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Identification and susceptibility of clinical isolates of *Candida* spp. to killer toxins

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Abstract

Although invasive infections and mortality caused by *Candida* species are increasing among compromised patients, resistance to common antifungal agents is also an increasing problem. We analyzed 60 yeasts isolated from patients with invasive candidiasis using a PCR/RFLP strategy based on the internal transcribed spacer (ITS2) region to identify different *Candida* pathogenic species. PCR analysis was performed from genomic DNA with a primer pair of the ITS2-5.8S rDNA region. PCR-positive samples were characterized by RFLP. Restriction resulted in 23 isolates identified as *C. albicans* using AlwI, 24 isolates as *C. parapsilosis* using RsaI, and 13 as *C. tropicalis* using XmaI. Then, a group of all isolates were evaluated for their susceptibility to a panel of previously described killer yeasts, resulting in 75% being susceptible to at least one killer yeast while the remaining were not inhibited by any strain. *C. albicans* was the most susceptible group while *C. tropicalis* had the fewest inhibitions. No species-specific pattern of inhibition was obtained with this panel of killer yeasts. *Metschnikowia pulcherrima*, *Pichia kluyveri* and *Wickerhamomyces anomalus* were the strains that inhibited the most isolates of *Candida* spp.

Keywords: Candida, killer yeasts, yeast antagonism.

Identificação e susceptibilidade de isolados clínicos de *Candida* spp. para toxinas assassinas

Resumo

Embora as infecções invasivas e a mortalidade causada por espécies de *Candida* estejam aumentando entre pacientes comprometidos, a resistência a agentes antifúngicos comuns também é um problema crescente. Analisamos 60 leveduras isoladas de pacientes com candidíase invasiva utilizando como estratégia PCR/RFLP baseada na região espaçadora transcrita interna (ITS2) para identificar diferentes espécies patogênicas de *Candida*. A análise por PCR foi realizada a partir de ADN genómico com um par de iniciadores da região ITS2-5.8S rDNA. As amostras PCR-positivas foram caracterizadas por RFLP. A restrição resultou em 23 isolados identificados como *C. albicans* usando AlwI, 24 isolados como *C. parapsilosis* usando RsaI e 13 como *C. tropicalis* usando XmaI. Em seguida, avaliou-se o grupo de todos os isolados quanto à sua susceptibilidade a um painel de leveduras killer previamente descritas, resultando em 75% sendo suscetíveis a pelo menos uma levedura killer, enquanto que as restantes não foram inibidas por qualquer cepa. *C. albicans* foi o grupo mais suscetível enquanto *C. tropicalis* teve o menor número de inibições. Não se obteve um padrão de inibição específico da espécie com este painel de leveduras killer. *Metschnikowia pulcherrima*, *Pichia kluyveri* e *Wickerhamomyces anomalus* foram as cepas que inibiram a maioria dos isolados de *Candida* spp.

Palavras-chave: Candida, levedura killer, antagonismo de leveduras.

1. Introduction

Invasive infections caused by primary and opportunistic fungal pathogens have increased in immunocompromised patients for over two decades (Beck-Sagué and Jarvis, 1993). Most of these life-threatening infections are caused by different *Candida* species, which represent the most commonly yeasts isolated from bloodstream infections in patients in intensive care units and are the second cause of mortality in such patients (Wisplinghoff et al., 2004). In Mexico, the most frequent species is *C. albicans* (70%), but the proportion of infections caused by Non-*albicans Candida* species (30%) has been increasing (Hernández-Hernández et al., 2003).

Mortality associated to *Candida* infections ranges from 40% to 60% (Nucci et al., 1997). Furthermore, in the last decade, the emergence of infections caused by *Candida* species resistant to common antifungal agents has been increasing, such as those caused by *C. krusei* and *C. glabrata* resistant to fluconazole. Therefore, proper identification at species level is essential to provide appropriate therapy and reduce mortality in patients with invasive candidiasis. Furthermore, efforts are being made in order to develop new antifungal therapies that could overcome such resistances with a potential candidate being killer toxins secreted from other yeasts.

