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Original Article

In-vitro assessment of first-line antifungal drugs against *Aspergillus* spp. caused human keratomycoses

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ABSTRACT

Background and objectives: Aspergillus keratitis are in the increasing trend and reported as the second most common cause of mycotic keratitis in developing countries. The present study was designed to isolate, identify *Aspergillus* spp. from the keratits/corneal ulcer patients attending a tertiary care eye hospital, Coimbatore, South India and to assess the minimum inhibitory concentrations (MICs) against ten clinically used first-line antifungal drugs.

Methods: A total of seventy-three *Aspergillus* strains isolated from corneal scrapings were included and assessed for a period of one year. All isolates were identified up to the species level by morphological observations. Antifungal drug susceptibilities were determined against a standard panel of antifungal agents.

Conclusions: Five different species of aspergilli, *A. flavus* (n=53), *A. fumigatus* (n=14), *A. terreus* (n=9), *A. tamarii* (n=6) and *A. niger* (n=3) were identified based on morphological features. Minimum inhibitory concentration analyses indicated that, voriconazole, natamycin, itraconazole, clotrimazole, econazole followed by ketoconazole shall be the order of choices for the effective treatment for *Aspergillus* keratitis.

Introduction

Keratomycosis/fungal keratitis is a suppurative, frequently ulcerative corneal infection, caused by molds and yeasts [1]. It is a major blinding eye disease representing 30–40% of all cases of culture positive infectious keratitis in India. Over the decades, there has been an increase in the percentage of keratitis caused by filamentous fungi [2–6]. The most common and frequently involved molds in mycotic keratitis are the species of *Fusarium* and *Aspergillus* in United States of America, South America and Africa as well as in developing countries such as India [7–10]. Tropical and sub-tropical climate plays an important role in determining the predominance of certain species in fungal keratitis [8]. *Aspergillus* species are saprophytic, aerobic, colonizing and pathogenic filamentous fungi, often implicated as the etiological agent of fungal keratitis, particularly in tropical countries such as India and China [7].

Aspergillus species that are isolated from keratitis include, *A. fumigatus*, *A. niger*, *A. tamarii* and *A. terreus* [11,12]. *Aspergillus* keratitis is a serious medical crisis, since infected patient frequently presents with extreme pain and significant loss of vision, and needs

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to be accurately diagnosed and promptly treated [13–17]. The corneal scrapings from the infected individuals with redness, pain, excess tears, blurred vision, photophobia, irritation, etc. are subjected to microbiological identification of the causative organism through staining procedures and culture methods. To treat ocular fungal infections more effectively, a choice between an apt antifungal agent and a most promising antifungal concentration cannot be compromised. Further, the limited availability of antifungal drugs and the lack of effective response among the treated patients lead to inaccurate treatment in a number of cases [18]. More importantly, the degree of antifungal susceptibility of *Aspergillus* strains could vary from one isolate to another towards different antifungal compounds [19]. Also, availability of newer antifungal agents necessitates standardizing the susceptibility testing precisely against the isolates [20]. Susceptibility testing is gaining therapeutic significance and therefore, the antifungal susceptibility is to be determined at species level for each isolate of *Aspergillus* and a regular documentation of the local susceptibility patterns of the endemic isolates against different types of antifungal drugs would be more appropriate. This would also be of help in monitoring the emergence of antifungal resistant *Aspergillus* spp. if any. Hence, the aim of the present study was to isolate, identify *Aspergillus* spp. from the keratits/corneal ulcer patients attending a tertiary care eye hospital, Coimbatore, South India and to assess the minimum inhibitory concentrations (MICs) against ten clinically used antifungal agents.

Methods

Isolation of Aspergillus *spp. from corneal ulcer*

The corneal scrapings of human keratomycosis patients attending Aravind Eye Hospital, Coimbatore, India were processed appropriately to isolate the causative agent [5]. The identification of *Aspergillus* isolates was based on growth morphology on potato dextrose agar (PDA) plates and microscopically using lacto-phenol cotton blue and potassium hydroxide (KoH) wet mount preparation [21].

