Trailing end-point phenotype of *Candida* spp. in antifungal susceptibility testing to fluconazole is eliminated by altering incubation temperature

According to the Clinical and Laboratory Standards Institute (CLSI) (formerly the National Committee for Clinical Laboratory Standards) standardized method for antifungal susceptibility testing M27-A2, the MIC for fluconazole is defined as the lowest concentration of drug causing an 80 % inhibition of growth relative to a drug-free control (CLSI, 2002). Proposed breakpoints suggest that susceptible isolates have an MIC $\leq 8 \mu\text{g ml}^{-1}$, susceptible-dose dependent isolates have an MIC of $16\text{--}32 \mu\text{g ml}^{-1}$ and resistant isolates have an MIC $\geq 64 \mu\text{g ml}^{-1}$ (CLSI, 2002). Standard testing reports an MIC at 48 h, and this result is usually within 1–2 doubling dilutions of the 24 h value. Most isolates of *Candida albicans* are susceptible to fluconazole with a 48 h MIC $\leq 1 \mu\text{g ml}^{-1}$ (Pfaffer & Diekema, 2004). However, when tested by the CLSI method, certain isolates have been found to be susceptible at 24 h, usually with an MIC $\leq 1 \mu\text{g ml}^{-1}$, but highly resistant at 48 h with an MIC $\geq 64 \mu\text{g ml}^{-1}$, which may be referred to as a trailing end point or tolerance (Pfaffer & Diekema, 2004). In one study, pH was found to affect trailing in certain isolates (Marr *et al.*, 1999). Mechanisms have been proposed to explain trailing, including activation of calcineurin and altered regulation of genes mediating resistance (Sanglard *et al.*, 2003; Lee *et al.*, 2004). Various studies have suggested that these isolates are actually clinically susceptible (Revankar *et al.*, 1998; Rex *et al.*, 1998). Other *Candida* species may also demonstrate trailing end points. We evaluated whether altering the incubation temperature in the CLSI method may affect the *in vitro* susceptibility testing of such isolates to fluconazole. The clinical isolates used were known to exhibit trailing end points by previous testing.

Strains used in this study were all clinical isolates, primarily from human

immunodeficiency virus positive patients with oropharyngeal candidiasis. Fourteen isolates were studied, eight with trailing end points (six *C. albicans*, one *Candida tropicalis*, one *Candida glabrata*), three susceptible (all *C. albicans*) (MIC $< 8 \mu\text{g ml}^{-1}$) and three non-susceptible (one *C. albicans*, one *C. glabrata*, one *Candida krusei*) (MIC $\geq 16 \mu\text{g ml}^{-1}$). Isolates were stored in sterile distilled water and subcultured on Sabouraud dextrose agar for 48 h prior to antifungal susceptibility testing. Susceptibility to fluconazole was tested by the CLSI method M-27A2, with temperature adjusted to 25 and 42 °C versus 35 °C as per the CLSI method. An MIC $\leq 8 \mu\text{g ml}^{-1}$ at 48 h was considered susceptible, and $\geq 16 \mu\text{g ml}^{-1}$ was considered non-susceptible. A trailing phenotype for fluconazole was an MIC $< 8 \mu\text{g ml}^{-1}$ at 24 h and $\geq 64 \mu\text{g ml}^{-1}$ at 48 h. Growth rates of the isolates were assessed by taking hourly optical density measurements with a spectrophotometer for 12 h at 530 nm. Isolates were incubated at 35 °C in RPMI media. Measurements were plotted on semi-logarithmic graph paper and doubling times determined by the slope of the growth curve in exponential phase.

All testing with variations in the standard CLSI method was done with fluconazole. Experiments were performed in triplicate. Isolates were initially tested at the standard temperature of 35 °C, and then were retested at 25 and 42 °C. All eight isolates that exhibited trailing at 35 °C lost the phenotype (appeared susceptible) at 25 and 42 °C. No differences were observed between results at 25 and 42 °C. The MICs of susceptible and resistant isolates did not change more than two dilutions. As different variables that may have had an effect on growth appeared to eliminate the trailing effect, we considered whether trailing isolates had a more rapid growth rate than non-trailing isolates. However, the mean doubling time of the trailing

isolates was 89 min and that of the non-trailing isolates was 95 min, which was not statistically different.

The current standardized antifungal susceptibility testing method may produce trailing end points in certain isolates of *Candida*. As this is observed consistently, it may be a fundamental characteristic of these isolates. Trailing is interpreted by the CLSI method as resistance, though testing by other *in vitro* methods, animal models and clinical experience reveals these isolates to be susceptible (Marr *et al.*, 1999; Revankar *et al.*, 1998; Rex *et al.*, 1998). The latest reference method also suggests that using the 24 h result may be more appropriate for such isolates (CLSI, 2002). We have shown that alteration of temperature in the testing methodology eliminates the occurrence of the trailing end point. When other isolates, which did not exhibit trailing end points, were tested under the same altered conditions, no significant change was seen in their susceptibilities. The reason for such dramatic changes in MICs for trailing isolates is not clear, but does not appear to be related to altered growth rates. However, these conditions may affect the expression of certain genes that have been associated with the trailing phenomenon. Ultimately, determining the optimal method to test and report the susceptibilities of trailing isolates will improve the correlation between *in vitro* testing and clinical outcome.

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