COMPARISON OF YEAST AND FUNGUS FORM *IN VITRO* SUSCEPTIBILITIES OF *SPOROTHRIX SCHENCKII* IN MALAYSIA

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Abstract. *Sporothrix schenckii* causes sporotrichosis, but the sensitivities of this organism's yeast and fungus forms in Malaysia to antifungals are not well studied. In this study, we aimed to determine the minimum inhibitory concentrations (MIC) of the yeast and fungus forms of *S. schenckii* obtained clinically to evaluate if there is a difference in susceptibility testing between these two forms: yeast and fungus. We retrospectively reviewed the susceptibilities of 96 clinical specimens of *S. schenckii* obtained during 2020-2021. Yeast and fungus specimens were identified through microscopic, macroscopic and molecular methods. The susceptibility of each isolate was determined following the Clinical and Laboratory Standards Institute (CLSI) M27-A4 and M38 guidelines. The tested antifungals were amphotericin B, terbinafine, posaconazole, voriconazole, itraconazole, ketoconazole, ravuconazole and isavuconazole. For each antifungal, the geometric mean (GM) MICs, MIC_{50} and MIC_{90} were determined. A comparison between the GM MICs for yeast and fungus forms was conducted using the Wilcoxon Sign Rank test with significance set at $p<0.05$. The GM MICs of amphotericin B, terbinafine, posaconazole, voriconazole, itraconazole, ketoconazole, ravuconazole and isavuconazole against the fungus form were 1.93 μg/ml, 0.13 μg/ml, 0.17 μg/ml, 0.68 μg/ml, 0.20 μg/ml, 0.09 μg/ml, 0.16 μg/ml and 0.18 μg/ml, respectively and against the yeast form were 0.23 μg/ml, 0.05 μg/ml, 0.04 μg/ml, 0.04 μg/ml, 0.04 μg/ml, 0.04 μg/ml, 0.04 μg/ml and 0.04 μg/ml, respectively. The MIC_{50} and MIC_{90} for ketoconazole against the fungus form were the lowest at 0.03 μg/ml and 0.5 μg/ml, respectively and the MIC_{50} and MIC_{90} against the yeast form were also the lowest at 0.03 μg/ml and 0.06 μg/ml, respectively. The MIC_{50} and MIC_{90} of amphotericin B against fungus form were the highest at 4 μg/ml and 16 μg/ml, respectively and against the yeast form were 0.03 μg/ml and 16 μg/ml, respectively. The MICs for each antifungal were significantly higher against the fungus form than the yeast form ($p<0.05$). The GM MIC and MIC_{90} results of the tested antifungals

were significantly lower against the yeast than the fungus forms, but the MIC_{50} results were not significantly different between the yeast and fungus forms. However, the best growth form (fungus or yeast) of *S. schenckii* that should be used for sensitivity testing to best represent the clinical response has yet to be determined. Further studies are needed to determine if these findings can inform clinical decision-making.

Keywords: susceptibility, *Sporothrix schenckii*, fungus, yeast

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INTRODUCTION

Sporotrichosis, called Gardener's Disease, occurs commonly among those who cultivate roses (Engle *et al*, 2007). This infection can become subacute or chronic and infect the fascia, lymphatics, cartilage, muscles and bones of animals and humans (Barros *et al*, 2011; Han and Kano, 2021; Marimon *et al*, 2008). Sporotrichosis is endemic and common in tropical and subtropical areas, including Malaysia (Kamal Azam *et al*, 2020; Chakrabarti *et al*, 2015).

Sporotrichosis is caused by a thermally dimorphic fungus called *Sporothrix schenckii sensu lato* (*S. schenckii*) (Davis, 1996; Feeney *et al*, 2007). It exists in the fungus form at 25°C and in the yeast form at 37°C (Barros *et al*, 2011). Although the yeast form is pathogenic, contraction of infection is usually due to exposure to the conidia of the fungus form (Chakrabarti *et al*, 2015). The best method of susceptibility testing to give the best clinical treatment outcome for this dimorphic fungus (yeast and fungus) has not yet been established (Kohler *et al*, 2006). The yeast form is often used since this is the form most often isolated from human tissue (Barros *et al*, 2011; Tovikkai *et al*, 2020). Conversion of the fungal to the yeast form in the lab is problematic because it requires a temperature of 35°C and 5% CO_2, and this conversion can take time to occur (Casali and Hamdan, 1997; Kohler *et al*, 2004; Kohler *et al*, 2006). The fungal form of this organism can more easily be grown in the laboratory due to its low nutrient requirement, and it can be grown at 25°C (Kohler *et al*, 2006).

