Saudi Journal of Biological Sciences 15 (2) 189-197 December, 2008
ISSN 1319-562X
The Official Journal of the Saudi Biological Society
htt:www.saudibiosoc.com

In vitro, Susceptibilities of Clinical Yeast Isolates to Antifungal Drugs of Polyene, Pyrimidine, and Azoles, and their Effect in Yeast Adhesion and Mycelial Formation

Saleh A. Kabli

Department of Biological Sciences, Faculty of Science,
King Abdulaziz University, P.O. Box: 80203, Jeddah, Saudi Arabia E-mail: sakabli@yahoo.com

Abstract

In vitro, susceptibilities were determined for 107 clinical isolates of Candida albicans obtained from the laboratory of King Abdulaziz University Hospital (UHC). The agents tested included amphotericin B (as a polyene), flucytosine (as a pyrimidine), and fluconazole (as an azole). MICs were determined by the broth microdilution technique following National Committee for Clinical Laboratory Standards document, using RPMI 1640 broth medium supplemented with 3 g glucose l-1, and the E-test with solidified RPMI 1640 supplemented with 18 g glucose l-1. The susceptibility tests indicated that amphotericin B is the most active fungicidal drug, fluconazole was with less activity and flucytosine was more effective than the later drug. The appropriate rank order of best agreement between the MIC technique, E-test and disk diffusion methods was amphotericin B>flucytosine> fluconazole. The influence of the tested drugs in adherence of C.albicans on phenyl sepharose (resin) column indicated that amphotericin B was with high antiadherence activity, followed by fluconazole and flucytosine (47, 33 and 18%, respectively.) The effect of the drugs on mycelial formation by the tested C.albicans isolates revealed that amphotericin B showed 71% inhibition of mycelial formation, fluconazole 58% and flucytosine showed about 40% inhibition of mycelial formation.

Key Words: C.albicans, yeast adhesion, susceptibilities, antifungal drugs

Introduction

Candidiasis, which is caused by the pathogenic yeast *Candida albicans,* is the most frequent fungal infection. *C.albicans* normally exists as a common organism and is the most frequent opportunistic fungal infection of man and can cause either systemic or mucosal infection (Soll, 2002). Three classes of antifungal drugs are used to fight *Candida* infections. The fungicidal polyene drugs such as amphotericin B act by binding to sterols in the plasma membrane, disrupting membrane function. The fungistatic azoles, such as fluconazole, act by inhibiting the largest enzyme lanosterol demethylase in the ergosterol biosynthetic pathway (White *et al.,* 1998). Flucytosine is one of the pyrimidine drugs that inhibit nucleic acids formation (John *et al.,* 1995).

All over the world, invasive fungal infections are steadily increased in frequency and clinical importance. Even more disturbing is the growing number of reports on resistance of fungi against commonly used antifungal drugs (Odds, 1993). Consequently, there is a need for a precise, clinically relevant easy to perform in vitro susceptibility testing method to guide antifungal therapy and monitor local resistance patterns (Ghannoum, 1996; Martins & Rex, 1996; Hoffman & Pfaller, 2001; Pfaller & Yu, 2001 and; Rex & Pfaller, 2002). A standard reference procedure has been described by the National Committee for Clinical Laboratory Standards (NCCLS, 1995). That reference procedure is a macrotube dilution technique (MIC) which is also cumbersome for use in most clinical laboratories. A broth microdilution adaptation of that procedure has found to be acceptable (Espinel-Ingroff *et al.,* 1992; Pfaller *et al.,* 1994; and Sewell *et al.,* 1994). The E-test, (AB Biodisk, Solna, Sweden) is a proprietary test that has also been found to be capable of giving reliable results (Sewell *et al.,* 1994; and Colombo *et al.,* 1995). For use in a clinic laboratory, a standardized disk diffusion test that is widely used to test

antibacterial agents (Barry & Brown, 1996).

The adhesion of pathogenic microorganisms to their target cells is recognized as the first event prior to colonization and infection of host tissues (Ofek & Beachey, 1980). *C.albicans* is a dimorphic yeast-like fungus that adheres to many epithelial and to some resins based in their cell hydrophobic surfaces. (King *et al.,* 1980; Segal& Savage, 1986; Kennedy *et al.,* 1987; and Polaquini *et al.,* 2006). Several environmental and biophysical factors have been seen to influence yeast binding to epithelial cells (Samaranayake & MacFarlane, 1982; Persi *et al.,* 1985; Kennedy & Sandin, 1988; Hazen, 1989; and Mehentee &Hay, 1989). Also, the host cell membrane receptors have a role in adhesion (Brassart *et al.,* 1991).

