DNA fingerprinting of serial *Candida albicans* isolates obtained during itraconazole prophylaxis in patients with AIDS


*Indiana University School of Medicine, Indianapolis, IN, USA; †Histoplasmosis Reference Laboratory, Indianapolis, IN, USA; ‡Research Medical Center, Kansas City, MO, USA; §University of Alabama at Birmingham, Birmingham, AL, USA; University of Michigan and Veterans Affairs Medical Center, Ann Arbor, MI, USA; ¶Roudebush Veterans Affairs Medical Center, Indianapolis, IN, USA

During a randomized double-blind placebo-controlled study testing the efficacy of itraconazole for prophylaxis of systemic and mucosal fungal infections in patients with acquired immune deficiency syndrome, 298 patients were enrolled with 295 evaluable. Of those, 46 patients were considered prophylaxis failures because of recurrent oral or esophageal candidiasis. Oropharyngeal fungal cultures were taken at the time of suspected thrush or *Candida* esophagitis, but not at baseline. All of the *Candida* spp. isolates were cultured on CHROMagar Candida medium then identified using API 20 AUX strips. Antifungal susceptibility testing was performed following the National Committee for Clinical Laboratory Standards M-27A guidelines. Sequential isolates were genotyped using randomly amplified polymorphic DNA. Polymerase chain reaction fingerprints were generated using two repetitive sequence primers, (GGA)_7 and (GACA)_4. The study group consisted of 23 patients, nine from the itraconazole arm and 14 from the placebo arm, who were prophylaxis failures and had more than two *C. albicans* isolates. Five of 23 had isolates showing a ≥4-fold reduction in susceptibility; four of these patients were in the itraconazole prophylaxis arm and one was in the placebo arm. Three of the five had yeast isolations showing changes in banding patterns over time. Such changes may indicate genetic changes in the same strain that could be linked to acquired resistance to itraconazole, or acquisition of a new strain, or emergence of a previously minor component of the original population.

**Keywords** AIDS, *Candida*, fingerprinting, itraconazole

**Introduction**

Oral candidiasis caused by *Candida albicans* is one of the most common mycotic opportunistic infections in persons with human immunodeficiency virus (HIV), occurring in 43–93% of patients at some time during the course of HIV infection [1–5]. As HIV infection advances, the frequency of thrush increases [5]. Relapse following treatment with antifungals is very common in this patient population [6]. Concerns exist that long-term suppressive therapy or prophylactic regimens may precipitate the emergence of antifungal resistance [1,2,4,7]. Antifungal pressure can be a major influence on changes in susceptibility [2,8]. Sangeorzan et al. [8]...
have shown that long-term use of fluconazole led to the development of resistance in a strain that was previously susceptible to fluconazole. Bart-Delabesse et al. [2] and Pfaller et al. [9] noted similar findings when they correlated fluconazole minimum inhibitory concentration (MIC) with DNA banding patterns on isolates from HIV-positive patients. Although they used other typing methods, namely karyotype and restriction fragment length polymorphism (RFLP), polymerase chain reaction (PCR) has been shown to be equally or more discriminatory than alternate DNA typing methods, as well as more time efficient and cost effective [10,11].

Genotype determinations made by randomly amplified polymorphic DNA (RAPD) PCR have been shown to be reproducible and to discriminate among strains of C. albicans [10–12]. A prospective trial comparing itraconazole versus placebo for antifungal prophylaxis has provided opportunities to further evaluate genotyping as a means of elucidating some of the factors involved in the development of antifungal drug resistance among Candida species isolates. The Mycoses Study Group protocol (MSG-28) was a prospective, randomized, double blind trial evaluating the ability of itraconazole to prevent histoplasmosis, other systemic mycoses and oropharyngeal candidiasis [13]. A secondary objective was to assess the impact of prophylaxis on susceptibility of selected fungal pathogens, including Candida species. Patients enrolled had absolute CD4 lymphocyte counts < 150 cells μl⁻¹ and were randomized to itraconazole capsules 200 mg daily versus placebo. Of the 295 evaluable patients, 46 were considered failures because of recurrent or refractory oral or esophageal candidiasis. The work presented in this paper is an analysis to determine if recurrent episodes of candidiasis in patients receiving itraconazole or placebo were caused by the same or different strains of C. albicans.

