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Original Research Article

Amphotericin B, itraconazole, posaconazole and isavuconazole minimum inhibitory concentration against 85 strains of *Rhizopus* spp, isolated from the patients having COVID-19 associated mucormycosis in North India

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ABSTRACT

Background: Mucormycosis cases are managed by extensive debridement of the affected tissues, with correction of predisposing risk factors and antifungal drugs. Amphotericin B is the drug of choice; however, few azoles also have a good activity against Mucorales. Therefore, the present study was done to determine the minimum inhibitory concentration (MIC) of antifungal drugs against Mucorales, causing COVID-19 associated mucormycosis in North India.
Methods: After obtaining written and informed consent, we processed the received tissue, sputum, and gastric lavage samples as per standard mycological procedures. Subsequently, we determined itraconazole, posaconazole, isavuconazole and amphotericin B MIC against the isolated Mucorales (one from each patient) by broth microdilution using CLSIM38A_3 guidelines.
Results: We received 615 samples from the enrolled 269 patients with CAM. We observed broad aseptate hyphae in 329 fresh tissues, ten sputum and one gastric lavage sample, whereas 163 follow-up excised tissue had broad aseptate hyphae. In addition, 209 Mucorales were isolated with a predominance of *Rhizopus arrhizus* (n=183), followed by *Rhizopus microsporus* (n=21) and *Rhizopus homothallicus* (n=5). We determined MIC against 77 and 8 strains of *R. arrhizus* and *R. microsporus*, respectively. Posaconazole had the least MIC. 0.25, 1, 0.5, and 2µg/ml were the MIC_{50} of posaconazole, amphotericin B, isavuconazole and itraconazole against *R. arrhizus* strains, respectively, whereas it was 0.125, 0.1875, 0.5 and 2 µg/ml against *R. microsporus*, respectively.
Conclusions: Lower posaconazole MIC makes it a preferred drug for managing the mucormycosis cases; however, availability and cost are the limitations. Thus, amphotericin B and itraconazole may be used in such conditions.

Keywords: CAM, *R. homothallicus*, *R. arrhizus*, *R. microsporus*, SARS-CoV-2

INTRODUCTION

An upsurge in mucormycosis cases has been observed during the second wave of SARS-CoV-2 infection in India.[1] This upsurge has been significantly attributed to recent SARS-CoV-2 infection, uncontrolled diabetes mellitus and steroid-induced hyperglycemia.[2,3] In addition, persistent neutropenia, patient receiving iron chelator and IL-6 inhibitors-tocilizumab, unforeseen use of industrial oxygen, use of broad spectrum antibiotics,

and multivitamins have been reported to be associated with this upsurge.[2,4]

The spores of the Mucorales enter the human body through inhalation, primarily infecting the paranasal sinuses. In patients having underlying predisposing risk factors, the spores germinate into the coenocytic hyphae that invade the blood vessels, resulting in vaso-occlusion with subsequent infarction of the affected area.[1,2]

Mucormycosis cases are managed by a multidisciplinary team where extensive surgical debridement of the affected region is done, with administering antifungal agents in appropriate doses with correction of predisposing risk factors.[2]

Despite being toxic, amphotericin B is a drug of choice for mucormycosis, as fluconazole and voriconazole do not have a good activity against the causative Mucorales.[5] However, itraconazole seems to have some activity. In such background, we did a prospective study to determine MIC of amphotericin B, itraconazole, posaconazole, and isavuconazole against the isolated mucorales causing COVID-19 associated mucormycosis (CAM).

METHODS

Sample collection

We received different samples; middle turbinate scrapping, excised nasal and paranasal sinus tissue, excise orbital tissue, gastric lavage etc., obtained from the patients having CAM between 1st April 2021-15thAugust 2021. In addition, early morning sputum samples for two consecutive days were received from suspected pulmonary mucormycosis cases. We received these samples in normal saline for mycological work-up.

Sample processing and identification

All the samples were processed as per the standard mycological procedures. Briefly, the tissue samples were cut aseptically into multiple small pieces. Then, we did the KOH wet mount to observe the broad aseptate hyphae. Concomitantly, the small pieces were inoculated on freshly prepared Sabouraud dextrose agar (SDA) and blood agar plates, which were incubated at 25^0C in a BOD incubator.

Once Mucorales grew over the medium, the isolate was identified as per standard protocol using growth rate, colony characteristics, and microscopic features on lactophenol cotton blue (LPCB) wet mount. Finally, these strains were stocked in sterile distilled water for further work-up.[6]

For confirmation, we also sent one batch of stocked strains to the Postgraduate Institute of Medical Education and Research (PGIMER) Chandigarh, a reference centre for mycology, India.