Candida albicans is dimorphic yeast has the ability to switch from yeast cells (blastospores) to hyphae. Hyphae are thought to be an important virulence factor that promotes invasion of cells into the mucosa and that allows *Candida* cells to resist macrophage and neutrophil engulfment (Odds, 1988). The effects of azoles, polyene and pyrimidine antifungal agents on the yeast cell/hypha transition in susceptible isolates were studied (Odds *et al.,* 1985; Abu-El-Teen *et al* 1989). At subinhibitery concentrations of drugs, hyphal branching was inhibited and at clinically relevant concentrations, hyphal development was arrested and the cells remained as yeast cells. This inhibition was independent of the inhibition growth (Ghannoum *et al.,* 1992; and Ha & White, 1999).

In a previous work the author (Kabli, 2006) study the pattern of growth and mycelial formation of two *C.albicans* isolates and in another work (Kabli, 2007) the conditions for induction of mycelial formation by *C.albicans* (ATCC10231) was elucidated. The present work aimed to test, in vitro, the efficiency of the antifungal drugs susceptibility tests (MIC, Disk-diffusion, and E-test) for *C.albicans* and the correlation between polyene (amphotericin B), azoles (fluconazole), and pyrimidine (flucytosine)drugs resistance and adhesion of *C.albicans*, as well as, its hyphal formation.

Materials and Methods

Organisms

One hundred seven clinical isolates of *Candida albicans* were obtained from the laboratory of King Abdulaziz University Hospital (UHC), Jeddah, Saudi Arabia. They were identified with the API 20C system (Bio Merieux, Marcy l'Etoile, and France). If needed, microscopical examination of morphology on control agar (Oxoid) was used to confirm identity. Two reference strains with known susceptibility patterns (*C.albicans* ATCC 90028) susceptible strain (NCCLS, 1995) and *C.albicans* ATCC 64550 resistant strains (Martinez & Rodriguez, 1996) were selected for comparative susceptibility testing with amphotericin B, flucytosine and fluconazole.

For short-term storage, isolates were grown on Sabouraud's agar slants (Sanofi Diagnostic, Pasteur) and stored at 2-5^{0}C temperature. Subcultures were repeated every 6 months and the isolates viability was examined regularly.

Susceptibility testing methods

The NCCLS macro tube dilution procedure (NCCLS, 1995) was used as standard method for evaluation of the other methods. MICs were read after 24 and 48h, the 48-h MIC was used as the reference end point. Antifungal agents disks (MAST Diagnostics) of amphotericin B 20 µg, flucytosine 1 µg and fluconazole 25 µg were tested. Every disk-test was accompanied by an E-test strip (AB Biodisk) applied to the same plate, thus providing another MIC for evaluation of the disk procedure. The E-test (disk and E) were read after 24 and 48h, although 48-h MICs were used for evaluation.

Antifungal Agents

Amphotericin B (Sigma A2422), flucytosine (Sigma F-7129) and fluconazole (Pfizer, Sandwich, United Kingdom). Amphotericin B was dissolved in dimethyl sulfoxide at a concentration of 1,280 µg/ml^{-1}, flucytosine, and fluconazole each was dissolved in 0.85% saline also at a concentration of 1,280 µg/ml^{-1}. Stock solutions were stored at -7^{0}C until used.

Assay Media

RPMI broth was prepared from RPMI 1640 broth medium (Sigma R 7880) supplemented with 0.3g of glutamine (Janssen, Beerse, Belgium) per liter, buffered with 34.6g of morpholine-propanesulfonic acid (MOPS) per liter and adjusted to pH 7.6. This medium contains 2g glucose l^{-1} and was used for the NCCLS broth macrodilution method. RPMI agar for disk-diffusion test and diffusion E-test was prepared by the same way as RPMI broth and supplemented with 18 g of glucose/l^{-1} and 15g of Bacto-agar/l^{-1} (Van Eldere *et al.,* 1996).

Inoculum

Prior to testing, each isolate was grown on Sabouroud's dextrose agar (Sanofi Diagnostic, Pasteur) for 24 h at 36^0 C. Suspensions were prepared from individual colonies (diameter $\geq$ 1mm) in 5ml of sterile 0.85% saline to a density of a 0.5 McFarland standard (1-5 x 10^6 CFU/ml). Dilutions of that inoculum suspension were prepared and quantitatively subcultured to confirm the actual number of CFU/ml. The tests were repeated if the inoculum was not within the range of 0.5-2.5 x 10^3 CFU/ml (NCCLS, 1995). RPMI-glucose agar in a 15-cm-diameter Petri plate was inoculated with a swab moistened in an inoculum suspension adjusted to match a McFarland 0.5 turbidity standard. One drug E-test strip and one antifungal drug disk was applied to each inoculated plate.

