Susceptibility of Filamentous Fungi to Voriconazole in Malaysia Tested by Sensititre YeastOne and CLSI Microdilution Methods

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Abstract

Background: Voriconazole is a trizaole antifungal to treat fungal infection. In this study, the susceptibility pattern of voriconazole against filamentous fungi was studied using Sensititre® YeastOne and Clinical & Laboratory Standards Institute (CLSI) M38 broth microdilution method.

Methods: The suspected cultures of Aspergillus niger, A. flavus, A. fumigatus, A. versicolor, A. sydowii, A. calidoutus, A. creber, A. ochraceopetaliformis, A. tamarii, Fusarium solani, F. longipes, F. falciferus, F. keratoplasticum, Rhizopus oryzae, R. delemar, R. arrhizus, Mucor sp., Poitrasia circinans, Syncephalastrum racemosum and Sporothrix schenckii were received from hospitals. Their identification had been confirmed in our lab and susceptibility tests were performed using Sensititre® YeastOne and CLSI M38 broth microdilution method. The significant differences between two methods were calculated using Wilcoxon Sign Rank test.

Results: Mean of the minimum inhibitory concentrations (MIC) for Aspergillus spp. and Fusarium were within 0.25 μg/mL-2.00 μg/mL by two methods except A. calidoutus, F. solani and F. keratoplasticum. Moreover, mean of MIC for S. schenkii were around 3.00 μg/mL by two methods. In contrast, mean of MIC for Rhizopus spp., Mucor sp., P. circinans and S. racemosum were ≥6.00 μg/mL by two methods. Generally, the MIC obtained by Sensititre YeastOne was one two-fold increase or decrease compared with the results obtained by CLSI method. The overall agreement between Sensititre YeastOne and CLSI methods to test susceptibility testing of voriconazole was more than 70% except A. sydowii. The significant differences between two methods were significant when tested on A. niger, A. flavus, A. fumigatus, A. versicolor, A. sydowii, F. solani and S. schenkii.

Conclusions: In conclusion, Sensititre YeastOne method appears to be an alternative procedure for antifungal susceptibility testing for some Malaysian moulds.

Background

Voriconazole is a potent new triazole drug with a broad spectrum of antifungal activity against many opportunistic fungal pathogens [1–6]. Previous studies reported that voriconazole can enhance clinical efficacy and coupled with lower toxicity [7]. It had prevented or delayed the mortality in infected animals [8, 9].

To date, much work on susceptibility has been done on yeast, especially for the Candida species, but susceptibility data on moulds is still limited. This could be due to the lack of established breakpoints for moulds, cost of antifungal reagents and laborious laboratory procedures [10]. A commercial panel named Sensititre® YeastOne (Thermo Fisher Scientific, Cleveland, United States) had been widely used in many routine microbiology laboratories recently [11, 12]. It is a commercial colorimetric panel that contains dried serial dilutions of antifungal agents in a disposable tray [13]. The MIC from this panel is based on the visible colour change which caused by an oxidation-reduction indicator, Alamar Blue [14].
In this study, MICs of voriconazole against isolated moulds were determined. It will be beneficial to clinicians in monitoring and selecting appropriate therapy for patients. The present study had assessed the performance for in vitro susceptibility testing of voriconazole against several moulds in Mycology laboratory, Bacteriology Unit, Institute for Medical Research using Sensititre® YeastOne and compared with Clinical and Laboratory Standards Institute (CLSI) broth microdilution method M38 [15].

**Methods**

**Ethic**

Ethical review was conducted and approved by the Medical Research and Ethics Committee, Ministry of Health of Malaysia, Malaysia (NMRR-20-207-53607).

