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Comparison of In-Vitro Antifungal Sensitivity Testing Methods - Broth Microdilution and Modified Agar Dilution: An Analytical Study on Clinical Candida Isolates

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Original Research Article

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Abstract: The increasing incidence of clinically significant *Candida* infections and raise in antifungal resistance among these isolates necessitates routine antifungal susceptibility testing in clinical laboratories. The broth dilution method recommended by Clinical Laboratory Standards Institute (CLSI) is very much labour-intensive to be used regularly in such laboratories. In our study, we have compared two in-vitro antifungal sensitivity testing methods and the concordance between these methods was evaluated. This was a comparative analytical study conducted in the Department of Microbiology. *Candida* isolates obtained during July-December 2017 from various clinical samples were included in the study. The minimum inhibitory concentration (MIC) of Amphotericin B and Fluconazole was evaluated using the two methods viz. broth microdilution method and modified agar dilution method. The discrepancies between the two methods were noted and inter-test agreement was analyzed using Cohen's weighted kappa statistic, concordance correlation coefficient and Bland-Altman analysis. A total of 25 *Candida* isolates obtained during the study period were subjected to the two test methods. Agar dilution method showed a moderate level of agreement with reference broth dilution method when tested for fluconazole as well as amphotericin B. The disagreements were found to be as a result of correctable systematic error i.e greater precison (≅ 0.9) and lesser accuracy (≅ 0.5). The trend of systematic error was quantified and with proper adjustments, this can be reduced to a greater extent. Since the clinical laboratory needs reliable and less labour-intensive alternative methods for the determination of the MICs of antifungal agents, we compared these two methods. The findings of this study need to be confirmed with more number of isolates and proposed modifications, to be adopted for regular use in laboratories.

Keywords: Candida, broth-dilution, agar dilution, antifungal susceptibility, comparison.

INTRODUCTION

The importance of invasive fungal infections have increased more than ever before. The epidemiology of these infections is constantly evolving and there have been remarkable changes concerning the host factors, the infecting fungi, and the antifungal agents in the past two decades [1, 2]. Among the invasive fungal infections, *Candida* infections constitute a major proportion [1]. The increasing incidence of clinically significant *Candida* infections and raise in antifungal resistance among these isolates necessitates routine antifungal susceptibility testing in clinical laboratories. There is a need for development of simpler and standard *in-vitro* antifungal susceptibility assays to be used in these laboratories. The reference broth dilution method recommended by Clinical Laboratory Standards Institute (CLSI) is very much labour-intensive to be used regularly in such laboratories. The aim of our study is to test the utility of a modified agar dilution method, a relatively simpler method, in place of broth dilution test for antifungal susceptibility testing in our laboratory. In our study, we have compared a modified agar dilution method against the standard reference method and the concordance between these methods was evaluated.

MATERIALS & METHODS

Study design

This was a comparative analytical study conducted in the Department of Microbiology. The same isolates were subjected to both tests viz. broth dilution and agar dilution, and the minimum inhibitory

concentration (MIC) for every isolate from both tests was recorded for comparative analysis as described below.

Isolates

Candida isolates obtained from various clinical samples from July to December 2017 were selected for the analysis. Isolates were identified and speciated by using standard conventional microbiological techniques namely, Gram stain, germ tube test, Corn-meal agar morphology and HiCrome *Candida* agar morphology [3, 4].

Antifungal susceptibility testing

The invitro antifungal susceptibility testing of the study isolates was done using two methods. Two antifungal agents namely, amphotericin B and fluconazole were used and their MIC was evaluated using broth dilution method and modified agar dilution method. Standard powders of amphotericin B (Product code-CMS462) and fluconazole (Product code-CMS8387) were procured from HiMedia Laboratories Pvt. Ltd. The stock solutions of the drugs were made after standardizing for assay potency as appropriate. Distilled water was used as solvent for fluconazole and dimethyl sulfoxide (DMSO) was used solvent for amphotericin B. The stock solutions were made at 10x concentration for fluconazole (640 µg/ml) and 100x concentration for amphotericin B (1600 µg/ml) in their respective solvents. Aliquots of stock solutions were stored at -20°C. Drug dilution range of 0.12-64 µg/ml and 0.03-16 µg/ml were tested for fluconazole and amphotericin B respectively in both testing methods [5].

