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In vitro activity of fluconazole and voriconazole against clinical isolates of *Candida* spp. by E-test method

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Abstract. The *in vitro* susceptibility of clinical *Candida* isolates towards fluconazole and voriconazole was determined using the E-test method. A total of 41 clinical isolates recovered from patients since 2004 until 2009 from two local hospitals in Kuala Lumpur, Malaysia were used. These comprised *Candida tropicalis*, *Candida albicans*, *Candida krusei*, *Candida parapsilosis*, *Candida rugosa*, *Candida dubliniensis* and *Candida glabrata*. Strains from American Type Culture Collection were used as quality control. Lawn cultures of the isolates on RPMI-1640 agar medium supplemented with 2% glucose were incubated with the E-test strips at 35°C for 48 h. Our results show that 71% were susceptible to fluconazole and 90% were susceptible to voriconazole. All strains of *C. krusei* were resistant to fluconazole and 50% were susceptible in a dose-dependent manner to voriconazole. There were 66% and 33% of *C. glabrata* that were resistant to fluconazole and voriconazole. Our study revealed that majority of the clinical *Candida* isolates was susceptible to fluconazole and voriconazole with a small percentage being resistant to both the drugs.

INTRODUCTION

Lately, there have been reports of resistance to azole drugs among clinical isolates of non-albicans *Candida* species. Standard treatment for *Candida* infections in the past was with amphotericin B. Azole drugs like fluconazole are suitable alternative because they are less toxic (Wilson & Gisvold, 1998). Azoles such as itraconazole, voriconazole and posaconazole are also used in the treatment of fungal infections (MIMS, 2008). Species like *Candida krusei* and *Candida glabrata* are becoming difficult to treat as they are known to be intrinsically resistant or have decreased susceptibility to fluconazole and itraconazole (Borg-von Zepelin *et al.*, 2007; Gonzales *et al.*, 2008; Quindos *et al.*, 2008). Although new triazoles like voriconazole and posaconazole are known to be more effective as treatment for *Candida* infections, there have been reports of resistance among a few of these clinical *Candida* strains especially from *C. glabrata* towards voriconazole (Diekema, 2009). It was also reported that the efficacy of the treatment in patients with candidiasis using voriconazole was only 57.5% (Perfect *et al.*, 2003). The aim of this study was to determine the susceptibility of clinical *Candida* isolates towards fluconazole and voriconazole from two local hospitals. We have employed the E-test method as it is documented as comparable to Clinical Laboratory Standard Institute's microdilution, macrodilution and disk-diffusion methods (Espinel-Ingroff *et al.*, 1996; Pfaller *et al.*, 1996, 1998, 2000,

2002, 2003; Barry *et al.*, 2002; Maxwell *et al.*, 2003).

MATERIALS AND METHOD

Isolates

Candida species isolated from clinical specimens of two local hospitals in Kuala Lumpur, Malaysia from 2004 until 2009 were studied. These isolates were from blood (3), nail (1), skin (1), sputum (1), urine (1), vagina (4) and unknown sites (30). Stock cultures were prepared in 20% glycerol and were stored at -80°C. They were thawed and cultured on Sabouraud's dextrose agar (Difco, USA) at least twice to ensure their purity and viability before antifungal susceptibility tests were performed. The isolates were re-identified using CHROMagar™ Candida (Becton Dickinson, USA) and RAPID Yeasts Plus System (Remel, USA) to confirm the species. Total number of clinical isolates used was 41. The quality control strains used in every batch of test were *Candida parapsilosis* ATCC 22019, *C. krusei* ATCC 6258 and *Candida albicans* ATCC 90028 (CLSI, 2000).

