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Full Length Research Paper

Commercial tests for *in vitro* antifungal susceptibility testing of *Candida* species compared to standard (NCCLS) broth microdilution

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It is of value to evaluate the commercial susceptibility methods as possible alternatives to standard one for routine fungal susceptibility testing. So we aimed to compare the NCCLS microdilution method with each of commercial Etest and candifast kit. A total of 30 *Candida* isolates were included and species identification was confirmed by morphological appearance on Sabouraud's dextrose agar (SDA), Gram stain, and candifast kit. The *in vitro* antifungal susceptibility testing of amphotericin B (AMB) and fluconazole (FCZ) were performed by 3 methods (standard broth microdilution, Etest and candifast) on the tested *Candida* species. The overall percentage of agreement of Etest with standard method was 90% for each of AMB and FCZ. For AMB the agreement of Etest with standard broth method was 100% in tested species except *Candida glabrata* (85.7%) and *Candida parapsilosis* (66.6%), while the percentage of agreement of candifast was100% in all species except *C. glabrata* (85.7%). For fluconazole the percentage of agreement of Etest, with the standard method, was 100% for each of *C. glabrata* and *Candida tropicalis* and was 92.8, 66.6% for *Candida albicans* and *Candida parapsilosis* respectively. The percentage of agreement of candifast method was 100% in all species except *C. albicans*, *C. glabrata* which was 92.8 and 85.7% respectively. Etest method is an alternative but cannot be considered as a substitute for the NCCLS reference method. The antifungal susceptibility method not greatly influenced by the type of tested antifungal agent.

Key words: NCCLS, Etest, candifast, antifungal drugs.

INTRODUCTION

Antifungal drug susceptibility testing has become more important due to the increase in serious fungal infections and the concomitant emergence of resistance to antifungal agents (Rex et al., 2001).

In 1997, the National Committee for Clinical Laboratory standards (NCCLS) published an approved reference procedure (document M27-A) for the *in vitro* testing of five antifungal agents against *Candida* species and *Cryptococcus neoformans*. The NCCLS document describes a broth macrodilution method and its microdilution modifications, specifies a defined test medium as well as a standardized inoculum, and recommends the visual determination of the MIC end points after incubation at 35°C for 48 h for *Candida* species. By this method the end point is defined as the lowest drug concentration at which a "prominent decrease in turbidity" is observed compared with the growth in the control drug-free medium (National Committee for Clinical Laboratory Standards, 1997).

Etest is susceptibility, agar-based, quantitative diffusion method which is based on the diffusion of a continuous concentration gradient of the antifungal agent tested from a plastic strip into an agar medium and provides minimum inhibitory concentration (MIC) endpoints instead of inhibition zone diameters. The MIC by the Etest is the lowest drug concentration at which the border of the elliptical inhibition zone intercepted the scale on the antifungal strip (Pfaller et al., 2004). It is of value to evaluate the commercial susceptibility methods as

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possible alternatives for routine fungal susceptibility testing. So we aimed to compare the NCCLS microdilution method with each of commercial Etest and with candifast kit.

MATERIALS AND METHODS

Organisms used in this study were clinical isolates obtained from 6 blood cultures for patients with different hematological malignancies, and 24 patients with different fungal skin and nail infections. A total of 30 *Candida* isolates were included and species identification was confirmed by morphological appearance on SDA, Gram stain, and candifast kit (International Microbio. Stago Group – Parc d'activities – Alleg d'Athenes 3870 Signes (France).

Analytical methods

Preparation of antifungal agents: Stock solutions were prepared in absolute dimethylsulfoxide (DMSO) for amphotericin B (AMB) and in sterile water for fluconazole .They were 5120 µg/ml for each drug. On performing susceptibility testing, further dilutions of each antifungal agent were prepared with RPMI 1640 medium which had been buffered to pH 7.0 with 0.165 M of morpholinopropanesulfonic acid (MPOS). The final drug concentrations in two fold serial dilutions ranged from 0.03 to 16 and 0.125 to 64 ug/ml for AMB and FCZ respectively (Baran et al., 2000; Arthington–Skaggs et al., 2002).