METHOD 1 - BROTH MICRODILUTION

The standard reference method recommended by CLSI was used as briefed below. The test medium Roswell Park Memorial Institute (RPMI) 1640 broth medium with 2% dextrose was employed. All isolates were freshly subcultured onto Sabouraud dextrose agar a day before and incubated at 37°C. The inoculum was prepared by picking five colonies of ~1 mm in diameter from 24-hour old cultures and suspended in 5 mL of sterile normal saline(0.85%). The resulting suspension was vortexed for 15 seconds and the cell density adjusted with a turbidometer by adding sufficient sterile saline to increase the transmittance to that produced by a 0.5 McFarland standard. This procedure would yield a yeast stock suspension of 1 x 10^6 to 5 x 10^6 cells per mL. A working suspension was made by a 1:50 dilution followed by a 1:20 dilution of the stock suspension with RPMI 1640 broth medium, which would result in 1 x 10^3 to 5 x 10^3 cells per mL (2X needed concentration). Test was performed by using sterile, disposable, 96-well microdilution plates. The 2x drug concentrations were dispensed into the wells in 100-µL volumes and each well of a microdilution tray was inoculated with 100 µL of the corresponding 2x diluted inoculum suspension. The growth control wells contained 100 µL of sterile, drug-free medium and were inoculated with 100 µL of the corresponding diluted (2x) inoculum suspensions. The microdilution plates were incubated at 37 °C and observed for the presence or absence of visible growth. A numerical score, which ranges from 0 to 4, was given to each well using the following scale: 0, optically clear; 1, slightly hazy; 2, prominent decrease in turbidity; 3, slight reduction in turbidity; and 4, no reduction of turbidity. The MIC for amphotericin B was defined as the lowest concentration in which a score of 0 (optically clear) was observed and, for fluconazole, as the lowest concentration in which a score of 2 (prominent decrease in turbidity) was observed. The readings were taken at the end of both 24 hour and 48 hour [5-7].

METHOD 2 – AGAR DILUTION

The test medium used was Mueller-Hinton agar with 2% dextrose. The medium was autoclaved and 9 ml was poured onto each petri-plate containing 1ml of appropriately diluted (10x) working solution of fluconazole. For amphotericn B, 9.9 ml was poured onto each petri-plate containing 0.1ml of appropriately diluted (100x) working solution. A similar ten set of plates ranging 0.12-64 µg/ml for fluconazole and 0.03-16 µg/ml for amphotericin B were prepared. The test isolates were subcultured and inocula were prepared in the same manner described for broth microdilution method. Ten microliter of standardized suspension was inoculated on the media incorporated with antifungal agents along with control plates. The inoculated plates were read at 24 hours and 48 hours. The MIC was the lowest drug concentration preventing growth of macroscopically visible colonies on drug-containing plates when there was visible growth on the drug-free control plates

STATISTICAL ANALYSIS

The MIC value for each isolate obtained from both methods were recorded using MS-Excel. The frequency distribution and descriptive parameters were calculated using the same. The comparative analysis between the two different methods was done using MedCalc v18. The discrepancies between the two methods were noted and inter-test agreement was analyzed using Cohen's weighted kappa statistic and concordance correlation coefficient. Bland-Altman plot was constructed to analyse the disagreement graphically with a supplementary Passing & Bablok regression analysis.

RESULTS

A total of 25 isolates obtained during the study period were included for this comparative analysis. The MIC values measured using broth microdilution method were used as standard for comparative analysis. The mean MIC of fluconazole for all the 25 isolates was 6.59 µg/ml (± 8.77 µg/ml). The MIC_{50} and MIC_{90} values were observed to be 4 µg/ml and 16 µg/ml respectively. The mean MIC of amphotericin B for all the 25 isolates was 0.1878 µg/ml (± 0.21 µg/ml). The MIC_{50} and

MIC_{90} values were observed to be 0.1250 µg/ml and 0.5 µg/ml respectively The species-wise descriptive summary is shown in Table-1 & 2 for fluconazole and amphotericin B respectively.