Another problem is that the protocol for testing the susceptibility of *S. schenckii* against antifungals has

only been developed for the fungal form of this organism (CLSI, 2017a). In addition, epidemiologic cutoff values (ECVs) for amphotericin B, itraconazole, posaconazole and voriconazole were proposed only for the fungal form of *S. schenckii* (Espinel-Ingroff *et al*, 2017). Hence, most susceptibility testing studies are performed using only the fungal form (Alvarado-Ramirez and Torres-Rodriguez, 2007; Marimon *et al*, 2008; Pereira Silveira *et al*, 2009).

The susceptibilities of the yeast and fungus forms of *S. schenckii* to antifungals are unclear. Therefore, in this study, we aimed to determine the minimum inhibitory concentrations (MIC) of the yeast and fungus forms of *S. schenckii* obtained clinically to determine if there is a difference in susceptibilities.

MATERIALS AND METHODS

Isolates

We retrospectively reviewed the charts of patients diagnosed with sporotrichosis during 2020-2021, and in whom *Sporothrix schenckii sensu lato* (*S. schenckii*) was isolated and sent to the Institute for Medical Research, Malaysia. The minimum number of samples calculated for this study was 97, following the calculation recommended by Ariffin (2013). Each isolate was identified using multiple methods. Isolates were identified using an amplified fragment of the calmodulin (CAL) gene as described previously (Marimon *et al*, 2006), evaluating for primers CL1 and CL2 as described previously (O'Donnell *et al*, 2000). The fungal form was identified by examining colonies grown on potato dextrose agar (PDA) agar at 25°C for 7 days. The yeast form was identified by examining colonies grown on Brain Heart Infusion (BHI) agar at 37°C for 7 days. The growth was inspected daily. Finally, a lactophenol cotton blue wet mount was used to stain mature colonies using the Scotch-tape technique (Leck, 1999).

Susceptibility testing

The minimum inhibitory concentrations (MICs) of amphotericin B, terbinafine, posaconazole, voriconazole, itraconazole, ketoconazole, ravuconazole and isavuconazole were determined using twofold serial dilutions in the broth microdilution technique (at concentrations of 0.03-16 μg/ml) using the M38 technique (CLSI, 2017a) for the fungus form and the M27 technique for the yeast form (CLSI, 2017b). The MIC required to inhibit growth by 50% (MIC50) and the MIC required to inhibit growth by 90% (MIC90) were calculated for each tested antifungal.

The inoculum sizes used to conduct the MIC testing of the fungus and yeast forms were 0.4-5.0 x 10^4 CFU/ml and 5.0 x 10^2-2.5 x 10^3 cells per ml, respectively.

Each test included two reference strains for quality control: *Aspergillus*

Table 1

Minimum inhibitory concentrations of selected antifungals against the fungal and yeast forms of Sporothrix schenckii (N = 97)

Antifungals	GM MIC (μg/ml)		MIC_{50} (μg/ml)		MIC_{90} (μg/ml)		Significant difference between FF and YF
	FF	YF	FF	YF	FF	YF	
Amphotericin B	1.93	0.23	4	0.03	16	16	0.0038
Terbinafine	0.13	0.05	0.03	0.03	2	0.06	0.0155
Posaconazole	0.17	0.04	0.03	0.03	2	0.06	0.0155
Voriconazole	0.68	0.04	1	0.03	16	0.03	0.0069
Itraconazole	0.20	0.04	0.03	0.03	16	0.03	0.0009
Ketoconazole	0.09	0.04	0.03	0.03	0.5	0.06	0.0429
Ravuconazole	0.16	0.04	0.03	0.03	4	0.03	0.0031
Isavuconazole	0.18	0.04	0.03	0.03	2	0.03	0.0002

GM: geometric mean; FF: fungus form; MIC: minimum inhibitory concentration; μg/ml: micrograms per milliliter; MIC_{50}: the lowest concentration of the tested antifungal at which 50% of the isolates were inhibited; MIC_{90}: the lowest concentration of the antifungal at which 90% of the isolates were inhibited; YF: yeast form

Table 2

Differences in GM MIC, MIC_{50} and MIC_{90} values for a combination of the 8 tested antifungals against the fungal forms and yeast forms of the studied organism

Parameter	*p*-value
GM MIC	0.0117
MIC_{50}	0.1795
MIC_{90}	0.0176

GM MIC: genetic mean minimal inhibitory concentration; MIC_{50}: minimal inhibitory concentration required to suppress growth by 50%; MIC_{90}: minimal inhibitory concentration required to suppress growth by 90%.

flavus ATCC 204304 and *A. fumigatus* ATCC 204305.

Statistical analysis

The significant difference of geometric mean (GM) MICs, MIC_{50} and MIC_{90} for the yeast and fungus forms were compared using the Wilcoxon Sign Rank test. Statistical calculations were conducted using the Statistical Package for Social Sciences (SPSS), version 20.0 (IBM®, Armonk, NY). Statistical significance was set at $p<0.05$.

