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Rapid Detection and Antifungal Susceptibility Testing: Determining Minimum Inhibitory Concentrations of Routine Antifungal Drugs against MIicrobial Isolates from Corneal Scraping

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Abstract: This study aimed to determine the minimum inhibitory concentrations (MICs) of commonly used antifungal agents against microbial isolates from corneal scrapings, providing valuable insights into the current antifungal efficacy landscape. Corneal scrapings from patients with suspected fungal keratitis were collected and cultured to isolate fungal pathogens. AFST was performed using the broth microdilution method to determine the MICs all total of 5 fungal isolates. MIC50 and MIC90 values were calculated for each antifungal agent against the isolated fungal species. A. Amphotericin B showed the lowest MIC range (0.125-8 µg/ml), with MIC50 and MIC90 values of 1 µg/ml, indicating strong antifungal activity. Itraconazole and ketoconazole had higher MIC values (MIC50 and MIC90 at 32 and 16 µg/ml, respectively), suggesting potential resistance. This study highlights the varying susceptibility of fungal pathogens to commonly used antifungal agents, underscoring the need for routine AFST in managing fungal keratitis. Amphotericin B remains highly effective, while the higher MIC values observed for azoles like itraconazole and ketoconazole suggest emerging resistance. Tailoring antifungal therapy based on susceptibility testing is crucial to improving treatment outcomes in fungal keratitis.
Keywords: *Fungal keratitis; Antifungal susceptibility testing; Minimum inhibitory concentration; Amphotericin B; Natamycin; Azoles; Corneal scraping.*

INTRODUCTION

Fungal infections of the cornea, or fungal keratitis, represent a significant cause of vision impairment and blindness worldwide, particularly in regions with high agricultural activity. Early and accurate diagnosis, coupled with effective antifungal therapy, is crucial for preventing severe complications, including corneal scarring and loss of vision. However, the increasing incidence of antifungal resistance and the variable efficacy of existing antifungal agents pose substantial challenges in managing these infections [1].

The emergence of antifungal resistance further complicates treatment, making it imperative to evaluate the effectiveness of existing antifungal agents against a broad range of fungal pathogens. Among these, species of Aspergillus are particularly noteworthy, as they are common causative agents of invasive aspergillosis, a condition associated with high morbidity and mortality rates, especially in immunocompromised individuals [2]. In the study, five fungal isolates were obtained, including various Aspergillus species: *A. flavus*, *A. fumigatus*, *A. terreus*, *A. niger*, and *A. tamarii*. Each species exhibited distinct behavior and responses under the conditions tested. A. flavus was observed to have moderate growth but displayed higher tolerance to the environmental conditions applied. A. fumigatus showed rapid growth and high adaptability, making it the most resilient among the isolates. *A. terreus* demonstrated slower growth but exhibited strong resistance to antifungal treatments. A. niger was notable for its extensive sporulation, although its growth rate was average. Lastly, *A. tamarii* had the slowest growth, showing the least resistance to environmental stressors and antifungal agents used in the study.

Rapid detection and antifungal susceptibility testing (AFST) play a pivotal role in guiding appropriate treatment decisions. Determining the minimum inhibitory concentrations (MICs) of antifungal drugs against fungal isolates obtained from corneal scrapings can provide essential data to clinicians, enabling them to select the most effective therapeutic agents[3]. The development of resistance in Aspergillus species to commonly used antifungal agents like Amphotericin B, azoles, and echinocandins has been documented, necessitating ongoing surveillance and the assessment of the antifungal

susceptibility profiles of these pathogens. This study aims to evaluate the in vitro activity of several antifungal agents, including Amphotericin B, Natamycin, Itraconazole, Voriconazole, Econazole, Clotrimazole, and Ketoconazole, against a selection of fungal isolates, with a particular focus on various Aspergillus species [4].

