Antifungal Activity of Voriconazole on Local Isolates: an In-vitro Study

Karina Q. De Sagun-Bella, MD,[1] Archimedes Lee D. Agahan, MD,[1]
Leo DP. Cubillan, MD,[1,2] Noel S. Carino, MD,[1] Roslyn De Mesa-Rodriguez, RMT, PhD[2]

[1]Department of Ophthalmology and Visual Sciences
 Philippine General Hospital
 University of the Philippines Manila
 Manila, Philippines

[2] Institute of Ophthalmology
 National Institutes of Health
 University of the Philippines Manila
 Manila, Philippines

Correspondence: Karina Q. De Sagun-Bella, MD
Department of Ophthalmology and Visual Sciences
Philippine General Hospital
Taft Avenue, Ermita
Manila, Philippines
Tel. no.: +632-3365203
Email: kqds_md@yahoo.com

ABSTRACT

Objective: To determine the in-vitro activity of voriconazole and compare it with amphotericin B, fluconazole, itraconazole, ketoconazole, and caspofungin against local yeast and mold clinical isolates *Candida albicans*, *Candida* sp., *Aspergillus terreus, Aspergillus niger,* and *Fusarium cylindrocarpone.*

Methods: Review of the Institute of Ophthalmology microbiology records were done and was the basis for the local isolates included in the study. Mean inhibitory concentration (MIC) was determined using YeastOne Sensititre Microtitre Colorimery method (TREK Diagnostic Systems, England). Two-way ANOVA, Duncan, and Pearson chi-squared tests were used to analyze the data.

Results: All isolates tested were sensitive to voriconazole. Eighty percent (80%) of the isolates were sensitive to amphotericin B and 25% showed resistance to itraconazole. Yeast pathogens were all sensitive to amphotericin B and voriconazole. More than 50% of the yeast pathogens were resistant to ketoconazole. Molds or filamentous fungi showed higher susceptibility to voriconazole than amphotericin B and the other antifungals.

Conclusion: Voriconazole exhibited good in-vitro activity against the isolates tested. It has the same efficacy on yeast pathogens (*Candida albicans* and *Candida* sp.) when compared with amphotericin B. It has superior efficacy on filamentous fungi (*Aspergillus* and *Fusarium*). There is a role for voriconazole in the treatment of ocular infections, especially in the setting of poor antifungal drug availability.

Keywords: Voriconazole, Amphotericin B, Antifungal susceptibility, *Candida*, *Fusarium*, *Aspergillus*

Fungal ocular infections most commonly occur in the form of central microbial keratitis. It accounts for 6-20% of all infectious keratitis and is one of the most difficult to treat.[1] If untreated, fungal keratitis can lead to corneal perforation and the need for penetrating keratoplasty. Presently, the range of common antifungal agents available for fungal keratitis remains inadequate and is generally associated with poor clinical outcomes. This is partly because the increase in the variety of fungal pathogens has not been matched by a corresponding increase in the number of antifungal agents available for their treatment.

Amphotericin B remains the gold standard in treating fungal infections caused by yeast (*Candida* sp.), while natamycin addresses those caused by molds or filamentous fungi (*Aspergillus, Fusarium*). However, limitations in the efficacy and/or tolerability of these agents have prompted a search for new drugs that may be effective in the management of patients with infections due to filamentous fungi and yeast pathogens.

At present, natamycin is the only FDA-approved topical antifungal for the treatment of fungal keratitis. It has good efficacy against filamentous fungi but does not penetrate well into the cornea. This is an acceptable, commonly-used, antifungal agent. However, there is a problem with inconsistent availability of the drug locally.

