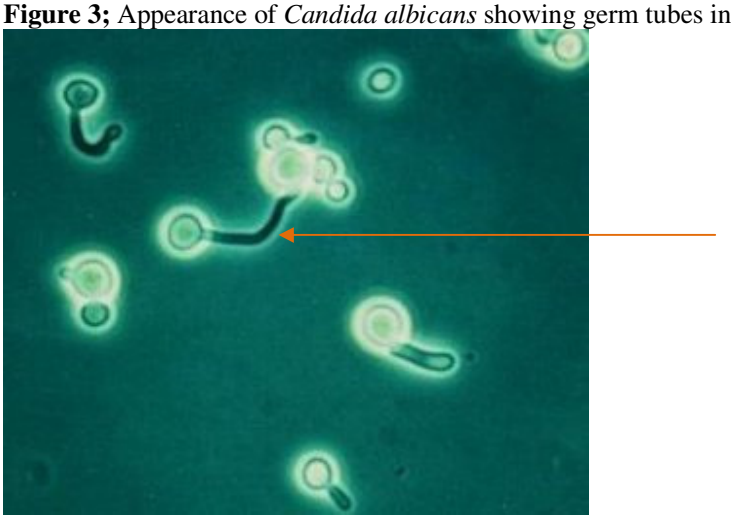


Figure 4; CHROMagar Candida plate showing colonies of (a) *C. parapsilosis, (b) C. albicans and (c) C. tropicalis*

