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The Etest Performed Directly on Blood Culture Bottles Is a Reliable Tool for Detection of Fluconazole-Resistant *Candida albicans* Isolates

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ABSTRACT We assessed the ability of the Etest performed directly on positive blood cultures (ET_{DIR}) to detect fluconazole susceptibility in 6 fluconazole-resistant and 12 fluconazole-susceptible *Candida albicans* isolates, according to CLSI M27-A3 and EUCAST EDef 7.2 procedures. Categorical agreement between ET_{DIR} and broth microdilution was 100% when the trays were incubated at 25°C and trailing effect was ruled out. ET_{DIR} is a reliable procedure when screening for the presence of fluconazole resistance in *C. albicans*.

KEYWORDS *Candida albicans*, fluconazole, resistance, Etest

Fluconazole and echinocandins are the backbone of antifungal treatment for candidemia and invasive candidiasis (1, 2). Different rates of fluconazole resistance have been reported in population-based studies (3–5), and prior exposure to azoles seems to be a risk factor for the development of resistance (6, 7). In Spain, the overall fluconazole resistance rate is below 10% in *Candida* spp. and below 2% in *Candida albicans* in particular (5, 8). Although resistance to fluconazole is infrequent in *Candida albicans* strains isolated from blood, it may complicate the management of patients. A correlation has been detected between mortality and delayed initiation of effective antifungal therapy in patients with candidemia, including cases in which the dose of fluconazole used is suboptimal (5, 6, 9–13).

The mechanisms responsible for azole resistance in *C. albicans* involve mutations in the *ERG11* and *ERG3* genes, overexpression of *ERG11*, overexpression of genes encoding efflux pumps, or a combination of the three (14, 15). Fluconazole-resistant *C. albicans* isolates can be detected in the clinical microbiology laboratory using gold standard broth microdilution methods (CLSI and EUCAST), commercially available broth microdilution microtiter systems (Sensititre YeastOne), and agar-based methods (Etest) (16, 17).

Conventional methods for detecting fluconazole-resistant isolates delay results for at least 48 h after the detection of *Candida* spp. in blood cultures. Antifungal susceptibility based on agar diffusion tests performed directly on positive bottles has reduced the time from positivity of blood culture, making it possible to obtain preliminary fluconazole susceptibility values (18–20); a similar approach using marketed microdilution systems (e.g., Vitek, Sensititre YeastOne, and flow cytometry) has proven unsuccessful (21–23).

We previously showed that when performed directly on positive blood culture bottles, the Etest reliably detected fluconazole resistance in non-*albicans Candida* isolates approximately 24 h after the diagnosis of candidemia is confirmed (19).

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However, the role of this procedure for the detection of fluconazole resistance in *C. albicans* has not been properly assessed, because no resistant isolates have been tested to date. In this study, we assessed the ability of the Etest performed directly on positive blood culture bottles to detect fluconazole-resistant *C. albicans* isolates.

(Data from this study were presented at the 26th European Congress of Clinical Microbiology and Infectious Diseases in Amsterdam, The Netherlands [abstr P1613] [24]).

Isolates and fluconazole susceptibility testing. We studied 6 fluconazole-resistant *C. albicans* isolates from patients admitted to our hospital (Hospital Gregorio Marañón, Madrid, Spain). As controls, we used 12 fluconazole-susceptible *C. albicans* isolates showing different degrees of trailing (25) and 24 fluconazole-resistant non-*albicans Candida* isolates (*C. glabrata*, $n = 4$; *C. parapsilosis*, $n = 1$; *C. lusitaniae*, $n = 2$; *C. krusei*, $n = 13$; *C. guilliermondii*, $n = 3$; and *C. inconspicua*, $n = 1$) from blood samples. All isolates were identified by sequencing the internal transcribed spacer (ITS1-5.8S-ITS2) regions (26).

The *in vitro* susceptibility to fluconazole was assessed for the 42 isolates, according to the CLSI M27-A3 and EUCAST EDef 7.2 microdilution procedures (16, 17). The MIC value was defined as the lowest concentration of drug that inhibited ≥50% of growth compared with the growth in the control well. Isolates were considered fluconazole resistant according to the current EUCAST and CLSI breakpoints (fluconazole MIC, >4 mg/liter).

