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Antifungal Susceptibility Testing of *Candida* Species by Flow Cytometry

The feasibility of flow cytometric antifungal susceptibility testing has been studied using the fluorescent anionic membrane potential probe, bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$]. The in vitro antifungal susceptibility testing of amphotericin B was performed on 8 *Candida* isolates from clinical specimens and 2 ATCC strains by flow cytometry with the results compared to those of the National Committee of Clinical Laboratory Standards (NCCLS) M27-T, broth macrodilution method. The flow cytometric method is based on an increase of fluorescence given out by $DiBAC_4(3)$ in fungi when they are killed by antifungal agents. Minimum inhibitory concentration (MIC) of amphotericin B ranged from 0.25 to 1 μg/mL. All results agreed within ± 2 dilution between the flow cytometric method and the M27-T method. MIC with ATCC strains were within recommended ranges of M27-T. The new flow cytometric method revealed a clear and distinct reproducible test end point. A four hr of incubation was sufficient for the test. In conclusion, flow cytometry using $DiBAC_4(3)$ is a rapid and accurate in vitro antifungal susceptibility testing method.

Key Words : *Flow cytometry, Antifungal agents, Fluorescent dyes*

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INTRODUCTION

As a therapeutic modality, including antimicrobial and immune suppressive therapies, rapid advances in fungal infections have increased many fold in recent years (1, 2). Although *Candida albicans* is considered to be the most prevalent species isolated from clinical specimens, the last decade marked a shift in the spectrum of *Candida* infections (3, 4). These *Candida* species except *C. albicans* are usually less vulnerable to antifungal agents (5-8). Therefore, reliable in vitro susceptibility testing can be useful for selecting appropriate and effective antifungal therapeutic agents. The National Committee for Clinical Laboratory Standards (NCCLS) proposed in 1992 a broth macrodilution method for which variable parameters have been defined (9). Unfortunately, this method is time-consuming and tedious to perform. In addition, the critical MIC end point determination relies on subjective visual evaluation of growth inhibition. Moreover, its reliability in identifying amphotericin B-resistant isolates has been questioned (10, 11).

Recently, there have been studies made on cell membrane potential (MP) changes induced by antibiotic activities. Cells as well as micro-organisms may respond to a change in living conditions by showing an increase or decrease in their cell MP. Cell death and/or membrane damage usually results in the collapse of electrical MP (12, 13). It has been reported that cell MP can be measured by flow cytometry after using electrical potential sensitive dyes or probes (14). Bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$], a lipophilic anion, is known to enter eukaryotic cell membranes only when the MP has collapsed (13). Mason et al. (15) have reported that this dye can be used to determine the killing effect of antibiotics on *E. coli*. However, studies on using $DiBAC_4(3)$ in antifungal susceptibility testing have not yet been reported.

Therefore, this study was conducted to evaluate the feasibility of flow cytometric antifungal susceptibility testing by using $DiBAC_4(3)$, a fluorescent anionic MP probe. *Candida* species were used for the study and results were compared to those of MIC by NCCLS M27-T, using the broth macrodilution method (16).

MATERIALS AND METHODS

Test organisms

Eight clinical isolates and 2 reference strains were used

for antifungal susceptibility testing by NCCLS M27-T and flow cytometric methods. The species represented were 2 isolates each of *Candida tropicalis* and *Torulopsis glabrata* and 4 isolates of *Candida albicans*, respectively. The reference strains were *C. albicans* ATCC 90028 and *T. glabrata* ATCC 90030. Test organisms were identified using conventional methods and the Vitek system (bioMerieux-Vitek, St. Louis, MO, USA). All clinical isolates were obtained from blood cultures of patients in the hematooncology ward.

Antifungal agent

Amphotericin B was obtained from Sigma Chemical Company (St. Louis, MO, USA). The stock solution of amphotericin B was prepared in dimethyl sulfoxide at 1,600 μg/mL.

Reference antifungal susceptibility testing

Broth macrodilution susceptibility testing was performed as described in NCCLS M27-T protocol (16) by using RPMI 1640 medium (Sigma Chemical Co.) buffered to pH 7.0 with 0.165 M MOPS (morpholinepropanesulfonic acid). Drug dilutions were prepared to 10 times the strength of the final concentration with the medium as the diluent (0.06-32 μg/mL). The yeasts were grown on Sabouraud dextrose agar for 24 hr at 35℃. Stock inoculum suspensions were made in sterile 0.85% saline and spectrophotometrically adjusted the transmittance to that produced by a 0.5 McFarland standard at 530 nm. A working suspension is made by a 1:100 dilution followed by a 1:20 dilution of the stock suspension with RPMI 1640 broth, which results in 0.5×10^3 to 2.5×10^3 cells per ml. Except for the growth control, the final inoculum was added in 0.9 mL aliquots to tubes containing 0.1 mL of drug dilutions. Incubation was done at 35℃ for 48 hr. The MIC was defined according to NCCLS criteria (16).

