



Antifungal Resistance Profile of Fungal Isolates from Fungal Rhinosinusitis Patients: A Study from Tertiary Care Hospital

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Abstract

Objective Fungal rhinosinusitis is on the rise worldwide and it is endemic especially in North India. The main purpose of this study was to determine the antifungal resistance profile of fungal isolates from the cases of fungal rhinosinusitis.

Methods Antifungal susceptibility testing of isolated fungi to fluconazole, amphotericin B, itraconazole, and voriconazole was determined by standard CLSI broth microdilution method.

Results Sixty-eight fungal isolates of *Aspergillus spp.* ($n = 49$), *Rhizopus spp.* ($n = 9$), *Candida spp.* ($n = 4$), *Penicillium spp.* ($n = 2$), *Mucor spp.* ($n = 2$), *Bipolaris spp.* ($n = 1$), and *Alternaria spp.* ($n = 1$) were obtained from 60 different clinical samples as exudate from nasal mucosa ($n = 28$), allergic mucin ($n = 8$), nasal lavage ($n = 2$), tissue biopsy from nasal polyps ($n = 14$), and intraoperative nasal mucosa ($n = 8$). Of the 68 isolates, 75% were resistant to fluconazole, 13.23% were resistant to itraconazole, 2.94% to amphotericin B, and none were resistant to voriconazole. *Aspergillus flavus* (5%) was the only fungi found resistant to amphotericin B, while against itraconazole, *A. flavus* (7.5%) and *A. niger* (100%) were found resistant. All the isolates of *A. flavus*, *A. fumigatus*, *A. niger*, and *Penicillium spp.* were resistant to fluconazole.

Conclusion Although amphotericin B stills remains to be the most effective drug, more prospective studies are needed for the requirement of knowledge of the sensitivity pattern for optimal treatment and reduction in morbidity in the long run.

Keywords

- ▶ antifungal resistance
- ▶ fungal sinusitis
- ▶ antifungal susceptibility

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Introduction

Fungal rhinosinusitis (FRS) is a disease that produces significant morbidity and one of the most challenging forms of sinonasal pathology is invasive fungal rhinosinusitis (IFRS), which is difficult to manage, presenting most commonly in immunocompromised patients.^{1,2} For the treatment of IFRS, mainly three types of antifungals have been used: polyenes, azoles, and newer classes, such as lipid complex nystatin and echinocandins.² Azoles apart from fluconazole are useful against all filamentous fungi except *Mucorales*. However, posaconazole has shown activity against *Mucorales*. Except *Mucorales*, echinocandins are highly active antifungals against all the fungal agents but are yet to be used widely, especially in India. Fluconazole and 5-flucytosine are inactive against filamentous fungi.³

Concurrent with the increase in fungal infections, a variety of antifungal drugs are seen with a different spectrum of activity. Therefore, there is a need to determine the antifungal susceptibility of isolates to the available drugs.⁴ NCCLS (now Clinical and Laboratory Standard Institute, CLSI) has recommended standard antifungal susceptibility testing methodologies (CLSI M27-A3 document) to be used for yeasts and CLSI M38-A2 document for filamentous fungi to give reproducible results.⁴⁻⁶

The resistance to antifungal agents has been increasing significantly affecting the morbidity, mortality, and health-care costs of the patients. Hence, a detailed understanding of mechanisms of resistance, development of newer antifungals for resistant organisms, and methods to prevent emergence and spread of resistance are needed. Detection of resistance by standardized methods for broth microdilution is used to establish the minimum inhibitory concentrations (MICs) of new and established antifungal drugs.^{7,8} CLSI and European Committee on Antimicrobial Susceptibility Testing (EUCAST) have laid down reference methods that give reproducible results and either of them is used worldwide including India.^{3,5-7} The purpose of this study was to determine the antifungal resistance profile of fungal isolates from the clinically suspected cases of FRS to choose the appropriate antifungal drug for the treatment.