Susceptibility was also assessed using the Etest according to the standard manufacturer's instructions (ET_{SD}), with yeast suspensions adjusted to a 0.5 McFarland standard streaked across the surface of the agar plates. The Etest was performed directly from positive blood culture bottles (ET_{DIR}), as previously described (19). Briefly, a 0.5-ml suspension (0.5 McFarland standard) of each isolate was inoculated into Bactec FX bottles (Becton Dickinson, Cockeysville, MD, USA) and reincubated in the automatic system. When growth of yeast was detected in Gram stains performed in bottles flagged as positive, 10 to 20 drops of broth were poured and streaked onto RPMI 1640 agar plates supplemented with 2% glucose (bioMérieux, Marcy l'Etoile, France). All plates were incubated at 35°C for 24 h before the fluconazole MIC was determined.

Sequencing and gene expression. The presence of previously reported fluconazole resistance mechanisms was studied in the 18 *C. albicans* isolates. *ERG11* and *ERG3* were amplified and sequenced as previously reported (15). The relative expression levels of *ERG11*, *CDR1*, *CDR2*, and *MDR1* were also studied after total RNA extraction, reverse transcription, and reverse transcription-quantitative PCR (RT-qPCR). For each isolate, the expression level of the gene was evaluated using the $2^{-\Delta\Delta CT}$ method, where the C_T was the average threshold cycle obtained in 3 independent experiments for the above-mentioned genes. The normalized C_T (based on the C_T of a housekeeping gene, *ACT1*) was further compared with that obtained after calculating the mean C_T values measured in 3 residual trailing isolates. Relative gene expression between fluconazole-resistant isolates (CA-1 to CA-6) and fluconazole-susceptible isolates (CA-7 to CA-18) was compared using the Mann-Whitney test.

Data analysis. Categorical agreement between the 4 antifungal susceptibility testing methods was calculated, using CLSI M27-A3 and EUCAST EDef 7.2 as the gold standards. Errors were categorized as very major errors (VMEs) or false susceptible when the ET_{SD} or ET_{DIR} classified an isolate as susceptible and the gold standard classified it as resistant, and as major errors (MEs) or false resistance when an isolate was classified as resistant by ET_{SD} or ET_{DIR} and susceptible by the gold standard (19).

Ethical considerations. This study (protocol no. 157/16) was approved by the ethics committee of Hospital Gregorio Marañón (CEIC-A1). The need for informed consent was waived, owing to the retrospective design of the study.

Table 1 shows the fluconazole susceptibilities of the 18 *C. albicans* isolates obtained by the 4 procedures studied, the mutations found in *ERG11* and *ERG3*, and the relative expression levels of these genes. According to both the EUCAST and the CLSI proce-

TABLE 1 Fluconazole MICs, gene mutations, and gene expression levels

Isolate	Classification[a]	Fluconazole MIC (mg/liter)				Gene mutation(s)		Relative gene expression[b]			
		EUCAST guideline	CLSI guideline	ET_{SD}	ET_{DIR}	*ERG11*	*ERG3*	*CDR1*	*CDR2*	*ERG*	*MDR*
CA-1	Resistant	8	16	12	16	E266D, V488I	V351A, A353T	1.61	2.20	1.32	1.05
CA-2	Resistant	8	32	32	32	A114S[c]	V351A	1.53	46.91	1.81	2.16
CA-3	Resistant	8	8	32	32	A114S[c], G464S[c]	Wild type	2.06	43.25	0.71	0.59
CA-4	Resistant	128	256	0.125	0.125	D116E, E266D, V488I	H28Y, D219N, S265F, V351A	1.54	10.60	2.48	4.44
CA-5	Resistant	128	256	0.094	0.125	D116E, V481I	H28Y, D219N, S265Y	0.42	4.52	0.96	0.67
CA-6	Resistant	128	256	0.38	0.75	Wild type	Wild type	0.22	3.09	0.57	0.60
CA-7	Trailer (heavy)	0.25	0.125	0.38	0.5	D153E	Wild type	0.62	11.19	1.14	1.19
CA-8	Trailer (heavy)	0.125	0.125	0.25	0.5	D116E, K128T	V351A	0.42	12.59	1.37	5.57
CA-9	Trailer (heavy)	0.125	0.062	0.125	0.19	R246C	V351A	0.72	6.19	1.22	1.45
CA-10	Trailer (moderate)	0.25	0.25	0.38	0.5	D116E, V437I	Wild type	0.53	27.60	0.94	0.30
CA-11	Trailer (moderate)	0.25	0.25	0.75	0.75	Wild type	V351A	0.53	13.40	1.51	2.54
CA-12	Trailer (moderate)	0.25	0.5	0.5	0.38	Wild type	V351A	0.45	2.14	1.21	4.29
CA-13	Trailer (slightly)	0.125	0.25	0.25	0.38	D116E, K128T	N62S	1.65	1.31	0.68	0.81
CA-14	Trailer (slightly)	0.125	0.062	0.125	0.19	D116E, V437I	H28Y	0.84	1.47	0.61	0.43
CA-15	Trailer (slightly)	0.25	0.25	0.75	0.75	Wild type	Wild type	0.71	10.10	1.22	1.13
CA-16	Trailer (residual)	0.125	0.062	0.25	0.25	D116E, V488I, E266D	Wild type	NA	NA	NA	NA
CA-17	Trailer (residual)	0.125	0.125	0.5	0.5	E266D	V351A	NA	NA	NA	NA
CA-18	Trailer (residual)	0.25	0.5	1	0.5	E266D, V488I	V351A	NA	NA	NA	NA

