CHAPTER 4

IN VITRO STUDIES-
ANTIFUNGAL
SUSCEPTIBILITY TESTING
4.1 INTRODUCTION

The treatment of mycotic infections is a perplexing problem since fungi are eukaryotic organisms with a structure and metabolism that is similar to those of their eukaryotic hosts. For this reason the antimicrobial agents presently available for the treatment of fungal infections can damage the host as well as destroy the fungal pathogen. Unlike many antibacterial agents that work intracellularly, most of the antifungal drugs exhibit a restricted spectrum of action because they work at the level of cell membrane. Contributing to this perplexity is the fact that most of the antimycotic agents are relatively unstable and insoluble in biological fluids.

Before 1950, no reliable or safe treatment existed for deep fungal infections, and treatment of superficial infections depended on empirical topical preparations. During the 1950s nystatin was introduced for the topical treatment of candidiasis, and use of amphotericin B (AMB) transformed the treatment of most forms of deep fungal infections. With the introduction of 5-fluorocytosine (5-FC) for treatment of candidiasis, drug resistance became an important mycological problem for the first time. The 1970s and 1980s have seen the introduction of a large number of broad-spectrum antifungal agents that are N-substituted imidazole or triazole compound. The earliest members of this group, clotrimazole (CTZ) and miconazole (MCZ), are useful for superficial forms of candidiasis. However, miconazole is also available for parenteral use, and some of the more recent azole compounds eg. ketoconazole (KTZ), itraconazole (ITZ) and fluconazole (FCZ), are effective after oral administration.

The continuing introduction of new antifungal drugs viz., polyene (amphotericin B); imidazoles: (miconazole, clotrimazole, fenticonazole, econazole, ketoconazole and saperconazole); and pyrimidenes: (5-fluorocytosine) etc. have increased the urgency for adequate methods of in vitro testing that could predict the effects of compounds in vivo. As with antibacterial drugs, tests designed to ascertain the minimum amount of drug needed to inhibit the growth of organisms in vitro i.e., minimum inhibitory concentration (MIC) have been used to predict the clinical results of treatment. If growth of the organism is inhibited in vitro at drug concentrations lower than levels attained in vivo,
the organism is regarded as "sensitive" to that drug. However, if growth of an organism is not inhibited at drug concentrations attained in vivo, the organism is regarded as "resistant". The results of treatment are often difficult to assess in infected patients. However, in situation where it can be assessed, there is often a good correlation between this interpretation and the clinical results of treatment with antibacterial drugs\textsuperscript{1,2}. In contrast, with most antifungal drugs, the correlation is either poor or absent.

So far, as the number of effective antifungal agents were limited, routine susceptibility testing of pathogenic or opportunistic fungi involved in such infections was not considered necessary. In the modern era of medicine a good number of antifungal agents are available globally. Primary and secondary resistance to 5-FC\textsuperscript{3,4} and even to amphotericin B (AMB), nevertheless, exceptionally considered as inductor of resistant strains, as well as to some azoles\textsuperscript{5}. Although, such resistant strains rarely observed previously are now much more common. For topical antifungal chemotherapy of superficial candidiasis a routine evaluation of the sensitivity of the responsible fungi is not necessary because these agents are applied at doses 100 to 1000 times superior to the MIC. However, such testing may be necessary for optimal management of patients with systemic candidiasis or with chronic recurrent candidiasis as reviewed by other workers\textsuperscript{6-9}. Consequently, a routine rapid evaluation of the sensitivity to the systemic antifungal agents seems to be helpful, however, none of the antifungal susceptibility tests (both macro-and micro-dilution and agar diffusion techniques), are standardized. These difficulties are due to the properties of the fungus (morphological complexity, amount of inoculum), the drug (solubility, various mode of action), the medium (composition, necessity to be free from antagonistic or obfuscating agents) and to the methodology adopted. Due to its morphological complexity, Candida represents the first difficult step on the way towards standardization. The yeast (Y) and mycelium (M) phases of Candida albicans have been found to be more or less equisensitive to a number of established antifungal agents, but with azoles (imidazoles and triazoles) this generalization does not hold\textsuperscript{10}. As shown by Ryley and Rathmell\textsuperscript{10} and Van Cutsem\textsuperscript{11} that the M-phase which is aggressive, invasive,
deep penetrating tissue phase in candidosis is more sensitive than the Y-phase to miconazole, clotrimazole and particularly to ketoconazole, the MIC by several orders of magnitude is dependent on culture conditions. If the initial screening of antifungal agents for M- and Y-phases of *C. albicans* have to be included for sensitivity for hospital routine work, the most sensitive medium for both phases must be selected. In order to allow the *in vitro* activity of the antifungals, the medium composition has to be free from antagonists or obfuscating agents which can only be recognised by a deep knowledge of the mode of action of the drug particularly, for azoles. Table 1 summarizes for each type of antifungals, mode of their action, and *in vitro* antagonists or obfuscating agents now recognized and Table 2 summarizes the year of origin of different antifungals used.

