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Antibiogram of *Candida* Species using Different Susceptibility Testing Techniques: A Systematic Review

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Abstract
Susceptibility pattern determination is a valuable key towards successful treatment of infectious diseases. Susceptibility of Candida species to different antifungal agents are also a common practice, however, different methods are employed to achieve the same goal. Some of the different techniques include: Disc diffusion method, Epsilometre test (E-test), Candfast, Microdilution technique among others. Fluconazole, Voriconazole, Itraconazole and Amphotericin B are some of the antifungal agents commonly used for treatment of infections due to Candida species. Most of these antifungals are also covered in this review. The commonly encountered Candida species in clinical settings include: C. parapsilosis, C. tropicalis, C. albicans, C. krusei, and C. glabrata. This review also gave some insight into their variable susceptibility pattern, as it affects different methods of susceptibility testing. There were reports of resistance from researchers from different regions across the globe; this therefore, signifies the importance of availability of data with regards to susceptibility of these species.
Key words: *Susceptibility, Candida species, Antifungal agents, testing techniques*

INTRODUCTION

Knowledge of drug susceptibility pattern is an important factor of help, in the progress of treatment and other aspects of patient management. In case of mycotic diseases, various antifungal agents are employed in treatment and their efficacy is continually being tested using various methods like disk-diffusion method, which involved the use filter disc impregnated with known concentration of antifungal agent, Epsilometer test (E- test) which a strip containing exponential gradient of the agent to be tested, Broth microdilution method that involved dilution of different concentration of the agent and the Candifast microbroth kit consists of a tray with two rows of eight wells each to test against the organism, among other methods (Aher, 2014).

It has been observed over the years that, *Candida* species have become prominent nosocomial pathogens (Abi-Said *et al.*, 1997). Over the years, available data suggested a shift from *Candida albicans* as the major cause of invasive fungal infections toward non-*C. albicans* species (Pfaller *et al.*, 2000 Kao *et al.*, 2001).

The Centers for Disease Control and Prevention (CDC) conducted population-based surveillance for *Candida* bloodstream infections over two different time periods: in 1992 and 1993 and from 1998 to 2000, it was discovered in the first surveillance period that, *C. albicans* accounted for 52% of the isolates while *C. glabrata* accounted for only 12%. In the second round of the surveillance period, *C. albicans* accounted for 45% but *C. glabrata* had a shift up, to 24% (Punithavathy and Nalina, 2012)

Another study also showed that, among the non albicans species, *Candida glabrata*, recorded higher incidence, which account for the decrease in dominance of *Candida albicans* as the cause of invasive fungal disease due to *Candida* species (Trick *et al.*, 2002).

There is a document developed by Clinical and Laboratory Standards Institute (CLSI) on *Candida* species-specific clinical breakpoints for some antifungal agents like echinocandins, fluconazole and voriconazole and is believed that use of such breakpoints can change the previously known *Candida* species sensitivity impact patterns and consequently, the management of the patients (Pfaller and Diekema, 2012).

Antifungal treatment selection is a factor of the causative agent identification and *in vitro* susceptibility testing which provide valuable information for patient management. In resource-limited settings, echinocandins, voriconazole and liposomal formulations of amphotericin B are either unvailable or if available, not affordable.

Presently, fluconazole and amphotericin B deoxycholate are the drugs most commonly used in some nations. Several studies indicated that *Candida* species distribution and in vitro drug susceptibility vary among nations and even in different regions of the same country (Nishikaku *et al.,* 2010; Cleveland *et al.,* 2012), even though most of these researches were done in the United States and/or in Europe, they revealed a consistently decreased levels of susceptibility to fluconazole by both *Candida albicans* and non-albicans *candida* species and such is expected even in other parts of the world unless proved otherwise (Arendrup *et al.,* 2013).