Determination of MIC values

The following clinically important and commonly available antifungal drugs were used for the tests: amphotericin B (AMB), miconazole (MCZ), ketoconazole (KTZ), fluconazole (FLZ) and nystatin (NYT) [HiMedia, Mumbai, India]; itraconazole (ITZ) and natamycin (NTM) [Sigma-Aldrich, St. Louis, MO, USA]; clotrimazole (CLZ), econazole (ECZ) and voriconazole (VCZ) [Aurolab, Madurai, India]. The solutions of these drugs were prepared in RPMI 1640 medium; the test drug concentrations ranged from 0.125 to 64 μg ml^{-1} and the stocks were kept at −20 °C until required. Fungal inoculum preparation and determination of the MICs of the test antifungal agents were done following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) M38-A2 document [22]. Experiments in triplicates were carried out along with the reference strain *A. flavus* ATCC 204304, for quality control. The MIC was defined as the lowest concentration of the antifungal drug that inhibited the visible growth of an isolate.

Calculation of MIC_{50} and MIC_{90}

For calculating MIC_{50} and MIC_{90}, the observed data were sort out in ascending (lowest to highest) order. From the arranged data the values were determined by the following mathematical formula.

$$MIC_{50} = N_{50}^{th} \text{observation, where, } N_{50} = \frac{Nx50}{100}; MIC_{90} = N_{90}^{th} \text{observation, where } N_{90} = \frac{Nx50}{100}$$

Results

Of a total of 1628 corneal scrapings examined during the study period, 624 (38.3%) yielded various microbial pathogens among which 73 (12 %) were confirmed as *Aspergillus* isolates. The analysis of morphology of *Aspergillus* isolates on PDA plates and lactophenol cotton blue wet mount preparation, suggested the involvement of five different species of aspergilla. Majority of the *Aspergillus* isolates were found to be the members of *A. flavus* (n = 47) followed by *A. fumigatus* (n = 13), *A. tamarii* (n = 6), *A. terreus* (n = 4) and *A. niger* (n = 3). The MIC results (Table 1) evidenced that, *A. flavus* isolates were susceptible to AMB (98%; MIC: 0.25–4 μg ml^{-1}), ITZ (98%; MIC: 0.25–1 μg ml^{-1}), CLZ (91%; MIC: 0.125–1 μg ml^{-1}), KTZ (89%; MIC: 0.125–1 μg ml^{-1}), VCZ (79%; MIC: 0.25–0.5 μg ml^{-1}) and MCZ (93%; MIC: 0.5–1 μg ml^{-1}). For the same isolates, ECZ showed an MIC range of 0.25–8 μg ml^{-1} and FLZ, NTM and NYT (2–32 μg ml^{-1}) were found to be least effective. The most effective drugs for *A. fumigatus* isolates were ITZ (0.125–1 μg ml^{-1}), VCZ (0.125–2 μg ml^{-1}), ECZ (0.25–2 μg ml^{-1}), CLZ (0.25–2 μg ml^{-1}), AMB (0.5–2 μg ml^{-1}) followed by KTZ (0.25–4 μg ml^{-1}).

ITZ (0.25–0.5 μg ml^{-1}) and VCZ (0.25–1 μg ml^{-1}) were noted to be the best antifungal drugs against *A. niger* followed by CLZ (0.5–1 μg ml^{-1}), ECZ (0.5–1 μg ml^{-1}), KTZ (1–2 μg ml^{-1}) and AMB (0.5–2 μg ml^{-1}). *A. tamarii* was found to be susceptible to VCZ (0.25–0.5 μg ml^{-1}), CLZ (0.25–0.5 μg ml^{-1}), ITZ (0.25–0.5 μg ml^{-1}), KTZ (0.25–1 μg ml^{-1}), ECZ (0.5–1 μg ml^{-1}) followed by AMB (1–2 μg ml^{-1}). *A. terreus* isolates were susceptible to ITZ (0.25–0.5 μg ml^{-1}), VCZ (0.25–1 μg ml^{-1}), AMB (0.5–1 μg ml^{-1}), ECZ (0.5–1 μg ml^{-1}), CLZ (0.5–1 μg ml^{-1}) and KTZ (1–2 μg ml^{-1}). The results also proved that FLZ, NYT, NTM and MCZ were least effective antifungal drugs against *A. fumigatus*, *A. niger*, *A. tamarii* and *A. terreus*.