Inoculum Preparation

The inocula were prepared following the guidelines of Clinical Laboratory Standards Institute (CLSI, 2000) Document M27-A2. After preparing 24 h cultures of the isolates at 37°C, 5 colonies were suspended in a sterile test tube containing 1 mL of 0.85% NaCl for each isolate. The mixture was vortexed at low speed to obtain homogeneity. The absorbance of this suspension was measured using a spectrophotometer to obtain cell density that is equivalent to 0.5 McFarland standards, which gave approximately 0.5 x 10^3 to 2.5 x 10^3 cells/ mL. An absorbance at 625 nm should be within 0.08 and 0.10 for the 0.5 McFarland standards.

Antifungal Agents

E-test strips containing fluconazole and voriconazole with continuous concentration gradient were purchased from AB Biodisk, Sweden. Fluconazole concentration ranged from 0.016 – 256 µg/ mL and voriconazole concentration ranged from 0.002 – 32 µg/ mL. All strips were stored at -20°C and thawed at room temperature before use.

Antifungal Susceptibility Testing

All isolates were tested against fluconazole and voriconazole according to the manufacturer's guidelines. Lawn cultures of the *Candida* isolates were prepared on RPMI-1640 agar medium supplemented with 2% glucose (pH 7.0) in Petri dishes. The plates were allowed to dry for several minutes at room temperature. E-test strips were gently placed on the lawn cultures with the MIC scale facing upwards using a sterile forcep. The Petri dishes were incubated at 35°C for 48 h as recommended by the manufacturer. The MIC values were read where the inhibition ellipse intersected the strip which was interpreted as the lowest concentration at which 80% of the growth was inhibited. Growth of micro-colonies throughout a discernable inhibition ellipse was ignored. The acceptable MIC ranges for fluconazole and voriconazole are shown in Table 1.

RESULTS

Overall, the most predominantly isolated species was *Candida tropicalis* (n=10), followed by *C. albicans* (n=7), *C. parapsilosis* (n=6), *C. krusei* (n=6), *Candida rugosa* (n=6), *Candida dubliniensis* (n=3) and *C. glabrata* (n=3). The geometric mean values of MIC for control and clinical strains are shown in Table 2. The quality control strains with each batch of test were within the control limits for fluconazole and voriconazole. For all clinical isolates, the MIC values for voriconazole were lower compared to fluconazole. The highest MIC value obtained for voriconazole was 6 µg/mL and fluconazole was more than 256 µg/mL. All species of *Candida* showed *in vitro* susceptibility towards voriconazole except for one strain of *C. glabrata* that was resistant to voriconazole with the MIC of 6

Table 1. MIC interpretive guidelines for *in vitro* susceptibility testing of *Candida* species (CLSI, 2000)

Antifungal Agent (MIC µg/ mL)	Susceptible (µg/ mL)	Susceptible Dose-dependent (µg/ mL)	Resistant (µg/mL)	Quality Control Strains (µg/ mL)
Fluconazole (0.016-256)	≤8	16–32	≥64	*C. parapsilosis* ATCC 22019 (1 – 8) *C. krusei* ATCC 6258 (128 – ≥ 256) *C. albicans* ATCC 90028 (0.125 – 0.5)
Voriconazole (0.002-32)	≤1	2	≥4	*C. parapsilosis* ATCC 22019 (0.016 – 0.064) *C. krusei* ATCC 6258 (0.25 – 1) *C. albicans* ATCC 90028 (0.004 – 0.016)

Table 2. MIC values for *Candida* spp.