Materials and Methods

A prospective observational study was conducted over a period of one and half years after obtaining approval from the Ethical Committee of the Institute. This study included the antifungal susceptibility testing of fungal isolates from the clinically suspected cases ($n = 75$) of FRS. Specimens included nasal/sinus biopsy tissue and discharge. They were subjected to fungal culture on two Sabouraud's dextrose agar and were incubated at 28°C and at 37°C. Fungal growths were identified by colony morphology and microscopy using mounts with lactophenol cotton blue stain. A slide culture was performed for growth where sporulation was delayed. Antifungal susceptibility testing of the clinical isolates and the standard ATCC reference strains was per-

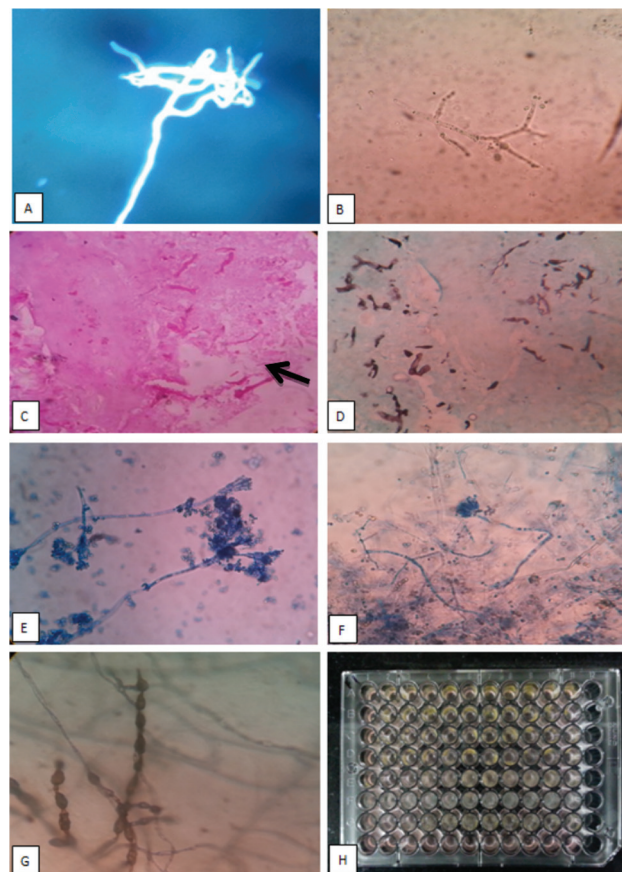


Fig. 1 (A) Calcofluor mount of nasal mucin sample showing fungal hyphae at 40X. (B) Potassium Hydroxide (KOH) mount of the nasal sample showing septate branching hyphae. (C) Hematoxylin and eosin staining of nasal biopsy sample showing fungal hyphae (arrow) at 40X. (D) Gomori methanamine silver staining showing fungal hyphae (black) in a sample nasal biopsy (40X). (E) LPCB mount showing *Penicillium* spp (40X). (F) LPCB mount showing *Aspergillus fumigatus* at 40X. (G) Microscopy of slide culture block showing chains of conidia of *Alternaria* spp under 40X. (H) Antifungal susceptibility testing by microbroth dilution technique. Microtiter plate showing susceptibility testing of isolates against fluconazole.

formed by in-house standardized broth microdilution method using the CLSI-M38-A3 method for filamentous fungi and CLSI-M27-A3 method for *Candida* species^{5,6} (→ Fig. 1H).

CLSI-M27-A3 Method

The in vitro MICs for *Candida* spp. were determined by broth microdilution method according to CLSI-M27-A3 document using RPMI-1640 medium (with glutamine and phenol red, without bicarbonate) supplemented with 0.2% glucose and MOPS (3-[N-morpholino] propane sulfonic acid). The concentration ranges tested were 0.125 to 128 µg/mL for fluconazole and 0.031 to 16 µg/mL for itraconazole, voriconazole, and amphotericin B. The ATCC (American Type Culture Collection) strains of *C. parapsilosis* (ATCC22019) and *C. krusei* (ATCC6258) were used as quality control with each batch of clinical isolates. Readings were taken according to CLSI guidelines as MICs ≤ 1.0 µg/mL as susceptible, MIC = 2.0 µg/mL as intermediate susceptible, and ≥ 4.0 µg/mL as resistant.⁵