Table-1: Distribution summary of fluconazole MIC values categorized for different *Candida* species

Organism	Mean (± S.D)	MIC_{50}	MIC_{90}
Candida albicans	1.92 (± 1.54 µg/ml)	1.5 µg/ml	4 µg/ml
Candida parapsilosis	3.2 (± 1.09 µg/ml)	4 µg/ml	4 µg/ml
Candida tropicalis	5.1 (± 3.97 µg/ml)	8 µg/ml	8 µg/ml
Candida glabrata	20.8 (± 10.73 µg/ml)	16 µg/ml	32 µg/ml

Table-2: Distribution summary of amphotericin B MIC values categorized for different *Candida* species

Organism	Mean (± S.D)	MIC_{50}	MIC_{90}
Candida albicans	0.17 (± 0.14 µg/ml)	0.1250 µg/ml	0.3750 µg/ml
Candida parapsilosis	0.11 (± 0.08 µg/ml)	0.06 µg/ml	0.25 µg/ml
Candida tropicalis	0.15 (± .09 µg/ml)	0.1250 µg/ml	0.25 µg/ml
Candida glabrata	0.35 (± 0.41 µg/ml)	0.1250 µg/ml	1 µg/ml

The MIC values from broth microdilution method and agar dilution method were compared. The agreement between the two tests were compared using Cohen's weighted *kappa* statistic (κ) and the measures are shown in Table-3. Agar dilution method showed a moderate level of agreement with reference broth dilution method when tested for fluconazole as well as amphotericin B. The concordance correlation co-efficient between the two methods for determination of fluconazole MIC was 0.4821 and for amphotericin B it was 0.4346 as shown in Table-4. The disagreements in both cases where found to be as a result of correctable systematic error showing greater precison (≅ 0.9) and lesser accuracy (≅ 0.5). A scatter diagram for each drug showing the correlation and trend of the agreement between two tests is shown figure-1 and figure-2.

Table-3: Inter-method agreement analysis using Cohen's weighted kappa statistic (κ) for both drugs

	Fluconazole	Amphotericin B
Kappa*	0.4821	0.4303
Standard error	0.0693	0.0850
95% CI	0.3464 to 0.6179	0.2638 to 0.5968

Cohen's weighted kappa (κ) (quadratic weights)

Table-4: Concordance correlation coefficient computed between the two methods for both drugs

	Fluconazole	Amphotericin B
Concordance correlation coefficient	0.4821	0.4346
95% Confidence interval	0.2884 to 0.6380	0.2517 to 0.5874
Pearson ρ (precision)	0.8574	0.8643
Bias correction factor C_b (accuracy)	0.5623	0.5028

Bland-Altman plot was constructed to quantify the systematic error observed in agar dilution method. The ratio between the MIC values found in agar dilution method and broth microdilution method were plotted in Y-axis against the broth microdilution MIC values in X- axis as shown in Figure 3 and 4 for fluconazole and amphotericin B respectively.

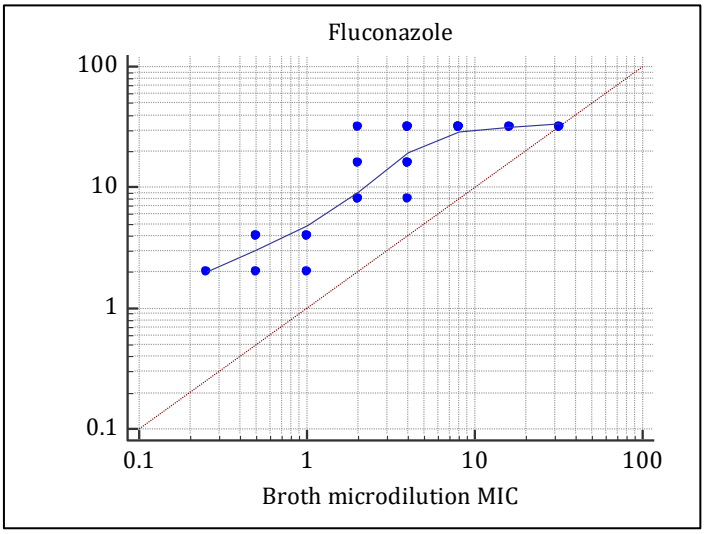

Fig-1: Scatter diagram with trend line (after logarithmic transformation) showing a correctable systematic error in agar dilution method for determination of fluconazole MIC