Antifungal MIC determination against isolated Mucorales

MIC of amphotericin B, posaconazole, itraconazole and isavuconazole was determined against the isolated strains of Mucorales by broth-microdilution method using CLSI $M38A_3$ guidelines. *Candida parapsiolosis* ATCC 22019 and *C. krusei* ATCC 6258 strain was used to standardize the protocol.[7] Briefly, we procured the antifungal drug powders from sigma pharmaceuticals, India. The drug powders were dissolved in DMSO to have a concentration of 1mg/ml, which were further diluted in RPMI 1640 (having 0.165M/L MOPS but without bicarbonate) to have a concentration of 32 ug/ml for amphotericin B, posaconazole and isavuconazole, and 128 ug/ml for itraconazole. These diluted drugs were kept at -20°C.

Mucorales strains were sub-cultured on potato dextrose agar (PDA) and incubated at 37^0C in a BOD incubator for 72 hrs. Then we prepared the spore suspension of ~10^4cells/ml in RPMI 1640. MIC was determined in 96 well flat bottom microtitre plates where 100µl of each diluted drug and the spore suspension were added. The plate was incubated at 35^0C. After 24 hours of incubation, MIC was observed for all four tested antifungal drugs. The minimum drug concentration, which resulted in 100% growth inhibition compared to a negative control, was noted as the MIC. We performed the biostatistical analysis of the MIC by calculating the median with interquartile range. Subsequently, we categorized the isolated Mucorales into wild and non-wild stains per their epidemiological cutoff.[8]

Biostatistical analysis

Biostatistical comparative analysis of in-vitro MIC of tested antifungal drugs against the isolated Mucorales was done by Kruskal-Wallis test as the generated data were non-parametric. We also made Box and Whisker Plots to represent in-vitro MIC results of tested antifungal drugs.

RESULTS

During the study period, 269 patients having CAM were admitted to the Mucor ward of SS hospital, IMS BHU Varanasi, India. Among these enrolled patients, males were predominant with M:F ratio of 2.05:1. 235 (87.4%) enrolled patients were more than 50 years of age. We received 615 samples from 269 patients with COVID-19 associated (258 ROCM, ten pulmonary and one GI) mucormycosis. The samples (n=523) were mostly excised tissue from the maxillary sinus, followed by 65 orbital tissue, 25 sputum and two gastric lavage samples.

We observed broad aseptate hyphae in 395 middle turbinate scrapping/excised sinus or orbital tissues, ten sputum and one gastric lavage sample. However, we isolated only 209 strains of Mucorale, out of which *R. arrhizus* (n=183) was the predominant pathogen,

followed by *R. microsporus* (n=21), and *R. homothallicus* (n=5). These strains were identified by LPCB wet mount and slide culture, which were further confirmed in PGIMER Chandigarh, India. We determined MIC of amphotericin B, itraconazole, posaconazole and isavuconazole against 85 strains of Mucorales (*R. arrhizus* n=77 and *R. microsporus* n=8).

In Table 1, we have documented the number of the Mucorales strains having different MIC for the above tested antifungals. Overall, posaconazole had the least MIC_{50} and MIC_{90} compared to itraconazole, isavuconazole and amphoter 0.25 (0.0925-0.5) µg/ml posaconazole MIC_{50} against *R. arrhizus* in comparison to 1 (0.5-2) µg/ml for amphotericin B, 0.5 (0.1875-1) µg/ml for isavuconazole MIC and 2 (1.5-6) µg/ml itraconazole MIC, respectively (Kruskal-Wallis: H=152.61; p<0.0001; df=3, Figure 1); whereas, posaconazole MIC_{50} against *R. microsporus* was 0.125 (0.06-0.1875) µg/ml in comparison to 0.1875 (0.0925-0.375) µg/ml for amphotericin B, 0.5 (0.25-0.75) µg/ml for isavuconazole MIC and 2 (1.25-4) µg/ml itraconazole MIC, respectively (Kruskal-Wallis: H=19.28; p<0.0001; df=3, Figure 2).