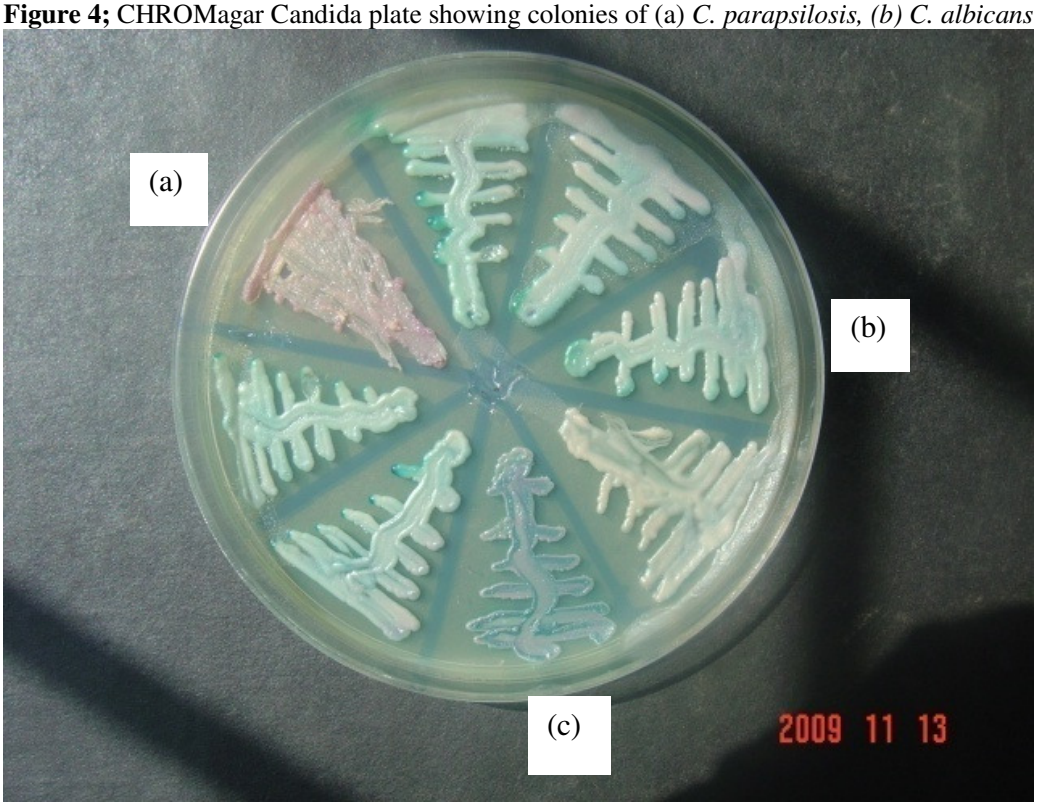


Figure 5; Appearance of smooth colonies of *Candida albicans* on Pal's agar

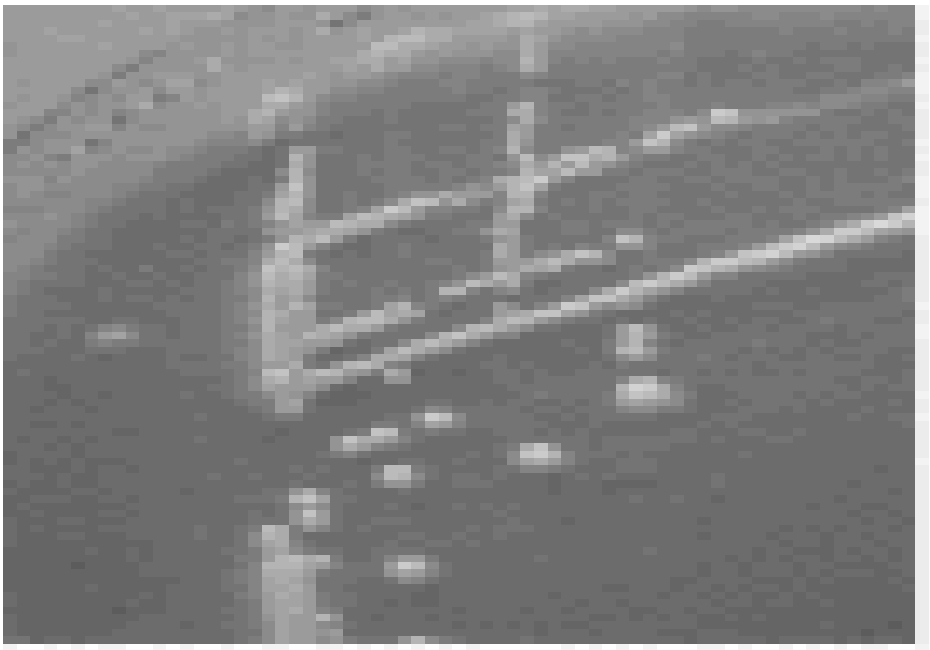


Figure 6: Appearance of rough colonies with a hyphal fringe of *Candida krusei* on Pal's agar