[a]Fluconazole-susceptible isolates (CA-7 to CA-18) were classified according to trailing using a previously reported score: residual trailers, 0.1 to 5%; slight trailers, 6 to 10%; moderate trailers, 11 to 15%; and heavy trailers, >15% (25).
[b]NA, not applicable. Residual trailing isolates were used as controls to determine gene expression.
[c]Mutations previously reported as conferring fluconazole resistance (28).

dures, 6 isolates (CA-1 to CA-6) were fluconazole resistant (MIC, >4 mg/liter) and showed 2 different growth patterns in the microdilution trays. When the CLSI procedure was used, setting the fluconazole MIC endpoint was easy against isolates CA-1, CA-2, and CA-3, whereas the very prominent growth at all fluconazole concentrations found in isolates CA-4, CA-5, and CA-6 led to an MIC above the highest fluconazole concentration tested (Fig. 1a). Agitation of the plates according to the CLSI procedure is optional and may facilitate the MIC setting in isolates showing heavy trailing (27). However, we retested the isolates (CA-1 to CA-6) and set the MIC after agitating the plates, but it did not have a significant impact on the MICs. According to the EUCAST procedure, the growth inhibition curve kinetics were also different (persistent growth slightly above 50% or sharp inhibition of growth at fluconazole concentrations of ≥8 mg/liter), and both patterns matched those observed with the CLSI procedure (Fig. 2a).

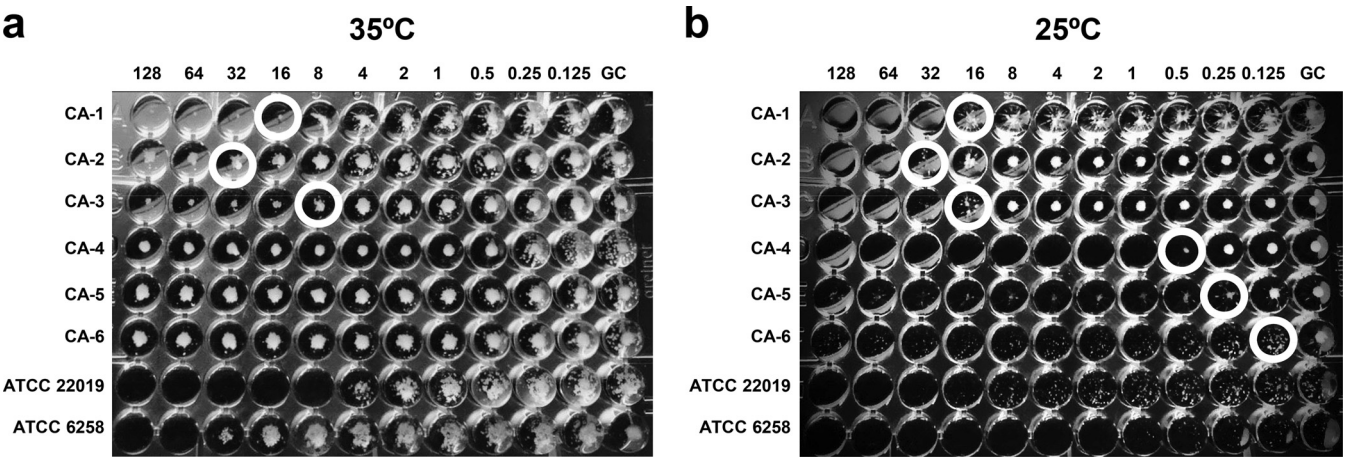

FIG 1 Tray showing the fluconazole MICs (top, in milligrams per liter) against the 6 fluconazole-resistant *C. albicans* isolates (CA-1 to CA-6) by CLSI procedure after 24 h of incubation at 35°C (a) and at 25°C (b). Wells in circles indicate the MIC. GC, growth control well.