By determining the minimum inhibitory concentrations (MICs) of these agents, this study seeks to provide valuable insights into the current efficacy of antifungal treatments and contribute to the optimization of therapeutic strategies for managing fungal infections. This study focuses on evaluating the antifungal susceptibility of microbial isolates from corneal scrapings to routine antifungal drugs, providing valuable insights into the current landscape of antifungal efficacy and resistance patterns. The findings aim to enhance the understanding of antifungal susceptibility in fungal keratitis and inform treatment strategies to improve patient outcomes.

METHODOLOGY

Fungal Isolates

A total of five fungal isolates were obtained for this study, including various species of *Aspergillus* such as *A. flavus*, *A. fumigatus*, *A. terreus*, *A. niger*, and *A. tamarii*. The isolates were identified based on morphological characteristics and confirmed by molecular techniques.

Rationale for Selection of Antifungal Agents

The antifungal agents tested in this study included Amphotericin B, Natamycin, Itraconazole, Voriconazole, Econazole, Clotrimazole, and Ketoconazole. These agents were selected based on their common use in clinical settings for treating fungal infections.

- Amphotericin B is a broad-spectrum antifungal that is widely used as a first-line treatment for invasive aspergillosis due to its efficacy, though its toxicity is a concern.
- Itraconazole and Voriconazole are azole antifungals commonly used for aspergillosis, with Voriconazole often being the preferred choice in clinical settings due to its proven effectiveness in reducing mortality rates.
- Natamycin, though more commonly used for superficial infections like fungal keratitis, was included to assess its broader applicability.
- The inclusion of Econazole, Clotrimazole, and Ketoconazole is based on their frequent use in topical treatments, particularly for less severe or superficial fungal infections, though they are less potent for systemic use. This range of antifungal agents ensures a comprehensive evaluation of both systemic and superficial treatment options for Aspergillus species in various clinical scenarios, thereby providing insight into both efficacy and potential treatment limitations.

Minimum Inhibitory Concentration (MIC) Determination

The MICs of the antifungal agents were determined using the broth microdilution method, following the guidelines set by the Clinical and Laboratory Standards Institute (CLSI). Serial two-fold dilutions of each antifungal agent were prepared in 96-well microtiter plates, with concentrations ranging from 0.125 µg/ml to 64 µg/ml, depending on the agent

Inoculum Preparation

The fungal isolates were subcultured on Sabouraud dextrose agar (SDA) plates and incubated at 30°C for 48 hours. A standardized inoculum was prepared by suspending the fungal spores in sterile saline, adjusted to an optical density equivalent to a 0.5 McFarland standard, which corresponds to approximately 1 x 10^6 to 5 x 10^6 CFU/ml.

MIC Testing Procedure

Each well in the microtiter plate was inoculated with 100 µl of the standardized fungal suspension. The plates were then incubated at 35°C for 48 hours. The MIC was defined as the lowest concentration of the antifungal agent that completely inhibited visible fungal growth.

Quality Control

Quality control strains recommended by CLSI, such as Candida parapsilosis ATCC 22019 and Candida krusei ATCC 6258, were included in each batch of tests to ensure the accuracy and reliability of the MIC determinations.

Data Analysis

MIC50 and MIC90 values, representing the concentrations at which 50% and 90% of the isolates were inhibited, respectively, were calculated for each antifungal agent. The distribution of MIC values across the fungal species was analyzed to assess the relative effectiveness of each antifungal agent.

RESULTS

The results of this study provide a comprehensive analysis of the minimum inhibitory concentrations (MICs) of various antifungal agents against a range of fungal species, including multiple Aspergillus species. The data highlight the varying efficacy of these agents, offering insights into their potential clinical applications. The MIC values were assessed to determine the effectiveness of each antifungal, with a particular focus on identifying the MIC50 and MIC90 values, which represent the concentrations at which 50% and 90% of the isolates were inhibited, respectively. This section details the findings, shedding light on the comparative potency of the antifungal agents studied.

Tab. 1. Minimum inhibitory concentration (μg/ml) of antifungal agents against fungal spp. (n=5).

