

**INFECTION BY *CANDIDA PARAPSILOSIS*:
MULTIFACTORIAL ANALYSIS OF AN EMERGING MEDICAL PROBLEM**

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“A mais fiel de todas as companheiras da alma é a esperança.”

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LIST OF PUBLICATIONS

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- I. Silva AP, Costa-de-Oliveira S, Silva-Dias A, Rodrigues AG, Pina-Vaz C. Dynamics of *in vitro* acquisition of resistance by *C. parapsilosis* to different azoles. **FEMS Yeast Research** 2009; 9:626-633.
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- IV. Ricardo E, Silva AP, Gonçalves T, Costa-de-Oliveira S, Granato C, Martins J, Rodrigues AG, Pina-Vaz C. *Candida krusei* reservoir in a neutropaenia unit: molecular evidence of a foe? **Clinical Microbiology and Infection** 2011; 17:259-263.
- V. Costa-de-Oliveira S, Miranda I, Silva R, Silva AP, Rocha R, Amorim A, Rodrigues AG, Pina-Vaz C. *FKS2* mutations associated with decreased echinocandin susceptibility of *Candida glabrata* following anidulafungin therapy. **Antimicrobial Agents and Chemotherapy** 2011; 55:1312-1314.
- VI. Silva AP, Miranda IM, Guida A, Synnott J, Rocha R, Silva R, Amorim A, Pina-Vaz C, Butler G, Rodrigues AG. Transcriptional profiling of azole-resistant *Candida parapsilosis* strains. **Antimicrobial Agents and Chemotherapy** 2011; 55:3546-3556.
- VII. Silva AP, Rocha R, Pina-Vaz C, Rodrigues AG. Aerosolized hydrogen peroxide dry-mist system for hospital environmental disinfection: assessment of *in vitro* and *in situ* efficacy. Submitted to **Infection Control and Hospital Epidemiology**.

- VIII. Costa-de-Oliveira S, Silva AP, Miranda IM, Salvador A, Azevedo MM, Rodrigues AG, Munro CA, Pina-Vaz C. A new approach for chitin cell wall content in fungal cells by flow cytometry. Submitted to *Eukaryotic Cell*.

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- I. Silva AP, Miranda I, Guida A, Synnott J, Silva-Dias A, Rodrigues AG, Pina-Vaz C, Butler G. Transcriptional profile highlight the increased expression of virulence attributes in azole resistant *C. parapsilosis*. **50th ICAAC** 2010; M-1083.
- II. Silva AP, Pina-Vaz C, Carvalho T, Rodrigues AG. Efficacy of hydrogen peroxide dry-mist disinfection system for hospital environment disinfection. *Journal of Hospital Infection* 2010; 76:S23.
- III. Silva AP, Figueiredo S, Lisboa C, Rocha R, Rodrigues AG. Antifungal activity of chemical agents against *Candida*. *Journal of Hospital Infection* 2010; 76:S23.
- IV. Silva AP, Silva-Dias A, Miranda I, Pina-Vaz C, Rodrigues AG. Correlation between azole resistance and virulence attributes displayed by *C. parapsilosis*. *Clinical Microbiology and Infection* 2010; 16:S577.
- V. Silva AP, Costa-de-Oliveira S, Miranda I, Pina-Vaz C, Rodrigues AG. Fungaemia by *Candida parapsilosis*: *in vivo* induction of azole resistance due to prolonged therapeutic exposure. *Clinical Microbiology and Infection* 2010; 16:S216.
- VI. Costa-de-Oliveira S, Silva AP, Miranda IM, Salvador A, Azevedo MM, Munro CA, Rodrigues AG, Pina-Vaz C. Easy quantification of yeast chitin cell wall content by flow cytometry. *Mycoses* 2011; 54:66.

LIST OF ABBREVIATIONS

AMB - amphotericin B

ANF - anidulafungin

CFU - colony forming unit

CFW - calcofluor white

CLSI - clinical and laboratory standards institute

CSF - caspofungin

CV - cristal violet

DMSO - dimethyl sulfoxide

FLC - fluconazole

h - hours

HS - hot spot

Ibu - ibuprofen

ITC - itraconazole

MCF - micafungin

MFS - major facilitator superfamily

MIC - minimal inhibitory concentration

min - minutes

MLC - minimal lethal concentration

mtDNA RE - mitochondrial DNA restriction endonuclease

MOPS - morpholine propanesulfonic acid buffer

NS - nonsusceptible

PBS - phosphate-buffered saline

PCR - polymerase chain reaction

PI - propidium iodide

R - resistant

RAPD - random amplified polymorphic DNA

RFLP - restriction fragment length polymorphism

Rh-6G - rhodamine 6G

RPMI - roswell park memorial institute

RT-qPCR - real-time quantitative chain reaction

S - susceptible

s - seconds

S-DD - susceptible-dose dependent

UV - ultra-violet

Vera - verapamil

XTT - 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl amino) carbonyl]-2H-tetrazolium hydroxide

YEPD - yeast extract peptone dextrose

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INTRODUCTION

GENERAL CONSIDERATIONS

Fungi have emerged as a major cause of human disease, particularly among immunocompromised patients and patients with serious underlying medical conditions [1], which has led to increased research in this area. Invasive candidosis now is widely recognized as an important public health problem, with considerable morbidity, mortality, and associated health care costs [2].

Candida species represent the fourth most common group of isolates from bloodstream infections in the United States; recent reports also suggest an increase of candidaemia in Europe [2-5].

C. parapsilosis was first isolated from the stools of a patient with diarrhea in Puerto Rico in 1928 [6, 7]. The species was named *Monilia parapsilosis* to distinguish it from the more common isolate, *Monilia psilosis*, known today as *C. albicans*. In 1932, *Monilia parapsilosis* was renamed *Candida parapsilosis*.

C. parapsilosis cells display an oval, round, or cylindrical shape. The colonies, on Sabouraud dextrose agar, are white, creamy, shiny, and smooth or wrinkly. *C. parapsilosis* can exist in either a yeast shape or pseudohyphal form. *C. parapsilosis* is typically a commensal of the human skin, but is also a disease agent. Over the past decade, the incidence and clinical relevance of *C. parapsilosis* has increased. It is of special concern among critically ill neonates [8-10].

Lodderomyces elongisporus was previously believed to be the teleomorph of *C. parapsilosis* due to similarities between: (1) GC nuclear content; (2) conserved rRNA portions and (3) behavior in biochemical tests and substrate assimilation [11, 12]. Nowadays, they are considered distinct species, although very closely related [13-15]. This is an example of a masquerading species, becoming molecular identification and genotyping more needed in clinical practice. It is quite possible that there are a number of new yeast species in clinical material that cannot be distinguished phenotypically from a more common species; implications could arise due to distinct antifungal susceptibility patterns.

Given the continued emergence of *C. parapsilosis*, comprehensive studies regarding its epidemiology, virulence traits, clinical manifestations, genetics, and antimicrobial susceptibility are needed.

EPIDEMIOLOGY

At present, *C. parapsilosis* stands out as the second most common yeast species isolated from patients with bloodstream infections in Latin America and Asia [16, 17], but it is also

commonly found in European surveys [1, 17, 18]. Similar results were found in Portugal, according to a prospective, observational study of bloodstream fungal infections [19]. *C. parapsilosis* outranks *C. albicans* in some European [20], Asian [21, 22], and South American [23] hospitals.

In comparison to other *Candida* species, *C. parapsilosis* has a more ubiquitous distribution in nature. *C. parapsilosis* is not an obligate human pathogen, having been isolated from nonhuman sources [7] such as domestic animals, insects, soil, and marine environments [24]. *C. parapsilosis* is also a normal human commensal, and it is one most frequent fungal isolate from the subungueal space of human hands, as is the case with the hands of health care workers. Such finding suggests its role as a potential reservoir and route for nosocomial transmission [25, 26].

The latest study concerning *Candida* bloodstream infections in 79 medical centers worldwide by Pfaller et al. [2] reported that 17.1% of infections were caused by *C. parapsilosis*, after *C. albicans* (48.4%) and *C. glabrata* (18.2%). Moreover, the study distinguished the infections in community-onset and nosocomial bloodstream infections; *C. parapsilosis* was more common among nosocomial episodes.

RISK FACTORS

Invasive disease caused by *C. parapsilosis* can occur without prior colonization and is frequently transmitted horizontally via contaminated external sources such as medical devices or fluids, the hands of health care workers, prosthetic devices, and central venous catheters. The increase in the frequency of *C. parapsilosis* infections has been attributed to a variety of risk factors, including the organism selective growth capability in hyperalimentation solutions and its affinity for intravascular devices and indwelling prosthetic materials. Immunocompromised individuals such as AIDS and surgical patients, particularly those submitting to surgery of the gastrointestinal tract, are at high risk for infection with *C. parapsilosis*. Additionally, patients requiring prolonged use of a central venous catheter or indwelling devices are at increased risk for infection with *C. parapsilosis*. The population at greatest risk for nosocomial infection with *C. parapsilosis* is that of extremely low-birth-weight neonates. Colonization of the skin or gastrointestinal tract is frequently the first step in the pathogenesis of invasive candidal disease, and neonates are especially prone to disease given their compromised skin integrity, increased susceptibility to gastrointestinal tract infection, the long-term needed for central venous catheters, and prolonged endotracheal intubation [8, 27]. An observational study addressing the epidemiology of invasive fungal infections among infants admitted to Neonatal Intensive Care

Units (NICUs) of one Italian region showed that *C. parapsilosis* was the most common isolated species (61,9%); the main potential risk factors were related to a central venous catheter placed, length of stay superior to 7 days and total parenteral nutrition for more than 5 days [28]. A study about the occurrence of *Candida* spp. during a three-year period in two NICUs, in Hungary, reported the following species distribution: *C. albicans* (73%), *C. parapsilosis* (24%) and *C. glabrata* (3%). The study also concludes that the mortality rate for *C. parapsilosis* was higher than for *C. albicans* [29]. A study regarding *Candida* spp. distribution among bloodstream infections by patient age revealed that *C. parapsilosis* was most common among the 0-19 year age group [30].

The hands of health care workers are major vectors for the exogenous acquisition of *C. parapsilosis*. As a normal commensal of human skin, *C. parapsilosis* represents a major threat to patients interacting with colonized health care workers, particularly when breaches in standard hand-washing protocols occur. Although the percentages vary among studies, multiple reports described *C. parapsilosis* as the yeast organism most commonly isolated from health care workers hands [31, 32]. Molecular typing methods have illustrated the link between hand carriage of *C. parapsilosis* and the horizontal transmission and outbreak of nosocomial infections by *C. parapsilosis* [33]. For example, *C. parapsilosis* genotyping results in 3 fungaemia outbreaks in NICUs in the southeastern United States, supplied clear evidence of horizontal transmission in 2 outbreaks; in the third outbreak, the bloodstream isolates corresponded to 2 distinct genotypes circulating in the unit; one genotype was shared by one infected patient and the hands of a healthcare worker [34]. In an investigation of a cluster of *C. parapsilosis* infections involving six patients in a Brazilian cancer ward, *C. parapsilosis* was recovered from the hands of three health care workers; two of such isolates were molecularly identical to the outbreak strain [25].

CLINICAL MANIFESTATIONS

C. parapsilosis is responsible for a broad variety of clinical manifestations that generally occur in individuals with impaired immune system, neutropenia, or burns and in patients admitted to neonatal or surgical intensive care units. *C. parapsilosis* causes invasive disease (fungaemia) worldwide. Several studies addressing fungaemia reported this species [18, 19, 21-23, 35-47]. *C. parapsilosis* fungaemia can lead to the seeding of tissues and multiple organs, resulting in deep-seated infections [48], and results in mortality rate ranging from 4% [49] to approximately 45% [3, 45, 46]. *Candida* species account for 94.1% of fungal endocarditis cases, many of which develop following cardiac surgery [50]; *C. parapsilosis* is associated with 17% of

the identified cases, making it the second most common species after *C. albicans* [51]; overall, mortality ranges from 41.7% [52] to 65% [7]. Fungal peritonitis results in serious morbidity and in a mortality rate of up to 44% [53]. The major predisposing factor for fungal peritonitis is treatment of previous bacterial peritonitis with antibiotics, which presumably promotes fungal overgrowth [54]. Although *C. albicans* is credited as the most common *Candida* species causing peritonitis, numerous papers have reported that *C. parapsilosis* is the predominant species associated with such disease [53, 55-57]. *C. parapsilosis* is associated with invasive ocular diseases such as endophthalmitis (particularly postoperative infection) and keratiti [58-60]. Otomycosis is a relatively uncommon infection causing otitis media or externa. However when it occurs, *C. parapsilosis* is the first or second most common isolate [61, 62]. *C. parapsilosis* was seldom mentioned as an agent causing pathological lesions of the nails, but it has gained increasing recognition as the most common etiological agent causing *Candida* onychomycosis [63, 64]. *C. parapsilosis* is an infrequent agent of fungal meningitis [65-68] or fungal arthritis [7]. Vaginal candidosis is the second most common genital infections in fertile age women in the United States and Europe, after bacterial vaginosis [69], and *C. albicans* is associated with 85% to 95% of such cases. Surprisingly, 30% of recurrent vulvovaginal candidosis cases are caused by non-*C. albicans* species like *C. parapsilosis* [70]. Therefore, proper identification of the *Candida* species should be undertaken in patients with recurrent or complex vulvovaginitis before initiation of shortcourse antifungal treatment, which may be less effective against non-*C. albicans* species [71-73]. It is noteworthy that although *C. parapsilosis* is infrequently isolated from vaginal cultures, it nevertheless may be isolated from asymptomatic women [72, 73]. Among *Candida* species, *C. parapsilosis* is not a frequent agent of urinary tract infection [74].

GENETICS

The increasing clinic relevance of *C. parapsilosis* promoted a deeper analysis into its genome. The sequencing of the entire genome was carried out by Sanger Institute (<http://www.sanger.ac.uk/sequencing/Candida/parapsilosis/>). Recently it was reported the genome sequences of six *Candida* species (including *C. parapsilosis*) and compared these and related pathogens and non-pathogens [75]. *C. parapsilosis* is a diploid pathogen with an estimated genome size of 13.1 megabases and 5733 open reading frames; the genome is organized in 8 chromosomes.

Genetic and/or genomic heterogeneity is known to occur among *C. parapsilosis* isolates [13, 76, 77]. The high level of genetic variation was shown by several techniques. On the basis of

randomly amplified polymorphic DNA (RAPD) analysis [78], multilocus enzyme electrophoresis [13], analysis of the internal transcribed spacer sequences of DNA encoding ribosomes [13], DNA relatedness analysis [79], morphotyping [80], analysis of mitochondrial DNA sequence differences [81], DNA topoisomerase II gene sequence analysis [82], and analysis with an oligonucleotide probe used for fingerprinting of *C. parapsilosis* isolates [83], it was found that *C. parapsilosis* forms a complex composed of three genetically distinct groups (groups I, II, and III). Although genetically heterogeneous, these groups are phenotypically indistinguishable. In 2005, Tavanti et al. [84] proposed the replacement of the existing designations of *C. parapsilosis* group II and group III by *C. orthopsilosis* and *C. metapsilosis*, respectively, and the retention of the designation *C. parapsilosis* for the former group I isolates. In order to distinguish between the three species, Tavanti et al. [84] proposed the analysis of the restriction polymorphism of the *SADH* gene, which encodes a secondary alcohol deshydrogenase and which is common to all three species. Strains from each group are found in samples from human patients; clinical isolates are predominantly of group I. However, there is substantial genetic variation even within group I isolates. This variation is associated with several molecular determinants of virulence, such as the ability of the fungus to colonize distinct hosts and niches (i.e., humans or animals and skin, blood, or sputum), the appearance of specific phenotypes (i.e., antibiotic resistance), and the ability to generate biofilm [76, 85]. The separation of the previous groups I, II, and III into species leads clinical publications to distinguish between them. Recent studies have shown that some clinical isolates formerly identified as *C. parapsilosis* in fact correspond to isolates of the closely related species *C. orthopsilosis* and *C. metapsilosis* [13, 84, 86, 87] which account for about 10% of the total number of former *C. parapsilosis* isolates [87]. As more data regarding the different species are collected, further differences between these species will likely accumulate. These two new species may inhabit important niches in certain patient populations, thus stressing the need for continued surveillance in each country to monitor the prevalence and distribution of these three species.

Interestingly, *C. parapsilosis* lacks nucleotide sequence diversity [84]. The frequency of mutation in *C. albicans* is about 1/140 bp in examined coding regions [88, 89]. In contrast, studies involving *C. parapsilosis* only found two polymorphic nucleotide sites among 7.5 kbp sequences [84] and only four mutations among more than 36 kbp [90]. The lack of nucleotide sequence diversity suggests that *C. parapsilosis* is highly clonal and has only recently, within the past one million years, emerged as a species.

Indeed, molecular methods used to distinguished *C. parapsilosis* strains only evidence a very few types. Different techniques are applied in molecular differentiation studies, especially

in outbreak situations: RAPD analysis [33, 86, 91], restriction fragment length polymorphism (RFLP) analysis [33], sequence analysis of the intergenic transcribed spacer gene [86, 91], microsatellite analysis [92, 93], electrophoretic karyotyping [94], and southern blot assay [34] among others. Different methods provide different results, a fact that underscores the importance of combining multiple typing methods, of testing several control isolates, and the need to correlate the results with careful epidemiological assessment.

VIRULENCE ATTRIBUTES

C. parapsilosis pathogenicity has been attributed to several intrinsic attributes, including promoted adhesion to medical devices and/or host cells, biofilm formation, and secretion of hydrolytic enzymes.

Adhesion is likely a critical step in yeast pathogenesis. This property might be expected for *C. parapsilosis*; following its acquisition from an exogenous source, it subsequently adheres to medical indwelling devices, and finally invades the host. However, several studies addressing intraspecies variation in adherence found no relationship between invasiveness and adherence [95-97]. In fact, Panagoda et al. [97] described that superficial *C. parapsilosis* isolates displayed 51.5% greater avidity for buccal epithelial cells than systemic isolates; nonetheless the result was not considered statistically significant. The first large-scale study comparing adhesion of *C. albicans* and *C. parapsilosis* (12 and 24 isolates, respectively) documented a 20.6% greater avidity of *C. parapsilosis* for buccal epithelial cells and a 143.7% greater adhesion to acrylic material, although the differences between the first values were not significant due to the large range of *C. parapsilosis* adhesion values (23.50 to 154.30 per 50 buccal epithelial cells) [97]. The large number of adherent *C. parapsilosis* cells reported in this study may result from coadherence among yeast cells causing aggregates at epithelial surfaces, a trait more often observed for this species than for *C. albicans*. Conversely, other studies reported an 80% to 95% higher adhesion of *C. albicans* to buccal epithelial cells comparing to *C. parapsilosis* [98, 99].

Clinical isolates of *C. albicans* form biofilm [100], which may be an important fact for sustaining infection. Biofilm formation may be especially important for *C. parapsilosis*, since medical indwelling devices appear to be the predominant route of infection [25, 101]. The biofilm-forming potential was cited as a reason why patients with *C. parapsilosis*-infected catheters should have the device promptly removed [102]. *C. parapsilosis* biofilms can occur on diverse medical indwelling devices, including central and peripheral venous catheters, hemodialysis and peritoneal dialysis catheters, intracardiac prosthetic devices, and prosthetic joints [103]. As a commensal of human skin, the organism can come into contact with medical

devices during its insertion or during its management, particularly in health care settings where lapses in proper hand hygiene may occur. It is noteworthy that *C. parapsilosis* isolates with increased biofilm ability have been associated with outbreaks. According to Kuhn et al. [95] biofilm formation is an important component of a strain's ability to cause an infections outbreak. Biofilm formation confers significant resistance to antifungal therapy by limiting the penetration of substances through the matrix and by protecting cells from the host immune responses. Biofilm-forming *C. parapsilosis* isolates have been associated with significantly higher mortality rates in patients at an Italian university hospital compared to clinical isolates incapable to develop biofilm (71.4% versus 28%, respectively) [104]. The capacities of different *C. parapsilosis* isolates to cause disease in various tissues may be influenced by its respective ability to form biofilm. Studies appointed that *C. parapsilosis* blood isolates are capable of forming more biofilm, compared to isolates from other body sites [105, 106]. Two recent studies documenting the generation of *C. parapsilosis* homozygous lipase knockout mutants found that the mutants have a decreased ability to form biofilm [107, 108].

Phenotypic switching occurs in *C. parapsilosis*; four phenotypes are described for *C. parapsilosis*: smooth, concentric, crepe and crater [83]. Cells from crepe and concentric phenotypes are almost entirely pseudohyphal, whilst cells from smooth and crater phenotypes are mostly yeast-like [109]. Hypha development from yeast cells is critical for adherence, an essential first step in microbial colonization, which is in turn a key event in the initiation of the pathogenic process [110, 111]. In response to attachment to a surface, fungal cells produce biofilm, that contributes to the infectious process and antibiotic resistance [112]. In *C. albicans* the generation of biofilm is associated with the dimorphic switch from yeast to hyphal growth. The structure of the formed biofilm involves two distinct layers: a thin, basal yeast layer and a thicker, less compact hyphal layer [113]. In contrast, *C. parapsilosis* strains produce quantitatively less and structurally a less complex biofilm than *C. albicans* [100, 114]. However certain pseudohyphal *C. parapsilosis* phenotypes generate more biofilm and are more agar invasive than strains remaining predominantly in the yeast form [109].

Fungal secreted enzymes may play a relevant role in its pathogenesis. Phospholipases, lipases and proteases are three of the best-characterized of such enzymes. Although the role of phospholipase B, lipases and secreted aspartyl proteases (SAPs) are well studied in *C. albicans* [115, 116], the relationship between *C. parapsilosis* virulence and these enzymes remains unclear. SAPs facilitate invasion and colonization of the host tissues by disrupting host mucosal membranes and degrading important immunological and structural defense proteins [117, 118]. The function of phospholipases during infection is not yet well understood, although it is

believed that they are involved in the disruption of host membranes [115, 119]. The putative roles of microbial extracellular lipases include the digestion of lipids for nutrient acquisition, adhesion to host cells and tissues, synergistic interaction with other enzymes, unspecific hydrolysis due to additional phospholipolytic activities, initiation of inflammatory processes by impairing immune cell functions, and self-defense mediated by lysing competing microflora [120, 121]. Regarding *C. parapsilosis*, no correlation was found between phospholipase activity and the site of isolation or with other virulence attributes, in isolates from an outbreak [95]. The same study [95] did not support the concept that SAPs expression is a critical virulence attribute for *C. parapsilosis*. De Bernardis et al. [96] found an inverse relationship between SAPs expression and invasiveness since, *C. parapsilosis* cutaneous isolates had higher SPAs activity than blood isolates. However, Gacser et al. [122] showed that lipase inhibitors significantly reduce tissue damage during *C. parapsilosis* infections of reconstituted human tissues.

Regarding the other related species, *C. metapsilosis* and *C. orthopsilosis*, several studies characterized their virulence attributes [122-126]. *C. metapsilosis* was the less virulent member of the *C. parapsilosis* groups based on the interaction with immune cells [126]. A study addressing the effects of the three species upon reconstituted human oral epithelial and epidermal tissues concluded that *C. orthopsilosis* caused damage similar to *C. parapsilosis* isolates, whereas *C. metapsilosis* was the less virulent [122]. Concerning biofilm formation, the available results contradict; whilst de Toro et al. [124] described that *C. orthopsilosis* and *C. metapsilosis* are not able to form biofilm, Lattif et al. [123] described that all species are able to form biofilm.

