

Brief report

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Fluconazole-containing agar Sabouraud dextrose plates are not useful when screening for susceptibility in *Candida albicans*

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ABSTRACT

Introduction. Fluconazole is an alternative for candidemic patients who are not critically ill. Fluconazole is mainly fungistatic and does not completely inhibit visual *Candida albicans* growth. We studied the usefulness of fluconazole-containing Sabouraud dextrose agar plates for detecting susceptibility to fluconazole in *C. albicans*.

Material and methods. Adjusted inocula of 19 isolates were transferred directly onto fluconazole-containing Sabouraud dextrose plates (concentrations ranging from 0.125 mg/L to 128 mg/L). The fluconazole MIC in fresh isolates and after growth on the fluconazole-containing plate at 128 mg/L was recorded following the EUCAST EDef 7.2 guidelines. Then isolates were classified according to their degree of trailing production, based on microdilution procedure.

Results. All isolates were able to grow on all fluconazole-containing plates, even those isolates susceptible to fluconazole. In fact, we selected isolates with different degrees of trailing based on microdilution procedures. 50% of isolates classified as heavy trailers, 35.71% as moderate trailers, and 14.28% as slight trailers.

Conclusions. The use of fluconazole-containing Sabouraud dextrose agar plates was not a reliable method to detect fluconazole susceptibility in *C. albicans* isolates; growth of the isolates was a trailing effect rather than true resistance.

Key words: Trailing, fluconazole, *Candida albicans*, EUCAST, agar plates

Las placas de agar dextrosado de Sabouraud con fluconazol no son útiles para la detección de resistencia en *Candida albicans*

RESUMEN

Introducción. Fluconazol es el antifúngico de elección en pacientes con candidemia que no están en estado crítico. Fluconazol es un fármaco fungistático y no inhibe por completo el crecimiento de *Candida albicans*. En este estudio se evalúa la posibilidad de emplear placas de agar dextrosado de Sabouraud con diferentes concentraciones de fluconazol como método para evaluar la sensibilidad a este fármaco de aislados de *C. albicans*.

Material y métodos. El inóculo ajustado procedente de 19 aislados de *C. albicans* se transfirió directamente a placas que contenían concentraciones de fluconazol comprendidas entre 0,125 mg/L y 128 mg/L. La CMI de fluconazol se calculó en los aislados originales y en los aislados crecidos en la placa de fluconazol de 128 mg/L, según el protocolo de EUCAST EDef 7.2. Los aislados se clasificaron según su grado de producción de efecto de arrastre, siguiendo el procedimiento de microdilución.

Resultados. Todos los aislados fueron capaces de crecer en todas las placas con diferentes concentraciones de fluconazol, incluso las cepas sensibles. Los aislados mostraron diferentes grados de efecto de arrastre. El 50% de los aislados se clasificaron como altamente productores, el 35,71% como moderadamente productores y el 14,28% como poco productores de efecto de arrastre.

Conclusión. El uso de placas de agar dextrosado de Sabouraud con fluconazol no demostró ser un método útil para el estudio de la sensibilidad de *C. albicans* a este fármaco ya que el crecimiento de los aislados fue interpretado como efecto de arrastre y no como una verdadera resistencia.

Palabras clave: Fenómeno de arrastre, fluconazol, *Candida albicans*, EUCAST, placas de agar.

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INTRODUCTION

Candida albicans is the main cause of fungemia in most population-based and nationwide studies on candidemia¹. Although current guidelines recommend echinocandins as the first-line agents for the treatment of fungemia, fluconazole is an alternative for patients who are not critically ill or have not previously received azoles^{2,3}. Although resistance to fluconazole is infrequent in *C. albicans* isolates causing fungemia⁴, its rapid detection may help to optimize antifungal therapy⁵. Fluconazole has been also widely used for the treatment of superficial infections caused by *C. albicans*⁶.

