DIFFERENCES BETWEEN AGAR DILUTION, BROTH MACRODILUTION (CLSI M27-A3) AND E TEST (AB BIODISK) FOR FLUCONAZOLE RESISTANCE IN CLINICAL *CANDIDA ALBICANS* ISOLATES.

E Robledo-Leal [1], JM Adame-Rodriguez [1], GM González [2], ET Aréchiga-Carvajal[1*].

[1]Unidad de Manipulación Genética, Laboratorio de Micología y Fitopatología, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León (San Nicolás de Los Garza, N.L., México); [2]Departamento de Microbiología, Facultad de Medicina, Universidad Autónoma de Nuevo León (Monterrey, N.L., México).

E-mail: elva.arechigacr@uanl.edu.mx

Introduction

Reliable lab tests must be installed in order to monitor the incidence of *in vitro* antifungal resistance. There are currently multiple options for testing *in vitro* antifungal drug susceptibility of *Candida albicans.* Reference standard tests remain to be few and resource consuming, such as those from the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). E test (AB BIODISK) offers more simplicity and celerity in the application of the test, being its cost a disadvantage particularly for small or newly started laboratories. Another factor that could represent a disadvantage especially in the case of vulvovaginal candidosis is the fact that colonization occurs in the tissue surface and not in a planktonic suspension, reason why CLSI reference method and other commercially available ones could not simulate entirely the growth conditions at which the drug will have to act. We believe that antifungal response of *C. albicans* seems affected by the way the organism colonizes tissues, cellular density and metabolism changes due to the organism's cellular age. Results obtained from susceptibility tests based on planktonic fungal growth does not certainly indicate the behavior of the infection in the living tissue thus opening the possibility of mis-correspondence between the minimal inhibitory concentrations (MIC) and the real susceptibility of the organisms in an infective state. In consequence, therapeutic schemes based on susceptibility tests could be imprecise suggesting ineffectively low doses or unnecessary high ones.

To test this theory, we decided to employ agar dilution assays such as the widely used for salt tolerance (1,2,3) in order to submit *C. albicans* inocula to different concentrations of fluconazole (FLC) and analyze the possible differences in the isolates' susceptibility compared with results obtained with M27-A3 protocol and E test.

Materials and Methods

Isolates: 40 isolates of *C. albicans* (CA01-CA40) were obtained from patients with vulvovaginitis sampled from the Hospital Universitario at Universidad Autónoma de Nuevo León. Strains included in the study were obtained from patients who met the conditions of not being pregnant, not being cursing any antibiotics treatment at the moment of the sampling and not being in the age of menopause according to Mexican Society of Nutrition and Endocrinology, all these in order to ensure as much as possible that the infection didn't occur due to a predisposal factor. Strains were identified by biochemical, physiological and morphological tests.

Susceptibility tests: CLSI reference method (M27-A3): Broth macrodilution (BMD) assays were performed according to CLSI guidelines (4). *C. albicans* strains were cultured in potato dextrose agar (PDA) for 24 h at 35°C. The inocula were adjusted afterwards to a final concentration of $0.5x10^3$ to $2.5x10^3$ cells per ml in RPMI 1640 medium (Sigma). For each strain a set of 10 test tubes was previously arranged containing 100 µl of FLC in concentrations ranging from 1.25 µg/ml to 640 µg/ml. 900µl of each adjusted inoculum was added to each of the tubes containing the drug resulting in final drug concentrations ranging from 0.125 to 64 µg/ml. An extra tube without antifungal drug was added for each set as a positive growth control.

Results were determined visually after 48 hours of incubation and MIC (minimum inhibitory concentration) was defined as the lowest drug concentration which resulted in a reduction of 80% in the turbidity compared to the drug-free growth control. As quality controls, *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 strains were used in accordance with CLSI document M27-A2.

E test (AB Biodisk): The E test was performed in accordance to the manufacturer's instructions (AB Biodisk, 1993). Briefly, 24h old cultures were adjusted to the turbidity of a 0.5 McFarland standard sterile saline solution. The medium used was RPMI 1640 agar supplemented with 2% glucose. The plates were inoculated by pouring 500µl of the adjusted inoculums and then streaking across the entire surface of the agar in three directions using a sterile cotton swab. The plates were dried at room temperature for 20 minutes before the E test strips were applied (Figure 1B), then they were incubated at 35°C and read after 48 h. The E test MIC was determined as the drug concentration at which the border of the elliptical inhibition zone intersected the scale on the antifungal test strip. Any growth throughout a discernible inhibition ellipse was ignored. *C. parapsilosis* ATCC 22019 was used as a quality control.