CLSI-M38-A2 Method

The in vitro MICs for filamentous fungi were determined by broth microdilution method according to the CLSI-M38-A3 document using RPMI-1640 medium (with glutamine and phenol red, without bicarbonate) supplemented with 0.2% glucose and MOPS (3-[N-morpholino] propane sulfonic acid). Inoculum suspensions of nongerminated conidial and sporangiospores were prepared spectrophotometrically and adjusted to an optical density at 530 nm that ranges from 0.09 to 0.13 for *Aspergillus spp.*, 0.15 to 0.17 for *Fusarium*, *Rhizopus* and other zygomycetous species and 0.25 to 0.3 for *Bipolaris* and *Alternaria spp.* The concentration ranges tested were 0.125 to 128 µg/mL for fluconazole and 0.031 to 16 µg/mL for itraconazole, voriconazole, and amphotericin B. The quality control strains were also used with each batch of clinical isolates. Readings were taken for all strains twice on alternate days as recommended by CLSI guidelines.⁶

Results

Out of total 75 clinically suspected cases of FRS, 69 confirmed cases of FRS were enrolled in the study based on culture and histopathological findings (►Fig. 1A–D). A total of 68 fungal isolates of species hyphomycetes (*Aspergillus flavus*, *Aspergillus niger*, *Aspergillus fumigatus*, *Alternaria spp.*, *Bipolaris spp.* and *Penicillium spp.*), order mucorales (*Rhizopus arrizhus* and *Mucor spp.*) and Saccharomycetes (*Candida albicans*) were obtained from 60 patients (►Fig. 1E–G). Rest nine patients had histopathological evidence of FRS but cultures were negative.

Antifungal susceptibility of these isolates showed that 75% were resistant to fluconazole, 13.23% were resistant to itraconazole, 2.94% to amphotericin B, and none were resistant to voriconazole (►Table 1). ►Table 2 shows MIC90/MIC50/range of different drugs (amphotericin B, fluconazole, itraconazole, and voriconazole) of fungal isolates.

Antifungal susceptibility testing against amphotericin B showed that 2 isolates (5%) of *Aspergillus flavus* were resistant, 12 isolates (30%) showed intermediate sensitivity, and 26 isolates (65%) were sensitive. One out of 9 (11.1%) isolates of *Rhizopus arrizhus* showed intermediate sensitivity, and rest 8 (88.8%) were sensitive. All the isolates of *A. fumigatus*, *A. niger*, *Mucor spp.*, *Penicillium spp.*, *Alternaria spp.*, *Bipolaris spp.* and *C. albicans* were sensitive.

All four (100%) isolates of *C. albicans* were susceptible. *Alternaria spp.* and *Bipolaris spp.* (one each) showed intermediate sensitivity, while all the isolates of *A. flavus*, *A. fumigatus*, and *A. niger* were found to be resistant to fluconazole.

Three (7.5%) isolates of *A. flavus* and 6(100%) isolates of *A. niger* were resistant to itraconazole. However, majority of *A. flavus* 37 (92.5%), all the isolates of *A. fumigatus*, *Penicillium spp.*, *Alternaria spp.*, *Bipolaris spp.*, and *C. albicans*, were sensitive.

All the isolates of *A. flavus*, *A. fumigatus*, *A. niger*, *Penicillium spp.*, *Alternaria spp.*, *Bipolaris spp.*, and *C. albicans* were sensitive to voriconazole.

Sixty-seven out of total 69 patients underwent surgical intervention like debridement and functional endoscopic

Table 1 In vitro antifungal susceptibility of isolated fungi against various drugs

Species	Amphotericin B		Fluconazole		Itraconazole		Voriconazole	
	≥ 4 µg/mL R, n (%)	2 µg/mL IS, n (%)	≤ 1 µg/mL S, n (%)	≥ 4 µg/mL R, n (%)	2 µg/mL IS, n (%)	≥ 4 µg/mL R, n (%)	2 µg/mL IS, n (%)	≤ 1 µg/mL S, n (%)
<i>Aspergillus flavus</i> (n = 40)	2(5)	12(30)	26(65)	40(100)	0	0	0	40(100)
<i>Aspergillus fumigatus</i> (n = 3)	0	0	3(100)	3(100)	0	0	0	3(100)
<i>Aspergillus niger</i> (n = 6)	0	0	6(100)	6(100)	0	0	0	6(100)
<i>Rhizopus arrizhus</i> (n = 9)	0	1(11.1)	8(88.8)	NA	NA	NA	NA	NA
<i>Mucor spp.</i> (n = 2)	0	0	2(100)	NA	NA	NA	NA	NA
<i>Penicillium spp.</i> (n = 2)	0	0	2(100)	2(100)	0	0	0	2(100)
<i>Alternaria spp.</i> (n = 1)	0	0	1(100)	0	1(100)	0	0	1(100)
<i>Bipolaris spp.</i> (n = 1)	0	0	1(100)	0	1(100)	0	0	1(100)
<i>Candida albicans</i> (n = 4)	0	0	4(100)	0	0	0	0	4(100)

Abbreviations: IS, intermediate susceptibility; NA, not applicable; R, resistant; S, sensitive.

