Bloodstream and non-invasive isolates of *Candida glabrata* have similar population structures and fluconazole susceptibilities

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We have compared multilocus sequence typing (MLST) and fluconazole susceptibility profiles of *Candida glabrata* bloodstream isolates obtained during active, population-based surveillance to those obtained from non-sterile sites of individuals with no evidence of fungal disease (i.e., non-invasive isolates) in the same US city during an overlapping time period. In each of the two populations, different proportions of the same six major sequence types (STs) encompassed 82% of the isolates. One ST was more prevalent in the candidemia population and two other STs were more prevalent in the non-invasive population, but the overall allelic frequencies within the groups suggested little, if any, genotypic diversity between them. Fluconazole susceptibility profiles of isolates from the patients in the two groups were not significantly different and were not associated with a particular sequence type. Our results support the hypothesis that *C. glabrata* strains causing bloodstream infections are genetically indistinguishable from those normally residing in/on the host, suggesting that relative pathogenicity may be closely tied to commensalism.

**Keywords** *Candida glabrata*, antifungal resistance, population structure, MLST

**Introduction**

In a recent survey, *Candida* spp. were the fourth most common organism causing hospital-based bloodstream infections, surpassed only by *Staphylococcus* and *Enterococcus* species [1]. While *Candida albicans* remains the species most frequently isolated, the incidence of *Candida glabrata* infections have been increasing steadily so that it is now the second most commonly isolated *Candida* species in the United States [2–4]. Although considered a commensal of the human epithelia, *C. glabrata* is capable of causing serious systemic infections in susceptible hosts. It has been shown to have reduced susceptibility to azole drugs, which may be a contributing factor to the increased proportion of infections caused by this species during the recent era of antifungal drug use [5,6].

During the past two decades the United States Centers for Disease Control and Prevention (CDC) with partners in the Emerging Infections Program has conducted active, population-based surveillance to determine the incidence of candidemia, most recently during 2008–2010 [7,8; Ahlquist A. Epidemiology of Candidemia in Metropolitan Atlanta and Baltimore City and County: Preliminary results of Population-Based active, Laboratory Surveillance – 2008 – 2010. ICAAC, San Francisco, CA, 2009 Abstract M-1241]. During each of the surveillance periods, incident bloodstream isolates from residents of a defined geographic area were collected from local hospitals and assessed for species type. In 1992–1993 and 2008–2010 this surveillance was conducted in metropolitan Atlanta, GA. While *C. glabrata* isolates comprised a minor percentage of the overall isolates in the 1992–1993 surveillance [7], they represented almost a third of those collected during the 2008–2010 surveillance [Ahlquist A. Epidemiology of Candidemia in Metropolitan Atlanta and Baltimore City and County: Preliminary results of Population-Based active, Laboratory Surveillance – 2008–2010. ICAAC, San Francisco, CA, 2009 Abstract M-1241].

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We recently performed MLST analysis in order to genotype *C. glabrata* bloodstream isolates collected from the Atlanta, GA area in 2008–2010 [9]. MLST allows highly reproducible strain discrimination, as well as the development of genotypic strain archives that can be stored digitally for both prospective and retrospective analysis [10,11]. We showed for Atlanta, GA, that the bloodstream isolates were composed of a relatively few sequence types (STs), and was dominated by STs (ST16 and ST3) that had increased from 22% of the population in 1992–1993 to 60% of the population in 2008–2009 [9]. This suggested the question of whether these sequence types were more abundant in candidemia cases because they were more prominent in the population of all circulating *C. glabrata* strains in hospitalized patients in Atlanta or whether those STs had some intrinsic quality that made them more effective at causing candidemia. To test this hypothesis, a second set of non-invasive *C. glabrata* isolates collected in an Atlanta area hospital during the same time period was also analyzed by MLST to determine the relatedness of those isolates to those recovered from bloodstream infections. In addition, antifungal susceptibility testing was performed to assess the role of antifungal drug resistance as a contributing factor in the population structure or strain abundance in either of the two populations.

**Materials and methods**

**Isolates**

In this study 90 incident *C. glabrata* bloodstream isolates from an ongoing, population-based surveillance (2008–2010) in metro Atlanta were tested of which 62 of these have been previously characterized [9]. In addition, 111 isolates from non-sterile body-sites (referred to as non-invasive in this manuscript) were selected at random from a collection maintained by a metro Atlanta hospital participating in the Atlanta candidemia surveillance. These were selected so that: (i) there was only one isolate per patient, (ii) isolates were collected during 2007–2008, (iii) patients did not have any indication of *C. glabrata* candidiasis including localized or deep-seeded infection during their hospital stay (isolates were from sputum, stool, or urine) and (iv) there was no known epidemiologic relationship among patients. Prior to their use all isolates were stored in glycerol at −70°C. Isolates were identified as *C. glabrata* by conventional biochemical means and by Luminex assay for bloodstream isolates [12]. The final identity of all isolates was confirmed by positive MLST analysis. The closely related species *Candida nivariensis* and *Candida bracarensis* were ruled out by a lack of amplification with the MLST primer set [9].