The killer phenomenon was described by Bevan and Makower (1963) and consists of the secretion of proteinaceous compounds (killer toxins) from yeasts that inhibit the growth of other yeasts. These killer toxins exhibit variations in their inhibition spectrum, mechanism of action, molecular size, optimum pH and temperature, and they have been described in more than 20 genera of yeast from both the ascomycetes and basidiomycetes phyla (Golubev, 1998), from either clinical or natural sources (Souza Cabral et al., 2009; Arroyo-Helguera et al., 2012). Reports from Magliani et al. (2004) and Polonelli et al. (2003) have shown that killer toxins pose an interesting strategy for the development of new antifungal treatments that could overcome antifungal resistance due to the evolutionary origin of these interactions.

In this study, we used a PCR/RFLP strategy based on the ITS2 region to identify at species level 60 clinical *Candida* isolates and then subjected them to a panel of previously isolated killer yeasts of diverse origin in order to evaluate their potential therapeutic application and look for patterns that may differentiate among species, as a potentially fast and low-cost identification technique.

2. Material and Methods

2.1. Killer yeasts

A panel of 19 previously isolated killer yeasts was selected, isolated and identified by Dr. Marcela Sangorrín (Table 1). The killer yeasts were identified by ITS1-5.8S-ITS2 rDNA PCR-RFLP analysis as described by Esteve-Zarzoso et al. (1999). Patterns obtained for each isolate after digestion with the restriction enzymes Cfo I, Hae III and Hinf l were compared with those of reference strains available in the yeast identification database (Esteve-Zarzoso et al., 1999). Yeast identity was confirmed by sequencing the D1/D2 domains of the 26S rRNA gene (Kurtzman and Robnett, 1998). The sequences obtained for yeast isolates were compared with those published at GenBank database (available at the National Center for Biotechnology Information, NCBI) using BLAST.

Table 1. Killer yeasts evaluated for their inhibitory activity of clinical isolates of *Candida* spp.

Strain No.	ID
1250	*Pichia membranifaciens*
1127	*Metschnikowia pulcherrima*
1144	*Metschnikowia pulcherrima*
1123	*Metschnikowia pulcherrima*
1023	*Metschnikowia pulcherrima*
1025	*Wickerhamomyces anomalus*
1027	*Wickerhamomyces anomalus*
1015	*Wickerhamomyces anomalus*
1018	*Wickerhamomyces anomalus*
1026	*Wickerhamomyces anomalus*
1033	*Torulaspora delbruecki*
1036	*Torulaspora delbruecki*
1147	*Pichia kluyveri*
1150	*Pichia kluyveri*
1151	*Pichia kluyveri*
1153	*Pichia kluyveri*
1148	*Pichia kluyveri*
1263	*Cryptococcus victoriae*
1268	*Cryptococcus weringae*

2.2. Clinical isolates

A total of 60 clinical isolates were obtained from the mycology laboratory of the Centro Regional para el Control de Enfermedades Infecciosas at the Universidad Autónoma de Nuevo León. All *Candida* strains were grown on YPD broth (1% yeast extract, 2% casein peptone and 2% dextrose) for 24 hours at 37°C with shaking (230 rpm). Yeast identification was previously performed using the API 20 C AUX test (bioMérieux Vitek, Hazelwood, MO, USA) according to manufacturer's instructions.

2.3. DNA isolation

Yeast DNA was obtained by the technique described by Sambrook and Russell (2001). For cell wall disruption and subsequent extraction of DNA, we used the method described by Jin et al. (2004), with modifications. In brief, cells of each fungal culture were homogenized in 500 µL of lyticase lysis buffer (50 mMtris, pH 7.6; 1 mM EDTA, 0.2% 2-mercaptoethanol, 10 µL of lyticase 5 U/µL) and incubated for 10 minutes at 37°C. Cell wall disruption was performed by mixing with glass beads in a vortex. Extraction of DNA was carried out by precipitation with phenol-chloroform and 70% ethanol. Finally, DNA was resuspended in 20 µL of TE buffer (10 mMTris-HCl,

pH 8.0 and 0.1 mM EDTA), 2 µLof RNase (10 mg/mL) and stored at -20°C until used.

