Flow cytometry susceptibility testing for conventional antifungal drugs and Comparison with the NCCLS Broth Macrodilution Test

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ABSTRACT

Introduction: During the last decade, the incidence of fungal infection has been increased in many countries. Because of the advent of resistant to antifungal agents, determination of an efficient strategic plan for treatment of fungal disease is an important issue in clinical mycology. Many methods have been introduced and developed for determination of invitro susceptibility tests. During the recent years, flow cytometry has developed to solving the problem and many papers have documented the usefulness of this technique.
Materials and methods: As the first step, the invitro susceptibility of standard PTCC (Persian Type of Culture Collection) strain and some clinical isolates of *Candida* consisting of *Candida albicans*, *C. dubliniensis*, *C. glabrata*, *C. kefyer* and C. parapsilosis were evaluated by macrodilution broth method according to NCCLS (National Committee for Clinical Laboratory Standards) guidelines and flow cytometry susceptibility test.
Results: The data indicated that macro dilution broth methods and flow cytometry have the same results in determination of MIC (Minimum Inhibitory Concentration) for amphotericin B, clotrimazole, fluconazole, ketoconazole and miconazole in *C. albicans* PTCC 5027 as well as clinical *Candida* isolates, such as *C.albicans*, *C.dubliniensis*, *C.glabrata C.kefyr*, and *C.parapsilosis*.
Discussion: Comparing the results obtained by macrodilution broth and flow cytometry methods revealed that flow cytometry was faster. It is suggested that flow cytometry susceptibility test can be used as a powerful tool for determination of MIC and administration of the best antifungal drug in treatment of patients with Candida infections.

Key words: Flow cytometry, Susceptibility, NCCLS, Macrodilution

INTRODUCTION

The number of patients predisposed to infections with opportunistic microorganisms such as *Candida* strains especially in immunocompromised patients has increased significantly over the last decade. Even more disturbing are frequent reports that fungi are developing resistance to antifungal agents (1-5). This has facilitated efforts to develop a more reliable in vitro susceptibility test to predict clinical outcome. Because of the advent of resistant to antifungal agents, determination of an efficient plan for treatment of fungal disease is an important issue in clinical mycology.
A number of investigators have used flow cytometry methods to obtain rapid susceptibility results for *Candida* spp. (1- 11) The method is based on the use of sodium deoxycholate for permeability and propidium iodide (PI), a membrane-impermeant DNA-intercalating dye, to detect increase in permeability of the cell membrane after antifungal treatment. A flow cytometry (FC) method was used in the present study for susceptibility testing of *Candida albicans* PTCC 5027, as well as clinical *Candida* isolates, including *C.albicans, C.dubliniensis, C.glabrata, C.kefyr* and *C.parapsilosis* based on accumulation of the DNA binding dye propidium iodide (PI). The results were compared with MIC results obtained for amphotericin B, clotrimazole, fluconazole, ketoconazole and miconazole by NCCLS broth macrodilution method (M27-A).

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MATERIAL AND METHODS

Organisms

Candida albicans PTCC 5027 was obtained from Institute of Standard and Industrial Research of Iran and *C.albicans, C.dubliniensis, C.glabrata, C.kefyr and C.parapsilosis* were isolated from the urine of diabetic patients at Zanjan (IRAN) province hospitals.

The clinical isolates were identified to the strains level by API 20C yeast identification system (API 20 C AUX, boimerieux). Prior to testing, each isolate was passaged at least twice on sabouraud dextrose agar to ensure purity and optimal growth.

Antifungal agents

Amphotericin B was purchased from sigma chemical company ($C47H73No_{13}$-A_{4888}-10T47H1247), fluconazole (Batch no: 104/70) was a gift from Pars Daru company and cloterimazole (Lot Number = 200211060136), ketoconazole (Batch Number: Det "50" 02-03) and miconazole (Lot: ZRO14889pud67) were gifts from Behvazan Rasht Co.