**DNA extraction, PCR amplification and sequencing**

After two passages of each isolate on Sabouraud dextrose agar plates, DNA was extracted using the Mo Bio Microbial DNA Isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The six oligonucleotide primer sets used for MLST analysis were those described by Dodgson *et al.* [13]. PCR reactions were performed exactly as discussed previously from our laboratory [9]. PCR products were purified using Exo SAP-IT as described by the manufacturer (USB, Cleveland, OH, USA). Sequencing reactions were performed using BigDye terminator technology (ABI, Foster City, CA, USA) with an ABI Prism 3730 DNA sequencer. All loci were sequenced in both forward and reverse directions with the same primers as those used for the PCR reactions.

**Antifungal susceptibility testing**

*C. glabrata* antifungal susceptibility testing was performed by broth microdilution using fluconazole, voriconazole, posaconazole, and caspofungin as described by Clinical Laboratory Standards Institute (CLSI) M27-A3 document guidelines [14] using RPMI microbroth trays custom manufactured by TREK Diagnostics (Cleveland, OH, USA). Amphotericin B MIC values were determined by Etest (Biomerieux, Inc, Durham, NC, USA). All results were read visually after 24 h of incubation for the echinocandins and after 48 h for the azoles and amphotericin B.

**Data analysis**

Nucleotide sequences were determined by alignment of the forward and reverse sequences with Sequencher™ 4.7 software (Genecodes Inc., Ann Arbor, MI, USA), and polymorphisms were confirmed by visual examination of the sequence traces. Sequences were then compared to the *C. glabrata* MLST database (http://cglabrata.mlst.net) to assign allele numbers and sequence types. All new alleles were submitted to the *C. glabrata* database curator for inclusion in the database. Population analysis was performed using the GENALEX 6 software environment [15] for principal coordinate analysis, Nei’s genetic distance and genetic identity, and for analysis of molecular variance. F ST values were calculated using Popgene (v1.31) [16].

**Results**

A total of 90 *Candida glabrata* bloodstream isolates collected in 2008–2009 in Atlanta, GA were analyzed by MLST [9]. In addition, we studied 111 non-invasive
isolates collected in the same time period from one of the Atlanta surveillance institutions. MLST typing of the non-invasive isolates revealed one ST that had been recently described in a Maryland population [9] but not in Atlanta, and two new STs. The first was the result of a novel combination of existing alleles and was represented twice in the non-invasive isolate population. The second was a result of novel alleles at NMT1, UGP1, and TRP1. The NMT1 and UGP1 loci displayed new alleles as the result of new combinations of existing SNPs. In addition to a new combination of existing SNPs, TRP1 had a new mutation at position 210 consisting of a G→A non-synonomous 1st position transversion (ala→thr). There was equal ST diversity in the candidemia and non-invasive populations with 16 different STs in each population (Fig. 1). Both populations had exclusive STs, i.e., 8% of the candidemia STs were not found in the non-invasive population and 10% of the non-invasive STs were not found in the candidemia population. Three major sequence types (ST16, ST3 and ST19) predominated in the candidemia population, and together comprised 64% of this population (Table 1), while the same three STs represented 43% of the non-invasive population. Although ST8, ST10 and ST15 comprised another 39% of the non-invasive isolates, they were only 18% of the candidemia isolates. Together these six sequence types accounted for 82% of each of the two populations, with other unique STs comprising the other 18%. There were three statistically significant differences in the percentage of isolates in each population, i.e., the proportion of ST16 in the candidemia population \( P = 0.024 \) and the proportions of ST8 \( P = 0.024 \) and ST15 \( P = 0.03 \) in the non-invasive population (Fisher’s exact test).

Nei’s genetic distance and Nei’s genetic identity values are used to measure the differences in frequencies of alleles among the two populations. Although the STs may be different, the arbitrarily-assigned ST numbers do not reflect the allelic diversity or identity within populations. A genetic distance value of 0, or a genetic identity value of 1, indicates that the populations share all of the alleles, as well as the allelic frequencies. For the candidemia and non-invasive populations, we calculated values of 0.137 for genetic distance and 0.872 for genetic identity which suggests that the two populations are very similar and that they share both alleles and allele frequencies. Wright’s fixation index (\( F_{ST} \)) is a measure of genetic divergence among populations at individual loci. A value of 0 indicates no genetic divergence between the populations, while 1 indicates fixation of alleles in divergent populations. An \( F_{ST} \) value of \( > 0.15 \) is generally used to indicate significant differentiation between two populations. An analysis of allelic similarity between the candidemia and non-invasive populations gave an \( F_{ST} \) value of 0.010, again indicating very little differentiation between the two populations and a general lack of alleles fixed in one population or the other.

