Original Article

Modification of Antifungal Susceptibility Testing for Aspergillus Species

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ABSTRACT

Background: In resource constrained laboratories, determination of antifungal susceptibility as described by CLSI and EUCAST guidelines is not always feasible. So, we have modified the method of Antifungal susceptibility testing (broth microdilution method) done for Aspergillus sp. to see whether the MIC values obtained are comparable with that of CLSI and EUCAST methods.

Methods: MICs for 30 isolates of Aspergillus sp. were determined against locally available drugs like amphotericin B injection (diluent-distilled water) and itraconazole granules (diluent-Dimethyl sulfoxide). SDA broth (pH 7) and RPMI 1640 are used as test medium. Broth microdilution method was followed and MICs were read at 48hrs, compared with references in CLSI and EUCAST standards. Minimum inhibitory concentration is taken as 100% inhibition of growth visually.

Result: MIC ranges observed for amphotericin B in µg/ml for A.flavus(8) is 0.5-1, A.fumigatus(6) 0.25-0.5, A.niger(10) 0.5-1, A.terreus(6) 0.5-1. Similarly MIC ranges for itraconazole are A.flavus(8) 0.5-1, A.fumigatus(6) 0.5-1, A.niger(10) 0.25-2, A.terreus(6) 0.5-1.Control strains were kept to check the quality control. These ranges are comparable to CLSI and EUCAST standards.

Conclusion: MIC ranges obtained in the study are within the ranges as published by CLSI and EUCAST guidelines. The observations in this pilot study will help for extending the method on larger number of isolates of filamentous fungi for standardisation.

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Introduction

Invasive fungal infections have not only increased in frequency but also new fungal species have been reported to cause infection, especially in immunocompromized patients. Concurrent with the increase in fungal infections, a large variety of antifungal drugs are available with different spectrum of activity. Therefore, there is a need to determine the antifungal susceptibility of isolates to available drugs[1].

Among the fungal infections, invasive aspergillosis has emerged worldwide as an important cause of infection among patients undergoing cancer chemotherapy, hematopoetic stem-cell transplantation, or solid organ transplantation. So, there is a requisite for determination of antifungal susceptibility patterns to know the MIC(Minimum Inhibitory Concentration)s at our community level. CLSI(Clinical Laboratory Standards Institute) has standardised disc diffusion method for yeasts and broth microdilution method for filamentous fungi.

To follow the broth microdilution method, procuring pure powder form of drugs is quite expensive, and also to preserve stock solutions of diluted drugs at -70˚C is not feasible in resource constrained laboratories. To overcome these, we made an attempt to simplify the broth microdilution method for *Aspergillus* sp which is user friendly, done with locally available drugs but yet comparable with that of CLSI and EUCAST[2](European committee for Antimicrobial Susceptibility Testing) references.

Aims and Objectives: Determination of MICs for *Aspergillus* species by broth microdilution method using

- locally available drugs.
- simple basic media.

Comparision of MICs of our study with those of CLSI and EUCAST.

Materials and Methods

MICs for 30 isolates of *Aspergillus* sp were determined. This includes *A.niger* 10, *A.flavus* 8, *A.fumigatus* 6 and *A.terreus* 6. Two control strains *Aspergillus flavus* ATCC 204305, *Aspergillus fumigatus* ATCC 204304 were used for quality control. The procedure followed was according to CLSI M38A reference method for antifungal susceptibility testing for filamentous fungi with some modifications.

According to CLSI M38A, medium used is RPMI 1640 broth, pure powder form of drug is used and the inoculum is set to $0.4x10^4$ - $0.5x10^5$ CFU/ml standardised spectrophotometrically[3].

In our study, initially RPMI 1640 broth was used and the procedure was later compared using SDA (Sabourauds Dextrose Agar) broth with pH 7.

