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Original Research Article

Applicability of Disc Diffusion Method for Antifungal Sensitivity Testing of Candida Isolates in a Clinical Microbiology Laboratory

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ABSTRACT

Invasive Candidiasis is associated with high morbidity and mortality, thereby underscoring the importance of early initiation of appropriate antifungal agents. In view of increasing resistance to antifungal agents among *Candida species*, routine antifungal susceptibility testing is becoming increasingly necessary. We, therefore, planned to corroborate the findings of the technically simpler disc diffusion method with the broth dilution method.

We selected 59 consecutive clinical isolates of *Candida*, and subjected them to antifungal susceptibility testing against Fluconazole and Ketoconazole by both the methods, in accordance to the corresponding CLSI guidelines. We observed significant inter-test agreement between the 2 methods for both *C. albicans* and non-albicans isolates. This is of significance since reliable results obtained in a less laborious test, like the disc-diffusion technique, would offer a scope of implementing this method in clinical laboratories for routine performance of antifungal sensitivity testing; similar to the practice adopted for bacterial isolates.

Keywords: Antifungal drug resistance, Antifungal Sensitivity Testing, Disc diffusion method, Invasive Candidiasis, Candida.

INTRODUCTION

Antifungal resistance has been evolving lately as a burgeoning health care problem among *Candida species.* [1] This is associated with a relative rise in the proportion of non-albicans Candida isolates. This bears important therapeutic implications, in view of the intrinsic resistance observed among several non-albicans Candida species towards specific antifungal agents. *C. glabrata* and *C. krusei* isolates are intrinsically resistant to Fluconazole, while *C. lusitaniae* demonstrates similar intrinsic resistance towards Amphotericin B. [2] Moreover, there has been a documented increase in fluconazole resistance even among other *Candida spp*., including *C. albicans, C. lusitaniae*, *C. tropicalis* and C. *dubliniensis*, which has been partially attributed to the popular use of fluconazole as empirical antifungal therapy since the 1990s. [3]

Preclinical and clinical studies have shown an association between the timely initiation of appropriate antifungal therapy and infection outcome. [4] This underscores the importance of performing antifungal susceptibility testing in clinical laboratories in order to guide the appropriate choice of antifungal drugs. [1] However, unlike

antibacterial susceptibility testing, antifungal susceptibility testing is not routinely practiced in most clinical laboratories, owing to the involvement of cumbersome technical processes, and an empirical approach is usually followed in prescribing antifungal agents. Though recent guidelines from Clinical and Laboratory Standards Institute (CLSI) have attempted to standardize antifungal susceptibility testing, limitations still exist as a result of the incomplete correlation between in vitro susceptibility and clinical response to treatment. [1] With this background the proposed study was aimed at making a comparative assessment of the two principal methods of performing antifungal susceptibility testing in Candida isolates recovered in the operational setting of a diagnostic Microbiology laboratory.

MATERIALS AND METHODS

Disc diffusion testing of Ketoconazole and Fluconazole was performed in accordance with CLSI document M44-A. [5] Mueller Hinton agar plates supplemented with 2% Glucose and 0.5 µg Methylene blue dye per ml at a depth of 4.0 mm (pH 7.2- 7.4) were used. Agar surface was inoculated by using the swab dipped in the cell suspension adjusted to 0.5 McFarland turbidity standard. Ketoconazole (Kt) (10 µg), Fluconazole (Fl) (10 µg) discs were placed on the surface of the inoculated plates and the plates were incubated at 37^0C and read after 20-24 hrs of inoculation. Zone diameter end points were read at 80% growth inhibition against the illuminated light. Reading at 48 hrs was taken, if insufficient growth was seen at 24 hrs. Zone size of 18-22 mm was considered susceptible for Ketoconazole and Fluconazole antifungal discs.