Agar dilution assays: For agar dilution assays we applied a modification to the Gaxiola *et al* method (5). Solidified RPMI 1640 medium was mixed with FLC to final concentrations of 0.5, 2, 8 and 64 µg/ml. Plates without antifungal drug were used as positive growth control. Inocula were obtained by incubating strains in YPD broth medium for 48h. Each inoculum was adjusted to a final concentration of $1x10^6$ cells per ml in RPMI 1640 medium and 5 µl of each strain were deposited in drug containing plates. Fungal growth was registered after 48 h of incubation at 35°C. MICs were defined as the minimum concentration at which growth was 80% inhibited compared to the positive growth control (Figure1A). MICs were determined visually. As quality controls, *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 strains were used.

Analysis of results: Results were compared to determine the agreement between each of the three susceptibility methods for each strain individually. Agreement was defined as results falling within ± 2 dilutions of the MIC results. Because E test strips contain a continuous gradient instead of the $\log_2$ drug dilution scheme, E test MICs were elevated to the next drug concentration that matched the macrodilution scheme to make the comparison of the results easier

Results

Table 1 shows the MICs of the *C. albicans* isolates produced by each of the three methods. All 40 isolates showed agreement between E-test and BMD. 19 isolates showed agreement between all three methods. 20 out of 21 unagreed MICs were higher in AD and 15 of those MICs were ≥64 µg/ml. Only 1 (CA36) of the un-agreed MICs was lower for AD than the other two tests. Although it agreed with the other tests, the MIC for strain CA12 was the only other result that was lower for AD. CA35 and CA40, both considered resistant according to their MIC were also resistant in AD.

Table 1. MICs of all three methods and agreement between them at 48 h.

Strain	BMD	E test	AD	Agreement
CA01	0.25	0.125	0.5	*
CA02	0.5	1	2	*
CA03	0.25	0.25	0.5	*
CA09	0.125	0.125	0.5	*
CA11	2	2	8	*
CA12	2	2	0.5	*
CA17	0.25	0.125	0.5	*
CA19	0.25	0.25	0.5	*
CA22	0.5	0.25	0.5	*
CA27	0.125	0.125	0.5	*
CA28	0.125	0.125	0.5	*
CA31	0.125	0.125	0.5	*
CA32	0.5	0.5	2	*
CA33	2	1	8	*
CA35	64	32	64	*
CA36	8	4	8	*

CA37	0.5	0.25	2	*
CA38	0.5	0.5	2	*
CA39	0.5	0.5	2	*
CA40	64	64	64	*
CA04	0.25	0.125	2	**
CA05	0.25	0.25	8	**
CA06	0.25	0.25	2	**
CA07	0.25	0.125	64	**
CA08	0.25	0.25	2	**
CA10	0.25	0.5	8	**
CA13	0.5	0.5	64	**
CA14	0.5	0.25	64	**
CA15	0.5	0.25	64	**
CA16	2	1	64	**
CA18	0.5	0.5	64	**
CA20	0.125	0.125	64	**
CA21	0.125	0.125	64	**
CA23	1	2	64	**
CA24	1	0.5	64	**
CA25	0.125	0.125	64	**
CA26	0.125	0.125	64	**
CA29	0.125	0.25	2	**
CA30	0.125	0.125	2	**
CA34	0.125	0.125	64	**

* Agreement between the three methods

** Disagreement between AD and the other two methods, with higher AD MIC

Figure 1 exemplifies the comparison made between AD and E test. While some strains exhibited accordance between all methods, such as CA35 in this figure, others like CA34 didn't agree between E test and neither AD or BMD. Strain CA33 shows a ≥80% inhibition at 8 µg/ml compared to the drug-free control in the AD and for the E test it shows a MIC of 1 µg/ml, for it is that value the one that intersects with the inhibition halo. Following this rubric, strains CA34, CA35 and CA36 show MICs of 64, ≥64 and 8 µg/ml respectively in AD and 0.125, 32 and 4 µg/ml respectively in E test.

