

# Association between fluconazole susceptibility and genetic relatedness among *Candida tropicalis* isolates in Taiwan

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Among the 162 *Candida tropicalis* isolates collected in the Taiwan Surveillance of Antimicrobial Resistance of Yeasts in 1999, 23 (14.2%) had fluconazole MICs  $\geq 64$  mg l<sup>-1</sup>, and thus fulfilled the definition of resistance. Random amplified polymorphic DNA assay showed that all 23 fluconazole-resistance *C. tropicalis* isolates collected from different hospitals around Taiwan were closely related. Two distinct pulsotypes associated with fluconazole susceptibility were identified when these 23 resistant isolates, along with 13 susceptible ones, were analysed by PFGE.

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## INTRODUCTION

Although *Candida albicans* is the most frequently isolated yeast pathogen causing morbidity in seriously immunocompromised hosts (Cheng *et al.*, 2004; Pfaller *et al.*, 2000; Yang *et al.*, 2004), there has been a shift toward the more treatment-resistant non-*albicans* *Candida* species (Sanglard & Odds, 2002; Walsh *et al.*, 2004; Yang *et al.*, 2004). We initiated a nationwide surveillance, the Taiwan Surveillance of Antimicrobial Resistance of Yeasts (TSARY), in 1999 to investigate the distribution and drug susceptibility of *Candida* species (Yang *et al.*, 2003, 2004). A total of 53 among the 632 isolates collected in the TSARY (in 1999) had fluconazole MICs  $\geq 64$  mg l<sup>-1</sup>. Thus, they were considered as resistant isolates (Clinical and Laboratory Standards Institute, 1997). Among them, 23 were *Candida tropicalis*, collected from 10 of the 22 participating hospitals.

Clinically, the increase in the rate of fluconazole resistance in *C. tropicalis* is of considerable importance since this is one of the most commonly isolated non-*albicans* *Candida* species (Cheng *et al.*, 2004; Pfaller *et al.*, 2000; Yang *et al.*, 2004, 2005). Information regarding the genetic background of the resistant and susceptible isolates may provide further insight into the distribution pattern and origin of the resistance. In this study, we hoped to determine whether

these resistant isolates of *C. tropicalis* are genetically related using two methods, random amplified polymorphic DNA (RAPD) assay and PFGE analysis.

## METHODS

**Organisms and media.** Yeast isolates were collected from 22 hospitals participating in the TSARY in 1999 (Lo *et al.*, 2001). These hospitals were located around the island and covered all four geographical regions: the North, Middle, South and East districts. Each hospital was asked to submit up to 10 *C. albicans* and 40 non-*albicans* *Candida* species between 15 April and 15 June 1999. Duplicate isolates from the same patients were excluded. All isolates were stored frozen at -70 °C in bead-containing Microbank cryovials (Pro-Lab Diagnostics). In total, the susceptibilities to fluconazole of 162 *C. tropicalis* isolates were determined. Then, the genetic relatedness of all 23 fluconazole-resistant isolates, which were collected from 10 hospitals, along with 13 susceptible ones, were determined. These 10 hospitals included four in the North district, three in the South district, two in the East district and one in the Middle district of Taiwan. The number of resistant isolates from each hospital ranged from one to five.

**RAPD.** RAPD assays were performed according to a modified protocol specifically for *C. tropicalis* (Roilides *et al.*, 2003). Amplification reactions (50 µl) were performed with the RP02 (5'-GCG ATC CCC A-3') primer and in accordance with the manufacturer's protocol, except that 5 units *Taq* DNA polymerase (New England Biolabs) and 1 µl genomic DNA were added to the reaction. PCR was performed as follows: 94 °C for 5 min; 36 °C for 5 min and 72 °C for 5 min for 4 cycles; 94 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min for 30 cycles; and a 10 min extension period

Abbreviations: RAPD, random amplified polymorphic DNA; TSARY, Taiwan Surveillance of Antimicrobial Resistance of Yeasts.

at 72 °C. The RAPD reactions (10 µl) were analysed on a 2% agarose gel containing  $0.5 \times$  TBE plus ethidium bromide ( $0.4 \text{ mg l}^{-1}$ ).

