Evaluation of gradient concentration strips for *in vitro* combination testing of antifungal combinations against *Candida* spp.

Maria Siopi, Antigoni Elefanti, Nikolaos Siafakas, Loukia Zerva and Joseph Meletiadis

NATIONAL & KAPODISTRIAN UNIVERSITY OF ATHENS

UNIVERSITY GENERAL HOSPITAL ATTIKON

Clinical Microbiology Laboratory, Attikon University General Hospital, Medical School, National and Kapodistrian University of Athens, Athens, Greece

Correspondence: Joseph Meletiadis, 1 Rimini str, Haidari 124 62, Athens Greece, Tel: +30-210-583-1909, Email: jmeletiadis@med.uoa.gr

INTRODUCTION

Antifungal combination therapy may be used in an attempt to improve treatment outcomes for fungal infections. Nevertheless, clinical isolates are seldom tested *in vitro* beforehand for drug interactions in the routine laboratory practice. Methods for determining synergistic activity are largely unstandardized for antifungal agents, reference guidelines are not available and considerable debate on the value of these tests in the clinical setting remains.

The most widely accepted technique of assessing antifungal combinations is the checkerboard dilution; however it is difficult to implement routinely in clinical microbiology laboratories since its design complexity is poorly suited to use on a case-per-case basis. As commercially available systems to provide simple alternative methodologies offering relative ease of use and accurate results are of great interest, the objective of this study was to evaluate the performance of gradient concentration strips versus standard checkerboard method for *in vitro* testing of antifungal combinations against *Candida* spp.

MATERIALS AND METHODS

Test organisms. A total of 6 clinical isolates of *Candida* spp. were studied, including 1 *C. albicans*, 1 *C. glabrata*, 1 *C. kefyr*, 1 *C. tropicalis* and 2 reference strains (*C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019) as quality controls in order to monitor the testing conditions. All isolates were stored in normal saline with 10% glycerol at -70°C until the study was performed. Prior to testing each isolate was revived by subculturing it twice onto Sabouraud dextrose agar (SDA) with chloramphenicol plates at 30°C for 24 hours.

Antifungal agents. Amphotericin B (AMB) and voriconazole (VRC) were dissolved in dimethyl sulfoxide, whereas caspofungin (CAS) in sterile distilled water and stock solutions were prepared based on EUCAST recommendations.

Inoculum preparation. For the two-drug microdilution checkerboard technique yeast suspensions were prepared following the EUCAST EDef 7.2 guidelines in order to obtain double the final concentration of $0.5\text{-}2.5 \times 10^5$ CFU/mL in RPMI 1640 medium supplemented with 2% glucose and buffered to pH 7.0 with 0.165M MOPS. For the agar diffusion method inoculum suspensions equivalent to a 0.5 McFarland standard were prepared in normal saline according to the manufacturer's instructions for Liofilchem® MIC Test Strips (MTS). CFU counts were affirmed each time by spread plate counts on SDA plates.

***In vitro* combination testing. i) Checkerboard method.** For the assessment of drug interactions using a two-dimensional broth microdilution checkerboard (CHECK) technique, the minimal inhibitory concentrations (MICs) of the individual agents were determined in an exploratory study for each strain as outlined in the EUCAST EDef 7.2 document in order to choose the appropriate range of concentrations to be tested. Twofold serial dilutions of AMB, CAS and VRC were prepared in the assay medium so as to yield the 4x desired concentrations, which finally ranged from 0.06 to 4, 0.004 to 4 and 0.002 to 2 mg/L., respectively. A 50 µL aliquot/well of each drug solution of the appropriate concentration was dispensed into sterile flat-bottom 96-well trays with the purpose of obtaining two different CHECK designs: AMB plus VRC and VRC plus CAS. Each well was inoculated with 100 µL of the 2x corresponding yeast suspension, while drug- and inoculum-free (blank) controls were included. After agitation for 15 s, the plates were incubated at 35°C for 48 hours. Readings were performed spectrophotometrically at 530 nm after 24 and 48 hours of incubation with the aid of a microplate reader. The percentage of growth was calculated based on the optical density (OD) of each well with the equation: $100 \times (OD_{well} - OD_{blank}) / (OD_{drug\text{-}free\ well} - OD_{blank})$. The MIC of AMB was defined as the lowest concentration that inhibited growth by ≥90% compared with that of untreated control (MIC-0), while the MICs of CAS and VRC as the lowest drug concentration giving rise to an inhibition of growth of ≥50% (MIC-2).