**Sample**

The suspected culture of *Aspergillus niger* (n=24), *A. flavus* (n=13), *A. fumigatus* (n=12), *A. versicolor* (n=8), *A. sydowii* (n=4), *A. calidoutus* (n=3), *A. creber* (n=1), *A. ochraceopetaliformis* (n=1), *A. tamarii* (n=1), *Fusarium solani* (n=6), *F. longipes* (n=2), *F. falciferus* (n=1), *F. keratoplasticum* (n=3), *Rhizopus oryzae* (n=2), *R. delemar* (n=1), *R. arrhizus* (n=1), *Mucor* sp. (n=2), *Poitrasia circinans* (n=1), *Syncephalastrum racemosum* (n=2) and *Sporothrix schenckii* (n= 9) in potato dextrose agar were received from Malaysian hospitals in year 2019. Their identifications were carried out by both macroscopic and microscopic methods. The moulds were inoculated on Sabouraud dextrose agar and potato dextrose agar. Following that, the agar plates were incubated in air at 30°C and their growth were inspected daily. The lactophenol cotton blue wet mount was used to stain the mature colony with scotch-tape technique.

**Culture medium**

RPMI-1640 (Sigma-Aldrich, St. Louis, United States) with glutamine and phenol red, without sodium bicarbonate and buffered with 0.165 mol/L 3-morpholinopropanesulfonic acid (MOPS) (Sigma-Aldrich, St. Louis, United States) at pH 7.0, was used as the basal medium. The procedure of preparing RPMI-1640 was following the procedures described in CLSI M38 [15].

**CLSI method**

Voriconazole (VOR) (Pfizer, North Carolina, USA) was dissolved with Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, United States) and diluted into RPMI media as mentioned in CLSI M38 [15]. Antifungal dilutions were ranged from 32.0000 to 0.0625 µg/ mL.

For the reference broth microdilution testing, conidial suspensions were prepared as described in the CLSI M38 document [15]. The plates were incubated at 30°C, and MIC were defined as the lowest drug concentration that prevents any discernible growth.

**Sensititre YeastOne**
A colorimetric microdilution method was performed using commercially available Sensititre YeastOne panels (Thermo Fisher Scientific, Cleveland, United States) according to the manufacturer’s recommendations. Colony-forming unit counts were affirmed each time by spread plate counts on potato dextrose agar. The range of concentration of voriconazole in the panel was 0.008 to 8 µg/mL.

**Quality controls.**

Each test had included two reference strains; *A. flavus* ATCC 204304, *A. fumigatus* ATCC 204305, in order to detect any alteration in the antifungal agent should the MIC obtained fail to fall within the reference range.

**Agreement between two methods**

The 2-fold differences and the level of agreement between the two methods were calculated for each species/drug/combinations as the proportion of the Sensititre YeastOne colour endpoints determined for each strain that fell within ±1 twofold dilutions of the corresponding MICs of the CLSI method.

**Analysis of data**

The significant differences between two methods were calculated using Wilcoxon Sign Rank test. The tests were performed for species that have more than three isolates. Any *p* values less than 0.05 were considered as statistically significant.

**Results**

In vitro susceptibility testing was carried out using voriconazole against *Aspergillus* spp. (*n* = 67), *Fusarium* spp. (*n* = 12), *Rhizopus* spp. (*n* = 4), *Mucor* sp. (*n* = 2), *P. circinans* (*n* = 1), *S. racemosum* (*n* = 2), and *S. schenckii* (*n* = 9). The MIC range, mean, MIC<sub>50</sub> and MIC<sub>90</sub> results for both Sensititre® YeastOne and reference CLSI method are shown in the Table 1. The *Aspergillus* spp. was the most common moulds that we received. The mean MICs of voriconazole for *Aspergillus* spp. were ≤ 2 µg/mL, with exception of *A. calidoutus*, which recorded 8 µg/mL with Sensititre® YeastOne; while 4 µg/mL with CLSI reference method. Moreover, the percentage of agreement between two methods were high (≥ 90%) when tested against *A. niger*, *A. flavus*, *A. fumigatus*, *A. calidoutus*, *A. creber*, *A. ochraceopetaliformis*, *A. tamarii* and *A. ochraceopetaliformis*, although the number of some tested species tested were not significant.