**PFGE.** PFGE analysis was performed as described in our previous report (Chen *et al.*, 2005). The plug slices were placed in 200 µl buffer 3 solution (100 mM NaCl, 50 mM Tris/HCl, 10 mM MgCl<sub>2</sub>, 1 mM DTT) (New England BioLabs) and incubated for 1 h at 50 °C. Then they were transferred to 200 µl buffer 3 solution containing 4 units BssHII and incubated at 50 °C overnight. Electrophoreses were performed with a Biometra Rotaphor at pulse time 6–50 s, angle 120°, 180 V in 0.8% agarose gel with  $0.5 \times$  TBE for 36 h. After the electrophoresis, the gel was stained in ethidium bromide solution ( $0.4 \text{ mg l}^{-1}$ ) for 15 min and destained in distilled water.

Dendrogram analysis was performed with Bionumerics software, version 3.0 (Applied Maths). The similarity values of the fingerprints were based on the presence or absence of bands between each profile pair compared. The band inclusion window was adjusted by the size reference markers. The position tolerance was set at 1% and optimization was set at 0%. The Dice coefficient was used to analyse the similarities ( $S_{AB}$ ) of the band patterns. UPGMA was used for the cluster analysis.

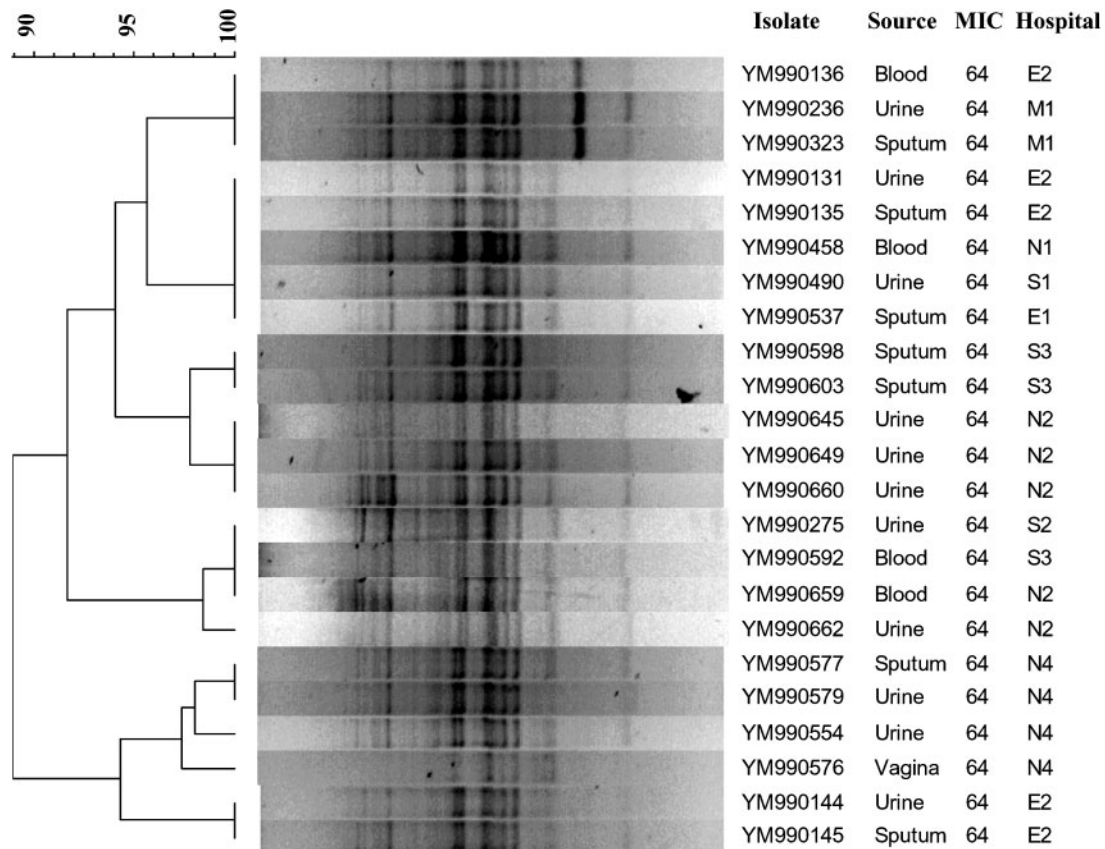
## RESULTS AND DISCUSSION

First of all, the genetic relatedness of all 23 resistant isolates was analysed by RAPD assay as described by Roilides *et al.*