ii) Gradient strip diffusion method. MTS agar diffusion testing was performed as recommended by the manufacturer, using strips with AMB, CAS and VRC concentrations ranging from 0.002 to 32 mg/L and solidified RPMI (2% glucose, buffered

with MOPS) agar plates as the test medium. Plates were inoculated by pouring a 1:5 dilution of the standardized yeast suspension onto the agar. After allowing 1-2 min for the suspension to achieve a uniform distribution, excess moisture was absorbed into the agar, the surface was left to dry completely (15-20 min at room temperature) and the MTS were applied to the center of each inoculated plate. Prior to synergy set-up MICs of the individual agents were defined in an preliminary study for each strain. Synergy testing was performed by placing the strips onto the agar surface in a cross formation, with the strips intersecting in a 90⁰ angle at the MICs of each drug (**Figure 1**). The plates were incubated at 35°C and endpoint readings were performed after 24 and 48 hours of incubation. AMB MICs were determined as the drug concentration at which the border of the elliptical zone of 100% inhibition intersected the strip, while CAS and VRC MICs were recorded as the lowest concentration at which the border of the elliptical zone of 80% inhibition intersected the strip, ignoring trailing growth or microcolonies throughout a discernible ellipse.

Isolates were tested in parallel by both methodologies. All experiments were carried out in duplicate and were independently performed on two different days with individually prepared inocula.

FIC index analysis. Drug interaction for each *in vitro* combination was determined by the fractional inhibitory concentration (FIC) index expressed as follows: FIC index = $FIC_A + FIC_B = (MIC_{AB} / MIC_A) + (MIC_{BA} / MIC_B)$, where MIC_A and MIC_B: MICs of drugs tested alone, MIC_{AB}: MIC of drug A in the presence of B and vice versa for MIC_{BA}. Regarding its interpretation synergy, additivity and antagonism was defined when the values were ≤0.5, >0.5 - <4.0 and ≥4, respectively. For checkerboard data both MIC-0 and MIC-2 were used to calculated the FIC-0 and FIC-2, respectively.

Data analysis. Checkerboard FIC-0 and FIC-2 indices were correlated with MTS FIC indices after 24h and 48h with Pearson correlation analysis after log10 transformation.
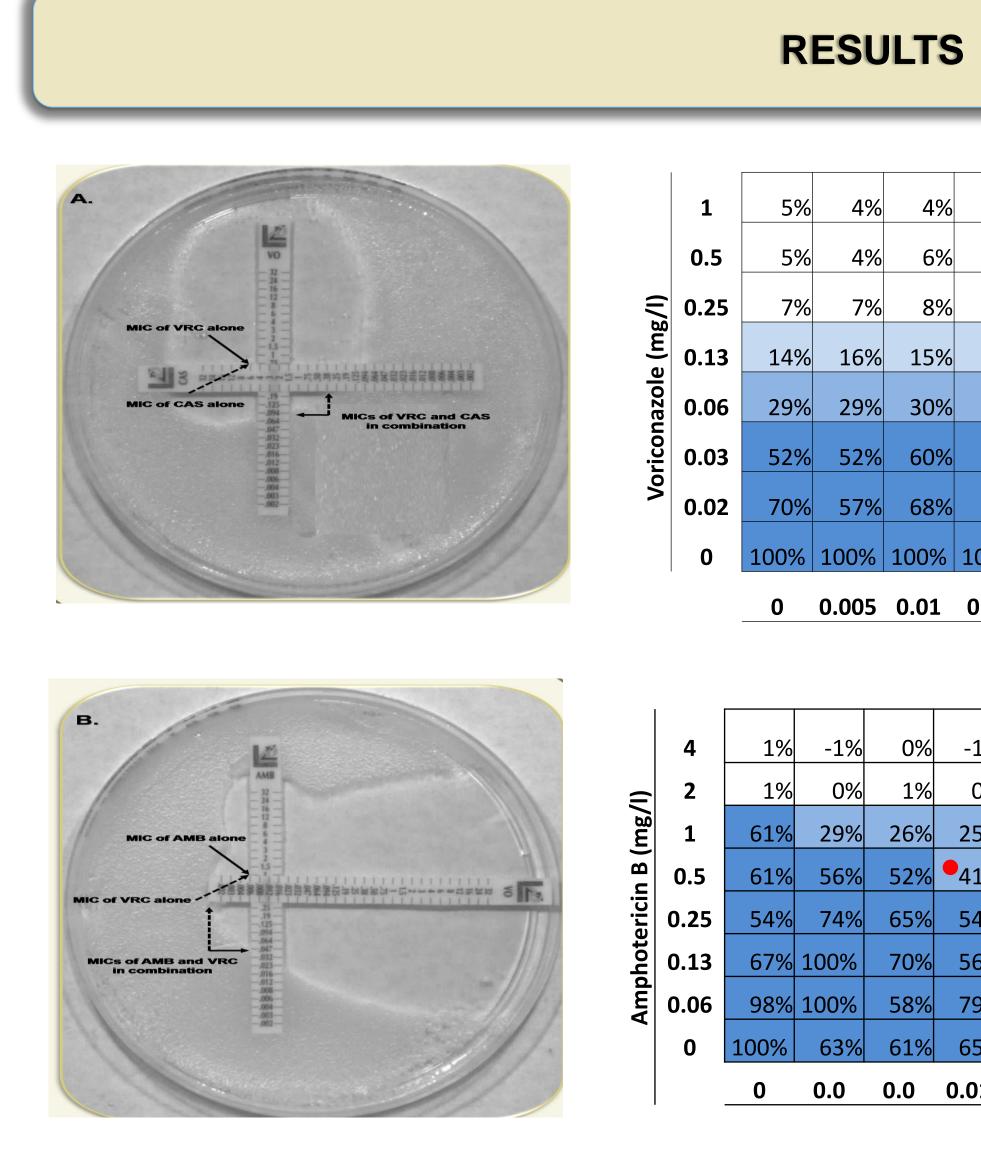
RESULTS