While analysing the values of MIC_{50} and MIC_{90} (Table 2) it was found that, lowest MIC_{50} (0.25 μg ml^{-1}) was recorded withKTZ for *A. flavus*, followed by 0.5 μg ml^{-1} of ITZ, CLZ & VCZ. For the same isolates, MIC_{90} of 1 μg ml^{-1} ITZ, CLZ & VCZ, was noted. Simlarly, ITZ & VCZ MIC_{90} of 1 μg ml^{-1} was determined against *A. fumigatus*. For *A. niger* isolates, ITZ MIC_{90} of 0.5 μg ml^{-1} followed by CLZ, ECZ & VCZ MIC_{90} of 1 μg ml^{-1} were obtained. For *A. tamarii*, the lowest MIC_{90} (0.5 μg ml^{-1}) was exhibited by ITZ, CLZ & VCZ. With *A. terreus* isolates, MIC_{90} ITZ of 0.5 μg ml^{-1} followed by 1 μg ml^{-1} of AMB, CLZ, ECZ & VCZ were recorded.

On the whole, it was found that, AMB MIC_{50} & MIC_{90} against *Aspergillus* species was 2 μg ml^{-1} and 4 μg ml^{-1}, respectively. Lowest MIC_{90} values of 1 μg ml^{-1} and 2 μg ml^{-1}, was recorded for ITZ & VCZ and CLZ, respectively.

Discussion

Aspergillus species has a worldwide distribution; most probably results from the production of numerous airborne conidia, which easily disperse by air movements and by insects. Atmosphere composition has a great impact on mould growth, with humidity being the most influencing variable [23]. The presence of *Aspergillus* in the air is a major risk factor for both invasive and allergic aspergillosis [24]. Bharathi et al. [12] from South India reported 12% of *Aspergillus* keratitis among 1126 culture positive cases of keratitis which was in line with the present study.

Among polyenes, natamycin and amphotericin B were the commonly used antifungal ophthalmic drug. Natamycin, a naturally occurring macrolide polyene antifungal agent is reported with a broad-spectrum activity against various fungi, including *Aspergillus*

Table 1
Distribution of *Aspergillus* isolates (n = 73) based on the MIC values of antifungal agents.