Recently, the National Committee for Clinical Laboratory Standards (NCCLS) subcommittee on antifungal susceptibility tests recommended visual reading criteria scored from 0 to 4+ according to the rate of growth in the control (drug-free) well. By the broth microdilution method, the inhibitory concentration (90 IC) is the lowest drug concentration that is capable of inhibiting a determined percentage of growth, and an objective value for assessing the final point of inhibition is achieved. Turbidimetric methods can determine the minimum drug concentration which inhibits 50% of growth (IC$_{50}$) or the lowest drug concentration at which growth is less than 30% of that in the positive control well (30 IC). The available antifungals mentioned above have not yet been properly studied *in vitro* on account of inhibitors in media used. There are certain problems still remain unsolved possibly because the methodology of testing antifungal activity has not been yet well studied. Therefore, an attempt has been made to carry out a systematic *in vitro* study on some antifungals in different media adopting modern techniques which could be useful for better guidelines in therapy. Various factors—pH, inoculum size, morphology, temperature etc., are defined according to the MIC studies by serial dilutions of drug in agar or broth. An automated microtitre technic using microplates and a registering spectrophotometer for reading is described.
Table 1. *Amphotericin B, 5-fluorocytosine and imidazole derivatives their mode of action and in vitro antagonists or obfuscating agents*

<table>
<thead>
<tr>
<th>Type of antifungal agent</th>
<th>Mode of action</th>
<th>In vitro antagonists</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B (Polyene)</td>
<td>Binding to ergosterol of plasmic membrane-impairment of selective permeability</td>
<td>Sterols K⁺ and Na⁺ in high concentration reducing agents, e.g. cysteine</td>
</tr>
<tr>
<td>5-Fluorocytosine (pyrimidine)</td>
<td>Cytosine permease disturbance of protein synthesis, inhibition of DNA, RNA synthesis</td>
<td>Cytosine, adenine, hypoxantine uridine</td>
</tr>
<tr>
<td>Imidazoles (Azole)</td>
<td>Interaction with unsaturated phospholipids of cell membranes, impairment of membrane activities</td>
<td>Unsaturated phospholipids Ca⁺⁺ and Mg⁺⁺</td>
</tr>
</tbody>
</table>

Table 2. *Antifungal drugs used and the year of origin*

<table>
<thead>
<tr>
<th>Antifungals</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyene</td>
<td></td>
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<tr>
<td>Amphotericin B</td>
<td>1958</td>
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<tr>
<td>Pyrimidine</td>
<td></td>
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<tr>
<td>5-fluorocytosine</td>
<td>1972</td>
</tr>
<tr>
<td>Imidazoles</td>
<td></td>
</tr>
<tr>
<td>Clotrimazole</td>
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<td>Miconazole</td>
<td>1971</td>
</tr>
<tr>
<td>Imazalil</td>
<td>1973</td>
</tr>
<tr>
<td>Econazole</td>
<td>1974</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>1981</td>
</tr>
<tr>
<td>Fenticonazole</td>
<td>1987</td>
</tr>
<tr>
<td>Saperconazole</td>
<td>1990</td>
</tr>
</tbody>
</table>
4.2 MATERIALS AND METHODS