Amphotericin B was supplied as a lyophilized powder for intravenous administration by Bristol-Myers Squib (Squib-Egypt). Fluconazole was supplied as capsule for oral administration by Global Napi (Global Napi pharmaceutical Egypt).

Prior to antifungal susceptibility testing, each isolate was subcultured on SDA plates to insure purity. Five colonies were suspended in 0.9% saline and adjusted to an 0.5 McFarland standard (corresponds to 1×10^6 to 5×10^6 cfu/ml). This stock solution was diluted 1:100 in RPMI 1640 medium buffered to pH 7.0 obtain a 2× test concentration.

MIC by broth microdilution (National Committee for Clinical Laboratory Standards M27-A method 1997)

One hundred microliters of the 2× inoculum was pipetted in the wells of sterile microtitration plate to which 100 ul of the each drug dilution were added to the corresponding well. Ten wells were used for each test and additional control well was used cotaining 100 ul of organism suspension and 100 ul of drug free medium. This achieves a final concentration of 0.5×10^3 to 2.5×10^3 cfu/ml in a final test volume of 200 µl.

Microwell plates were incubated at 35°C for 48 h. MICs were determined visually. The MICs values were defined as the lowest drug concentration which resulted in reduction of 80% in turbidity in comparison with the drug free growth control well for fluconazole, while for Amphotericin B the MIC value was defined as the lowest drug concentration for which the well was optically clear. Break points for fluconazole were interoduced and validated by Pfaller et al. (2006) and Ostrosky-Zeichner et al. (2008). These breakpoints for fluconazole are susceptible <16µg/ml; susceptible dose-dependent, 16 to 32 µg/ml; resistant ≥ 64 µg/ml. The susceptibility for amphotericin B is <2.0 µg/ml and resistance is ≤2.0 µg/ml.

Etest (AB BIODISK, Solana, Sweden)

Medium for Etest was prepared by RPMI 1640, buffered to pH 7.0 by MOPS buffer, supplemented with 20 g/L 0f glucose and 15 g/L of agar base according to Etest technical guide. It was poured into 15 cm diameter sterile plates. The prepared 0.5 Mc Farland suspention of each isolate was applied on the agar surface with cotton swab.

Plates were allowed to dry for 15 min before application of Etest strips and then incubated at 35°C for 48 h. The MIC was taken as the lowest drug concentration at which the border of the elliptical inhibition zone intercepted the scale on the antifungal strip.

Candifast

Principally the determination of susceptibility of *Candida* to antifungal agent is based on presence or absence of growth of inoculated *Candida*.100 ul of inoculated standardized reagent 1 (dilution identification reagent) were inoculated into Reagent 2 (susceptibility reagent), from which 100 ul were dispensed into each well of corresponding susceptibility row and covered with 2 drops of paraffin oil, sealed and incubated at 35°C for 48 h, it was read visually. If the well was red, orange or orange – red, the strain was inhibited by the drug in that well. The organism was considered resistant to the antifungal in the well when the color changes to yellow, presence of turbidity, or even sediment.

RESULTS

Identification of *Candida* isolates (30) by candifast revealed the flowing distribution:

C. albicans 46.7% (n=14), followed by *C. glabrata* 23.3% (n=7), *C. parapsilosis* 20% (n=6) and *C. tropicalis* 10% (n=3).

Table 1 summarizes the *in vitro* antifungal susceptibility of AMB and FCZ on 30 *Candida* species by 3 methods (Standard broth dilution, Etest and candifast). We well comment on AMB and FCZ only in candifast plate.

For AMB the agreement of Etest with standard method was 100% in tested species except *C. glabrata* (85.7%) and *C. parapsilosis* (66.6%). The number of susceptible isolates in those 2 species was lower when tested by Etest method than standard method. The percentage of agreement of candifast was 100% in all species except *C. glabrata* (85.7%). The overall modal MICs obtained was 1.0 µg/ml for both standard method and E test.