SUSCEPTIBILITY

Several classes of antimycotic drugs with different modes of action and target spectra are at present available to treat clinical fungal infections, including *C. parapsilosis* infections [127]. The base analog flucytosine, which is taken up into the cell by a specific transporter and then converted into 5-fluoro-uridine monophosphate by the sequential action of the enzymes cytosine deaminase and uracil phosphoribosyltransferase, causes the production of aberrant RNA and also inhibits DNA synthesis. The polyenes, the most important member of which is amphotericin B (AMB), bind to ergosterol, the major sterol in the fungal cell membrane, and form pores, thereby causing membrane damage. The azoles impair sterol biosynthesis by inhibiting sterol 14 α -demethylase, which results in ergosterol depletion and accumulation of toxic methylated sterols. Finally, the echinocandins impair cell wall synthesis by inhibiting β -1,3-glucan synthase.

Fungi can become resistant to the described antifungal drugs by specific mechanisms. Mutations that cause defects in the uptake of flucytosine or in its intracellular conversion are a frequent cause of resistance to this drug [128-130]. A decrease in the membrane ergosterol content can lead to reduced polyene susceptibility [131-133]. Three distinct mechanisms have been so far described regarding azole resistance: (1) failure to accumulate the drug intracellularly, which may be caused by the lack of drug penetration due to changes in membrane lipids and sterols [134] or by active efflux of drugs resulting particularly from overexpression of genes *CDR1*, *CDR2* and *MDR1* [135-138]; (2) increased production of the azole target enzyme [139-141]; (3) point mutations in genes coding for the target enzyme, the *ERG* genes, thus reducing azole target affinity [142]. The overexpression of efflux proteins and the associated increased activity is considered to be the most relevant mechanism of azole resistance, conferring cross-resistance to several azoles. Two types of efflux pumps, localized in the cytoplasmic membrane, that transport antifungal drugs are known: the ATP-binding cassette transporters, which use ATP as the energy source to drive transport, and the major facilitators, which are energized by the proton gradient across the cell membrane [143]. Mutations in the *FKS1* and *FKS2* genes, encoding a subunit of the β -1,3-glucan synthase complex, result in resistance to echinocandin drugs [144]; such mutations confer resistance to all clinically used echinocandins. Recently, the compensatory increase in the production of chitin, the second structural cell wall polysaccharide, has been proposed as a mechanism causing reduced efficacy of echinocandins [145, 146].

C. parapsilosis is not considered particularly prone to the development of antifungal resistance [36, 95, 147, 148]. However, recent reports suggest that its decreased susceptibility to azoles and to echinocandins might become a cause for clinical concern [147-151]. The emergence of *C. parapsilosis* strains that have decreased susceptibility to fluconazole (FLC) and that may be transmitted throughout the hospital environment has been related to the extensive use of fluconazole in combination with suboptimal hand hygiene and central venous catheter care in a population of seriously ill patients [18]. Few studies have yet addressed the antifungal susceptibility profile of *C. parapsilosis* [7, 18, 147] and to our knowledge none has addressed the inherent resistance mechanisms.

There is currently no consensus about the treatment of invasive *C. parapsilosis* disease, although the therapeutic approach typically involves the removal of any foreign body and the administration of systemic antifungal. Historically, amphotericin B has been the most frequently used antifungal [38, 152, 153]. The main issue with conventional amphotericin B therapy is the drug's poor aqueous solubility and toxicity [154]. Fluconazole is the most frequently

administered alternative to amphotericin B. However, clinical resistance in *C. parapsilosis* has been reported [104, 151]. Interestingly, the long-term use of fluconazole to control *C. parapsilosis* bloodstream infections in a Finland NICU eventually led to the emergence of a fluconazole-resistant strain that was responsible for cross-infections over a 12-year period [151]. However, other studies addressing fluconazole prophylaxis have not identified fluconazole-resistant strains [155-157]. *In vitro* resistance to itraconazole (ITC) has been described [38, 158]. Minimal inhibitory concentration (MIC) values of voriconazole (VRC) are lower than of older azoles [35, 38, 40, 159, 160]. *In vitro* resistance to voriconazole is rare, with early reports showing 100% susceptibility [161]. Overall, *C. parapsilosis* isolates are also susceptible to posaconazole (PSC), the newer triazole [162].

Regarding echinocandins, the newest class of antifungal agents, MIC values are higher for *C. parapsilosis* than those for other *Candida* species [38, 152, 163, 164]. Although the basis for echinocandin resistance is not yet clearly understood, structural differences in the components of the cell wall, a reduced affinity for the glucan synthase protein complex due to mutations in *FKS* genes, or a difference in its regulatory network may be involved [164]. Despite elevated echinocandin MIC levels, several studies reported *in vitro* and *in vivo* activity against *C. parapsilosis* [95, 165-169], particularly in clinical episodes involving isolates resistant to amphotericin B, to fluconazole, or to both compounds [170]. Echinocandins can fail during treatment of *C. parapsilosis* bloodstream infections, in which the echinocandins MIC values are low [149]. Also, breakthrough infections by *C. parapsilosis* have occurred in individuals administered echinocandins for other indications [171]. At concentrations above the breakpoint for echinocandins, these drugs can paradoxically promote the *in vitro* growth of some isolates of *C. parapsilosis* and other *Candida* species [172]. Additionally, recent epidemiological studies have found an association between increasing caspofungin usage and an increased incidence of *C. parapsilosis* bloodstream infections [173].

The latest available study concerning the antifungal resistance pattern of *Candida* bloodstream isolates [174] only identified 14 resistant *C. parapsilosis* isolates (among a total of 232 isolates) to fluconazole. All isolates were susceptible to voriconazole, posaconazole, anidulafungin (ANF), caspofungin (CSF) and micafungin (MCF); the study incorporated 2085 bloodstream infections isolates of *Candida* spp. from 79 medical centers throughout the world between January 2008 and December 2009.

Few studies addressed the antifungal susceptibility profiles of the distinct *C. parapsilosis* groups. Chen et al. [175] demonstrated that most isolates within *C. parapsilosis* groups were susceptible to flucytosine, azoles, amphotericin B and echinocandins; however, *C. metapsilosis*

isolates related to significantly higher MIC values of both fluconazole and voriconazole comparing to *C. parapsilosis* sensu stricto and *C. orthopsilosis*. On the other hand, *C. metapsilosis* isolates related to significantly lower MIC values of caspofungin than *C. parapsilosis* sensu stricto and *C. orthopsilosis*. Micafungin MIC values were also significantly higher for *C. parapsilosis* sensu stricto isolates than for *C. orthopsilosis* and *C. metapsilosis*. Usually, *C. metapsilosis* is described as the less susceptible species to fluconazole [87, 148]. Diekema et al. [176] found that all the isolates of the less common species within the *C. parapsilosis* groups were susceptible to voriconazole, posaconazole, anidulafungin, caspofungin, and micafungin. Similar results were described in other studies [87, 177]. Additionally, a surveillance study in Spain revealed that the new species were highly susceptible to all the antifungals (amphotericin B, flucytosine, azoles and echinocandins) in particular to echinocandin compounds (MIC values were two- to threefold lower) and amphotericin B, comparing with *C. parapsilosis*. These differences were especially notable for anidulafungin [178].

A large amount of data demonstrates the resistance of *Candida* biofilms to antimycotic drugs [179]. Despite its less complex structure, *C. parapsilosis* biofilm is similarly resistant to *C. albicans* biofilm to antifungals such as amphotericin B and azole compounds [106, 180]. However, therapeutic levels of echinocandins can inhibit metabolic activities of *C. parapsilosis* biofilms [152, 180, 181]; lipid formulations of amphotericin B were shown to have activity against *C. parapsilosis* biofilm [152].

While the endogenous reservoir remains the most important mechanism for acquisition of candidaemia, patient-to-patient spread by indirect transmission and the nosocomial acquisition of *Candida* species from environmental sources is being increasingly suspected [182]. The role of the hospital environment as a potential reservoir for *Candida* species was suggested by prospective epidemiological studies. In these studies, *Candida* isolates had been recovered from inanimate surfaces prior to the acquisition of the same strains by patients [183]. Although the primary reservoir of *C. parapsilosis* in the hospital setting is yet unknown, carriage on the skin of healthy individuals, particularly on the hands of health care workers, has since long been recognized [25, 184]. Prophylactic measures are necessary to control the nosocomial microbial population and to reduce the incidence of hospital infections. Few studies have addressed the *in vitro* activity of antiseptics and detergents against *Candida* spp.. The degree of *in vitro* inhibition of growth varied according to *Candida* species [185]. Traoré et al. [186] showed that products with sufficient potency to exhibit tuberculicidal proprieties produced substantial reductions in the titre of *C. albicans*, although some showed a lesser reduction in titre of *C. parapsilosis*, which

may reflect differences in cell hydrophobicity between *Candida* species. These findings highlight the need for particular care in selecting a suitable surrogate for disinfectant testing assays.

FINAL REMARKS

The emergence of *C. parapsilosis* as the leading non-*C. albicans* *Candida* species poses a major threat for the future since, unfortunately, the incidence of *C. parapsilosis* infections may continue to rise. Advances in health care will allow for higher survival rates among neonates admitted at NICUs, and the increased use of immunomodulatory medications for diverse indications will place more people at risk of infection with *C. parapsilosis*. This pathogen has a high affinity for parenteral nutrition, frequently colonizes the hands of health care workers, and forms biofilm on prosthetic surfaces, central venous catheters and medical indwelling devices. Its recognition as a major cause of numerous diseases across the globe, particularly among neonates and its survival hospital environments, merits additional compressive research efforts concerning its epidemiology, genetics, and antimicrobial susceptibility.

The main goal of this work was to achieve a better understanding of the genesis of nosocomial infection by *Candida parapsilosis*. A special emphasis was directed to the following topics:

1. Assessment of the incidence and distribution of *C. parapsilosis* groups (including *C. metapsilosis* and *C. orthopsilosis*) in a large university hospital.
2. *C. parapsilosis* genotyping, in order to establish a relationship regarding the identity and similarity of different isolates (including clinical and environmental isolates).
3. Definition of the antifungal susceptibility patterns to azoles, amphotericin B and echinocandins by *C. parapsilosis*, *C. metapsilosis* and *C. orthopsilosis* in a large university hospital.
4. Assessment and characterization of the potential for *in vitro* induction of resistance by distinct azole antifungals among *C. parapsilosis* isolates.
5. Elucidation about the native and induced (*in vitro* and *in vivo*) antifungal drug resistance mechanisms developed by *C. parapsilosis* to azoles and echinocandins.
6. Assessment of the activity of several chemical germicides and physical agents against *C. parapsilosis* (clinical and environmental isolates).
7. Monitoring of the expression of virulence attributes by distinct *C. parapsilosis* strains (susceptible *versus* resistant).

MATERIAL AND METHODS

STRAINS

The study included clinical and environmental *Candida parapsilosis* strains. In some experiments strains from the American Type Culture Collection (ATCC) and other *Candida* species were included.

Until testing, all strains were stored in Brain-Heart broth (Difco Laboratories, Sparks, MD) with 5% glycerol at 70°C. For each experiment, each strain was subcultured twice on Sabouraud agar medium (Difco Laboratories) at 35°C for 48 h.

CLINICAL *C. PARAPSILOSIS* STRAINS

All the clinical *C. parapsilosis* strains used were obtained from patients admitted at different care units from Hospital S. João, Porto, Portugal, namely from oncologic, burn, transplant, infectious and pediatric departments, among others. The strains were isolated from different body sites, like blood, urine, respiratory tract, bile fluid, mucosal surfaces, spinal fluid, peritoneal fluid, among others and also from central venous catheters.

The clinical strains used also included strains deposited at the collection of the Microbiology Department, Faculty of Medicine, University of Porto that was constructed throughout the years.

The set of clinical strains shows considerable diversity since the isolates were obtained at different dates and from different patients and anatomic sites.

All isolates were biochemically identified as *C. parapsilosis* by Vitek 2 YBC identification cards (bioMérieux, Paris, France).

ENVIRONMENTAL *C. PARAPSILOSIS* STRAINS

The environmental group of strains included strains isolated from the air of care units of Hospital S. João and from food given to patients admitted at the same hospital.

Air samples were collected in different critical care units. Samples were obtained by air filtration with a MAS-100 Eco instrument (Merck Eurolab, Switzerland). The instrument was placed in the center of the room, one meter above the floor. Five hundred liters of air was filtered; the agar medium used was Dichloran Glycerol 18 (Difco Laboratories). The air particles were deposited at the agar surface of a 90 mm Petri plate. Afterwards, plates were incubated at 30°C during 48 h for assessment of fungal growth.

Food samples were obtained according to Portuguese guidelines for yeasts and moulds counting in food [187, 188]. A sample from a daily meal of patients was evaluated; fungi were recovered in the Cooke Rose Bengal agar medium (Difco Laboratories).

All the isolates were biochemically identified as *C. parapsilosis* by the use of Vitek 2 YBC identification cards.

CONTROL STRAINS

The Reference strains ATCC 22019, ATCC 96139, and ATCC 96144, from the American Type Culture Collection, were used as controls for *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis*, respectively.

The Reference strains *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* ATCC 14990 were used as positive and negative controls for coagulase expression.

In some experiments, specific *Candida* species had been used as controls (mentioned in the appropriate section). Two *C. albicans* strains (kind gift from Dr. Ted White, Seattle, WA, USA) with well characterized antifungal resistance mechanisms were used: *C. albicans* strain 95-68, with overexpression of *CDR1* and *CDR2* genes (ATP-dependent efflux pumps), and *C. albicans* strain 2-76, showing low levels of resistance genes expression. *C. albicans* wild type strain SC5314 and the chitinase deleted strain Δ chs3 with low chitin content (kind gift from Dr. Carol Munro, Aberdeen, United Kingdom), were used as controls for chitin content determination assays.

OTHER MICROBIAL STRAINS

Regarding the assessment of hydrogen peroxide dry-mist system efficacy (Susceptibility to Physical and Chemical Agents section) other bacteria and fungi were used. The Reference strains *S. aureus* ATCC 25279, *Acinetobacter baumannii* ATCC 25393, *Escherichia coli* ATCC 32218, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 2912, *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258, *Aspergillus fumigatus* ATCC 204305 and *Penicillium marneffei* ATCC 18224 and one clinical isolate (from Hospital S. João patients) of each of the mentioned species were used; in addition, three clinical isolates and ATCC 27294 type strain of *Mycobacterium tuberculosis* were also included in this study.

Additional yeasts strains were used in chitin studies (Virulence Attributes section), namely, *C. glabrata* (6 strains), *C. krusei* (4 strains), *C. tropicalis* (4 strains), *C. albicans* (3 strains) and *Cryptococcus neoformans* (1 strain). All these strains had been isolated from patients admitted at Hospital S. João and from different body sites.

MOLECULAR DISTINCTION BETWEEN *C. PARAPSILOSIS*, *C. ORTHOSILOSIS* AND *C. METAPSILOSIS*

In order to distinguish between *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis*, Tavanti et al. [84] proposed the analysis of the restriction polymorphism of the *SADH* gene, which encodes a secondary alcohol desidrogenase and is common to all three species. The restriction profile obtained is singular by species.

Type strains ATCC 22019, ATCC 96139, and ATCC 96144 were used as controls for *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis*, respectively.

DNA EXTRACTION

Yeast genomic DNA was extracted as described by Hoffman and Winston [189], with some adaptations. Briefly, yeast cells grown overnight in YEPD broth (1% yeast extract, 2% peptone, 2% glucose) at 30°C with agitation were harvested, washed with water and lysed by vortexing for 10 min with 0.3 g of glass beads (diameter 0.45 to 0.52 mm; Sigma, St. Louis, MO), 200 μ l of lysis buffer (100 mM Tris-HCl, pH 8.0, 2% Triton X-100, 1% sodium dodecyl sulfate, 1 mM EDTA), and 200 μ l of phenol-chloroform-isoamyl alcohol 25:24:1 (Sigma). After vortexing the mixture, 200 μ l of TE (1 mM EDTA, 10 mM Tris-HCl, pH 8.0) was added to the lysate; the mixture was microcentrifuged at 20,000 g for 10 min and the aqueous phase was transferred to a new vial. DNA was precipitated following the addition of 1 ml of ethanol and afterwards was treated with RNase (10 mg.ml⁻¹; Sigma) for 1 h at 37°C; the DNA was reprecipitated and resuspended in 100 μ l of TE.

The concentration, quality, and integrity levels of DNA samples were evaluated with a BioPhotometer (Eppendorf, Madrid, Spain).

SADH GENE AMPLIFICATION

Amplification of the *SADH* gene was performed by Polymerase Chain Reaction (PCR) with the primers *SADH*-F and *SADH*-R (Table 1, page number 74) as previously described by Tavanti et al. [84]. The amplification conditions were as follows: a first denaturation cycle of 2 min at 94°C, followed by 35 cycles at 94°C for 30 s (denaturation step), 50°C for 30 s (annealing step), and 72°C for 30 s (elongation step) and an extension final step of 10 min at 72°C. PCRs were performed in a Realplex Mastercycler instrument (Eppendorf).

SADH GENE RESTRICTION

The amplified *SADH* genes was digested with the *BanI* enzyme (New England Biolabs, Hitchin, United Kingdom) in a 40 µl volume containing 20 µl of the PCR product and 40 U of *BanI*. The digestion products were separated on a 2% agarose gel containing ethidium bromide (Sigma) 0.5 mg.ml⁻¹; the running buffer was TAE (40 mM Tris acetate [pH 8.0], 1 mM EDTA) and a 100-bp DNA ladder (Invitrogen, Barcelona, Spain) was used as a molecular size marker. DNA fragments were visualized in an UVITEC instrument (Cambridge, United Kingdom) and strains distinguished between *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis*.

Amplified fragments of the *C. orthopsilosis* strains were sequenced (as described in Antifungal Resistance Mechanisms section) to exclude the hypothesis that a point mutation exists in the restriction site of *C. parapsilosis*.

After this procedure *C. parapsilosis* strains were named *C. parapsilosis* sensu stricto. The following experiments were all based upon *C. parapsilosis* sensu stricto strains.

GENOTYPING OF *C. PARAPSILOSIS* STRAINS

RANDOM AMPLIFIED POLYMORPHIC DNA ANALYSIS

The technique performed allows concluding about genetic similarity between strains from the species *C. parapsilosis*. Genotyping was performed following random amplified polymorphic DNA (RAPD) analysis method, based upon randomly amplification of unknown sequences of genomic DNA using a specific primer. All *C. parapsilosis* isolates (clinical and environmental) were screened by RAPD test with the primer *RPO2* (Table 1, page number 74) [14]. Genomic DNA was extracted as described above (Molecular Distinction between *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* section). Reaction volumes of 50 µl contained 100 ng of genomic DNA, 1.5 U of *Taq* polymerase (Fermentas, Maryland, USA), 5 µl of 10x magnesium-free buffer, 3 µl of 25 mM MgCl₂, a 200 µM concentration of deoxynucleoside triphosphates, and a 10 µM concentration of the primer. The amplification conditions were as follows: a first denaturation cycle of 2 min at 94°C, followed by 35 cycles at 94°C for 1 min (denaturation step), 36°C for 1 min (annealing step), and 72°C for 75 s (elongation step) and an extension final step of 10 min at 72°C. PCRs were performed in a Realplex Mastercycler instrument. Amplified DNA products were separated by electrophoresis in a 2% agarose gel containing ethidium bromide (0.5 mg.ml⁻¹); the running buffer was TAE (1 mM EDTA, 40 mM Tris acetate, pH 8.0) and a 100-bp DNA ladder was used as a molecular size marker. DNA fragments were visualized with an UVITEC instrument.

MITOCHONDRIAL DNA RESTRICTION ENDONUCLEASE ANALYSIS

The mitochondrial DNA restriction endonuclease (mtDNA RE) analysis is another convenient and powerful tool that allows valid comparisons between isolates of the same species. This technology was applied to all *C. parapsilosis* isolates (clinical and environmental). For each sample, a reaction mixture was prepared containing 1x *HinfI* enzyme reaction buffer (Metabion, Planegg, Germany), 1 µg.µL⁻¹ RNase, 0.5 U.µL⁻¹ *HinfI* restriction enzyme (Metabion), approximately 25-30 µg of total DNA and DNase-RNase free water up to 20 µL final volume; reaction tubes were incubated overnight at 37°C. Restriction was ended upon *HinfI* inactivation by incubating for 20 min at 80°C. DNA fragments were separated by electrophoresis in a 1% agarose gel containing ethidium bromide (0.5 mg.ml⁻¹); the running buffer was TAE and a 1-kbp DNA ladder (Invitrogen) was used as a molecular size marker. DNA fragments were visualized with an UVITEC instrument.

ANTIFUNGAL SUSCEPTIBILITY TESTING

ANTIFUNGALS

Antifungal drugs used in this study were obtained from the respective manufacturer as standard and pure powders. Four azoles (fluconazole-FLC, voriconazole-VRC, itraconazole-ITC and posaconazole-PSC), three echinocandins (caspofungin-CSF, anidulafungin-ANF and micafungin-MCF) and amphotericin B (AMB) were used. Fluconazole, voriconazole and anidulafungin were obtained from Pfizer (Groton, CT, USA); itraconazole was obtained from Janssen-Cilag (Titusville, NJ, USA); posaconazole was obtained from Schering-Plough (Kenilworth, NJ, USA); caspofungin was obtained from Merck (Rahway, NJ, USA); micafungin was obtained from Astellas Pharma (Tokyo, Japan); and amphotericin B was obtained from Bristol-Myers Squibb (New York, NY, USA).

The solvent of fluconazole, caspofungin and micafungin was sterile distilled water; for voriconazole, itraconazole, posaconazole, anidulafungin and amphotericin B was dimethyl sulfoxide (DMSO; Merck). For all antifungals, successive dilutions to a final concentration of 6400 $\mu\text{g}\cdot\text{ml}^{-1}$ were prepared in Roswell Park Memorial Institute 1640 medium (RPMI 1640; Sigma) buffered to pH 7.0 with 0.165 M Morpholine Propanesulfonic Acid Buffer (MOPS; Sigma) and stored at -70°C until use.

Itraconazole was used only for susceptibility evaluation of isolates from *in vivo* cases (*In Vivo* Induction of Antifungal Resistance section). Micafungin, the new antifungal from the echinocandin class, was used for susceptibility evaluation of *in vitro* induced echinocandin nonsusceptible strain (*In Vitro* Induction of Antifungal Resistance section), as well as for strains used in *FKS1* gene sequencing (Antifungal Resistance Mechanisms section) and chitin assessment (Virulence Attributes section) studies.

SUSCEPTIBILITY TESTING

The minimal inhibitory concentration (MIC) of each antifungal drug was determined according to the M27-A3 protocol and the M27-S3 supplement of the Clinical and Laboratory Standards Institute [CLSI, [190, 191]]. The MIC values were registered after 24 h of incubation with echinocandins, and after 48 h with azoles and amphotericin B. MIC value was the lowest concentration at which a visual reduction of growth of approximately 50% was registered for azoles and echinocandins. Regarding amphotericin B, MIC value was the lowest concentration at which no discernible growth was observed. The susceptibility breakpoints were those

established by CLSI. For fluconazole, the MIC for susceptibility (S) was $\leq 8 \mu\text{g.ml}^{-1}$, the MIC for susceptible-dose dependent (S-DD) was 16 to $32 \mu\text{g.ml}^{-1}$, and the MIC for resistance (R) was $\geq 64 \mu\text{g.ml}^{-1}$; for voriconazole, the MIC for S was $\leq 1 \mu\text{g.ml}^{-1}$, the MIC for S-DD was $2 \mu\text{g.ml}^{-1}$, and the MIC for R was $\geq 4 \mu\text{g.ml}^{-1}$; for itraconazole, the MIC for S was $\leq 0.125 \mu\text{g.ml}^{-1}$, the MIC for S-DD was $0.25\text{--}0.5 \mu\text{g.ml}^{-1}$, and the MIC for R was $\geq 1 \mu\text{g.ml}^{-1}$. For posaconazole and amphotericin B, strains inhibited by $\leq 1 \mu\text{g.ml}^{-1}$ of each antifungal were considered to be S. For the echinocandins, susceptibility was considered for a MIC $\leq 2 \mu\text{g.ml}^{-1}$. Additionally, standard MIC₅₀ and MIC₉₀ values were calculated for echinocandins, which are the MIC values at which 50% and 90% of the isolates were inhibited, respectively.

