



Original Research Article

Evaluation of E Test Strips EASYMIC of Fluconazole & HICOMB MIC of Ketoconazole for Antifungal Susceptibility Testing of Trichophyton Species

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ABSTRACT

In vitro antifungal susceptibility testing is important to guide the clinicians to choose the correct antifungal for patients. Broth microdilution method (BMD) recommended by CLSI is the reference method for antifungal susceptibility testing of filamentous fungi. However, the method is time consuming and labour intensive and not practical for clinical laboratories. Therefore alternate approaches are in demand. E test is surface agar based quantitative MIC determination method. We evaluated EASYMIC (Fluconazole) and HICOMB MIC (Ketoconazole) manufactured by HIMEDIA, Mumbai for MIC determination against 60 isolates of Trichophyton species against BMD. MIC ranges for *T. rubrum*, *T. mentagrophyte* and *T. tonsurans* for Fluconazole were 4-256, 1-64 and 0.5-2 respectively and MIC range of 0.25-2 for Ketoconazole for three species. Determination of MIC by E test seems to be easy alternative to BMD for Fluconazole and Ketoconazole. MICs noted for Ketoconazole by E test correlated well with BMD method but not for Fluconazole.

Keywords: antifungal susceptibility, E test, MICs, Dermatophytoses, Trichophyton,

INTRODUCTION

Dermatophytes have the capacity to invade keratinized tissues producing dermatophytosis. *Trichophyton rubrum* and *Trichophyton mentagrophyte* are two of the most frequently isolated dermatophytes. ⁽¹⁾ Although an increasing number of antimycotics has become available for the treatment of dermatophytosis, there are reports suggesting recalcitrance to therapy or possibly even resistance of dermatophytes to antimicrobial agents. ⁽²⁾ Successful treatment depends on the ability of given antimycotic agents to eradicate the fungus.

In order to predict this ability, in vitro susceptibility testing becomes helpful to guide clinicians to choose the correct treatment for patients. ⁽¹⁾ Broth microdilution method recommended by CLSI is the reference method for antifungal susceptibility testing of filamentous fungi. ⁽³⁾ However, this method is time consuming and labour intensive and not practical for clinical laboratories. Therefore alternate approaches are in demand. E test is a surface agar based quantitative MIC method. E test commercially available from various manufactures are evaluated for

Trichophyton species⁽²⁾ ESYMIC and HICOMB MIC manufactured by HIMEDIA has not been evaluated for Trichophyton species. Therefore the present study was undertaken to evaluate the ESYMIC and HICOMB for MIC determination against Trichophyton species.

MATERIALS AND METHODS

A total of 60 strains of Trichophyton species (30 strains of *Trichophyton rubrum*, 14 of *Trichophyton mentagrophyte* and 9 of *Trichophyton tonsurans*) isolated from skin scrapping, hair, and nail were included in the study. Isolates of Trichophyton were identified as per standard laboratory techniques.⁽⁴⁾ Each Trichophyton isolate was maintained in sterile distilled water at room temperature (25^o C) before testing. Strains were subcultured on potato dextrose agar and incubated at 30^o C for 7 days before testing to ensure viability of the inoculums. Control strains of *Aspergillus fumigatus* and *Candida parapsilosis* were also included in the study as reference strains and were tested thrice with both methods.

Antifungal susceptibility testing was performed by broth microdilution method⁽³⁾ and by E test method using EZYMIC for Fluconazole and HICOMB MIC for Ketoconazole. The pure antifungal powders of the (Fluconazole and Ketoconazole) and E test strips (ESYMIC and HICOMB) were procured from HIMEDIA, Mumbai. E test strips were stored at -20^oC until use. Medium: RPMI-1640 agar (1.5%) supplemented with glucose (2%) and buffered to pH 7.0 with MOPS was used.

