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Use of phylogenetical analysis to predict susceptibility of pathogenic *Candida* spp. to antifungal drugs

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ABSTRACT

Successful treatment of a *Candida* infection relies on 1) an accurate identification of the pathogenic fungus and 2) on its susceptibility to antifungal drugs. In the present study we investigated the level of correlation between phylogenetical evolution and susceptibility of pathogenic *Candida* spp. to antifungal drugs. For this, we compared a phylogenetic tree, assembled with the concatenated sequences (2475-bp) of the *ATP2*, *TEF1*, and *TUF1* genes from 20 representative *Candida* species, with published minimal inhibitory concentrations (MIC) of the four principal antifungal drug classes commonly used in the treatment of candidiasis: polyenes, triazoles, nucleoside analogues, and echinocandins. The phylogenetic tree revealed three distinct phylogenetic clusters among *Candida* species. Species within a given phylogenetic cluster have generally similar susceptibility profiles to antifungal drugs and species within Clusters II and III were less sensitive to antifungal drugs than Cluster I species. These results showed that phylogenetical relationship between clusters and susceptibility to several antifungal drugs could be used to guide therapy when only species identification is available prior to information pertaining to its resistance profile. An extended study comprising a large panel of clinical samples should be conducted to confirm the efficiency of this approach in the treatment of candidiasis.

1. Introduction

Fungi within the *Candida* genus are typically asexual (anamorphs) and their yeast-like cells proliferate by budding. These organisms are part of the normal human gut flora but they may cause serious infections in immune-compromised patients (Hube, 2004; Pilmis et al., 2016). *Candida* species are responsible for a large proportion of fungal infections (Rapp, 2004; Nguyen et al., 2016; Imabayashi et al., 2016) and account for between 8% and 10% of all blood culture isolates (Maschmeyer and Ruhnke, 2004). Historically, *Candida albicans* was considered the causal organism of most candidiasis. However, over the past three decades, an important increase in the quantity of nosocomial infections caused by non-*albicans Candida* species, has triggered the development of various surveillance programs (Wingard, 1995; Nguyen et al., 1996; Pfaller et al., 1998b, 1999, 2000; Rex et al., 1998; St-Germain et al., 2001; Slavin et al., 2004). These programs have generated important information related to antifungal susceptibility and species distribution (Pfaller and Diekema, 2002, 2004) and have highlighted the fact that it is no longer sufficient to refer to *Candida* isolates as simply *albicans* or non-*albicans*.

A few years ago, identification methods for members of this group were based on their morphological and biochemical characteristics. However, these techniques were time-consuming and not always reliable (Becker, 2000; Bretagne, 2004). In contrast to culture-based biochemical tests, the use of DNA-based techniques provides highly accurate tools for identification, and an insight into the genetic diversity and phylogenetic relationships between *Candida* species (Diezmann et al., 2004; Larriba, 2004). Phylogenetic work is facilitated by the accessibility of taxonomic information deposited in public databases (Berbee and Taylor, 1999; Tautz et al., 2003). Multigenic approaches improved the phylogeny of medically important *Candida* species over previous analyses based on partial sequences from a single ribosomal gene (Diezmann et al., 2004; Tsui et al., 2008; Schmalreck et al., 2014).

Rokas et al. (2003) showed that yeasts phylogenetic trees with a greater degree of resolution are obtained by concatenating several

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highly conserved genes. A similar approach was subsequently used by Diezmann et al. (2004), Tsui et al. (2008), and Schmalreck et al. (2014) to investigate the relationship between different *Candida* species. However, these studies used at least 5 different genes to produce phylogenetic trees. Furthermore, they only analyzed the antifungal susceptibility of limited antifungal drug classes.

In the present study we concatenated only 3 different conserved genes (*ATP2* (coding for the F-ATPase beta-subunit), *TEF1* (coding for the GTPase cytoplasmic elongation factor 1α (EF-1α)), and *TUF1* (coding for the GTPase mitochondrial elongation factor *Tu* (EF-Tu)) to 1) find an easier way to investigate phylogenetic relationships between the most clinically important *Candida* species and 2) examine whether species within the resulting phylogenetic clusters share similar susceptibility to a more extended panel of antifungal drugs i.e. the four principal classes of systemic antifungal agents used in the treatment of candidiasis (polyenes, triazoles, nucleoside analogues and echinocandins; Nagata et al., 1983; Chiron et al., 2005; Saltzgaber-Muller et al., 1983). A close relationship between phylogenetical evolution and susceptibility of *Candida* spp. to antifungal drugs as well as an easier way to generate results could accelerate, maximize, and improve the treatment of candidiasis.

2. Materials and methods

2.1. Fungal strains and genomic material

With the exception of *Candida dubliniensis*, which was obtained from the National Collection of Pathogenic Fungi (Bristol, UK), all fungal strains used (see below for species list) were type strains obtained from the American Type Culture Collection (Manassas, Va, USA). Identification of the 20 *Candida* species was either confirmed in-house using conventional ID-32C phenotypical techniques (bioMérieux Canada, Inc., St-Laurent, Québec, Canada), or by a provincial reference laboratory (Laboratoire de santé publique du Québec). All *Candida* species in the present study have the potential to cause candidiasis in humans. Genomic DNA was extracted using the G-NOME Yeast DNA extraction kit from Qbiogene (Carlsbad, CA, USA).