Analysis of molecular variance (AMOVA) is another method for partitioning diversity within and between populations. AMOVA analysis revealed that 97% of the variance occurred within each population while only 3% of the variance occurred between the two populations. We used the AMOVA genetic distance matrix to perform principal coordinate analysis (PCA) between the two populations. PCA is a multivariate analysis that allows the two dimensional visualization of the major components of variation between complex data sets and is plotted according to population. Fig. 2 shows that although there is some separation of the candidemia and non-invasive populations, the two populations had generally overlapping distributions, and that in general the division by principal components of variation was again within the two populations and not between the two populations.

![Fig. 1](image)

Fig. 1 Distribution of sequence types between the two populations.
Antifungal susceptibility of candidemia and non-invasive isolates

Fluconazole resistance (MIC ≥ 64 μg/ml) was shown in 22 isolates in the total collection and they were evenly distributed, with 11 each in the candidemia and non-invasive populations (Table 2). These resistant isolates were distributed among nine STs, mostly in ST3 or ST16 (n = 12), although this distribution was not statistically significant. Twenty-five isolates were voriconazole resistant or susceptible-dose dependent (MIC ≥ 2 μg/ml) with 15 in the candidemia population and 10 in the non-invasive population. Fifteen of the voriconazole resistant isolates were ST3 or ST16, and this distribution was statistically significant (P = 0.05). The voriconazole geometric mean MIC values for the candidemia and non-invasive populations were the same, but the MIC distributions were different. Both populations had an MIC90 of 0.5 μg/ml but the MIC90 of the non-invasive isolates was 1 μg/ml and the MIC90 of the candidemia isolates was 4 μg/ml. This difference was statistically significant (P = 0.02).

Only five isolates in the total population had elevated caspofungin MIC values (MIC ≥ 0.5 μg/ml). All of these isolates were in the candidemia population and they were distributed among four of the major STs. The only other significant difference in the MIC distributions between candidemia and non-invasive isolates was for amphotericin B. However, since all isolates were well within the range considered clinically susceptible (MIC ≤ 1 μg/ml), this difference is probably not clinically relevant.

Discussion

Candida glabrata has long been recognized as a human commensal, and colonization has been shown to increase as a function of host age [17]. Its increasing importance as an opportunist in immunocompromised individuals receiving prophylaxis has been well documented [2,18], particularly because of its innate ability to acquire antifungal resistance [19,20]. Little is known about the epidemiology of Candida glabrata, particularly the degree of genetic relatedness between bloodstream isolates and non-invasive isolates. An assessment of this relationship could help address the question of whether every Candida glabrata isolate is capable of becoming a successful pathogen, or whether pathogenicity is limited to specific genetic lineages that disproportionately undergo clonal expansion. In order to address this question, we previously assessed the ST distribution of a population of bloodstream isolates from the Atlanta, GA metropolitan area. We determined that ST3, ST16 and ST19 were the major sequence types recovered from candidemia isolates in Atlanta [9]. In this study, we added ST data from isolates from the same geographic region and time period that were known not to be causing invasive disease. We found that non-invasive populations of C. glabrata are also enriched in the same three STs (ST3, ST16, and ST19), and that the isolates from the candidemia and non-invasive collections had very similar population structure. Although a combination of

Table 1 Abundance of the major sequence types in the candidemia and non-invasive populations.

<table>
<thead>
<tr>
<th>Sequence type</th>
<th>Candidemia N = 90</th>
<th>Non-invasive N = 111</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>26 (29)</td>
<td>18 (16)</td>
<td>0.024</td>
</tr>
<tr>
<td>3</td>
<td>20 (22)</td>
<td>18 (16)</td>
<td>0.184</td>
</tr>
<tr>
<td>19</td>
<td>12 (13)</td>
<td>12 (11)</td>
<td>0.148</td>
</tr>
<tr>
<td>8</td>
<td>3 (3)</td>
<td>13 (12)</td>
<td>0.024</td>
</tr>
<tr>
<td>10</td>
<td>7 (8)</td>
<td>12 (11)</td>
<td>0.315</td>
</tr>
<tr>
<td>15</td>
<td>6 (7)</td>
<td>18 (16)</td>
<td>0.030</td>
</tr>
<tr>
<td>Total percentage</td>
<td>82%</td>
<td>82%</td>
<td></td>
</tr>
</tbody>
</table>
The genotypes ST16, ST3 and ST19 were predominately found in both populations (43% of the non-invasive population and 64% of the candidemia population), ST16 was significantly enriched in the candidemia population. In a recent study, Enache-Angoulvant et al. [21] showed through microsatellite typing that there were more unique genotypes among digestive tract isolates than among bloodstream isolates in their European C. glabrata populations. Their study was limited by the fact that their two populations were not temporally and geographically linked. We found that the diversity was similar between the two groups with each group having 16 STs in total and six unique STs.