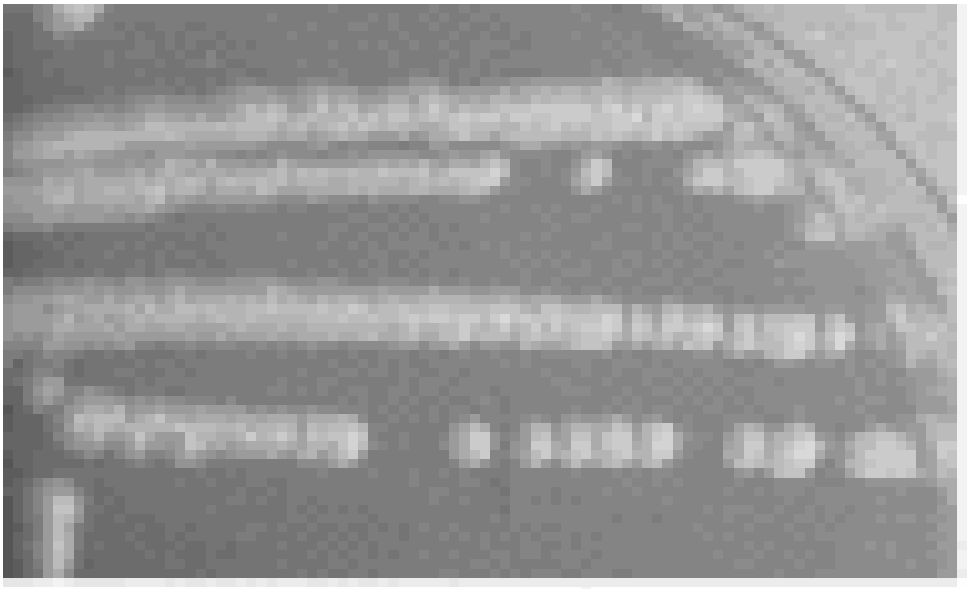
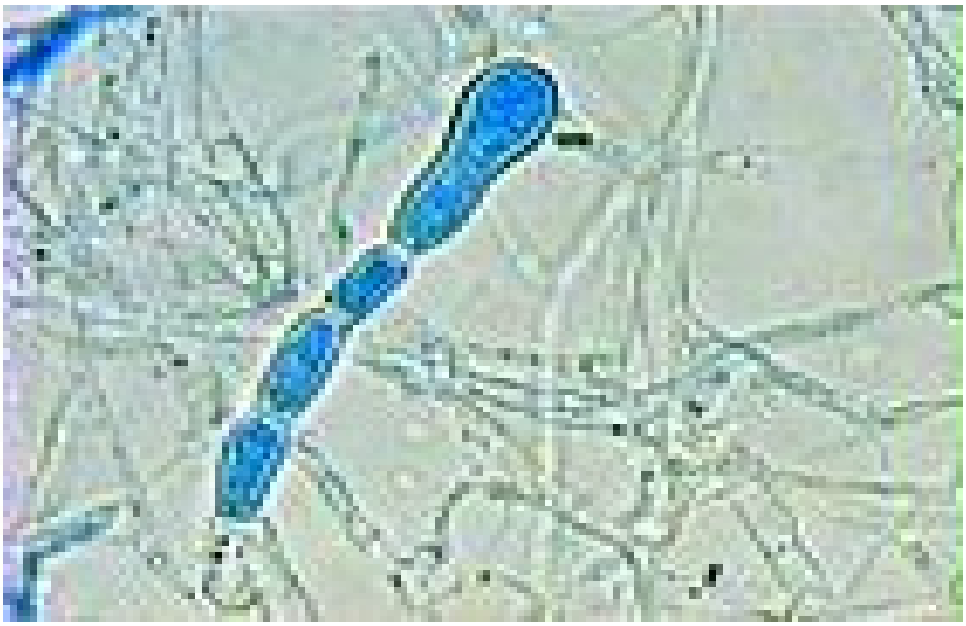
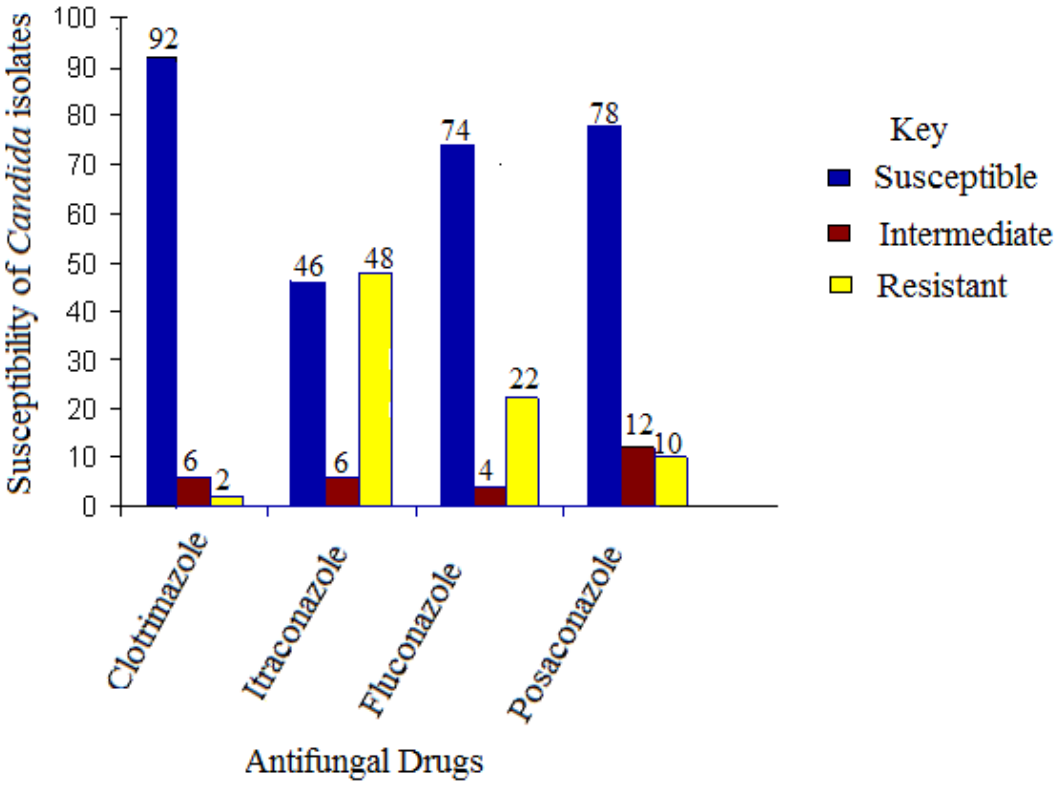


Figure 7; Microscopic appearance of Chlamydospores in singles of *C. albicans* in corn meal agar.



