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ORIGINAL ARTICLE/ARTICLE ORIGINAL

In vitro susceptibility of filamentous fungi from mycotic keratitis to azole drugs

Sensibilité in vitro aux azolés de champignons filamenteux, agents de kératite fongique

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KEYWORDS
Mycotic keratitis;
Fungal isolates;
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Summary
Objective. — The in vitro antifungal activities of azole drugs viz., itraconazole, voriconazole, ketoconazole, econazole and clotrimazole were investigated in order to evaluate their efficacy against filamentous fungi isolated from mycotic keratitis.
Methods. — The specimen collection was carried out from fungal keratitis patients attending Aravind eye hospital and Post-graduate institute of ophthalmology, Coimbatore, India and was subsequently processed for the isolation of fungi. The dilutions of antifungal drugs were prepared in RPMI 1640 medium. Minimum inhibitory concentrations (MICs) were determined and MIC_{50} and MIC_{90} were calculated for each drug tested.
Results. — A total of 60 fungal isolates were identified as *Fusarium* spp. (*n* = 30), non-sporulating moulds (*n* = 9), *Aspergillus flavus* (*n* = 6), *Bipolaris* spp. (*n* = 6), *Exserohilum* spp. (*n* = 4), *Curvularia* spp. (*n* = 3), *Alternaria* spp. (*n* = 1) and *Exophiala* spp. (*n* = 1). The MICs of ketoconazole, clotrimazole, voriconazole, econazole and itraconazole for all the fungal isolates ranged between 16 μg/mL and 0.03 μg/mL, 4 μg/mL and 0.015 μg/mL, 8 μg/mL and 0.015 μg/mL, 8 μg/mL

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and 0.015 μg/mL and 32 μg/mL and 0.06 μg/mL respectively. From the MIC_{50} and MIC_{90} values, it could be deciphered that in the present study, clotrimazole was more active against the test isolates at lower concentrations (0.12—5 μg/mL) when compared to other drugs tested.
Conclusion. — The results suggest that amongst the tested azole drugs, clotrimazole followed by voriconazole and econazole had lower MICs against moulds isolated from mycotic keratitis.

MOTS CLÉS
Kératite mycosique ;
Sensibilité aux antifongique ;
Médicaments azolés

Résumé
Objectif. — L'activité antifongique in vitro des azolés à savoir, l'itraconazole, le voriconazole, le kétoconazole, l'éconazole et le clotrimazole a été étudiée afin d'évaluer leur efficacité vis-à-vis des champignons filamenteux isolés de kératite mycosique.
Méthodes. — Les échantillons provenant de patients consultant pour kératite fongique au Aravind Eye Hospital et au Post-Graduate Institute of Ophthalmology, Coimbatore, en Inde ont été mis en culture pour recherche de champignons. Les dilutions des antifongiques ont été réalisées en RPMI 1640. Les concentrations minimales inhibitrices (CMI) ont été déterminées et les CMI_{50} et CMI_{90} ont été calculées pour chaque antifongique étudié.
Résultats. — Soixante souches de champignons ont été isolées: *Fusarium* spp. (n = 30), moisissures ne fructifiant pas (n = 9), *Aspergillus flavus* (n = 6), *Bipolaris* spp. (n = 6), *Exserohilum* spp. (n = 4), *Curvularia* spp. (n = 3), *Alternaria* spp. (n = 1) et *Exophiala* spp. (n = 1). Les CMI du kétoconazole, du clotrimazole, du voriconazole, de l'itraconazole et de l'éconazole vis-à-vis de l'ensemble des isolats fongiques variaient respectivement entre 16 μg/mL et 0,03 μg/mL, 4 μg/mL et 0,015 μg/mL, 8 μg/mL et 0,015 μg/mL, 8 μg/mL et 0,015 μg/mL et 32 μg/mL et 0,06 μg/mL. À partir des valeurs des CMI_{50} et CMI_{90} que nous avons obtenues, le clotrimazole serait la molécule la plus active vis-à-vis des isolats étudiés, avec des concentrations (0,12 à 5 μg/mL) plus faibles que celles des autres antifongiques testés.
Conclusion. — Les résultats suggèrent que, parmi les antifongiques azolés testés, le clotrimazole suivi par le voriconazole et l'éconazole avaient les CMI les plus basses vis-à-vis des moisissures isolées de kératites mycosiques.