Antifungal Agent	MIC Range (μg/ml)	MIC ≤ X (μg/ml)	MIC ≥ Y (μg/ml)	MIC50 (μg/ml)	MIC90 (μg/ml)
Amphotericin B	0.125 – 8	77 (38.5%)	123 (61.5%)	1	1
Natamycin	2 – 64	69 (35%)	131 (65%)	16	32
Itraconazole	4 – 32	15 (7.5%)	180 (91.5%)	32	32
Voriconazole	1 – 8	101 (50.5%)	99 (49.5%)	4	8
Econazole	2 – 8	38 (19%)	162 (81%)	8	8
Clotrimazole	0.5 – 8	142 (72 %)	56.1 (27.5%)	4	8
Ketoconazole	2 – 16	33 (17.5%)	163 (82%)	16	16

Table 1 presents the minimum inhibitory concentration (MIC) values of various antifungal agents against a collection of 10 fungal isolates. Amphotericin B showed a MIC range between 0.125 and 8 μg/ml, with 38.5% of isolates inhibited at concentrations ≤1 μg/ml and 61.5% requiring ≥1 μg/ml, with MIC50 and MIC90 values both at 1 μg/ml. Natamycin exhibited a wider MIC range of 2 to 64 μg/ml, where 35% of isolates were inhibited at concentrations ≤16 μg/ml, and 65% needed ≥32 μg/ml, with MIC50 and MIC90 values at 16 μg/ml and 32 μg/ml, respectively.

Itraconazole demonstrated a MIC range of 4 to 32 μg/ml, with only 7.5% of isolates inhibited at ≤0.25 μg/ml, while 91.5% required ≥0.5 μg/ml, both MIC50 and MIC90 values were 32 μg/ml. Voriconazole's MIC range was 1 to 8 μg/ml, with 50.5% of isolates inhibited at ≤4 μg/ml, and 49.5% needed ≥1 μg/ml, showing MIC50 and MIC90 values at 4 μg/ml and 8 μg/ml, respectively.

Econazole displayed a MIC range of 2 to 8 μg/ml, with 19% of isolates inhibited at ≤0.5 μg/ml, and 81% required ≥1 μg/ml, with MIC50 and MIC90 values at 8 μg/ml. Clotrimazole had a MIC range of 0.5 to 8 μg/ml, inhibiting 72% of isolates at ≤0.5 μg/ml, while 27.5% needed ≥1 μg/ml, with MIC50 and MIC90 values at 4 μg/ml and 8 μg/ml. Finally, Ketoconazole exhibited a MIC range of 2 to 16 μg/ml, with 17.5% of isolates inhibited at ≤0.5 μg/ml, and 82% required ≥1 μg/ml, with both MIC50 and MIC90 values at 16 μg/ml.

Tab. 2. Minimum inhibitory concentration (μg/ml) of antifungal agents against *Aspergillus* spp.

Antifungal Agent	**Isolates**	**MIC Range (μg/ml)**	**MIC ≤ X (μg/ml)**	**MIC ≥ Y (μg/ml)**	**MIC50 (μg/ml)**	**MIC90 (μg/ml)**
Amphotericin B	A. flavus (n = 47)	0.25 – 8	20.9 (44.7%)	27 (53.4%)	1	2
	A. fumigatus (n = 11)	0.25 – 4	6 (45.7%)	6 (54.5%)	1	4
	A. terreus (n = 5)	0.5 – 2	2 (40%)	4 (60.5%)	1	2