The *C. parapsilosis* ATCC 22019 type strain was used for quality control of antifungal susceptibility testing, according to the same CLSI protocol.

PARADOXICAL EFFECT

During chitin assessment studies, the existence of a paradoxical growth phenomenon in the presence of high caspofungin levels was checked for *C. parapsilosis* strains. In this case, a range of concentrations ranging from 0.03 to $256 \mu\text{g.ml}^{-1}$ was tested (rather than 0.015 to $8 \mu\text{g.ml}^{-1}$) and MIC was determined using prominent inhibition, corresponding to 50% as an end point. The paradoxical effect was defined as a progressive increase in cell growth occurring at least two drug dilutions above the MIC after 48 h of incubation [172].

***IN VITRO* INDUCTION OF ANTIFUNGAL RESISTANCE**

Induction studies with two different antifungal classes were performed: azoles and echinocandins. All experiments started by incubating a randomly selected colony from a susceptible *C. parapsilosis* strain in 10 ml of RPMI 1640 overnight in a rotating drum at 150 rpm, 35°C. Next day, an aliquot of this culture, containing 10^6 cells, was transferred to different vials, each containing 10 ml of culture medium without or with an antifungal drug at specific concentration and reincubated as described.

Each day, during the assay, an aliquot (1 ml) from each subculture was mixed with 0.5 ml of 50% glycerol and frozen at -70°C for later testing.

INDUCTION OF AZOLE RESISTANCE

Azole resistance was induced with three different antifungals: fluconazole, voriconazole and posaconazole. Four blood culture isolates of *C. parapsilosis* (BC014, BC011, BC190 and BC237) and one *C. parapsilosis* ATCC strain were used in this study.

The susceptibility profile to fluconazole, voriconazole, posaconazole, amphotericin B and caspofungin was monitored during the assay.

Two different approaches were followed:

- i) Incubation for 30 days with constant concentrations of fluconazole ($16 \mu\text{g}.\text{ml}^{-1}$), voriconazole ($2 \mu\text{g}.\text{ml}^{-1}$) or posaconazole ($1 \mu\text{g}.\text{ml}^{-1}$); these concentrations are described as corresponding to therapeutic blood levels obtained during antifungal treatment [192]. Strain BC014 was used in microarray assays (Antifungal Resistance Mechanisms section); the strains obtained after incubation with fluconazole, voriconazole and posaconazole were named BC014R_{FLC}, BC014R_{VR}C and BC014R_{PSC} respectively and the initially susceptible strain was named BC014S.
- ii) Incubation with fluconazole, voriconazole and posaconazole twice the concentration of the MIC value, for 15 days, and afterwards four times the concentration until the 30th day.

INDUCTION OF ECHINOCANDINS RESISTANCE

One *C. parapsilosis* susceptible strain (blood culture isolate BC041) was incubated with anidulafungin at constant concentration of 2 $\mu\text{g.ml}^{-1}$, during 30 days; this concentration is described as corresponding to therapeutic blood level obtained during antifungal treatment [193]. The initial susceptible strain was named BC014S and the nonsusceptible final strain was named BC041NS. The susceptibility profile to fluconazole, amphotericin B, caspofungin, anidulafungin and micafungin was monitored during the assay.

ASSESSMENT OF THE STABILITY OF THE ANTIFUNGAL RESISTANCE PHENOTYPE

The stability of the resistance phenotypes obtained following *in vitro* induction assays was assessed by subculturing the resistant strains in 10 ml of RPMI 1640 medium overnight in a rotating drum at 150 rpm, 35°C, without any antifungal drug for a total of 30 days. Daily, an aliquot containing 10^6 cells of the previous day's culture was inoculated into 10 ml of fresh medium while another aliquot was taken and mixed with 0.5 ml of 50% glycerol and frozen at -70°C. After thawing, the MIC values for antifungals were re-determined for each of the frozen isolates.

POPULATION ANALYSIS

After *in vitro* induction assays and in order to determine whether the induced resistance in *C. parapsilosis* isolates was associated with selection of a resistant subpopulation, analysis of resistance within the cellular population was performed both with susceptible and induced resistant strains. Population analysis would allow assessing whether it existed or not a homogeneous population (containing only susceptible cells or a mixture between susceptible and resistant cells) both in the resistant induced strains and in the initial susceptible strain. This could exclude the selection by induction of an initial resistant clone that already existed in initial susceptible population.

This assay was performed as described previously by Marr et al. [194]. A single colony of the initially susceptible strain and one of each of the final resistant strains were cultured overnight in YEPD broth at 35°C; a suspension containing 10^3 cells was prepared in 1 ml of RPMI 1640 and 50 μl were plated in Sabouraud agar medium without and with fluconazole (1, 4, 16 and 64 mg.ml^{-1}), voriconazole (0.125, 0.5, 2 and 8 mg.ml^{-1}) and posaconazole (0.25, 1, 4 and 16 mg.ml^{-1}) for azole resistant strains and without and with anidulafungin (0.5, 1, 2 and 4 mg.ml^{-1})

for echinocandin resistant strain. Growth was quantified after 48 h of incubation at 35°C. The number of colonies growing in the presence of the drug at each concentration, relatively to the number growing in its absence was calculated and plotted.

***IN VIVO* INDUCTION OF ANTIFUNGAL RESISTANCE**

During the time study three *in vivo* episodes of *C. parapsilosis* bloodstream infection were studied. All the episodes involved patients admitted at different care units of Hospital S. João. Previous therapy with fluconazole and decreased azole susceptibility during the treatment were findings common to these cases.

From each patient, *C. parapsilosis* isolates were recovered from blood before, during and/or after the treatment with fluconazole. All isolates were initially identified as *C. parapsilosis* using Vitek 2 YBC identification cards. They were also identified molecularly as *C. parapsilosis* sensu stricto and not the related species *C. orthopsilosis* or *C. metapsilosis* by a DNA-based test (Molecular Distinction between *C. parapsilosis*, *C. metapsilosis* and *C. orthopsilosis* section). Molecular typing was performed for all isolates collected from the same patient (Genotyping of *C. parapsilosis* Strains section). MIC values of different azoles were determined for all isolates (Antifungal Susceptibility Testing section); stability of the *in vivo* acquired susceptibility phenotype was assessed as described above (*In Vitro* Induction of Antifungal Resistance section) and the resistant mechanism developed was explored (Antifungal Resistance Mechanisms section). Moreover, microarray technique was applied in order to compare gene expression profile of the initial and the final strains from patient A.

CASE REPORT A

A 26-year-old male patient was admitted in October 2007 with a diagnosis of congestive heart failure. In addition to chronic kidney failure, under hemodialysis, he had Crohn's disease. Neutrophil count and glucose blood level were normal; leukocyte count, hemoglobin, albumin and protein blood levels were below normal. A thoracic surgical procedure was performed to the patient. Renal support was provided. After surgical procedure, bacteria were isolated from blood. He was started a cephalosporin and aminoglycoside, ending the infection. In January 2008, the patient developed fever and antifungal prophylaxis was initiated with liposomal amphotericin B, 80 mg intravenous/day. The first blood culture positive for *C. parapsilosis* (isolate BC_{A1}) was obtained three days later after initiation of the antifungal therapy. Following four days, amphotericin B was replaced by oral therapy with fluconazole 400 mg/day. Two other *C. parapsilosis* isolates were recovered from blood, after fifteen days (isolate BC_{A2}) and seventeen days (isolate BC_{A3}) away following the first positive blood culture. *C. parapsilosis* was

not detected in cultures of other biological products. He died in April 2008; death was associated with candidaemia.

CASE REPORT B

A 68-year-old male patient was admitted in January 2008 with a diagnosis of duodenal stenosis, being submitted to a gastrojejunostomy. Neutrophil count and glucose blood level were normal; leukocyte count, hemoglobin, albumin and protein blood levels were below normal. During hospital admission, he developed a nosocomial pneumonia and bacteremia and received treatment with several antibiotics, like vancomycin, ciprofloxacin, linezolid, amikacin, imipinem and amoxicillin + clavulanic acid. Since admission the patient was placed a central venous catheter. The first isolation of *C. parapsilosis* (isolate BC_{B1}) was in April; he was started six days later intravenous fluconazole (400 mg/day), during eight days. The second (BC_{B2}) and the third (BC_{B3}) *C. parapsilosis* isolates were obtained after three days and on the last day of fluconazole therapy respectively. Then the therapy was changed to intravenous liposomal amphotericin B (80 mg/day). *C. parapsilosis* was also found in the central venous catheter. He died in June; death was considered to be candidaemia-related.

CASE REPORT C

A 70-year-old male patient was admitted in January 2008 with a diagnosis of colon cancer, anemia and pneumonia. Glucose blood level was normal; leukocyte count, hemoglobin, albumin and protein blood levels were below the normal. He was submitted to a colostomy and ileostomy. Since admission the patient was placed a central venous catheter. Bacteria were recovered from the catheter, urine, blood, respiratory tract and cutaneous exsudate; therapy with azithromycin, ciprofloxacin, vancomycin, imipinem, ceftriaxone, tazobactam and piperacillin was administered. *C. parapsilosis* (isolate BC_{C1}) was recovered for the first time in July; he was immediately started with fluconazole 400 mg/day for twelve days. Three, five and nine days after from the first isolation of *C. parapsilosis* other three isolates were obtained (isolates BC_{C2}, BC_{C3} and BC_{C4}). *C. parapsilosis* were also recovered from others specimens, like urine, bile fluid and cutaneous exsudate. In urine and cutaneous exsudate, *C. albicans* was concomitant with *C. parapsilosis*. Death occurred within 10 days and was associated with candidaemia.

ANTIFUNGAL RESISTANCE MECHANISMS

Different experimental approaches were followed in order to elucidate about resistance mechanisms expressed by *C. parapsilosis* to different antifungals. Phenotypic and genotypic studies were undertaken with resistant strains obtained from *in vitro* induction studies, from the *in vivo* cases and with other available resistant clinical strains.

EVALUATION OF EFFLUX ACTIVITY

Two different approaches were considered for evaluation of the efflux activity of resistant *C. parapsilosis* strains. Strains obtained after induction of susceptible strains (BC014, BC011, BC190 and BC237; *In Vitro* Induction of Antifungal Resistance section) with different azoles and isolate BC_{A3} from the *in vivo* case (*In Vivo* Induction of Antifungal Resistance section) were studied.

EFFLUX PUMPS BLOCKERS

Several compounds were described as being able to block efflux pumps from the yeast cell membrane. The blocker prevents the antifungal to be expelled, allowing its inhibitory effect. There is a synergic effect between the antifungal and the blocker. To highlight this effect, susceptibility testing in the presence of the efflux blocker was evaluated. Two methodologies were undertaken:

- i) Verapamil (Sigma) 100 μ M and ibuprofen (Sigma) 100 mg.l⁻¹ were used as efflux pumps blockers of ATP-binding cassette efflux pumps according to Pina-Vaz *et al.* [195]. Stock solutions were prepared in sterile distilled water and then added to yeast cell suspensions. Susceptibility to azoles (fluconazole, voriconazole and posaconazole) was evaluated in the presence of these two blockers, at constant concentrations, according to the M27-A3 protocol from CLSI for antifungal susceptibility testing of yeasts [190, 191]. *C. albicans* strain 95-68, with overexpression of *CDR1* and *CDR2* genes (ATP-dependent efflux pumps), and *C. albicans* strain 2-76, showing low level of resistance gene expression, were used as controls.

- ii) Agar disk diffusion assay was performed with tacrolimus (FK506), another efflux blocker, according to Marchetti *et al.* [196]. Strains were incubated overnight in YEPD broth at 37°C, 150 rpm. The inoculum of 10^6 cells.ml⁻¹ was prepared in sterile distilled water. The yeast suspensions were spread onto YEPD agar medium containing fluconazole at a supra-MIC value (128 µg.ml⁻¹), and without fluconazole. Blank paper disks, 6mm (Becton Dickinson, NJ, USA), were impregnated with an FK506 solution in serial concentrations (10-fold dilutions ranging from 0.1 to 100 mg.ml⁻¹) and with DMSO and allowed to dry for 2 h. For each tested strain, the disks were applied in the inoculated agar plates (after previous assessment of FK506 nontoxicity upon yeast cells). A plate containing only fluconazole (no FK506 disk) was used as a control for cell viability in the presence of fluconazole supra-MIC values. The agar plates were incubated at 37°C, and the results were evaluated after 24 h and 48 h. *C. albicans* strain 95-68, with overexpression of *CDR1* and *CDR2* genes (ATP-dependent efflux pumps) was used as control.

EFFLUX PUMPS FLUORESCENCE SUBSTRATE

Flow cytometric analysis using Rhodamine 6G (Rh-6G; Sigma), a specific substrate of efflux pumps, was performed as described previously [197, 198] to compare initial susceptible strain with induced resistant final strains.

Briefly, yeast cells were grown to logarithmic phase in 5 ml of YEPD broth at 35°C, 150 rpm. Labeling of cells was conducted in 1 ml of YEPD broth, containing 10^7 cells and 10 mM Rh-6G for 30 min at 35°C, after which the reaction was stopped by placing the tubes on ice. The mixture was then diluted 40-fold with cold sterile 0.1 M phosphate-buffered saline (PBS; Sigma) at pH 7.0 and analysed in a FACSCalibur flow-cytometer equipped with a 15 mW 488 nm argon laser. From each yeast suspension, 30.000-50.000 cells were analysed; cell fluorescence was determined at FL1 fluorescence channel (515 nm). All experiments were performed in duplicate.

The final strains were also assayed with and without verapamil. Before labeling the yeast cells, they were incubated with verapamil (100 µM) for 90 min [199].

C. albicans strain 95-68, with overexpression of *CDR1* and *CDR2* genes, was used as control and tested for efflux pump activity in the presence of verapamil.

GENE SEQUENCING

Genes involved in antifungal resistance were amplified and then sequenced for assessment of point mutations.

GENE AMPLIFICATION

ERG11 GENE

Genomic DNA from one azole susceptible *C. parapsilosis* strain (BC_{A1}) and one azole resistant *C. parapsilosis* strain (BC_{A3}) from an *in vivo* induction case (*In Vivo* Induction of Antifungal Resistance section) was extracted as described previously (Molecular Distinction between *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* section). Afterwards, DNA sequence analysis of the *ERG11* gene was performed. The primers used were *ERG11*-F1 and *ERG11*-R1 (Table 1, page number 74). A 1470 bp fragment was amplified. The amplification conditions were as follows: a first denaturation cycle of 2 min at 98°C, followed by 40 cycles at 98°C for 10 s (denaturation step), 64°C for 30 s (annealing step), and 72°C for 30 s (elongation step) and an extension final step of 7 min at 72°C. PCRs were performed on the Realplex Mastercycler instrument.

TRANSCRIPTION FACTOR GENES

The transcription factors *MRR1*, *UPC2* and *NDT80* from the susceptible strain (BC014S) and resistant strains (*MRR1* for BC014R_{FLC} and BC014R_{VRC}, *UPC2* and *NDT80* for BC014R_{PSC}) from the *in vitro* induction assay were amplified. Genomic DNA was extracted as described previously (Molecular Distinction between *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* section). The primers used were *MRR1*-F1 and *MRR1*-R1 for *MRR1* gene, *NDT80*-F1 and *NDT80*-R1 for *NDT80* gene, and *UPC2*-F1 and *UPC2*-R1 for *UPC2* gene (Table 1, page number 74). The amplification conditions were as follows: a first denaturation cycle of 2 min at 94°C, followed by 30 cycles at 94°C for 30 s (denaturation step), 60°C for *MRR1*, 58°C for *UPC2* or 62°C for *NDT80* for 30 s (elongation step), and 72°C for 50 s (elongation step) and an extension final step of 7 min at 72°C. PCRs were performed on the Realplex Mastercycler instrument.

HOT SPOT 1 REGION OF *FKS1* GENE

C. parapsilosis strains [six echinocandin nonsusceptible clinical strains (BC011, BC017, BC022, BC043, BC101 and BC132), and the initial echinocandin susceptible strain (BC041S) and the final echinocandin nonsusceptible strain (BC041NS) from the *in vitro* induction assay (*In Vitro* Induction of Antifungal Resistance section)] were studied. Genomic DNA was extracted as described previously (Molecular Distinction between *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* section) and DNA sequence analysis of the hot spot 1 (HS1) region of *FKS1* gene was performed. The primers used were *FKS1HS1-F* and *FKS1HS1-R* (Table 1, page number 74). A 796 bp portion of *FKS1* was amplified. The amplification conditions were as follows: a first denaturation cycle of 2 min at 96°C, followed by 40 cycles at 96°C for 30 s (denaturation step), 69,6°C for 30 s (annealing step), and 72°C for 30 s (elongation step) and an extension final step of 10 min at 72°C. PCRs were performed on the Realplex Mastercycler instrument.

SEQUENCING OF AMPLIFIED PRODUCTS

PCR products amplified were treated with ExoSAP-IT (USB Corporation, OH, USA) and used as template for the sequencing reactions, performed with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington, United Kingdom). The DNA products were purified with Sephadex G-50 Fine (GE Healthcare Europe GmbH, Freiburg, Germany) and sequenced in an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). The results were analyzed with the Sequencing Analysis 5.2 software from Applied Biosystems.

ALIGN SEQUENCES

The coding sequence of the susceptible strain (BC_{A1}) *ERG11* gene was aligned with that obtained for the resistant strain (BC_{A3}).

The coding sequences of the susceptible strain (BC014S) *MRR1*, *UPC2* and *NDT80* genes were aligned with those obtained for the resistant strains (BC014R_{FLC}, BC014R_{VRC} or BC014R_{PSC}).

The coding sequences of the clinical strains (BC011, BC017, BC022, BC043, BC101 and BC132) HS1 region of *FKS1* gene were aligned with the *C. parapsilosis* sequence obtained

from Broad Institute (<http://www.broadinstitute.org/>). Regarding the induced nonsusceptible strain (BC041NS), the sequence was aligned with that obtained from the susceptible initial strain (BC041S).

The software used was the MUSCLE software [200]. Alignments were analyzed in BioEdit (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>).

MICROARRAY ASSAYS (1)

In order to compare gene expression profile between different *C. parapsilosis* strains (susceptible *versus* resistant), microarray technology was applied. The microarray platform provides the global constitutive changes in gene expression, which can shed some light on genes that might explain resistant profiles. All laboratory and data analysis work was performed in School of Biomolecular and Biomedical Science, Conway Institute, University College Dublin, Dublin, Ireland, in collaboration with Professor Geraldine Butler.

This technology was applied on susceptible strain (BC014S) and resistant strains (BC014R_{FLC}, BC014R_{VR}C and BC014R_{PS}C) obtained from the *in vitro* induction assay (*In Vitro* Induction of Antifungal Resistance section). It was also performed with BC_{A1} and BC_{A3} strains from the *in vivo* induction case (*In Vivo* Induction of Antifungal Resistance section).

RNA EXTRACTION

A 50 ml *C. parapsilosis* suspension with an OD_{600nm} of 0.2 was obtained after dilution of an overnight culture in YEPD broth at 35°C, 150 rpm. The suspension was incubated in the same conditions until an OD_{600nm} of approximately 1. After centrifugation, the pellet was used for RNA extraction using a RiboPure Yeast Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions.

The concentration, quality, and integrity levels of RNA samples were determined with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer's instructions. Only samples yielding 28S/18S rRNA ratios between 1.6 and 2.2 and showing absence of degradation were used in subsequent analysis.

MICROARRAY PLATFORM

All the experiments were carried out using *C. parapsilosis* microarrays manufactured by Agilent Technologies. The microarray platform is described on the NCBI Gene Expression

Omnibus Database (GEO) with the ID GPL13192. The oligonucleotides were designed with eArray from Agilent Technologies using an in-house annotation of 5,834 putative open reading frame. Each gene is represented by two sets of probes, both spotted in duplicate. Probes are randomly distributed. Four copies of each array were printed on a single slide (4x44k), and hybridized individually [201].

PROBE PREPARATION AND MICROARRAY HYBRIDIZATION

Twenty-four micrograms of total RNA were first incubated with 200 pmol of anchored oligonucleotide deoxyribosylthymine (Invitrogen, Carlsbad, California, USA) for 10 min at 70°C. First-strand buffer (1x; Invitrogen); 1.5 mM dATP, dTTP, and dGTP; 25 µM dCTP; 10 mM dithiothreitol; 2 µl Superscript II reverse transcriptase (Invitrogen); and 37.5 µM Cy3-dCTP or 37.5 µM Cy5-dCTP (GE Healthcare Europe GmbH) were mixed in a final volume of 40 µl. The mixture was incubated at 42°C for 2 h. An additional 1 µl of Superscript II was added to the mixture, and incubation continued for 1 h at 42°C. Residual RNA was degraded with 1 µl of RNase A (QIAGEN, Hilden, Germany) at 50 µg.ml⁻¹ and 1 µl of RNase H (Invitrogen) at 0.05 unit.µl⁻¹, for 30 min at 37°C. The labeled cDNAs were purified using a QIAquick PCR purification kit (QIAGEN), according to the manufacturer's instructions; then, labeled cDNAs were pooled in 45 µl of hybridization buffer (supplied with microarray). This mixture was denatured at 95°C for 2 min, immediately cooled with ice, applied to the DNA microarray and covered with a 24-mm X 60-mm coverslip. Slides were placed in a hybridization chamber (Corning Incorporated, Palo Alto, CA, USA) and incubated at 42°C for approximately 16 h. Slides were washed twice with oligo aCGH wash buffer 1, oligo aCGH wash buffer 2 and with stabilization and drying solution from Agilent. The microarray analysis of the resistant induced strains was performed in distinct experiments; comparisons were made with the initial susceptible strain. In each experiment, four biological replicates (including two dye swaps) were prepared.

DATA ANALYSIS

The microarrays were scanned with a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA, USA) at 10 µm resolution. Data were acquired with the GenePix Pro 5.0 software (Molecular Devices), and low-quality spots were automatically flagged. In addition, saturated spots or spots without background corrected intensities greater than 20 in the Cy3 or the Cy5 channel were flagged. In each experiment, four biological replicates were

performed. In order to estimate and adjust any probe-specific dye effect, two arrays were dye-swapped in each experiment. Data was analyzed using the LIMMA package [202] from the Bioconductor Project (<http://bioconductor.org>). Three datasets were preprocessed using Loess normalization and no background correction, as suggested by Zahurak et al. [203]. Duplicate probes within each array were considered as technical replicates. This assumption allows taking full advantage of the platform design, by analyzing the within-array replicate spots using a pooled correlation method. Quality controls were performed in order to assess the good quality of the arrays and to verify any presence of chromosomal-bias expression [204].

GENE ONTOLOGY ANALYSIS

A total of 5,091 *C. parapsilosis* orthologs of *C. albicans* genes (87.3% of *C. albicans* genome) were extracted from the Candida Gene Order Browser [205]. Gene ontology enrichment analysis was performed using the web tool GoTermFinder, available on the website of the Candida Genome Database (<http://www.candidagenome.org/cgibin/GO/goTermFinder>).

CLUSTERING ANALYSIS

The datasets were preprocessed using the LIMMA package from the Bioconductor Project. Loess normalization was used to normalize the ratio of the intensities between the two channels for each array separately (within-array normalization), followed by quantile normalization to normalize the ratio of the intensities to be compared across arrays. Hierarchical clustering was performed on the normalized datasets using euclidean distance and complete linkage [206]. Clustering approaches to identify gene expression patterns from DNA microarray data were performed [206].

MICROARRAY DATA ACCESSION NUMBER

Raw microarray data for BC014S strain and BC014R_{FLC}, BC014R_{VRC} and BC014R_{PSC} strains and the description of the array have been deposited in the Gene Expression Omnibus Database, under the accession number GSE27409 (superseries). The link is <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE27409>. The same did not happen for BC_{A1} and BC_{A3} strains due to its low quality.