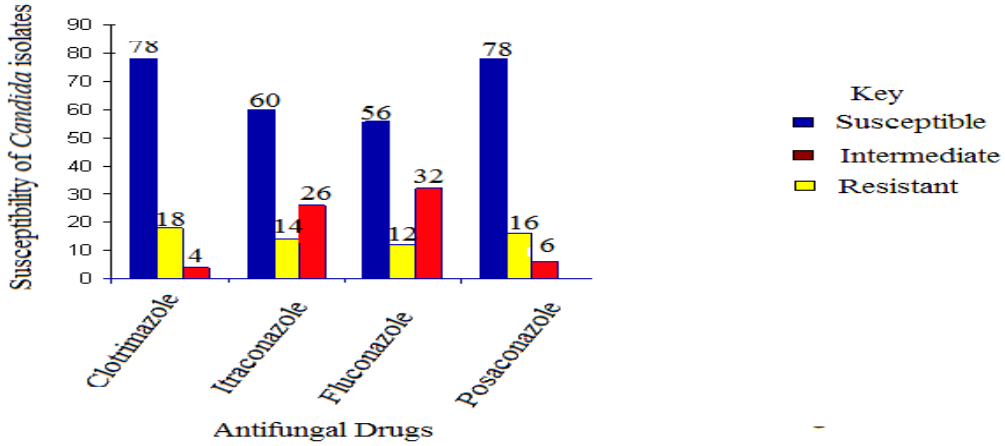
Susceptibility

Figure 8; Susceptibility of *Candida* isolates to azoles using Disc Diffusion Method



In disc diffusion method , clotriazole showed the highest level of susceptibility (92%) and the least level of resistance (2%). Posaconazole and itraconazoles' susceptibilities were also high (78% and 74%) respectively. However resistance levels in fluconazole and itraconazole were considerably high (48% and 22%) respectively. Intermediary susceptibilities were almost constant in the four drugs (Fig. 8)

Figure 9; Susceptibility of *Candida* isolates to azoles using Epsilometer-test



In E-test method, posaconazole and clotrimazole had the greatest levels of susceptibilities (78%) and very 26% were resistant. Itraconazole had the highest level of resistance (32%) and only 58% susceptibility (Fig. 9).

Discussion

Candida species can be recovered from almost any site in a human body (Walsh and Dixon, 1996). In this research study, strains of *Candida* species originated from sputum, urine, High Vaginal Swabs (HVS), pus swabs, throat swabs and necrotic wounds. Most isolates of *Candida* were recovered from urine (42%) whereas those from swabs were <30%. As *Candida* species differ in their antifungal susceptibility, it is essential to identify and differentiate them. *Candida albicans* once accounted for most serious nosocomial candidal infections (Pfaller, 2000). Over the last decade *Candida* infections, specifically candidemia, due to *C. albicans* have declined. Other non-*albicans Candida* species are now responsible for about half of candidemias and other deep *Candida* infections (Walsh and Dixon, 1996). In this study, *C. albicans* was found to be the most prevalent strain (90%) of *Candida* species. Out of the 50 isolates of *Candida* that were used in this study, 45 were identified as *Candida albicans,* 1 as *Candida glabrata,* 1 as *Candida famata* and 3 as *Candida parapsilosis*. This indicates that most infections of candidiasis were due to *Candida albicans* (>90%). Infections due to non *albicans candida* were less significant (<10%).

Gram staining technique was used as a preliminary test prior to identification just to ensure that the archived *Candida* species were still viable and free of contamination. Gram negative isolates were regarded as contaminated with bacteria as all yeasts are gram positive.

Culture on SDA was done to obtain pure colonies that could be used for identification and later susceptibility testing. In this study, morphocultural characterization of the isolated *Candida* species showed cream-colored, circular and convex colonies with large, spherical cells. The difference in colony sizes could have been attributed to the individual characteristics of the different species of *Candida.* However their sufficient growth indicated a proper adaptation of *Candida* species to the contents of the medium. *Candida albicans* produced short one piece germ tubes in serum in varied times (30-60 minutes). This happened because the different subtypes of *C.albicans* absorbed essential growth nutrients required for germ tube formation at unequal rates and proportions (Richardson *et al.,* 1981). CHROMagar *Candida* was used as a primary medium for differentiating between various *Candida* species based on their colony colour and morphology (rough/smooth) (Pfaller *et al.,* 1996). Pal's agar was considered as a secondary medium for differentiation of *Candida* species. In this study, colonies of *C.krusei* produced a hyphal fringe on Pal's agar. Ability to produce hyphal fringe on Pal's agar is attributed to pseudohyphae production by *C.krusei*.