Ethical approval

Ethical approval for this study was obtained from the Medical Research and Ethics Committee, Ministry of Health for Malaysia (NMRR-20-207-53067).

RESULTS

Isolates were obtained from a total of 97 subjects, 58% male. The median (range) age of study subjects was 48 (20-65) years. The most common symptoms among subjects were small skin papules (90%) and sores or ulcers at the site of infection (74%). Ninety-five percent of subjects had lymphocutaneous/cutaneous sporotrichosis, 4% had mucosal sporotrichosis, and 1% had systemic sporotrichosis. Ninety-one percent of subjects had a history of contact with domestic cats or dogs, and 50% had a history of exposure to soil or plants.

The GM MICs for amphotericin B, terbinafine, posaconazole, voriconazole, itraconazole, ketoconazole, ravuconazole and isavuconazole against the fungus form were 1.93 μg/ml, 0.13 μg/ml, 0.17 μg/ml, 0.68 μg/ml, 0.20 μg/ml, 0.09 μg/ml, 0.16 μg/ml and 0.18 μg/ml, respectively and against the yeast form were 0.23 μg/ml, 0.05 μg/ml, 0.04 μg/ml, 0.04 μg/ml, 0.04 μg/ml, 0.04 μg/ml, 0.04 μg/ml and 0.04 μg/ml, respectively (Table 1). The GM MIC results show that the antifungals were significantly (p=0.0117) more effective against the yeast forms than the fungus forms (Table 2).

The MIC_{50} for amphotericin B, terbinafine, posaconazole, voriconazole, itraconazole, ketoconazole, ravuconazole and isavuconazole against the fungus form were 4 μg/ml, 0.03 μg/ml, 0.03 μg/ml, 1 μg/ml, 0.03 μg/ml, 0.03 μg/ml, 0.03 μg/ml and 0.03 μg/ml, respectively and against the yeast form were all 0.03 μg/ml (Table 1). There was no significant difference (p=0.1795) in the MIC_{50} for the tested antifungals between the fungus and yeast forms (Table 2).

The MIC_{90} for amphotericin B, terbinafine, posaconazole, voriconazole, itraconazole, ketoconazole, ravuconazole and isavuconazole against the fungus form were 16 μg/ml, 2 μg/ml, 2 μg/ml, 16 μg/ml, 16 μg/ml, 0.5 μg/ml, 4 μg/ml and 2 μg/ml, respectively and against the yeast form were 16 μg/ml, 0.06 μg/ml, 0.06 μg/ml, 0.03 μg/ml, 0.03 μg/ml, 0.06 μg/ml, 0.03 μg/ml

and 0.03 μg/ml, respectively (Table 1). The yeast form was significantly (p=0.0176) more sensitive to the tested antifungals than the fungus form (Table 2).

Ketoconazole and amphotericin B exhibited the lowest and highest GM MIC against *S. schenckii* for fungus and yeast forms (Table 1). The GM MICs of ketoconazole and amphotericin B against the fungus form were 0.09 μg/ml and 1.93 μg/ml, respectively and against the yeast form were 0.04 μg/ml and 0.23 μg/ml, respectively. Ketoconazole and amphotericin B had the lowest and highest MIC_{50} values against *S. schenckii*. The MIC_{50} for ketoconazole and amphotericin B against the fungal form was 0.03 μg/ml and 4 μg/ml, respectively and against the yeast form was 0.03 μg/ml. Ketoconazole and amphotericin B also had the lowest and highest MIC_{90} values against *S. schenckii*, respectively. The MIC_{90} for ketoconazole and amphotericin B against fungus form was 0.5 μg/ml and 16 μg/ml, respectively and against the yeast form was 0.06 μg/ml and 16 μg/ml, respectively.

The epidemiological cutoff values (ECV) proposed for amphotericin B, posazonazole voriconazole and itraconazole are: 4 μg/ml, 2 μg/ml, 64 μg/ml and 2 μg/ml, respectively (Espinel-Ingroff *et al*, 2007). For analytical purposes, we used an ECV of ≤2 μg/ml. The percentages meeting this ECV for the fungus form of *S. schenckii* were 92% for ketoconazole 90% for terbinafine, 90% for posaconazole, 90% for isavuconazole, 87% for ravuconazole, 82% for itraconazole, 79% for voriconazole and 47% for amphotericin B and for the yeast form were 97% for ketoconazole, 97% for itraconazole, 97% for ravuconazole, 97% for isavuconazole, 96% for terbinafine, 95% for posaconazole, 97% for voriconazole and 73% for amphotericin B.