MIC of Fluconazole and Ketoconazole were determined by broth Macrodilution Method in accordance with CLSI document M27-A2. [6] All isolates were tested in RPMI 1640 (with glutamine, without bicarbonate, and with phenol red as indicator) buffered to a pH of 7.0 at 25^0C, using MOPS buffer [3-(N-morphine) propanesulfonic acid]. MIC performance characteristics of each batch of broth were evaluated using a standard set of quality control organisms, *C. albicans*-ATCC-5314 and *C. krusei*-ATCC- 6258. Stock solution for Fluconazole was prepared at concentration 6400µg/ml and for Ketoconazole was prepared at concentration of 1600µg/ml. The drug concentration range for Ketoconazole was 0.0313 to 16 µg/ ml and Fluconazole was 0.125 to 64 µg/ml. Inoculum was prepared from growth on SDA sub cultures at 35ºC for 24 to 48 hours depending on species. Colonies were suspended in 0.85% saline and the turbidity was adjusted to a 0.5 McFarland standard. A working suspension was made by diluting the original suspension 1:100 dilution and then 1:10 in RPMI 1640 broth medium which resulted in 1.0 x 10^3 to 5.0 x 10^3 cells/ml. Before adjusting the inoculum, 0.1 ml of the various antifungal concentrations were placed in 12x75 mm tubes. In growth control tube 0.1 ml of drug diluents without antifungal agent was added. Within 15 minutes after the inoculum had been standardized, 0.9 ml of the adjusted inoculum was added to each tube in the dilution series and mixed. This resulted in 1:10 dilution of each antifungal concentration and 11% dilution of the inoculum. The tubes were incubated at 35ºC for 48 hrs in ambient air. As per the definition, MIC was taken as the lowest concentration of an antifungal agent that substantially inhibited the visible growth of an organism after overnight incubation. The amount of growth in the tubes containing the agent was compared with the amount of growth in the growth control tubes used in each set of tests. The concentration of antifungal agents that demonstrated 80% inhibition of growth was considered as MIC.

Statistical analysis: Fisher's Exact Test and Kappa test were done, using SPSS software version 21.0, to ascertain the inter-test agreement between M27-A2 and M44-A procedures.

RESULTS

In our study, out of 32 *C. albicans* isolates, 28 (87.5%) were sensitive to Ketoconazole, and 29 (90%) were sensitive to Fluconazole by disc diffusion method (DDM). Thirty out of 32 *C. albicans* isolates had MICs of <8.0 µg/ml for Ketoconazole. The remaining two *C. albicans* isolates had MIC between 8-32 µg/ml. All the isolates with MIC below 8.0 µg/ml for Ketoconazole were also found to have MICs < 8.0µg/ml for Fluconazole. The two *C. albicans* isolates, which were found to have MIC in the sensitive-dose dependent range for Ketoconazole, were resistant to Fluconazole (Table 1). Of the 19 isolates of *C. tropicalis*, 16 (84.2%) were sensitive to Ketoconazole and Fluconazole by disc diffusion method (DDM); whereas 16 (84.3%) were found to have MIC below 8.0 µg/ml for Ketoconazole and Fluconazole by BDM. There were five isolates of *C. glabrata*, of which four were sensitive to Fluconazole and all were sensitive to Ketoconazole by DDM while all the five isolates were found to have MIC below 8.0µg/ml for Ketoconazole. One isolate of *C. glabrata* was found to have MIC > 64 µg/ml for Fluconazole, though its MIC for Ketoconazole was < 8.0 µg/ml. All the three recovered isolates of *C. parapsilosis* were sensitive to Ketoconazole and Fluconazole by both the methods (Table 2).

Table1: Comparison of the results of Antifungal susceptibility testing to Fluconazole & Ketoconazole by Broth Macrodilution and Disc Diffusion methods for *C. albicans*.

		Broth Dilution for Fluconazole			Fisher's Exact Test (Exact Sig. 2 sided)	Kappa value (Approx. Sig)
		Susceptible	Susceptible-Dose Dependent	Resistant		
Disk Diffusion for Fluconazole	Susceptible	29	-	-	0.006	0.784 (0.000)
	Susceptible-Dose Dependent	-	-	-		
	Resistant	1	-	2		
		Broth Dilution for Ketoconazole			Fisher's Exact Test (Exact Sig. 2 sided)	Kappa value (Approx. Sig)
		Susceptible	Susceptible-Dose Dependent	Resistant		
Disk Diffusion for Ketoconazole	Susceptible	28	-	-	0.012	0.304 (0.000)
	Susceptible-Dose Dependent	-	-	-		
	Resistant	2	2	-		

Table2: Comparison of the results of Antifungal susceptibility testing to Fluconazole and Ketoconazole by Broth Macrodilution and DiscDiffusion methods for non-albicans Candida