2.4. PCR amplification

Primers ITS3 (5′–GCA TCG ATG AAG AAC GCA GC–3′) and ITS4 (5′–TCC TCC GCT TAT TGA TAT GC–3′) previously reported were used to amplify the ITS2-5.8S rDNA from the isolates (White et al., 2003). Amplification was performed using 5-10 ng of fungal DNA as template in a 50µL mix containing 100 ng of each primer, 10 µM of deoxyribonucleotide triphosphate mixture, 2.5 mM of magnesium chloride, 5 µL of 10X PCR buffer (200 mM Tris, pH 8.4; and 500 mM KCl) and 2.5 U of TaqDNA polymerase (Invitrogen, Carlsbad, CA, USA). A reaction mix without a template was used as a negative control. Amplification consisted of an initial denaturation step of 5 minutes at 94°C, followed by 35 cycles. Each cycle consisted of a denaturation step at 95°C for 1 minute, annealing at 55°C for 1 minute and elongation at 72°C for 1.5 minutes, with a final elongation step at 72°C for 5 minutes, in a PTC-100 thermocycler (MJ Research, Waltham, MA, USA). All PCR products were separated by electrophoresis in 3.5% agarose electrophoresis gels stained with ethidium bromide and visualized with the software LabImage 1D version 3.4.0 (Kapelan Bio-Imaging, Leipzig, Germany). Afterwards, samples were stored at -20°C until used.

2.5. Selection of restriction enzymes

The nucleotide sequences of the ITS2-5.8S rDNA of various *Candida* species were analyzed first *in silico* using the NEBcutter software (New England BioLabs, Ipswich, MA, USA) to determine the different restriction patterns generated by the endonucleases. We selected *Rsa*I, *Alw*I,, *Xma*I,, *Xho*I, and *Aat*II due to the differential restriction patterns generated by them (Table 2).

2.6. Restriction fragment length polymorphisms

PCR-amplified ITS2-5.8S rDNA fragments from clinical isolates were digested with selected endonucleases in the appropriate buffer provided by the manufacturer (New England Biolabs, Ipswich, MA, USA). Digestion was carried out for 12 hours at 37°C, in a total volume of 20 µL containing 10 µL of the PCR product, 5 U of each restriction enzyme and the respective buffer solution. Digested materials were separated by electrophoresis in 8.0% polyacrylamide gel stained with ethidium bromide and the restriction fragments were analyzed using the LabImage 1D version 3.4.0 (Kapelan Bio-Imaging, Leipzig, Germany).

2.6. Killer assays

The screening of killer activity was performed as reported previously (Robledo-Leal et al., 2012), using YEPD-MB agar (0.3% Yeast extract, 0.3% Malt extract, 0.5% Peptone, 2% Glucose, 2% Agar and 0.003% Methylene blue, adjusted to pH 4.5 with 0.1 M citrate-phosphate buffer). Twenty-four-hour-old cultures of the clinical isolates were mixed with the YEPD-MB agar to obtain a final concentration of $1x10^6$ cells per mL. After homogenization, media was poured onto Petri plates. Killer strains were streaked as thick smears over the sensitive lawn and replicates were made for incubation at 25°C for up to 72 h. The appearance of an inhibition zone surrounding the killer yeast bordered with a halo of dark-blue-stained cells was considered a positive indication of the presence of killer activity (Figure 1). Once the results were obtained, each assay was repeated for confirmation and reproducibility.

