Factors influencing the trailing endpoint observed in *Candida albicans* susceptibility testing using the CLSI procedure

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ABSTRACT

The trailing endpoint phenotype observed during testing of *Candida albicans* susceptibility to azoles according to the CLSI procedure is defined as a difference in MIC depending on whether the result is obtained after 24 or 48 h. This study investigated whether intrinsic differences between the EUCAST and CLSI methods could explain trailing growth. The glucose concentration in the medium and the shape of the microtitre plate wells were both found to be involved. In order to reduce the incidence of trailing growth according to the CLSI procedure, the use of higher glucose concentrations and flat-bottomed microtitre plates could be valuable improvements.

Keywords *Candida albicans*, CLSI method, EUCAST method, fluconazole, susceptibility testing, trailing endpoint

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Several standardised antifungal susceptibility tests have been developed [1], including the CLSI M27-A2 microdilution method [2] and the EUCAST antifungal susceptibility testing method for fermentative yeasts [3]. Despite differences between these methods, they generally reveal similar MICs [4]. However, the so-called 'trailing endpoint phenotype' observed when using the CLSI method can make the interpretation of MIC values difficult [5]. The trailing endpoint phenotype is defined as a difference in MIC depending on whether the result is obtained after incubation for 24 or 48 h; after 24 h, the yeast appears to be susceptible, but it appears to be resistant after 48 h [6]. The frequency of *Candida albicans* isolates displaying trailing growth with fluconazole can be quite high (5–18%) [7,8], but fungal isolates exhibiting a trailing endpoint phenotype appear to remain susceptible *in vivo* [6,8]. The exact cause of trailing growth has not been determined, although it has been suggested that the pH of the medium [9], the glucose concentration [10], the inoculum size [10], the incubation temperature [11], and strain-dependent characteristics, e.g., regulation of expression of resistance genes [12], could be involved. Trailing is not observed when using the EUCAST method, as reading after 48 h is not normally recommended. The present study investigated whether other differences between the two methods could explain the trailing endpoint phenotype.

Non-trailing (NT) *C. albicans* isolate IHEM 9559 was obtained from the BCCM/IHEM Collection (Brussels, Belgium), and trailing (T) isolate 98/472 was provided by M. Cuenca-Estrella (National Centre for Microbiology, Madrid, Spain). Susceptibility testing to fluconazole (Pfizer, Brussels, Belgium) was performed according to published guidelines [2,3], but the plates were also read after 48 h when using the EUCAST method. The medium used was RPMI-1640 with L-glutamine and without sodium bicarbonate (Life Technologies, Gent, Belgium), buffered to pH 7.0 with 0.165 M morpholinepropanesulphonic acid. Doubling times were determined spectrophotometrically. Optical densities were measured using a Wallac Victor reader (Perkin Elmer LAS, Waltham, MA, USA). For both methods, the MIC was considered to be the lowest fluconazole concentration that resulted in an inhibition of growth of ≥50% of that of the drug-free control.

According to the EUCAST procedure, the MIC was the same for both isolates (0.25 mg/L). In contrast, according to the CLSI procedure, the MIC was 0.25 mg/L (at both time-points) for the NT isolate, but increased after 48 h for the T isolate (Fig. 1). Using the EUCAST procedure, the shape of the well had no influence on the turbidity and MIC value for both isolates, but when using the CLSI method and the NT isolate, flat-bottomed wells resulted in a lower relative

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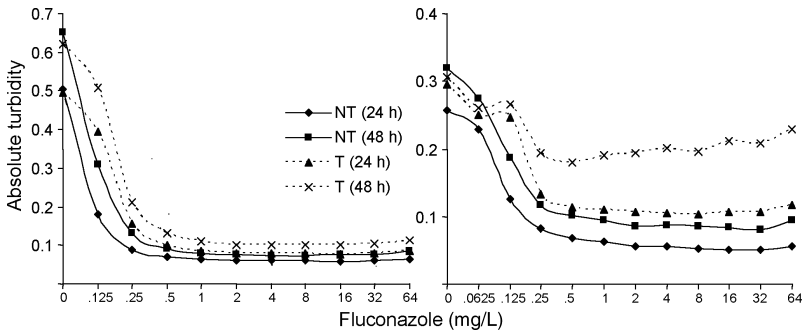

Fig. 1. Effect of fluconazole on the absolute turbidity obtained according to the EUCAST (left) and CLSI (right) methods for trailing (T) and non-trailing (NT) isolates of *Candida albicans*. Note that reading the plate after 48 h is only recommended in the CLSI procedure (data for 48 h obtained with the EUCAST procedure are shown for comparison only).

turbidity compared to round-bottomed wells, although this had no effect on the MIC. With the CLSI method and the T isolate, the relative turbidity was higher in plates with round-bottomed wells, and the relative turbidity after 48 h had increased more in round-bottomed than in flat-bottomed wells, resulting in a shift in MIC from 0.25 to >64 mg/L (data not shown).