Meanwhile, the mean MICs of voriconazole for *Fusarium* spp. were slightly higher than most of the *Aspergillus* spp. The minimum and maximum MIC mean for *Fusarium* was 0.75 µg/mL and > 8.00 µg/mL respectively. Some of *F. solani* and *F. keratoplasticum* have high MIC (≥ 8.00 µg/mL) compared with *F. longipes* and *F. falciferus* which have lower MIC (≤ 2.00 µg/mL). In general, Sensititre MIC<sub>50</sub> and MIC<sub>90</sub> were one two-fold dilution higher or lower than those of the CLSI. Finally, the percentage of agreement between two methods were perfect (100%) and it was higher compared with *Aspergillus* spp.
All of the *Rhizopus* spp. had given the similar results when tested by Sensititre or CLSI method. The mean MICs of voriconazole for *Rhizopus* spp. were higher than *Aspergillus* spp. and most of the *Fusarium* spp. However, the exact MICs were not able to be determined by Sensititre as it had exceeded the tested range. Therefore, the MIC\textsubscript{50} and MIC\textsubscript{90} were not able to be determined for *Rhizopus* spp. On the other hand, the percentage of agreement between two methods were perfect and achieved 100.00%.

In addition, the mean MICs of voriconazole against *Mucor* sp., *P. circinans*, *S. racemosum* and *S. schenkii* were higher than most of the *Aspergillus* spp. and *Fusarium* spp. too. The MICs by Sensititre method were one double dilution different than those of the CLSI method, except *S. schenkii* which recorded similar value. One of the *Mucor* sp. and *P. circinans* were found unable to be inhibited by the highest tested concentration of voriconazole by both methods. However, the percentage of agreement for both methods to determine MIC against *S. schenkii* (77.78%) was lower than *Mucor* sp., *P. circinans*, and *S. racemosum* (100.00%).
Table 1
Susceptibilities of isolates to voriconazole and agreement between Sensititre YeastOne and reference microdilution method

| Species                  | MIC by Sensititre® YeastOne (µg/ mL) | MIC by CLSI (µg/ mL) | Agreement (± one 2-fold dilution) (%) | p value |
|--------------------------|--------------------------------------|----------------------|--------------------------------------|---------|
| **Range**                | **Mean**                             | **MIC₅₀/MIC₉₀**       | **Range**                            | **Mean** | **MIC₅₀/MIC₉₀** | **p value** |
| A. niger (n = 24)        | 0.03-2.00                            | 0.60                 | 0.50/1.00                           | < 0.03-1.00 | 0.25/0.50       | 91.67 *       |
| A. flavus (n = 13)       | 0.25-1.00                            | 0.67                 | 0.50/1.00                           | 0.25-1.00 | 0.25/0.50       | 92.31 0.887   |
| A. fumigatus (n = 12)    | 0.25-1.00                            | 0.44                 | 0.50/0.50                           | 0.13-1.00 | 0.25/0.50       | 91.67 0.439   |
| A. versicolor (n = 8)    | 0.03-2.00                            | 0.88                 | 0.50/2.00                           | 0.25-2.00 | 1.00/2.00       | 75.00 0.443   |
| A. sydowii (n = 4)       | 1.00-2.00                            | 1.25                 | 1.00/2.00                           | 0.25-1.00 | 0.56            | 50.00 0.180   |
| A. calidoutus (n = 3)    | 8.00                                 | 8.00                 | -                                   | 4.00      | -               | 100.00 0.250  |
| A. creber (n = 1)        | 0.50                                 | 0.50                 | -                                   | 1.00      | -               | 100.00         |
| A. ochraceo-petaliformis (n = 1) | 0.50                              | 0.50                 | -                                   | 1.00      | -               | 100.00         |
| A. tamarii (n = 1)       | 0.25                                 | 0.25                 | -                                   | 0.50      | -               | 100.00         |
| F. solani (n = 6)        | 0.50-8.00                            | 3.75                 | 4.00/4.00                           | 0.50-16.00 | 5.75           | 4.00/8.00 0.180 |
| F. longipes (n = 2)      | 2.00                                 | 2.00                 | 2.00/2.00                           | 0.50-1.00 | 0.75            | 100.00         |
| F. falciferus (n = 1)    | 2.00                                 | 2.00                 | -                                   | 1.00      | -               | 100.00         |
| F. keratoplasticum (n = 3) | 2.00- >8.00                       | *                   | > 8.00/ >8.00                       | 2.00-8.00 | 6.00            | 8.00/8.00 *    |