It has been observed that limited information is available from many parts of the world, which means no large studies that evaluated species distribution and antifungal susceptibility pattern of yeast isolates in some infections. The only limited information at hand mostly suggests that non-*C. albicans* species are in most cases the aetiologic agents of blood stream infections (BSIs) in some countries than expected (Becerra *et al.,* 2010).

The information may have serious implications for infection treatment against the *Candida* species, because *Candida glabrata* is also considered a fluconazole acquired resistant species, *Candida parapsilosis* may display high MIC values for echinocandins while *C. krusei* is also considered to be a resistant specie to fluconazole irrespective of its MIC (Pfaller *et al.*, 2012a).

In a swift effort, the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing, the (AFST-EUCAST) has developed a standard broth microdilution protocol for the determination of antifungal MICs for fermentative yeast species (Rodriguez-Tudela *et al.*, 2003). The standard developed was based on the guidelines of National Committee for Clinical Laboratory Standard (NCCLS), the procedure described in document M27-A2 (NCCLS, 2002), but the procedure includes some modifications that allows for automation of the method and also permit the incubation period to be reduced from 48 to 24 h. Reproducibility of 94% has been observed in the EUCAST procedure for antifungal susceptibility testing according to a multicentre evaluation study (Cuenca-Estrella *et al.*, 2003). Additionally, a panel of 109 bloodstream isolates of *Candida* species were evaluated against some antifungal agents: fluconazole, itraconazole, amphotericin B, and flucytosine by a two-laboratory study which correlated between the NCCLS M27-A and EUCAST microdilution procedures, the study demonstrated an overall agreement rate of 92% (Cuenca-Estrella *et al.*, 2002).

Because they involved rather complex methods for susceptibility testing, standard reference procedures are mostly not practicable routinely in clinical laboratories, for this reason most microbiologists engage other systems that have numerous advantages, like rapid results, ease of performance, and reduced financial implication. For these reasons therefore, several techniques like the use of a colorimetric oxidation–reduction indicator and those based on agar diffusion are used. Accordingly, some of these techniques are commercially made available which are rapid and simple alternatives to those procedures developed by either the NCCLS or the EUCAST (Arikan *et a*l., 1997).

The significance of use of reference procedures is to provide a standard measure from which other methods can be developed and compared, as a results, many studies have analysed the correlation between the NCCLS procedure and various commercially available systems (Espinel-Ingroff *et al.*, 1999), this includes some suitable for susceptibility testing of *Candida* species.

The expanding use of some new antifungal agents, and the emergence of antifungal resistance as one of the known important clinical problems, calls for the need of reproducible, and clinically relevant antifungal susceptibility testing, due also to continued challenge by the increasing number of invasive fungal infections (Pfaller, 2012).

It is of value that, the collaborative efforts of numerous researchers and the Clinical and Laboratory Standards Institute (CLSI), known before as, National Committee for Clinical Laboratory Standards (NCCLS), Subcommittee on Antifungal Susceptibility Testing have generated an agreed consensus documents describing standardized methods for broth- and agar-based antifungal susceptibility testing (CLSI 2009; CLSI, 2010 CLSI, 2017). Based on these, *in vitro* antifungal susceptibility testing remain a model, which: (i) Guides therapeutic decision making (ii) Serves as an antifungal resistance tracker in epidemiologic studies and (iii) Aid in drug development studies (Kanafani and Perfect 2008). The aim of the review was to survey some susceptibility testing techniques used some regions in the world.