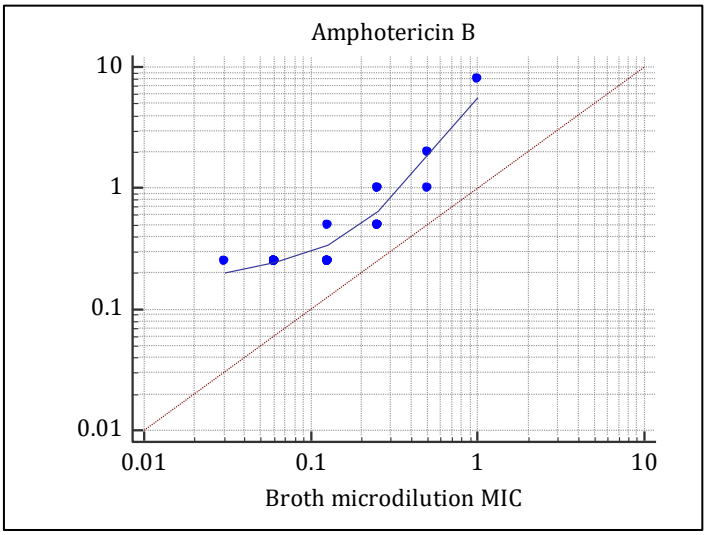

Fig-2: Scatter diagram with trend line (after logarithmic transformation) showing a correctable systematic error in agar dilution method for determination of amphotericin B MIC

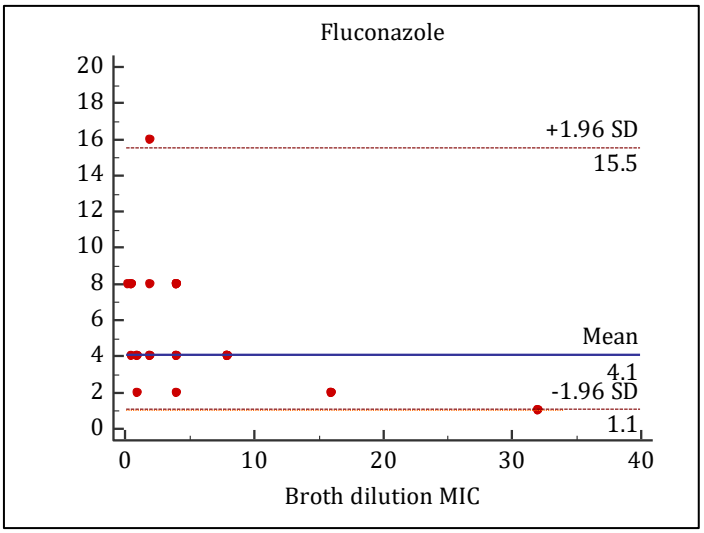

Fig-3: Bland-Altman plot for fluconazole MIC

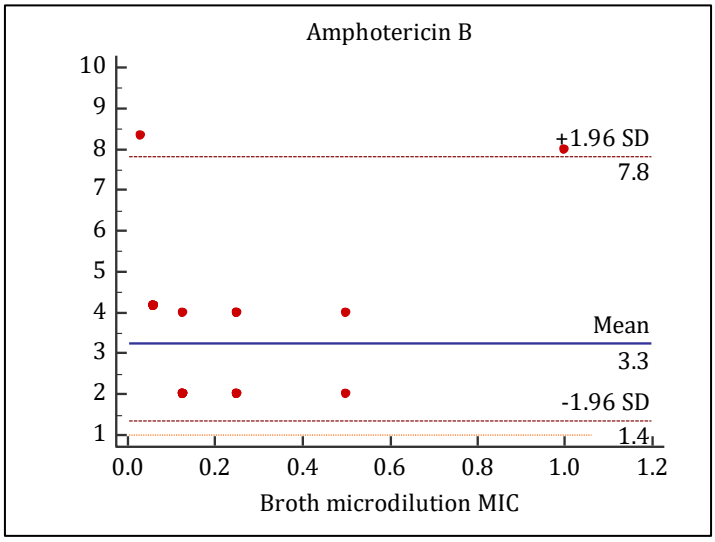

Fig-4: Bland-Altman plot for amphotericin B MIC

In determination of fluconazole MIC, the agar dilution method showed 3.26 times (95% CI: 2.71 to 3.92; p<0.0001) higher MIC than broth microdilution method and similarly with amphotericin B, the agar dilution method showed 4.11 times (95% CI: 3.12 to 5.44; p<0.0001) higher MIC than broth microdilution method. In both cases, approximately 4 fold higher MIC values were obtained with agar dilution method compared to broth dilution method. As a supplementary model for Bland-Altman plot, Passing & Bablok regression was computed for both drugs as shown in Table 5. The graphical representation of regression is shown in Figure-5.