For FCZ the percentage of agreement of Etest with the standard method was 100% for each of *C. glabrata* and *C. tropicalis* and 92.8, 66.6% for *C. albicans* and *C. parapsilosis* respectively. The number of susceptible isolates in those 2 species was lower when tested by Etest than standard method. The percentage of agreement of candifast method was 100% in all species except *C. albicans*, *C. glabrata* which was 92.8 and 85.7% respectively. The overall modal MICs obtained for fluconazole were 0.25 µg/ml for the standard methods and 0.5 µg/ml for Etest.

The overall percentage of agreement of Etest with standard method was 90% for each of AMB and FCZ. The overall percentage of agreement of candifast with standard broth method was 96.6% for AMB and 93.3% for FCZ.

Table 1. Comparison of susceptibility results by different methods for 30 *Candida* isolates for amphotericin B and fluconazole.

Isolates	NCCLS	E test	Candifast
Candida albicans **(14)**			
AMB:			
No of sensitive strains (%)	14 (100)	14 (100)	14 (100)
Range (µg/ml)	0.06-1	0.06-1	–
Percentage of agreement	–	100	100
FCZ :			
No of sensitive strains (%)	8 (57)	7 (50)	9 (64)
Range (µg/ml)	0.25-64	0.5 – 64%	–
Percentage of agreement	–	92.85	92.85
Candida glabrata **(7)**			
AMB:			
No of sensitive strains (%)	4 (57)	3 (43)	3 (43)
Range (µg/ml)	0.25 – 16	0.5 – 16	–
Percentage of agreement	–	85.70	85.70
FCZ:			
No of sensitive (%)	5 (71)	5 (71)	6 (86)
Range (µg/ml)	0.25 – 64	0.5 – 64	–
Percentage of agreement	–	100	85.70
Candida parapsilosis **(6)**			
AMB:			
No of sensitive strains (%)	5 (83)	3 (50)	5 (83)
Range (µg/ml)	0.06 – 16	0.125 – 16	–
Percentage of agreement	–	66.60	100
FCZ :			
No of sensitive strains (%)	6 (100)	4 (66)	6 (100)
Range (µg/ml)	0.25 – 16	0.5 – 64	–
Percentage of agreement	–	66.60	100
Candida tropicalis **(3)**			
AMB :			
No of sensitive (%)	2 (66)	2 (66)	2 (66)
Range (µg/ml)	0.25 – 16	0.25 – 16	–
Percentage of agreement	–	100	100
FCZ:			
No of sensitive (%)	1 (33)	1 (33)	1 (33)
Range (µg/ml)	0.25 – 64	0.5 – 64	–
Percentage of agreement	–	100	100
Total % of agreement			
AMB:			
Mode (µg/ml)	1	1	–
Percentage of agreement	–	90	96.60
FCZ:			
Mode (µg/ml)	0.25	0.5	–
Percentage of agreement	–	90	93.30

DISCUSSION

In the past, *in vitro* testing of antifungal agents was regarded as problematic. The development of reliable and reproducible broth dilution reference procedures against *Candida* species has, however, enabled MICs to be correlated with clinical outcomes and has permitted interpretive breakpoints to propose for the drugs (Rex et al., 1997, 2001).

Although the NCCLS M27-A reference method remains the standard by which all other methods are Judged, it is impossible for a modest-size laboratory to perform the test on a routine basis (Chang et al., 2001). There have been many alternatives developed over the past several years including flowcytometic (Wenisch et al., 1997) and MIC diffusion strips (Etest) (Simor et al., 1997; Pfaller et al., 1998).

In this study, (Table 1) on examining AMB on different *Candida* species, by Etest compared with the reference NCCLS, the number of susceptible species was the same except in *C. glabrata* and *C. parapsilosis* which were lower in Etest.

The overall agreement percentage among Etest and standard MIC method was 90% according to the MIC breakpoints recommended by M27-A method for each AMB and FCZ.

Etest has introduced as an easier testing procedure and an alternative for the NCCLS method (Pfaller et al., 1995; Ambler et al., 2001). The great advantage of Etest is the simplicity of the methodology. However not all antifungal agents are available in Etest and there is difficulty associated with endpoint interpretation (Koga-Ito et al., 2008).