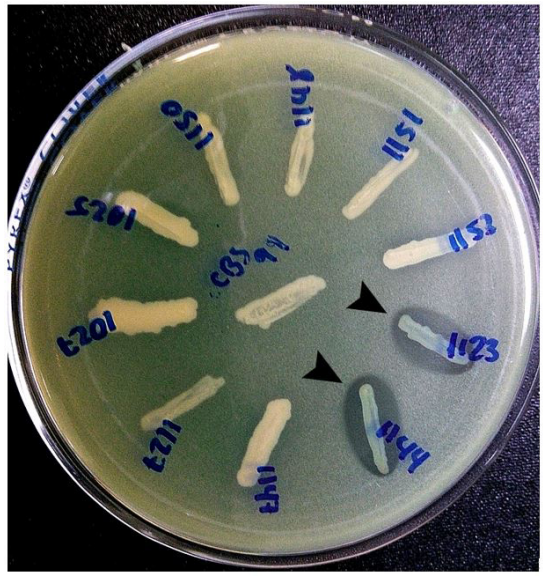

Figure 1. Sample of a killer assay. Strains 1123 and 1144 in the plate (arrowheads) exhibit the killer phenotype.

Table 2. Differential restriction patterns employed in this study.

Species	GenBank accession number	ITS2, 5.8S and 28S partial sequence of rDNA (nt)	Restriction enzyme	Restriction fragments length (nt)	No. of clinical isolates identified
C. rugosa	EU663569	271	RsaI	234, 37	0
C. albicans	EU663565	335	AlwI	208, 127	23
C. tropicalis	EU796734	327	XmaI	186, 141	13
C. lusitaniae	EU663568	255	XhoI	159, 84, 12	0
C. guilliermondii	EU663566	378	RsaI	210, 168	0
C. parapsilosis	ATCC 22019	311	RsaI	228, 83	24

ITS: Internal Transcribed Spacer; nt: nucleotides.

3. Results

Amplification of the ITS2-5.8S rDNA region of the different reference *Candida* species generated PCR products ranging 271 to 378 base pairs (bp) in size (Figure 2). Enzymes that gave differential restriction fragment lengths *in silico* were employed *in vitro* to evaluate their differentiation of this strategy; electrophoresis in 8.0% polyacrylamide gel showed that the target bands corresponded to those predicted *in silico*.

To evaluate the reliability of the PCR/RFLP strategy used to identify clinical isolates of *Candida,* we extracted genomic DNA from 60 *Candida* isolates obtained from invasive infection cases. At least 100 ng of DNA were used for the ITS2-5.8S rDNA amplification. Restriction resulted in 23 isolates identified as *C. albicans* using *Alw*I, 24 isolates as *C. parapsilosis* using *Rsa*I, and 13 as *C. tropicalis* using *Xma*I.

3.1. Susceptibility to killer yeasts

All of the isolates were assayed against killer yeasts as described above (Table 3). Forty-five isolates (75%) were sensitive against at least one killer yeast and the 15 remaining isolates were not inhibited by any killer strain. *Candida albicans* was the most susceptible group, with 142 combinations resulting in inhibition, compared to 53 in *C. parapsilosis* and 27 in *C. tropicalis*. With the exception of CP21, *C. parapsilosis* had the narrowest range of susceptibility, with only 6 different strains being able to inhibit them, while for *C. albicans* and *C. tropicalis* as many as 12 different killer yeasts were able to inhibit them. Killer strains 1123, 1127 and 1144 were the most active, inhibiting the growth of 24 clinical isolates, including every one of *C. albicans*. None of the other killer yeasts was able to inhibit a whole species group. The most susceptible strains were also within the *C. albicans* group, being inhibited by 12 different killer strains. No species-specific pattern of inhibition was obtained with this panel of killer yeasts.