Antifungal agent	Isolates	MIC Range (μg ml^{-1})	Minimum inhibitory concentration (μg ml^{-1})										
			0.125	0.25	0.5	1	2	4	8	16	32	64	>64
	A. flavus (n = 47)	0.25–8		1	3	12	16	14	1				
	A. fumigatus (n = 13)	0.5–2			1	4	8						
AMB	*A. niger* (n = 3)	0.5–2			2		1						
	A. tamarii (n = 6)	1–2				1	5						
	A. terreus (n = 4)	0.5–1			3	1							
	A. flavus (n = 47)	0.5–4			6	13	25	3					
	A. fumigatus (n = 13)	8–16							9	4			
MCZ	*A. niger* (n = 3)	16–32								1	2		
	A. tamarii (n = 6)	2–64					1		4			1	
	A. terreus (n = 4)	8–64							1	1	1	1	
	A. flavus (n = 47)	0.25–2		9	26	11	1						
	A. fumigatus (n = 13)	0.125–1	3	1	6	3							
ITZ	*A. niger* (n = 3)	0.25–0.5		1	2								
	A. tamarii (n = 6)	0.25–0.5		2	4								
	A. terreus (n = 4)	0.25–0.5		1	3								
	A. flavus (n = 47)	0.125–4	3	21	5	1	12	5					
	A. fumigatus (n = 13)	0.25–4		1	1	6	4	1					
KTZ	*A. niger* (n = 3)	1–2				2	1						
	A. tamarii (n = 6)	0.25–1		1	3	2							
	A. terreus (n = 4)	1–2				3	1						
	A. flavus (n = 47)	2–32					3	1	3	16	24		
	A. fumigatus (n = 13)	16–32								8	5		
NTM	*A. niger* (n = 3)	8–16							1	2			
	A. tamarii (n = 6)	32–64									5	1	
	A. terreus (n = 4)	NA									4		
	A. flavus (n = 47)	0.125–4	3	7	26	7	3	1					
	A. fumigatus (n = 13)	0.25–2		2	3	3	5						
CLZ	*A. niger* (n = 3)	0.5–1			2	1							
	A. tamarii (n = 6)	0.25–0.5		4	2								
	A. terreus (n = 4)	0.5–1			1	3							
	A. flavus (n = 47)	0.25–8		1	14	13	10	5	4				
	A. fumigatus (n = 13)	0.25–2		1	3	7	2						
ECZ	*A. niger* (n = 3)	0.5–1			1	2							
	A. tamarii (n = 6)	0.5–1			1	5							
	A. terreus (n = 4)	0.5–1			2	2							
	A. flavus (n = 47)	16–64								1	7	39	
	A. fumigatus (n = 13)	≥64										7	6
FLZ	*A. niger* (n = 3)	32–65									1	2	
	A. tamarii (n = 6)	NA								6			
	A. terreus (n = 4)	32–64									3	1	
	A. flavus (n = 47)	2–32					1	14	16	14	2		
	A. fumigatus (n = 13)	32–64									10	3	
NYT	*A. niger* (n = 3)	32–64									2	1	
	A. tamarii (n = 6)	16–64								1	2	3	
	A. terreus (n = 4)	32–64									3	1	
	A. flavus (n = 47)	0.25–32		13	24	9					1		
	A. fumigatus (n = 13)	0.125–2	3	4	3	2	1						
VCZ	*A. niger* (n = 3)	0.25–1		1	1	1							
	A. tamarii (n = 6)	0.25–0.5		4	2								
	A. terreus (n = 4)	0.25–1		2	1	1							

Table 2
Calculated MIC_{50} and MIC_{90} values in μg ml^{-1} against species of *Aspergillus* from keratitis.

Antifungal drugs	*Aspergillus* spp. (n = 73)		*A. flavus* (n = 47)		*A. fumigatus* (n = 13)		*A. niger* (n = 3)		*A. tamari* (n = 6)		*A. terreus* (n = 4)	
	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
AMB	2	14	2	4	2	2	0.5	2	2	2	0.5	1
MCZ	2	16	2	4	8	16	32	32	8	64	16	64
ITZ	0.5	1	0.5	1	0.5	1	0.5	0.5	0.5	0.5	0.5	0.5
KTZ	1	2	0.25	4	1	2	1	2	0.5	1	1	2
NTM	32	32	32	32	16	32	16	16	32	64	32	32
CLZ	0.5	2	0.5	1	1	2	0.5	1	0.25	0.5	1	1
ECZ	1	4	1	4	1	2	1	1	1	1	0.5	1
FLZ	64	64	64	64	64	>64	64	64	16	16	32	64
NYT	16	64	8	16	32	64	32	64	32	64	32	64
VCZ	0.5	1	0.5	1	0.25	1	0.5	1	0.25	0.5	0.25	1

species [25,26]. The present study determined a higher NAT MIC between 8 and 64 μg ml^{-1} against the test isolates of *Aspergillus* species and was in accordance with the results of Lalitha et al. [27]. Similarly, Xuguang et al. [28] from China reported as much as 72.4% *Aspergillus* isolates susceptible to natamycin. Rahman et al. [26] stated that a majority of the *Aspergillus* spp., isolated from keratits cases in Bangladesh was susceptible to NAT with MICs ranging between16 μg ml^{-1} and 32 μg ml^{-1} of NAT.