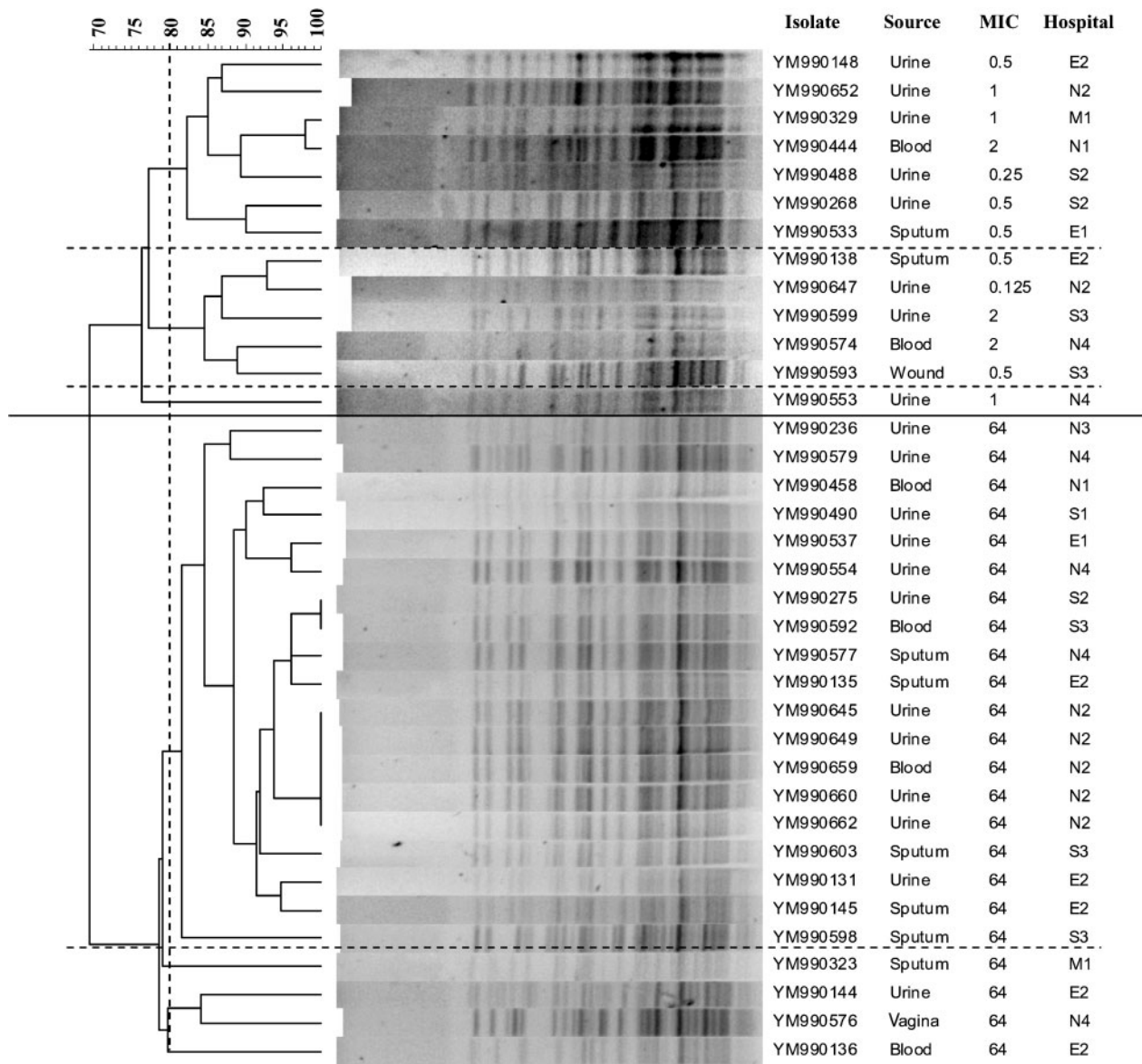
(2003). Interestingly, all the resistant isolates were closely related (Fig. 1). There are two possible explanations for this result. One is that all *C. tropicalis* isolates are genetically related due to the intrinsic stability of their genomes. The other is that all resistant ones were related due to clonal spreading. To distinguish between these two possibilities, we performed PFGE analysis as described previously (Chen *et al.*, 2005) to determine the genetic relatedness of the 23 resistant isolates along with 13 susceptible ones.