In a similar study by Matsumoto et al. (2007) for comparison of Etest and standard microdilution method susceptibility of bloodstream yeasts, they recorded that results presented a greater agreement between Etest MICs and the standard NCCLS. The percentage of agreement was 98% for FCZ in each of *C. albicans* and *C. parasilosis*. However for AMB, the agreement between methods was low for all species. In another study on fluconazole and itraconazole (Koga- Ito et al. 2008), they reported poor agreement for fluconazole (53.33%) after incubation for 24 h.

Pfaller et al. (1998) obtained the best agreement between the NCCLS reference method and the Etest using RPMI agar with 2% glucose for AMB susceptibility testing, good correlation was observed between the tested method at 24 h. (66.6%) and 48 h (71.6). Interestingly, best agreement percentage was observed after 48 h (Koga-Ito et al., 2008).

By comparing candifast susceptibility method to the reference method for AMB in this study, the percentage of agreement was 100% in all tested *C. species* except *C.glabrata* (85.7%) and the overall percentage of agreement was 96.6%. For FCZ, the overall percentage of agreement was 93.3%. We could report that candifast is a rapid, easy, reproducible method for simultaneous identification and susceptibility testing.

Candifast method was used previously by Waller et al. (1993) who assayed the susceptibility of *C. albicans* to different antifungals.

The poor agreement of Etest than candifast could be explained on the basis that the determination of endpoints in Etest is a significant factor in the variability of MIC results with FCZ, the Etest often produces inhibitory zones with diffuse edges. In addition, the MICs of an isolate of *C. albicans* and an isolate of *C. parapsilosis* were 16 µg/ml in reference method (dose dependent susceptibility strain), while they were 64 µg/ml by Etest (resistant). So the MIC near the resistant endpoint should be reported cautiously and further validation of the data is important.

In a similar study by Claudino et al. (2008) they reported that agreement between MICs provided by the Etest and reference Clinical Laboratory Standard Institute (CLSI) method was 100% for AMB and 96.6% for FCZ. Their study demonstrated the adequacy of Etest method using Muller Hinton agar to evaluate AMB and FCZ susceptibility of clinical isolates of *Candida* species. We could conclude that Etest method is an alternative but cannot be considered a substitute for the NCCLS reference method. The antifungal susceptibility method not greatly influenced by the type of tested antifungal. Candifast is a simple, rapid, method for simultaneous identification and susceptibility of *Candida* species. It has also a precise endpoint which is important in results interpretation.

REFERENCES

Ambler JE, Kerawala M, Yaneza A, Drabu YJ (2001). Evaluation of CHROM agar *Candida* for rapid identification and Etest for antifungal susceptibility testing in a district hospital laboratory. J. Clin. Path., 54: 158-159.

Arthington–Skaggs BA, Lee–Yang W, Ciblak MA, Frade JP, Brandt ME, Hajjeh RA, Harrison LH, Sofair AN, Warnock DW (2002). Candidemia Active Surveillance Group. Comparison of visual and spectrophotometric methods of broth microdilution MIC end point determination and evaluation of sterol quantitation method for *in vitro* susceptibility testing of fluconazole and itraconazole against trailing and non trailing Candida isolates. Antimicrob. Agents Chemother., 46(8): 2477-2481.

Baran J, Klauber E, Barczak J, Riederer K, Khatib R (2000). Trends in antifungal susceptibility among *Candida* spp. Urinary isolates from 1994 and 1998. J. Clin. Microbiol., 370-371.

Chang HC, Chang JJ, Chan SH (2001). Evaluation of Etest for direct antifungal susceptibility testing of yeasts in positive blood cultures. J. Clin. Microbiol., 39: 1328-1333.

Claudino AL, Peixoto RF Jr, Melhem MS, Szeszs MW, Lyon JP, Chavasco JK, Franco MC (2008). Correlation between CLSI, EUCAST and Etest methodologies for amphotericin B and fluconazole antifungal susceptibility testing of *Candida* spp. clinical isolates. Pharmazie, 63(4): 286-289.