Materials and methods

Clinical study design

Patients were considered prophylaxis failures because of candidiasis if they fell into one of four categories: (i) oropharyngeal or vaginal candidiasis requiring systemic treatment for more than 2 weeks; (ii) systemic treatment for oropharyngeal or vaginal candidiasis on more than one occasion; (iii) esophageal candidiasis requiring systemic treatment for more than 3 weeks; or (iv) systemic treatment for esophageal candidiasis on more than one occasion. Some patients had a positive oral culture for yeast but did not meet the above definition of a failure. These patients were allowed to remain on the study protocol. Patients were not eligible for enrolment if they had an active fungal infection, and baseline fungal cultures from the mouth and throat were not collected. Patients were assessed for mycotic infections at weeks 6 and 12 and then every 3 months for the remainder of the study. Suspicion of a mycotic infection at any time during the course of the trial warranted a culture. Patients who were not considered prophylaxis failures were followed for a minimum of 12 months.

Fungal isolates

The isolates were collected from 298 patients who had clinical evidence of mucosal or systemic fungal infections at seven participating sites, processed at local hospital laboratories following usual isolation practices and techniques, then shipped to the Histoplasmosis Reference Laboratory (Indianapolis, IN, USA). Upon receipt, all fungal isolates were subcultured on potato dextrose agar (Remel, Lenexa, KA, USA) then frozen in lactose-glycerol media (146 mm β-lactose and 1:1 m glycerol) and kept under liquid nitrogen until the study was completed and unblinded. At that time all Candida spp. isolates were identified using CHROMagar Candida medium (Hardy Diagnostics, Santa Maria, CA, USA) and API 20 C AUX strips (bioMerieux, Marcy l’Etoile, France). Antifungal susceptibility testing was then performed on all of the Candida spp. isolates following the National Committee for Clinical Laboratory Standard (NCCLS) M27-A guidelines for broth microdilution [14].

DNA extraction

Genomic DNA extraction was performed by a modification of the procedure of Schöni et al [15]. Yeasts were grown overnight in Brain Heart Infusion broth (Difco, Detroit, MI) at 37 °C. The cells were pelleted by centrifugation and lysed with 5 m 2-(p-toluidino)-naphthalene-6-sulfonic acid, sodium salt (TNS) (Aldrich Chemical, Milwaukee, WI, USA); 3 m 4-aminosalicylic acid, sodium salt dihydrate (PAS) (Aldrich Chemical) and phenol in an extraction buffer of 1 m Tris-HCl, pH 8.0; 1.25 m NaCl and 250 mM EDTA, pH 8.0. Addition of chloroform was followed by centrifugation at 3685 g, after which the aqueous phase was subjected to a further 1:2 phenol:chloroform extraction. After a final chloroform extraction, the aqueous phase had 3 m sodium acetate added to a final concentration of 100 mM. The DNA was then precipitated with ice-cold absolute ethanol. The DNA pellet was resuspended in Tris-EDTA (10 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA, pH 8.0) and stored at 4 °C. The purity of the DNA samples was determined by the A260/A280 ratio. Ratios of ≥1.6 were considered acceptable.
Primers and PCR