Most fungi play an important role in the utilization of various substrates including hydrocarbons, nuceic acids, carbohydrates and uric acid (Buesching *et al.,* 1979). *Candida* species utilise these substrates for carbon sources (Buesching *et al,* 1979). The API 20C AUX (bio Merieux) system was used in this study and was able to provide the correct identification for all the isolates tested after 48 h of incubation, because it also incorporated the results of a morphological assessment. In this study, all *Candida* species utilized glucose, galactose, sucrose and maltose. All the non *albicans candida* except *Candida famata* utilized starch. *Candida albicans* utilized raffinose in addition to the four sugars in common. Many laboratories now use extensive commercial identification systems to determine the physiological profiles of yeast isolates hence capable of identifying a wide range of taxa. However, a morphological assessment of isolates remains essential to avoid errors in the identification of organisms with identical biochemical profiles. Likewise a few incorrect identifications may be obtained with morphological assessment (Buchaille *et al.,* 1998). For instance, in this research study; one chlamydospore-forming *Candida* spp. was misidentified as *C. famata* during this evaluation but later confirmed with API AUX (Bio Meriux).

Imidazoles have been effective in the treatment of mycotic infections over the past years but emergence of resistance is continually increasing. Thus, there is a big challenge in the future treatment of fungal infections which makes it necessary for constant monitoring of antifungal drugs to detect further emergency of resistance.

However, patients with AIDS present quite a different venue for these imidazoles. Chronic and repetitive use of antifungal azoles to treat protracted opportunistic infections in HIV infected patients predisposes to the development of resistance (Law *et al.,* 1994). The appearance of secondary azole resistance to this date has been demonstrated most commonly in HIV positive patients who previously received fluconazole (Millon *et al*., 1994).

Clotrimazole has been shown to inhibit all the major fungi causing systemic infections at a concentration of 1 ug / ml particularly *Candida, Histoplasma* and *Aspergillus spp* (Plempel *et al.,* 1969). Consequently most isolates of *Candida* species showed low MIC's in E-test and large zone sizes in disc diffusion method. Clotrimazole was the most effective antifungal as there was resistance in only one out of the 50 isolates of *Candida* species tested in disc (table 1), and only two in E-test methods(table 2). As much as the drug is old generation there is a clear manifestation it is still strong and can be used to treat *Candida* infections.

Fluconazole revealed a lot of resistance (Fig 1 and 2). In the disc test 48% of the isolates were resistant to the drug while 46% were susceptible (table 1) whereas in E-test 26% were also resistant (table 2). MIC_{90} value was >140 µg/ml which is far above the breakpoint (64.000 µg/ml). This indicates that this is a very ineffective drug especially in the treatment of mycoses due to *Candida albicans* as these accounted for 90% of all species isolated. As an old generation drug, the resistance could be due to the over exposure of the *Candida spp*. to the drug.

Posaconazole is a new member of the triazoles class of antifungals with broad spectrum antifungal activity. Based on the findings of this study; it could be predicted that posaconazole would be active against the agents responsible for the majority of moderate and severe mycoses detected in Kenya. Its low MIC_{90} of 0.600 µg/ml is a clear indication that the drug is very effective in the treatment of *Candida* infections (Table 2). It has demonstrated clinical efficacy in the treatment of Oropharyngeal Candidiasis. In addition, data from randomized controlled studies support its efficacy for use in prophylaxis of invasive fungal infections in patients who are severely immune-compromised (Kauffman, 2006).

Itraconazole is active against serious fungal or yeast infections, such as Oropharyngeal Candidiasis (oral thrush), and esophageal Candidiasis (Candida esophagitis) (M cGinnis and Rinaldi, 1996). Performance of itraconazole also indicated considerable resistance (Fig 1 and 2). All the resistance to the drug occurred in *Candida albicans* whereas *C.famata, C.parapsilosis and C. glabrata* were susceptible to the drug in disc method. Given that 90% of the species were *C. albicans* there could be a possibility that the drug is effective on other *Candida* species like *C.famata, C.parapsilosis* and *C. glabrata* and not *C.albicans.*

The conventional morphocultural methods (germ tube test, CHROMagar Candida, slide culture and Pal's agar) re-identified the *Candida* spp. The biochemical tests (API 20C AUX) further confirmed the isolates as *C. albicans, C. famata, C. parapsilosis* and *C. glabrata* in agreement with the identification based on their morphocultural characteristics. One isolate, initially identified as *C. albicans* using the germ tube test was later identified as *C.famata* using the API 20C AUX profile. Thus the combined application of morphocultural and biochemical methods proved useful in the identification and confirmation of *Candida* species.