It was only until the recent past that studies have suggested the better efficacy of a new generation triazole antifungal agent, namely, voriconazole. Inhibition of fungal cytochrome P-450-dependent 14-alpha-sterol dimethylase-mediated synthesis of ergosterol is the mechanism of action of voriconazole. Its chemical structure is a modification of fluconazole by replacement of one triazole moiety by a fluoropyrimidine grouping and alpha methylation.[2] Recent in-vitro and in-vivo studies have demonstrated the effectiveness of voriconazole against certain opportunistic filamentous and dimorphic fungi (molds) and yeasts.[3] Some of these previous in-vitro studies have evaluated a limited number of isolates and species.

In the local setting, complicating the management of fungal ocular infections is the inconsistent and fluctuating supply of the more commonly used antifungals. Moreover, antifungal susceptibility testing remains to be relatively unstandardized and unreliable in directing therapy. There is a lack of local studies to provide guidelines on antifungal sensitivity testing, a reason why it is not done routinely.

Thus, this study established the in-vitro efficacy of voriconazole and documented its sensitivity to local fungal isolates recovered from ocular media during the months of May to August 2012. It also compared the minimum inhibitory concentrations (MIC) of voriconazole, amphotericin B, fluconazole, itraconazole, ketoconazole, and caspofungin using the YeastOne Sensititre Microtitre Colorimetry method.

METHODOLOGY

The study was conducted at the Ocular Microbiology Laboratory of the Institute of Ophthalmology - National Institutes of Health, University of the Philippines, Manila. The records on fungal ocular infections during the months of May to August 2012 were reviewed and were the basis of including the isolates into the study. Susceptibility testing was done on the local isolates recovered from cornea and/or ocular fluids using the YeastOne Microtitre Colorimetry method (TREK Diagnostic Systems, England).

The reference isolate used in the study was (ATCC) *Candida albicans.* Local isolates were categorized as being either a yeast or a mold. Yeast included *Candida albicans* and *Candida* sp., while molds were *Aspergillus terreus, Aspergillus niger,* and *Fusarium cylindrocarpone.*

Antifungal Sensitivity Testing

Sensitivity testing was done using Sensititre® YeastOne™ Test Panel (manufactured by TREK Diagnostic Systems and supplied locally by Levins Philippines). This was a microtitre broth dilution method that provided qualitative and quantitative minimum inhibitory concentration (MIC) results in a dried plate format. Each test consisted of a disposable microtitre plate that contained dried serial dilutions of antifungal agents, namely, amphotericin B (range 0.008-16 mg/mL), fluconazole (range 0.125-256 mg/mL), itraconazole (0.008-16 mg/mL), posaconazole (0.008-8 mg/mL), ketoconazole (0.008-16 mg/mL), flucytosine (0.03-64 mg/mL), caspofungin (0.008-16 mg/mL), and voriconazole (range 0.008-16 mg/mL). The wells contained Alamar Blue as a colorimetric indicator, which produced the end point color change from blue to pink. Results were expressed as MIC.

The specimen were inoculated into BHIB broths and 100 mcg were inoculated into the YeastOne wells using micropette pipette. Three replicates per isolate

were done. The YeastOne plates were incubated at room temperature.

Mean inhibitory concentrations (MICs) were determined by visual examination at 48 hours. The microdilution wells were visualised with the aid of a reading mirror and the growth in each well compared with that of the growth control. Growth in the antifungal solutions was evident as a change in the colorimetric growth indicator from blue (negative) to red (positive). Results were tallied as either POSITIVE if the well turned red and NEGATIVE if the color was blue.

The MIC is the lowest concentration of an antifungal agent that substantially inhibits growth of the organism as detected by a color change.[4] The amount of color change in the wells containing the agent was compared to the color of the positive growth- control wells. No growth occured when there was no change in the blue indicator in any dilution of an antifungal. The organism was susceptible to the lowest concentration of the antifungal and the MIC was recorded. The organism was resistant to the highest concentration of the antifungal when growth was seen in all the wells. The MIC endpoint was recorded as "greater than" (">") the highest concentration. The reference breakpoints determining whether an isolate was sensitive, resistant, or intermediately susceptible (taken from National Committee for Clinical Laboratory Standards (CLSI)) is shown in Table 1.