4.2.1 Antifungal agents (Fig. 1). The amphotericin B (AMB), Squibb, USA, miconazole (MCZ), ketoconazole (KTZ), fenticonazole (FTZ), imazalil (IMZ), saperconazole (SCZ) Janssen Pharma, Belgium, econazole (ECZ), Cilag, USA and canesten (clotrimazole, CTZ), Bayer; fresh solution were made. A 10 mg of each drug (pure) was dissolved in 5 ml of dimethylsulfoxide (DMSO, Sisco Research Labs., India) and a stock solution (1 mgml\(^{-1}\)) was completed after dilution with 5 ml triple distilled water (TDW). The serial drug dilutions were made in assay tubes with broth medium. The highest drug concentration was 100 µgml\(^{-1}\) and serially diluted to 0.04 µgml\(^{-1}\). For 5-fluorocytosine (5-FC), Roche, Switzerland, 5 mg pure powder was dissolved in 5 ml TDW at 60°C and stock (1 mgml\(^{-1}\)) was used for serial dilutions in respective media. Blotting paper (Whatman No. 1) discs of 6 mm diameter were charged with 13, 6.5, 3.25, 1.62, 0.8 µg for all the drugs mentioned above for antibiotic disc agar diffusion technique.

4.2.2 Media used. The fungal media used were Sabouraud dextrose (chloromycetin) broth (SDB) or agar (SDA; Himedia), pH 5.6; yeast nitrogen base broth (YNB) or agar (YNBA, Difco ref. 0939-15), pH 5.6, and RPMI-1640 (Sigma), pH 7.0.

4.2.3 Culture and inoculum preparation. In vitro activity of nine antifungals was determined against 19 clinical isolates (Str. Nos. CA-1-19) obtained from immunocompromised patients including one reference strain of Candida albicans (Str. No. CA-14, NCPF 3153A, Serotype A). All isolates were identified to a species level by the AUX system\(^{18}\) supplemented as needed with conventional morphologic and biochemical methods (described in Chapter 2), and maintained on Sabouraud dextrose agar (SDA) slants at 4°C until tested. The inocula of all Candida species tested were obtained by picking a colony of the yeast by a loop (1 mm diam) from 24-48 hr old cultures at 28±1°C. The yeast blastospores were suspended in sterile saline (0.85%) and vortexed for 20 mts. This suspension was further diluted to 1:100 in assay medium. Colony counts were performed to confirm the number of colony forming units (cfu) approx. (5x10\(^{5}\) cfuml\(^{-1}\)) on SDA plates of all pathogenic strains.
4.2.4 *In vitro* testing technique. Three techniques viz., (i) macrobroth two-fold serial dilution technique (TFSD), (ii) microbroth microtitre technique (MT), and (iii) disc agar diffusion (DD) techniques were employed for antifungal susceptibility testing as follows:

4.2.4(i) Macrobroth Two-Fold Serial Dilution (TFSD) technique. The drug was diluted two-folds in a series of tubes (assay tubes 100x10 cm, corning) with seeded (10^5 cfu ml^-1) macrobroth. Initially 1.8 ml of seeded broth was added with 0.2 ml of antifungal drug, and at least 10-12 such dilutions were made serially. Suitable growth and solvent (DMSO) controls were maintained under identical conditions. The set of tubes were incubated at 28±1°C and the MICs (µgml^-1) of antifungals based upon visual appearance of the growth was recorded after 24, 48 and 72 hr. The geometric mean MIC (µgml^-1) of drugs was calculated.

4.2.4(ii) Microbroth microtitre (MT) technique. This technique in principle is essentially the same as that of the TFSD technique. Briefly, the microbroth quantities 270 µl per well were dispensed in an ELISA microtitre plate with 96 (12x8) wells. The drug (30 µl) was added to the first well and then diluted serially with a multichannel appendorf pipette (titeretek). The test inoculum (20 µl containing 10^5 cfu) was added separately in each well. Appropriate controls (broth, inoculum, solvent and drug) were set under identical condition and the titre plate was incubated at 28±1°C as described above. The microtitre MIC was recorded on an automated ELISA reader (Flow Labs., Scotland) based upon optical density (OD at 492 nm, matrix 0.2) after 24, 48 and 72 hr. The geometric mean MIC (µgml^-1) of drugs was calculated.

4.2.4(iii) Disc agar diffusion (DD) technique. The test compound 13 µg per disc) was impregnated in standard filter paper (Whatman No.1) disc (6 mm diam). The medium (SDA) 20 ml per petriplate (90 mm diam) was flooded with 5-10 ml broth of the test inoculum (10^5 cfu) by floatation and then excess broth drained off. The petriplates were desiccated 15-20 min at room temp for adequate drying. The dried antifungal discs were kept at least 15 mm apart from each other (4-5 discs can be placed in a 90 mm diam
petridish) and then left for 30 min at room temp at horizontal surface. The petri plates were incubated at 28±1°C and the zone of inhibition (mm diam) was measured after 24, 48 and 72 hr post-incubation.