Flow cytometric assay

The assay medium was RPMI 1640 buffered to pH 7.0 with 0.165 M MOPS buffer. Drug dilutions were prepared to 10 times the strength of the final concentration with the medium as the diluent (0.06-32 μg/mL). The yeasts were grown on Sabouraud dextrose agar for 24 hr at 35℃. Stock inoculum suspensions were made in sterile 0.85% saline and spectrophotometrically adjusted at 530 nm to a final transmission of 70 to 75%. The adjusted yeast suspensions were diluted 1:10 with the medium achieving a final concentration of 5×10^5 to 8×10^5 CFU/mL. Except for the growth control, the final inoculum was added in 0.9 mL aliquots to tubes containing 0.1 mL of drug dilutions. Incubation was performed at room temperature for 4 hr. $DiBAC_4$ (3) (Molecular Probes, Inc, Eugene, OR, USA) was then added to all tubes for a final concentration of 100 μg/mL. Flow cytometric analysis was initiated 10 min after adding the dye to the first tube. Flow cytometry was performed on a FACScan (Becton Dickinson Immunocytometry Systems, Mountain View, CA, U.S.A.) with Lysis II software for data acquisition and analysis. Instrumental parameters were forward light scatter (FSC, log), sideward light scatter (SSC, log, 488/10 filter), and fluorescence (FL1, log, 554 PMT voltage, 530/30 filter). Ten thousand cells per sample were analyzed. The measured mean channel fluorescence (MCF) was converted to the Arithmetic/linear mean of fluorescence using Becton Dickinson software. The MIC was defined as a concentration which produced a 50% increase in fluorescence mean compared to the fluorescence mean of the drug free control (MCF of test/MCF of control>1.5).

RESULTS

Cell membrane depolarization in amphotericin B-treated cells was shown by increased uptake of $DiBAC_4(3)$, resulting in a dose-dependent increase in cellular fluorescence intensity. A dose-response curve was modeled for each strain (Fig. 1). Amphotericin B MIC by flow cytometry ranged from 0.25 to 1 μg/mL. MICs obtained by flow cytometry were compared with the NCCLS broth macrodilution analysis for all 8 strains of *Candida* species (Table 1). The overall agreement ±1 and 2 dilution were 88% and 100%, respectively. The ATCC strains showed MICs within the recommended ranges. Fig. 2 and 3 show representative flow cytometric histograms for ATCC strains that were exposed to amphotericin B.

Table 1. MIC of amphotericin B for 10 *Candida* strains determined by flow cytometry and NCCLS procedure

	MIC (μg/mL)									
Method	ATCC 90028	ATCC 90030	A*1	A2	A3	A4	T□1	T2	G□1	G2
Flow cytometry	0.5	0.5	0.5	0.25	0.5	0.25	1	0.5	0.5	0.25
M27 procedure	0.5	0.5	0.5	1	0.5	0.5	1	0.5	1	0.5

*A, *C. albicans*; □T, *C. tropicalis*; □G, *T. glabrata*

DISCUSSION

The traditional antifungal susceptibility testing was influenced by a variety of factors such as inoculum concentration, preparation, pH and composition of media, incubation temperature, and the physical and chemical properties of antifungal agents (17-19). Also, these methods involve semiquantitative and subjective assessments of yeast growth quantities and therefore, required incubation of 48 hr or longer to produce accurate results. Recently, flow cytometry