Figure 1. Sample images of the results obtained with agar dilution (A) and E test (B). In agar dilution assays, 5µl of each inocula were placed in plates with different fluconazole concentrations (A). E test MICs were determined as the drug concentration at which the border of the elliptical inhibition zone intersected the scale on the antifungal test strip. Any growth throughout a discernible inhibition ellipse was ignored (B).

Table 2 shows the distribution of the MICs for the different methods. BMD and E Test exhibit very similar MIC number in their concentration range overall, while AD MICs locate mostly at the highest concentration used. MIC90 for BMD and E Test resulted in 2 µg/ml while for AD it dramatically increases to 64 µg/ml.

Table 2. Distributions of fluconazole MICs for different susceptibility methods.	
Method	No. of isolates for which indicated MIC (µg/ml) was:

	0.125	0.25	0.5	1	2	4	8	16	32	64
BMD	11	10	10	2	4		1			2
E Test	14	10	7	3	3	1			1	1
AD			10		10		5			15

Discussion

It could be suspected that the big differences between MICs obtained with AD compared with BMD were being caused by the way in which cells are grown, i.e. planktonic in BMD and sessile in AD but in E test however, cells are grown conceptually in the same way as in AD and E test agreement with BMD is absolute for this study. Although both AD and E test target cells in agar based growth, there is a greater ratio of cells per area in AD than in E test which could mean that the a bigger concentration of cells are exposed to the same drug concentration. The roll of cell density has been studied before (6,7) with results that support and reject the idea of cell density as a contributor for antifungal resistance. In the present study there is an overall tendency suggesting that cell density could indeed have contributed to fluconazole resistance although almost half of the results agreed among the three methods tested. There could be several explanations for strains that showed notorious increase in fluconazole resistance. Enjalbert and Whiteway demonstrated the quick and significant induction of drug resistance genes in medium containing farnesol, particularly drug efflux components and stress response genes (8). Since farnesol is produced depending only on cell growth and not on a particular carbon or nitrogen source and it is produced in equivalent amounts by cells growing in either yeast or mycelial modes (9), it is possible that the strains in our study that showed an increased resistance to fluconazole may have had a series of upregulated drug efflux pumps and/or activated stress response mechanisms conferring them with the remarked resistance. However, it has been proven that drug efflux pumps are not required for the development of resistance associated with cell density (10) and it's suggested that this increased resistance is produced by factors others than molecular mechanisms or biofilm architecture. From this perspective it becomes equally interesting to discover the reason for which the other strains did not develop resistance and even more the reason for which one strain was more susceptible in this higher cell density conditions.

Microbiological and molecular biology tests need to be done to further research this. AD has been proved to be useful in antifungal resistance evaluations for scientific works (11,12,13,14,15). Although it may not be reliable as a substitute for neither E test nor the CLSI reference method, because of its cost and efficiency it can be considered to be used as a preliminary resistance test, and it could be used to classify and/or search specific and possibly undescribed sources of resistance and susceptibility in *Candida* isolates under a condition not considered before.

Acknowledgements

We acknowledge the personnel of Centro Regional de Control de Enfermedades Infecciosas in Nuevo León for their technical help and support.

Abstract

Susceptibility assays performed by agar dilution (AD) were compared to M27-A3 broth macrodilution (BMD) methodology (Clinical Laboratory and Standards Institute) and E test (AB BIODISK) in order to determine the feasibility and reliability of agar dilution as an unexpensive test for fluconazole (FLC) susceptibility assays against C*andida albicans*. A total of 40 strains were used. All of the isolates resulted in agreement for BMD and E test. 19 isolates showed agreement between all three methods. Of the 21 divergent results, 20 resulted in higher MIC for AD and 15 of those were ≥64 µg/ml. Cell density due to proportional contact of cells with the drug in medium may be responsible for the results obtained and the divergence of AD compared to the other tests. AD can't reliably substitute neither BMD nor E test, but it may be helpful as a preliminary resistance test for strain susceptibility classification in laboratory research.