We categorized the clinical Mucorale isolates into wild and non-wild strains per their epidemiological cut off (ECV). Taking 4 µg/ml amphotericin B MIC and 2 µg/ml posaconazole MIC as epidemiological cut off (ECV), all the *R. arrhizus* strains were classified as wild type. In contrast, only 50.6% of the *R. arrhizus* strains were the wild type for itraconazole, taking 2 µg/ml MIC as ECV. On the other hand, taking 2 µg/ml MIC as ECV for both amphotericin B and posaconazole, all the eight *R. microsporus* strains were wild-type.[7]

Table 1: Amphotericin B, itraconazole, posaconazole and isavuconazole MIC against isolated Mucorales from the patients suffering from COVID-19-associated mucormycosis.

Antifungal drug Mucorales	≤0.03 µg/ml	0.06 µg/ml	0.125 µg/ml	0.25 µg/ml	0.5 µg/ml	1 µg/ml	2 µg/ml	4 µg/ml	8 µg/ml	≥16 µg/ml
Amphotericin B										
R. arrhizus, (n=77)	0	2	4	5	10	19	27	10	0	0
R. microspores, (n=8)	1	1	2	2	2	0	0	0	0	0
Itraconazole										
R. arrhizus, (n=77)	0	0	0	3	6	10	20	19	13	6
R. microspores, (n=8)	0	0	0	0	2	0	2	3	1	0
Posazonazole										
R. arrhizus, (n=77)	7	12	18	16	20	4	0	0	0	0
R. microspores, (n=8)	0	3	3	1	1	0	0	0	0	0
Isavuconazole										
R. arrhizus, (n=77)	2	5	12	13	16	20	9	0	0	0
R. microspores, (n=8)	0	0	3	3	1	1	0	0	0	0

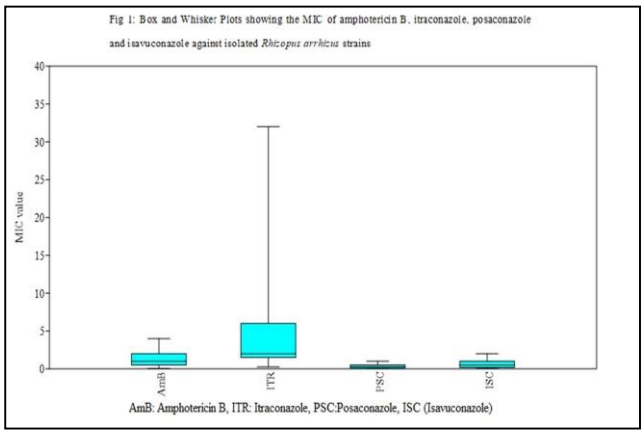

Figure 1: Box and Whisker plots showing the MIC of amphotericin B, itraconazole, posaconazole and isavuconazole against isolated *Rhizopus arrhizus* strains.

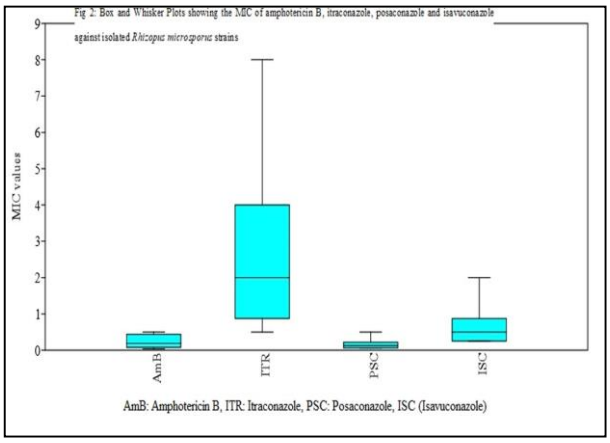

Figure 2: Box and Whisker plots showing the MIC of amphotericin B, itraconazole, posaconazole and isavuconazole against isolated *Rhizopus microsporus* strains.

DISCUSSION

During the second wave of SARS-CoV-2 infection in India, a rapid upsurge in mucormycosis cases was observed. This upsurge has been attributed to a higher number of patients having hyperglycemia, either due to diabetes or induced hyperglycemia by corticosteroids, zinc uptake and oxygen-requirement by patients suffering from SARS-CoV-2 infection.[9] Corticosteroid administration reduces the complication and mortality by reducing the intensity of the inflammatory response, observed in form of elevated levels of inflammatory markers (IL1, IL6, TNF-α) in patients suffering from SARS-CoV-2 infection.[10] Hyperglycemia has been reported as a predisposing risk factor for invasive mucormycosis as inhaled spores of Mucorales easily germinate into the coenocytic hyphae, which invade the blood vessels, resulting in hematogenous dissemination with subsequent infarction of the affected region.[11] In addition, hyperglycemia reduces the mobilization and degranulation of the neutrophil, a major defense mechanism against the Mucorales.[12]

Rapid diagnosis of mucormycosis cases is usually done by demonstrating broad aseptate hyphae in KOH wet mount of the excised tissue. However, the sensitivity of the KOH wet mount is poor, which can be increased by adding calcofluor white stain, or multiple wet mounts with their examination by different observers.[13] As in our study, out of 615 received samples from 269 CAM cases, 385 had broad aseptate hyphae on KOH wet mount. Thus, rapid diagnosis helps in determining the extent of the debridement of the affected tissue and selecting an appropriate antifungal drug.