GENE EXPRESSION BY REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION

In order to quantify the expression level of certain genes involved in azole resistance mechanisms, real-time quantitative polymerase chain reaction (RT-qPCR) was performed using the susceptible strain (BC014S) and resistant induced strains (BC014R_{FLC}, BC014R_{VR}, BC014R_{PSC}) from *in vitro* studies and the susceptible strain (BC_{A1}) and the resistant strain (BC_{A3}) from the *in vivo* induction case. Moreover, this technique was applied after microarrays data analysis in order to confirm its findings.

cDNA SYNTHESIS

First-strand cDNA was synthesized from 100 ng of total RNA in a 20 µl reaction volume using a SuperScript III Reverse Transcriptase kit (Invitrogen) according to the manufacturer's instructions. RNA was extracted as described previously.

GENES

For BC014R_{FLC} strain, expression level of *ERG11*, *GRP2*, *ALD5*, *MDR1*, *MRR1* and *RPL82* genes were determined. For BC014R_{VR} strain, expression level of *GRP2*, *ALD5*, *ERG11*, *MDR1* and *MRR1* genes were determined. For BC014R_{VR} strain, expression level of *ERG11*, *RPL82*, *ERG6*, *UPC2*, *SOD4* and *NDT80* genes were determined. For BC_{A3} strain, expression level of *ERG11*, *ERG6*, *NDT80* and *UPC2* genes were determined.

The oligonucleotides used were designed using Oligo Explorer program (<http://www.genelink.com/>) and are listed in Table 1 (page number 74).

POLIMERASE CHAIN REACTION

For each RT-qPCR five replicates for each strain were integrated. The amplification conditions were as follows: a first denaturation cycle of 1 min at 95°C, followed by 35 cycles at 95°C for 15 s (denaturation step), 60°C for 30 s (annealing step), and 60°C for 30 s, and an extension final step of 5 min at 60°C. PCRs were performed using PerfeCTa SYBR Green Fast Mix (Quanta Biosciences, Gaithersburg, MD, USA) on the Realplex Mastercycler instrument. The signal obtained for each gene was normalized with the *TUB4* signal.

SUSCEPTIBILITY TO PHYSICAL AND CHEMICAL AGENTS

Eight *C. parapsilosis* clinical strains and eight environmental strains were exposed to physical and chemical agents. Regarding chemical agents, either pure compounds or commercial solutions commonly used as disinfectants were tested. Moreover, a new product, a hydrogen peroxide dry-mist system for hospital disinfection, was tested against *C. parapsilosis* strains and other microorganisms.

SUSCEPTIBILITY TO PHYSICAL AGENTS

C. parapsilosis strains were exposed to different physical agents in order to determine the inhibitory effect in cell growth. Exposition occurred at different times to obtain a kinetic of the effect. At the end of the treatment with the physical agent, 50 µl of serial dilutions (10^{-1} till 10^{-6}) of the different suspensions were plated on Sabouraud agar medium. Plates were incubated for 76 h at 37°C and CFUs counted afterwards.

HEATING AT 60°C

Glass vials containing 5 ml of cell suspensions in 0.1 M PBS adjusted to 10^6 cells.ml⁻¹ were incubated in a hot bath at 60°C for 0, 5, 15, 30 and 45 min. To ensure the metabolic stability of cells after the treatment, vials were immersed in ice for 30 min.

MICROWAVE IRRADIATION

Microwave irradiation was performed by exposing 60 ml of cell suspensions in PBS adjusted to 10^6 cells.ml⁻¹ in glass vials for 0, 20, 40, 60 and 90 seconds to 2540 MHz, at maximum power (800 W) in a microwave (Crown, Japan). After treatment, vials were immersed in ice for 30 min.

ULTRAVIOLET IRRADIATION

Glass vials containing 1 ml of cell suspensions in 0.1 M PBS adjusted to 10^6 cells.ml⁻¹ were exposed to ultraviolet irradiation for 1, 2, 3, 5 and 10 min at 8 mW.cm⁻², 312 nm in the UVITEC instrument.

SUSCEPTIBILITY TO CHEMICAL AGENTS

CHEMICAL AGENTS

PURE COMPOUNDS

Chemicals were tested following the CLSI protocol for antifungal susceptibility testing [190, 191]. Chlorhexidine (Sigma; 0.004-2%), ethanol (Merck; 0.08-40%), sodium hypochlorite (Merck; 0.04-20%), hydrogen peroxide (Merck; 0.002-1%), potassium permanganate (Sigma; 0.0008-0.4%) and acid boric (Sigma; 0.002-1%) were studied. Serial dilutions of the initial solutions were prepared with RPMI 1640 medium.

COMMERCIAL SOLUTIONS

The commercial solutions of chemical agents as they are used in clinic practice were tested by time-kill assay. The solutions were: Handsrub (chlorhexidine 0.4%; Paracelsia, Porto, Portugal), Aniosgel 85 NPC (ethanol 70%; ANIOS, Hellemmes-Lille, France), Solution Dakin (0.5% sodium hypochlorite; Proclinica, Odivelas, Portugal), Hydrogen Peroxide (3%; Labesfal, Amadora, Portugal), Potassium Permanganate (0.01%; Uniphar, Dublin, Ireland) and Acid Boric (3%; Fecofar, Lisboa, Portugal).

MIC AND MLC VALUES DETERMINATION

The MIC and the minimal lethal concentration (MLC) values of chemical agents (pure compounds) were determined according to the M27-A3 protocol and the M27-S3 supplement from CLSI for antifungal susceptibility testing of yeasts [190, 191]. The value was established by visual reading at 24 and 48 h. MIC value was the lowest concentration at which a visual reduction in growth of approximately 50% was registered. After reading MIC value, 20 µl of each suspension were plated on Sabouraud agar medium and incubated at 30°C for 48 h. MLC value was the lowest concentration at which a growth of 10 or less colonies was registered.

TIME-KILL ASSAY

One ml of a 10^6 yeast cell suspension of each *C. parapsilosis* strain was exposed to each commercial solution for 1, 5, 10, 15 and 30 min at room temperature. Afterwards, the chemical was removed, the cells resuspended in sterile distilled water, serially diluted and 50 μ l of each dilution plated on YEPD agar medium. Plates were incubated at 30°C for 5 days and growth assessed by CFUs counting. Growth inhibition kinetic was determined comparing the test plates with control plate (not exposed to chemicals).

EVIDENCE OF CELL MEMBRANE LESION BY FLOW CYTOMETRY

The goal of this task was to evaluate the lesion of the membrane caused by each agent. Cytometric evaluations after staining with the fluorescence probe propidium iodide (PI) were performed. As shown previously, PI is a fluorescence probe that only stains nucleic acids of cells with serious cytoplasmic membrane damage, i.e., dead cells [207].

A final concentration of 10^6 cells.ml⁻¹ of one *C. parapsilosis* strain was incubated with RPMI 1640 medium containing the chemical agent or was exposed to the physical agent. After the incubation time, cells were washed and resuspended in 0.1 M PBS with PI 1 μ g.ml⁻¹ for 30 min at room temperature. Yeast suspensions were analyzed in a FACSCalibur flow-cytometer equipped with a 15 mW 488 nm argon laser. From each suspension a 30.000-50.000 cells were analysed; cell fluorescence was determined at FL3 fluorescence channel (650 nm). All experiments were performed in triplicate.

CHEMICAL AGENTS

Cell suspensions were exposed to commercial solutions for 1, 5, 10, 15 and 30 min at room temperature. After incubation, cells were washed and stained with PI.

PHYSICAL AGENTS

Cell suspensions were exposure to heating at 60°C for 45 min, microwave irradiation for 90 s and ultraviolet irradiation for 10 min. Afterwards yeast cells were washed and staining with PI.

HYDROGEN PEROXIDE DRY-MIST SYSTEM

Glosair™ 400® system (ASP, Irvine, CA, USA) is a programmable dry-mist generator which combines with the disinfectant solution Glosair™ 400® Cartridge, diffusing hydrogen peroxide and silver cations as small size particles (approximately 10 µm in diameter). These droplets circulate freely in the air as a dry aerosol. The Glosair™ 400® Cartridge is composed by hydrogen peroxide 5%, phosphoric acid <50 ppm, silver cations <50 ppm, gum arabica <1 ppm and bi-osmotic water 95%. Diffusion times are calculated according to the room size in order to reach the concentrations mentioned.

IN VITRO ANTIMICROBIAL ACTIVITY

Type strain *C. parapsilosis* ATCC 22019 and one clinical isolate (BC014) were included in this study. Yeast suspensions containing approximately 10^6 cells.ml⁻¹ were prepared in 0.1 M PBS. Serial dilutions from 10^6 cells.ml⁻¹ to 10^1 cells.ml⁻¹ were prepared and 100 µl from each suspension spread on BH agar medium. The plates were then left open in a laboratory room in different positions and heights, and exposed to one single cycle of dry-mist decontamination. Afterwards, the plates were removed and incubated at 25°C for 5 days. CFUs were quantified and compared to control plates (not exposed to decontamination but incubated in similar conditions). The reduction in growth was calculated. All experiments were performed in duplicate.

The Reference strains *S. aureus* ATCC 25279, *Acinetobacter baumannii* ATCC 25393, *Escherichia coli* ATCC 32218, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 2912, *C. ATCC 90028*, *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258, *Aspergillus fumigatus* ATCC 204305 and *Penicillium marneffeii* ATCC 18224 and one clinical isolate of each of the mentioned species were also included in this study (manuscript VII in Publications section).

DETERMINATION OF MIC VALUES

The determination of MIC value of the disinfectant solution was performed according to the M27-A3 protocol and the M27-S3 supplement from CLSI for antifungal susceptibility testing of yeasts [190, 191] regarding *C. parapsilosis* ATCC 22019 type strain. The MIC values were established by visual reading at 48 h of incubation; MIC value was the lowest concentration at which a visual drastical reduction in growth ($\geq 90\%$) was registered. The

disinfectant solution was successively diluted (1:2) in RPMI 1640 medium; ten concentrations were tested, ranging from 5% to 0.01% of hydrogen peroxide and <50 ppm to <0.1 ppm silver cations.

MIC values regarding the ATCC type strains of the other species mentioned above were also calculated according to the respective protocol for susceptibility testing. Activity against three clinical isolates and ATCC 27294 type strain of *M. tuberculosis* was evaluated according to the standard procedures of the BD BACTEC MGIT 960 (Becton Dickinson, Franklin Lakes, NJ, USA) using Mycobacteria Growth Indicator Tubes (MGIT) (manuscript VII in Publications section).

EVIDENCE OF CELL MEMBRANE LESION BY FLOW CYTOMETRY

A final concentration of 10^6 cells.ml⁻¹ of *C. parapsilosis* was prepared in 1 ml of disinfectant solution. After 5 min of incubation at room temperature, cells were washed and re-suspended in 0.1 M PBS with propidium iodide (PI) at final concentration of 1 µg.ml⁻¹. Yeast suspensions were analyzed in a FACSCalibur flow-cytometer equipped with a 15 mW 488 nm argon laser. From each suspension a 30.000-50.000 cells were analysed; cell fluorescence was determined at FL3 fluorescence channel (650 nm). All experiments were performed in triplicate. Intact and damaged cells were photographed under an optical microscope (Axioskop 40, Zeiss) with camera system AxionCam MRc5 Zeiss (AxionVision Release 4.6.3. SP1 program).

VIRULENCE ATTRIBUTES

In order to study the correlation between drug resistance and virulence attributes, we compared susceptible and resistant *C. parapsilosis* strains. The first nine topics involved the azole susceptible (BC014S) *versus* resistant strain (BC014R_{pSC}); the last two were performed with echinocandin susceptible strains (BC041S strain and BC002, BC021, BC034 clinical isolates) *versus* nonsusceptible strains (BC041NS strain and BC011, BC017, BC022 clinical isolates). BC014R_{pSC} and BC014NS were obtained from *in vitro* induction studies (*In Vitro* Induction of Antifungal Resistance section).

GROWTH KINETIC

For the determination of growth curves, *C. parapsilosis* cell suspensions (from a smooth colony) in 100 ml YEPD broth with an initial OD_{600nm} of 0.1 were incubated at 37°C under agitation for 48 h; growth was assessed by measuring optical density along time, up to 48 h.

ANALYSIS OF PHENOTYPIC SWITCHING

C. parapsilosis cell suspensions obtained after overnight growth, of cells from a smooth colony, at 30°C, 150 rpm, in YEPD broth were prepared in the same medium, to a final concentration of 10³ cells.ml⁻¹. Approximately 50 µl were plated onto YEPD agar medium (ten plates per strain) and incubated at 30°C for 96 h; the presence of distinct colony phenotypes was checked visually. Colonies were photographed under a stereoscopic binocular microscope (520-LAB1, Quirumed, Valencia, Spain) with AxionCam MRc5 Zeiss (AxionVision Release 4.6.3. SP1 program) camera system. Cells from each colony were resuspended in sterile distilled water and photographed under an optical microscope (AxiosKop 40, Zeiss, Barcelona, Spain) with AxionCam MRc5 Zeiss camera system.

To assess the switching frequencies of colonies, cells were removed from individual colonies, suspended in water, counted and plated at approximately 100 cells (CFUs) per plate (ten plates of YEPD agar medium). These plates were incubated for 72 h at 30°C and the different colonies morphologies were counted and classified.

AGAR INVASION

C. parapsilosis suspensions obtained after overnight growth, of cells from a smooth colony, at 30°C, 150 rpm, in YEPD broth were serially diluted to 10^3 cells.ml⁻¹. Approximately 50 µl were plated onto YEPD agar medium and incubated at 30°C for 96 h. Different colonies were photographed under a stereoscopic binocular microscope with AxionCam MRc5 Zeiss camera system. Those on the surface were removed by washing under running water. The cells remaining under the agar were then photographed.

PSEUDOHYPHAL DEVELOPMENT

C. parapsilosis cells (from a smooth colony) were grown in RPMI 1640 medium with 10% fetal calf serum (Invitrogen) for 24 h at 37°C; pseudohyphal development was checked and photographed under an optical microscope with AxionCam MRc5 Zeiss camera system.

ADHERENCE ASSAY

Adherence ability of *C. parapsilosis* cells (from a smooth colony) was evaluated using two different methods: the classic method and a new method developed by Silva-Dias et al. [208] based upon a flow cytometric assay.

CLASSIC METHOD

C. parapsilosis cells were grown at 30°C to stationary phase in Sabouraud broth, washed three times with ice-cold deionized water and adjusted to 2×10^6 cells.ml⁻¹ in sterile 0.1 M PBS). A stock solution of blue, unmodified polystyrene microspheres was prepared by mixing 6 µl of a 10% solids suspension (Molecular Probes, Invitrogen) in 2 ml of 0.1 M PBS, resulting in a concentration of 9×10^8 microspheres.ml⁻¹. Equal volumes (100 µl) of yeast cells and microsphere suspensions were placed in acid-washed glass test tubes and equilibrated at room temperature (final concentrations, 10^6 cells.ml⁻¹ and 4.5×10^8 microspheres.ml⁻¹). The mixture was vigorously vortexed for 30 s and the percentage of yeast cells with three or more attached microspheres was assessed by microscopy (300 cells per sample). The assay was performed in triplicate.

FLOW CYTOMETRIC ASSAY

A mixture of green fluorescent polystyrene beads (1×10^5 beads. ml^{-1}) (Molecular Probes, Invitrogen) and *C. parapsilosis* cells (10^6 cells. ml^{-1}) in 0.1 M PBS was analyzed in a FACSCalibur flow-cytometer equipped with a 15 mW 488 nm argon laser. From each yeast suspension, 30.000-50.000 cells were analysed; cell fluorescence was determined at FL3 fluorescence channel (650 nm). All experiments were performed in triplicate. The numerical results were expressed as a percentage of cells associated with microspheres population.

BIOFILM FORMATION

Biofilm formation was assessed in 12-well tissue culture plates by incubation of 1 ml of a 10^6 cells. ml^{-1} *C. parapsilosis* cells suspension (from a smooth colony) in RPMI for 24 h and 48 h at 37°C. After biofilm formation, the medium was aspirated, and nonadherent cells were removed by thoroughly washing the biofilms three times with sterile 0.1 M PBS. The biofilm formation capacity was evaluated by: i) a 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl amino) carbonyl]- 2H-tetrazolium hydroxide assay (XTT; Sigma) which measures the metabolic activity [209] and ii) a crystal violet assay (CV) which measures the biomass formation [210]. XTT was dissolved in sterile 0.1 M PBS at 0.5 g. l^{-1} . The solution was sterilized by filtration through a 0.22 μm pore size filter. Prior to each assay, the XTT solution was thawed and supplemented with menadione (10 mM stock dissolved in acetone; final concentration 1 μM ; Sigma), an aliquot of 1 ml of the XTT-menadione solution was added per well, and the plates were incubated in the solution at 37°C for 2 h. A sample (500 μl) was then transferred from each well into a fresh 12-well plate (to eliminate interference of cells with colorimetric readings), and the colorimetric change resulting from XTT reduction was measured at 490 nm. The assays were performed in triplicate. For fixation of the biofilms, 100 μl 99% methanol was added (15 min), after which supernatants were removed and the plates were air-dried. Then, 100 μl of a 0.02 % CV solution was added to all wells. After 20 min, the excess CV was removed by washing the plates under running tap water. Finally, bound CV was released by adding 150 μl of 33% acetic acid (Sigma). The absorbance was measured at 590 nm.

COAGULASE ACTIVITY

Coagulase production was assessed by the formation of a clot that could not be resuspended by gentle shaking after inoculation each strain [controls and *C. parapsilosis* strains

(smooth colony)] in 500 µl of EDTA-rabbit plasma and incubation for 4 h (or 24 h if no clot was formed), at 35°C.

Type strains *S.aureus* ATCC 25923 and *S.epidermidis* ATCC 14990 were used as positive and negative controls.

CATALASE ACTIVITY

C. parapsilosis suspensions (from a smooth colony) adjusted to 10^6 cells.ml⁻¹ were exposed to hydrogen peroxide solution at concentration of 25 mM. After 1 h of incubation at 30°C, yeast cells were washed, resuspended in sterile distilled water and serially diluted. Approximately 50 µl were plated onto YEPD agar medium and incubated at 30°C for 72 h; afterwards CFUs were counted and the results compared with plates sampled with suspensions not exposed to hydrogen peroxide. Assays were performed in triplicate.

MICROARRAY ASSAYS (2)

To get an insight whether there is an effective relation between drug resistance and virulence attributes for *C. parapsilosis*, we compared large-scale gene expression profile of susceptible and resistant strains (smooth phenotypes) using microarray analysis, as described previously (Antifungal Resistance Mechanisms section).

CELL WALL CHITIN CONTENT

CALCOFLUOR WHITE RESISTANCE

Calcofluor white (CFW; Sigma) was incorporated into YEPD agar plates at 50 µg.ml⁻¹. *C. parapsilosis* cells were grown to late log phase in YEPD broth and serially diluted to a final concentration ranging from 10^6 to 10^2 cells.ml⁻¹ in fresh YEPD broth. YEPD agar plates were inoculated with 5 µl drops of each cell suspension and incubated for 72 h at 30°C.

FLOW CYTOMETRIC ASSAY

Chitin content was measured using by a new method developed by Costa-de-Oliveira et al. (manuscript VIII in Publications section) based on flow cytometric analysis.

CFW binds to chitin and cellulose from cell wall of yeasts. Since it is a fluorescent probe, it is possible to correlate the mean of intensity of fluorescence emitted by cells with the amount of chitin. A cytometric protocol was developed in order to measure cell wall chitin content and distinguish chitin content among different cell populations.

C. parapsilosis cells were grown in YEPD broth at 35°C under agitation until late *log* phase. A 10^6 cells.ml⁻¹ suspension in sterile distilled water was stained with CFW 2.5 µg.ml⁻¹, a specific chitin dye (excitation at 365 nm and emission at 430 nm), for 15 min at room temperature, in the dark. Yeast cells were washed by centrifugation and the blue fluorescence (Pacific blue channel) was quantified in a BD FACSCanto II flow cytometer (Becton Dickinson, Madrid, Spain). BD FACSCanto II system consists of an excitation source with up to three lasers: blue (488-nm, air-cooled, 20-mW solid state), red (633-nm, 17-mW HeNe), and violet (405-nm, 30-mW solid state). The mean intensity of fluorescence, from three independent experiments, emitted from stained (positive population) and non-stained (autofluorescence - negative population) yeast cell populations were analyzed and processed with FACSDiva software. For each experiment a staining index (SI) was calculated as follows: [mean positive population – mean negative population]/ 2 x standard deviation of the negative population; [211]).

C. albicans cells SC5314 [212] and Δ chs3, a chitinase deleted strain with low chitin content [213], were used as controls for chitin content determination assays. Other yeasts were used: 6 strains of *C. glabrata*, 4 strains of *C. krusei*, 4 strains of *C. tropicalis*, 3 strains of *C. albicans* and 1 strain of *Cryptococcus neoformans* (manuscript VIII in Publications section).

EPIFLUORESCENCE MICROSCOPY

In order to confirm the results obtained from flow cytometry, *C. parapsilosis* cells were examined under epifluorescence microscopy. Yeast cells were grown and prepared as described for flow cytometric assays and stained with CFW 25 µg.ml⁻¹ for 15 min. Upon staining, 30 µl of the cell suspension were placed on a slide and overlapped with vectashield fluorescence mounting media (Vector Laboratories, Peterborough, UK). The stained suspensions were observed under an epifluorescence microscopy (400X) imager Z Apotome (Zeiss).

Table 1. Sequences of primers.

Primer name	Primer sequence
ALD5-F	5'-ACTTACAAACAACCAACGGG-3'
ALD5-R	5'-ATCTTCTCCAATGCTTCA-3'
ERG6-F	5'-GAAACAAAGAGATTGAGCGACT-3'
ERG6-R	5'-GAAAGAAGAACCCAGCC-3'
ERG11-F1	5'-ATTTGGTTTACTTGTGTTTGCTCC-3'
ERG11-R1	5'-ATTCTGCTGGGTGTTGAGGTA-3'
ERG11-F2	5'-GGTTTACTTGTGTTTGCTCCT-3'
ERG11-R2	5'-GTCCATAAGATACGGCTGAAC-3'
FKS1HS1-F	5'-AATGGGCTGGTGCTCAACAT-3'
FKS1HS1-R	5'-CATCAAGTTCATCTGAAATTGAAGG-3'
GRP2-F	5'-GTTGTATTCATCAGTGGAGCC-3'
GRP2-R	5'-GACCAGCAGACTTGAGCA-3'
MDR1-F	5'-TTCGTGATAGTTTTGGTGGTAG-3'
MDR1-R	5'-TGAACCTGGAGTGAATCTTGT-3'
MRR1-F1	5'-CCCTTCTTCCGCAGATTTC-3'
MRR1-R1	5'-CGTTGTAAAGATGGCGTGGT-3'
MRR1-F2	5'-ACAATGGTCTGAGCAATGAA-3'
MRR1-R2	5'-GGCAATACTGGTGATGGAA-3'
NDT80-F1	5'-TCTCGTGCCCGATATCTTTG-3'
NDT80-R1	5'-TCTCGCATACACAGTCTCTC-3'
NDT80-F2	5'-CTCTGGTGGTGCTAATGATG-3'
NDT80-R2	5'-ATGTTGTTGAGGGAGTGAAGA-3'
RPL82-F	5'-TTGCTTCTGCTGATGAGG-3'
RPL82-R	5'-CCATAATACCACCACCCC-3'
RPO2	5'-GCGATCCCCA-3'
SADH-F	5'-GTTGATGCTGTTGGATTGT-3'
SADH-R	5'-CAATGCCAAATCTCCCAA-3'
SOD4-F	5'-TCAGGAAGACACGGTAAGATT-3'
SOD4-R	5'-GCAAAGTGGATAACAATGGA-3'
TUB4-F	5'-TGTATTCCACAATGATGCCT-3'
TUB4-R	5'-TGCCTTGAAACGAAGTAG-3'
UPC2-F1	5'-TTTGCCGGTAAACCATCC-3'
UPC2-R1	5'-TTTCCTCCACCCCTATTGTAG-3'
UPC2-F2	5'-ATTGGAGTGTGGGTATCTTCAT-3'
UPC2-R2	5'-CCTTCGCCTTCTCAGTTC-3'

RESULTS

INCIDENCE AND DISTRIBUTION OF *C. PARAPSILOSIS*, *C. ORTHOPSILOSIS* AND *C. METAPSILOSIS*

Details regarding the 175 *C. parapsilosis* isolates are depicted in Table 1. Amplification of *SADH* gene fragment was performed using the genomic DNA of all isolates as template. Isolates displaying a fragment with a size other than 716 bp were excluded from this study. Six strains (3.4%), corresponding to 4 blood and 2 mucosal isolates, were not confirmed to belong to *C. parapsilosis* group (e.g. Fig. 1a, lane 3). For *C. parapsilosis* group a fragment of *SADH* gene (716 bp) was amplified by PCR (e.g. Fig. 1a, lane 1,2,4,5 and 6). According to the *BanI* restriction pattern of the above fragment, isolates were molecularly discriminated as *C. parapsilosis sensu stricto*, *C. orthopsilosis* and *C. metapsilosis* (Fig. 1b): *C. parapsilosis sensu stricto* has one *BanI* restriction site, at position 196; *C. orthopsilosis* has none; and *C. metapsilosis* has three *BanI* restriction sites (at positions 96, 469, and 529). The *SADH* fragments of the previously identified *C. orthopsilosis* isolates were sequenced (data not shown); all four sequenced isolates correspond in fact to *C. orthopsilosis*. Therefore, misidentification due to possible point mutation in *C. parapsilosis sensu stricto* was excluded.