Fluconazole is mainly fungistatic and does not completely inhibit visual *C. albicans* growth in microdilution susceptibility tests. Trailing is a well-known phenomenon that is defined as reduced but persistent growth of *Candida*-susceptible isolates at fluconazole concentrations above the MIC. This phenomenon is commonly observed in broth dilution procedures, particularly with *C. albicans* and *C. tropicalis*^{7,8}. In theory, resistance to fluconazole by *C. albicans* could be potentially ruled out by the lack of growth of susceptible isolates on fluconazole-containing plates, as previously reported with *A. fumigatus* and azoles⁹. However, trailing may not preclude susceptible *C. albicans* isolates from growing on the fluconazole-containing plates, even at high concentrations, although no solid data have been reported.

The aim of the present study was to analyze whether fluconazole-containing Sabouraud dextrose agar plates can be used to determine susceptibility to fluconazole. We assessed whether trailing interfered with fungal growth of fluconazole-susceptible *C. albicans* using a set of isolates showing different degrees of trailing according to our recently proposed cut-off¹⁰.

MATERIALS AND METHODS

We studied 13 *C. albicans* isolates recovered from blood cultures of patients with candidemia admitted to Ramón y Cajal Hospital, Madrid, Spain from October 2012 to September 2013. Six fluconazole-resistant isolates previously characterized and ATCC90028 isolate, which is known to produce trailing, were included as a control¹¹.

The isolates were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS, Bruker Daltonics, Germany). All isolates were tested for antifungal susceptibility to fluconazole (Pfizer Pharmaceutical Group, New York, USA) according to the EUCAST EDef 7.2 microdilution procedure¹². The isolates were grown on CHROMAgar plates (Tec-Laim S.A., Madrid, Spain) and incubated for 24 hours at 35°C to check for purity. A loopful of yeast was suspended in 10 mL of yeast peptone dextrose broth medium (YPD, Becton Dickinson, Madrid, Spain) and incubated overnight at 30°C. Suspensions were adjusted to 3-9 x10⁸ CFU/mL (mean of 6.09 ± 1.51 x10⁸) and 100 µL of the inocula was streaked on homemade Sabouraud-dextrose agar plates (DIFCO,

Madrid, Spain) containing two-fold fluconazole concentrations ranging from 0.125 mg/L to 128 mg/L. Plates were incubated at 35°C and visually inspected for growth after 24 hours.

We studied the EUCAST MICs of fluconazole before (MIC_{initial}) and after growth on the plates containing the highest fluconazole concentration (128 mg/L) (MIC_{final}). We calculated the geometric mean and range of MIC_{initial} and MIC_{final}. Trailing was defined as the presence of residual yeast growth in wells containing fluconazole concentrations above the MIC. For each isolate, we calculated the percentage of trailing detected in each well, starting with the first well containing a two-fold concentration above the MIC and finishing with the well containing 64 mg/L of fluconazole. According to our proposed cut-off, isolates were classified according to mean trailing as slight trailers (6-10% residual growth), moderate trailers (11-15% residual growth), and heavy trailers (>15% residual growth)¹⁰.

RESULTS AND DISCUSSION

All isolates were able to grow on the Sabouraud-dextrose fluconazole-containing agar plates. Fluconazole-susceptible isolates were able to grow on fluconazole-containing agar plates, regardless of the concentration (table 1). Moreover, fungal growth on the plates was not attenuated even at concentrations above the breakpoint (>4 mg/L), and both single colonies and surface film were observed. The MIC_{final} and MIC_{initial} did not differ significantly in both, fluconazole-resistant or fluconazole-susceptible isolates (table 1).