The different susceptibility pattern studies in some regions of the world

India (Asia): A study conducted by Sumana *et al*. (2017), 50 isolates of different *candida* species were exposed to variety of antifungal agents and pattern of susceptibility using agar disc diffusion method to these antifungals were noted: Resistance to Fluconazole been observed were in the following rates; 4% for *C. parapsilosis*, 4% for *C. tropicalis* , 4% for *C. albicans*, 2% for *C. krusei,* and 2% for *C. glabrata*, while the greater percentage of the species 84% were sensitive to it. For Voriconazole, 2% each, of *C. albicans*, *C. tropicalis*, *C. glabrata*, and *C. haemulonii* exhibited resistance while 92% were sensitive it. In the same vein, 4% of *C. parapsilosis*, 2% of *C. albicans*, 2% of *C. glabrata*, 2% of *C. tropicalis*, and 2% of *C. haemulonii* were resistant to Amphotericin B, but 88% proved sensitive to it. Susceptibility to Ketoconazole was observed to be as follows: 2% of *C. albicans* and 2% of *C. haemulonii* were found resistance while 96% were sensitive. The next antifungal agent, Itraconazole proved the most effective in vitro with all isolates (100%) sensitive to it. A sample of disc diffusion technique used is given in Fig. 1

Fig 1: Disc Diffusion Method, Source: Rosco diagnostica, 2011

Das *et al*. (2016) in India identified variable efficacy of some antifungal agents when tested against different *Candida* isolates using disc diffusion method, they found 9.3% (n=6) of *C. albicans* isolates to be resistant to both voriconazole and fluconazole where as Fungitest method revealed no fluconazole resistance among these isolates, except one intermediate result. However, none of the non-albicans *Candida* isolates showed resistance to fluconazole and voriconazole using disk-diffusion method, but some of these isolates were found to have intermediate result to azoles by Fungitest method. In the same vein, (100%) of the isolates were found to be susceptible to amphotericin B and fluorocytosine. It was also discovered that none of the *Candida* species isolates from HIV positive subjects with previous exposure to antifungal agents show resistance to any antifungal agent tested in the study.

Brazil (Latin America): A study in Brazil that involved 70 *Candida* species, in which, 67% of them were from infection (either candiduria or candidemia) and 33% were neither of the two, in the University Hospital (UH), Maringa, a 116 beds school hospital in Parana,-Brazil. According to the results, non *Candida albicans*-candida species (NCAC) were found to overtake *candida albicans* in the study, with up to 61% of the yeasts isolates. From the results, among the NCAC species, *C. glabrata* and *C. tropicalis* were identified to be more prevalent than the other NCAC species. Using Epslometre test (E-test), amphotericin B proved the only antifungal drug which had MIC values under the reference limit (01 mg/ml) for all isolates except one *C. albicans* and one *C. glabrata* with (MIC532mg/ml) and (MIC56ug/ml) respectively. Incidentally, all tested strains, when assessed using E-Test (ET) method, revealed higher MICs compared with Microdilution (MD) method for: fluconazole, itraconazole, and amphotericin B. When the isolates were compared, on the basis of colonization (COL) and infection (INFC), it was found that COL yeasts were more susceptible to the 3 antifungal agents than the INFC yeasts in the ET method. It was also observed that, NCAC species exhibited smaller inhibitory zone diameters when disc diffusion method was used in relation to *C. albicans*. Among the three

methods studied, itraconazole proved to be having the highest number of resistant NCAC The overall concordance (based on the MIC value obtained within two dilutions) between the ET and MD methods was 83% for amphotericin B, 63% for itraconazole, and 64% for fluconazole. Considering the breakpoint, E-species.

Test and DD methods had high categorical agreement (Negri *et al.*, 2009). A sample of The Epslometre test (E-test) is given below in figures 2 and 3:

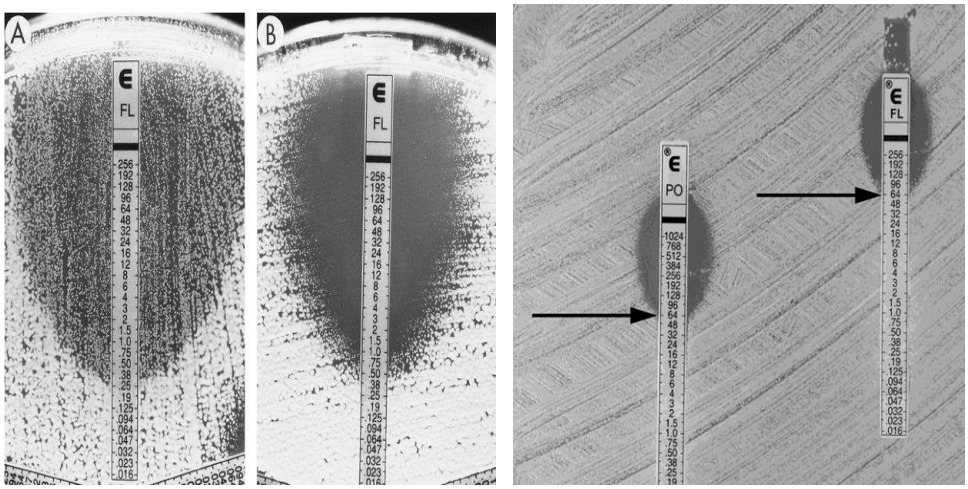

Fig 2:Fluconazole (FL) Etest reading patterns for C. albicans . (A) Growth of microcolonies inside the entire inhibition zone (ellipse); MIC, 0.38 ug/ml. (B) Clear ellipse on Casitone agar; MIC, 0.5 ug/ml. The numbers on the scale correspond to the fluconazole concentrations on the strip (in micrograms per milliliter). Pfaller et al., Evaluation of the Etest Method for Determining Fluconazole Susceptibilities of 402 Clinical Yeast Isolates by Using Three Different Agar Media M. A. Pfaller,1 S. A. Messer,1 Å. Karlsson,2 and A. Bolmstro ¨M21998. Fig.3: The Epslometre test (E-test) pointing to MIC point on the test strips, Source: (George et al., 2014).*

U.S.A. (America): A study on different methods of susceptibility based on MICs and zone diameters and for 24 and 48 h incubation period was reported, by Matar *et al*. (2003), based on the result, it was generally observed that, ET method revealed slightly higher fluconazole MICs than the MD method at both incubation times, whereas the voriconazole MICs by the MD and ET methods were similar at both time points. It was also observed that, the overall levels of agreement between the MICs obtained according to both ET and MD methods at 24 and 48 h were satisfactory for both fluconazole and voriconazole. For both drugs, the agreement between the ET, MIC and the reference (MD) MIC, at 48 h were 93%. However, a better agreement percentage was noticed for the readings obtained at 24 h, when the rate of agreement was 98% for the two drugs. For some isolates, there was some difference in which the ET MICs were found to be lower while the MD method was lower at 24 h but the MICs tend to be elevated at 48 h. Comparisons of the results as indicated by the different methods showed that isolates susceptible by the disc diffusion method or ET method at either time points had a 96% likelihood of testing susceptible or susceptible dose dependent by the MD method. On the other hand the MICs for isolates resistant by the disc diffusion and ET method tend to be almost always in the susceptible-dose dependent or resistant category by the MD method, whereas isolates testing resistant by the MD method produced results by the agar-based method ranging from susceptible to resistant. After 48 h, trailing growth, for fluconazole tend to generate higher MICs. Use of Mueller-Hinton agar flooded with glucose-methylene blue (GMB) enhanced growth and simplified reading relative to the MD method.

This suggest that, the results for isolates that appear to be resistant by any method should be carefully reviewed and that, such isolates may merit repeat testing and/or testing by an alternative method. Although more work needs to be done with less susceptible isolates, the aggregate data suggest that agar-based methods appear to produce a more consistent in *vitro-in vivo* correlation than the reference Microdilusion method, example of the microdilusion test can be seen in Figure 4.