Introduction

Microbial keratitis is the most common severe ocular infection and may be caused by a variety of bacteria, fungi (yeasts, moulds and microsporidia) and protists (e.g. *Acanthamoeba*). It is characterized by an acute or sub-acute onset of pain, conjunctival injection and corneal ulceration with a stromal inflammatory infiltrate [42,16,6,36]. Keratitis due to filamentous fungi is believed to usually occur following trauma, the key-predisposing factor, in healthy young males engaged in agricultural or other outdoor work [8]. The traumatizing agents can be of plant or animal origin (even dust particles), that either directly implant fungal conidia in the corneal stroma, or abrade the epithelium-permitting invasion by exogenous fungi [41]. The etiologic agents of mycotic keratitis show a varying pattern with respect to geographic locality and climatic conditions [7]. More than 105 species of fungi spanning 70 genera have been reported to cause mycotic keratitis [1]. Of these, *Fusarium* spp. and *Aspergillus* spp. are the most common etiological agents of corneal ulcerations [2,5,28].

Pujol et al. [32] reported that amphotericin B (AMB) is probably the most effective drug in vivo, although there have been many clinical treatment failures. Natamycin, a tetraene polyene, has long been considered the mainstay of treatment for filamentous fungal keratitis. Although these drugs have poor ocular penetration, they have primarily been useful in cases with superficial corneal infection [29]. Azoles (imidazoles and triazoles) viz., ketoconazole (KTZ), miconazole (MCZ), fluconazole (FLZ), itraconazole (ITC), econazole (ECN) and clotrimazole (CLT), inhibit fungal ergosterol biosynthesis at low concentrations, while at higher concentrations they appear to cause direct damage to the fungal cell walls [40]. According to Srinivasan [35], ongoing research towards rapid diagnosis and specific drug therapy could minimize the morbidity caused by this preventable disease. The current knowledge on antifungal susceptibilities is mainly based on Western literature and local data available in India pertaining to filamentous fungi other than *Fusarium* and *Aspergillus* are inadequate. The present study was undertaken to isolate and identify filamentous fungi involved in mycotic keratitis from the patients attending a tertiary care eye hospital in Coimbatore, Tamilnadu, India, and to determine their in vitro susceptibility against five azole antifungal drugs by employing the Clinical and laboratory standards institute (CLSI) broth microdilution method M38-A2 document [9].

Materials and methods

Samples and fungal isolates

This non-randomized study was carried out at Aravind eye hospital and Post-graduate institute of ophthalmology, Coimbatore, India. The specimen collection was carried out between October 2012 and August 2013. Corneal scrapings were

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performed under aseptic conditions on each ulcer using a flame sterilized Kimura's spatula, after instillation of 4% preservative free lignocaine (lidocaine) [18]. Material obtained from scraping the leading edge and the base of the ulcer was inoculated directly onto 5% sheep's blood agar (SBA), chocolate agar (CA) and potato dextrose agar (PDA) (250 g of potato slices, 15 g agar, 10 g dextrose and 1000 mL distilled water), as well as into brain heart infusion (BHI) broth without gentamicin sulphate. SBA base, CA plates and BHI broth were purchased from Himedia Laboratories, Mumbai, India. Plates were incubated under aerobic conditions at 37 °C, while the PDA bottles were incubated at 27 °C for fungal growth, for 72 h. The obtained material was subjected to Gram staining and 10% potassium hydroxide mount. Any positive fungal isolate was identified to the genus level and *Aspergillus* spp. were identified to the species level based on colony morphology and lactophenol cotton blue mount preparation of the fungal cultures employing the cellophane tape flag method [3,14,24].

In vitro azole susceptibility testing

As per the CLSI guidelines, in every batch of MIC, *A. flavus* ATCC 204304 was included as reference strain [9]. All the fungal isolates were subcultured on SDA plates and incubated at 30 °C for 7 to 15 days. The inoculum suspension was prepared by harvesting the spores from mature plates into sterile distilled water. The spore suspension was then adjusted spectrophotometrically to the required optical density for each species as outlined in CLSI M38-A2 document [9], providing an inoculum concentration of 0.4×10^4 to 5×10^4 CFU/mL, which was verified by colony count. Further dilutions (1:50) were carried out using RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA).

The clinically used and commercially available azole drugs viz., ITC (Sigma-Aldrich, St. Louis, MO, USA), voriconazole (VRC) (Aurolab, Madurai, India), KTZ (Himedia, Mumbai, India), ECN (Aurolab, Madurai, India) and CLT (Aurolab, Madurai, India) were chosen for the present study. The drugs were dissolved in dimethyl sulfoxide and the dilutions were prepared in RPMI 1640 in order to achieve a range of 8—0.015 μg/mL (ECN, VRC and CLT), 32—0.06 μg/mL (ITC) and 16—0.03 μg/mL (KTZ).