4.2.5 Statistical analysis
The data were analysed by correlation coefficient and regression analysis for determination of geometric mean MICs.

4.3 RESULTS

The in vitro susceptibility-geometric mean (GM) MIC (µgml⁻¹) of antifungals is presented in Figs. 2 to 10. The AMB showed best activity (GM MIC 0.12 µgml⁻¹) in SDB by the TFSD technique at 24 hr. Likewise by MT technique GMMIC was 0.29 µgml⁻¹, in YNBA upto 48 hr and by DD technique it was derived to >1.0 µgml⁻¹ upto 72 hr. The equivalent activity of AMB was found in YNBA and RPMI at 24 hr by both TFSD and MT technique. In DD technique better results were obtained with YNBA compared to SDA (Fig. 2). For MCZ the GM MIC was 0.11 µgml⁻¹ at 24 hr by TFSD and 0.17 µgml⁻¹ by MT in both YNB and RPMI. The activity was comparatively poor in SDB in order of decreasing trend in MT and TFSD. In DD the SDA was found better compared to YNBA. Overall DD showed poor activity (GM MIC 7.4-13 µgml⁻¹) irrespective of agar media used (Fig. 3). In case of KTZ the highest order of activity was found in RPMI-1640 at 24 hr by TFSD (GM MIC 0.13 µgml⁻¹) and by MT (GM MIC 0.23 µgml⁻¹), whereas, in SDB by both the above techniques GM MIC was >1.0 µgml⁻¹. In DD the best GM MIC was 5.6 µgml⁻¹ and 0.3 µgml⁻¹ at 24 hr in SDA and YNBA respectively (Fig. 4). In FTZ the best activity was found in RPMI at 24 hr by TFSD (GM MIC 0.37 µgml⁻¹) followed by MT (GM MIC 0.54 µgml⁻¹). In YNB the best activity was found at 24 hr in MT (1.1 µgml⁻¹) followed by TFSD (2.0 µgml⁻¹). The activity after 24 hr was found to decrease with respect to incubation period. DD showed lowest activity (GM MIC 2.9-10.8 µgml⁻¹) irrespective of the media used, however, in case of YNBA the activity was better as compared to SDA particularly at 24 hr but beyond 48 hr there was no remarkable difference in the activity (Fig. 5). Fig. 6 shows that imazalil (IMZ) was not a drug of choice for Candida strain as was evident from the GM MIC data (Fig. 6). The best MIC was found by TFSD in broth (GM MIC 21.60 µgml⁻¹). The
data evinced that SDB was better compared to YNB or RPMI, particular at 24 hr. The DD showed almost comparable results in SDA and YNBA (GM MIC >10 µgml⁻¹) during 24-72 hr. In case of SCZ the best susceptibility was found by TFSD (GM MIC 0.16 µgml⁻¹) in RPMI followed by MT (GM MIC 0.28 µgml⁻¹) at 24 hr incubation. The activity got reduced during 48-72 hr by both the techniques. Comparable results were obtained with SDA and YNBA (GM MIC 4.8-13.0 µgml⁻¹) during 24-72 hr (Fig. 7). The geometric mean MIC for econazole (ECZ) was also found to be best in RPMI in TFSD (GM MIC 0.12 µgml⁻¹) which was close to MT (GM MIC 0.2 µgml⁻¹) at 24 hr, whereas, by DD the GM MIC was derived to be 10.43 µgml⁻¹ in SDA compared to YNBA (GM MIC 0.5 µgml⁻¹) at 24 hr (Fig. 8). In case of CTZ also RPMI showed excellent results by both TFSD and MT (GM MIC 0.2 µgml⁻¹) during 24-48 hr and, thereafter, the activity was slightly reduced during 72 hr. MT was found to be comparatively better than TFSD and DD techniques (GM MIC 3.9 µgml⁻¹) (Fig. 9). For 5-FC the synthetic medium YNB was the choice of media compared to RPMI (Fig. 10) at 24 hr. YNB showed best results by MT (GM MIC 0.3 µgml⁻¹) followed by TFSD (GM MIC 0.6 µgml⁻¹) compared to RPMI by both the techniques (GM MIC 1 µgml⁻¹ and 1.4 µgml⁻¹) respectively. In DD technique the GM MIC was found to be 1.7 µgml⁻¹ irrespective of incubation period upto 72 hr in YNBA as compared to SDA (GM MIC 7.2 µgml⁻¹ to 7.5 µgml⁻¹) (Fig. 10).