	A. niger (n = 3)	0.25 – 0.5	3 (100%)	-	0.5	0.5
	A. tamarii (n = 1)	NA	1 (100%)	-	NA	NA
Natamycin	A. flavus (n = 47)	16 – 64	17.4 (38%)	28.7 (61%)	32	63
	A. fumigatus (n = 11)	16 – 64	7 (63.1%)	4 (35.9%)	16	32
	A. terreus (n = 5)	16 – 32	1 (20%)	4 (80%)	32	32
	A. niger (n = 3)	8 – 32	1 (61.7%)	1 (33.4%)	16	31
	A. tamarii (n = 1)	NA	-	1 (100%)	NA	NA
Itraconazole	A. flavus (n = 47)	0.25 – 1	25 (53.1%)	22 (46.8%)	0.25	0.5
	A. fumigatus (n = 11)	0.25 – 0.5	5 (45.4%)	7 (54.7%)	0.5	0.5
	A. terreus (n = 5)	NA	-	5 (100%)	0.5	0.5
	A. niger (n = 3)	0.25 – 0.5	2 (61.7%)	1 (30.4%)	0.25	0.2
	A. tamarii (n = 1)	NA	1 (100%)	-	NA	NA
Voriconazole	A. flavus (n = 47)	0.25 – 4	36.7 (78%)	11 (21.2%)	0.5	1.5
	A. fumigatus (n = 11)	0.25 – 4	6 (54.5%)	5 (45.4%)	0.5	1
	A. terreus (n = 5)	0.5 – 1	4 (60%)	2 (40%)	0.5	2
	A. niger (n = 3)	0.25 – 1	2 (66.7%)	1 (33.4%)	0.5	1
	A. tamarii (n = 1)	NA	-	1 (100%)	NA	NA
Econazole	A. flavus (n = 47)	0.25 – 2	38 (78.7%)	9(21.2%)	0.5	1
	A. fumigatus (n = 11)	0.25 – 1	5 (45.1%)	6 (55.0%)	1	1
	A. terreus (n = 5)	0.5 – 1	2 (40%)	3 (60%)	0.5	1
	A. niger (n = 3)	0.25 – 2	1 (33.9%)	2 (67.1%)	2	2
	A. tamarii (n = 1)	NA	-	1 (100%)	NA	NA
Clotrimazole	A. flavus (n = 47)	0.125 – 1	31 (65.9%)	16 (34%)	0.5	1
	A. fumigatus (n = 11)	0.125 – 1	7(72.7%)	3 (27.2%)	0.5	1
	A. terreus (n = 5)	0.5 – 1	1 (20%)	5 (80%)	1	1
	A. niger (n = 3)	0.5 – 1	1 (33.4%)	2 (66.7%)	1	1
	A. tamarii (n = 1)	NA	1 (100%)	-	NA	NA
Ketoconazole	A. flavus (n = 47)	0.5 – 8	13.7 (27.7%)	33 (70.2%)	1	4
	A. fumigatus (n = 11)	0.125 – 4	4 (36.3%)	7 (63.6%)	1	2
	A. terreus (n = 5)	1 – 4	-	5 (100%)	2	4
	A. niger (n = 3)	0.5 – 4	1 (33.4%)	2 (66.7%)	1	4
	A. tamarii (n = 1)	NA	-	1 (100%)	NA	NA

Table 2 provides insights into the Minimum Inhibitory Concentration (MIC) of various antifungal agents against different Aspergillus species. Amphotericin B was effective against A. flavus, A. fumigatus, A. terreus, and A. niger, with MIC50 values ranging from 0.5 to 2 µg/ml. Voriconazole also demonstrated potency, particularly against A. flavus and A. fumigatus, with MIC50 values of 0.5 µg/ml for both species.

Itraconazole was most effective against A. flavus, with a MIC50 of 0.25 µg/ml, while clotrimazole showed similar efficacy, especially against A. fumigatus and A. flavus, with MIC50 values of 0.5 µg/ml. Natamycin and ketoconazole, although less effective, had MIC50 values ranging from 1 to 32 µg/ml, showing varied activity across the Aspergillus species. Econazole demonstrated moderate effectiveness, with MIC50 values between 0.5 and 1 µg/ml across the tested species.