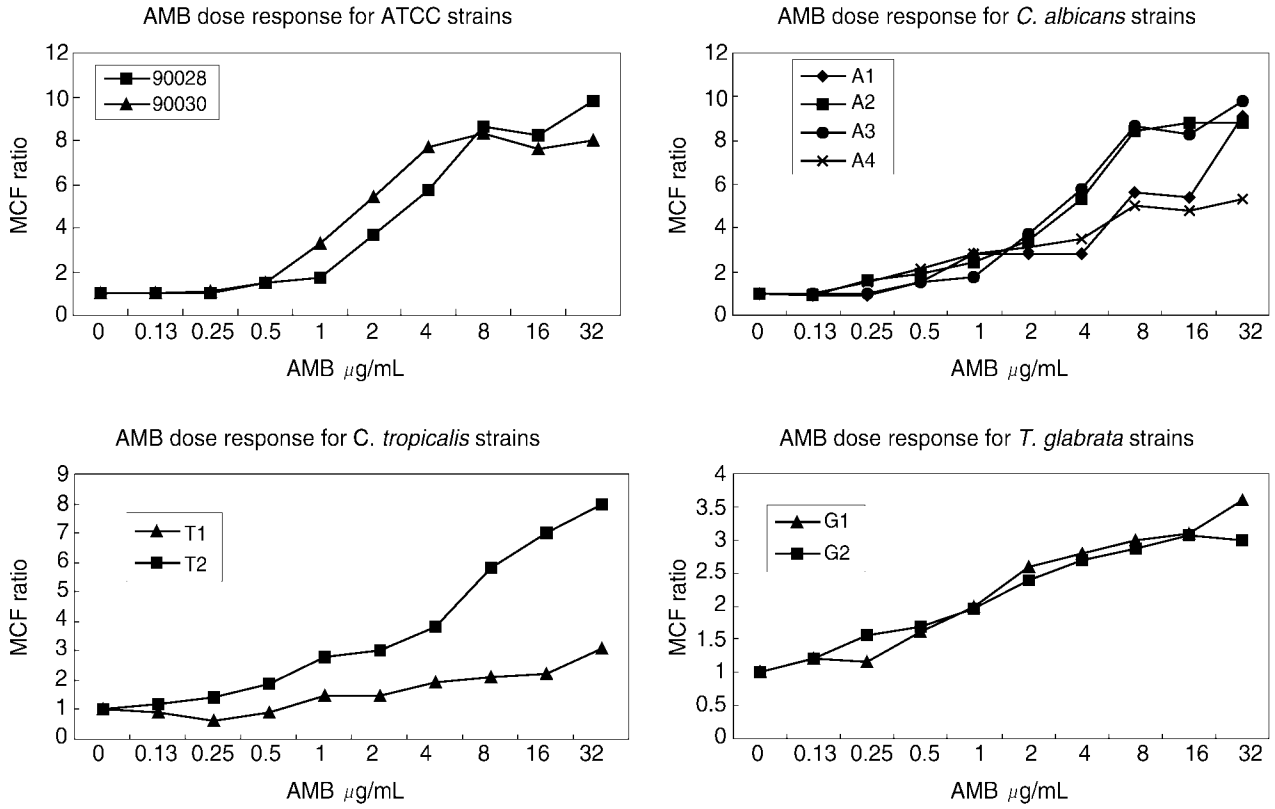

Fig. 1. Dose response to amphotericin B for *Candida* species. AMB: amphotericin B.

has increasingly been used to perform susceptibility tests for bacteria and yeasts (20-26). Flow cytometric antifungal susceptibility does not rely on the growth of yeast cells, but instead measures antibiotic-induced membrane damage. Therefore this method offers rapid and reproducible results. The lipophilic fluorescent dye, $DiBAC_4(3)$, has a high-voltage sensitivity and enters depolarized cells, where it binds to lipid-rich intracellular components (27). Therefore, cells that possess a membrane potential will exclude oxonol and show weak fluorescence due to the bound dye. Once the membrane potential is lost, the dye enters the cell and fluorescence increases (28, 29). Exposure to dye for 10 min at room temperature was enough for yeast to reach maximum fluorescence intensity. The $DiBAC_4(3)$ fluorescence distribution of a representative experiment with the two ATCC *Candida* strains used in this study is shown in Fig. 2 and 3. A fluorescence intensity increment was clearly visible. It was found that length of incubation, needed to acquire NCCLS comparable results, was 4 hr for amphotericin B. Results of this study indicated that MICs obtained by flow cytometry were equal to or within two dilutions of those obtained by the NCCLS broth macrodilution test and the MICs obtained by flow cytometry were lower than those by the NCCLS method. Those results were similar to the results of the studies of Kirk et al. (24) and Wenisch et al. (23) that were equal to or within two dilution of those obtained by NCCLS test. In their studies, the MICs obtained by flow cytometry were lower than those by the NCCLS method. However Ramani et al. (26) reported that the MICs obtained by flow cytometry were higher than those by the NCCLS method. The criteria that determine cutoff points for the MIC were readily established by MCF. Flow cytometric susceptibility testing has been reported previously (20-26). In those studies, many methods for determining the cutoff of MIC were introduced, i.e. "negative gating" based on a combination of ethidium bromide fluorescence, and forward light scatter to assess antibiotic effect (20) and sensitivity index (22). In this study, membrane integrity, the target of antifungal agents, was measured. Damaged yeast cells collapsed membrane potential and thus produced a higher MCF. The measured MCF was determined by the logarithmic scale, and each decade was expanded on the low end and compressed on the high end. Therefore, to prevent misrepresentation, MCF was converted to the linear mean of fluorescence with the Becton Dickinson software. The MIC was defined as a concentration which produced a 50% increase in fluorescence mean compared to the fluorescence mean of the drug-free control (MCF of test/MCF of control>1.5). At the concentrations less than this cutoff point, yeast cells showed a fluorescence mean 10% less compared