In the present investigation the activity of most of the antifungals was found to reduce in media with respect to prolongation of incubation period beyond 24 hr. Correlation coefficient and regression equation on susceptibility of C. albicans strains with antifungals by DD technique showed poor correlation between MICs derived from inhibition zone diameter (mm) with antifungals (Table 3). The regression lines for various strains were parallel and lie close to each other (Fig. 11 and 12). The regression equation (Y) and correlation coefficients (r) showed deviation of the individual strains from the overall regression or total correlation coefficient. It is evident from the Table 3 that these deviations depend on the MIC of individual strains, based on media, kind of drug, technique and incubation. The 5-FC showed better correlation coefficient (r) r = 0.85 both at 24-48 hr and -0.71 at 72 hr in YNBA based upon zone diameter inhibition (Table 3).
Overall, the best medium for susceptibility testing was found to be RPMI-1640 followed by YNB at 24 hr incubation period; and MT technique was the most sensitive, quantitative, reproducible, rapid and economic compared to macrobroth dilution or DD technique.

4.4 DISCUSSION

It seems logical that methods for determination of MICs should be selected on the basis of good correlation with clinical results of treatment. In practice, there have been few attempts to test the extent of the correlation between MIC tests with antifungal drugs and the results of their administration to patients with fungal infection. Stiller et al.\textsuperscript{20} tested the effects of 5-FC against 40 isolates of \textit{C. albicans} in vitro and in a murine model of candidiasis. Their results demonstrated a significant correlation between clinical response and broth dilution test and agar diffusion methods for MICs. The clinical response of several of the most sensitive isolates overlapped with those of several of least susceptible isolates. Plempel\textsuperscript{21} tested the effects of triazole against 59 isolates of \textit{C. albicans} in vitro and in a murine model of candidiasis. MICs read after 48 hr of incubation showed no correlation with the results in the animal model. Polak et al.\textsuperscript{22} tested the effects of ketoconazole against 58 isolates of \textit{C. albicans} in vitro and in a murine candidiasis model. No significant correlation were detected among a number of methods of \textit{in vitro} testing and the results \textit{in vivo}.

Thus, the current applicability of \textit{in vitro} antifungal susceptibility tests is limited by inadequate standardization and insufficient correlation of \textit{in vitro} test results with clinical outcome. The poor correlation between MICs of antifungal drugs \textit{in vitro} and their effects \textit{in vivo} is due, at least in part, to the tremendous variation in MICs obtained under different test conditions. For instance, tests with \textit{C. albicans} isolates have produced MIC ranges for miconazole with mid points of 0.5-35.0 mgL\textsuperscript{−1} depending on the conditions under which the tests were performed\textsuperscript{22}. With ketoconazole, even greater variations in MICs have been recorded, with mid points in tests with \textit{C. albicans} ranging from 1 to 80 mgL\textsuperscript{−1} \textsuperscript{23}. The \textit{in vitro} techniques necessitate standardization of factors including size of
inoculum, media used, pH, duration of incubation, temperature, solubility of antifungal agents, chemical stability and the tendency to produce partial inhibition of growth over a wide range of concentration\textsuperscript{24, 25}. Studies attest to the fact that inoculum size affects MIC test results with 5-fluorocytosine\textsuperscript{26} and the azoles\textsuperscript{27}. In general, the MIC increases with the size of the fungal inoculum. In contrast, inoculum size appears to have minimal effects on the MICs of amphotericin B\textsuperscript{28}. The composition of the test medium has a pronounced effect on MIC results with 5-FC\textsuperscript{29}. However, identical results have been obtained in tests in which the MICs of \textit{C. albicans} and \textit{C. tropicalis} isolates were determined with liquid and solid media of similar composition\textsuperscript{29}. Reports have shown that medium composition has a marked effect on MIC results with imidazole drugs\textsuperscript{29}. However, in several investigations in which MICs were determined with liquid and solid media of similar composition under standardized conditions, identical results were obtained in most instances\textsuperscript{30}. Inclusion of serum in the medium leads to higher MICs\textsuperscript{31}.