* Unable to be calculated
| Species             | MIC by Sensititre® YeastOne (µg/ mL) | MIC by CLSI (µg/ mL) | Agreement (± one 2-fold dilution) (%) | p value |
|---------------------|--------------------------------------|----------------------|--------------------------------------|---------|
|                     | Range Mean MIC<sub>50</sub>/MIC<sub>90</sub> | Range Mean MIC<sub>50</sub>/MIC<sub>90</sub> |                                      |         |
| R. oryzae (n = 2)   | 8.00-<8.00 * 8.00/8.00 8.00-8.00 8.00 | 8.00/8.00 8.00       | 100.00                               |         |
| R. delemar (n = 1)  | >8.00 * - 16.00 16.00 - | 100.00 - |         |         |
| R. arrhizus (n = 1) | >8.00 * - 16.00 16.00 - | 100.00 - |         |         |
| Mucor sp. (n = 2)   | 8.00-<8.00 * 4.00-16.00 * | 100.00 - |         |         |
| P. circinans (n = 1)| >8.00 * - >16.00 * | 100.00 - |         |         |
| S. racemosum (n = 2)| 8.00 8.00 8.00/8.00 4.00-8.00 6.00 | 4.00/8.00 100.00 - |         |         |
| S. schenkii (n = 9)| 0.50-8.00 3.28 4.00/4.00 1.00-8.00 2.78 | 2.00/4.00 77.78 0.302 |         |         |

* Unable to be calculated

**Discussion**

Voriconazole is a new broad-spectrum triazole antifungal agent with fungicidal activity against *Aspergillus* spp. [1]. It has good bioavailability and well tolerated by humans [16]. Previous clinical studies of invasive aspergillosis have provided encouraging results [17, 18]. Voriconazole was demonstrated well absorbed following oral administration and was highly effective in preventing or delaying mortality in an experimental model of pulmonary aspergillosis [9]. The initial results from animals’ trials also suggested it was effective in disseminating the *Aspergillus* infection [8, 19]. The survival was proved greater with voriconazole treatment compared to itraconazole treatment [9]. Moreover, the superiority of voriconazole to amphotericin B for the treatment of invasive aspergillosis also has been reported [20, 21]. In May 2002, voriconazole had been approved by the Food and Drug Administration for the treatment of invasive aspergillosis [5].

The breakpoints of voriconazole against mould have not been determined by CLSI [15]. However, European Committee on Antimicrobial Susceptibility Testing Subcommittee on Antifungal Susceptibility Testing (EUCAST-AFST) has determined breakpoints for voriconazole against *A. fumigatus* where susceptibility ≤ 1 mg/L and resistance > 1 mg/L [22]. The voriconazole MICs for *Aspergillus* showed no
significant difference among species [23]. This is parallel with our finding except for A. calidoutus. In addition, the ratio of Sensititre MIC<sub>50</sub> to MIC<sub>90</sub> for A. niger and A. flavus in this study were one two-fold dilution higher than Linares et al. [24]; however, the CLSI ratio for A. fumigatus and A. niger were same. On the other hand, the ratio of CLSI MIC<sub>50</sub> to MIC<sub>90</sub> of A. niger [9] were same with the result in this study; however, its ratio for A. fumigatus and A. flavus were lower. Furthermore, the CLSI mean and MIC<sub>90</sub> for A. fumigatus, A. niger and A. flavus reported by Espinel-Ingroff were one two-fold dilution higher than our finding [25].