2.2. PCR primers

Sequences of the *ATP2*, *TEF1*, and *TUF1* genes were retrieved from public databases and aligned using the GCG package (version 8.0, Accelrys, San Diego, CA, USA). Sequencing PCR primers were designed from highly conserved regions of the multiple sequence alignment using the Oligo primer analysis software (version 5.0, National Biosciences Inc., Plymouth, MN, USA). When required, primers containing inosines or degeneracies were used to allow amplification of polymorphic regions. Oligonucleotide primers were synthesized using a model 394 DNA/RNA synthesizer (PE Applied Biosystems, Foster City, CA, USA). The PCR primers used to amplify and sequence the three conserved genes from *Candida* species are shown in Table 1.

2.3. DNA sequencing

Conserved portions of the *ATP2*, *TEF1*, and *TUF1* genes were amplified from 20 *Candida* species using a previously described method (Ke et al., 1999), slightly modified by the use of 1 μL of purified genomic DNA at 5 ng/μL for each species. After electrophoresis, the gel was stained with methylene blue and PCR products having the predicted size were recovered using the QIAquick gel extraction kit (Qiagen Inc., Mississauga, Ontario, Canada). The purified PCR products were then sequenced using an automated DNA sequencer ABI 3730/XL and the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA, USA). To exclude the possibility of sequencing errors attributable to misincorporations by *Taq* DNA polymerase, each strand was sequenced twice using PCR products obtained from two independent PCR reactions.

Table 1
PCR primer pairs used to amplify and sequence conserved genes of *Candida* species.

Primer	Sequence	Nucleotide position[a]	Amplicon length (bp)[a]
ATP2			
ATP2-1	RTIATIGGIGCIGTIRTIGAYGT	145–167	1339
ATP2-2	CCICCIACCATRTARAAIGC	1465–1484	
ATP2-3	ATIGCIATGGAYGGIACIGARGG	283–305	1201
ATP2-4	CCICCIACCATRTARAAIGC	1465–1484	
TEF1			
TEF1-1	CARYTIATHGTIGCIGTIAAYAARATGGA	439–467	808
TEF1-2	TARTCIGTRAAIGCYTCIACRCACAT	1222–1247	
TEF1-3	TCITTYAARTAYGCITGGGT	157–176	1144
TEF1-4	CCGACRGCRAYIGTYTGICKCAT	1279–1301	
TUF1			
TUF1-1	CATGTCAAYATTGGTACTATTGGTCATGT	142–170	1129
TUF1-2	CCACCYTCICTCAMGTTGAARCGTT	1247–1271	
TUF1-3	CATGTCAAYATTGGTACTATTGGTCATGT	142–170	1064
TUF1-4	CATYTCRAIRTTGTCACCTGG	1186–1206	
TUF1-5	AAYATGATIACIGGIGCIGCICARATGGA	379–407	901
TUF1-6	ACIGTICGGCCRCCCTCACGGAT	1258–1280	

[a] The nucleotide positions and expected amplicon length given are for the *Saccharomyces cerevisiae* S288C *ATP2*, *TEF1*, and *TUF1* sequences (GenBank accession numbers NC_001142, NC_001148 and NC_001147, respectively).

2.4. Phylogenetic analysis

Multiple sequence alignments were performed using CLUSTAL W software (version 1.83; Thompson et al., 1994) and gaps, indels, or ambiguities were removed from the phylogenetic analysis using the GCG SeqLab editor. Phylogenetic trees were obtained by neighbor-joining and parsimony methods using MEGA3 software (version 3.0; Kumar et al., 2004). Evolutionary distances of either individual or concatenated *ATP2*, *TEF1*, and *TUF1* genes, were estimated using Kimura's two-parameter substitution model for nucleotide sequences or the JTT (Jones, Taylor and Thornton) correction for protein sequences (Nei and Kumar, 2000). Phylogenetic tree topologies were assessed by 1000 bootstrap replicates for both nucleotide and protein sequences. Finally, *ATP2*, *TEF1*, and *TUF1* sequences from the unrelated Ascomycete *Neurospora crassa* were used as an outgroup.

2.5. Nucleotide sequence accession numbers

GenBank accession numbers for the sequences of fungal species used in this study are DQ447205 to DQ447224 for *ATP2* gene (1014-bp), DQ447225 to DQ447244 for *TEF1* gene (742-bp), and DQ447245 to DQ447264 for *TUF1* gene (719-bp). Sequences of *ATP2*, *TEF1*, and *TUF1* genes from *Saccharomyces cerevisiae* S288C genome project were retrieved respectively from the chromosome X (accession number NC_001142), chromosome XVI (NC_001148) and the chromosome XV (NC_001147). For the ongoing genome project *Neurospora crassa* N150, sequences of the three conserved genes were found by BLAST homology search on the Broad Institute website (http://www.broad.mit.edu).

2.6. Compilation of in vitro susceptibilities of Candida species to antifungal agents

Epidemiologic studies published since 1952, show that five *Candida* species are consistently and globally responsible for the majority of candidiasis. These are, in descending order: *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, and *C. krusei* (Wingard, 1995; Nguyen et al., 1996; Pfaller et al., 1998b, 1999, 2000, 2005b; Rex et al., 1998; St-Germain et al., 2001; Slavin et al., 2004). The remaining clinical