Preparation of inoculums: All Trichophyton species were sub cultured on Sabouraud dextrose agar with cycloheximide to ensure purity and viability. For conidia formation in *Trichophyton mentagrophyte* and *Trichophyton tonsurans* PDA was used. As conidia formation in *T. rubrum* was very poor in PDA, we used oat meal agar for

inducing conidia formation as recommended by the study.⁽⁵⁾ Trichophyton colonies grown on PDA and oat meal agar were covered with 1 ml of sterile 0.85% saline and gently probed with the sterile swab. Suspension was prepared and allowed to settle for 5 to 10 minutes. Conidia were counted with hemocytometer. Suspension was then diluted 1:50 in RPMI-1640 to standardize the inoculums density 0.5 McFarland standard was used. It corresponds to density of approximately 0.4×10^4 to 5×10^4 CFU/ml.

Procedure: All strains were tested against three antifungal agents by E test according to manufactures instructions. Inoculum suspension was prepared as follows. Plates of 90mm with 4-5 mm of depth were used. The agar surface was streaked dipping a sterile swab into inoculums suspension and streaked in to three directions. After plate dried, the test strip was applied to each plate. The plates were incubated at 30^o C. The results were read after 72 hours for *Trichophyton mentagrophyte* and after 96 hours for all other trichophyton species.⁽⁶⁾

MIC endpoints: MIC was defined as the lowest drug concentration at which the border of the elliptical inhibition zone intercepted the MIC scale on the E test strip. In case of ImmunoComb, comb showing lower MIC was considered.

Broth microdilution method: Antifungal agents: Pure powders of Ketoconazole and Fluconazole were obtained from HIMEDIA, Mumbai. Stock solutions of drugs were prepared at 1600 ug and 1200 ug for Fluconazole using DMSO and were kept at -20^oC until use.

Medium: RPMI-1640 broth at pH 7.0 with MOPS was used for broth microdilution.

Procedure: CLSI⁽³⁾ reference method was used. Inoculum suspension was prepared in RPMI broth and adjusted to final concentration of 103-104 cfu/ml. Microtitre plate was incubated at 30^o C and were read

after 72 hours for *Trichophyton mentagrophyte* and after 7 days for other *Trichophyton* species. MIC was defined as lowest concentration of an antifungal drug that substantially inhibits the growth of the organism as detected visually. For broth micro dilution procedure, the amount of growth in each well is compared with that of growth control (drug free medium). Each well was then given a numerical score as follows: 4- no reduction in growth, 3- slight reduction or approximately 75% of the growth control, 2- prominent reduction in

growth or 50% of the growth control, 1- slight growth or approximately 25% of the growth control and 0- optically clear or absence of growth. (3)

Data analysis: E test MIC value falling in between two fold dilutions was rounded to next upper two fold value for purpose of comparison with broth microdilution method.

RESULTS

All the strains of *Trichophyton* species were grown on RPMI glucose agar plate.

Table1: MIC's of Trichophyton species by E test and broth microdilution method

species	No of strains tested	Antifungal agent	MIC by E test		MIC by BMD*	
			Range	GM**	Range	GM
<i>T. rubrum</i>	31	FCZ	4-256	34.58	1-64	6.6
		KTZ	0.25-2	0.81	0.25-1	0.49
<i>T. mentagrophyte</i>	21	FCZ	1-64	8.02	0.5-32	4.64
		KTZ	0.25-2	0.97	0.25-2	0.97
<i>T. tonsurans</i>	08	FCZ	0.5-1.5	1.12	0.25-.75	0.53
		KTZ	0.5-2	1.03	0.125-0.75	.39

*BMD- Broth Microdilution, **GM- Geometric mean

The data was reported as MIC ranges and geometric mean MIC values. In broth microdilution method, Fluconazole was the least active drug. For E test, high MICs were noted for Fluconazole and low MICs were reported for Ketoconazole. MIC end points were difficult to read in Ketoconazole. (Photos:1,2,3)

Table2: Percentage agreement between E test and broth microdilution methods

species	No strains tested	Agreement(%) FCZ	KTZ
<i>T. rubrum</i>	31	24(77.41)	31(100)
<i>T. mentagrophyte</i>	21	21(100.00)	20(95.23)
<i>T. tonsurans</i>	08	8(100.00)	8(100.00)

The highest level of agreement was obtained with Ketoconazole and lowest was seen with Fluconazole in three species of *Trichophyton*.