*Fusarium* spp. are resistant *in vitro* to many antifungal compounds [26]. The management of fusariosis is not well defined; therefore, susceptibility test might be useful in choosing the suitable antifungal therapy [26]. The satisfactory response for voriconazole against fusariosis was 45% [27]. Voriconazole MICs for *Fusarium* spp. were higher than other genus including *Aspergillus* spp. [23, 29]. Arikan et al. suggested that it might due to the use of 100% growth reduction endpoint instead of 50% growth reduction endpoint [23]. The MIC of *Fusarium* spp. was usually ranged 1 to 4 µg/mL [2, 23, 29]. However, among several tested *Fusarium* spp., *F. solani* was found the most resistant species to various drugs including amphotericin B, itraconazole, posaconazole and voriconazole [30]. Both of their MIC<sub>50</sub> and MIC<sub>90</sub> of voriconazole against *F. solani* were recorded > 8.0 µg/mL. A similar result also obtained by Alastrauey-Izquierdo et al. where their MIC was ranged 4–16 µg/mL [26]. Interestingly, their findings are similar with our observations except one of the samples had low MIC which recorded 0.50 µg/mL.

Besides *Aspergillus* and *Fusarium*, *Rhizopus* can also cause severe and fatal infections in immunocompromised patients [31, 32]. The treatment of zygomycosis is problematic and frequently associated with suboptimal therapeutic outcomes [33]. Voriconazole possesses no meaningful activity against *Rhizopus* strains [34–36]. This is parallel with our finding. Instead, posaconazole and amphotericin B were found active and potent against *Rhizopus* [36, 37]. The MIC from the combination of both of these drugs were lower than those from single drug [36]. Therefore, this combination could be tested in our future study.

Similar to *Rhizopus* spp., voriconazole has shown no reliable activity against *Mucor* spp. [34, 37]. As mucormycosis is less common than aspergillosis and the course is progressively rapid; therefore, the effectiveness of antifungal treatment in small case studies is difficult to evaluate. Since Mucorales are resistant in vitro to many antifungals [38], its treatment with fluconazole, flucytosine, ketoconazole, echinocandins, itraconazole and voriconazole were reported not effective in many cases [39–43]. On the other hand, data on the antifungal susceptibility of Mucorales spp. are limited, and MIC testing remains investigational [44]. The means of voriconazole MIC were higher than 32 µg/ mL [34, 37] and the MIC<sub>90</sub> was even > 64 µg/mL [37]. These MIC were much higher than the finding in this study. However, more samples are needed to determine the accuracy of the result.

*Poitrasia circinans* is fall under the order Mucorales. Both Sensititre and CLSI methods had shown that voriconazole had inactive activity against this mould. The result of this sample was similar with *Mucor* sp.
Meanwhile, *S. racemosum* is an opportunistic pathogen and rarely caused infection in human [40]. Thus, research related with its susceptibility testing was limited. Chowdhary et al. reported that the MIC$_{50}$ and MIC$_{90}$ were 8 and 16 µg/mL respectively by CLSI method [45]. This result was four-fold higher than the CLSI result obtained in this study.

Sporotrichosis is a subacute or chronic infection which caused by the dimorphic fungus *Sporothrix schenckii* [46]. The antifungal drugs which commonly used are itraconazole for cutaneous or lymphocutaneous fixed forms [47], and amphotericin B for disseminated cases [48, 49]. However, these antifungal drugs are not always efficient and may lead to chronicity and disseminate in immunocompromised patients [50]. Several studies have searched and tested for alternatives including voriconazole; however, the MIC$_{50}$ and MIC$_{90}$ obtained were varied among them. By using CLSI method, MIC$_{50}$ was reported 32, 16 and 8 µg/mL; while the MIC$_{90}$ was reported 32, >16 and 16 µg/mL by Marimon et al., Rodrigues et al. and Córdoba et al. respectively [51, 52, 50]. However, both of the CLSI MIC$_{50}$ and MIC$_{90}$ in this study were lower than these reported findings.