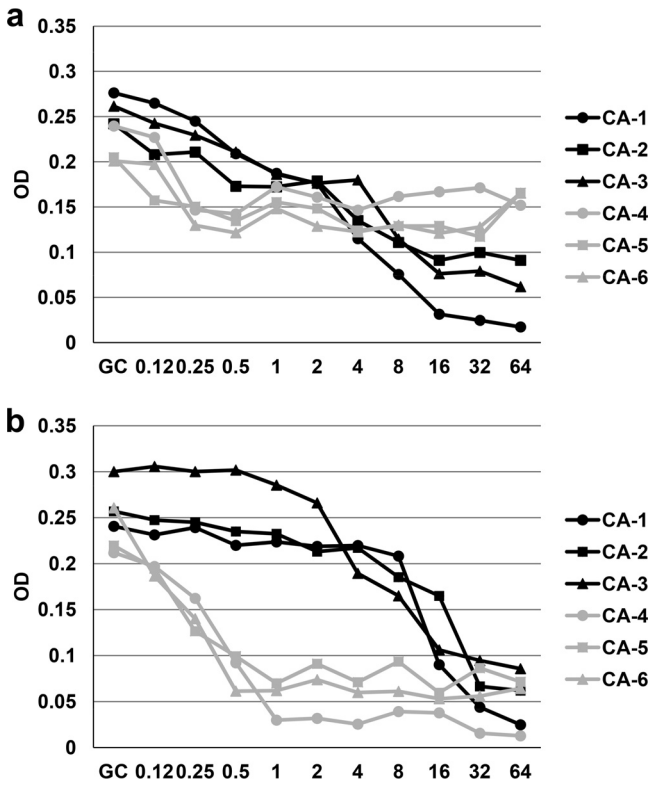

FIG 2 Growth inhibition curves of the 6 fluconazole-resistant *C. albicans* isolates (CA-1 to CA-6) by EUCAST procedure after 24 h of incubation at 35°C (a) or 25°C (b). *x* axis, concentration in milligrams per liter. *y* axis, optical density (OD).

Point mutations in *ERG11* and *ERG3* were found in most isolates, although only 2 isolates had mutations in *ERG11*, which has been reported to confer resistance (15, 28, 29). The remaining mutations were also previously described in fluconazole-susceptible isolates and do not seem to play a major role in resistance (15, 30–32). No differences in gene expression were observed between the fluconazole-resistant isolates and the fluconazole-susceptible isolates ($P > 0.05$) (Table 1). However, CA-2 and CA-3 had higher expression levels of *CDR2* and were also resistant to voriconazole and posaconazole (data not shown). The lack of a clear correlation between phenotypic and molecular resistance was not surprising, as the molecular explanation for fluconazole resistance in *Candida* is based on single mechanisms or simultaneous multiple mechanisms, and there might be other unknown underlying mechanisms that play a role in the resistance of these isolates (33–36). Furthermore, there may be an association between the specific resistance mechanisms and the anatomical site at which the isolate has become resistant (37).

Categorical agreement between ET_{DIR}, ET_{SD}, and broth microdilution was 100% for fluconazole-susceptible *C. albicans* isolates (no MEs) and for fluconazole-resistant non-*albicans Candida* isolates (no VMEs), thus confirming our previous observations (19). However, ET_{DIR} and ET_{SD} classified only 3 out of the 6 fluconazole-resistant isolates as resistant (isolates CA-1, CA-2, and CA-3; Tables 1 and 2), which yielded 50% of VMEs. Interestingly, inhibition of growth was sharply reduced in the EUCAST curves, and the MIC was easily interpreted using CLSI, whereas the remaining 3 isolates, which showed apparently "false" susceptibility, displayed persistent growth, even at high fluconazole concentrations in both microdilution methods (isolates CA-4, CA-5, and CA-6; Fig. 1a and 2a).