Incubation

All tests were incubated in an ambient air at 36^0C, and the results were recorded after 24h and again after 48h of incubation.

Endpoint determination

For the NCCLS reference test, the MIC was determined as the lowest concentration inhibiting at least 90% of the growth (Espinel-Ingroff *et al.,* 1994; and NCCLS, 1995). That was determined by comparing each tube to a 1:5 dilution of the growth control tube. For agar-based tests, inhibitory zones were measured at the point where there was a sharp decline in the amount of the growth (approximately 90% inhibition). Similar guidelines were used for reading E-test according to the manufacturer's instructions.

For the MIC, the isolate considered resistance for amphotericin or flucytosine if MIC $\geq$ 4 $\mu g/ml^{-1}$, and for fluconazole if MIC $\geq$ 8 $\mu g/ml^{-1}$ (Fugn *et al.,* 1995; and Goff *et al.,* 1995).

Adhesion of *C.albicans* to phenyl sepharoseresin

The method was based on cell surface hydrophobicity of *C.albicans* (Polaguini *et al.,* 2006) using hydrophobic interaction chromatography through column of phenyl sepharose (Sigma P-7892). *C.albicans* cultivated in Sabouraud's agar medium for 48h at 36^0 C was inoculated into flasks each containing 50ml RPMI 1640 medium and incubated under shaked conditions at 36^0 C for 24h. Under aseptic conditions, the yeast cells separated by centrifugation, washed three times with phosphate buffer saline PBS (Ghannoum *et al.,* 1992). In phosphate buffer saline (PBS) cell density of O.D=1 was prepared and 5 ml was passed through 30 cm phenyl sepharose column, using Pasteur pipette. The yeast cells were collected and counted as optical density using spectrophometer (470nm). The tested antifungal drug (5ml) was added at its subminimal inhibitory concentration, thereafter the cell count as O.D. was determined.

Effect of Antifungal drugs on hyphal formation

A random collection for *C. albicans* isolates (nine) were analyzed for hyphal formation in the absence and presence of antifungal drugs, at a concentration equal half its MIC (Ghannoum *et al.,* 1992). A standard protocol for large-scale hyphal formation was used. The cells were grown to stationary phase (48h) at 30^0 C followed by washing in PBS and resuspension to a final concentration of less than $3x10^6$ cells/ml in an induction medium (M199) at 37^0 C, under shaking conditions (240 rpm). After 3h over 250 cells were examined microscopically with a haemocytometer (Ha & White, 1999).

Results and discussion

Susceptibility of *C.albicans* to tested antifungal drugs: The results (Fig.1) indicated that about 21.5% of the investigated *C.albicans* isolates with high susceptibility to amphotericin B (inhibition zone $\geq$ 22 mm) and also the same percentage of isolates showed low susceptibility to the same drug (zone of inhibition $\leq$ 17 mm). However, 57% of the isolates showed inhibition zone ranged between 18-22mm (moderate susceptibility). While about 92.5% of the same tested isolates were with high susceptibility to flucytosine (inhibition Zone $\geq$ 23 mm) and only 12.8% of lower susceptibility (18-22mm inhibition zone). On the other hand, 4.7% of yeast isolates were resistant to the flucytosine of pyrimidine nature. As for the other azole drug, fluconazole, only 15% of the isolates were highly susceptible (inhibition zone $\geq$ 36 mm) and about 43% showed moderate susceptibility (inhibition zone 27- <36mm), while 15% showed low susceptibility, thus inhibition zone ranged between 10-26 mm. On the other hand, about 26.1% of the isolates are extremely resistant to the drug.

As the MIC is considered, the isolates of *C.albicans* showed different susceptibilities to the tested antifungal drugs (Fig.2). Thus, only 3% of the isolates were resistance

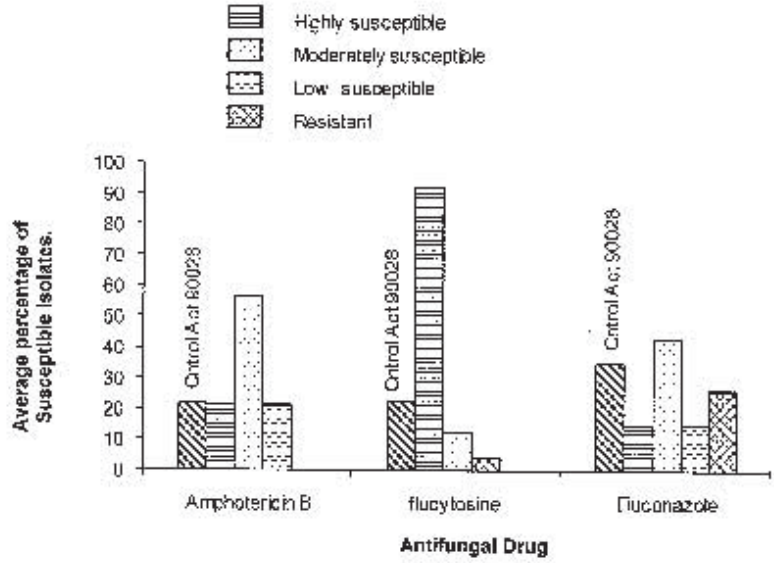

Fig.1 Average of susceptibility of 107 *C.albicans* isolates to amphotericin B, flucytosine, and fluconazole using disk diffusion test.