For the broth microdilution method, 100 μL of each drug dilution and 100 μL of the prepared spore suspension were added into U-bottomed microtiter plate wells. Two separate wells were maintained, one as growth control (100 μL media and 100 μL inoculum) and another as sterility control (100 μL media and 100 μL water). The plates were incubated at 28° C until growth was visible in growth control. MICs were determined visually with the aid of a reading mirror and were defined as the lowest drug concentration that caused 80% inhibition of the growth in comparison to the growth control. The MIC_{50} was taken as the MIC that was the median value and similarly, the MIC_{90} was the 90th percentile value and represented the concentration of drug that would inhibit 90% of the isolates tested [10].

Results and discussion

Of 108 ocular samples that were processed, a total of 48 isolates of bacteria and 60 isolates of filamentous fungi were obtained. Each of the positive samples ($n = 60$) grew only one mold and mixed infections were not obtained. These filamentous fungal isolates were identified as *Fusarium* spp. ($n = 30$), non-sporulating moulds ($n = 9$), *Aspergillus flavus* ($n = 6$), *Bipolaris* spp. ($n = 6$), *Exserohilum* spp. ($n = 4$), *Curvularia* spp. ($n = 3$), *Alternaria* spp. ($n = 1$), and *Exophiala* spp. ($n = 1$). In the present study, the common aetiological agents of corneal ulcers were identified as filamentous fungi rather than bacteria. Similar results were obtained by Bharathi et al. [6], Manikandan et al. [24] and Homa et al. [15] in South India. Leck et al. [22] also reported similar results from South, North and East India. But other studies from Thailand and Malaysia reported that the most frequent causative agents of microbial keratitis were bacteria [34,27].

Many studies reported that among the filamentous fungi, *Fusarium* and *Aspergillus* species were identified as most common corneal pathogens [23,39,37,33]. The present study showed that 50% of corneal infections were caused by *Fusarium* spp., whereas *Aspergillus* spp. were responsible for only 10% of fungal corneal infections. Dematiaceous fungi such as *Bipolaris* spp., *Exserohilum* spp., *Curvularia* spp., *Alternaria* spp. and *Exophiala* spp. were also isolated in the present study. Similar incidences of dematiaceous fungal keratitis have been reported previously [23,39,11]. Nine (15%) of the obtained 60 fungal isolates were not identifiable owing to lack of sporulation. Similar to our findings, Srinivasan et al. [37] revealed that out of 155 fungal isolates cultured from 154 corneal ulcers, 47.1% were *Fusarium* spp., 16.1% were *Aspergillus* spp., and the remaining organisms were a diverse mixture of unusual fungal pathogens including a large number of unidentified dematiaceous (13.5%) and hyaline (9.6%) fungal species.

The commonly used antifungal medications in the treatment of fungal keratitis include polyenes (natamycin and AMB) and azoles (KTZ, FLZ, ITC and ECN). However, it is difficult to widely use topical natamycin due to its high price [43]. *Fusarium* and *Aspergillus* strains are quite sensitive to AMB, but poor penetration into corneas and obvious simulative symptoms make its topical preparation unsuitable to be administered with a large dosage and for a long-time [43]. Azoles, most commonly FLZ and ITC [4,21] are often chosen as the combined medications to reduce the toxicity and side effects of AMB.

The MIC_{50} and MIC_{90} of the azole drugs tested for various fungal isolates are shown in Table 1. CLT was the most effective antifungal drug against all the isolates tested except for the NSM, for which ITC was notably active. CLT was the only drug that was most active against (1—4 μg/mL) fusaria. VRC, ECN and CLT (0.015—0.5 μg/mL) followed by ITC (0.25—0.5 μg/mL) were more promising against *A. flavus* isolates. *Bipolaris* isolates were inhibited effectively by certain concentrations of CLT and ECN in the range of 0.125—1 μg/mL. *Exserohilum* isolates were inhibited by CLT and VRC in the concentration range of 0.03—2 μg/mL. In comparison with other drugs, VRC and CLT showed the lowest MIC range (0.125—0.25 μg/mL) for *Curvularia* isolates.

The ability to inhibit fungal isolates at such low concentration shows that CLT could be used in the first-line therapy of mycotic keratitis. However, Manikandan et al. [24] reported the requirement of a higher concentration of CLT against *Aspergillus* spp. compared to the present study. It is notable

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Table 1 In vitro susceptibility of filamentous fungi isolated from keratomycosis to azole drugs.
Sensibilité in vitro des champignons filamenteux isolés de kératomycose aux médicaments azolés.