Table-5:

	Fluconazole	**Amphotericin B**
Regression equation	y = 1.07 + 3.73 x	y = 0.13 + 2.00 x
Slope	3.7333	2.0000
Deviation from linearity	No significant deviation from linearity (P=0.32)	No significant deviation from linearity (P=0.15)

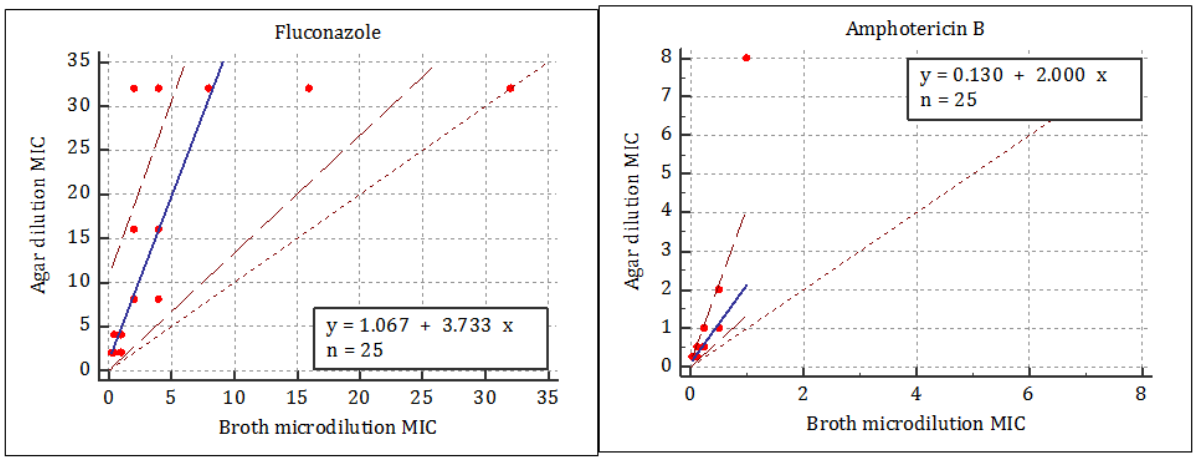

Fig-5: Passing & Bablok regression model for fluconazole MIC & amphotericin B MIC

DISCUSSION

As antifungal usage becomes more widespread and resistance develops, antifungal susceptibility testing remains an area of rapid development with a high impact on clinical practice. Accurate and timely susceptibility information ensures that patients will receive the most appropriate therapy with the highest chance for clinical success [8].

In this study, various *Candida* isolates from clinical specimens were used to compare a modified agar dilution method to the CLSI reference method. The comparison between the two methods was done for determination of fluconazole MIC and amphotericin B MIC. In all the instances which showed discrepancy, the agar dilution method showed higher MIC values

than the reference broth microdilution method. In a study by Werk *et al*., they observed higher MIC values in broth dilution method when compared with agar dilution method [9]. However, in another study by *Yoshida et al*., agar dilution method showed higher MIC values in agar dilution for most of their test isolates [10]. This contrast could be attributed to the difference in the agar media and inoculum density between the studies, which necessitates validation of these methods in respective laboratories. In most of the studies they observed non-significant differences in the MIC values between the two methods and the existing differences were attributed to difference in media composition [9-11]. In our study, there was a significant difference between the two methods. However, the differences were in terms of accuracy (i.e lower accuracy) and the agar dilution method showed greater precision when tested for both drugs. This indicates the existence of a systematic error which can be corrected by adjusting the inoculum density and media concentration appropriately. If adjusted, agar dilution method for both these drugs can give results with higher accuracy and precision.

LIMITATION

Few studies have reported the influence of oxygen and CO_2 concentration in the outcome of these MIC determination methods which was not considered in our study [10]. This analysis should be repeated with more number of isolates and necessary modifications in the inoculum density / media composition to be adopted for regular use in our laboratory.

CONCLUSION

Since the clinical laboratory needs reliable and less labour-intensive alternative methods for the determination of the MICs of antifungal agents, we compared these two methods. The modified agar dilution test evaluated in our study as an antifungal susceptibility testing method can be a reasonable method for determination of minimum inhibitory concentration (MIC) in the line of reference broth dilution method recommended by Clinical Laboratory Standards Institute. However, it should be evaluated more prospectively with necessary modifications proposed, to be implemented as a routine procedure in the laboratory.

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