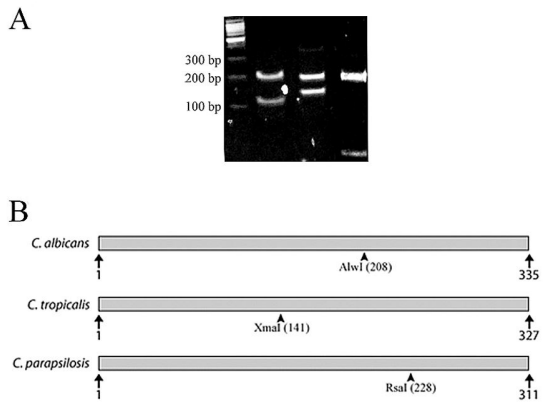

Figure 2. PCR/RFLP of the ITS2-5.8S region of the rRNA gene from different *Candida* species. (**A**) PCR-amplified fragments of the mentioned region plus restriction enzyme used. Lanes: 1: *C. albicans* + *Alw*I (208 and 127 bp); 2: *C. tropicalis* + *Xma*I (186 and 141 bp); 3: *C. lusitaniae* + *Xho*I (84, 96 and 159 bp); 4: *C. guilliermondii* + *Rsa*I (210 and 168 bp); 5: *C. parapsilosis* + *Rsa*I (228 and 83 bp); 6: *C. rugosa* + *Rsa*I (37 and 234 bp). (**B**) Predicted (*in silico*) length and restriction map of the PCR-amplified fragments of the ITS2-5.8S region from different *Candida* species.

4. Discussion

The incidence of pathogenic fungal species has a critical impact on clinical outcome in patients with invasive fungal infections, especially in immunocompromised patients. Although most of the clinically relevant fungal infections are caused by *Candida* species, it is important to identify the specific etiological agent to make an adequate choice of the antifungal therapy (Messer et al., 2009; Bourgeois et al., 2010). In order to provide a more rapid and reliable method for the identification of fungal species in invasive candidiasis, a number of molecular techniques have been developed. Several methods for specific fungal species identification are based on ITS2 ribosomal region variability, which is flanked by conserved sequences of rDNA (Fujita et al., 2001; Ferrer et al., 2001; Trost et al., 2004). The identification of fungal species using the ITS2 region has been described previously by Landlinger et al. (2009). They established a strategy based on PCR-fragment length analysis of the ITS2 region to identify fungal species from clinical specimens. The PCR/RFLP technique differentiated seven clinically important species of *Candida*. A limitation of a single ITS2-5.8S rDNA PCR fragment length analysis is the low variation of the fragment sizes between certain species, such as *C. albicans* and *C. tropicalis*, but they exhibit variation in their nucleotide sequences that are exploitable for differentiation using restriction enzymes. The site *Alw*I was conserved in 23 clinical isolates that had been previously identified by microbiological techniques as *C. albicans*, and the site *Xma*I was present in 13 isolates identified as *C. tropicalis*. Killer activity depends on various factors being temperature and pH some of the most relevant, but the nature of the sensitive yeast may be the most important. Our experiments exhibit the strain-related property of the killer phenomenon as described by Golubev (1998). While some killer toxins have a broad spectrum of activity, in theory there is no such thing as a "universal" killer yeast, for it depends on receptors and thus phylogenic relatedness. Yeasts may be resistant to certain killer toxins due to the lack of receptors, or immune due to either mutations or cross-protection resulting from the secretion of a similar toxin. *Metschnikowia pulcherrima* strains 1023, 1123 and 1144 showed a complete inhibition of the *C. albicans* group; while this species' antimicrobial effect has been related to the production of pulcherrimic acid which is converted to the iron sequestering compound pulcherremin, instead of a proteinaceous agent, differences in the inhibition spectrum suggest the possibility of a killing factor different from or additional to this. On the other hand, strains 1153 (*P. kluyveri*) and 1025 (*W. anomalus*) exhibited a broad spectrum of inhibition as well (16 and 15 inhibitions respectively); both species have been reported before as producers of killer toxins and while these strains were

Table 3. Interactions between killer yeasts and clinical isolates (only killer yeasts with positive reactions are shown).