to the polyene amphotericin B, while 11% were resistance to the pyrimidine flucytosine. However, as much as 63% of the isolates were resistance to the azole drug of fluconazole.

The results of E-test susceptibility of the tested *Calbicans* isolates indicated that all the isolates were susceptible to the amphotericin B and 9% were resistance to flucytosine. On the other hand, as high as 63% of isolates, were resistances to the azole drug (fluconazole). The results indicated that, in vitro, disk-diffusion test gave, to some extent, false indication for *Calbicans* susceptibility to the tested polyene (amphotericin B) or pyrimidine (flucytosine) and azole (fluconazole) antifungal drugs, while MIC technique and to lesser extent E-test technique gave to some extent different susceptibility figures for the tested isolates than disk diffusion test (Fig. 3). The data indicated that the polyene, amphotericin B, is the most active fungicidal drug against *Calbicans* isolates, and the azole drug was with less fungicidal effect, while the pyrimidine drug (flucytosine) was more effective than fluconazole. Therefore, the appropriate rank order of best agreement between the MIC technique, E-test and disk diffusion methods was amphotericin B>flucytosine>flucon azole. The disk diffusion test, MIC and E-test methods were used successfully by many workers to test the susceptibility of *C. albicans* isolates (Braga *et al.*, 2007; Steenkamp *et al.*, 2007; De Logu *et al.*, 2005; Ostrosky-Zeichner *et al.*, 2003; Paniagua *et al.*, 2002; Ha & White, 1999; Ruhnke *et al.*, 1996; Van Eldere *et al.*, 1996; and Barry & Brown, 1996). In accordance with our findings that MIC test was the most precise method to test *C.albicans* susceptibility, most the above workers recommended the use of MIC technique to test, in vitro, the susceptibility of *C.albicans* to the antifungal drugs.

Effect of the tested antifungal drugs in adherence of *C.albicans* isolates

The first step of candidiasis is correlated to the adherence of *C.albicans* cells to epithelial host cells (Osullivan *et al.*, 1997; and Segal *et al.*, 1997). The effect of subminimal inhibitory concentrations (SMIC) of amphotericin B, flucytosine and fluconazole in adherence of 10 randomly by chosen *C.albicans* isolates on phenyl sepharose column (Fig. 4) indicated that the tested drugs noticeably decreased the adherence of the isolates on phenyl sepharose by 47, 18, and 33%, respectively. This indicates that amphotericin B drug with high anti adherence activity on the tested isolates, followed by fluconazole (33%), while flucytosine have low antiadherence activity in *C.albicans* isolates. These findings for fluconazole and flucytosine are vice versa the results of susceptibility of the yeast isolates to the same drugs using disk diffusion, MIC and E-test techniques. In accordance with these results, it was reported that

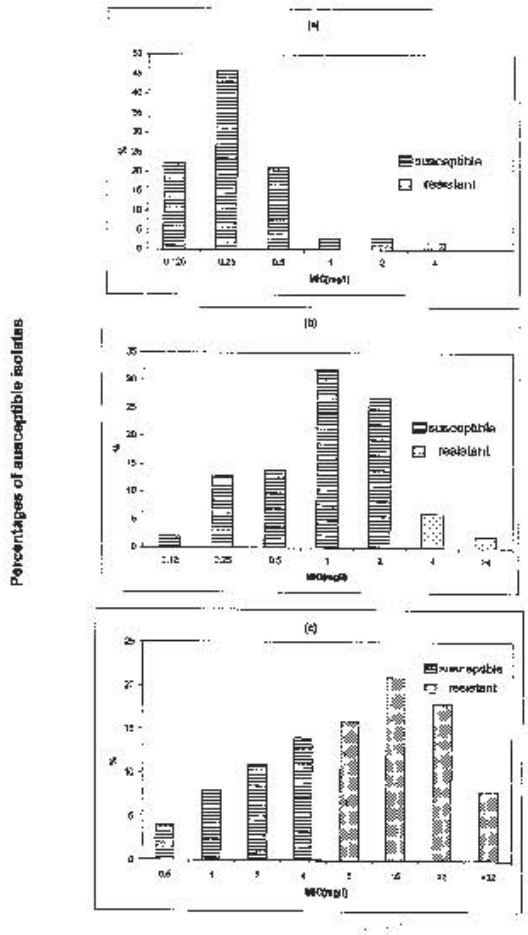

Fig.2. Percentage of susceptible isolates of the tested c. albicans (107 isolates) as influenced by different concentration of antifungal drugs of amphotericin B(a), fluconazole (c) using MIC susceptibly test.

