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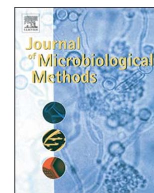

A quest to find good primers for gene expression analysis of *Candida albicans* from clinical samples

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ABSTRACT

Biofilm production contributes to several human diseases, including oral candidiasis. Among the *Candida* species, *Candida albicans* is the most prevalent. The expression of virulence genes is implicated in the pathogenic potential of *Candida* biofilms. However, the evaluation of microbial gene expression from *in vivo* biofilm samples is not trivial, specifically, assessment *via* quantitative PCR (qPCR) can be a challenge because of several species present in clinical samples. Hence, the necessity of primers specificity. The aim of this study was to evaluate through *in silico* and *in vitro* analyses the specificity of published primers and newly designed primers for *C. albicans* virulence genes: ALS1, CAP1, CAT1, EFG1, HWP1, LIP3, PLB1, SAP1, SAP4, SOD1, SOD5 and ACT1 (normalizing gene). *In silico* analysis was performed through a PubMed search of articles with primer sequences that evaluated gene expression of *C. albicans*. Then, the sequence similarity of twenty-eight primers was checked through BLASTn and ClustalW2. The analysis of secondary structures was performed using mfold. When the primers did not present satisfactory characteristics (absence of secondary structures, not discrepant Tm of forward and reverse sequences and specificity) following *in vitro* analysis (*i.e.*, end point PCR), new primers were designed using Beacon Designer™ and sequences obtained from the "*Candida Genome Database*". The selected primers were tested *in vitro* by end point PCR using a panel of genomic DNA from five different *Candida* species (*C. albicans*, *Candida glabrata*, *Candida dubliniensis*, *Candida krusei*, and *Candida tropicalis*). The resulting PCR products were visualized on agarose gel. qPCR reactions were performed to determine primers' optimal concentration and PCR efficiency. End point PCR demonstrated that published primers for the SAP1 and HWP1 were specific for *C. albicans* and the one for SOD1 reacted with *C. albicans* and *C. dubliniensis*. The sequence of primers designed for ACT1, ALS1 and HWP1 genes were specific for *C. albicans*, while the ones for CAP1, CAT1, EFG1, LIP3, and PLB1 were detected in *C. albicans* and *C. dubliniensis*. After optimization, all primers presented a single peak on melt curves, correlation coefficient of ≅1 and qPCR reaction efficiency of 90–110%, with slope of ≅−3.3. Therefore, these primers should be suitable for future gene expression analyses from clinical samples.

1. Introduction

Oral candidiasis is the most common soft tissue fungal infection of the oral cavity in humans caused by *Candida* spp. (Akpan and Morgan, 2002; Sardi et al., 2013). An overgrowth of these fungi can cause superficial, cutaneous, mucosal, and invasive infections (Sardi et al., 2013). *Candida albicans* is considered the most prevalent pathogenic species in the oral microbiota (Kulak et al., 1994), growing as yeast, pseudohyphal or hyphal forms. The yeast morphology of *C. albicans* is considered commensal in healthy humans but can cause systemic infection in immunocompromised patients, mainly because of their ability to adapt to different niches (Sardi et al., 2013). Even though the yeast form is considered a less harmful morphology, there is an increase in resistant of this fungus to antifungal drugs, contributing to human disease once the immune system is repressed, *C. albicans* can prevail and act as an opportunistic fungus, causing infection especially when the host microbiota is modified by certain predisposing factors (Akpan and Morgan, 2002; Nobile and Johnson, 2015). Besides the high prevalence of *C. albicans*, other species have been detected in human infections. The most commonly described are: *Candida glabrata*, *Candida dubliniensis*, *Candida krusei*, and *Candida tropicalis* (Lyon et al., 2006). In addition to these species, *Candida auris* is an emerging multidrug resistant fungal pathogen (Chatterjee et al., 2015).

A major virulence factor of *C. albicans* is its capacity to form biofilms on biotic or abiotic surfaces (Gulati and Nobile, 2016; Mayer et al., 2013). Biofilms can be described as surface-associated communities of

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microorganisms embedded within an extracellular matrix (Silva et al., 2011). The biofilm formation by *Candida* spp. is an important virulence factor because it protects microbial cells from host immune responses, limits the penetration of substances through the matrix, thereby conferring significant resistance to conventional antifungal therapy (Gulati and Nobile, 2016; Silva et al., 2011). In addition to the ability to form biofilms, there are others important factors to the virulence of *C. albicans*: the morphological transition between yeast and hyphal forms; the expression of adhesins and invasins on the cell surface; thigmotropism; phenotypic switching; secretion of hydrolytic enzymes; resistance to changes in environmental pH; metabolic flexibility; powerful nutrient acquisition systems (glucose, lipids, proteins and amino acids); and response to oxidative stress (Mayer et al., 2013).

The increased incidence of superficial and systemic infections caused by *Candida* spp. has been attributed to resistance to antifungals and expression of many virulence factors that these fungi present after exposure to antifungals to infection treatment (Haynes, 2001; Sardi et al., 2013). Therefore, understanding the virulence and resistance mechanisms associated with these species is relevant. Moreover, the knowledge about the expression of virulence factors genes and how therapeutic approaches affect the expression of those genes can be an indicator of treatment/intervention effectiveness.