The AMB is known for its efficacy against *Aspergillus* spp. and *Candida* spp. and has been used by systemic, topical, and intravitreal routes in the treatment of fungal keratitis. In the current study, MIC values of AMB against *A. flavus* were comparable with studies of Xie et al. [23], Alastruey-Izquierdo et al. [24] and Lalitha et al. [27]. Interestingly, Qiu et al. [29] results of AMB susceptibilities against *Aspergillus* spp. (44.4%) were relatively lower than the present findings. Although this study advocated AMB, ITZ, CLZ, KTZ and VCZ are the choices for *A. flavus* infections, Xie et al. [3] stated that poor penetration into corneas and obvious stimulative symptoms make AMB topical preparation unsuitable to be administered with a large dosage and for a long time. David et al. [30] reported 20.8% of filamentous fungi were resistant to AMB. The evaluation of antifungal susceptibilities of the present study, further revealed that when to compared *A. flavus* and *A. fumigatus*, complete susceptibility of *A. terreus*, *A. niger*, and *A. tamarii* to AMB (MICs 0.25 μg ml^{-1} to 4 μg ml^{-1}) was noted. The present study confirmed an MIC range of 0.5–2 μg ml^{-1} of AMB against *A. fumigatus*, *A. terreus*, *A. niger* and *A. tamarii* and 0.25–8 μg ml^{-1} against *A. flavus*. However, Manikandan et al. [5] and Theresa et al. [31] reported that *A. fumigatus* being more susceptible to AMB. Of five species of *Aspergillus* examined in the study, *A. fumigatus*, *A. terreus*, *A. niger* and *A. tamarii* were more susceptible to AMB than *A. flavus* (MIC$_{90}$ 4 μg ml^{-1}). Contrary to this, Hahn et al. [32] and Therese et al. [31] stated that all four species of were resistant to AMB.

Among imidazoles, Myoken et al. [33] reported miconazole MIC of 1.72 μg ml^{-1} against *Aspergillus* species and was closely similar to the present study's MCZ MIC range (0.5–4 μg ml^{-1}) against *A. flavus* isolates. The other four species viz., *A. fumigatus*, *A. terreus*, *A. niger* and *A. tamari* had relatively a higher concentration of MCZ MIC. In this context, the present study does not suggest dosages of MCZ against *Aspergillus* keratitis.

Manikandan et al. [5] reported comparatively lower KTZ MIC values between 0.25 and 1 μg ml^{-1} and all the 26 corneal *Aspergillus* isolates were susceptible to ketoconazole by the E-test method. Nevertheless, the present evaluation determined KTZ MIC range of 0.125–4 μg ml^{-1} and all the test *Aspergillus* isolates were found to be 'susceptible'. Manikandan et al. [5], reported higher ketoconazole MICs compared to the present study and also stated that the ketoconazole was more effective against *A. flavus.*

Hahn et al. [32] reported that *Aspergillus* isolates from mycotic keratitis had a higher susceptibility to CLZ than AMB which is parallel to the present findings. *A. tamarii* were more susceptible (MIC value of 0.25–0.5 μg ml^{-1}) to CLZ than other species of aspergilli in the present study. However, the limited utility of clotrimazole due to low susceptibility among filamentous fungi could be a clinical concern as reported by David et al. [30].

Guinet and Mazoyer [34] stated that econozole had exhibited the best *in vitro* activity (MICs of ≤3.12 μg/mL) against *Aspergillus* strains as a majority (96%) of the test isolates were susceptible. However, Gonawardena et al. [35] reported decreased susceptibility of keratitis fungi against econozole. Although Bernauer [36] emphasized econazole usage with other antifungals, Prajna et al. [37] reported no additional benefit even with a concurrent use of 5% natamycin and 2% econazole to manage fungal keratitis. Further, Manikandan et al. [5] described a difference in aspergilli econazole susceptibility with a highest against *A. flavus* compared to *A. fumigatus* in contrast to the present findings revealing an ascending ECZ susceptibility of *A. flavus*, *A. fumigatus*, *A. niger*, *A. tamarii* and *A. terreus*.