Antifungal Agents: We used two locally available antifungals amphoterecin B, itraconazole. According to CLSI guidelines, powdered form of pure drug is used with Dimethylsulfoxol (DMSO) as diluent. In our work, we used Injection amphoterecin B(AMP B) with its diluent distilled water. For the second antifungal itraconazole (ITR), we used granular form of drug in capsules with DMSO as diluent. Stock solutions were prepared 1600µg/ml for both Amp B, ITZ. 20µl of diluted drug solution(each concentration 800, 400, 200...) is added to 1ml of RPMI broth.

Inoculum Preparation*:* Inoculum suspensions are prepared from fresh, mature (2- to 5-day-old) cultures grown on Saborauds dextrose agar slants. Colonies are covered with approximately 1 ml of sterile water supplemented with 0.1% Tween 20. The inoculum is standardized spectrophotometrically to 0.5 Mc Farland. The conidia are collected carefully with a sterile cotton swab and transferred to a sterile tube.

Microtitre Plate Inoculation: 100µl of broth diluted drug is added in serial dilutions to the wells. Later 100µl of inoculum is added to each well. Two controls are kept. One is Quality control with 200µl of RPMI broth without inoculum, drug and the other is growth control with 100µl of RPMI broth with 100µl of inoculum without drug.

The microtitration plate is incubated in a humid atmosphere in a sealed container or bag at 35˚C for 48 hrs. Minimum Inhibitory concentrations(MIC) were read.

Reading and Interpretation of Results: After checking the growth in control tubes, the endpoint is read visually in a good light. The concentration of drug in the first well in which there is no growth is the MIC value. These MIC values are compared with that of CLSI and EUCAST guidelines.

Results

Table 1: Comparison of MIC of standard drugs against control strains.

Table 2: Comparison of MICs of RPMI 1640 and SDA broths.

Table 3: MIC ranges obtained in the study with comparison to CLSI and EUCAST standards.

Discussion

Antifungal susceptibility testing has evolved rapidly during the last decade and has now become a relevant tool. To determine the MICs, there are several methods. However, each has its own disadvantages like broth micro dilution method is cumbersome[3], E test is relatively expensive, disk diffusion is attractive method but no standard references are available for moulds, AFST with Semi-semisolid agar[1] even requires pure form of drug which is expensive.

Coming to our study, we replaced pure powdered form of drug (costly, to be procured, difficult to stock up) with easily available injectables and capsules which are economical and can be procured in all basic laboratories. Initially, AFST was done with modified drug using two control strains, *Aspergillus flavus* ATCC 204305, *Aspergillus fumigatus* ATCC 204304. MICs obtained are within the ranges of CLSI and EUCAST (table 1).

Later, the medium RPMI 1640 (to be procured) is modified with SDA broth (pH 7) which can be prepared in all laboratories. The MICs obtained are within the ranges of CLSI and EUCAST (table 2).

The modified study with local drugs as antifungals and SDA broth (pH 7) as medium was applied to the 30 isolates

Table 1: Comparision of MIC of standard drugs against control strains

Quality control strains	MIC(µg/dl)					
	AMP B			ITR		
	Study	CLSI[1]	EUCAST[1]	Study	CLSI[1]	EUCAST[1]
Aspergillus flavus ATCC 204305	0.5	0.5-4	0.5-2	0.25	0.25-0.5	0.12-0.5
Aspergillus fumigatus ATCC 204305	0.5	0.5-2	0.25-1	0.5	0.125-1	0.12-0.5

AMP B-amphotericin B, ITR-itraconazole, MIC - minimum inhibitory concentration, CLSI-Clinical Laboratory Standards Institute, EUCAST-European committee for Antimicrobial Susceptibility Testing.

Table 2: Comparison of MICs of RPMI 1640 and SDA broths.

Isolates	MICs for AMP B (µg/ml)			MICs for ITR (µg/ml)		
	RPMI 1640 Broth	SDA broth (pH 7)	CLSI[2] (RPMI)	RPMI 1640 Broth	SDA broth (pH 7)	CLSI[2] (RPMI)
A. flavus(8)	0.5-1	0.5-1	0.5-2	0.5-1	0.5-1	0.5-1
A. fumigatus(6)	0.25-0.5	0.25-0.5	0.12-0.5	0.5-2	0.5-1	0.5->8
A. niger(10)	0.5-1	0.5-1	0.5-4	0.25-2	0.25-2	0.25-8
A. terreus(6)	0.5-1	0.5-1	0.5-4	0.5-2	0.5-1	0.5->8

References quoted according to CLSI document M38A[3], EUCAST (v 8.0)[2]. AMP B-amphotericin B, ITR-itraconazole, MIC- minimum inhibitory concentration.