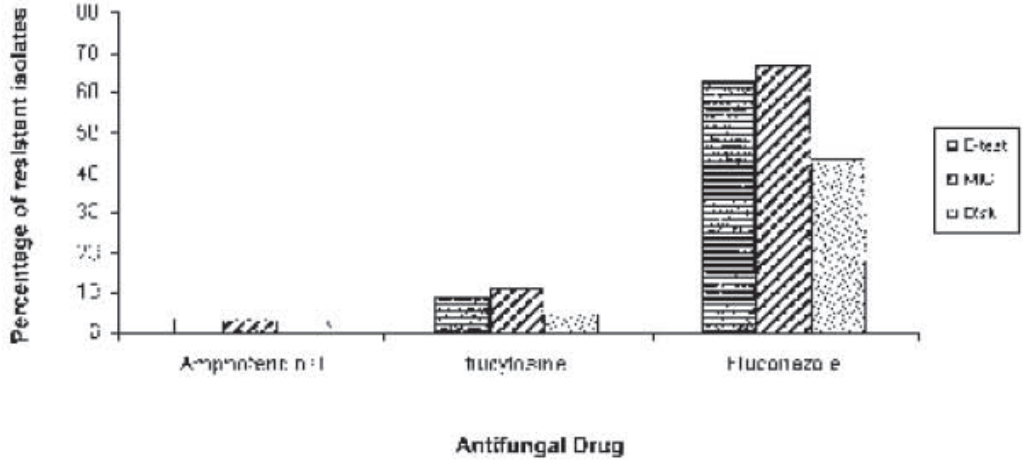

Fig.3 Comparison between the tested techniques used to study the susceptibility of *C.albicans* isolates for the antifungal drugs of amphotericin B, flucytosine and fluconazole.

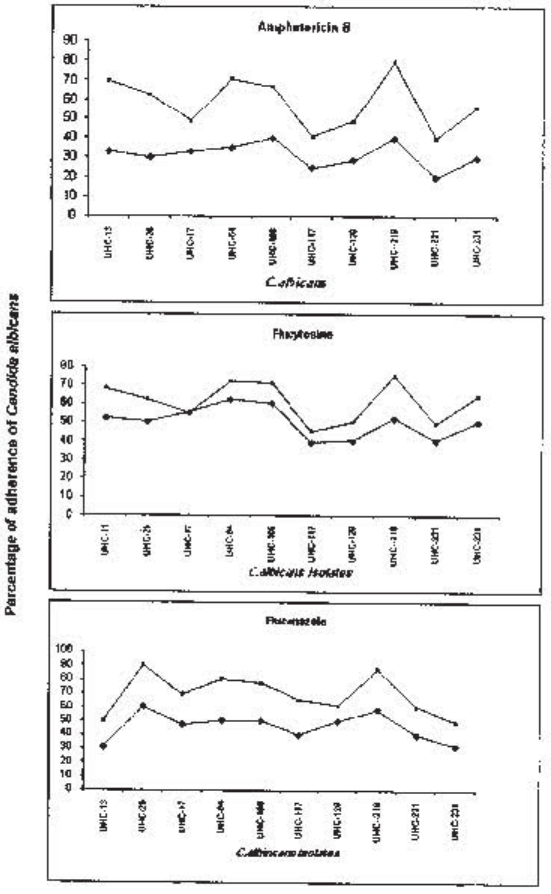

Fig.4: Effect of amphotericin B, flucytosine and fluconazole antifungal drugs on adherence of *Candida albicans* on phenyl sepharose.

no drug, Subinhibitory drug concentration

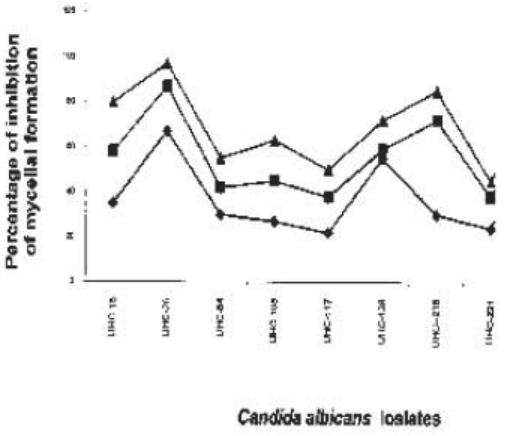

Fig.5 : The influence of amphotericin B ▲, flucytosine fluconazole ■ drugs on mycelial formation of C

exposure of *C.albicans* to subinhibitory concentrations of fluconazole and amphotericin B inhibited yeast adherence to endothelial cells by 22 and 91%, respectively(Ghannoum *et al.,* 1992). It was also reported that the antiadherence activity of flucytosine is more less that of amphotericin B (Hawser, 1996).