The repetitive sequence primers \((GGA)_7\) and \((GACA)_4\) were obtained from GibcoBRL (Grand Island, NY, USA). These short sequence primers anneal at multiple loci throughout the genome generating highly reproducible banding patterns [10,15]. Reaction mixes for RAPD PCR contained 25 ng of DNA, 30 ng of either primer, 2.5 U AmpliTaq DNA Polymerase (Perkin-Elmer Applied Biosystems, Foster City, CA, USA), 10 mM Tris-HCl pH 8.3, 50 mM KCl, 15 mM MgCl\(_2\), 0.01% w/v gelatin and 200 \(\mu\)M dNTP mixture in a 50 \(\mu\)l total volume. An additional 3 mM of magnesium acetate was included in the mix for primer \((GACA)_4\). Thermo-cycling was performed in a GeneAmp PCR System 2400 (Perkin-Elmer Applied Biosystems). The program for primer \((GGA)_7\) was one cycle for 5 min at 94 \(^\circ\)C followed by 40 cycles of 60 s at 94 \(^\circ\)C, 60 s at 25 \(^\circ\)C and 2 min at 74 \(^\circ\)C followed by one cycle of 10 min at 74 \(^\circ\)C and a final hold at 4 \(^\circ\)C. The program for primer \((GACA)_4\) was one cycle of 5 min at 94 \(^\circ\)C followed by 40 cycles of 20 s at 93 \(^\circ\)C, 60 s at 50 \(^\circ\)C and 20 s at 72 \(^\circ\)C followed by one cycle of 6 min at 72 \(^\circ\)C and a final hold at 4 \(^\circ\)C. The PCR products were electrophoresed overnight through a 1.3% agarose gel at 10 mA with a 1-kb ladder used as a marker. The DNA bands were visualized using ethidium bromide and photographed under UV light. RAPD PCR fingerprinting was performed on the sequential \(C\). \textit{albicans} isolates from the 23 patients who were considered failures. To ensure reproducibility, all isolates were typed twice. Isolates were considered to be different strains if the banding patterns showed an easily detectable difference of at least two bands [16].

Statistical analysis

The change in MIC was categorized by those increasing \(\geq 4\)-fold versus those not increasing 4-fold and compared to those isolates showing genotypic change. The Fisher’s exact test was used since the numbers tested were small.

Results

Clinical analysis

A total of 298 patients were enrolled in the MSG-28 study. Of the 295 evaluable patients, 149 were randomized into the itraconazole arm and 146 into the placebo arm. Forty-six patients were prophylaxis failures due to candidiasis, 23 in each arm of the study. Of those failures, 43 had cultures banked, including 23 patients who had two or more \(C\). \textit{albicans} isolates. The study group consisted of these 23 patients, which included nine patients who had been in the itraconazole prophylaxis arm and 14 who had been in the placebo arm. A total of 55 isolates were typed.

The majority (18) of the patients with banked isolates had only two \(C\). \textit{albicans} isolates; three patients had three isolates and one patient each had four and six isolates.

Correlation of genotype with itraconazole susceptibility changes

The patient isolate series were originally categorized into two groups, the first having a significant change in itraconazole MIC (\(\geq 4\)-fold reduction in susceptibility) and the second having a stable itraconazole susceptibility. Three of five series with a reduction in susceptibility to itraconazole showed changes in genotype, while three of 18 with a stable MIC had genotypic changes (Table 1; \(P = 0.078\)). The mean time that a patient received itraconazole prophylaxis before the first change in MIC occurred was 4 months. Results of susceptibility runs performed in duplicate were indistinguishable (data not shown).

Correlation of genotype with fluconazole susceptibility changes

The susceptibility testing we performed on the isolates from the 23 patients with at least two \(C\). \textit{albicans} cultures included fluconazole as well. Although patients did not receive fluconazole prophylaxis, changes in fluconazole MIC were observed. Fluconazole use was allowed for treatment of episodes of candidiasis in both the itraconazole prophylaxis and the placebo arms. The relationship of genotype to fluconazole MIC was evaluated. There was no statistically significant difference between genetic changes over time and fluconazole susceptibility (Table 1; \(P = 0.576\)).