		Broth Dilution for Fluconazole			Fisher's Exact Test (Exact Sig. 2 sided)	Kappa value (Approx. Sig)
		Susceptible	Susceptible-Dose Dependent	Resistant		
Disk Diffusion for Fluconazole	Susceptible	20	3	-	0.000	0.680 (0.000)
	Susceptible-Dose Dependent	-	-	-		
	Resistant	-	-	4		
		Broth Dilution for Ketoconazole			Fisher's Exact Test (Exact Sig. 2 sided)	Kappa value (Approx. Sig)
		Susceptible	Susceptible-Dose Dependent	Resistant		
Disk Diffusion for Ketoconazole	Susceptible	24	-	-	0.000	0.471 (0.000)
	Susceptible-Dose Dependent	-	-	-		
	Resistant	-	3	-		

DISCUSSION

In this paper, we compared the results of antifungal susceptibility testing by broth macro dilution and disc diffusion methods in *C. albicans* and non-albicans isolates against two antifungal agents, viz. Fluconazole and Ketoconazole, and observed significant agreement between them. These results, hence, underscore the feasibility of using the technically simpler Disc Diffusion method for routine antifungal susceptibility testing in Candida isolates within the operational setting of a diagnostic Microbiology laboratory.

Our findings assume significance in view of the increasing incidence of Candidemia and other invasive Candidiasis infections in the contemporary health care

scenario [7-9] and the continuing emergence of non-albicans Candida species as significant human pathogens. [9,10] The current guidelines of the Infectious Diseases Society of America (IDSA) have defined clear indications for the use of Fluconazole in Candida infections. Fluconazole is recommended as one of the initial agents for the treatment of Candidemia in non-neutropenic adult patients and less critical neutropenic patients who have not had recent azole exposure. Secondly, transition from echinocandins or Amphotericin B to Fluconazole has been recommended in stable patients and in patients with isolates that are likely to be susceptible to Fluconazole, e.g. *C. albicans*. Among non-albicans *Candida species*, use of Fluconazole has been recommended for *Candida parapsilosis* and for continuation therapy of patients who have clinically improved with initial fluconazole use, and whose follow-up culture results are negative. Fluconazole has also been recommended as an alternative to Amphotericin B for neonatal candidiasis and for prophylactic therapy of neonates weighing <1000 g in nurseries with high rates of invasive candidiasis and for prophylactic use in high-risk settings like solid-organ transplant recipients, ICU patients, neutropenic patients receiving chemotherapy and stem cell transplant recipients at risk of candidiasis. [11] Increasing use of Fluconazole has also been incriminated as one of the factors responsible for the rising incidence of Fluconazole resistance among Candida isolates. [12,13] The balance between prudent use and overuse of Fluconazole can be achieved by the incorporation of antifungal susceptibility testing within the routine workflow of a Clinical Microbiology laboratory. This calls for a technically simple and less cumbersome test, like the Disc Diffusion method, that delivers results comparable to the gold standard Broth Dilution Assay. Given the methodological similarity of the Disc Diffusion method with the widely practiced Kirby Bauer method of antibacterial sensitivity testing and considering the significant agreement between the two methods of antifungal sensitivity testing observed in the present study, it is imperative that the Disc Diffusion method can fulfill the existing gap in the routine performance of antifungal susceptibility testing. Routine performance of antifungal susceptibility testing can also assist in tailoring empirical antifungal regimens, based on locally prevalent susceptibility profiles.

Our findings are in agreement with previous authors who have also reported high rates of agreement between the two methods of antifungal susceptibility testing. Diekema et al in their study noted that the categorical agreement between the agar-based method and broth macro dilution results was 98%. [14] Similarly, Noake et al, in their study reported 94.7% agreement between the two methods. [15] Likewise, Basu et al reported 95.5% correlation between susceptibility results of disk diffusion test and BMD-MIC test. [16] A similar study by Pfaller et al showed that the agreement between the disk diffusion test results and BMD-MIC was only 87.4%. [17] Capoor et al reported 85.3% agreement between the BMD-MIC and DD method. [18]

However, our study suffered from several limitations. Firstly, we did not compare the two methods with respect to susceptibility of the recovered isolates to echinocandins and newer azoles like voriconazole and posaconazole. Secondly, the number of isolates belonging to the individual species of Candida was relatively small. Accordingly, it would be prudent to validate the findings of this pilot study with optimum number of isolates belonging to the different species of Candida and also to observe the agreement between the two methods for susceptibility to other antifungal agents.