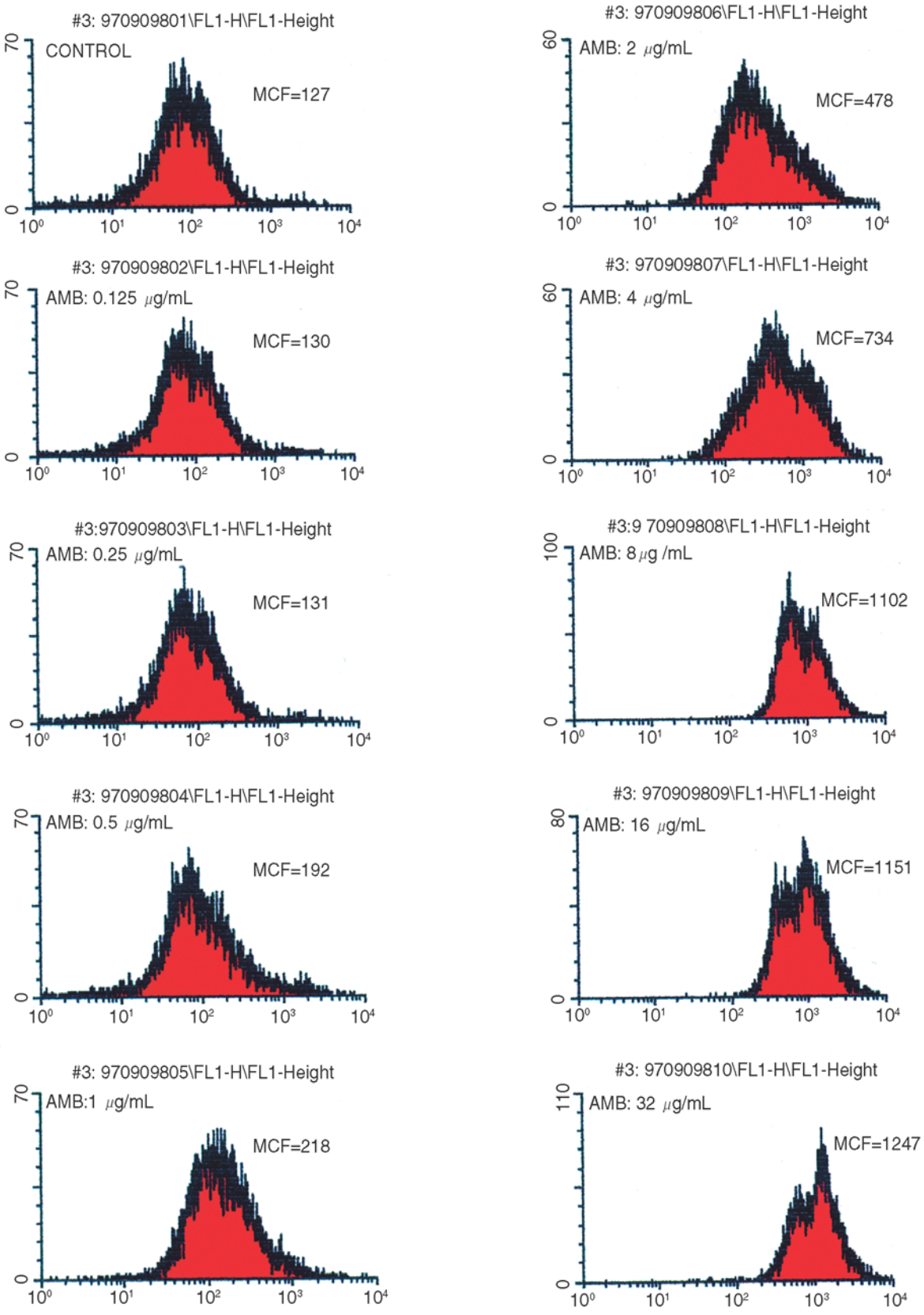

Fig. 2. Histogram profiles of mean channel fluorescence (MCF) values for *C. albicans* ATCC 90028. AMB: concentration of amphotericin B.