Table 1. Reference breakpoints of the antifungals.

DRUG (ug/mL)	S	R
Amphotericin B	≤1	≥ 4
Fluconazole	≤8	≥ 64
Itraconazole	≤0.12	≥ 1
Ketoconazole	≤0.12	≥ 0.5
Voriconazole	≤1	≥ 4
Caspofungin	≤1	≥ 2

Data Analysis

Two-way ANOVA was used considering drug and organism as independent variables. Alpha value was 0.05. Duncan test was used as post hoc analysis. Pearson's chi-square was used in analyzing the sensitivity or resistance of the strains.

RESULTS

All isolates produced sufficient growth to determine the MICs after 48 hours. The MICs of the antifungals to the isolates are listed in Table 2.

Table 2. MIC values of antifungals to standard and clinical isolates.

DRUG	REPLICATE	ATCC CAL	CAL 539	CAL 544	CAS 533	AST 511	ASN	FSH
Ampho B	1	2	0.5	0.5	1	8	0.5	2
Fluconazole	1	2	1	1	1	1	4	8
Itraconazole	1	0.5	0.06	0.25	1	0.12	0.06	0.06
Ketoconazole	1	0.25	0.06	0.25	0.25	0.25	0.25	0.06
Voriconazole	1	0.03	0.06	0.06	0.06	0.25	0.25	0.5
Caspofungin	1	0.12	0.03	0.06	0.06	4	1	0.12
Ampho B	2	0.25	0.03	0.25	0.25	0.5	0.5	0.5
Fluconazole	2	0.25	1	16	4	2	8	64
Itraconazole	2	0.5	0.03	2	0.25	1	0.25	1
Ketoconazole	2	0.5	0.06	0.12	0.06	1	0.25	0.06
Voriconazole	2	0.03	0.03	0.03	0.12	0.25	0.06	0.5
Caspofungin	2	0.5	0.06	2	0.06	0.06	0.06	0.12
Ampho B	3		0.5	1	1	0.5	1	4
Fluconazole	3		4	4	2	4	2	64
Itraconazole	3		0.25	0.25	0.25	4	0.12	0.06
Ketoconazole	3		0.25	0.25	0.06	0.5	0.12	0.25
Voriconazole	3		0.06	0.12	0.015	0.25	0.06	0.12
Caspofungin	3		0.06	0.25	0.12	0.06	1	0.25

ATCC CAL – ATCC *C. albicans*, CAL 539 – *C. albicans* #539, CAL 544- *C. albicans* #544, AST 511- *Aspergillus terreus*, ASN – *Aspergillus niger*, FSH – *Fusarium cylindrocarpone*

Drugs

Using Duncan's new multiple range test, mean MIC values were analyzed. Table 3 showed the MIC values at 48 hours. At alpha ≤0.05, the mean MICS of amphotericin B (1.239), itraconazole (0.6005), caspofungin (0.4995), ketoconazole (0.2425), and voriconazole (0.1427) were statistically lower than the mean MICs of fluconazole (9.6625). Voriconazole had the lowest MIC translating to greatest potency.

Table 3. Mean MIC at 48 hours.

DRUG	MIC 48H	Std. error
Amphotericin B	1.239	0.4087
Fluconazole	9.6625	4.2333
Itraconazole	0.6005	0.2097
Ketoconazole	0.2425	0.0497
Voriconazole	0.1427	0.032
Caspofungin	0.4995	0.2144

Organisms

Using Duncan's new multiple range test, susceptibility of the organism to the antifungal drugs were analyzed. At alpha ≤0.05, *Fusarium* had the highest MIC (8.0889) while all the other isolates showed homogenously lower MICs (Table 4).

Table 4. Mean MIC of the isolates.