There are a few of methods for gene expression analysis to identify changes of expression of important virulence factors responsible for the onset and development of infection in the host and for resistance to therapeutics. Among them is the qPCR (quantitative Polymerase Chain Reaction) technique, which can quantify mRNA expression of virulence genes. An ideal PCR reaction shows high specificity, yield and fidelity (Cha and Thilly, 1993). Therefore, it is important that the qPCR primers have satisfactory characteristics (*i.e.*, absence of secondary structures, resulting PCR product size…) and specifically anneal to improve the sensitivity of PCR. Although transcriptomic analysis using next-generation sequencing (NGS) approaches can provide an overall expression profile, subsequent validation of critical genes is almost invariably carried out by qPCR. A summary of considerations can be found in Bustin (2004) and Thornton and Basu (2011).

Therefore, to evaluate the expression of *C. albicans* genes in *in vivo* biofilm samples from patients, we first analyzed whether the published primers were suitable for use. Biofilms or clinical sample analysis can be challenging because several species may be present in a clinical sample and the primers need to be specific. In this context, the purpose of this study was to find and standardize published or newly designed primers for target *C. albicans* virulence genes, these primers needed to display satisfactory characteristics to be used in future *in vivo* studies. The primers pairs were tested through *in silico* and later with the *in vitro* tests, end point PCR and quantitative PCR.

2. Materials and methods

2.1. In silico analyses of selected C. albicans virulence genes

2.1.1. PubMed searches (Performed in July of 2015)

Initially, PubMed searches were performed to find significant genes associated with *C. albicans* virulence. The thirteen genes selected are detailed in Table 1. Subsequently, additional PubMed queries were performed using a combination of the following key words: 1) *Candida albicans*, qPCR, gene expression; 2) *Candida albicans*, specific gene name of genes listed in Table 1. The following publications were selected in which the authors described the primers and cited when these primers were target-specific for the chosen virulence genes (Alves et al., 2014; Chen and Lan, 2015; Dai et al., 2013; Green et al., 2004; Hnisz et al., 2012; Komalapriya et al., 2015; Martchenko et al., 2004; Naglik et al., 2003b; Nailis et al., 2010; Zhu et al., 2011). Next, all primers were subjected to a step wise validation approach described below.

2.1.2. Analysis using Basic Local Alignment Search Tool (BLASTn)

The sequences of the specific genes were obtained in FASTA format from the *Candida Genome Database* (http://www.candidagenome.org/) and PubMed database (for SAP1 gene). The primer sequences (forward and reverse) were identified in the downloaded gene sequence, and the predicted amplicons were analyzed using BLASTn to check sequence similarity of them to the intended target genes in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Johnson et al., 2008) for all currently known genes in other species. The primers were also analyzed for sequence similarity by BLAST against other fungal species *via* "*Candida Genome Database*" search (http://www.candidagenome.org/cgi-bin/compute/blast_clade.pl).

2.1.3. Alignment of target sequences by Clustal analysis

Clustal W2 was used to align multiple sequences (http://www.ebi.ac.uk/Tools/msa/clustalo) to evaluate the sequence similarities of primers against genes within gene families. The strength of interaction between homologous nucleotides was also observed.

2.1.4. MFOLD

The MFOLD program was used to detect possible secondary structures in the chosen primers and the resulting PCR products (http://unafold.rna.albany.edu/?q=mfold/DNA-Folding-Form). This tool has as main advantages the possibility of changing the ionic conditions of the primers ([Na+] and [Mg++]) and desired temperatures for annealing. The parameters used for the analysis of secondary structures were: folding temperature: 60 °C; [Na+]: 50 mM; and [Mg++]: 3 mm.

Finally, all published primers were also analyzed by Beacon Designer™ software (Premier Biosoft, version 8.14, Corina Way, Palo Alto, CA, USA). The parameters used to test were: Tm of forward and reverse (50 ± 5 °C); primer size (18–24 bp); product size (80–150 bp); GC% and absence of hairpins.

2.1.5. Design of new primers

When the published primers did not show the characteristics already described in the Introduction for clinical samples evaluation, new primers were designed by Beacon Designer™ software. The primers were designed using the following parameters: no identity with other species or gene; Tm around 50 °C; SYBR Green; primer size between 18 and 24 bp; PCR product size between 80 and 150 bp, no hairpins and GC% (around 50%). The designed primers were also analyzed by *in silico* methodology described earlier (*i.e.*, Blastn, Clustal, Mfold).

2.1.6. Primers selected for in vitro analyses

Ten published primers and eight newly designed primers were selected for *in vitro* analyses to test their specificity (Table 2).

2.1.7. In vitro analyses of published and newly designed primers

All the selected primers shown in Table 2 were evaluated for specificity to *C. albicans*. The cross-reaction was evaluated through the detection of the PCR products against a panel of genomic DNA isolated from four different *Candida* species (*C. glabrata*, *C. dubliniensis*, *C. krusei*, and *C. tropicalis*) in addition to *C. albicans* DNA. Those primers that did not cross react with other species (except *C. dubliniensis*) were used to determine optimal concentration for qPCR reaction. Moreover, the detection limit for each selected primer was evaluated using qPCR and isolated DNA as template.

2.1.8. Yeast strains and DNA isolation

A panel of five reference strains of *Candida* spp. associated with candidiasis, was used for genomic DNA isolation. Three strains were obtained from the American Type Culture Collection (ATCC; Rockville, MD): *Candida glabrata* (ATCC 2001), *Candida tropicalis* (ATCC 4563) and *Candida krusei* (ATCC 6258). *Candida dubliniensis* (CBS 7987) was obtained from Centraalbureau voor Schimmelcultures (CBS, Utrecht, Netherlands) and *C. albicans* (SC5314) was obtained from Stanford

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