Candida species (n)	Antifungal Agent	Mean MIC_{80} (µg/mL)	MIC range (µg/mL)
C. albicans ATCC 90028 (1)	Fluconazole Voriconazole	S = 0.5 S = 0.004	N/A
C. krusei ATCC 6258 (1)	Fluconazole Voriconazole	R = > 256 S = 0.25	N/A
C. parapsilosis ATCC 22019 (1)	Fluconazole Voriconazole	S = 1.5 S = 0.023	N/A
C. tropicalis (10)	Fluconazole Voriconazole	S = 0.5 S = 0.1	0.25 – 1.5 0.023 – 0.25
C. albicans (7)	Fluconazole Voriconazole	S = 1.35 S = 0.033	0.125 – 8 0.002 – 0.19
C. parapsilosis (6)	Fluconazole Voriconazole	S = 0.41; SDD = 12 S = 0.0298	0.023 – 12 0.004 – 0.064
C. krusei (6)	Fluconazole Voriconazole	R = > 192 S = 0.375; SDD = 1.83	64 - >256 0.50 – 2
C. rugosa (6)	Fluconazole Voriconazole	S = 6; SDD = 12 S = 0.049	0.094 – 12 0.003 – 0.094
C. glabrata (3)	Fluconazole Voriconazole	S =1.5; R =176 S = 0.142; R = 6	1.5 - >256 0.094 – 6
C. dubliniensis (3)	Fluconazole Voriconazole	S = 0.18 S = 0.006	0.047 – 0.25 0.004 – 0.008

*S = susceptible; SDD = susceptible dose-dependent; R = resistant; N/A = not applicable

µg/mL and three strains of *C. krusei* that were susceptible dose-dependent for voriconazole. As for fluconazole, two strains of *C. glabrata* with their MIC values of 96 µg/mL and more than 256 µg/mL and all six strains of *C. krusei* were resistant towards this antifungal drug. One species of *C. parapsilosis* and two strains of *C. rugosa* were susceptible dose-dependent for fluconazole with their MIC value of 12 µg/mL. Two different zones of growth (ellipse) were observed only for *C. albicans* isolates and the smaller ellipse with microcolonies was ignored as instructed by the manufacturer for the MIC values.

Among the 41 clinical isolates tested with E-test strips, 100% of *C. albicans*, *C. dubliniensis* and *C. tropicalis* were susceptible to both of the drugs *in vitro*. In addition, 100% *in vitro* susceptibility was found among *C. parapsilosis* and *C. rugosa* towards voriconazole where as the susceptibility of *C. krusei* and *C. glabrata* towards this drug were 50% and 67%, respectively. *In vitro* susceptibility of *C. glabrata*, *C. parapsilosis* and *C. rugosa* towards fluconazole were 33%, 83% and 67%, respectively. All *C. krusei* isolates (100%) were found resistant to fluconazole. From the total clinical isolates (41), we found 71%, 7% and 22% were susceptible, susceptible dose-dependent and resistant to fluconazole whereas 90%, 7% and 3% were susceptible, susceptible dose-dependent and resistant to voriconazole.

DISCUSSION

Antifungal susceptibility tests using E-test method is uncommon due to cost. Comparative studies done using E-test and broth microdilution tests were reported to be more than 90% in agreement with the MIC values obtained for fluconazole, itraconazole, ketoconazole and voriconazole, against *Candida* spp. (Colombo *et al*., 1995; Pfaller *et al*., 1998, 2000; Chryssanthou & Cuenca-Estrella, 2002; Matar *et al*., 2003). The overall intra- and interlaboratory concordance of E-test method with microdilution reference method was found to be 90% in Italy (Morace *et al*., 2002). Reproducibility of the MIC values using E-test on quality control strains of *Candida* was proven across four laboratories using five antifungal agents (Pfaller *et al*., 1996). The same quality control strains that were used in our study also suggested the reproducibility of the MIC values obtained using E-tests. The E-test method is also simple to perform compared to broth dilution method.