Demonstration of relationship of changes in susceptibility and genotype

Three representative patients were selected to illustrate the possible relationships between changes in MIC and
genotype. All three representative patients were in the itraconazole arm of the study. The first patient had six isolates, collected over a span of 132 days, that showed both genetic changes over time as well as an increase in MIC (Fig. 1). The last isolate collected from this patient (Fig. 1, lanes 6 and 13) showed a genetic difference of at least seven bands from all of the previous isolates. Both of the primers used in this study, \((GGA)_7\) and \((GACA)_4\), detected the change in strain. The changes in both itraconazole MIC and genotype seen in the isolates from patient 1 can be explained either by acquisition of a new strain or by occurrence of a genetic mutation associated with development of resistance mechanisms. In the latter scenario, the change was detected by RAPD PCR as it occurred over an extended period of prophylaxis, although it may be noted in Figure 1 that itraconazole MIC’s increased well before any genetic change was seen. Patient 2 had two isolates collected 130 days apart that had an increase in MIC but a stable genotype (Fig. 2), indicating selective pressure on a single strain. The data from patient 2 indicate that if a mutation occurred in the DNA, it was in an area that was not detected by either of the primers used. Patient 3 had two isolates collected 16 days apart with a stable MIC but a change in genotype (Fig. 3). In isolates from patient 3, the change in banding patterns between the first and last isolate was at least four bands. Both primers detected the genetic mutation. The genetic changes seen in the isolates from patient 3 are consistent with acquisition of a new strain or co-occurrence of two successively isolated strains, as the change occurred over a very short period of time.

**Correlation of genotype over study arms**

Changes in susceptibilities, either to itraconazole or fluconazole, demonstrated a possible trend closely approaching statistical significance between the arms of the study (Table 2; \(P \approx 0.056\)). Changes in genotype were equally spread between the itraconazole arm (3/9)

![Fig. 1](image-url) (a) RAPD PCR of the six sequential isolates from patient 1 with primer \((GGA)_7\) (lanes 1–6), primer \((GACA)_4\) (lanes 8–13) and control (water blank; lanes 7 and 14). Lane 1 is a 1-kb molecular weight ladder. (b) Graph of MIC values for both itraconazole and fluconazole for the sequential isolates from patient 1.
and the placebo arm (3/14) of the study (Table 2; \( P = 0.643 \)). Four of the five patients with significant changes in itraconazole MIC were in the itraconazole arm of the study.

**Discussion**

As HIV infected patients become more immunosuppressed, more occurrences of candidiasis occur, often leading to more frequent or prolonged exposure to azole antifungal agents. Extended use of azoles can lead to increased resistance and thereby reduce the efficacy of these drugs [17,18]. Several studies have reported that the extent of prior fluconazole exposure correlates with reduction in the susceptibility of *C. albicans* isolates [1,2,4,7]. Likewise, itraconazole resistance has occurred following long-term exposure [19,20]. Long-term exposure to the antifungal agent can select for mutations in the original strain, which may lead to reduced susceptibility in those isolates. In patients who are colonized or

![Fig. 2](image_url)  
(a) RAPD PCR of the sequential isolates from patient 2 with primer (GGA)\(_7\) (lanes 1 and 2), primer (GACA)\(_4\) (lanes 4 and 5) and control (water blank; lanes 3 and 6). Lane 1 is a 1-kb molecular weight ladder. (b) Graph of the MIC values for both itraconazole and fluconazole for the isolates from patient 2.

![Fig. 3](image_url)  
(a) RAPD PCR of the sequential isolates from patient 3 with primer (GGA)\(_7\) (lanes 1 and 2), primer (GACA)\(_4\) (lanes 4 and 5) and control (water blank; lane 3). Lane 1 is a 1-kb molecular weight ladder. (b) Graph of the MIC values for both itraconazole and fluconazole for the isolates from patient 3.