CONCLUSION

The results of this pilot study show that the less laborious disc-diffusion test demonstrates significant agreement with the

broth dilution method of antifungal susceptibility testing, thereby offering a scope of implementing this method in clinical laboratories; similar to the practice adopted for bacterial isolates.

REFERENCES

1. Kanafani ZA, Perfect JR. Resistance to antifungal agents: mechanisms and clinical impact. Clin Infec Dis. 2008; 46: 120-8.
2. Pfaller MA, Diekema DJ. Rare and emerging opportunistic fungal pathogens: concern for resistance beyond Candida albicans and Aspergillus fumigatus. J Clin Microbiol. 2004; 42: 4419-31.
3. Kotwal A, Biswas D, Sharma JP et al. An observational study on the epidemiological and mycological profile of Candidemia in ICU patients. Med Sci Monit. 2011; 17(11):663-68.
4. Badiee P, Hashemizadeh Z. Opportunistic invasive fungal infections: diagnosis & clinical management. Indian J Med Res. 2014; 139(2):195-204.
5. Clinical Laboratory Standards Institute (formerly-National Committee for Clinical Laboratory Standards). Method for Antifungal Disc Diffusion Susceptibility Testing of yeasts: Propose Guideline M44-A. NCCLS, Wayne, PA: 2004.
6. Clinical Laboratory Standards Institute (formerly- National Committee for Clinical Laboratory Standards). Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts: Approved standard - Second Edition. NCCLS document M27-A2. 2nd ed. Wayne, PA: 2002.
7. Kothari A, Sagar V. Epidemiology of candida bloodstream infections in a tertiary care institute in India. Indian J Med Microbiol. 2009; 27(2):171-2.
8. Xess I, Jain N, Hasan F et al. Epidemiology of candidemia in a tertiary care centre of north India: 5-year study. Infection. 2007; 35(4):256-9.
9. JK Oberoi. Invasive Candidiasis. JIMSA.2010; 23: 125.
10. Nazir A. Non albicans Candida in Neonatal Septicemia-An emerging clinical entity. International Journal of Biomedical Research. 2016; 7:2.
11. Pappas PG, Kauffman CA, Andes D et al. Infectious Diseases Society of America. Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. Clin Infect Dis. 2009; 48: 503-35.
12. Healy CM, Campbell JR, Zaccaria E et al. Fluconazole prophylaxis in extremely low birth weight neonates reduces invasive candidiasis mortality rates without emergence of fluconazole- resistant Candida species. Pediatrics.2008; 121:703-710.
13. Kaufman D, Boyle R, Hazen KC et al. Fluconazole prophylaxis against fungal colonization and infection in preterm infants. N Engl J Med.2001; 345:1660-1666.
14. Diekema DJ, Messer SA et al. Evaluation of E test and Disk diffusion Methods compared with Broth microdilution Antifungal Susceptibility Testing of Clinical Isolates of Candida species against posaconazole. J Clin Microbiol. 2007; 45: 1974-7.
15. Noake T, Kuriyama T, White PL et al. Antifungal Susceptibility Testing of Candida *species* using the Clinical and laboratory Standards Institute Disk diffusion and Broth microdilution Method. J Clin Microbiol. 2007; 19(3): 283-7.
16. Basu S, Chakraborti B, Das S. Susceptibility of Candida specie isolated from HIV infected and newborn candidemia patients to AMB. J Biol Sciences.2010; 10(2): 109-13.
17. Pfaller MA, Diekema DJ, Rinaldi MG et al. Results from the ARTEMIS DISK Global Antifungal Surveillance Study: a 6.5-Year Analysis of Susceptibilities of Candida and other yeast species to Fluconazole and Voriconazole as determined by CLSI Standardized Disk Diffusion. J Clinical Microbiol. 2005; 43: 5848-9.
18. Capoor MR, Mair D, Deb M et al. Emergence of non albicans Candida spp. and antifungal resistance in a tertiary care hospital. Jpn J Infect Dis. 2005; 58(6):344-8.

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