DISCUSSION

The best form of *S. schenckii* to be used in susceptibility testing is unclear (Kohler *et al*, 2006; CLSI, 2017a). The yeast form is the primary form found clinically, but the fungus form is easier to manipulate in the lab. In this study, we compared the susceptibilities of both forms against antifungals commonly used in clinical practice to determine if the susceptibilities of the yeast and fungal forms differed. If they do not, then the clinically more convenient fungal form could be used for testing. However, if the susceptibilities are significantly different, further studies are needed to determine which corresponds to clinical improvement and what ECV should be used.

In our study, the fungal forms of *S. schenckii* required significantly higher GM MIC (p=0.0117) and MIC_{90} (p=0.0176) and had lower susceptibilities (79-92%) to the antifungals tested than the yeast forms (95-97%). There was no significant difference in the MIC_{50} for

any of the tested antifungals between the fungus and yeast forms (p=0.1795) but there was a significant difference in the MIC_{90} for all the tested antifungals between the fungus and yeast forms (p=0.01796) (Table 2). The reason for this is unclear and may indicate two organism subpopulations with different susceptibilities against the tested antifungals (Schwarz *et al*, 2010).

In our study, ketoconazole and amphotericin B had the lowest and highest GM MIC against *S. schenckii* for both fungal and yeast forms, respectively (Table 1). The higher MIC for ketoconazole against the fungal form found in our study is similar to a previous study from India (Ghosh *et al*, 2001). In our study, amphotericin B had a high MIC_{90} that similar to studies from Iran (Mahmoudi *et al*, 2016) and India (Ghosh *et al*, 2001) but different from a study from Peru, Venezuela, Brazil, Uruguay and Spain (Pereira Silveira *et al*, 2009). This high MIC_{90} seen in our study could be caused by our samples being intrinsically resistant to amphotericin B, as was seen in another study from Malaysia (Han *et al*, 2017).

The difference in the MIC of ketoconazole and amphotericin B which seen in our study could be due to the facts that mechanisms of action are different between these two antifungals. Ketoconazole can inhibit the synthesis of lanosterol that acts as the precursor for ergosterol biosynthesis (Van Tyle, 1984). However, amphotericin B acts by binding to ergosterol and induces oxidative damage, pore formation and ergosterol sequestration (Mesa-Arango *et al*, 2012).

In our study we found differences in the MIC of terbinafine between the fungal and yeast forms of the organism, similar to the results of studies from Brazil (Kohler *et al*, 2006), Spain (Pereira Silveira *et al*, 2009) and Iran (Mahmoudi *et al*, 2016).

The GM MIC for posaconazole against the fungus form in our study is similar to that reported in a study from Spain (Pereira Silveira *et al*, 2009) but lower than studies from Brazil (Galhardo *et al*, 2008) and Iran (Marimon *et al*, 2008).

In our study, the GM MIC for voriconazole against the fungal form was much lower than that reported in studies from Spain and Brazil (Marimon *et al*, 2008; Oliveira *et al*, 2015; Galhardo *et al*, 2018) and the GM MIC for voriconazole against the yeast form was much lower than a previous study (Mahmoudi *et al*, 2016).

In our study, the low GM MIC of itraconazole against the fungal and yeast forms were like to those reported in studies from India (Ghosh *et al*, 2001), Iran (Mahmoudi *et al*, 2016), and Brazil (Kohler *et al*, 2006) suggesting this antifungal may be clinically useful for treating both the fungal and yeast forms in these countries.

A strength of our study was that this is the first study from Malaysia

to determine the MICs of the studied antifungals against the locally obtained clinical isolates of *S. schenckii* using a broth microdilution method. A weakness of our study was that we were unable to determine if the differences between the fungal and yeast forms were due to differences in the in the initial fungal and yeast inoculum or differences in *S. schenckii* genotypes. All the isolates used in our study were determined to be of the same species, similar to other studies (Ghosh *et al*, 2001; Alvarado-Ramírez and Torres-Rodríguez, 2007; Galhardo *et al*, 2008; Mahmoudi *et al*, 2016), but different isolates may have different susceptibilities.

The overall differences between our study results and those from other studies may be due to differences in strains by region (Galhardo *et al*, 2008; Pereira Silveira *et al*, 2009), differences in incubation temperatures and times (Trilles *et al*, 2005), differences susceptibility testing protocols (Galhardo *et al*, 2008; Pereira Silveira *et al*, 2009) and differences in organism sources (environment, human or animal) (Oliveira *et al*, 2011).

In summary, the GM MIC and MIC_{90} results of the tested antifungals were significantly lower against the yeast than the fungus forms, but the MIC_{50} results were not significantly different between the yeast and fungus forms. We conclude there is a difference between the fungal and yeast forms but the best growth form (fungus or yeast) of *S. schenckii* that should be used for sensitivity testing to best represent the clinical response has yet to be determined. Further studies are needed to determine if these findings can inform clinical decision-making.

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CONFLICT OF INTEREST DISCLOSURE

The authors declare no conflict of interest.

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