MIC values with E-test for the quality control strains recommended by CLSI and the manufacturer were within the established range using RPMI-1640 medium supplemented with 2% glucose for fluconazole and voriconazole. The use of RPMI-1640 for E-test method in this study was used as recommended by the manufacturer and was also found an optimum growth medium for various *Candida* species with excellent MIC correlation among many laboratories for azoles (Espinel-Ingroff *et al*., 1996; Pfaller *et al*., 1996, 2000). The results in our study show that voriconazole is still more effective than fluconazole in controlling the growth of *C. krusei* and *C. glabrata* which are two species of *Candida* that are known to be either intrinsically resistant or have decreased susceptibility to fluconazole. Voriconazole is a more effective azole compared to fluconazole in all the isolates tested except for one strain of *C. glabrata* that showed *in vitro* resistance towards voriconazole. Our results can be supported with a global surveillance study done across 26 countries between 1997 and 1998 (Meis *et al*., 2000). In this study, *C. albicans* had the highest susceptibility (99%) towards fluconazole, followed by *C. parapsilosis* (94%), *C. tropicalis* (90%), *C. glabrata* (67%) and *C. krusei* (26%), with the lowest. In another study conducted in Israel, local clinical isolates of *C. albicans*, *C. tropicalis*, *C. parapsilosis* and *C. glabrata* showed 100% susceptibility to voriconazole (Samra *et al*., 2005) which was the same in our study except for *C. glabrata*.

In another global surveillance study done between 2004 and 2007, the susceptibility of *Candida* isolates towards voriconazole still remained high for all species tested except for 95% of *C. glabrata* which demonstrated *in vitro* resistance towards voriconazole (Diekema *et al*., 2009). A French multicenter study reported the susceptibility of clinical *Candida* and *Aspergillus* isolates towards 4 antifungal agents using E-test (Mallie *et al*., 2005). In that report, voriconazole was found to be more effective than amphotericin B against *C. albicans*, *Candida kefyr*, *C. parapsilosis* and *C. tropicalis*. Voriconazole was also found to

inhibit the growth of the fluconazole-resistant and susceptible dose-dependent isolates of *Candida* isolates *in vitro* (Pfaller *et al.*, 2002).

However in another study, all the clinical *Candida* isolates tested *in vitro* (*C. albicans*, *C. glabrata*, *Candida guillermondii*, *Candida lipolytica*, *C. parapsilosis*, and *C. tropicalis*) were found susceptible to fluconazole (Pinto *et al.*, 2008). A 14-year study in a Spanish tertiary medical centre revealed that 100% *C. krusei* was resistant to fluconazole, which was the same in our study and 6.7% was resistant to voriconazole (Quindos *et al.*, 2008). However, Quindos *et al.* (2008) also reported that 85.7% and 92.9% of *C. glabrata* isolates were susceptible towards fluconazole and voriconazole respectively, which were much higher than our findings. A small percentage of *Candida* clinical isolates were found resistant to voriconazole in a phase III clinical study mainly among *C. albicans* (6.5%), *C. glabrata* (30%), *Candida inconspicua* (22%) and *C. tropicalis* (5.4%) (Johnson *et al.*, 2008). In our study, we found that one of the two strains of *C. glabrata* that were fluconazole-resistant was also resistant to voriconazole and three of the six fluconazole-resistant *C. krusei* were susceptible dose-dependent towards voriconazole. Reduced susceptibility to fluconazole and/ or itracononazole was also found among *C. glabrata* and *C. krusei* isolates of the Danish population (Arendrup *et al.*, 2005). There were also reports showing that clinical *Candida* isolates with decreased susceptibility towards fluconazole having high significant MIC values towards voriconazole and posaconazole (Rautemaa *et al.*, 2008). Although voriconazole is more effective than fluconazole against many fungal species, adverse effects such as transcient and reversible visual disturbances, changes in colour perception, photo-sensitivity, abonormal liver functions, diarrhoea, injection site reactions and infusion site reactions can be observed (MIMS, 2008).

E-test is a reliable and effective alternative to broth microdilution or disk-diffusion methods of MIC determination for azole drugs as it is simple to perform and does not require laborious procedures. Our study also shows that majority of our clinical *Candida* strains show *in vitro* susceptibility towards fluconazole and voriconazole with a small percentage that are resistant towards these drugs, except for *C. krusei* all of which are resistant towards fluconazole. However, there may be possibilities that fluconazole-resistant isolates of *C. krusei* and *C. glabrata* can be acquiring resistance towards voriconazole which would require a larger set of data over time to study their resistance pattern.

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