The pH of the testing media, specially at low pH, has been associated with lower MICs of \textit{AMB}\textsuperscript{32}. Effects of pH shifts are minimal in broth dilution with 5-FC for MIC tests\textsuperscript{33}. In control, the pH of the medium has a marked effect on azole MICs. Most reports indicate that a neutral pH gives the lowest MICs\textsuperscript{34}. Another problem encountered with imidazole and triazole compounds results from the fact that these drugs often cause partial inhibition of growth over a wide range of concentrations making visual determinations of endpoints in MIC test difficult\textsuperscript{35}. This effect is most pronounced with \textit{C. albicans}\textsuperscript{35} and appears to account for the similar MICs obtained with azole sensitive and azole-resistant strains of the fungus in some agar and broth dilution tests\textsuperscript{36}. The poor correlation of MICs for antifungal drugs \textit{in vitro} with effects of the compounds \textit{in vivo} has stimulated attempts to develop alternative methods of \textit{in vitro} testing that avoid the problems of inoculum dependence and endpoint determination.

Agar disc diffusion tests have been described for a number of antifungal drugs\textsuperscript{37}. Tests with 5-FC using \textit{C. albicans} have shown that there is a good correlation between the diameter of the zone of inhibition and the MIC determined with agar dilution method\textsuperscript{38}. The same cannot be said for other antifungal agents. With imidazoles the results of disc diffusion tests often fail to
correlate with broth or agar dilution MICs\textsuperscript{39} and substantial variations in results occur depending on the composition of the test medium\textsuperscript{40}. In our study we found that there is a good correlation between the zone diameter and the MIC determined among some drugs especially with 5-FC (Table 1). Although the results of disc diffusion tests of the antifungal drugs did not correlate with micro- or macro-broth technique. The demerits of disc diffusion technique is that it is time consuming and this technique does not eliminate the trailing effect that occurs when testing the susceptibility of strains of \textit{C. albicans} to azoles. Anaissie \textit{et al}\textsuperscript{41} reported that microtitre plate agitation yielded reproducible endpoints for \textit{C. albicans} that were independent of inoculum size, temperature of incubation, and time of reading and correlated well with the degree of fungal inhibition as determined spectrophotometrically. While reading the microplates it is important to produce a homogenous suspension in the wells. After the incubation period (24, 48 and 72 hr) the organisms form a pellet which is easily visualized through the MIC reading mirror at the bottom of the well. We found that the automated agitation of the wells on titertek (Flow labs.) for 5 min at speed also provides a dispersion of the sediment and the formation of different degree of turbidity depending upon the number of organisms present in the wells.

Although, the National Committee for Clinical Laboratory Standards (NCCLS) subcommittee established parameters for the visual reading of the MIC end points. It is often difficult to give a precise definition of the score due to partial growth inhibition (slight turbidity) produced by imidazoles derivatives. The technical problem confirms the importance of carrying out the reading of antifungal susceptibility testing in different media with different techniques using different incubation period. Several studies showed that the visual reading gave less objective data and these data are often in disagreement with the spectrophotometric reading\textsuperscript{42-44}.

From our study we found that there is no complete disagreement between turbidimetric and visual endpoint determinations\textsuperscript{45}, if for the latter criteria, the recommendations provided by the NCCLS subcommittee are followed. Our study suggests that the microbroth dilution test by MT technique using RPMI-1640 medium produced reproducible MIC results.
4.5 CONCLUSION

The microbroth technique is both efficient and economical, and it yields precise endpoints for MICs, particularly with automated plate-reading technology. The advantages of choosing the turbidimetric rather than the visual endpoint include objectivity, rapidity and elimination of subjective judgements concerning minimal turbidity. Thus, it may be concluded that for in vitro testing amphotericin B is the golden drug of choice for susceptibility testing against C. albicans strains. YNB was the best medium for 5-FC, whereas, RPMI-1640 was the best medium for imidazoles. An incubation period of 24 hr was the most suitable period for susceptibility testing against C. albicans strains. Microtitre technique (MT) was the most sensitive, rapid, quantitative, reproducible and economic technique for yeast susceptibility testing.
REFERENCES