Effect of the tested fungicides on mycelial formation by *C.albicans* isolates

The pathogenicity of *C.albicans* is usually due to its mycelial formation (morphogenesis dimorphism) (Gorman *et al.,* 1986). Therefore, the influence of subinhibitory concentrations of amphotericin B, flucytosine and fluconazole on the tested *C.albicans* isolates were investigated. The results (Fig.5, 6) indicated that the tested fungal drugs lowering significantly mycelial formation by the isolates. Amphotericin B has the most deleterious influence in mycelial formation, where abortion ranged between 46 to 96% (about 71% inhibition) was detected. In the second order fluconazole inhibit mycelial formation by 36 to 86% (about 58%), while flucytosine showed the lowest percentage of inhibition of mycelial formation 21-74% (about 40% inhibition). It was reported that amphotericin B was inhibitorier for mycelial formation more than fluconazole (Ghannoum *et al.,* 1992). The effect of antifungal drugs on mycelial formation was studied by many workers (Ha & White 1999; De Logu *et al.,* 2005; Braga *et al.,* 2007; and Cateau *et al.,* 2008).

The present work clearly indicated that, in vitro, amphotericin B as a polyene antifungal drug was the most effective (inhibitory) tested drug against *C.albicans* isolates growth, adhesion and mycelial formation, while the pyrimidine drug (flucytosine) was with lower activities on the same isolates under the experimental conditions. And fluconazole (azole) was inhibitorier for the same parameters than flucytosine. These finding may due to the experimental conditions as well as the mode of action of each drug.

Conclusion

The mentioned results revealed that, in vitro, amphotericin B is the most effective antifungal drug in response to susceptibility tests; adherence and mycelial formation of the tested *C.albicans* isolates. The pyrimidine drug (flucytosine) was more effective than the azole drug

(fluconazole) in susceptibility tests. While vice versa was recorded for adherence and mycelial formation of the yeast isolates.

References

Abu-El-Teen, K., Ghannum, M., and Strettan, R.J. 1989. Effects of subinhibitory concentrations of antifungal agents on adherence of *Candida* spp. to buccal epithelial cells *in vitro*. *Mycoses*. 32: 551-562.

Barry, A.L., and Brown S.D. 1996. Fluconazole disk diffusion procedure for determining susceptibility of *Candida* species. *J. Clinical Microbiology*. 34: 2154-2157.

Braga, P.C., Alfieri, M., Culici, M., and Dal Sasso, M. 2007. Inhibitory activity of thymol against the formation and viability of *Candida albicans* hyphae. *Mycoses*. 50: 502-506.

Brassart, D., Woltz, A., Golliard, M., and Neeser, J. 1991. In vitro inhibition of adhesion of *Candida albicans* clinical isolates to human buccal epithelial cells by Fucα1→ 2Ga1β-bearing complex carbohydrates. *Infect. and Immunit.* 59 (5): 1605-1613.

Cateau, E., Berjeaud, J., Rodier, M., and Imbert, C. 2008. Fungal biofilm inhibition by a component naturally produced by *Candida albicans* yeasts growing as a biofilm. *Int. J. Antimicrobial Agents,* 31: 166-170.

Colombo, A.L., Barchiesi, F., McGough, D.A., and Rinaldi, M.G. 1995. Comparison of E-test and National Committee for Clinical Laboratory Standards broth macrodilution method for azole antifungal susceptibility testing. *J. Clin. Microbiol.* 33: 535-540.

De Logu, A., Saddi, M., Cardia, M.C., Borgna, R., Saddi , B., and Maccioni, E. 2005. In vitro activity of 2-cyclohexylidenhydrazo-4-phenyl-thiazole compared with those of amphotericin B and fluconazole against clinical isolates of *Candida* spp. and fluconazole–resistant *Candida albicans*. *J. Antimicrob. Chemother*. 55: 692-698.

Espinel-Ingroff, A., Kish, G.W., Kerkering, T.M., Fromthing, R.A., Bartizal, K., Galgiani, J.N., Villareal, K., Pfaller, M.A., Gerarden, T., Rinaldi, M.G., and Fothergill, A. 1992. Collaborate comparison of broth macrodilution and microdilution antifungal susceptibility test. *J.Clin. Microbiol,* 30: 3138-3145.