When the glucose concentration used in the CLSI method was increased to 2% w/v after 24 h, the increased concentration resulted in a higher absolute turbidity at low fluconazole concentrations (<0.25 mg/L) for both isolates, but no difference in absolute turbidity was seen at high concentrations. For the NT isolate, changes in turbidity were minimal, and the same MIC was observed with and without glucose. However, for the T isolate, the addition of glucose resulted in a more pronounced decrease in the relative turbidity. Consequently, the MIC value in the presence of glucose was the same as the MIC determined after 24 h, while the MIC differed in the absence of glucose, indicating that glucose is important for the growth of *C. albicans*. To further investigate this finding, doubling times were calculated for both isolates in the presence and absence of glucose. The T isolate had a shorter doubling time (*c.* 140 min) than the NT isolate (*c.* 170 min), and also had a shorter lag phase. While the addition of glucose had no impact on the doubling time of either isolate, low-glucose conditions resulted in a shorter exponential phase for the T isolate, which reached stationary phase more rapidly (data not shown).

These results suggested that the use of wells with flat bottoms, as well as the addition of glucose, might suppress the trailing phenotype. Experiments were therefore performed, based on the CLSI method, but using glucose 2% w/v and round-bottomed wells. There was a marked increase in turbidity following incubation for 24 h and 48 h for the T isolate (Fig. 2), but this was less pronounced when using the standard CLSI method (Fig. 1) and no trailing was obser-

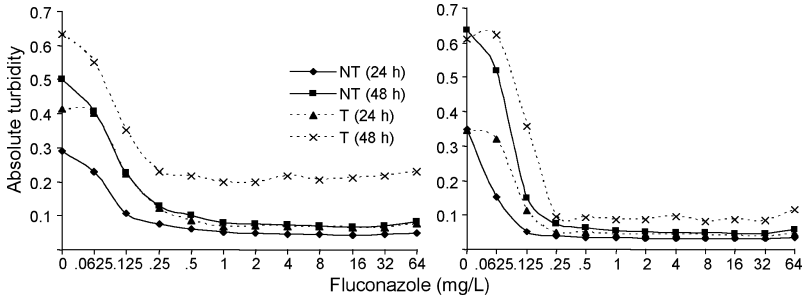

Fig. 2. Effect of fluconazole on the absolute turbidity according to the CLSI method in the presence of glucose 2% w/v using round-bottomed wells (left) and flat-bottomed wells (right) for trailing (T) and non-trailing (NT) isolates of *Candida albicans*.

ved. Using glucose 2% w/v and flat-bottomed wells, no difference in turbidity after incubation for 24 h and 48 h was revealed (Fig. 2), which is in agreement with the results obtained according to the EUCAST method (Fig. 1).

The lower maximal absolute turbidity in the growth control caused by a lower glucose concentration (and probably also by the 100-fold lower inoculum), the fact that the maximal absolute turbidity in the growth control was reached earlier in faster-growing isolates, and the fact that absolute turbidity in the fluconazole-treated wells increased more rapidly with faster-growing isolates, can explain the trailing phenomenon observed when using CLSI methodology. Trailing will occur in situations where the maximal absolute turbidity in the control wells has been reached, while absolute turbidity is still increasing in the fluconazole-treated wells, which results in a reduced difference between them. A trailing endpoint will be obtained only if this situation arises following incubation for between 24 h and 48 h, and if the difference in turbidity is sufficiently reduced. The odds of this situation occurring are increased at low glucose concentrations, because the growth control will reach a lower maximal absolute turbidity more rapidly. In addition, the odds of such a situation occurring between 24 h and 48 h are higher for faster-growing organisms. The results obtained in the present study show that trailing depends on isolate-specific characteristics, as well as certain aspects of the method used. The higher glucose concentration, as well as the use of flat-bottomed microtitre plates (as recommended in the EUCAST procedure), seems to suppress the trailing endpoint phenotype.

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