The incidence and distribution of the three species is detailed in Table 1; 160 isolates (91.4%) were identified as *C. parapsilosis sensu stricto*, 4 (2.3%) as *C. orthopsilosis* and 5 (2.9%) as *C. metapsilosis*; six isolates corresponded to other *Candida* species. *C. orthopsilosis* and *C. metapsilosis* species were found in biological products other than blood; *C. parapsilosis sensu stricto* species were found in all biological samples. All 8 environment strains (6 from air and 2 from food) were identified as *C. parapsilosis sensu stricto*.

RESULTS

Table 1. Distribution of *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis*.

Site of isolation	No. of <i>C. parapsilosis</i> sensu stricto	No. of <i>C. orthopsilosis</i>	No. of <i>C. metapsilosis</i>	No. of isolates
Urine	9	4	0	13
Respiratory tract	2	0	1	3
Central venous catheter	6	0	0	6
Blood	60	0	0	64
Mucosal surface	65	0	4	71
Peritoneal fluid	1	0	0	1
Bile fluid	2	0	0	2
Spinal fluid	1	0	0	1
Hospital environment	8	0	0	8
Unknown	6	0	0	6
Total (%)	160 (91.4%)	4 (2.3%)	5 (2.9%)	175 ^a (100%)

^aTotal number of isolates is higher than the sum of *C. parapsilosis* sensu stricto, *C. orthopsilosis* and *C. metapsilosis*, since the molecular analysis of six isolates showed that they corresponded to distinct *Candida* species.

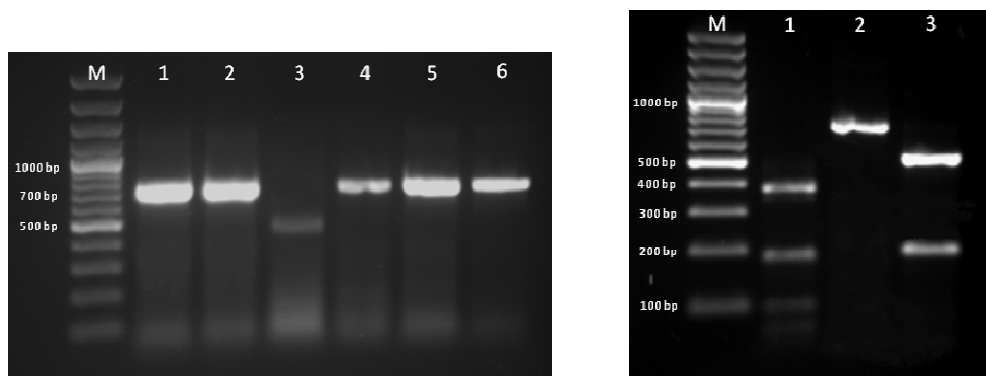


Figure 1. Distinction of *C. parapsilosis* sensu stricto, *C. orthopsilosis* and *C. metapsilosis* according to *SADH* gene restriction profile. a) Amplification of a 716 bp *SADH* gene fragment from genomic DNA of isolates provided genetic confirmation of *C. parapsilosis* group (lane 1, 2, 4, 5 and 6); smaller *SADH* gene fragments amplifications correspond to misidentified *C. parapsilosis* species (lane 3). b) Representative *SADH* gene restriction profile for *C. metapsilosis* (lane 1), *C. orthopsilosis* (lane 2), and *C. parapsilosis* (lane 3) type strains. Lane M, 100-bp DNA ladder.

GENETIC HOMOGENETY OF *C. PARAPSILOSIS* ISOLATES

Based on the major DNA fragments (ranging from 200 bp to approximately 2 kb) the RAPD profiles were defined. From the 160 *C. parapsilosis* isolates RAPD analysis generated 3 unique profiles (Fig. 2a). The isolates were identified as RAPD type A1 (151 isolates, 94.4 %), A2 (6 isolates, 3.8 %) and A3 (3 isolates, 1.8 %). All the environmental isolates belong to type A1.

These results were corroborated by mtDNA RE analysis. A representative example of the major type (which corresponds to RAPD type A1) is represented in Fig. 2b.

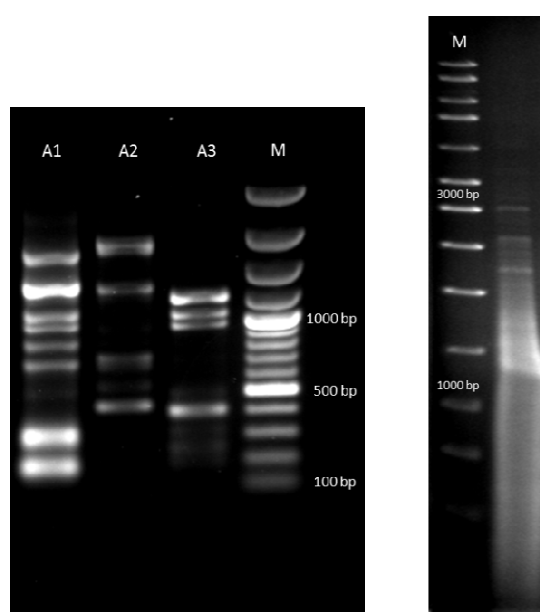


Figure 2. *C. parapsilosis* genotypes. a) RAPD profiles generated by primer *RPO2* among *C. parapsilosis* isolates included in this study. Lanes: 1, RAPD type A1, 2, RAPD type A2, 3, RAPD type A3 and M, 100-bp DNA ladder. b) Restriction endonuclease pattern of *HinfI*-digested DNA. The pattern corresponds to RAPD profile type A1. Lane M, 1-kbp DNA ladder.

ANTIFUNGAL SUSCEPTIBILITY PROFILES OF *C. PARAPSILOSIS*, *C. ORTHOPILOSIS* AND *C. METAPSILOSIS*

The susceptibility profiles of *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* concerning azoles and AMB are detailed in Table 2. The vast majority of the *C. parapsilosis* sensu stricto isolates (94.4%) were susceptible to FLC; 8 isolates (5%) were susceptible-dose dependent to FLC, and a single (0.6%) isolate was resistant to FLC. No FLC-resistant or FLC-susceptible-dose dependent strains were detected among the *C. orthopsilosis* and *C. metapsilosis* isolates; the highest MIC was 4 $\mu\text{g}.\text{ml}^{-1}$. A single isolate of *C. parapsilosis* sensu stricto (the same that was resistant to FLC) was resistant to VRC and PSC. All isolates of *C. orthopsilosis* and *C. metapsilosis* were susceptible to VRC and PSC. AMB MIC values were equal to or less than 1 $\mu\text{g}.\text{ml}^{-1}$ for all isolates tested, with the exception of two *C. parapsilosis* sensu stricto isolates, which were resistant. AMB MIC values for *C. orthopsilosis* and *C. metapsilosis* were lower than AMB MIC values for *C. parapsilosis* sensu stricto.

Table 2. Antifungal susceptibility profile of *Candida parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* isolates to different azoles and amphotericin B.

Species (no. of isolates)	Antifungal	MIC ($\mu\text{g}.\text{ml}^{-1}$) range	No. S (%)	No. S-DD (%)
<i>C. parapsilosis</i> sensu stricto (160)	FLC	0.125 - 64	94.4	5
	VRC	≤ 0.015 - 8	99.4	0
	PSC	≤ 0.03 - 2	99.4	NE
	AMB	0.06 - 2	98.8	NE
<i>C. orthopsilosis</i> (4)	FLC	0.5 - 1	100	0
	VRC	≤ 0.015 - 0.03	100	0
	PSC	≤ 0.03	100	NE
	AMB	0.5	100	NE
<i>C. metapsilosis</i> (5)	FLC	1 - 4	100	0
	VRC	≤ 0.015 - 0.125	100	0
	PSC	< 0.03 - 0.125	100	NE
	AMB	0.125 - 1	100	NE

S, susceptible; S-DD, susceptible-dose dependent; NE, not established.

The susceptibility pattern to echinocandins (CSF and ANF) is detailed in Table 3. A high number of *C. parapsilosis* sensu stricto isolates was nonsusceptible to CSF (38%); as a wide range of MIC values (4 to 32 $\mu\text{g}.\text{ml}^{-1}$) was found. All *C. orthopsilosis* and *C. metapsilosis* isolates were susceptible to CSF. Similar findings were registered for ANF: all *C. orthopsilosis* and *C. metapsilosis* isolates were susceptible to ANF, while 29% of the *C. parapsilosis* sensu stricto isolates were nonsusceptible to ANF; ANF MIC values (4 to 16 $\mu\text{g}.\text{ml}^{-1}$) were lower than those of CSF. For *C. parapsilosis* sensu stricto, the standard MIC50 and MIC90 values of both echinocandins were 2 and 4 $\mu\text{g}.\text{ml}^{-1}$, respectively. For *C. orthopsilosis*, the standard MIC50 of CSF was 2 $\mu\text{g}.\text{ml}^{-1}$ and that of ANF was 1 $\mu\text{g}.\text{ml}^{-1}$. For *C. metapsilosis*, the standard MIC50 value of both echinocandins was 1 $\mu\text{g}.\text{ml}^{-1}$. The MIC90 was not calculated because the number of *C. orthopsilosis* and *C. metapsilosis* isolates was less than 10.

Table 3. Antifungal susceptibility profile of *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* to echinocandins.

Species (no. of isolates)	Antifungal	Phenotype ^a	MIC Range ($\mu\text{g}.\text{ml}^{-1}$)
<i>C. parapsilosis</i> sensu stricto (160)	CSF	S (62%)	0.06 – 2
		NS (38%)	4 – 32
	ANF	S (71%)	0,125 – 2
		NS (29%)	4 – 16
<i>C. orthopsilosis</i> (4)	CSF	S (100%)	1 – 2
	ANF	S (100%)	1
<i>C. metapsilosis</i> (5)	CSF	S (100%)	1 – 2
	ANF	S (100%)	0.5 – 1

^aS, susceptible (MIC $\leq 2 \mu\text{g}.\text{ml}^{-1}$); NS, nonsusceptible.

***IN VITRO* INDUCTION OF ANTIFUNGAL RESISTANCE**

DEVELOPMENT OF *IN VITRO* RESISTANCE

Table 4 shows fluconazole, voriconazole and posaconazole MIC values before and after induction with different azoles, for all tested strains (ATCC 22019, BC014, BC011, BC190 and BC237). After exposure to therapeutic blood concentrations of the different azoles, distinct patterns of resistance were observed. Following 15 days of antifungal exposure, only the fluconazole susceptibility phenotype changed. This finding was observed with most strains exposed to fluconazole and with a few strains exposed to voriconazole; following posaconazole exposure, only minor MIC variations were registered after this period of time. Fluconazole and voriconazole increased MIC values to themselves and to each other, changing the susceptibility phenotype to resistant after 30 days, but not to posaconazole, which maintained the susceptible phenotype. Posaconazole induced resistance to all azoles only at the very end of incubation time (30 days). Therefore, the activity of fluconazole was the most impaired and it was the faster inducer of resistance.

In a different strategy to compare the ability of the same azole drug to induce resistance, incubation with two- and four-fold MIC values was assayed. A representative example (type strain ATCC 22019) of the development of resistance in such instances is detailed in Fig. 3. The lower concentration of fluconazole was sufficient to induce resistance to itself and to voriconazole (at day 30th) but not to posaconazole; the exposure of the yeast cells to the lower concentration of voriconazole only changed the fluconazole phenotype (susceptible strain changed to susceptible-dose dependent; at day 30th); only minor variations of MIC values were observed with the other tested azoles. Posaconazole had the minor effect on the MIC values of all azoles in such experimental conditions.

Incubation with these three azoles, independently on the tested concentration, did not induce resistance to AMB or to CSF.

Regarding the control assay, where the same strains were successively incubated without antifungals, the MIC values of all antifungals (FLC, VRC, PSC, AMB and CSF) tested remained constant throughout the duration of the study.

Concerning the BC041 strain incubated with anidulafungin, the MIC values for CSF, ANF and MCF changed from 0,25 µg.ml⁻¹, 1 µg.ml⁻¹ and 0,5 µg.ml⁻¹ to 8 µg.ml⁻¹, 16 µg.ml⁻¹ and 8 µg.ml⁻¹, respectively at the end of the incubation time. Fluconazole and AMB MIC values did not change after incubation with anidulafungin. In parell, the incubation throughout the 30 days without antifungal did not change the initial MIC values of all three echinocandins.

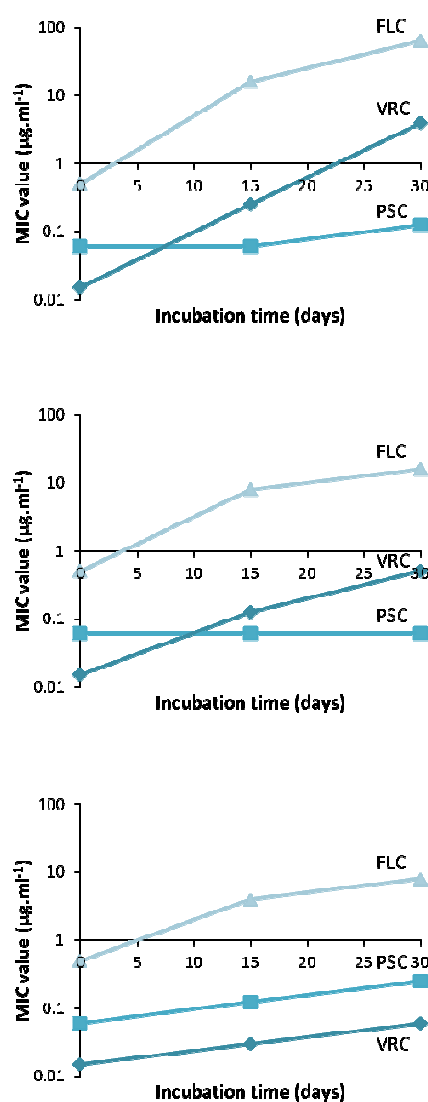


Figure 3. Variation of fluconazole (FLC), voriconazole (VRC) and posaconazole (PSC) MIC values for *Candida parapsilosis* ATCC 22019 type strain cultured in the presence of two- and four-fold MIC values of FLC (a), VRC (b) and PSC (c) for 30 days. —▲— MIC of FLC; —◆— MIC of VRC; —■— MIC of PSC.

STABILITY OF INDUCED RESISTANCE

Regarding the stability of the *in vitro* developed resistance, such resistant organisms were subcultured in drug-free medium for 30 days; the resistance pattern remained stable and no decrease greater than one single dilution was observed in MIC values of antifungals tested. These results are detailed in Fig. 4 for a representative example (type strain ATCC 22019).

The stability of echinocandin resistance acquired by BC041NS strain was also assessed. No decrease greater than one dilution was observed in MIC values of the 3 echinocandins tested (data not shown).

Table 4. MIC value ($\mu\text{g.ml}^{-1}$) and phenotype displayed by *Candida parapsilosis* type strain (ATCC 22019) and by BC014, BC011, BC237 and BC190 clinical strains before and after induction with fluconazole ($16\ \mu\text{g.ml}^{-1}$), voriconazole ($2\ \mu\text{g.ml}^{-1}$) and posaconazole ($1\ \mu\text{g.ml}^{-1}$).

MIC/Phenotype												
Induction with	Strain	Fluconazole			Voriconazole			Posaconazole				
		Day 0	Day 15	Day 30	Day 0	Day 15	Day 30	Day 0	Day 15	Day 30		
Fluconazole	ATCC	0.5/S	16/S-DD	64/R	0.015/S	0.25/S	2/S-DD	0.06/S	0.06/S	0.125/S	0.125/S	
	BC014	1.0/S	16/S-DD	128/R	0.03/S	0.5/S	4/R	0.03/S	0.25/S	0.5/S	0.5/S	
	BC011	1.0/S	8/S	128/R	0.03/S	0.25/S	2/S-DD	0.03/S	0.125/S	0.5/S	0.5/S	
	BC237	1.0/S	32/S-DD	128/R	0.03/S	0.25/S	4/R	0.06/S	0.125/S	0.5/S	0.5/S	
	BC190	0.5/S	16/S-DD	128/R	0.03/S	0.125/S	4/R	0.06/S	0.125/S	0.5/S	0.5/S	
Voriconazole	ATCC	0.5/S	16/S-DD	64/R	0.015/S	0.06/S	2/R	0.06/S	0.06/S	0.125/S	0.125/S	
	BC014	1.0/S	16/S-DD	128/R	0.03/S	0.06/S	4/R	0.03/S	0.06/S	0.5/S	0.5/S	
	BC011	1.0/S	4/S	64/R	0.03/S	0.25/S	2/S-DD	0.03/S	0.125/S	0.5/S	0.5/S	
	BC237	1.0/S	4/S	64/R	0.03/S	0.125/S	2/S-DD	0.06/S	0.125/S	0.5/S	0.5/S	
	BC190	0.5/S	2/S	64/R	0.03/S	0.125/S	4/R	0.06/S	0.125/S	0.5/S	0.5/S	
Posaconazole	ATCC	0.5/S	1/S	64/R	0.015/S	0.03/S	8/R	0.06/S	1/S	16/R	16/R	
	BC014	1.0/S	1/S	128/R	0.03/S	0.03/S	16/R	0.03/S	0.125/S	32/R	32/R	
	BC011	1.0/S	1/S	128/R	0.03/S	0.03/S	16/R	0.03/S	0.06/S	32/R	32/R	
	BC237	1.0/S	1/S	64/R	0.03/S	0.03/S	2/S-DD	0.06/S	0.06/S	8/R	8/R	
	BC190	0.5/S	1/S	128/R	0.03/S	0.06/S	16/R	0.06/S	0.125/S	32/R	32/R	

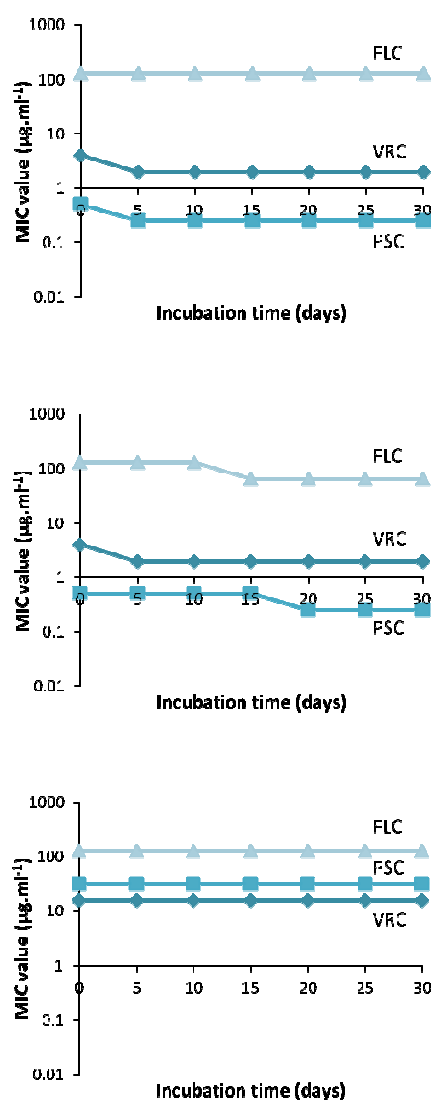


Figure 4. Variation of fluconazole (FLC), voriconazole (VRC) and posaconazole (PSC) MIC values for *Candida parapsilosis* ATCC 22019 type strain after induction with FLC (a), VRC (b) and PSC (c) cultured in the absence of antifungal for 30 days. —▲— MIC of FLC; —◆— MIC of VRC; —■— MIC of PSC.

HOMOGENITY OF CELLULAR POPULATIONS

To determine whether the induced resistance in *C. parapsilosis* isolates was associated with the selection of a resistant subpopulation, analysis of resistance within the cellular population was performed with the initial susceptible and *in vitro* induced resistant strains. Population analysis clearly demonstrated a homogeneous population both for the resistant induced strains and for the initial susceptible strain. A representative example of studies with induced azole resistant strains is shown for BC014S, BC014R_{FLC}, BC014R_{VRC} and BC014R_{PSC} strains in Fig. 5. The same was observed for BC041S and BC041NS (Fig. 6).

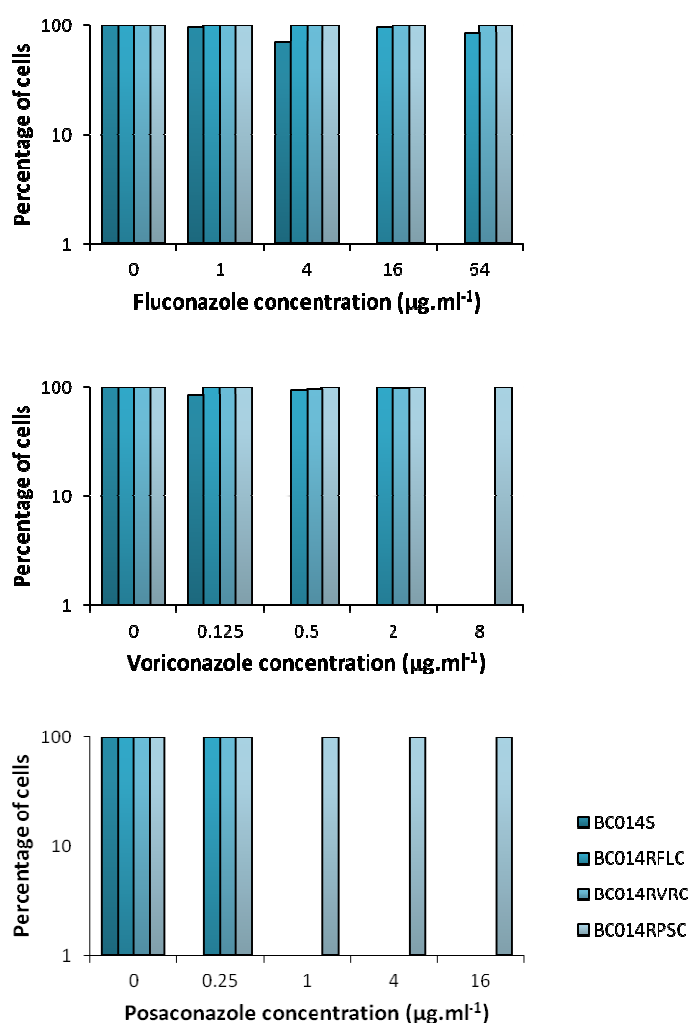


Figure 5. Population analysis (a representative example) of the parent susceptible (BC014S) and the respective resistant induced strains following 30 days of exposure to azoles (BC014R_{FLC}, BC014R_{VRC} and BC014R_{PSC}). The percentage of resistant cells (y-axis, log scale) is defined as the ratio of colonies with growth on plates containing the indicated concentration of fluconazole (a), voriconazole (b) and posaconazole (c) in relation to growth in the absence of drug.