Stock solutions of these antifungal drugs were prepared in dimethylsulfoxide (Sigma).

Antifungal susceptibility testing using NCCLS broth macrodilution test

The broth macrodilution test was performed in accordance with standard NCCLS, M27-A2 (12). Briefly, serial twofold dilutions of the antifungal agents were prepared with RPMI 1640 medium containing L-glutamin without bicarbonate (Sigma, St. Louis, MO, USA) buffered to pH 7.0 and supplemented with 0.2% glucose and 0.165 M MOPS (3-*N*-morpholinopropanesulfonic acid). The final concentrations of the antifungal agents ranged from 16 to 0.25 µg/mL for amphotericin B and 64 to 0.125 µg/mL for clotrimazole, fluconazole, ketoconazole and miconazole. The suspensions containing antifungal agents were then inoculated with 1×10^6 to 5×10^6 cells/mL and incubated at 35 ºC for 48 hrs. Drug-free and yeast-free controls were included. For the azoles, clotrimazole, fluconazole, ketoconazole and miconazole, the MIC was established as the lowest antifungal concentration that inhibited 80% of the control growth (0.2 mL of growth control plus 0.8 mL of uninoculated RPMI). For amphotericin B, endpoints were determined visually by recording the lowest concentration of the agent that prevented the appearance of visible growth (12, 13).

Flow cytometry susceptibility test

The condition of the FC method has been previously described by Ramani et al. (1, 7). Briefly, serial twofold dilutions of antifungal drugs were prepared in RPMI 1640 containing L-glutamin without bicarbonate (Sigma, St. Louis, MO, USA) buffered to pH 7.0 and supplemented with 0.2% glucose and 0.165 M MOPS (3-*N*-morpholinopropanesulfonic acid). The yeast isolate were grown on Sabouraud dextrose agar for 24 hrs at 35ºC and yeast suspensions were prepared in 0.85% sterile saline and adjusted spectrophotometrically to match on 0.5 McFarland density. This procedure yielded a stock suspension of 1×10^6 to 5×10^6 cells/mL.

The final concentrations of AMB ranged from 0.25 to 16 µg/mL and 64 to 0.125 µg/mL for clotrimazole, fluconazole, ketoconazole and miconazole.

One-half milliliter of serial twofold dilutions of each antifungal agent was placed in 12- 75-mm tubes (Falcon 2054; Becton Dickinson, Lincoln Park, N.J.). Yeast inocula (0.5 mL) were added, and incubated for 2 hrs for amphotericinB and 4-6 hrs for other drugs. The growth control tube contained no drugs. After incubation of the assay suspensions, 200 mL of the mixture of yeast and drug were placed in 12- 75-mm falcon tubes. Two hundred microliters of 25 mM sodium deoxycholate (Sigma Chemical Company) and 5 mL of PI (200 mg/mL) were added to each dilution and tubes were mixed by flicking with fingers. The samples were analyzed with a FAC Scan Flow Cytometer (Becton Dickinson) with Cell Quest software for data acquisition and analysis. The sample volume was 75 mL, and the sample flow rate was 10 mL/min.

The number of yeast cells analyzed for forward scatter (3.73 linear gain), side scatter (270-V log), log of red fluorescence, FL2 (457-V log), threshold value 52, and mean channel fluorescence (MCF; intensity of fluorescence of yeasts labeled with PI) was 10000 cells.

The MIC was defined as the lowest drug concentration that produced a 50% increased in MCF compared to that of growth control (1).

RESULTS

Table1. shows results of the antifungal effects of amphotericinB, clotrimazole, fluconazole, ketoconazole and miconazole on *C.albicans* PTCC 5027, as well as clinical *Candida* isolate, such as *C.albicans, C.dubliniensis, C.glabrata, C.kefyr* and *C.parapsilosis* by NCCLS broth macrodilution and flow cytometry techniques.

Figure 1. depicts results of the effect of amphotericinB on *C.albicans* PTCC 5027 by flow cytometry method.