Espinel-Ingroff, A., Steele-Moore, L., and Galgiani, J.N. 1994. Evaluation of 80% inhibition standards for determination of fluconazole minimum inhibition concentrations in three laboratories. Diagn. *Microbiol. Infect. Dis.* 20: 81-86.

Fugn, T.F., Beatrice, M., Elizabeth, H., Benjamin, K., Danielp B., and Roberte, K. 1995. In vitro antifungal and fungicide spectra of a new pradimicin derivative, BMS-181184. Antimicrob. Agents and Chemother. 39 (2): 295-300.

Ghannoum, M.A. 1996. Is antifungal susceptibility testing useful in guiding fluconazole therapy. *Clin. Infect.Dis.* 22 (2): s161-S165.

Ghannoum, M., Filler, S., Ibrahim, A., Fu, Y., and Edwards, J. 1992. Modulation of interactions of *Candida albicans* and endo thelial cells by fluconazole and amphotericin B. Antimicrob. Agents and Chemother. 36 (10): 2239-2244.

Goff, D.A., Koletar, S.L., Buesching, W.J., and Fass, R.J. 1995. Isolation of fluconazole-resistant *Candida albicans* from human immunodeficiency virus-negative patients never treated with azole. *Clin. Infect.Dis.* 20: 77-83.

Gorman, S.P., McCafferty, D.F., Woolfson, A.D., and Anderson, L. 1986. Inhibition of hypha development and kill of *Candida albicans* blastospores by noxythiolin in vitro. *J. App. Bacter*. 60: 319-325.

Ha, K. and White, T. 1999. Effects of azole antifungal drugs on the transition from yeast cells to hyphae in susceptible and resistant isolates of the pathogenic yeast *Candida albicans*. Antimicrob. Agents and Chemother. 43 (4): 763-768.

Hawser, S. 1996. Comparisons of the susceptibilities of planktonic and adherent *C.albicans* to antifungal agents: a modified XTT tetrazolium assay using synchronized *C.albicans* cells. *J. Med. Veter. Myco.* 34 (2): 144-152.

Hazen, K.C. 1989. Participation of yeast cell surface hydrophobicity in adherence of *C.albicans* to human epithelial cells. *Infect. Immune* 57: 1894-1900.

Hoffman, H. L. and Pfaller, M.A. 2001. In vitro antifungal susceptibility testing. *Pharmacotherapy*. 21: 111s-123s.

John, N. A., Nicole, M.L., and Popele 1995. Antifungal agent in neonatula systemic candidiasis. Antimicrob. Agents Chemother. 38 (7): 1391-1397.

Kabli, S. A. 2006. Morphogenesis of two *Candida albicans* strains as influenced by growth media, pH value and incubation temperature. Am-Eurasian *J. Agric and Environ. Sci:* 1(2): 127-132.

Kabli, S.A. 2007. Optimal conditions for induction of mycelial formation by *Candida albicans* (ATCC10231). *Bull. Fac. Sci. Alex. Univ.* 45(1): 1-9.

Kennedy, M.J., Volz, P.A. Edwards, C.A. and Yancey, R.J. 1987. Mechanisms of association f *C.albicans* with intestinal mucosa. *J. Med. Microbiol.* 24: 333-341.

Kennedy, M.J. and Sandin, R.L. 1988. Influence of growth conditions on *C.albicans* adhesion, hydrophobicity and cell wall ultrastructure. *J. Med. Vet. Mycol.* 26: 79-92.

King, R.D., Lee, J.C. and Morris, A.L. 1980. Adherence of *C.albicans* to mucosal epithelial cells. *Infect. Immun.* 27: 667-674.

Martins, M.D. and Rex, J.H. 1996. Resistance to antifungal agents in the critical care setting: problems and perspectives.

N. Horiz. 4: 338-344.

Martinez, U.J. and Rodriguez, L.U. 1996. In vitro activities of semisynthetic pneumocandin L-733, 560 against fluconazole resistant and susceptible *Candida albicans* isolates. Antimicrob. Agents Chemother. 40 (5): 1277-1279.

Mehentee, J.F. and Hay, R.J. 1989. In vitro adherence of *C.albicans* strains to murine gastrointestinal mucosal cells and explants and the role of environmental pH. *J. Gen. Microbial.* 135: 2181-2188.

National Committee for Clinical Laboratory Standards (NCCLS) 1995. Reference method for broth dilution antifungal susceptibility testing of yeasts. Tentative standard M27-T. National Committee for Clinical Laboratory Standards, Wayne.Pa.

Odds, F.C., Cockayne, A., Hayward, J. and Abbott, A.B. 1985. Effects of imidazole and triazole derivative antifungal compounds on the growth and morphological development of *Candida albicans* hyphae. *J. Gen. Microbiol.* 131: 2581-2589.