Figure 1. Representative checkerboard data and MIC test strips of voriconazole in combination with caspofungin against *C. glabrata* (A) or amphotericin B against *C. kefyr* (B) after 48h. Red dots correspond to the FIC-2.

RESULTS

➤ The range and the median value of FIC indices obtained from antifungal combinations with the CHECK and MTS methods are presented in **Table 1**. Representative checkerboard and MTS data are shown in **Figure 1**.

➤ Synergy was detected with the CHECK and MTS technique in 1 of 12 (8%) and 2 of 12 (17%) isolates, respectively. On the other hand, antagonism was not detected for any of the combinations tested with the microdilution method, but it was noted in 1 of 12 (8%) with MTS.

➤ Pearson correlation analysis showed a statistically significant correlation between checkerboard FIC-2 indices and MTS FIC indices after 48h of incubation (**Figure 2**).

Table 1. FCI indices of voriconazole (VRC) in combination with amphotericin B (AMB) and caspofungin (CAS) for 6 *Candida* spp. as determined by two different methods and with after 24h and 48h of incubation.

Species	Combinations	Incubation	CHECK FICs		MTS FICs
			FIC-0	FIC-2	
C. albicans	AMB + VRC	24h	2.00 (1.50-2.50)	1.25 (1.00-1.50)	4.34 (3.67-5.00)
		48h	1.50 (1.50-1.50)	2.00 (1.50-2.50)	4.34 (3.67-5.00)
	CAS + VRC	24h	2.50 (2.50-2.50)	2.50 (2.50)	1.25 (1.25-1.25)
		48h	2.00 (1.50-2.50)	2.00 (1.50-2.50)	1.25 (1.25-1.25)
C. glabrata	AMB + VRC	24h	1.03 (0.56-1.50)	3.28 (2.50-4.06)	1.63 (1.50-1.76)
		48h	1.25 (1.00-1.50)	0.88 (0.75-1.00)	1.88 (1.76-2.00)
	CAS + VRC	24h	0.62 (0.50-0.75)	0.69 (0.62-0.75)	0.58 (0.52-0.63)
		48h	0.62 (0.50-0.75)	0.88 (0.75-1.00)	0.80 (0.59-1.01)
C. kefyr	AMB + VRC	24h	1.25 (1.00-1.50)	0.69 (0.62-0.75)	0.42 (0.39-0.46)
		48h	2.80 (1.50-4.10)	0.88 (0.75-1.00)	0.50 (0.39-0.62)
	CAS + VRC	24h	0.50 (0.50-0.51)	1.78 (1.50-2.06)	1.33 (1.33-1.33)
		48h	0.75 (0.50-1.00)	1.78 (1.50-2.06)	1.33 (1.33-1.33)
C. krusei	AMB + VRC	24h	2.00 (1.50-2.50)	2.50 (2.50-2.50)	1.83 (1.33-2.33)
		48h	1.25 (1.00-1.50)	3.38 (2.25-4.50)	1.83 (1.33-2.33)
	CAS + VRC	24h	1.28 (0.50-2.06)	1.50 (1.50-1.50)	1.25 (1.17-1.33)
		48h	1.00 (1.00-1.00)	1.50 (1.50-1.50)	1.59 (1.33-1.85)
C. parapsilosis	AMB + VRC	24h	2.50 (2.50-2.50)	0.66 (0.56-0.75)	0.96 (0.83-1.08)
		48h	2.18 (2.12-2.25)	1.03 (0.56-1.50)	1.20 (1.08-1.33)
	CAS + VRC	24h	2.88 (1.50-4.25)	0.88 (0.75-1.00)	1.19 (1.19-1.19)
		48h	2.15 (2.06 -2.25)	1.25 (1.00-1.50)	1.41 (1.19-1.63)
C. tropicalis	AMB + VRC	24h	2.28 (2.06-2.50)	3.05 (1.60-4.50)	2.26 (2.02-2.50)
		48h	1.50 (1.50-1.50)	3.75 (2.50-5.00)	2.26 (2.02-2.50)
	CAS + VRC	24h	1.02 (0.53-1.5)	0.99(0.38-1.50)	0.17 (0.11-0.23)
		48h	1.00 (0.50-1.50)	0.41 (0.31-0.51)	0.28 (0.19-0.36)
All	AMB + VRC	24h	2(1.03-2.5)	1.87(0.66-3.28)	1.73(0.42-4.34)
		48h	1.5(1.25-2.8)	1.51(0.88-3.75)	1.85(0.5-4.34)
	CAS + VRC	24h	1.15(0.5-2.88)	1.64(0.69-2.5)	1.22(0.17-1.33)
		48h	1(0.62-2.15)	1.37(0.41-2)	1.29(0.28-1.59)