Most C. glabrata fungemias are thought to originate from the endogenous flora of the host [19,22], suggesting that in order to predominate as a pathogen, an isolate must first predominate as a commensal. Due to their relative abundance in the population, ST3, ST16, and ST19 may be more available when host factors or environmental conditions allow them to switch from a commensal state to a pathogenic state. Yet there is also some evidence that certain STs may be better pathogens. While we found that the percentage of the population comprised of ST3, ST16 and ST19 increased from commensalism to infection, the opposite was true for the other three abundant STs in our population. While ST8, ST10 and ST15 formed a major proportion of the non-invasive population (39%), these same STs formed a much smaller percentage of the candidemia population (18%). Although the six most prevalent STs (see Table 1) comprise the same proportion of isolates in the candidemia and non-invasive isolates, the STs are not equally distributed between the two groups. In the non-invasive population ST8, ST10 and ST15 are more prevalent and at least for ST8 and ST15 this difference is statistically significant. This may indicate that ST8, ST10 and ST15 are less fit as pathogens than as commensals.

However, ST10 was the third most prevalent ST from candidemia isolates in Baltimore [9]. This shift in population structure between commensalism and infection may be indicative of the fitness of certain sequence types for one or the other role.

Antifungal resistance does not seem to play a role in determining the population structure of either the candidemia or non-invasive populations. It had been previously shown that there was no difference in susceptibility to antifungal drugs between candidemia and colonizing isolates of C. glabrata in three hospitals in New York [23]. We similarly found no significant association between fluconazole resistance from either the candidemia or non-invasive population, nor did we find an association between fluconazole resistance and any of the major sequence types that comprise the majority of isolates within either population. This indicates that selection through antifungal resistance does not account for the increase in specific STs, but it does not mean that the enriched STs may not have a growth advantage under antifungal pressure, as this parameter could not be measured in this study. There were statistically significant differences associated with voriconazole resistance. It is puzzling as to why such association between voriconazole and either population would have occurred, since voriconazole is not one of the major antifungal drugs used to treat candidemia patients in Atlanta [A. Ahlquist and S. Lockhart, unpublished]. Voriconazole, a more potent azole, may exert more selective pressure than fluconazole.

Antifungal susceptibility and selection pressure has long been considered a driver for the increase in C. glabrata incidence in the US. Eleven percent of the C. glabrata isolates in this study were fluconazole resistant. This percentage is similar to that reported for C. glabrata in the US since at least the late 1990s [6,24], but has not been dramatically increasing as much as the prevalence of C. glabrata BSI has been increasing. We have documented an increase over time in the prevalence of the major C. glabrata STs (ST3, ST16 and ST19) in US populations [9]. One intriguing aspect of this increase is that the most prevalent sequence type in both candidemia and non-invasive populations is ST16, a ST that is exclusively found in the US [13,25,26]. At the same time, C. glabrata comprises a much smaller percentage of bloodstream isolates recovered from other countries around the world [24,27–31]. Perhaps the clonal expansion of a small set of STs has contributed to the increase in prevalence of C. glabrata in the US through enhanced commensalism.

There are several limitations to our study. The first is that we have treated the candidemia and non-invasive populations as geographically equivalent when that is not necessarily the case. While the candidemia isolates came from a collection of 20 hospitals within our catchment area, the non-invasive isolates came from a single hospital within...
that same area. It is not clear whether collecting non-invasive isolates from a broader swath of our catchment area would change the overall genetic diversity of that population or reveal significant differences in diversity. Furthermore, it is not clear to what extent the non-invasive collection reflects the actual numbers of isolates collected in this institution, whereas the bloodstream collection was collected through audited surveillance and is highly reflective of the full diversity of the bloodstream population.

Second, our conclusion that there is no significant association between sequence type and fluconazole resistance made the assumption that our genetic markers can be in some way genetically linked to markers directly associated with fluconazole resistance and that the linkage can be measured by our parameters but this is not necessarily the case. For some strains, antifungal resistance may be more epigenetic in nature and not associated with a specific mutation [32]. This epigenetic tendency would not necessarily be measurable in our assay, especially in isolates from patients not previously exposed to fluconazole. Another possibility is that the gene(s) for fluconazole resistance, such as efflux pumps, etc. [22], are located on chromosomes not associated with MLST markers or distally located such that linkage cannot be detected. Studies are ongoing to determine if surrogate markers can be developed for azole resistance and incorporated into an MLST typing scheme.

Third, MLST is of very low discriminatory power for distinguishing C. glabrata strains, so the isolates within any one subgroup may be more diverse than we can measure.

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