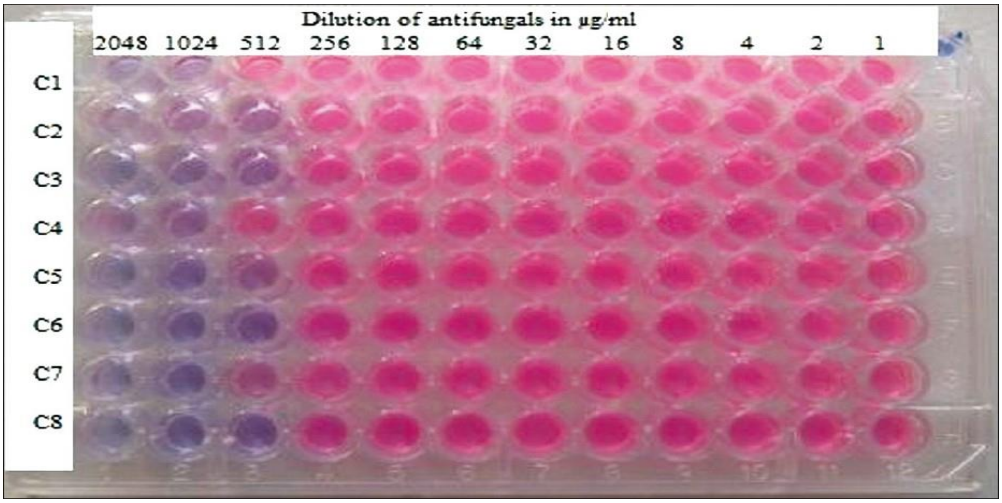

Figure 4: Microdilusion Technique, Source: (Punithavathy et al., 2012)

Egypt: El-Mashad *et al.*(2011), in a study on the *in vitro* antifungal susceptibility of ampotricin B (AMB) and Fluconazole (FCZ) on 30 *Candida* species by 3 methods (Standard broth dilution, E-test and candifast) found that, 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 E-test 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 were 1.0 µg/ml for both standard method and E test. For FCZ, the percentage of agreement of E-test with the standard method was 100% for each of *C. glabrata* and *C. tropicalis* and 92.8% and 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 0.25µg/ml for the standard methods and 0.5µg/ml for E-test. The overall percentage of agreement of E-test 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.

According to Lyon *et al.* (2011), a susceptibility testing based on 24-h incubation for the 6 commonly encountered *Candida* species (*C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, and *C. lusitaniae*), using microdilution method, revealed different MIC status of the involved isolates. The results were presented as the MIC ranges: The MIC50 and MIC90 values, the numbers of susceptible isolates, and the numbers of resistant isolates. Overall, fluconazole exhibited promising activity against most species isolated. In particular, *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. lusitaniae* were quite susceptible to fluconazole. In contrast, *C. glabrata* was less susceptible to fluconazole (MIC90, 16g/ml). The agents,echinocandins, caspofungin and micafungin, exhibited excellent activity against all species of *Candida*, with the overall rate of susceptibility of 99.8%. The rate of resistance was low, at 0.2%. The echinocandins were quite potent against all species except *C. parapsilosis*, for which the MIC90 was 1g/ml. When the epidemiological cutoff values (ECVs) were applied to the echinocandins, the overall rates of resistance increased slightly. However, most isolates were slightly more resistant to caspofungin than to micafungin. Overall, 133(2.3%) isolates were resistant to fluconazole (MIC 64 g/ml). The numbers of isolates of specific species resistant to fluconazole were as follows: *C. albicans*, 30(1.2%) isolates; *C. glabrata*, 87(5.9%) isolates; *C. parapsilosis*, 3(0.3%) isolates; *C. tropicalis*, 2(0.4%) isolates; and *C. lusitaniae*, 0(0%) isolates.

CONCLUSION

Among the reviewed techniques disc diffusion method remain the best for routine work, being the easiest and less expensive than E-test and microdillution techniques and it gives good results. Among the antifungal agents reviewed, fluconazole and itraconazole were shown to be promising against the *Candida* species among other agents.

According to available data on susceptibility pattern of *Candida* species from different regions of the world, it is evident that, these species display variable reactions (with different susceptibility techniques) to the conventional antifungal agents throughout the world, with many reports of resistance. There is therefore the need for global concerted effort to adequately handle diseases caused by these organisms.

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