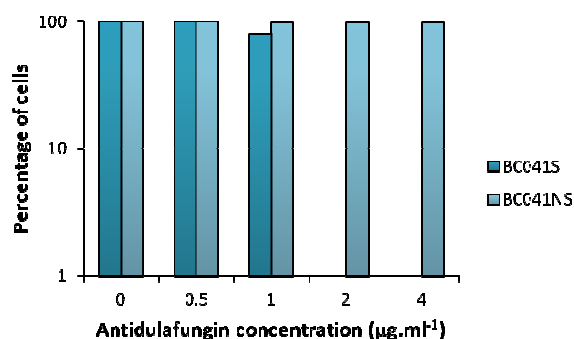


Figure 6. Population analysis of the parent susceptible (BC041S) and the respective nonsusceptible induced strain (BC041NS) following 30 days of exposure to anidulafungin. The percentage of resistant cells (y-axis, log scale) is defined as the ratio of colonies with growth on plates containing the indicated concentration of anidulafungin in relation to growth in the absence of the drug.

***In Vivo* INDUCTION OF ANTIFUNGAL RESISTANCE**

MOLECULAR IDENTIFICATION OF ISOLATES

Although *C. parapsilosis* isolates were identified by conventional methods, molecular testing was also performed in order to confirm identity. The *SADH* gene restriction polymorphism indicated that all the clinical isolates were in fact *C. parapsilosis* sensu stricto, since, as described by Tavanti et al. [84], a typical restriction profile of *SADH* gene of two bands was obtained (Fig. 7).

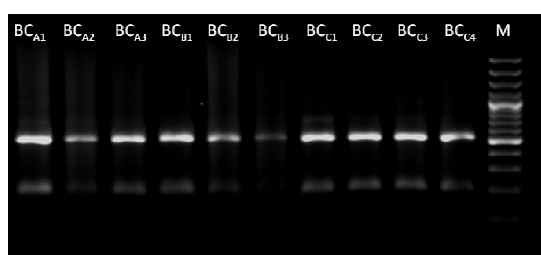


Figure 7. *SADH* gene restriction profile for *C. parapsilosis* strains from patient A (BC_{A1}, BC_{A2} and BC_{A3}), patient B (BC_{B1}, BC_{B2} and BC_{B3}) and patient C (BC_{C1}, BC_{C2}, BC_{C3} and BC_{C4}). Lane M, 100-bp DNA ladder.

Regarding genotyping results, RAPD analysis (Fig. 8) showed that isolates from the same patient shared identical profiles and were thus considered to belong to the same initial strain. Moreover, all strains exhibited the same profile (RAPD type A1).

For patient B and patient C, *C. parapsilosis* strains were also isolated from other body sites; RAPD analysis revealed that these strains were similar to blood strains (data not shown).

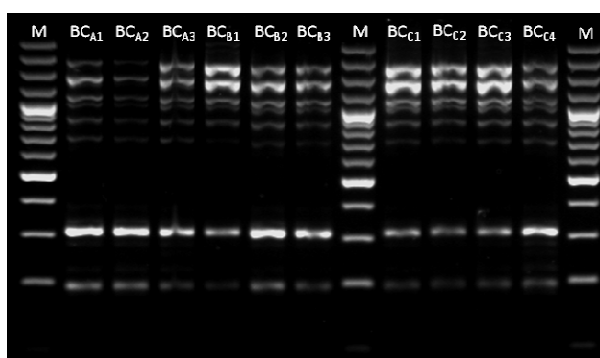


Figure 8. RAPD profiles produced for *C. parapsilosis* strains with the primer *RPO2*, isolated from blood cultures of patient A (BC_{A1}, BC_{A2} and BC_{A3}), patient B (BC_{B1}, BC_{B2} and BC_{B3}) and patient C (BC_{C1}, BC_{C2}, BC_{C3} and BC_{C4}). Lane M, 100-bp DNA ladder.

SUSCEPTIBILITY PROFILES

The isolates from the three different fungaemia episodes were tested for antifungal susceptibility to four azoles (Table 5). Overall, throughout time the initial susceptible isolates became progressively less susceptible to azoles, especially to fluconazole, itraconazole and voriconazole. In the first case (patient A), the last isolate (BC_{A3}), recovered after 14 days of FLC exposure, was resistant to all four azoles, with high MIC values. In the second case (patient B), although only a slightly increase in MIC values of all azoles was registered, the last isolate (BC_{B3}), which had been exposed to FLC for 8 days, changed the susceptibility phenotype from susceptible to susceptible-dose dependent, only for FLC. Regarding the third patient (patient C), the isolate BC_{C4} also became susceptible-dose dependent to FLC and VRC after 9 days of exposure to FLC.

Table 5. *In vitro* antifungal susceptibility [MIC value ($\mu\text{g.ml}^{-1}$) and phenotype] of *C.parapsilosis* blood culture isolates from patient A, patient B and patient C to different azoles.

Isolate	MIC/Phenotype			
	Fluconazole	Itraconazole	Voriconazole	Posaconazole
BC _{A1}	0.25/S	≤ 0.015 /S	≤ 0.015 /S	0.06/S
BC _{A2}	1/S	0.06/S	≤ 0.015 /S	0.06/S
BC _{A3}	> 64/R	> 4/R	> 8/R	>16/R
BC _{B1}	4/S	0.125/S	0.125/S	≤ 0.03 /S
BC _{B2}	16/S-DD	0.5/S-DD	0.5/S	0.06/S
BC _{B3}	32/S-DD	0.5/S-DD	0.5/S	0.06/S
BC _{C1}	8/S	0.125/S	0.25/S	≤ 0.03 /S
BC _{C2}	8/S	0.25/S-DD	0.25/S	≤ 0.03 /S
BC _{C3}	8/S	0.25/S-DD	0.25/S	≤ 0.03 /S
BC _{C4}	32/S-DD	0.5/S-DD	1/S	≤ 0.03 /S

The susceptibility phenotypes acquired *in vivo* were confirmed to be stable *in vitro*, since after sub-culturing the last isolates from the three cases (BC_{A3}, BC_{B3} and BC_{C4}) in drug-free medium, only slight variations were seen (maximum one dilution) in MIC values of all azoles, maintaining the resistant and susceptible-dose dependent phenotypes (data not shown). Such findings are similar to what happened with *in vitro* induced resistant strains.

ANTIFUNGAL RESISTANCE MECHANISMS

EFFECT OF EFFLUX BLOCKERS ON MIC VALUES OF AZOLES

After incubation with ibuprofen and verapamil, two ATP-binding cassette efflux pumps blockers, a decrease of MIC values of no more than one single dilution was registered, while the resistance pattern remained stable; such results were obtained with all strains studied. Results obtained with BC014 resistant strains, a representative example, are represented in Table 6. Regarding the control strain *C. albicans* 95-68, displaying overexpression of *CDR1* and *CDR2* genes, in the presence of verapamil and ibuprofen the MIC values of fluconazole, voriconazole and posaconazole decreased drastically; as expected for control strain *C. albicans* 2-76, with low levels of resistance genes expression, the MIC values of the three antifungals did not change.

In the presence of FK506 no synergistic effect was detected for all *C. parapsilosis* strains tested using the agar diffusion method. In Fig. 9, a representative example of this assay with BC014R_{PSC} strain is shown. In control plates (no fluconazole added to the medium) neither FK506 nor its solvent was able to inhibit *Candida* growth, which demonstrates that such compound does not exhibit antifungal activity. Regarding BC014R_{PSC} no synergistic effect was observed at any FK506 concentration tested. As expected for *C. albicans* 95-68, with overexpression of *CDR1* and *CDR2* genes (ATP-dependent efflux pumps), a clear synergistic effect was observed.

RH-6G STAINING

The steady state regarding Rh-6G fluorescence of the susceptible phenotype (strains incubated in drug-free medium) and of the resistant phenotypes obtained after 30 days of antifungal exposure (*in vitro* induction studies) were compared. Similar intensity of fluorescence was obtained with induced resistant strains and susceptible strains (Fig. 10a depicts a representative example for BC014S and BC014R_{FLC} strains). The fluorescence intensity did not increase in the presence of the efflux blocker verapamil (data not shown).

The final strain (BC_{A3}) and the initial strain (BC_{A1}) from *in vivo* induction (case report A) were also compared by flow cytometric analysis and similar results were obtained (data not shown).

Candida albicans strain 95-68, with overexpression of *CDR1* and *CDR2* resistance genes (ATP-dependent efflux pumps), behaved as expected, showing an increase in Rh-6G staining when incubated with verapamil (Fig. 10b), whereas *C. albicans* strain 2-76 showing low levels of efflux genes expression did not (data not shown), which validates the assays.

Table 6. MIC value ($\mu\text{g}\cdot\text{ml}^{-1}$) and phenotype displayed by *Candida parapsilosis* after induction with fluconazole (BC014R_{FLC} strain), voriconazole (BC014R_{VR} strain) and posaconazole (BC014R_{PSC} strain) and of *C. albicans* 95-68 and *C. albicans* 2-76 strains, determined in the absence and the presence of the efflux modulators verapamil (Vera) 100 μM and ibuprofen (ibu) 100 $\text{mg}\cdot\text{l}^{-1}$.

Strains	MIC/Phenotype							
	Fluconazole	Fluconazole + Vera	Fluconazole + Ibu	Voriconazole	Voriconazole + Vera	Voriconazole + Ibu	Posaconazole	Posaconazole + Vera + Ibu
BC014R _{FLC}	128/R	64/R	128/R	4/R	2/S-DD	4/R	0.5/S	0.5/S
BC014R _{VR}	128/R	64/R	128/R	4/R	2/S-DD	4/R	0.5/S	0.5/S
BC014R _{PSC}	128/R	128/R	128/R	16/R	16/R	16/R	32/S	32/S
<i>C. albicans</i> 95-68	>512/R	128/R	16/S-DD	64/R	16/R	0.5/S	>16/R	0.5/S
<i>C. albicans</i> 2-76	0.5/S	0.5/S	0.5/S	0.125/S	0.125/S	0.125/S	0.06/S	0.06/S

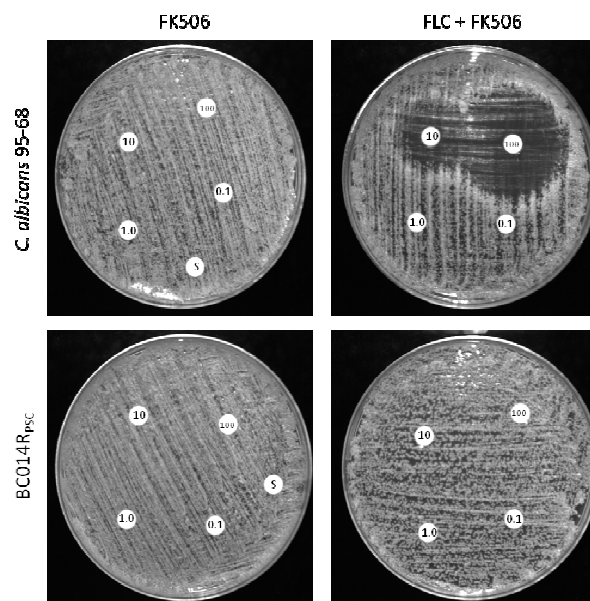


Figure 9. Disk diffusion assays with *Candida albicans* 95-78 control strain and *C. parapsilosis* resistant strain (BC014R_{PSC}) in the presence of FK506. *C. albicans* 95-78 control strain (with overexpression of genes that codify to efflux pumps) and BC014R_{PSC} strain were grown on YEPD agar medium with paper disks impregnated with serial dilutions of FK506 (0.1, 1.0, 10 and 100 mg.ml⁻¹) and DMSO (S-solvent), in the presence of fluconazole (FLC 128 µg.ml⁻¹) (right panel) or in its absence (left panel).

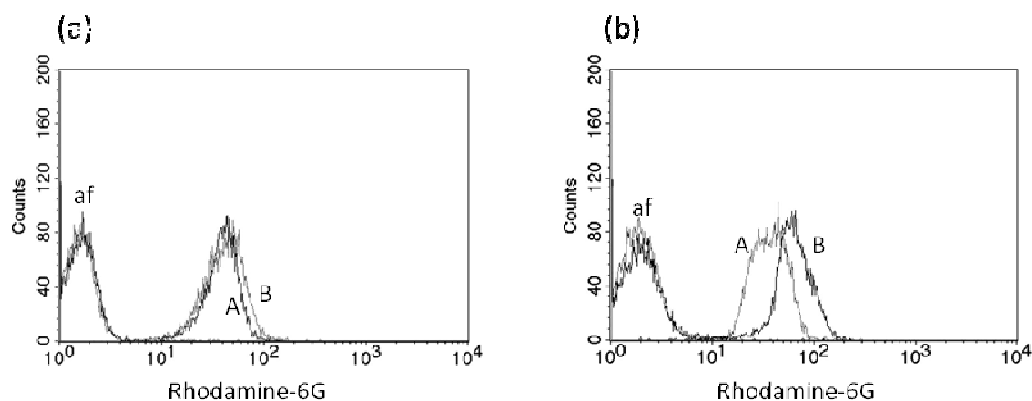


Figure 10. Flow cytometric histograms of yeast cells after Rh-6G staining (efflux pumps substrate). (a) Flow cytometric analysis at FL1 (515 nm) of BC014S strain incubated in drug-free medium (A) and of BC014R_{FLC} strain (B). (b) Flow cytometric analysis at FL1 (515 nm) of resistant *Candida albicans* 95-68 strain (overexpression of genes that codify to efflux pumps) without (A) and with (B) the efflux blocker verapamil. af, autofluorescence.

GENE SEQUENCING ANALYSIS

To determine whether resistance was associated with mutations in the target genes, genomic DNA extraction was performed and several genes were amplified and sequenced.

No sequence alterations were detected in *ERG11* gene when comparing the susceptible strain (BC_{A1}) and the resistant strain (BC_{A3}) from the *in vivo* case.

Regarding the sequence of *MRR1* gene in BC014R_{FLC} and BC014R_{VR}, a single nucleotide exchange was detected in both strains, when comparing with the *MRR1* sequence of the susceptible strain (BC014S). Regarding BC014R_{FLC} strain, a substitution of G1747A in BC014R_{FLC} strain, results in an amino acid exchange G583R (glycine to arginine). In BC014R_{VR} strain, a different mutation was found, A2619C, which results in the substitution of a lysine by an asparagine (K873N).

To determine whether the overexpression of the transcription factors *NDT80* and *UPC2* is associated with point mutations, the genes were sequenced both in BC014R_{PSC} and BC014S strains. No difference in the nucleotide sequence was detected between the susceptible (BC014S) and the BC014R_{PSC} strains.

MIC values concerning echinocandins for the six nonsusceptible clinical strains are represented in Table 7. No sequence alterations were observed in HS1 region of the *FKS1* gene for all strains, independently of the susceptibility phenotype for each echinocandin. Additionally, no sequence alterations were detected between the susceptible strain (BC041S) and the nonsusceptible strain (BC041NS) obtained from the *in vitro* induction assay.

Table 7. MIC value ($\mu\text{g}.\text{ml}^{-1}$) and susceptibility phenotype displayed by *C. parapsilosis* strains to echinocandins.

Strain	MIC/Phenotype ^a		
	CSF	ANF	MCF
BC011	4/NS	1/S	2/S
BC017	4/NS	8/NS	2/S
BC022	4/NS	8/NS	8/NS
BC043	8/NS	2/S	2/S
BC101	4/NS	4/NS	4/NS
BC132	4/NS	2/S	2/S

^aS, susceptible (MIC $\leq 2 \mu\text{g}.\text{ml}^{-1}$); NS, nonsusceptible.

MICROARRAYS (1) WITH *IN VITRO* INDUCED STRAINS

TRANSCRIPTIONAL PROFILING

In order to identify genes that are differentially regulated in *C. parapsilosis* resistant strains, we performed transcriptional profiling experiments using genomic microarrays.

Microarray analysis identified differential expression ($FC > 2$, $p < 0.05$) of 1128 (595 up-regulated and 533 down-regulated), 210 (138 up-regulated and 72 down-regulated) and 598 (341 up-regulated and 257 down-regulated) genes in BC014R_{FLC}, BC014R_{VRC} and BC014R_{PSC} strains respectively (Fig. 11a; the data is available in Tables S1-S3). Figure 11b represents the distribution of genes with differential expression in BC014R_{FLC} and BC014R_{VRC} strains, which were the strains with similar expression gene profiles.

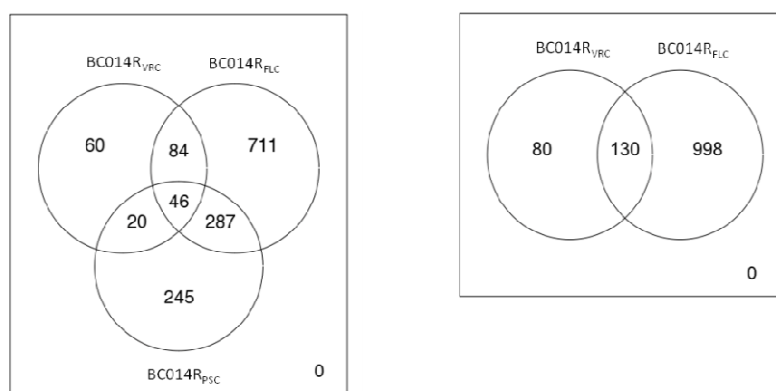


Figure 11. Distribution of genes with differential expression in BC014R_{FLC}, BC014R_{VRC} and BC014R_{PSC} strains (a) and in BC014R_{FLC} and BC014R_{VRC} strains (b).

GENE ONTOLOGY ANALYSIS

Gene Ontology (GO) analysis was used to identify similarities and differences between the resistant strains obtained after exposition to the different antifungals. GO terms assigned to both process and function analyses were used, and the process analysis provided more relevant results. The large number of genes affected makes it difficult to identify overrepresented categories. Figure 12 represents the processes overrepresented in BC014R_{FLC} strain; up to 13% of the up-regulated genes are associated with RNA metabolic process and ribosome biogenesis and 10% are associated with translation. GO terms overrepresented in BC014R_{VRC} and BC014R_{PSC} are

RESULTS

detailed in Table 8 (and Table S4). In BC014R_{VR} GO term associated with response to chemical stimulus is overrepresented, while in the BC014R_{PS} strain the GO terms associated with sterol biosynthesis, response to chemical stimulus and response to drugs are overrepresented.

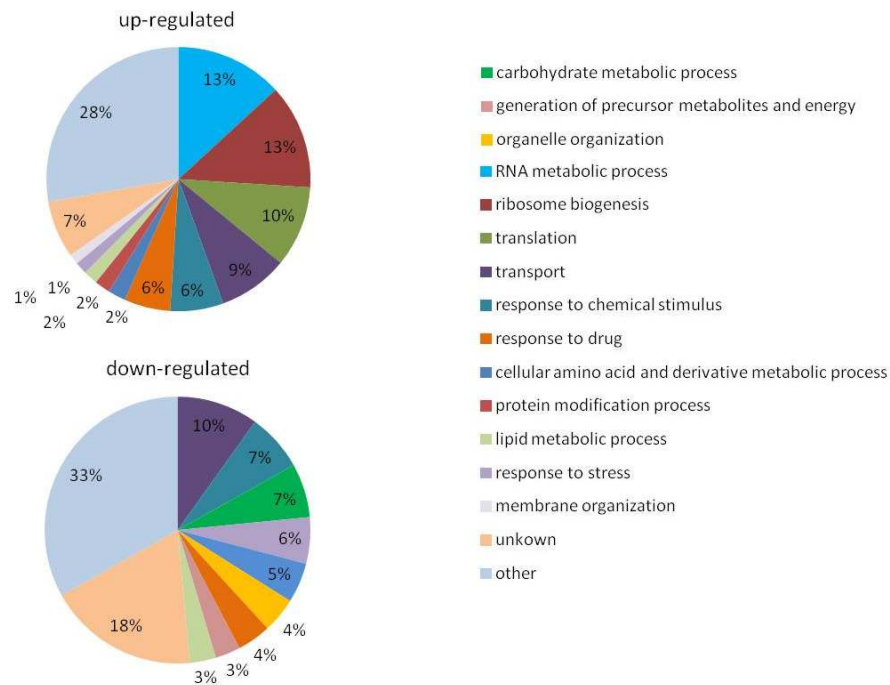


Figure 12. Selected process GO terms overrepresented in BC014R_{FLC} strain. The full list is available in Table S1.

Table 8. GO terms overrepresented in BC014R _{VR} and BC014R _{PS} strains.				
Cluster frequency ^a	Background frequency ^b	Corrected value	p-	GO term
BC014R _{VR}				
31/155	504/5085	0.0322		Response to chemical stimulus
BC014R _{PS}				
19/409	32/5085	1.17E-10		Sterol biosynthesis
70/409	504/5085	0.0014		Response to chemical stimulus
50/409	310/5085	0.0007		Response to drugs

^aCluster frequency: fraction of genes that are differentially regulated with homologs identified in *C. albicans* assigned to GO term.

^bBackground frequency: fraction of entire dataset with homologs identified in *C. albicans* assigned to GO term.

GENE EXPRESSION PROFILE OF BC014R_{FLC} AND BC014R_{VR} STRAINS

Because BC014R_{FLC} and BC014R_{VR} strains displayed a similar resistance profile, the genes that were differentially regulated in both strains were identified (Fig. 11b: 130 genes, representing 62% of total genes in BC014R_{VR}). Figure 13 shows the gene distribution into 5 clusters distinguished by the level of gene expression (all members of the clusters are listed in Table S5, following the same order depicted in Fig. 13). Clusters 1, 2 and 3 contain genes with increased expression in BC014R_{FLC} and BC014R_{VR} strains; cluster 3 showed the greatest change in gene expression (Table 9 describes the genes contained in these 3 clusters which included its description and FC value).

Cluster 2 contains *MDR1* and *CPAG_03790* genes, both members of the major facilitator superfamily (MFS) of multidrug transporters. Expression of a third member of the MFS family (*CPAG_01932*) was also increased, albeit in a lesser extent (cluster 1, Fig. 13, Table 9). Expression of *MDR1* is up-regulated by 19.43- and 40.22-fold in the BC014R_{FLC} and BC014R_{VR} strains, respectively (confirmed by RT-qPCR; Fig.16). In *C. albicans*, expression of *MDR1* is regulated by the transcription factor *MRR1* [214]. Interestingly, expression of *MRR1* is also up-regulated in *C. parapsilosis* BC014R_{FLC} and BC014R_{VR} strains (Cluster 1; Fig. 13 and Table 9); this finding was confirmed by RT-qPCR (Fig.16).

The expression of aldo-keto reductases and other genes associated with oxidative stress response is also regulated by Mrr1p transcription factor in *C. albicans* [214]. We observed a similar response in *C. parapsilosis*. Expression of several putative aldo-keto reductases and NADPH oxidoreductases (orthologs of *orf19.3442*, *OYE32*) is increased in both BC014R_{FLC} and BC014R_{VR} strains (Clusters 1 and 2, Fig. 13, Table 9). Cluster 3 contains the stress response gene *GRP2*, which is up-regulated 107.63- and 207.94-fold in BC014R_{FLC} and BC014R_{VR} strains, respectively (confirmed by RT-qPCR; Fig.16); the second member of cluster 3, *CPAG_02538* gene, has a high sequence identity to *GRP2*, and therefore is likely to encode for a protein in the same family.