Koga-Ito CY, Lyon JP, Resende MA (2008). Comparison between Etest and CLSI broth microdilution method for antifungal susceptibility testing of *Candida* albicans oral isolates Rev. Inst. Med. Trop. S. Paulo, 50(1): 7-10.

Matsumoto FE, Dias AL, Melhem MS, Szeszs MW, Auler ME, Ruiz LS, Gonçalves da Silva E, Gandra RF, Paula CR (2007). Antifungal susceptibility of bloodstream yeasts isolated at a public children's

hospital in Brazil: Comparison of the Etest and the AFST-EUCAST microdilution method. Can. J. Microbiol., 53(12): 1300-1306.

National Committee for Clinical Laboratory Standards (1997). Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard M27-A. National Committee for Clinical Laboratory Standards, Wayne, Pa.

Pfaller M A, Diekema DJ, Sheehan DJ (2006). Interpretive breakpoints for fluconazole and *Candida* revisited: A blueprint for the future of antifungal susceptibility testing. Clin. Microbiol. Rev., 19: 435-444.

Pfaller MA, Messer SA, Karlsson A, Bolmstrom A (1998). Evaluation of the Etest method for determining fluconazole susceptibilities of 402 clinical yeast isolates by using three different agar media. J. Clin. Microbiol., 36: 2586–2589.

Pfaller MA, Boyken L, Messer SA, Tendolkar S, Hollis RJ, Diekema DJ (2004). Evaluation of the Etest Method Using Mueller-Hinton Agar with Glucose and Methylene Blue for Determining Amphotericin B MICs for 4,936 Clinical Isolates of *Candida* species. J. Clin. Microbiol., 42(11): 4977-4979.

Pfaller MA, Messer SA, Coffman S (1995). Comparison of visual and spectrophotometric methods of MIC endpoint determinations by using broth microdilution methods to test five antifungal agents, including a new triazole. J. Clin. Microbiol., 33: 1094-1097.

Rex JH, Pfaller MA, Galgiani JN, Bartlett MS, Espinel-Ingroff A, Ghannoum M A, Lancaster M, Odds FC, Rinaldi MG, Walsh TJ, Barry AL, Subcommittee on Antifungal Susceptibility Testing of the National Committee for Clinical Laboratory Standards (1997). Development of interpretive breakpoints for antifungal susceptibility testing: Conceptual framework and analysis of *in vitro - in vivo* correlation data for fluconazole, itraconazole, and *Candida* infections. Clin. Infect. Dis., 24: 235-247.

Rex JH, Pfaller MA, Walsh TJ, Chaturvedi V, Espinel-Ingroff A, Ghannoum MA, Gosey LL, Odds FC, Rinaldi MG, Sheehan DJ, Warnock DW (2001). Antifungal susceptibility testing: Practical aspects and current challenges. Clin. Microbiol. Rev., 14: 643-658.

Simor AE, Goswell G, Louie L, Lee M, Louie M (1997). Antifungal susceptibility testing of yeast isolates from blood cultures by microbroth dilution and the Etest. Eur. J. Clin. Microbiol. Infect. Dis., 16: 693–697.

Waller J, Koenig H, Debsuyne M , Contant G (1993). Evaluation d'un nouveau milieu d'isoement des levures et de diagnostic rapide de *Candida* albicans. Rev. Fr. Lab., 252: 89-92.

Wenisch C, Linnau KF, Parschalk B, Zedtwitz-Liebenstein K, Georgopoulos A (1997). Rapid susceptibility testing of fungi by flow cytometry using vital staining. J. Clin. Microbiol., 35: 5–10.

Ostrosky-Zeichner LO, Rex JH, Pfaller MA, Diekema DJ, Alexander BD, Brown SD, Chaturvedi V, Ghannoum MA, Knapp CC, Sheehan DJ, Walsh TJ (2008). Rationale for Reading Fluconazole MICs at 24 Hours Rather than 48 Hours When Testing *Candida* spp. by the CLSI M27-A Standard Method. Antimicrob. Agents Chemother., pp. 4175-4177.