Observation of the ET_{DIR} plate in isolates CA-4, CA-5, and CA-6 revealed prominent growth of slime within the elliptic inhibition zone, thus suggesting that the disagreement between the methods could be due to heavy trailing in the microdilution trays

TABLE 2 Categorical agreement between the recommended conditions of incubation trays as per EUCAST and CLSI, with results obtained after modification of the incubation temperature of 25°C[a]

Isolate	Fluconazole susceptibility[a]			
	EUCAST (35°C/25°C)	CLSI (35°C/25°C)	ET_{SD}	ET_{DIR}
CA-1	R/R	R/R	R	R
CA-2	R/R	R/R	R	R
CA-3	R/R	R/R	R	R
CA-4	R/S	R/S	S	S
CA-5	R/S	R/S	S	S
CA-6	R/S	R/S	S	S

[a]R, resistant; S, susceptible.

rather than true fluconazole resistance (Fig. 3). Given the fungistatic nature of fluconazole, the trailing effect is frequently observed in *C. albicans* isolates and may complicate assessment of the MIC. This phenomenon is a consequence of the activation of calcineurin and altered regulation genes, although this effect cannot be explained accurately (38). Trailing can be misinterpreted as resistance in broth microdilution methods, particularly in isolates showing heavy trailing. However, animal models and clinical experience reveal these isolates to be truly fluconazole susceptible (39–41). In a previous report, we found that most of the *C. albicans* bloodstream isolates displayed fluconazole trailing to some extent: 26% were classified as heavy trailers, with the consequent potential to misclassify isolates as resistant (25).

In order to unravel whether the persistent growth pattern resembled true resistance or trailing, we modified the CLSI and EUCAST procedures by lowering the incubation temperature of the trays to 25°C and lowering the pH of RPMI broth medium to 4.5. These modifications were previously reported to minimize the interference of trailing when assessing the MIC using CLSI (39, 42). The modification of the broth medium to pH 4.5 did not enable growth of the isolates after 24 h of incubation or clear reduction of trailing (data not shown). However, when both microdilution trays were incubated at 25°C, the trailing effect was considerably reduced, and the 3 isolates classified as resistant by ET_{DIR} (CA-4, CA-5, and CA-6) switched from resistant to susceptible by both EUCAST and CLSI (Fig. 1b and 2b), thus leading to 100% categorical agreement between the 4 methods (Table 2). Previous reports have compared broth microdilution methods and the Etest for fluconazole susceptibility testing. Consistent with our data,

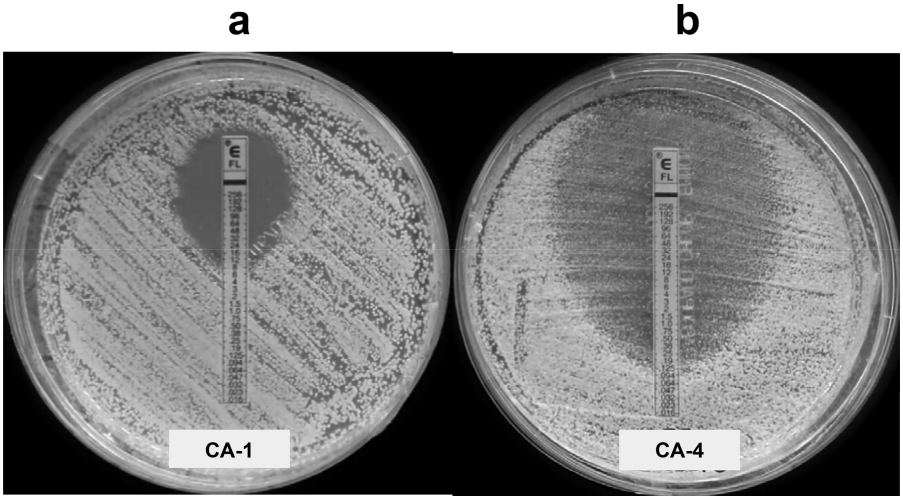

FIG 3 Fluconazole MICs obtained by ET_{DIR} against *C. albicans* isolates CA-1 and CA-4 after 24 h of incubation at 35°C. (a) True resistant isolate (CA-1), in which both ET_{DIR} and microdilution were in agreement. (b) Isolate (CA-4), in which ET_{DIR} classified the isolate as susceptible and microdilution as resistant, albeit with heavy trailing.

the findings were limited by the low number of resistant isolates, thus precluding an evaluation of the role of the Etest for detecting fluconazole resistance (20, 43–45). The small samples of fluconazole-resistant isolates in these studies are partially a consequence of the low frequency of isolation in the clinical microbiology laboratory (46).

We conclude that ET_{DIR} is a reliable procedure when screening for the presence of fluconazole resistance in *C. albicans* isolates causing candidemia. When using microdilution procedures, true fluconazole resistance should be proven after incubation of the microtiter trays at 25°C if the EUCAST shows a growth inhibition pattern consisting of a persistent growth slightly above 50% or heavy trailing, using CLSI guidelines.

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