Odds, F.C. 1988. *Candida* and candidiasis: a review and bibliography. Bailliere Tindall, London, United Kingdom.

Odds. F.C. 1993. Resistance of yeasts to azole-derivative antifungals. *J. Antimicrob. Chemother.* 31: 463-471.

Ofek, L., and Beachey, E.H. 1980. General concepts and principles of bacterial adherence in man and animals, p. 1-31. In E.H. Beachey (ed.), bacterial adherence. Chapman and Hall London.

Ostrosky-Zeichner, L., Rex, L.H., Pappas, P.G., Hamill, R.J., Larsen, R.A.; Horowitz, H.W., Powderly , W.G.; Hyslop, N.; Kauffman, C.A.; Clearly, J.; Mangino, J.E., and Lee, J. 2003. Antifungal susceptibility survey of 2000 bloodstream *Candida* isolates in the United States. Antimicrob. Agents and Chemother. 47 (10): 3149-3154.

Osullivan, J.M., Cannon, R.D., Sullivan, P.A., and Jnkinso, H.F. 1997. Identification of salivary basic proline rich protein as receptors for *C.albicans* adhesion. Microbiol. Readihg . 143(20): 341-384.

Paniagua, G.L., Monroy, E., Negrete, E., and Vaca, S. 2002. Susceptibility to 5-fluorocytosine, miconazole and amphotericinB of *C.albicans* strains isolated from the throat of non-AIDS patients. Rev.

Persi, M.A., Burnham, J.C., and Duhring, J.L. 1985. Effects of CO_2 and pH on adhesion of *C.albicans* to vaginal epithelial cells. Infect. Immun. 50: 82-90.

Pfaller, M.A., Grany, C., Morthland , V. and Rhine-Chalborg, C. 1994. Comparative evaluation of alternative methods for broth dilution susceptibility testing of fluconazole against *C.albicans*. *J. Clin. Microbiol.* 32: 506-509.

Pfaller, M. A. and Yu, W.L. 2001. Antifungal susceptibility testing. New technology and clinical applications. *Infect Dis. Clin. N. Am.* 15: 1227-1261.

Polaguini, S.R., Svidzinski, T.I., Kemmelmeier, C. and Gasparetto, A. 2006. Effect of aqueous extract from neem (*Azadirachta indica* A. Juss) on hydrophobicity, biofilm formation and adhesion in composite. resin by *C.albicans*. *Arch. Oral. Biol.* 51: 482-490.

Rex, J.H. and Pfaller, M.A. 2002. Has antifungal susceptibility testing come of age? Clin. Infect. Dis. 35: 982-989.

Ruhnke, M., Schmidt-Westhausen, A., Engelmann, E. and Trautmann, M. 1996. Comparative evaluation of three antifungal susceptibility test methods for *C.albicans* isolates and correlation with response to fluconazole drug. *J. Clin. Microbial.* 34: 3208-3211.

Samaranayake, L.T. and MacFarlane,T.W. 1982. Factors affecting the in vitro adherence of the fungal oral pathogen *C.albican* to epithelial cells of human origin. *Arch. Oral Biol.* 27:869-873.

Segel, E. and Savage, D.C. 1986. Adhesion of *C.albicans* to mouse intestinal mucosa in vitro: development of the assay and test of inhibitors. *J. Med. Vet. Mycol.* 24: 477-479.

Segel, E., Trygerman, O., Gov, Y., Samdovsky, L.H. and Berdicevsky, I. 1997. Adhesion of *C.albicans* to epithelial cells, effect of antimycotics. *J. Mycologie Medical.* 7(2)71-73.

Sewell, D.L., Pfaller, M.A., and Barry, A.L. 1994. Comparison of broth macrodilution, broth microdilution and E-test antifungal susceptibility tests for fluconazole. *J. Clin. Microbiol.* 32: 2099-2102.

Soll, D.R. 2002. *Candida* commensalisms and virulence: the evolution of phenotypic plasticity. *Acta Tropica.* 81: 101-110.

Steenkamp, V., Fernands, A.C., and Van Rensburg, C.E.J. 2007. Screening of Venda medicinal plants for antifungal activity against *C.albicans*. *South African J. Botany,* 73: 256-258.

Van Elder, J. , Joosten, L., Verhaeghe, A. and Surmont, I. 1996. Fluconazole and amphotericin B antifungal susceptibility testing by National Committee for Clinical Laboratory Standards broth macrodilution method compared with E-test and semiautomated broth microdilution test. *J. Clin. Microbiol.* 34(4): 842-847.

White, T.C., Marr, K.A. and Bowden, R. A. 1998. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. *Clin. Microbiol. Rev.* 11: 382-402.