Although the broth microdilution methods had improved the level of interlaboratory agreement of antifungal MIC endpoints; however, these procedures are laborious, inconvenient, labour intensive and inefficient for the clinical laboratory [13, 53, 54]. This is due to the main disadvantage of the M38 method is the need to prepare microdilution plates, which is time-consuming and impractical for routine use in clinical microbiology laboratories [55]. Sensititre® YeastOne is an adapted susceptibility system of the microbroth CLSI method based on the M27-A3 standard for yeasts and has been extensively evaluated for yeasts. It has been approved by the U.S. Food and Drug Administration (FDA) for *Candida* species but not for mould yet [56].

The level of agreement for the voriconazole between Sensititre and CLSI methods were inconsistent throughout publications. For example, Wang et al. and Mello et al. had found 100% agreement of Sensititre with the CLSI reference method for the voriconazole when they tested on all the *Aspergillus* spp [55, 57]. In contrast, Castro et al. reported that the overall agreement between Sensititre and CLSI methods for voriconazole was only 82.5% [13]. Moreover, the phenomenon of Sensititre® YeastOne test tended to increase or decrease the MIC by only one dilution when compared with the reference test also had been reported by Siopi et al., Castro et al., Sanchez Sousa et al. and Guinea et al. This phenomenon was consistent with the finding in this study [11, 13, 14, 54].

Based on in vitro susceptibility pattern in this study, voriconazole seems to have reliable activity against most of the species except *A. calidoutus, F. keratoplasticum, R. oryzae, R. delemar, R. arrhizus, Mucor* sp., *P. circinans, S. racemosum* and *S. schenkii* as their mean were more than 1 µg/mL. However, the correlation between MIC results and treatment outcome is not well defined [26, 58]. More data from clinical trials with voriconazole are required for this purpose. Despite voriconazole was shown well-tolerated in human [58]; however, the doses have to be monitored to minimize its side effects such as visual disturbances, skin rashes, elevations in several hepatic enzyme levels, headache, nausea and vomiting, diarrhoea and abdominal pain [4].
To our knowledge, this was the first study to compare the susceptibility of voriconazole against Malaysian moulds using both CLSI and commercial Sensititre YeastOne methods. Moreover, the research pertaining susceptibility pattern of *A. versicolor*, *A. sydowii*, *A. calidoutus*, *A. creber*, *F. keratoplasticum*, *Mucor* sp., *S. racemosum*, *P. circinans* and *S. schenckii* are still limited especially in Malaysia. In contrast, there are several limitations in this study. The sample sizes of some isolates were small and MIC$_{50}$ and MIC$_{90}$ were unable to be determined. In addition, the MICs were still not able to be interpreted as susceptible or resistant there are no official clinically correlated breakpoints for moulds according to CLSI method. However, these results could contribute to its limited antifungal database in Malaysia.

**Conclusions**

In conclusion, data derived from the present study support the claim that Sensititre® Yeast One method might be equivalent to the CLSI reference method for the determination of MIC of *A. flavus*, *A. fumigatus*, *A. versicolor*, *A. sydowii*, *A. calidoutus*, *F. solani* and *S. schenckii* against voriconazole. This is due to the Wilcoxon signed rank test had found there were no significant difference ($p > 0.05$) between these two methods when testing on these species. This is consistent with what were suggested by Castro et al., Guinea et al. and Martin-Mazuelos et al. [13, 54, 59].

**Abbreviations**

CLSI: Clinical & Laboratory Standards Institute; EUCAST: European Committee on Antimicrobial Susceptibility Testing Subcommittee on Antifungal Susceptibility Testing; FDA: Food and Drug Administration; MIC: Minimum Inhibitory Concentration; spp.: several species; U.S United States.

**Declarations**

**Acknowledgement**

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**Author’s contribution**

TXT contributed to the study design, experiments, and the writing the manuscript. SJG contributed to sample collection and mould identification. FbA conceived the study and assisted in experimental design. SbMS contributed to the study coordination. SbS helped to performs some experiments. All authors have read and approved the final manuscript.

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**Availability of data and materials**

The datasets used in the current study are available from the corresponding author on reasonable requests.

**Ethic approval and consent to participate**

The Medical Review & Ethics Committee (MREC) had approved this study.

**Consent for publication**

Not applicable

**Completing interest**

None

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