Genes in cluster 4 are differentially regulated in the BC014R_{FLC} and BC014R_{VR} strains, and are unlikely to play a role in azole resistance. Genes in cluster 5 are down-regulated in both strains. These include several genes linked to ergosterol synthesis (named below).

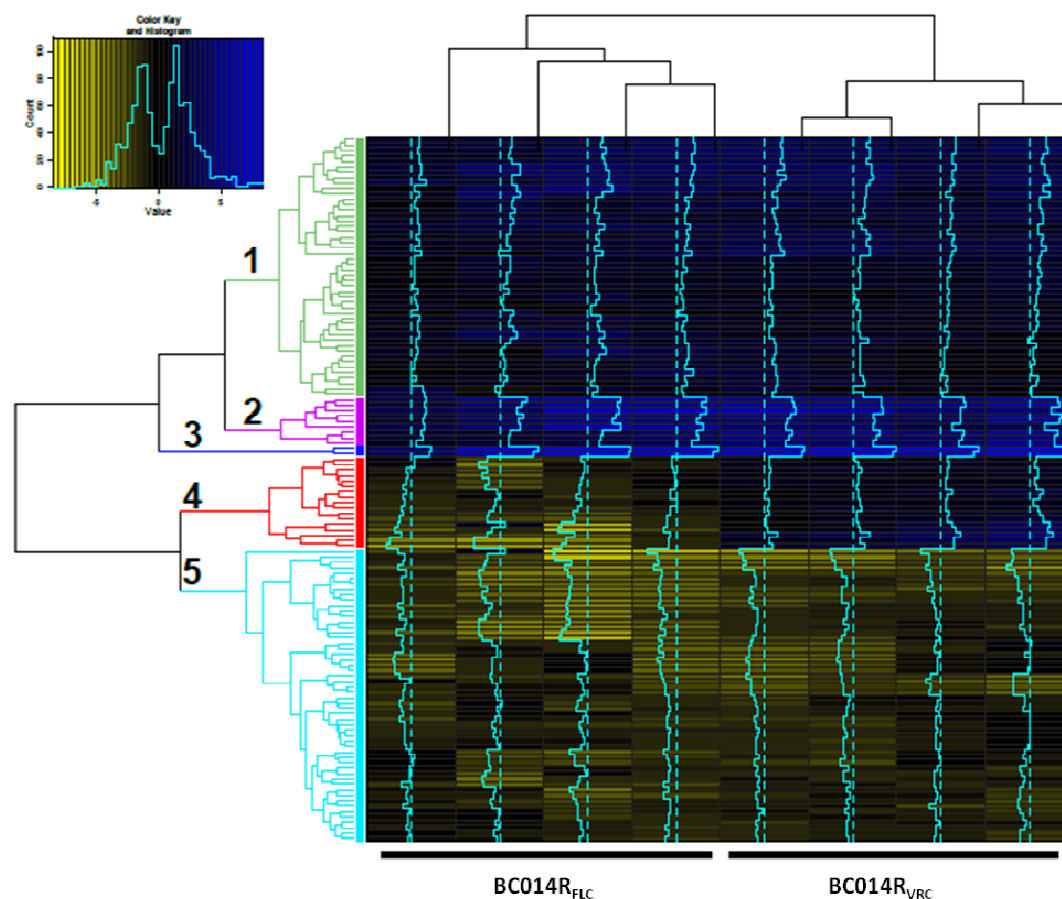


Figure 13. Cluster analysis of 130 genes that are differentially expressed in both BC014R_{FLC} and BC014R_{VRC} strains. Each cluster is represented by a different number, and the members of each cluster are listed in Table S5. The genes identified as *C. albicans* orthologs are shown. Genes with decreased and increased expression are marked in yellow and blue, respectively. Genes with unchanged expression are marked in black. The histogram in the upper and left side of the figure shows the distribution of the differentially expressed genes based on their logFC levels. The X axis represents the logFC levels in the heatmap. The Y (labeled as count) axis shows how many genes have a specific “value” or differential expression level.

Table 9. Genes with increased expression in BC014R_{FLC} and BC014R_{VRc} strains from Fig. 13.

CPAG ID ^a	<i>C. albicans</i> homolog ^b	Gene name ^c	Description ^c	BC014R _{FLC} ^d	BC014R _{VRc} ^d
<i>Cluster 1</i>					
CPAG_00168	orf19.7306		Aldo-keto reductase family	5.62	8.51
CPAG_00971	orf19.2446		Uncharacterized	5.78	7.73
CPAG_02192	orf19.6000*		Multidrug transporter of ATP-binding cassette (ABC) superfamily	5.94	9.58
cpar4417	orf19.6943*		Uncharacterized	4.69	7.11
CPAG_02875	orf19.7372	<i>MRR1</i>	Regulator of MDR1 transcription	5.24	5.70
CPAG_01174	orf19.345			9.19	9.00
CPAG_04671				9.71	6.82
CPAG_04730	orf19.6586		Uncharacterized	6.77	10.93
CPAG_02752	orf19.5517*		Alcohol dehydrogenases	6.82	8.57
CPAG_01932	orf19.5604*		Member of the MDR family of major facilitator transporter superfamily	7.41	5.06
CPAG_01173	orf19.5860*		Uncharacterized	6.54	4.63
CPAG_01317	orf19.5806	<i>ALD5</i>	Aldehyde dehydrogenase	3.12	4.72
CPAG_03039	orf19.6348		Not essential for viability	3.16	4.50
CPAG_03040	orf19.6349		Uncharacterized	3.63	3.92
CPAG_00536	orf19.909	<i>STP4</i>	Putative transcription factor	3.34	4.66

RESULTS

CPAG ID ^a	<i>C. albicans</i> homolog ^b	Gene name ^c	Description ^c	BC014R _{FLC} ^d	BC014R _{IRC} ^d
CPAG_01908	orf19.4907		Uncharacterized	3.51	3.68
CPAG_03665	orf19.1027	<i>PDR16</i>	Phosphatidylinositol transfer protein	5.17	3.68
CPAG_04263	orf19.7235		Uncharacterized	3.92	3.76
CPAG_04761	orf19.7166		Uncharacterized	2.97	6.77
CPAG_03215	orf19.4609		Uncharacterized	3.48	5.13
CPAG_05176	orf19.3131	<i>OYE32</i>	NAD(P)H oxidoreductase	2.69	8.75
CPAG_03284	orf19.4886		Uncharacterized	3.76	7.46
CPAG_02183	orf19.6671	<i>LAP4</i>	Similar to aminopeptidase I	2.55	3.12
CPAG_00234	orf19.3661		Uncharacterized	2.60	2.89
CPAG_00905	orf19.3045		Uncharacterized	2.68	2.55
CPAG_03631	orf19.6838		Uncharacterized	2.25	3.14
CPAG_00668	orf19.2726		Uncharacterized	2.13	2.62
CPAG_02710	orf19.3442		Uncharacterized	1.90	2.91
CPAG_03888	orf19.1663	<i>MNT2</i>	Alpha-1,2-mannosyl transferase	2.44	2.16
CPAG_03148	orf19.4592	<i>HXS11</i>	UDP-glucose:ceramide glucosyltransferase	2.25	1.99
CPAG_02044	orf19.1139		Uncharacterized	2.10	2.41
CPAG_00123	orf19.5611		Uncharacterized	1.99	2.17
CPAG_00563				1.93	2.36
CPAG_04323	orf19.6594	<i>PLB3</i>	Secreted phospholipase B	3.89	2.53

CPAG ID ^a	<i>C. albicans</i> homolog ^b	Gene name ^c	Description ^c	BC014R _{FLC} ^d	BC014R _{VRC} ^d
CPAG_05296	orf19.1240		Uncharacterized	2.83	2.66
cpar744	orf19.7372*		Zn-finger transcription factor, related to Mrr1 (partial)	3.46	2.00
CPAG_02639	orf19.1839	<i>RPA190</i>	Uncharacterized	5.13	2.16
CPAG_02927	orf19.4393	<i>CIT1</i>	Citrate synthase	3.16	2.68
CPAG_03981	orf19.4688	<i>DAG7</i>	Uncharacterized	3.56	2.85
CPAG_05645	orf19.4377	<i>KRE1</i>	Predicted GPI-anchor, role in 1,6-beta-D-glucan biosynthesis	3.76	2.17
CPAG_04458	orf19.2829		Uncharacterized	2.79	2.16
CPAG_03615	orf19.756	<i>SAP7</i>	Secreted aspartyl proteinase	2.55	2.16
CPAG_00669	orf19.2724		Uncharacterized	2.10	2.26
CPAG_00730	orf19.655*		High-affinity phosphate transporters	2.51	2.17
CPAG_02152	orf19.932		Uncharacterized	2.55	3.43
CPAG_04056	orf19.1430		Uncharacterized	1.88	3.39
CPAG_04294	orf19.4765	<i>PAG6</i>	Putative GPI-anchored cell-wall protein	2.25	2.07
CPAG_03868	orf19.5383	<i>PMA1</i>	Plasma membrane H ⁺ -ATPase; fluconazole induced	2.19	2.07
Cluster 2					
CPAG_02122	orf19.3544		Uncharacterized	32.22	35.02
CPAG_03963	orf19.5604	<i>MDR1</i>	Member of the MDR family of major facilitator transporter superfamily	19.43	40.22

CPAG ID ^a	<i>C. albicans</i> homolog ^b	Gene name ^c	Description ^c	BC014R _{FLC} ^d	BC014R _{VR} ^d
CPAG_00506	orf19.5517		Alcohol dehydrogenases	22.63	29.45
CPAG_00978	orf19.320		Uncharacterized	18.38	24.42
CPAG_00478	orf19.2812*		Member of orf19.308 family	11.71	21.86
CPAG_03790	orf19.5604*		Member of the MDR family of major facilitator transporter superfamily	21.70	9.58
CPAG_03994	orf19.2693*		GST2/URE2 family	6.19	30.91
CPAG_03636	orf19.771	<i>LPG20</i>	Aldo-keto reductase family	6.50	22.63
CPAG_04322	orf19.6594		Secreted phospholipase B	3.92	15.89
<i>Cluster 3</i>					
CPAG_02182	orf19.4309	<i>GRP2</i>	Similar to <i>S. cerevisiae</i> Gre2p (methylglyoxal reductase)	107.63	207.94
CPAG_02538	orf19.4309*		Similar to <i>S. cerevisiae</i> Gre2p (methylglyoxal reductase)	71.51	148.06

^aCPAG ID from genome annotation of *C. parapsilosis* [75]. Where no CPAG gene has been annotated, the cpar annotation is used <http://cgob.ucd.ie>). Genes are listed in the order shown in Fig. 13.

^b*C. albicans* orthologs are identified where possible, from <http://cgob.ucd.ie>. Genes with * are homologs but not orthologs.

^c*C. albicans* gene names and descriptions are taken from the Candida Genome Database.

^dAverage FC in gene expression.

GENE EXPRESSION PROFILE OF BC014R_{PSC} STRAIN

The specific genes which showed the greatest change in gene expression profile of BC014R_{PSC} strain are depicted in Table 10 (full list is available in Table S3).

We observed an increased expression of the transcription factors *NDT80* and *UPC2* in BC014R_{PSC}, confirmed by RT-qPCR (Fig. 16). Ndt80p and Upc2p were recently identified in *C. albicans* [139, 215] as transcription factors that bind to the promoter regions of ergosterol biosynthesis genes.

Expression of *SOD4* gene was also up-regulated (2.68-fold) in the BC014R_{PSC} strain (also confirmed by RT-qPCR; Fig.16).

ERGOSTEROL BIOSYNTHESIS GENES

Several genes displayed in the expression profiles of the three resistant strains are linked to ergosterol biosynthesis. Figure 14 represents a simplified ergosterol pathway which depicts the genes involved in the process; the regulation level of such genes in BC014R_{FLC}, BC014R_{VRC} and BC014R_{PSC} strains is shown in Table 11.

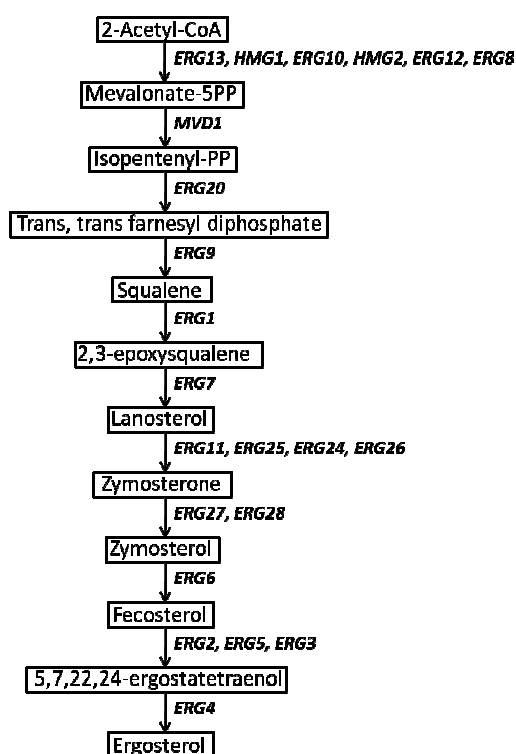


Figure 14. Simplified ergosterol metabolic pathway, as described for *S. cerevisiae* (<http://www.yeastgenome.org/>). Genes up-regulated or down-regulated in BC014R_{FLC}, BC014R_{VRC} and BC014R_{PSC} strains are indicated in the Table 11.

Table 10. Genes with increased expression in BC014R_{pSC} strain.

CPAG ID ^a	<i>C. albicans</i> homolog ^b	Gene name ^c	Description ^c	BC014R _{pSC} ^d
CPAG_00389	orf19.7077*		Protein with similarity to ferric reductase	10.27
CPAG_00124			Uncharacterized	6.68
CPAG_01721	orf19.3829	<i>PHR1</i>	Glycosidase of cell surface	5.78
CPAG_01381	orf19.5753*	<i>HGT10</i>	Glycerol permease involved in glycerol uptake	5.94
CPAG_05645	orf19.4377	<i>KRE1</i>	Predicted GPI-anchor, role in 1,6-beta-D-glucan biosynthesis	3.76
CPAG_01409	orf19.1031	<i>HMG1</i>	HMG-CoA reductase enzyme of sterol pathway	2.66
CPAG_01467	orf19.2311	<i>RPL82</i>	Predicted ribosomal protein	2.10
CPAG_02523	orf19.4336	<i>RP55</i>	Predicted ribosomal protein	2.87
CPAG_03665	orf19.1027	<i>PDR16</i>	Phosphatidylinositol transfer protein	2.93
CPAG_01511	orf19.1313	<i>CDR3</i>	Transporter of the Pdrp/Cdrp family of the ATP-binding cassette (ABC) superfamily	2.99
CPAG_02597	orf19.2501	<i>FLC1</i>	Protein involved in heme uptake	2.28
CPAG_05474	orf19.1813	<i>FLC2</i>	Protein involved in heme uptake	2.03
CPAG_04555	orf19.2062	<i>SOD4</i>	Copper- and zinc- containing superoxide dismutase	2.68
CPAG_03048	orf19.6026*	<i>ERG2</i>	C-8 sterol isomerase; enzyme of ergosterol biosynthesis pathway	6.50
CPAG_05078	orf19.1631	<i>ERG6</i>	Delta(24)-sterol C-methyltransferase; enzyme of ergosterol biosynthesis pathway	5.24
CPAG_04483	orf19.3732	<i>ERG25</i>	Putative C-4 methyl sterol oxidase; enzyme of ergosterol biosynthesis pathway	5.50
CPAG_03871	orf19.5379	<i>ERG4</i>	Protein described as similar to sterol C-24 reductase; enzyme of ergosterol biosynthesis pathway	4.14
CPAG_05162	orf19.1598	<i>ERG24</i>	C-14 sterol reductase; enzyme of ergosterol biosynthesis pathway	4.08
CPAG_00173	orf19.7312*	<i>ERG13</i>	Protein similar to <i>S. cerevisiae</i> Erg13p; enzyme of ergosterol biosynthesis pathway	3.68

CPAG ID ^a	<i>C. albicans</i> homolog ^b	Gene name ^c	Description ^c	BC014R _{PSC} ^d
CPAG_04530	orf19.1591	<i>ERG10</i>	Protein similar to acetyl-CoA acetyltransferase; enzyme of ergosterol biosynthesis pathway	2.91
CPAG_03624	orf19.767	<i>ERG3</i>	C-5 sterol desaturase; enzyme of ergosterol biosynthesis pathway	3.25
CPAG_03310	orf19.922	<i>ERG11</i>	Lanosterol 14- α -demethylase; enzyme of ergosterol biosynthesis pathway	2.89
CPAG_05249	orf19.3616	<i>ERG9</i>	Putative farnesyl-diphosphate farnesyl transferase; enzyme of ergosterol biosynthesis pathway	2.53
CPAG_03443	orf19.5178	<i>ERG5</i>	Putative C-22 sterol desaturase; enzyme of ergosterol biosynthesis pathway	2.08
CPAG_02967	orf19.3240	<i>ERG27</i>	3-keto sterol reductase of ergosterol biosynthesis; enzyme of ergosterol biosynthesis	2.20
CPAG_03183	orf19.4631	<i>ERG251</i>	Uncharacterized	2.14
CPAG_04608	orf19.2119	<i>NDT80</i>	Activator of <i>CDR1</i> induction by antifungal drugs and transcriptional regulator of ergosterol biosynthesis genes	2.53
CPAG_01906	orf19.391	<i>UPC2</i>	Transcriptional regulator of ergosterol biosynthesis genes and sterol uptake	1.93

^aCPAG ID from genome annotation of *C. parapsilosis* [75]. Where no CPAG gene has been annotated, the cpar annotation is used (see <http://cgob.ucd.ie>).

^b*C. albicans* orthologs are identified where possible, from <http://cgob.ucd.ie>. Genes with * are homologs but not orthologs.

^c*C. albicans* gene names and descriptions are taken from the Candida Genome Database.

^dAverage FC in gene expression.

Table 11. Genes from ergosterol biosynthesis process up-regulated (+) or down-regulated (-) in BC014R_{FLC}, BC014R_{VR}C and BC014R_{PSC} strains.

Gene name ^a	BC014 _{FLC} ^b	BC014 _{VR} C ^b	BC014 _{PSC} ^b
<i>ERG13</i>			+3.69
<i>HMG1</i>			+2.66
<i>ERG10</i>			+2.91
<i>ERG9</i>			+2.52
<i>ERG1</i>	-2.60	-2.28	
<i>ERG7</i>			+2.15
<i>ERG11</i>	-2.69	-2.01	+2.89
<i>ERG25</i>	-2.24		+5.52
<i>ERG24</i>			+4.10
<i>ERG27</i>			+2.20
<i>ERG6</i>			+5.24
<i>ERG2</i>	-2.01		+6.52
<i>ERG5</i>			+2.10
<i>ERG3</i>	-4.86		+3.24
<i>ERG4</i>			+4.14

^a*C. albicans* gene names are taken from the Candida Genome Database.

^bAverage FC in gene expression.

The expression of *ERG1* and *ERG11* is reduced in BC014R_{FLC} (2.60- and 2.69-fold respectively) and in BC014R_{VR}C (2.28- and 2.01-fold respectively). Additionally, the expression of *ERG2*, *ERG3* and *ERG25* is reduced in BC014R_{FLC} (2.01-, 4.86- and -2.24-fold respectively). The expression of *ERG11* gene was confirmed for both strains by RT-qPCR (Fig.16).

In contrast, BC014R_{PSC} strain displayed an over-expression of thirteen genes directly linked to ergosterol biosynthesis (Table 11, Fig. 14). The expression of *ERG11* and *ERG6* genes was confirmed by RT-qPCR (Fig.16).

ANTIFUNGAL TRANSPORTER GENES

The *C. parapsilosis* genome encodes at least 14 members of the ABC transporter family [75]. The expression of one of these transporters, *CPAG_0151*, is up-regulated (2.30-fold) in the BC014R_{PSC} strain (Table 10). Expression of *CPAG_02192*, another ABC transporter, is up-regulated both in BC014R_{FLC} and BC014R_{VR}C strains (Table 9). Expression of *CPAG_03665*, an ortholog of *C. albicans* *PDR16*, is up-regulated 5.17-, 3.68- and 2.93-fold in BC014R_{FLC}, BC014R_{VR}C and BC014R_{PSC} strains respectively (Table 9 and Table 10).

BC014R_{FLC} and BC014R_{VRC} strains displayed a decreased expression of *CPAG_03819* (Fig. 13 and Table S5); this gene is an ortholog of *C. albicans orf19.3902*, and its reduced expression is correlated with over-expression of *CDR1* and *CDR2* in *C. albicans* azole resistant isolates [216]. The expression of *CPAG_02274 (SNQ2)* was down-regulated in BC014R_{FLC} and BC014R_{VRC} strains (Fig. 13 and Table S5), while the expression of the other ABC transporter family members remained unchanged.

COPPER TRANSPORT AND IRON MOBOLIZATION RELATED GENES

The expression levels of several genes involved in copper and iron transportation were found to be changed in all three azole resistant *C. parapsilosis* strains: *CTR1* (copper transporter; 4.53-fold) and *FRE10* (ferric reductase; 2.08-fold) are up-regulated while *CRP1* (copper transporter; 30.70-fold) is down-regulated in BC014R_{FLC} strain; *FTR1* (iron membrane permease; 2.25-fold) is down-regulated in BC014R_{VRC} strain; *CTR1* (6.32-fold) is up-regulated while *CRP1* (28.25-fold) is down-regulated in BC014R_{PSC} strain (Tables S1-S3).

Expression of the two genes *FLC1* and *FLC2* involved in heme uptake [217] is increased 2.28- and 2.03-fold, respectively, in the BC014R_{PSC} strain (Table 10).

MICROARRAYS (1) WITH *IN VIVO* INDUCED STRAIN

Despite the low quality of the array that compared the susceptible strain (BC_{A1}) with the resistant strain (BC_{A3}) from the *in vivo* case, relevant data could be extracted. Microarray analysis revealed 198 genes (150 up-regulated and 48 down-regulated) differently expressed (all genes are listed in Table S6). GO analysis of processes is represented in Fig. 15; up to 27% of the up-regulated genes are associated with membrane transport. The total number of genes displaying expression changes is much lower than the number revealed by microarray analysis of BC014S *versus* BC014R_{FLC} strains. Moreover, the processes showing up- and down-regulation in the *in vivo* resistant strain are quite different comparing to the *in vitro* induced resistant strains.

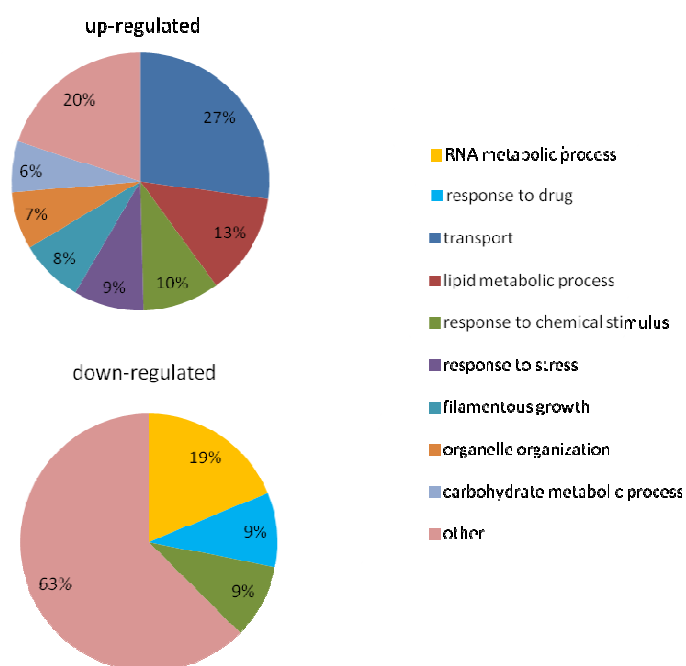


Figure 15. Selected process GO terms overrepresented (up- and down-regulated) in BC_{A3} strain. The full list is available in Table S6.