The result of the PFGE analysis is shown in Fig. 2. All of the 36 tested isolates were grouped into one of two pulsotypes, which were independent of sources and hospitals, but were closely associated with the fluconazole susceptibility. In fact, all the 13 susceptible isolates tested belonged to one pulsotype, while all the 23 resistant ones belonged to the other. Among the 13 susceptible isolates, two subgroups with >80% relatedness were identified, one with 7 and the other with 5 isolates. Among the 23 resistant ones, a major subgroup consisting of 19 (82.6%) isolates with >80% relatedness was also identified.

Molecular epidemiological surveillance of *Candida* species isolated from an intensive care unit has been performed



**Fig. 1.** Dendrogram of the 23 fluconazole-resistant *C. tropicalis* isolates. Cluster analysis was based on the patterns of the RAPD assay. MIC refers to fluconazole. An MIC of 64 indicates the resistant isolates with MIC  $\geq 64 \text{ mg l}^{-1}$ . E, East district; M, Middle district; N, North district; S, South district of Taiwan.



**Fig. 2.** Dendrogram of the 23 resistant and 13 susceptible *C. tropicalis* isolates. Cluster analysis of the 36 isolates was based on the patterns of *Bss*HIII restriction endonuclease analysis of genomic DNA. The long solid horizontal line separates the two major pulsotypes. The long dotted horizontal lines show the boundaries between subpulsotypes with >80% relatedness. MIC refers to fluconazole. An MIC of 64 indicates the resistant isolates with MICs  $\geq 64$  mg l<sup>-1</sup>. E, East district; M, Middle district; N, North district; S, South district of Taiwan.

using RAPD analysis (Ergon & Gulay, 2005). There were 20 patterns among the 38 *C. albicans* isolates tested, suggesting that the source of *C. albicans* was mostly endogenous, consistent with other reports (Chen *et al.*, 2001; Li *et al.*, 2006). In contrast, there were only 3 genotypes among a set of 15 *C. tropicalis* isolates (Ergon & Gulay, 2005). Furthermore, through multilocus sequence typing analysis on *C. tropicalis*, a set of clustered flucytosine-resistant isolates has been reported recently (Tavanti *et al.*, 2005). Interestingly, we have also found relatively few genotypes among the 36 isolates tested by PFGE analysis. In fact, they can be divided

into only two groups as showed in Fig. 2. Whether this phenomenon is due to clonal spreading or genome stability needs further investigation. Though resistant isolates were related, only one group consisted of more than two isolates exhibiting >90% relatedness. Therefore, if those resistant isolates were clonal, microevolution over time has diluted their genetic closeness. Alternatively, there was selective pressure that edited out other genetic patterns.

According to the guidelines of the CLSI (Clinical and Laboratory Standards Institute, 1997), the MICs to fluconazole



are defined as the MICs of drugs capable of reducing the turbidity of cells by >50% after incubation at 35 °C for 48 h. Isolates with MIC  $\geq 64$  mg l<sup>-1</sup> are considered to be fluconazole resistant. Among the phenomena associated with resistance, 'trailing' describes the reduced but persistent growth that some isolates exhibit at drug concentrations above the MIC in broth dilution tests with azole antifungal agents, such as fluconazole (Lee *et al.*, 2004). Trailing may interfere with the observation of resistance levels *in vivo*. The number of isolates exhibiting trailing with a particular MIC at 48 h is approximately fourfold higher than at 24 h. Therefore, the trailing growth can make an isolate that appears susceptible (MIC <64 mg l<sup>-1</sup>) after 24 h of incubation to appear resistant (MIC  $\geq 64$  mg l<sup>-1</sup>) at 48 h (Arthington-Skaggs *et al.*, 2002). Thus, we would also like to determine whether the 23 fluconazole-resistant isolates exhibit trailing. Of the 23 isolates with MICs  $\geq 64$  mg l<sup>-1</sup> at 48 h, only 5, including YM990236, YM990275, YM-990576, YM990579 and YM990649, exhibited MICs  $\geq 64$  mg l<sup>-1</sup> at 24 h. Nevertheless, the data obtained from *in vitro* susceptibility testing are not always correlated with *in vivo* outcome. Whether these 23 isolates with MICs  $\geq 64$  mg l<sup>-1</sup> will cause treatment failure needs further investigation.

The most important finding from this study was that the fluconazole-resistant *C. tropicalis* isolates appeared to be genetically related. Potentially, this will allow the development of easy and even rapid identification methods for clinical fluconazole-resistant or reduced-susceptibility *C. tropicalis* using the genotyping information.

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