ISOLATE	MIC 48H	Std. error
Aspergillus niger	1.0822	0.4669
Aspergillus terreus 511	1.5411	0.5025
ATCC *C. albicans*	0.5775	0.1985
Candida albicans 539	0.447	0.2219
Candida albicans 544	1.577	0.8817
Candida sp. 533	0.6419	0.2348
Fusarium cylindrocarpone	8.0889	4.8170

Table 5 showed the frequency of sensitive, intermediate, and resistant organisms to the anti-fungals tested. All isolates tested were sensitive to voriconazole. Eighty percent of the isolates were sensitive to amphotericin B. Ten percent were resistant and the remaining 10% were intermediately sensitive. Fluconazole and caspofungin showed similar in-vitro activity. There were more isolates intermediately susceptible to itraconazole and ketoconazole. There seemed to be an emerging resistance of local isolates towards itraconazole, with about 25% isolates resistant and 40% only intermediately susceptible. Ketoconazole shared an almost similar picture with more isolates only intermediately resistant. Eighty-five percent of all the isolates were sensitive to fluconazole and 10% resistant. Ninety percent of the isolates were sensitive to caspofungin. Note that the isolate *Aspergillus terreus* was resistant to the antifungals 1/3 of the time and *Fusarium cylindrocarpone* was resistant 1/5 of the time.

Table 5. Frequency of sensitive, intermediate, and resistant isolates to the antifungal drugs.

Drug	Frequency of Isolates		
	Intermediate	Resistant	Sensitive
Amphotericin B	2	2	16
Fluconazole	1	2	17
Itraconazole	8	5	7
Ketoconazole	9	3	8
Voriconazole	0	0	20

Susceptibility of the Yeasts

Susceptibility of the yeast pathogens (*Candida albicans* and *Candida* sp.) to all the antifungals were analyzed using Pearson's chi-square test. Table 6 showed the sensitivity of the yeast organisms to the different drugs. Results were statistically significant implying that the yeasts were sensitive to all the drugs tested. All the organisms were sensitive to amphotericin B and voriconazole. There was 1 yeast isolate resistant to caspofungin, and 1 yeast isolate intermediately sensitive to fluconazole. More than 50% of the isolates were resistant to ketoconazole and more than 50% intermediately sensitive to itraconazole.

Table 6. Sensitivity of yeast isolates to the antifungal drugs.

Drug	Frequency of Yeast Isolates		
	Intermediate	Resistant	Sensitive
Amphotericin B	0	0	9
Fluconazole	1	0	8
Itraconazole	5	2	2
Ketoconazole	4	0	5
Voriconazole	0	0	9
Caspofungin	0	1	8

Susceptibility of the Molds

Susceptibility of the molds (*Aspergillus* and *Fusarium*) to the antifungal agents were analyzed using Pearson's chi-square test. Table 7 showed the sensitivity of the molds to the different drugs. Results were statistically significant showing that all organisms were sensitive to voriconazole. Sixty-six percent of the filamentous fungi were sensitive to amphotericin B, 2 isolates resistant, and 1 intermediately sensitive. The filamentous fungi had similar sensitivities to fluconazole and caspofungin. There were more isolates intermediately resistant to ketoconazole than the other drugs.

Table 7. Sensitivity of filamentous fungi to drugs.

Drug	Frequency of Molds/ Filamentous Isolates		
	Intermediate	Resistant	Sensitive
Amphotericin B	1	2	6
Fluconazole	0	2	7
Itraconazole	1	3	5
Ketoconazole	4	2	3
Voriconazole	0	0	9
Caspofungin	0	1	8

DISCUSSION

This study showed that activities of in-vitro voriconazole, in general, were higher than amphotericin B and the other antifungal drugs. This was consistent with the few in-vitro comparisons of voriconazole to established agents against a similar spectrum of fungi.