Of 219 isolated Mucorale strains from the patients having CAM, *Rhizopus arrhizus* was the predominant isolate, followed by *Rhizopus microsporus* and *R. homothallicus*. *R. arrhizus* has been reported as a most common cause of mucormycosis in the literature.[14-16] However, during COVID-19 pandemic, rare Mucorales such as *Rhizopus microsporus* and *R. homothallicus* were also isolated from patients having CAM.[17] Thus, identification of the causative Mucorales helps in selecting the appropriate antifungal agents as varied MIC of tested antifungals were noted against the isolated Mucorales.

We determined *in-vitro* MIC of amphotericin B, itraconazole, posaconazole and isavuconazole against 85 strains of isolated Mucorales. Amphotericin B, posaconazole and isavuconazole are known for their anti-Mucorale activity. However, we also selected itraconazole to determine MIC, despite having poor activity against Mucorales. Itraconazole has been reported to achieve ~20 times higher drug levels in the skin compared to the plasma, thus might be effective in cutaneous mucormycosis.[18] We observed that posaconazole had the lowest MIC among the tested antifungals. Results of lower posaconazole and amphotericin B MIC against *R. arrhizus* are similar to a multicentric study by Espinel-Ingroff et al.[8]

In contrast, Borman et al reported the lowest amphotericin B MIC compared to itraconazole, posaconazole and isavuconazole against the isolated *Rhizopus arrhizus* strains; however, itraconazole and isavuconazole MIC were higher as similar to the present study.[19] This variation in MIC may be attributed to the availability and use of the antifungal drugs. In India, amphotericin B is most widely used in managing mucormycosis, invasive candidiasis, invasive pulmonary aspergillosis and leishmaniasis, whereas itraconazole is widely used in the management of dermatophytosis, histoplasmosis and chronic aspergillosis.[20] However, posaconazole and isavuconazole are rarely used in the Indian scenarios due to their availability and higher cost.

Among azoles, posaconazole is used widely as a salvage therapy for mucormycosis, whereas itraconazole is used as a prophylactic agent.[21] Recent studies have reported a good in-vitro efficacy of isavuconazole against the Mucorales. These azoles inhibit the fungal enzyme lanosterol 14α-demethylase, resulting in reduced ergosterol with the accumulation of toxic ergosterol precursors in the cytoplasm, with ultimate cell death.[22] Polyene, amphotericin B, a preferred drug for mucormycosis, binds to the ergosterol in the fungal cell wall, resulting in leakage and cell death. Mucormycosis is a serious life-threatening fungal infection where rapid acting fungicidal drug is needed. Administration of amphotericin B results in good survival rate in mucormycosis cases, however, crisis for amphotericin B was noted during the second wave of SARS-CoV-2 infection in India. Thus, at our institute, we managed the patients having COVID-19 associated mucormycosis with posaconazole. In addition, we administered liposomal amphotericin B as per availability. In the periphery, itraconazole may be administered despite having a higher MIC in ~50% of the isolated *R. arrhizus* strains in condition of amphotericin B crisis and non-availability of posaconazole.

Mucormycosis cases are managed by a multidisciplinary team, where extensive debridement of the affected tissues with antifungal drugs in appropriate doses with corrections of predisposing risk factors is done. In this present study, we only observed the in-vitro MIC of the tested antifungal against the isolated Mucorales. Despite having a lower number of tested pathogens, this study will enrich the available database regarding the use of antifungals in management of mucormycosis cases.

CONCLUSION

Rhizopus arrhizus is the predominant cause of COVID-19 associated mucormycosis. Direct microscopy of the clinical samples helps in early diagnosis. Amphotericin B, posaconazole and isavuconazole had the lower MIC against the isolated *Rhizopus arrhizus*. However, lower

posaconazole MIC makes it a preferred drug for managing the mucormycosis cases; however, availability and cost are the limitations.

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