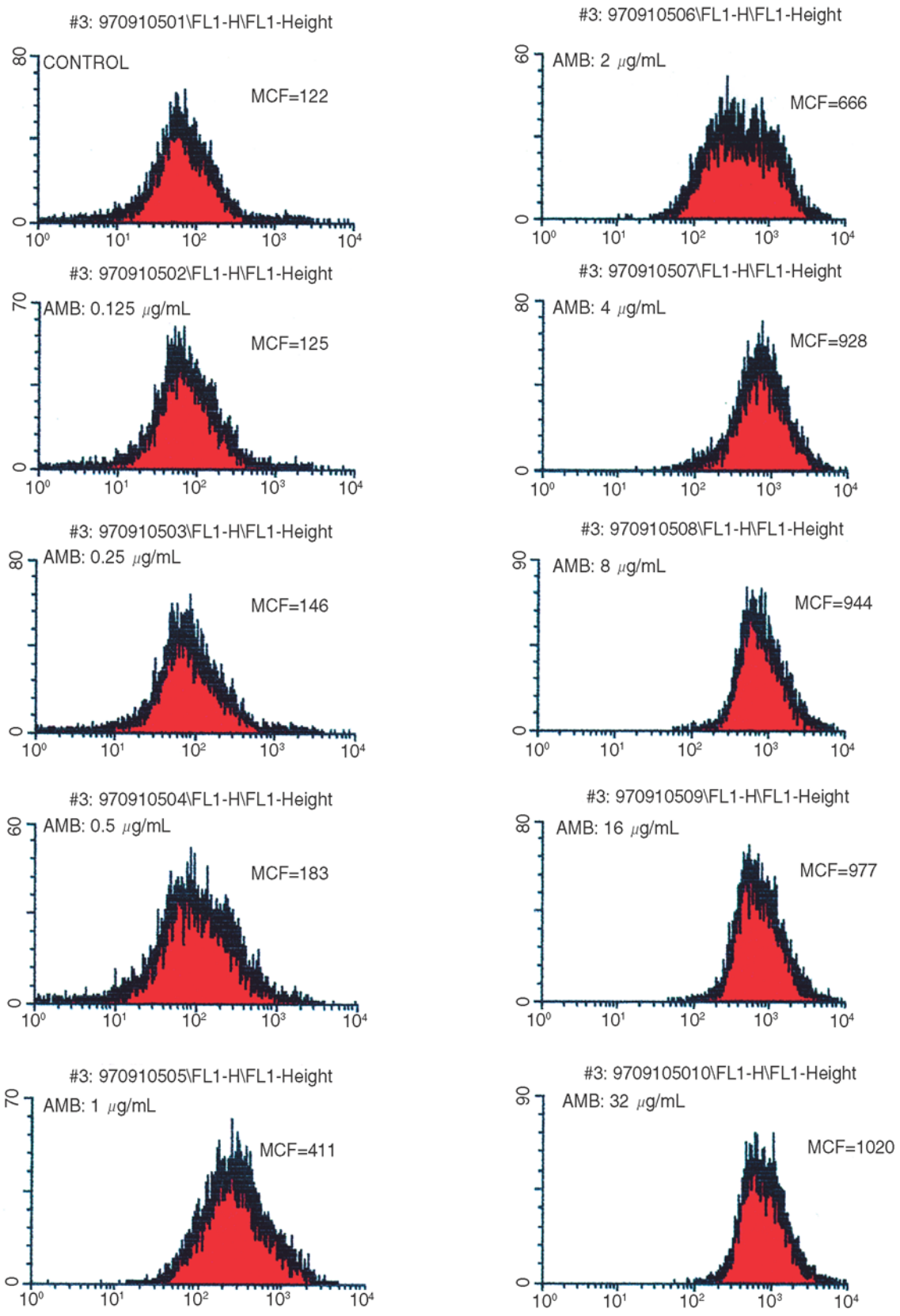

Fig. 3. Histogram profiles of mean channel fluorescence (MCF) values for *T. glabrata* ATCC 90030. AMB: concentration of amphotericin B.

with that of the drug free control. This calculating method is simple and clear. MICs obtained by this study were reproducible from day to day and comparable to the results obtained by NCCLS macrodilution method. However, flow cytometric antifungal susceptibility testing require instrument and cost is also stated to be a disadvantage. But the speed and quality of information obtained by flow cytometry improve quality of health care.

In conclusion, this study shows that flow cytometric antifungal susceptibility testing can be used to provide rapid and reproducible results, and may be a valuable alternative to traditional antifungal susceptibility testing methods. The assay is simple and useful for clinical applications.

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