CONCLUSIONS
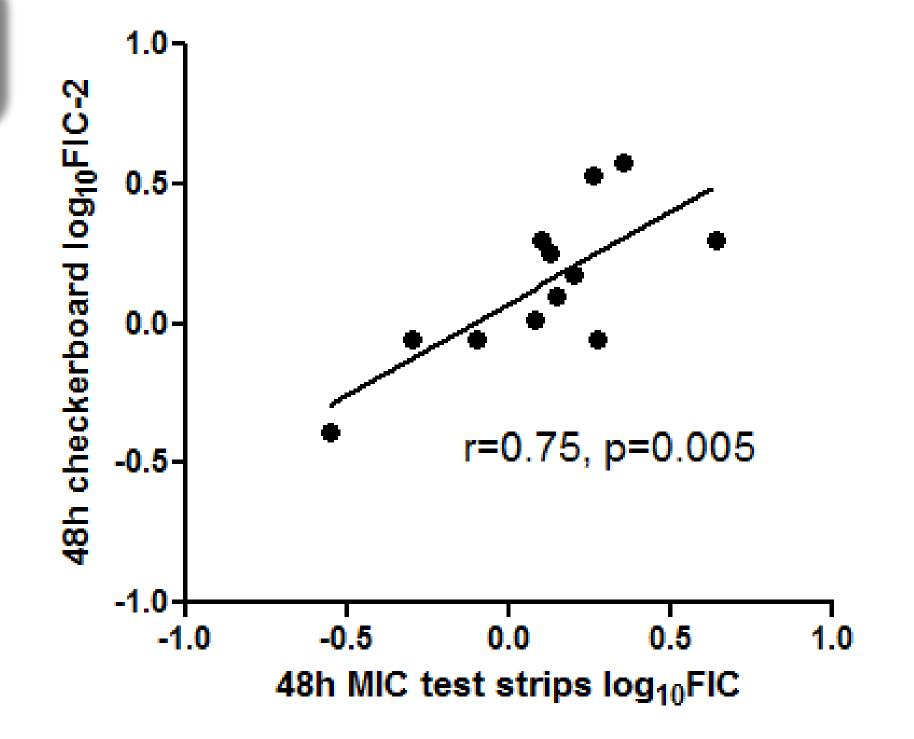
✓ A significant correlation of FIC indices was found between checkerboard and MIC test strips methods.

✓ The gradient concentration strip method was less laborious and time consuming than microdilution checkerboard technique and resulted in broader FIC ranges and more significant interactions.

✓ Optimization studies using a larger collection of isolates with synergistic and antagonistic interactions are required in order to improve the concordance of the methodologies tested.

✓ Validation of readily available, easy to use and reliable tests for drug interactions is of great interest as they might be helpful in the choice of combination therapy, especially given the greater availability of antifungal drugs with different mode of action and the emergence of resistance strains.

r=0.75, p=0.005

Figure 1. Pearson correlation analysis between between checkerboard FIC-2 indices and MTS FIC indices after 48h of incubation .