DISCUSSION

A reference microdilution method (M38-A2) is approved by the Clinical and Laboratory Standards Institute (CLSI) for

antifungal susceptibility testing of molds. (3) However, no agar-based susceptibility testing method has been standardized for the testing of dermatophytes. Advantages of a standardized disk diffusion-based assay for evaluating the antifungal susceptibility of dermatophytes include the ease of use, reproducibility, accuracy, and low cost. (7) So we performed antifungal susceptibility testing to determine the MIC using commercially available E test for two antifungal agents Fluconazole, and Ketoconazole. We compared E test and Broth microdilution methods to determine the in vitro susceptibility of *T. rubrum*, *T. mentagrophyte* and *T. tonsurans* to Fluconazole and Ketoconazole.

In our study, for MICs by E-test (Ezy MIC for Fluconazole and HiComb MIC for Ketoconazole was used. For *Trichophyton rubrum* MICs obtained were as follows: FCZ (4-256 µg/ml), and KTZ (0.25-2 µg/ml). For *Trichophyton mentagrophyte* FCZ (1-64 µg/ml) and KTZ (0.25-2 µg/ml)

and for *Trichophyton tonsurans* FCZ (0.5-1.5 µg/ml), and KTZ (0.5-2 µg/ml). Our results are comparable with Fernandez Torres et al (2003) ⁽⁶⁾ who reported MICs ranges by E test as follows: *Trichophyton rubrum* FCZ (8-256 µg/ml), and KTZ (0.25-2 µg/ml). For *Trichophyton mentagrophyte*, MICs ranges FCZ (4-256 µg/ml), and KTZ (0.25-2 µg/ml) our results were similar for FCZ and KTZ. For *Trichophyton tonsurans* MICs were FCZ (4-256 µg/ml), and KTZ (0.5-4 µg/ml). Our results are comparable with Fernandez Torres et al (2003) ⁽⁶⁾ for *Trichophyton ton-surans*. Similar results are reported by Solgum G, Findik D, Turk Dagi H, Arlan U et al (2011). ⁽⁸⁾ Increase in MIC values by E test as compared to broth microdilution was noted in this study. In our study percentage agreement between E test and a broth microdilution method was calculated as per Fernandez Torres et al (2003). ⁽⁶⁾ In case of *Trichophyton rubrum* good percentage agreement was found for Ketoconazole (100%), and least percentage agreement for Fluconazole (77.41%). In case of *Trichophyton mentagrophyte* we found good percentage agreement for Fluconazole (100%) and least percentage agreement for Ketoconazole (95.71%). However in case of *Trichophyton tonsurans*, good percentage agreement was seen for both Ketoconazole (100%), and Fluconazole (100%). However, it is difficult to compare results of the E test and broth microdilution methods due to variability in critical technical factors in different studies, including inoculum size, type of media, incubation temperature and time of reading, which may explain the different results in antifungal susceptibility testing obtained by various investigators and laboratories. ⁽²⁾

As per Fernandez Torres et al (2002) ⁽⁶⁾ the high level of disagreement seen with FCZ was ascribable to lower broth microdilution MICs for the majority of species tested than were seen in the E test.

This agrees with a previous study ⁽⁷⁾ in which they found that MICs generated using agar-based techniques (agar dilution) tended to be much higher than those produced by broth assays. These data suggest caution in interpreting the MICs of FCZ, which may falsely indicate resistance in the E-test and falsely suggest susceptibility in broth microdilution. However, there is no data on the clinical efficacy of FCZ that indicate which method is more predictive of successful outcome.

CONCLUSION

Determination of MIC by E-test seems to be easy alternative to BMD for Ketoconazole and Fluconazole. MICs noted for Ketoconazole by E test correlated well with BMD method but not for Fluconazole.

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