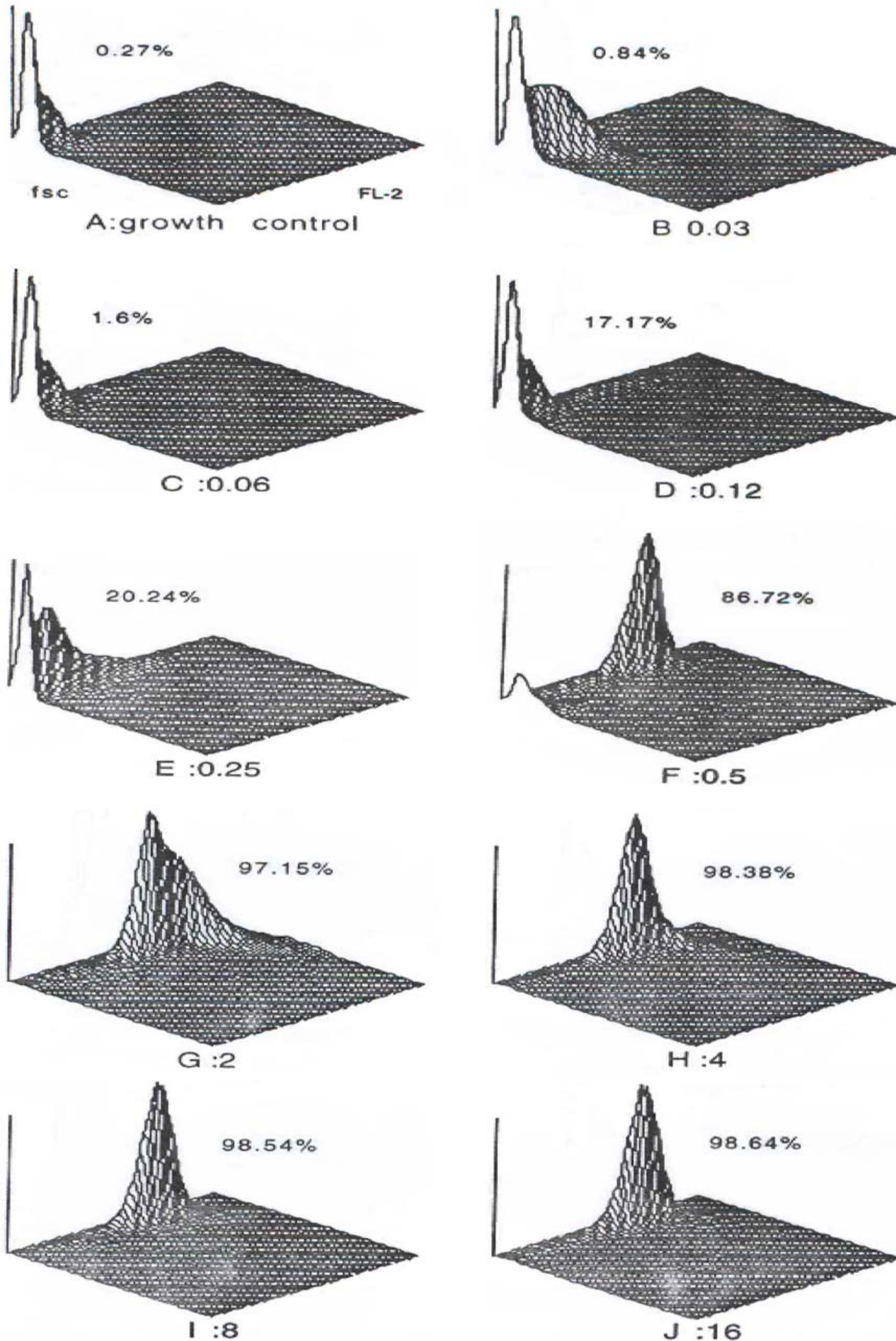

Figure. 1. Effect of amphotericin B on *C.albicans* PTCC 5027. (A) 3-D plot depicting growth control. (B to J) 3-D plot illustrating increasing concentration of amphotericin B, with death cells Indicated as percent MCF. The MIC of the isolate was the drug concentration at which the MCF was equal to or more than 50% in this isolate, an increase of percent MCF from 20.24% at 0.25 µg/mL to 86.72% at 0.5 µg/mL of amphotericin B was seen, therefore 0.5 µg/mL was considered the MIC.