DISCUSSION

The findings of this study provide significant insights into the antifungal susceptibility of various fungal species, particularly *Aspergillus* species, to a range of antifungal agents. The results reveal a varied spectrum of efficacy among the tested agents, underscoring the ongoing challenge of managing fungal infections, particularly in the context of rising antifungal resistance. Amphotericin B, a long-standing gold standard in antifungal therapy, showed strong activity against several Aspergillus species with MIC50 values from 0.5 to 2 µg/ml, affirming its ongoing relevance in treating invasive fungal infections. Voriconazole displayed potent activity, particularly against A. flavus and A. fumigatus with MIC50 values of 0.5 µg/ml, emphasizing its effectiveness in aspergillosis, though susceptibility testing is advised due to species-specific differences. Itraconazole proved effective against A. flavus (MIC50 of 0.25 µg/ml), but its higher MIC values for other species suggest limited broader application. Natamycin and Ketoconazole had less consistent efficacy, with MIC50 values of 1 to 32 µg/ml, indicating they may be less reliable as first-line treatments, especially for invasive aspergillosis. Econazole and Clotrimazole showed moderate to good activity with MIC50 values between 0.5 and 1 µg/ml, making them potential alternatives, particularly in cases of resistance to other agents. These findings, consistent with previous research, highlight the importance of antifungal susceptibility testing (AFST) in managing fungal keratitis and guiding effective treatment [6,7].

For instance, a study by Goncalves et al., (2016) [8] found that amphotericin B and voriconazole were among the most effective antifungal agents against Aspergillus and Fusarium species, which are commonly isolated from corneal infections. This supports our observation that amphotericin B showed low MIC values against a majority of the fungal isolates, indicating its continued relevance as a potent antifungal drug.

Similarly, a study conducted by Rai et al. (2021) [9] reported high MIC values for natamycin in cases of Fusarium keratitis, consistent with our findings where natamycin exhibited relatively higher MICs, suggesting limited efficacy against certain fungal species. This highlights the need for cautious use of natamycin, particularly in regions where Fusarium is a predominant pathogen.

Furthermore, a review by Kam et al. (2023) [10] discussed the emerging resistance patterns in ocular fungal infections, particularly noting the rising MIC values for azoles like itraconazole and ketoconazole. One notable study by Manikandan, et al. (2019) [11] similarly reported the efficacy of amphotericin B against *Aspergillus fumigatus*, showing MIC50 values in the range of 0.5–2 µg/ml, which aligns with our finding of an MIC50 of 1 µg/ml against *A. flavus*, *A. fumigatus*, and *A. terreus*. This suggests the sustained potency of amphotericin B across multiple studies. Finally, clotrimazole, which demonstrated MIC50 values of 0.5 µg/ml against A. flavus and A. fumigatus in our study, was similarly effective in the work of Kredics et al. (2015). Their findings reported comparable efficacy against various Aspergillus species, suggesting the robustness of clotrimazole's antifungal activity in both clinical and experimental settings (Sharma ,2021) [13]. Our study mirrors these concerns, as both itraconazole and ketoconazole demonstrated higher MIC values against the tested isolates, indicating potential resistance issues. The variation in MIC values among the different antifungal agents and fungal species underscores the importance of routine antifungal susceptibility testing in clinical settings. Such testing enables the tailoring of antifungal therapy to the specific pathogen involved, thereby improving treatment efficacy and reducing the risk of resistance development. Moreover, the study highlights the need for ongoing surveillance of antifungal resistance patterns. The evolving nature of fungal resistance necessitates continuous monitoring to ensure that treatment guidelines remain effective and up-to-date.

CONCLUSION

In conclusion, this study reinforces the importance of individualized antifungal therapy, guided by susceptibility testing, to achieve the best clinical outcomes. While traditional agents like Amphotericin B and Voriconazole remain highly effective, the potential for varying susceptibility across *Aspergillus* species emphasizes the need for careful agent selection. Future research should focus on exploring new antifungal agents and strategies to combat resistance and improve patient outcomes in the face of challenging fungal infections.

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