© 2001 ISHAM, Medical Mycology, 39, 207–213
infected with multiple strains or species, antifungal prophylaxis can select for the few organisms that are able to develop resistance, allowing them to flourish [9]. Other studies have demonstrated a pattern of cross-resistance to fluconazole and itraconazole in patients receiving either of the agents [1,4,21,22]. Itraconazole usage has increased in HIV-positive patients [20], and this increased exposure has had the potential to select for strains of *C. albicans* that are resistant to this agent, as has been seen with fluconazole.

Changes in banding patterns over time may be brought about by any of several different events. Antifungal selective pressure can cause genetic mutations that may be observable by changes in the DNA fingerprint generated when the DNA from the patient isolate is amplified with primers of high discriminatory power and electrophoresed through a gel. The technique of RAPD PCR has been shown to have such high discriminatory power that it may even be able to detect point mutations [23]. As RAPD PCR is capable of detecting single as well as multiple genetic changes, such changes brought about by antifungal selective pressure conferring resistance to the organism may be detectable during RAPD PCR fingerprinting. Second, observable genetic changes in banding patterns may have occurred by the acquisition of a new strain of the organism by the patient [24,25]. Third, among patients who are initially infected by multiple strains, antifungal selective pressure may lead to the selection of a resistant strain [19,26].

In a study conducted by Bart-Delabesse *et al.* [2] molecular typing (RFLP and karyotype) was performed on *Candida* isolates from HIV-positive patients that developed resistance to fluconazole. Results on isolates from three of the patients showed the maintenance of the original strain. The results of the isolates from patient 4 could be interpreted as selection of a resistant strain. The results from this and other studies [2,9] suggested that such patterns also needed to be studied for itraconazole. The present study was an initial attempt to ascertain if itraconazole prophylaxis had an effect on the genotype of *C. albicans* isolates obtained from HIV-positive patients.

Of the five patients whose isolates showed an increase in MICs, three showed genotypic changes while two remained stable. For the three strains that demonstrated genotypic changes, we cannot discriminate between new strain acquisition and selection for a minor component of the original population because limitations in the study design prevented analysis of multiple colonies from each initial patient isolate. Reports in the literature have shown that more than a single strain can cause a single clinical episode of candidiasis [9,17,18,26,27]. Acknowledging this limitation, RAPD PCR fingerprinting on these isolates indicates that three patients had strain replacement and two had selection for resistance in the same strain. Although no statistical correlation was shown between the use of itraconazole prophylaxis and the occurrence of relapse with the same genotype, this pattern occurred in two patients in this study.

We have shown that recurrent episodes of thrush in AIDS patients receiving long-term itraconazole prophylaxis may be caused by the same or a new strain of *C. albicans*. Although the sample size was small a trend was noted toward a change in genotype among isolates that demonstrated a $\geq 4$ fold increase in MIC as compared to those without an increase.

### Acknowledgements

We are grateful to Dr Richard Hafner, National Institute of Allergy and Infectious Disease, Division of AIDS, Rockville, Maryland, for collaboration in the development of the study and advice on preparation of the manuscript. Other contributors to this study, by site, include: Mycoses Study Group (NIAID contract #NO1-AI65296): C. Flanigan, H. Gutsch; Janssen Pharmaceutical: B. Weissinger, A. Baruch, A. Dine; Infectious Disease Associates of Kansas City; D. Smith, B. Lee; Indiana University School of Medicine: H. Nixon; University of Missouri, Kansas City; D. Bamberger, D. Simpson; Infectious Disease of Indiana: J. Black, S. Norris, T. Slama, S. Ryan; Vanderbilt University: J. Richardson, J. McKinsey; Methodist Hospital System: D. Lancaster, D. Ray; Infectious Disease Associates of Memphis, M. Threlkeld. This work was supported by grants from the National Institutes of Health AIDS Clinical Trials Group (U01-AI 25859) and Mycoses Study Group of the National Institute of Allergy and Infectious Diseases contract #NO1-AI65296, and The Histoplasmosis Reference Laboratory.

### References