Although David et al. [30], found only 25% of filamentous fungi isolated from corneal ulcer as itraconazole resistant, the report of Agarwal et al. [38] in which aspergilli were 100% susceptible to the drug were in consistent with the present study. Even, Hahn et al. [32] suggested that the use of itraconazole should be a primary consideration in the treatment of *Aspergillus* keratitis. Manikandan et al. [5] reported ITZ MICs of 4 μg ml^{-1} and 2 μg ml^{-1} against *A. flavus*. It was observed in the present study that all the isolates of *Aspergillus* species were susceptible to ITZ, *A. terreus*, *A. niger* and *A. tamarii* showed an ITZ MIC between 0.25 and 0.5 μg ml^{-1} and *A. flavus* (MIC$_{90}$ 1 μg ml^{-1}) and *A. fumigatus* between 0.25 and 2 μg ml^{-1}. Few other studies from different places were both congruent and contrasting against the present study. Based on E-test method, Qiu et al. [29] determined 100% (n = 9) of the test aspergilli as susceptible to ITZ and Manikandan et al. [39] noted MIC-values of 26 corneal *Aspergillus* isolates between 0.064 and 2 μg ml^{-1} with a single exception of an *A. terreus* isolate with a higher MIC of 32 μg ml^{-1}. Using Sensititre Yeast One Method, Marangon et al. [40] described the MIC values of four *Aspergillus* isolates as 0.256–1 μg/mL. However, Xie et al. [23] stated that 77.8% and 80% of *A. flavus* and *A. fumigatus*, respectively, were resistant to itraconazole.

In consensus, Marangon et al. [40] and Johnson et al. [41] reported that voriconazole was more effective against *Aspergillus* spp. Similar to the present findings, Lalitha et al. [42] reported VCZ MIC9O of 1 μg ml^{-1} against *Aspergillus*. Similarly, Pfaller et al. [43] and Lass-Flörl et al. [44] had determined comparatively lowest voriconazole concentrations required to inhibit the growth of aspergilla.

The study concluded the fact that a lowest MIC$_{50}$ value of 0.25 μg ml^{-1} was exhibited by ketoconazole against *A. flavus* isolates. Manikandan et al. [39] reported comparatively lower KTZ MIC values between 0.25 and 1 μg ml^{-1} against all the 26 corneal *Aspergillus* isolates. Precisely, 90% of *A. flavus* isolates were susceptible to ITZ, CLZ and VCZ, whereas MCZ exhibited MIC$_{90}$ of 2 μg ml^{-1}. Particularly, ITZ & VCZ had a lowest MIC value of 0.125 μg ml^{-1} against *A. fumigatus* and KTZ & CLZ against *A. flavus*. Against *A. niger* and *A. terreus*, the lowest MIC of 0.25 μg ml^{-1} was with ITZ and VCZ and was similar against *A. tamarii* by ITZ, KTZ, CLZ and VCZ. Most of the *Aspergillus* isolates were resistant to FLZ, NTM and NYT. While analyzing the MIC data, it was evident that, *Aspergillus* isolates exhibited an increasing trend in the resistance pattern towards ECZ, MCZ and AMB. Thus, from the current analyses it could be suggested that VCZ, ITZ, CLZ, ECZ followed by KTZ shall be used as the first line therapeutic agents against *Aspergillus* keratitis. Overall, *in-vitro* susceptibility testing would facilitate treatment decisions when performed in a timely manner and determination of MIC clinical breakpoints for every pathogen of *Aspergillus* spp. on case- to- case basis would certainly be an effective strategy both for a judicious therapy as well as to prevent further emergence of such pathogens.

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Conflict ofinterest

We have no conflict of interest to declare.

Ethical approval

Not required.

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