Table 2 Minimum inhibitory concentration values of various isolated fungi against different drugs

Species	Amphotericin B			Fluconazole			Ketoconazole			Itraconazole			Voriconazole		
	MIC ₉₀ (µg/mL)	MIC ₅₀ (µg/mL)	Range	MIC ₉₀ (µg/mL)	MIC ₅₀ (µg/mL)	Range	MIC ₉₀ (µg/mL)	MIC ₅₀ (µg/mL)	Range	MIC ₉₀ (µg/mL)	MIC ₅₀ (µg/mL)	Range	MIC ₉₀ (µg/mL)	MIC ₅₀ (µg/mL)	Range
<i>Aspergillus flavus</i> (n = 40)	2	1	0.25-4	≥64	32	16-≥64	0.5	0.25	0.0625-2	0.25	0.0625	0.0313-≥16	0.5	0.125	0.0313-0.5
<i>Aspergillus fumigatus</i> (n = 3)	0.5	0.5	0.25-0.5	≥64	≥64	32-≥64	0.5	0.5	0.125-0.5	0.0625	0.0625	0.0313-0.0625	0.5	0.25	0.25-0.5
<i>Aspergillus niger</i> (n = 6)	0.5	0.25	0.25-0.5	≥64	≥64	32-≥64	1	0.5	0.5-1	≥16	≥16	8-≥16	1	0.5	0.5-1
<i>Rhizopus arrhizus</i> (n = 9)	2	0.5	0.5-2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
<i>Mucor</i> spp. (n = 2)	1	0.5	0.5-1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
<i>Penicillium</i> spp. (n = 2)	1	0.25	0.25-1	≥64	8	8-≥64	0.25	0.25	0.25	0.25	0.125	0.125-0.25	0.5	0.25	0.25-0.5
<i>Alternaria</i> spp. (n = 1)	-	-	0.25	-	-	2	-	-	0.0625	-	-	0.25	-	-	0.5
<i>Bipolaris</i> spp. (n = 1)	-	-	0.25	-	-	2	-	-	0.0625	-	-	0.25	-	-	0.5
<i>Candida albicans</i> . (n = 4)	0.5	0.125	0.125-0.5	0.5	0.125	0.125-0.5	0.125	0.0625	0.0313-0.125	0.125	0.125	0.125	0.25	0.0625	0.0625-0.25

Abbreviation: MIC, minimum inhibitory concentration.

sinus surgery. Drug therapy using itraconazole was given in 71.01% patients and 18.84% received amphotericin B. Combinations of drugs were used in two patients. Outcome was favorable for 97.1% cases who were discharged on medication. Mortality was seen in two cases of acute invasive FRS.

Discussion

All the fungal isolates were tested against various drugs like amphotericin B, fluconazole, voriconazole, and itraconazole by broth microdilution method according to CLSI guidelines.^{5,6} Isolates were grouped susceptible (MIC ≤ 1 µg/mL), intermediate (MIC = 2 µg/mL), and resistant (MIC ≥ 4 µg/mL; ▶ **Table 1**). These are working breakpoints for analytical purposes only; the breakpoints have not been established for mold testing. The clinical relevance of testing this group of fungal pathogens remains unclear, and the breakpoints with proven relevance are yet to be identified or approved by CLSI or any regulatory agency.^{5,6,8} The data concerning correlation between MIC and outcome of treatment by amphotericin B for the filamentous fungi are scanty, MICs above 2 µg/mL have been associated with treatment failure, and MICs below 2 µg/mL have been associated with clinical cure.^{6,9} All the isolates in our study were sensitive to amphotericin B with MIC₉₀ ≤ 2 µg/mL. Two isolates of *A. flavus* had MIC ≥ 4 µg/mL. Twelve isolates of *A. flavus* and one isolate of *Rhizopus arrhizus* had MIC of 2 µg/mL. A study done in Chandigarh (India) in 2013 showed that all the isolates in their study were sensitive to amphotericin B.¹⁰ A study in Austria during 1996 to 2006 tested various isolates, the range of *A. flavus* to amphotericin B was found to be 1 to 4 µg/mL similar to our study where the range was 0.25 to 4 µg/mL.¹¹ In another study done in New Delhi (India) from 2002 to 2010, all the 47 isolates of *A. flavus* and 12 isolates of *A. fumigatus* were seen to be susceptible to amphotericin B. Also, a study done in Vellore also indicates all isolates being susceptible to amphotericin B with range 0.06 to 2.0 µg/mL except *Aspergillus terreus*.¹² This necessitates the correlation of resistance to amphotericin B and treatment failure.¹³