Susceptibility results show that there is emerging fungal resistance to azoles particularly fluconazole and new generation triazoles particularly itraconazole. However clotrimazole and posaconazole are still effective in the treatment of *Candida* infections.

The study therefore recommends the following:

- Further investigations on fungal resistance against various new generation tri-azoles using various susceptibility methods.
- Rational use of antifungal and antibiotics drugs to curb emerging resistance.
- Development of new drugs with different mechanisms of action from those of the azoles.
- Expansion of the spectrum of activities of the current antifungal drugs to cover a wide spectrum of *Candida* infections.

Acknowledgements

This work was supported by Dr. Christine Bii, Kenya Medical Research Institute, Dr. Gikunju, Jomo Kenyatta University of Agriculture and Technology, Centre for Microbiology Research-KEMRI staff , Director ITROMID- JKUAT, Professor Ng'ang'a and the Director ITROMID –KEMRI, Dr. Mpoke.

References

1. Barry AL, Pfaller MA, Brown SD, Espinel-Ingroff A, Ghannoum MA, Knapp C, Rennie RP, Rex JH and Rinaldi MG**.** Quality control limits for broth micro dilution susceptibility tests of ten antifungal agents. *Journal of Clinical Microbiology*. 2000; **38**: 3457-3459.
2. Bii CC, Ouko TT, Amukoye E and Githinji LW. Antifungal drug susceptibility of Candida albicans. *East African Medical Journal*. 2002; **79**: 3.
3. Buchaille L, Freydiere AM, Guinet R and Gille Y. Evaluation of six commercial systems for the identification of medically important yeasts. *Clinical Microbiology and Infectious Diseases.*1998; **17**: 479-488.
4. Buesching WJ, Kurek K and Roberts GD**.** Evaluation of the modified API 20C system

for identification of clinically important yeasts. *Journal of Clinical Microbiology.* 1979; **9**: 565-569.
5. Kauffman CA. Clinical efficacy of new antifungal agents. *Current Opinion in Microbiology.* 2006; **9**: 483-8.
6. Maenza JR, Keruly JC, Moore RD, Chaisson RE, Merz WG and Gallant JE. Risk factors for fluconazole-resistant candidiasis in human immunodeficiency virus-infected patients. *Infectious Diseases***.** 1996; **173**: 219-25.
7. M cGinnis M and Rinaldi MG**.** Antifungal drugs: mechanisms of action, drug resistance, susceptibility testing, and assays of activity in biological fluids. *Antibiotics in Laboratory Medicine*. 1996; **13**: 176-211.
8. Pfaller MA. Epidemiology of nosocomial candidiasis and the importance of molecular typing. *Clinical Microbiology.* 2000; **53**: 161–167.
9. Pfaller MA, Houston A and Coffmann S. Application of CHROMagar Candida for rapid screening of clinical specimens for *Candida albicans***,** *Candida tropicalis***,** *Candida krusei***,** and *Candida* **(***Torulopsis***)** *glabrata***.** *Clinical Microbiology*. 1996; **34**: 58-61.
10. Pfaller MA, Diekema DJ and Sheehan DJ. Interpretive breakpoints for fluconazole and *Candida* revisited: a blueprint for the future of antifungal susceptibility testing. *Clinical Microbiology*. 2006; **17**: 435–47.
11. Plempel M, Bartmann K, Bu¨che KH and Regel E. BAY b 5097, a new orally applicable antifungal substance with broad-spectrum activity. *Antimicrobiol agents and Chemotherapy.* 1969; **9**: 271–274.
12. Richardson MD and Smith H. Production of germ tubes by virulent and attenuated strains of Candida albicans. *Journal of Infectious Diseases*. 1981; **144**: 565 – 569.
13. Vendettuoli V, Vento G, Tirone C, Posteraro B and Romagnoli C. Antifungal prophylaxis: identification of preterm neonates at highest risk for invasive fungal infection. *Clinical Microbiology.* 2009; **123:** 368-9.
14. Walsh TJ and Dixon DM**.** Deep Mycoses. *Baron's Medical Microbiology*. 1996; **4**: 239-241.