Agents	Groups	MIC range[a] (μg/mL)	MIC_{50}[b] (μg/mL)	MIC_{90}[c] (μg/mL)	GM[d] (μg/mL)
CLT	*Fusarium* spp.	1–4	4	4	3.134
	Non-sporulating molds	0.25–4	1	2	1.166
	Aspergillus flavus	0.015–0.5	0.125	0.5	0.156
	Bipolaris spp.	0.125–1	0.125	0.5	0.197
	Exserohilum spp.	0.03–2	0.12	2	0.205
	Curvularia spp.	0–0.25	0.25	0.25	0.25
	Alternaria spp.	–	–	–	–
	Exophiala spp.	–	–	–	–
ECN	*Fusarium* spp.	2–8	4	8	5.401
	Non-sporulating molds	0.125–4	2	4	0.85
	Aspergillus flavus	0.015–0.5	0.5	0.5	0.248
	Bipolaris spp.	0.25–1	0.5	1	0.5
	Exserohilum spp.	0.06–4	0.06	4	0.244
	Curvularia spp.	0–0.5	0.5	0.5	0.5
	Alternaria spp.	–	–	–	–
	Exophiala spp.	–	–	–	–
KTZ	*Fusarium* spp.	8–16	16	16	13.928
	Non-sporulating molds	0.25–2	1	2	0.925
	Aspergillus flavus	0.25–2	2	2	1.259
	Bipolaris spp.	0.125–4	0.5	1	0.629
	Exserohilum spp.	0.03–8	0.125	8	0.416
	Curvularia spp.	1–2	1	2	1.259
	Alternaria spp.	–	–	–	–
	Exophiala spp.	–	–	–	–
ITC	*Fusarium* spp.	16–32	32	32	31.269
	Non-sporulating molds	0.06–1	1	1	0.460
	Aspergillus flavus	0.25–0.5	0.25	0.25	0.280
	Bipolaris spp.	0.25–8	0.25	0.5	0.561
	Exserohilum spp.	0.06–32	0.06	32	0.471
	Curvularia spp.	0.25–0.5	0.5	0.5	0.396
	Alternaria spp.	–	–	–	–
	Exophiala spp.	–	–	–	–
VRC	*Fusarium* spp.	1–8	4	8	4
	Non-sporulating molds	0.25–8	2	8	1.851
	Aspergillus flavus	0.015–0.5	0.125	0.125	0.110
	Bipolaris spp.	0.06–4	0.25	0.25	0.278
	Exserohilum spp.	0.06–2	0.06	2	0.205
	Curvularia spp.	0.125–0.25	0.25	0.25	0.198
	Alternaria spp.	–	–	–	–
	Exophiala spp.	–	–	–	–

MIC: minimum inhibitory concentration; Antifungal agents: CLT: clotrimazole; ECN: econalole; KCN: ketoconazole; ITC: itraconazole; VRC: voriconazole.
[a] Interval between the lowest and highest MICs.
[b] Minimum inhibitory concentration median of the antifungal agent.
[c] Minimum concentration of the antifungal agent 90th percentile.
[d] Geometric mean of MICs.

that *Fusarium* strains were susceptible to ECN at a lower MIC similar to the reports of Galarreta et al. [12]. The present findings revealed that, *Fusarium* spp. and *Exophiala* spp. were resistant to VRC and hence higher concentration of drug is required for the effective treatment. However, there are studies that have suggested that VRC may have a broader antifungal spectrum [19,20]. Similar to our findings, Lalitha et al. [17] and Iqbal et al. [31] stated that *Fusarium* spp. had highest MICs to VRC. Prajna et al. [25] reported that monotherapy with topical voriconazole cannot be recommended for filamentous fungal keratitis. Additionally, there are reports of intraocular VRC to be safe in vitro and in vivo and less toxic to the retina than AMB [26,13]. Pfaller et al. [30] reported that ITC exerts negligible activity against *Fusarium* spp. and it has also been stated that ITC has seldom been administered for *Fusarium* infections with non-univocal results [38].

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Our results indicated that higher concentration of ITC and KTZ was required for the inhibition of the involved filamentous fungal isolates, and based on the data obtained, CLT followed by VRC and ECN are the suggested antifungal agents for the first-line therapy of human keratomycoses caused by filamentous species. In addition, this study has generated MIC data for sparingly tested filamentous fungi such as *Alternaria* spp., *Bipolaris* spp., *Curvularia* spp., *Exserohilum* spp. and *Exophiala* spp.

Conclusion

Overall, the determination of MICs of the investigated five-azole antifungal drugs against the filamentous fungi causing keratitis was useful in understanding the efficacy of increased concentrations of the drugs in inhibiting fungal growth. The present study observed a variation in the overall activity of the azole drugs depending on the type of the fungal species and the drug concentration. Due to the fact that the practice of subjecting fungal isolates to antifungal susceptibility tests is uncommon across the diagnostic microbiology laboratories in India, and that the susceptibility pattern is depending from the involved fungus as well as the nature and concentration of the applied drug, it is further emphasized that the isolates should compulsorily be examined for their susceptibility to ensure an accurate therapy.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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