Filamentous fungi are not susceptible to fluconazole and most MICs are >64 µg/mL for these isolates.⁶ All the isolates in our study had MIC ≥ 4 µg/mL for fluconazole except *C. albicans*, *Alternaria*, and *Bipolaris* species, similar to the findings of Rudramurthy et al's study who reported a MIC range of 8 to >64 µg/mL for most of the isolates. *C. albicans* had a range in between 0.125 and 0.5 µg/mL in our study with MIC₉₀ of 0.5 µg/mL, while the range and MIC₉₀ for their isolates were 0.125 to 64 µg/mL and 0.25 µg/mL, respectively.¹⁰ The MIC of *Alternaria* and *Bipolaris* spp. was found to be 2 µg/mL almost in concordance to his study, where two isolates of *Bipolaris* spp. had MIC of 1 µg/mL.¹⁰

Further, resistance to itraconazole was shown by all the isolates of *A. niger* and three isolates of *A. flavus*. Rest all the isolates had MIC < 4 µg/mL. Preliminary data indicate that high itraconazole MIC (>8 µg/mL) is associated with clinical resistance to this agent.⁶ In a study in Austria, the ranges for itraconazole for various isolates were *A. flavus* (0.5-2 µg/mL), *A. niger* (2-4 µg/mL), and *Rhizopus* spp. (4 µg/mL).¹¹ These are

the isolates that were resistant in our study, the ranges being 0.0313 to >16 µg/mL in *A. flavus* and 8 to ≥16 µg/mL in *A. niger*. The MIC₉₀ for these two species was 0.25 µg/mL for *A. flavus* and >16 µg/mL for *A. niger* showing that most of the isolates of *A. flavus* were susceptible to itraconazole. The MIC₉₀ for *A. niger* against itraconazole was ≥16 µg/mL which indicated that these isolates may have clinical resistance to treatment. On the contrary, a study done in Vellore showed that *Aspergillus* species have low voriconazole and itraconazole MICs.¹²

Most of the isolates were susceptible to voriconazole in our study. *C. albicans* were sensitive to all the drugs tested having MIC < 4 µg/mL in concordance with the study done by Rudramurthy et al where 26 isolates of *C. albicans* from various clinical sample were sensitive to amphotericin B, itraconazole, voriconazole, and fluconazole.¹⁰ All *C. albicans* isolates were sensitive to these drugs in the study done in Austria too.¹¹

Conclusion

In conclusion, data of our study demonstrates that the amphotericin B is still the most effective drug for the treatment of fungal pathogens. Although the findings of our and other studies showed amphotericin B to be a good choice for the treatment of FRS, yet prospective studies are needed for the requirement of knowledge of the sensitivity pattern for optimal treatment and reduction in morbidity in the long run. The resistance to antifungal agents has been increasing significantly affecting the morbidity, mortality, and healthcare costs of the patients. Hence, a detailed understanding of mechanisms of resistance, development of newer antifungals for resistant organisms, and methods to prevent emergence and spread of resistance are needed.

Authors' Contributions

L.S. contributed to study design and concept, data acquisition, data analysis, manuscript preparation, editing, and review. R.K. helped in study design and concept, data analysis, manuscript preparation, editing, and review. M.S.D. contributed to data analysis, manuscript preparation, editing, and review. A.G. was involved in data acquisition, manuscript preparation, editing, and review.

Ethical Approval

Ethical approval has been obtained from institute's IEC under the number F.No./11/IEC/MAMC/2011/232 dated: 25/10/2013.

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None.

Conflict of Interest

None declared.

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