Clinical isolates	Killer yeasts												
	1250	**1127**	**1144**	**1123**	**1023**	**1025**	**1027**	**1015**	**1018**	**1026**	**1147**	**1153**	**1268**
CP01				+									
CP02			+										
CP03			+	+									
CP04													
CP05													
CP06			+	+									
CP07													
CP08		+	+	+	+								
CP09		+	+	+	+								
CP10			+	+	+								
CP11													
CP12													
CP13				+									
CP14		+	+	+									
CP15				+									
CP16			+	+									
CP17		+	+	+	+								
CP18		+	+	+	+								
CP19		+	+	+	+								
CP20		+	+	+									
CP21			+	+		+	+			+		+	+
CP22			+	+	+		+						
CP23			+	+	+								
CP24													
CA01			+	+	+								
CA02	+		+	+	+	+	+	+	+	+	+	+	+
CA03	+		+	+	+	+	+	+	+	+	+	+	+
CA04			+	+	+								
CA05			+	+	+	+	+					+	+
CA06	+		+	+	+	+	+			+		+	+
CA07			+	+	+								
CA08			+	+	+					+			
CA09			+	+	+							+	+
CA10	+		+	+	+	+	+			+		+	
CA11			+	+	+	+						+	
CA12			+	+	+								
CA13	+		+	+	+								
CA14	+		+	+	+	+	+	+	+	+	+	+	+
CA15			+	+	+								
CA16	+		+	+	+	+				+		+	+
CA17			+	+	+	+	+			+		+	
CA18			+	+	+								
CA19	+		+	+	+	+	+			+		+	+
CA20			+	+	+								
CA21			+	+	+								
CA22	+		+	+	+	+	+			+		+	+
CA23			+	+	+	+	+					+	+
CT01													
CT02	+		+	+	+	+	+			+		+	+

+: positive inhibition; CP: *Candida parapsilosis*; CA: *Candida albicans*; CT: *Candida tropicalis*.

Table 3. Continued...

Clinical isolates	Killer yeasts												
	1250	**1127**	**1144**	**1123**	**1023**	**1025**	**1027**	**1015**	**1018**	**1026**	**1147**	**1153**	**1268**
CT03													
CT04													
CT05													
CT06	+					+	+	+	+	+	+	+	+
CT07													
CT08													
CT09													
CT10													
CT11													
CT12	+					+	+	+	+	+	+	+	+
CT13													

+: positive inhibition; CP: *Candida parapsilosis*; CA: *Candida albicans*; CT: *Candida tropicalis*.

not able to inhibit any *Candida* species group completely, they encourage the potential application of killer yeasts from the same species with a broader activity towards new antifungal therapies, both topical and systemic.

Killer activity is present in a wide diversity of yeasts but since the appropriate sensitive strain has to be used, many yeast species may have been misidentified as non-killer. It has been possible to differentiate between species of pathogenic yeasts using the killer system (Coutinho and Paula 1998; Scheid et al. 2010; Boekhout and Scorzetti 1997), and we suggest that it could be done for *Candida* species as well if a more taxonomically diverse panel of killer strains is employed. This would represent a rapid and low cost diagnostic tool. The value of such strategy would have a direct impact on therapy, due to the antifungal susceptibility differences among species (Treviño-Rangel et al., 2012; Fothergill et al., 2014).

On the other hand, antifungal resistance is a widely-reported issue that has become increasingly important, which has promoted the search for non-synthetic alternative, including plant extracts (Höfling et al., 2010). Currently, there is a remarkable need for better antifungals with high specificity and reduced toxicity. In our results, 3 yeasts were able to kill every *C. albicans* strain, suggesting that their killer toxin could potentially be used as a therapeutic alternative via the production of "antibiododies" as described and demonstrated by Polonelli et al. (2011). While these yeasts were not able to kill every *Candida* strain in our study, the extraordinary diversity of yeasts makes it highly probable for the existence of strains with a wider range of action or a specific effect against other species of pathogenic fungi. Uncommon yeasts from non-conventional sites, such as those reported by Moraes et al. (2005) may prove useful in accomplishing this.

Follow-up experiments to investigate the inhibitory nature of *M. pulcherrima* strains and towards the search for a killer panel able to differentiate among *Candida* species, as well as new killer yeasts capable of inhibiting other pathogenic fungi, are ensured.

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