Key words: Candida albicans, fungal, drug resitance

Resumen

Ensayos de susceptibilidad realizadas por dilución en agar (AD) se compararon con M27-A3 macrodilución de caldo (BMD) metodología (Clinical Laboratory Standards Institute y) y la prueba E (AB BIODISK) con el fin de determinar la viabilidad y la fiabilidad de dilución en agar como una prueba de unexpensive para ensayos de susceptibilidad a fluconazol (FLC) contra Candida albicans. Un total de 40 cepas fueron utilizados. Todos los aislamientos resultaron en un acuerdo para la DMO y la prueba de evaluación. 19 aislados mostraron concordancia entre los tres métodos. De los 21 resultados divergentes, 20 resultaron en una mayor MIC para la EA y 15 de ellos fueron ≥ 64 mg / ml. La densidad de células debido a un contacto proporcional de las células con el fármaco en el medio puede ser responsable de los resultados obtenidos y la divergencia de AD en comparación con las otras pruebas. AD no son capaces de sustituir ni la DMO ni prueba E, pero puede ser útil como una prueba de resistencia a la tensión preliminar para la clasificación de susceptibilidad en la investigación de laboratorio.

Palabras clave: Candida albicans, hongo, resitencia de drogas

References

1. de Nadal E., F Calero, J Ramos, and J Arino. 1999. Biochemical and genetic analyses of the role of yeast casein kinase 2 in salt tolerance. J. Bacteriol; 181:6456-6462.

2. Gaxiola R, IF De Larrinoa, JM Villalba and R Serrano. 1992. A novel and conserved salt-induced protein is an important determinant of salt tolerance in yeast. EMBO Journal; 11:3157–3164.

3. Ruiz A, L Yenush and J Arino 2003. Regulation of ENA1 Na+-ATPase Gene Expression by the Ppz1 Protein Phosphatase is Mediated by the Calcineurin Pathway. Eukaryotic Cell; 2: 937-948.

4. National Committee for Clinical Laboratory Standards. 2002. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard, 2nd ed. M27-A2. National Committee for Clinical Laboratory Standards, Wayne, Pa.

5. Gaxiola R *et al, Op. cit.*

6. Seneviratne CJ, LJ Jin, YH Samaranayake and LP Samaranayake 2008. Cell Density and Cell Aging as Factors Modulating Antifungal Resistance of *Candida albicans* Biofilms. Antimicrob Agents Chemother; 52(9):3254-66.

7. Palani P, M Satish, and W LaJean 2007. Role for Cell Density in Antifungal Drug Resistance in *Candida albicans*Biofilms. Antimicrob Agents Chemother; 51(7):2454-63.

8. Enjalbert B and M Whiteway 2005. Release from Quorum-Sensing Molecules Triggers Hyphal Formation during*Candida albicans* Resumption of Growth. Eukaryot Cell; 4(7): 1203–1210.

9. Nickerson, KW, AL Atkin, and JM Hornby. 2006. Quorum sensing in dimorphic fungi: farnesol and beyond. Appl. Environ. Microbiol. 72:3805-3813.

10. Jia XM, ZP Ma, Y Jia, PH Gao, JD Zhang, Y Wang, YG Xu, L Wang, YY Cao, YB Cao, LX Zhang and YY Jiang 2008. RTA2, a novel gene involved in azole resistance in Candida albicans. Biochem Biophys Res Commun; 373(4):631-6.

11. Dunkel N, TT Liu, KS Barker, R Homayouni, J Morschhäuser and PD Rogers 2008. A gain-of-function mutation in the transcription factor Upc2p causes upregulation of ergosterol biosynthesis genes and increased fluconazole resistance in a Eukaryot Cell;7(7):1180-90.

12. Ferrari S, F Ischer, D Calabrese, B Posteraro, M Sanguinetti, G Fadda, B Rohde, C Bauser, O Bader and D Sanglard 2009.Gain of function mutations in CgPDR1 of Candida glabrata not only mediate antifungal resistance but also enhance virulence. PLoS Pathog; 5(1):e1000268.

13. Jia XM, *et al, Op.cit.*

14. Shukla S, SV Ambudkar and R Prasad 2004. Substitution of threonine-1351 in the multidrug transporter Cdr1p of Candida albicans results in hypersusceptibility to antifungal agents and threonine-1351 is essential for synergic effects of calcineurin inhibitor FK520. J Antimicrob Chemother; 54(1):38-45.

15. Vandeputte P, G Tronchin, G Larcher, E Ernoult, T Bergès, D Chabasse *et al* 2008. A nonsense mutation in the ERG6 gene leads to reduced susceptibility to polyenes in a clinical isolate of Candida glabrata. Antimicrob Agents Chemother; 52(10):3701-9.