Table 3. Statistical data on correlation-coefficient (r) and regression analysis with antifungals in SDA and YNBA media on different incubation hour

<table>
<thead>
<tr>
<th>Antifungals</th>
<th>Media</th>
<th>Correlation coefficient (r)</th>
<th>Regression equation (Y)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>incubation (hr)</td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>Amphotericin B (AMB)</td>
<td>SDA</td>
<td>0.12</td>
<td>-0.26</td>
</tr>
<tr>
<td></td>
<td>YNBA</td>
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<td>-0.04</td>
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<tr>
<td>Miconazole (MCZ)</td>
<td>SDA</td>
<td>0.15</td>
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<td>YNBA</td>
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<td>-0.3</td>
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<td>Ketoconazole (KTZ)</td>
<td>SDA</td>
<td>-0.71</td>
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<td>YNBA</td>
<td>-0.61</td>
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<td>Fenticonazole (FTZ)</td>
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<td>5-Fluorocytosine (5-FC)</td>
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SDA = Sabouraud dextrose agar, YNBA = yeast nitrogen base agar

Y* = a+bx, Y=Zone diameter (mm), X = loge MIC + 9
ANTIFUNGAL DRUGS

AMPHOTERICIN B

CLOTRIMAZOLE

FLUCYTOSINE

IMAZALIL

ECONAZOLE

FENTICONAZOLE

MICONAZOLE

KETOCONAZOLE

SAPERCONAZOLE
Figure 2. Geometric mean MIC of amphotericin B against
*C. albicans* (19 strains) by two-fold serial dilution
microtitre and disc diffusion techniques using
different media at varying incubation periods (hr)

Figure 3. Geometric mean MIC of miconazole against *C. albicans*
(19 strains) by two-fold serial dilution, microtitre
and disc diffusion techniques using different media at
varying incubation periods (hr)
Figure 4. Geometric mean MIC of ketoconazole against *C. albicans* (19 strains) by two-fold serial dilution, microtitre and disc diffusion techniques using different media at varying incubation periods (hr).

Figure 5. Geometric mean MIC of fenticonazole against *C. albicans* (19 strains) by two-fold serial dilution, microtitre and disc diffusion techniques using different media at varying incubation periods (hr).
Figure 6. Geometric mean MIC of imazalil against *C. albicans* (19 strains) by two-fold serial dilution, microtitre and disc diffusion techniques using different media at varying incubation periods (hr)

Figure 7. Geometric mean MIC of saperconazole against *C. albicans* (19 strains) by two-fold serial dilution, microtitre and disc diffusion techniques using different media at varying incubation periods (hr)
Figure 8. Geometric mean MIC of econazole against *C. albicans* (19 strains) by two-fold serial dilution, microtitre and disc diffusion techniques using different media at varying incubation periods (hr)

Figure 9. Geometric mean MIC of clotrimazole (Canesten) against *C. albicans* (19 strains) by two-fold serial dilution, microtitre and disc diffusion techniques using different media at varying incubation periods (hr)
Figure 10. Geometric mean MIC of 5-fluorocytosine against C. albicans (19 strains) by two-fold serial dilution, microtitre and disc diffusion techniques using different media at varying incubation periods (hr)
80
60
40
20
0

100
90
80
70
60
50
40
30
20
10
0

GEOMETRIC MEAN MIC (µg ml⁻¹)

INCUBATION TIME (hr)

5-FLUOROCYTOSINE

24
48
72
Figure 11. Regression line analysis of *C. albicans* strains, for econazole (ECZ), canesten (CNS), fenticonazole (FTZ), miconazole (MCZ), amphotericin B (AMB), 5-fluorocytosine (5-FC), saperconazole (SCZ), ketoconazole (KTZ) and imazalil (IMZ) in Sabouraud chloromycetin agar at 24 hr incubation period.

Figure 12. Regression line analysis of *C. albicans* for fenticonazole (FTZ), miconazole (MCZ), amphotericin B (AMB), 5-fluorocytosine (5-FC), saperconazole (SCZ), ketoconazole (KTZ) and imazalil (IMZ) in Sabouraud chloromycetin agar at 24 hr incubation period.