Table 3: MIC ranges obtained in the study with comparison to CLSI and EUCAST standards.

Isolates	MIC(µg/ml)					
	AMP B			ITR		
	Study	CLSI	EUCAST	Study	CLSI	EUCAST
A.flavus(8)	0.5-1	0.5-2	-	0.5-1	0.5-1	0.25-8
A.fumigatus(6)	0.25-0.5	0.12-0.5	-	0.5-1	0.5->8	0.25-2
A.niger(10)	0.5-1	0.5-4	-	0.25-2	0.25-8	0.25-4
A.terreus(6)	0.5-1	0.5-4	-	0.5-1	0.5->8	0.25->8

References quoted according to CLSI document M38A[3], EUCAST (v 8.0)[1]. AMP B-amphotericin B, ITR-itraconazole, MIC- minimum inhibitory concentration.

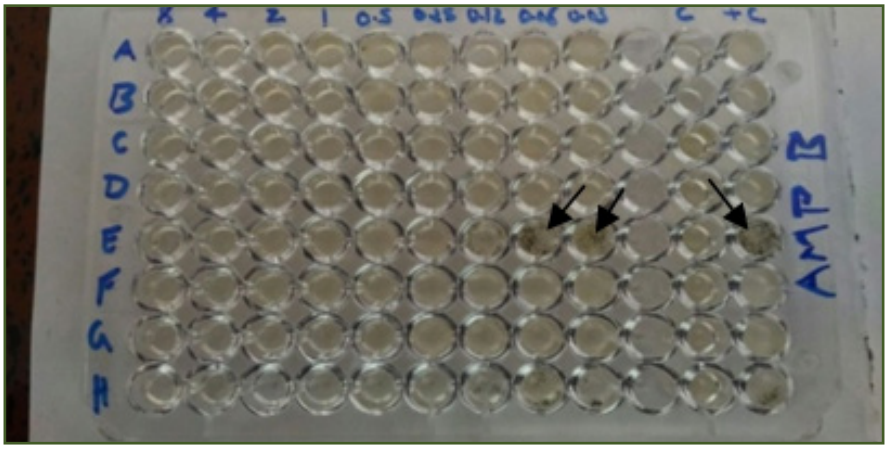

Fig. 1: Microtitre plates showing growth of Aspergillus niger in positive control wells and in wells with lower drug concentrations.

of *A.flavus*, *A.fumigatus*, *A.niger* and *A.terreus*. The MICs obtained are within the ranges of CLSI and EUCAST (table 3).

Conclusion

The MICs obtained with both RPMI 1640 and SDA broth are comparable with CLSI references where they used RPMI 1640 broth. The MICs obtained in the study for both amphotericin B (Injection) and itraconazole (Capsule) are within the reference ranges given by CLSI and EUCAST where pure powder form of drug is used.

The procedure adopted in our study is easy to perform and cost-effective. This study is helpful to adopt in all resource constrained laboratories. The observations in this pilot study will help for extending the method on larger number of isolates of filamentous fungi for standardization.

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Competing Interests

None declared

References

1. Khan S, Singhal S, Mathur T, Upadhyay DJ, Rattan A. Antifungal susceptibility testing method for resource constrained laboratories. Indian journal of medical microbiology, (2006) 24 (3):171-6.
2. C. Lass-Florl, M. Cuenca-Estrella, D. W. Denning & J. L. Rodriguez-Tudela. Antifungal susceptibility testing in aspergillus spp. according to EUCAST methodology. Medical mycology 2006, 44, s319-s325.
3. Wayne PA. National committee for clinical laboratory standards. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi approved standard. 2002 M38A.