Table 1. MICs of amphotericinB, clotrimazole, fluconazole, ketoconazole and miconazole for *candida* species by flow cytometry and the NCCLS broth macrodilution test

Candida SPP.	AmphotericinB		Clotrimazole		Fluconazole		Ketoconazole		Miconazole	
	FCM	**NCCLS**	**FCM**	**NCCLS**	**FCM**	**NCCLS**	**FCM**	**NCCLS**	**FCM**	**NCCLS**
***C. albicans* PTCC 5027**	0.5	0.5	1.0	2.0	16.0	8.0	1.0	2.0	1.0	1.0
C. albicans	0.5	0.125	0.5	0.5	8.0	8.0	2.0	4.0	2.0	2.0
C. dubliniensis	1.0	0.25	0.5	0.5	2.0	4.0	64.0	32.0	0.5	1.0
C. glabrata	0.25	0.125	8.0	8.0	4.0	4.0	4.0	8.0	1.0	1.0
C. kefyr	1.0	0.5	1.0	0.5	4.0	2.0	64.0	32.0	1.0	0.5
C. parapsilosis	0.5	0.5	4.0	4.0	1.0	2.0	1.0	2.0	2.0	1.0

DISCUSSION

During the last decade, the incidences of infections from various Candida species and azole-resistant candidiasis have been increased (1-5, 13). Therfore availability of information rapidly is helpful in directing appropriate treatment for seriously ill patients. MICs can play an important role in the management of candidiasis. Susceptibility testing of yeasts with flow cytometry is a flexible method that can be modified for fungistatic and fungicidal reactions to provide rapid and accurate MIC results for guiding the treatment of seriously ill patients (6, 14).

In this study it was shown that flow cytometry antifungal susceptibility test was useful for susceptibility testing of amphotericinB, clotrimazole, fluconazole, ketoconazole and miconazole with *C. albicans*, *C. dubliniensis*, *C. glabrata*,*C. kefyer* and *C. parapsilosis*.

In this study sodium deoxycholate was used to facilitate the diffusion of PI into the yeast cell membranes damaged by antifungal drugs. PI is one of the most popular fluorescence probes which is used for susceptibility testing (15). The growth controls did not show dye uptake in the presence of deoxycholate. However, the technique as used is only applicable to the antifungal agents that directly or indirectly affect fungal membrane integrity (1, 7). Previously, Green et al. (8) used PI without sodium deoxycholate and obtained results (MICs) after 6 hrs. In the present study, the combination of PI with sodium deoxycholate gave faster results, perhaps because deoxycholate enhanced PI penetration.

In this report, it is shown that susceptibility testing of *candida* species could be accomplished rapidly by using flow cytometry.

In conclusion, FC antifungal susceptibility testing provided rapid, reproducible results that were comparable to those obtained by the NCCLS method. The time required to obtain MICs by FC susceptibility testing varied from 2 to 6 hrs compared to the 24 to 72 h required in the NCCLS broth macrodilution method. The FC procedure is simple and can be useful in research and clinical practice by providing precise MIC cutoff points. One obvious drawback of this approach is the need for specialized equipment, which limits its use in routine laboratories. Further evaluations are necessary to assess the usefulness of FC as a technique for antifungal susceptibility testing.

On the whole, the results of this study in comparison with other investigations (1,2,5,9,16,17,18,19) show that flow cytometry correspond with standardized methods for determination of antifungal susceptibility and can be used as a rapid alternative to traditional methods to study antifungal susceptibility.

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