For *Candida albicans* and *Candida* sp., in-vitro activities of voriconazole were comparable to amphotericin B, which remains the gold standard in treating these yeast infections. This was in agreement with previous studies on voriconazole activity against yeasts.[5,6,7] Fluconazole and caspofungin were seen to be appropriate alternatives if there was unavailability of amphotericin B or voriconazole. This study also demonstrated that MIC endpoints for voriconazole were comparable to or less than those of the established agents for the common yeast pathogens, including some isolates for which the amphotericin B and itraconazole MICs were high, as well as fluconazole-resistant (MICs of 64 ug/mL). It has been reported that voriconazole was active against all *Candida* species, including *C. krusei*, strains of *C. glabrata* that were inherently fluconazole-resistant and strains of *C. albicans* that had acquired resistance to fluconazole.[8]

Findings of this study were conflicting with the results of Espinel-Ingroff, where in-vitro activity of voriconazole against yeast infections were less than those of amphotericin B and fluconazole, including some isolates for which the amphotericin B and itraconazole MICs were high (>2 µg/mL), as well as fluconazole-resistant (MICs >64 µg/mL) and susceptible-dose-dependent (MICs of 16 to 32 µg/mL) *Candida* spp. strains.[6] A possible reason for this conflict was in the methodology. The mentioned study used macrodilution method of sensitivity testing and interpretative breakpoints used were different. In general, antifungal sensitivity testing remained unstandardized and this was where the confusion or difference in results lie.

For *Aspergillus terreus, Aspergillus niger,* and *Cylindrocarpone*, voriconazole was shown to be superior to all the other drugs tested, including amphotericin B. Published studies on voriconazole activity against *Aspergillus* and *Fusarium species* were less compared to those involving yeast pathogens. Voriconazole has been tested mainly on *Candida* species and this finding of its remarkable activity against the molds or filamentous fungi was invaluable. It supported the earliest study of Nguyen and colleagues, who found that voriconazole was more active in-vitro than amphotericin B against *Aspergillus* sp.[9] The mean MICs of voriconazole and amphotericin B against *Aspergillus* spp. were 0.36 microg/mL and 0.64 microg/mL, respectively. In that study, voriconazole also demonstrated fungicidal activity against *Aspergillus* sp., with 86% of isolates exhibiting minimum lethal concentrations of ≤4 mcg/mL. In the study by Espinel-Ingroff, the in-vitro activities of voriconazole were similar to or better than those of itraconazole and amphotericin B against *Aspergillus* spp. and *Fusarium* spp.,[6] supporting the findings of this study.

Many studies stated that the treatment of choice for infections caused by filamentous fungi is natamycin[10] (Natacyn, Alcon) and it is this study's limitation that it was not able to investigate on natamycin in-vitro activity. However, this study clearly showed the superiority of voriconazole over other antifungals in the treatment of infections caused by the molds. In circumstances where natamycin was unavailable, voriconazole was an obvious alternative. Further studies to compare voriconazole and natamycin would be needed to elucidate whichever was superior.

This study also suggested a possible emerging resistance of local isolates towards itraconazole, with about 25% isolates resistant.

Of the organisms tested, *Fusarium* needed higher amounts of all the antifungals tested. This might be due to the fact that *Fusarium* is an intrinsically pathogenic organism, as opposed to opportunistic. *Fusarium* is a filamentous fungus with true hyphae that rendered the organism more resistant to lower amounts of the antifungal. Higher MIC values for *Fusarium* seen in this study was consistent with findings of previous studies.[11]

In conclusion, local fungal isolates proved to be sensitive to voriconazole and other antifungals. At present, there seemed to be no emerging resistance among the local isolates to the antifungals. However, this study clearly showed that voriconazole might be as effective or even more superior in the treatment of fungal infections, both from yeasts and filamentous organisms alike. For yeast infections, voriconazole was comparable to amphotericin B activity. For mold infections, voriconazole proved to be even more superior to amphotericin B.

This study also recommended further studies on the following: (1) antifungal sensitivity testing using ocular drug concentrations, (2) sensitivity testing including natamycin, and lastly (3) more replicates included to further strengthen the conclusion.

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