Fluconazole is a fungistatic agent that is administered to treat infections caused by *Candida* spp., and isolates commonly show a trailing effect when EUCAST or CLSI microdilution procedures are used^{7,8,10}. In the present study, we also showed that trailing is observed when the isolates are cultured on agar plates containing different fluconazole concentrations. The exposure of the isolates to several different concentrations of fluconazole did not have an impact on the MIC of this drug, proving that growth of the isolates was a trailing effect and not phenotypic resistance. In fact, we selected isolates with different degrees of trailing based on microdilution procedures (mean percentage of trailing was 14.15%) with 50% of isolates classified as heavy trailers, 35.71% as moderate trailers, and 14.28% as slight trailers (table 1). However, the scope of trailing did not affect the growth of the isolates on fluconazole-containing plates.

Although fluconazole resistance is not frequent, it should not be disregarded, to ensure that prompt and appropriate antifungal treatment can be administered⁵. Fluconazole susceptibility testing may take days when performed on pure cultured isolates. Alternatively, methods using solid agar plates performed directly on positive blood culture bottles, such as MIC gradient tests (e.g. Etest), have been able to speed up susceptibility testing¹³. If fluconazole-containing agar plates had proven useful for assessing susceptibility to fluconazole in pure cultured isolates, the results could have been extrapolated to blood culture bottles. Unfortunately, this was not the case, and

Isolates	MIC _{initial}	MIC _{final}	Percentage of fluconazole trailing (GM±SD) ^a	Trailing effect
CA ₁	0.125	0.125	8.37±0.77	Slight
CA ₂	0.125	0.125	8.85±1.25	Slight
CA ₃	0.125	0.125	11.20±0.86	Moderate
CA ₄	0.125	0.125	11.63±0.53	Moderate
CA ₅	0.125	0.125	11.72±0.40	Moderate
CA ₆	0.125	0.125	13.31±0.45	Moderate
CA ₇	0.25	0.125	14.86±1.79	Moderate
CA ₈	0.125	0.125	15.45±0.66	Heavy
CA ₉	0.125	0.125	15.76±0.54	Heavy
CA ₁₀	0.125	0.125	16.03±0.86	Heavy
CA ₁₁	0.125	0.125	16.65±1.19	Heavy
CA ₁₂	0.125	0.125	18.05±1.58	Heavy
CA ₁₃	0.125	1	24.91±1.65	Heavy
ATCC 90028	0.25	0.125	19.71±2.78	Heavy
CA ₁₄	>64	>64	NA	
CA ₁₅	8	8	NA	
CA ₁₆	8	8	NA	
CA ₁₇	>64	16	NA	NA
CA ₁₈	8	8	NA	
CA ₁₉	>64	>64	NA	
GM (range) ^b	32 (0.125–128)	14.49 (0.125–128)	14.15±0.93	

^aPercentage of trailing calculated as the geometric mean of residual growth compared with the drug-free well at each fluconazole concentration.

^bGM, Geometric mean of the MIC_{initials}, MIC_{finals} and percentage of trailing obtained for each isolate.

NA: No applicable. For fluconazole resistant isolates we did not calculate the percentage of trailing because the growth was >50%.

fluconazole-containing agar plates are not a suitable alternative to the Etest applied directly on solid medium.

Previous studies showed that either fluconazole-containing chromogenic agar media or disk diffusion with fluconazole onto chromogenic media plates proved useful to detect fluconazole resistance in *Candida*^{14–16}. We here evaluated whether Sabouraud was also useful considering the low price and availability of this medium but according to our results, the use of this medium for this purpose cannot be recommended. The discrepancy among our results and those previously reported may be explained by the lower inocula previously used (10³–10⁴ CFU/mL vs 10⁸ CFU/mL)^{14,15}. The reason to use such a high inoculum was to search for the presence of potential poorly represented mutants in the suspension. The fact that the MIC_{final} was similar to MIC_{initial} allowed us to exclude this possibility. We conclude that growth of *C. albicans* on Sabouraud dextrose agar plates containing various fluconazole concentrations reflected a trailing phenomenon rather than phenotypic resistance. Therefore, we do not

recommend this method for the screening of fluconazole-susceptible *C. albicans* isolates in the clinical microbiology laboratory.

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All other authors: no conflicts to declare.

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