GENE EXPRESSION

The expression level of certain genes involved in azole resistance mechanisms and others that were found altered in microarray analysis of BC014R_{FLC}, BC014R_{VRC} and BC014R_{PSC} strains was assessed by RT-qPCR. Results are presented in Fig. 16. These results confirm the gene expression results obtained with microarray assays, already mentioned throughout the text.

Regarding the *in vivo* strains, the expression of *ERG11*, *ERG6*, *NDT80* and *UPC2* genes for BC_{A3} strain was quantified (Fig. 17). The expression level for resistant strain is higher.

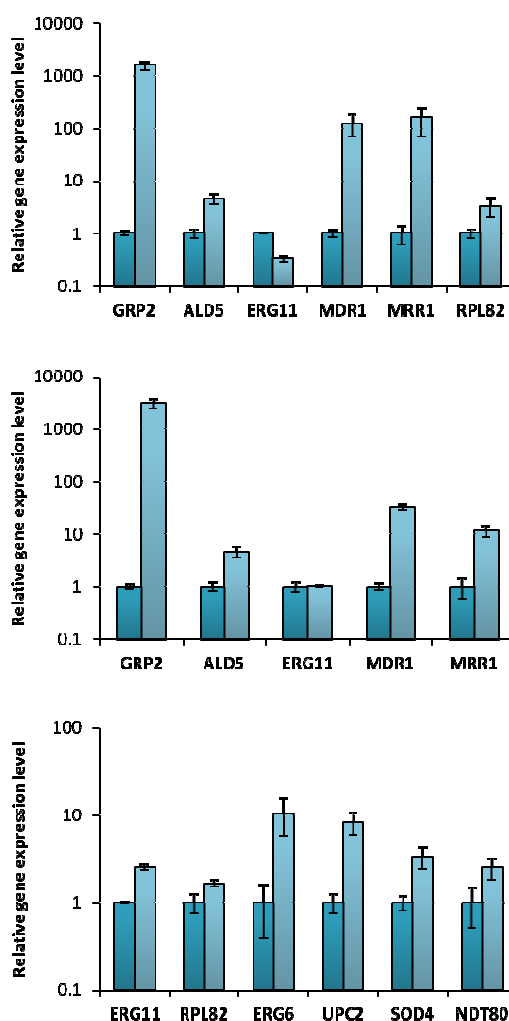


Figure 16. RT-qPCR analysis of genes implicated in antifungal resistance (*ERG5*, *ERG11*, *MDR1*, *MRR1*, *NTD80*), stress response (*GRP2*) and metabolism (*ALD5*, *RPL82*). Blue dark bars represent the susceptible strain (BC014S); blue light bars represent BC014R_{FLC} (a), BC014R_{VRC} (b) and BC0114R_{PSC} (c) resistant strains. The y axis scale is logarithmic due to the large variance in gene expression levels. The expression level shown for each gene represents the variation in gene expression relative to the susceptible strain and is expressed as average with standard deviation of five independent experiments. Each mean value was normalized to the amplification efficiency and to the amount of *TUB4* (endogenous reference gene). All genes displayed similar levels of expression to microarray results.

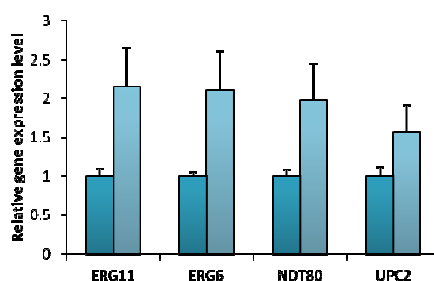


Figure 17. RT-qPCR analysis of *ERG11*, *ERG6*, *NDT80* and *UPC2* genes. The blue dark bar represents the susceptible strain (BC_{A1}) and the blue light bar represents the resistant strain (BC_{A3}). The expression level shown for the gene represents the variation in gene expression relative to the susceptible strain and is expressed as average with standard deviation of five independent experiments. The mean value was normalized to the amplification efficiency and to the amount of *TUB4* (endogenous reference gene). All genes displayed similar levels of expression to microarray results.

SUSCEPTIBILITY TO PHYSICAL AND CHEMICAL AGENTS

ANTIFUNGAL ACTIVITY OF PHYSICAL AGENTS

The effect of heating at 60°C, microwave and ultraviolet irradiation against *C. parapsilosis* strains is shown in Fig. 18. Graphics were obtained after plotting the number of CFUs obtained after a specific time of treatment. After 5 min of exposure at 60°C the number of CFUs decreased 3 *log* for clinical and environmental strains. After 45 min the number decreased 5 *log* for clinical strains and 4 *log* for environmental strains. Regarding microwave irradiation treatment, after 20 and 40 s of exposure, the reduction is scarce independently the strains origin, however, after 60 s and 90 s the reduction was 3 *log* for clinical strains and 5 *log* for environmental strains. When exposed to ultraviolet irradiation there was a reduction of 1 *log* after 2 min for clinical strains and 3 min for environmental strains. With the maximum time of exposure (10 min) the reduction among clinical strains was higher than the reduction of environmental strains (7 and 4 *log*, respectively).

ANTIFUNGAL ACTIVITY OF CHEMICAL AGENTS

The results of *in vitro* susceptibility of *C. parapsilosis* strains to chemical agents (pure compounds) are shown in Table 12; for each chemical agent MIC and MLC values range is represented. No significant differences were found regarding the antifungal effect of chemical agents against clinical and environmental strains. The concentrations of 0.06% of chlorhexidine, 10% of ethanol, 2.5% of sodium hypochlorite, 0.06% of hydrogen peroxide, 0.1% of potassium permanganate and 1% of acid boric, inhibited all *C. parapsilosis* strains growth, independently of its endogenous or exogenous origin. Due to the proximity between MIC and MLC values (only one or two dilutions apart) all chemical seems to have a fungicidal effect upon *C. parapsilosis*.

Table 12. MIC and MLC values range for chemical agents (pure compounds) against all 16 *C. parapsilosis* strains.

Chemical agent	MIC range (%)	MLC range (%)
Chlorhexidine	0.004-0.06	0.008-0.06
Ethanol	5-10	5-10
Sodium hypochlorite	0.625-2.5	0.625-2.5
Hydrogen peroxide	0.03	0.06
Potassium permanganate	<0.0008-0.1	0.0015-0.1
Acid Boric	0.125-0.25	0.25-1

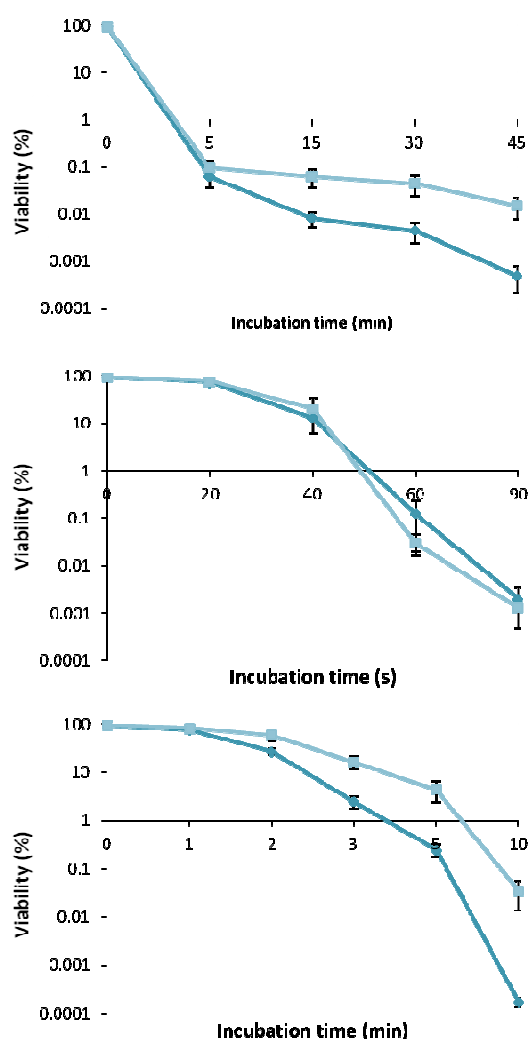


Figure 18. Effect of heating at 60°C (a), microwave irradiation (b) and ultraviolet irradiation (c) upon the survival of clinical (—◆—) and environmental (—■—) *C. parapsilosis* strains. Each point represents the geometric mean of the number of CFUs for all strains.

Figure 19 depicts the result of the time-kill assays with commercial solutions for all 16 *C. parapsilosis* strains. No differences were found between clinical and environmental strains. Handscrub, Aniosgel 85 NPC and Solution Dakin revealed to be the most active compounds.

Considering the MLC values achieved with the pure compounds, the results with Handscrub (chlorhexidine 0.4%) and Angiosgel 85 NPC (ethanol 70%) solutions were expected, since the solution concentration is higher than MLC values. Conversely, the results obtained with Hydrogen Peroxide 3% and Acid Boric 3% were quite different; despite the solution concentration is higher than MLC values it could not kill all cells at least in 30 min; Solution Dakin

(0.5% sodium hypochlorite) and Potassium Permanganate 0.01% were efficient even at a lower concentration than MLC values (the highest value from MLC range).

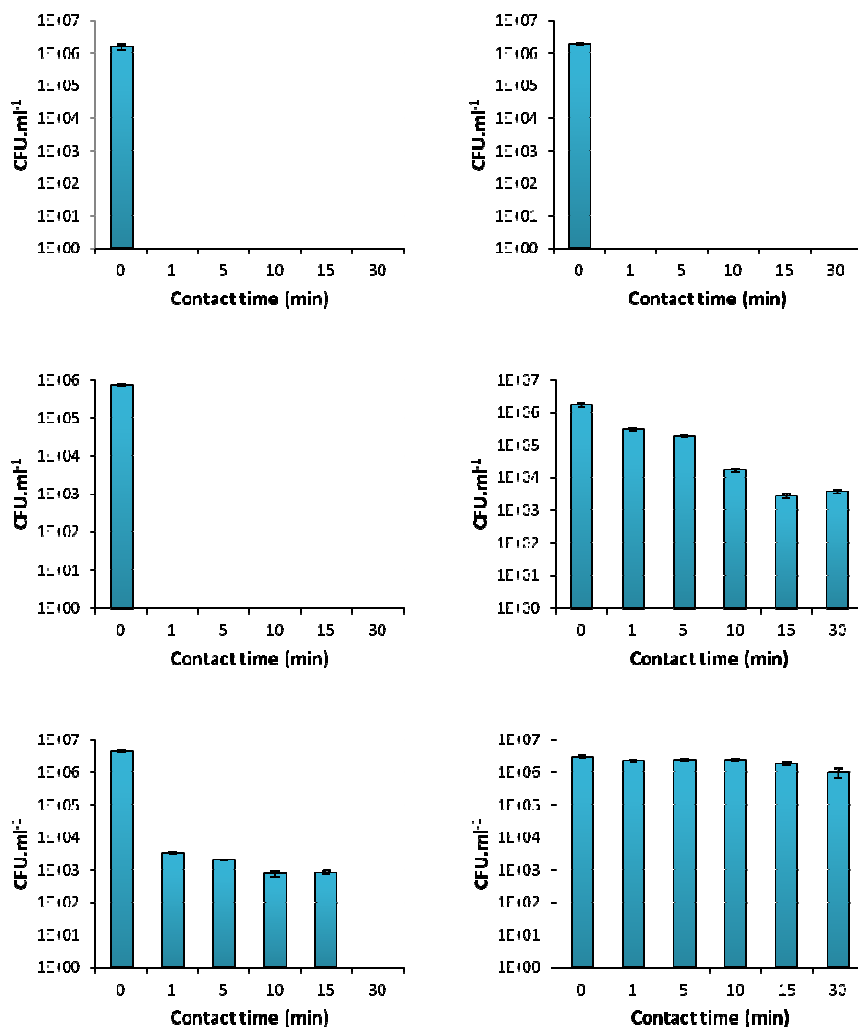


Figure 19. Effect of different commercial solutions upon the reduction of *C. parapsilosis* CFUs according to the contact time. Growth inhibition was determined comparing the test plates with control plates unexposed to solutions (contact time 0). a, Hanscrub; b, Aniosgel 85 NPC; c, Solution Dakin; d, Hydrogen Peroxide; e, Potassium Permanganate; and f, Acid Boric.

IP STAINING

The cell membrane lesion resulting from the activity of physical and chemical agents (commercial solutions) was characterized by flow cytometry. Regarding physical agents, both heating at 60°C and microwave irradiation caused evident cell membrane lesion (Table 13 and Fig. 20). Regarding chemical agents, only after Hydrogen Peroxide exposure an increase in PI stain was observed during time (Fig. 21).

Table 13. Propidium iodide (PI) staining (%) of one *C. parapsilosis* strain tested (values represent geometric means), following treatments with physical agents.

Treatment with	PI staining (%)
Heating at 60°C (45 min)	89.75±7.12
Microwave irradiation (90 s)	90.54±12.36
Ultraviolet irradiation (10 min)	14.53±2.54
Control live cells	1.50±0.45
Dead live cells	81.38±14.87

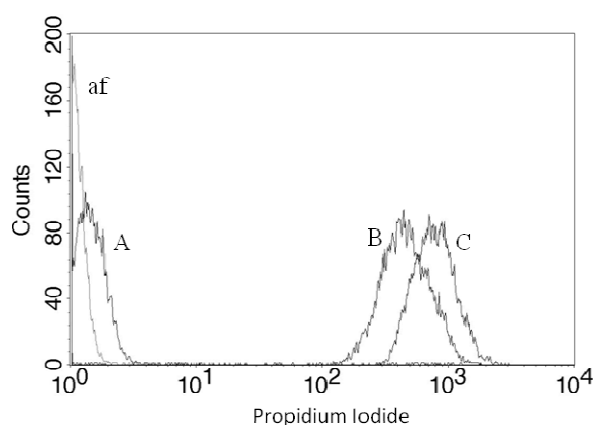


Figure 20. Propidium iodide staining of one *C. parapsilosis* strain after treatment with microwave irradiation. Histogram obtained by flow cytometric analysis at FL3 (650 nm). af, autofluorescence; A, control live cells; B, control dead cells; and C, treated cells with microwave irradiation for 90 s.

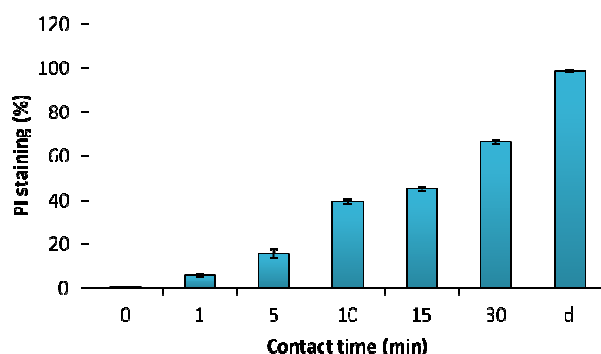


Figure 21. Propidium iodide staining (%) of one *C. parapsilosis* strain after exposed (at different times) to Hydrogen Peroxide 3%. 0, control live cells; d, control dead cells.

EFFICACY OF HYDROGEN PEROXIDE DRY-MIST SYSTEM

In order to evaluate the antimicrobial efficacy of hydrogen peroxide/silver cations dry-mist system against *C. parapsilosis*, *in vitro* studies were performed. A reduction in growth of 5 *log* was observed following exposure; such results were obtained both with ATCC and clinical strains. For other fungi tested the reduction was also 5 *log*; for bacteria was 7 *log* (manuscript VII in Publication section).

The MIC value obtained when testing *C. parapsilosis* ATCC 22019 was 0.02% of hydrogen peroxide and <0.2 ppm of silver cations (Table 14). The results concerning other species tested are detailed in Table 14.

The microbicidal activity of disinfectant solution was confirmed by flow cytometry. Figure 22 represents the percentage of PI staining cells of *C. parapsilosis* untreated and treated during 5 min with the disinfectant; this cell population was significantly stained, meaning that dead was due to membrane lesion induced by exposure to the disinfectant.

Table 14. Results of susceptibility testing of disinfectant solution against selected bacterial and fungal species. For each strain the dilution and the respective concentration of the disinfectant that inhibited growth corresponding to the MIC value is represented.

Strain	Dilution	Concentration (hydrogen peroxide % and
		silver cations ppm)
<i>A. baumannii</i>	1:128	0.04 and <0.4
<i>E. coli</i>	1:128	0.04 and <0.4
<i>S. aureus</i>	1:128	0.04 and <0.4
<i>P. aeruginosa</i>	1:128	0.04 and <0.4
<i>E. faecalis</i>	1:128	0.04 and <0.4
<i>C. krusei</i>	1:512	0.01 and <0.1
<i>C. parapsilosis</i>	1:256	0.02 and <0.2
<i>C. albicans</i>	1:256	0.02 and <0.2
<i>A. fumigatus</i>	1:256	0.02 and <0.2
<i>P. marneffeii</i>	1:256	0.02 and <0.2
<i>M. tuberculosis</i>	1:280	0.02 and <0.2

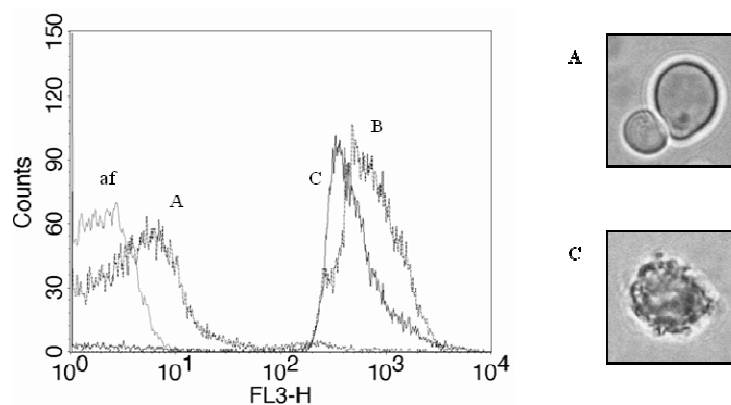


Figure 22. (a) Histogram obtained by flow cytometric analysis at FL3 (650 nm) of *C. parapsilosis* cells stained with propidium iodide, following 5 min of treatment with disinfectant solution. af, autofluorescence; A, control live cells; B, control dead cells; and C, cells treated with disinfectant solution. (b) Optical microscopy photograph of control live cells (A) and of cells treated with disinfectant solution (C).

VIRULENCE ATTRIBUTES

GROWTH OF STRAINS

Resistant strain (BC014R_{PSC}) was compared to its parent susceptible strain (BC014S) for growth capacity at 37°C. BC014R_{PSC} strain had a prominent log phase compared to BC014S and its total growth yield after 24 h to 48 h was higher; nonetheless, the two strains displayed almost overlapping growth curves in the lag phase (Fig. 23).

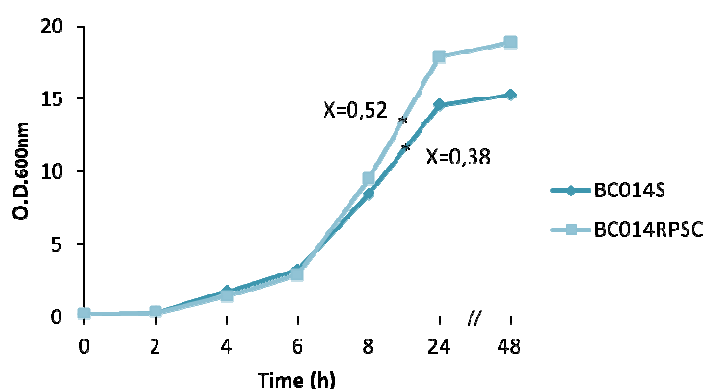


Figure 23. Growth curves of BC014S and BC014R_{PSC} strains. The growth curves were obtained from cultures at 37°C in YEPD broth. The growth rate (X) is represents for each strain.

IDENTIFICATION OF COLONY PHENOTYPES

For the susceptible strain BC014S two phenotypes (smooth and crepe) were identified (Fig. 24a). For the resistant strain BC014R_{PSC} two phenotypes (smooth and concentric) were also identified (Fig. 24a). Cells from each phenotype were photographed (Fig. 25). Moreover, for BC014R_{PSC} we identified other cell-phase forms, namely white-opaque (Fig. 24b), considered here smooth phenotype, and myceliated-unmyceliated (Fig. 24c), giving rise to sectorized colonies. Under microscopy, it was evident that the cell morphology of the phenotypes varies considerably (Fig. 25). Whilst the crepe and concentric phenotypes correspond to mostly pseudohyphae, cells from the smooth phenotype are small and yeast-shaped. In opaque section of BC014R_{PSC} colonies cells have a pseudohyphal form.

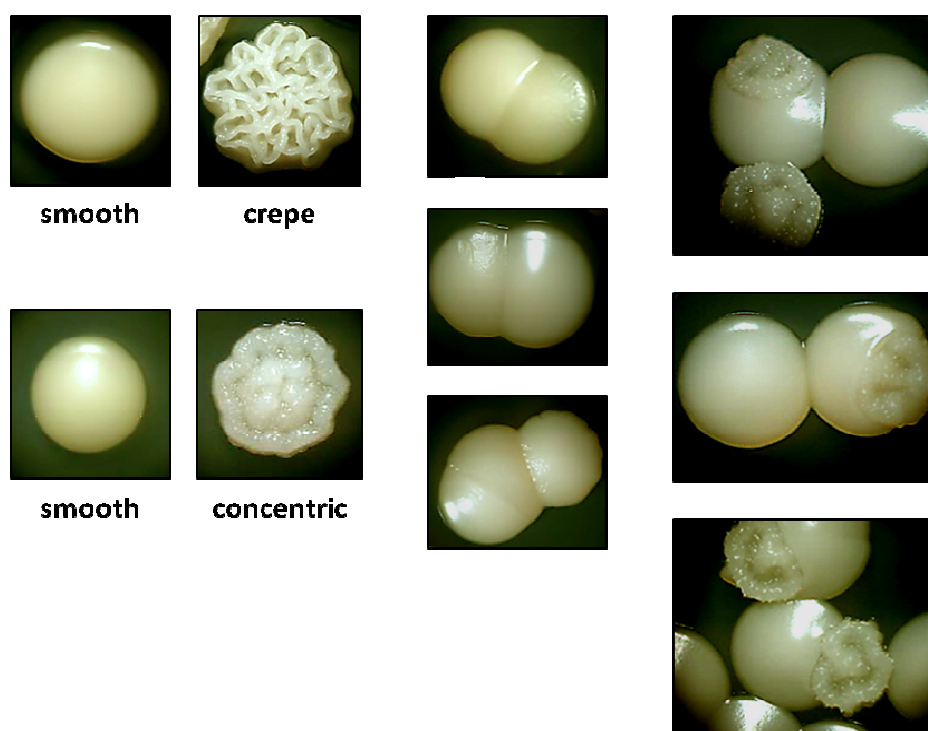


Figure 24. Colony morphotypes of BC014S and BC014R_{PSC} strains. (a) Representative colonies of phenotypes from BC014S strain (up) and from BC014R_{PSC} strain (down). (b) White-opaque form in BC014R_{PSC} strain. (c) Myceliated-unmyceliated form in BC014R_{PSC} strain.

To address the frequency of switching, single nonsectored colonies from each morphology type were disaggregated, and cells from the individual colonies were plated. In each case, we determined the fraction of new colonies (excluding mixed colony types) that resembled the parental colony morphology as well as the fraction of spontaneous variants. Figure 26 summarizes the switching results. Regarding smooth and crepe phenotypes, the majority of the new colonies exhibited the same colony morphology seen in the parent colony. The same was not observed for the concentric phenotype of BC014R_{PSC}.

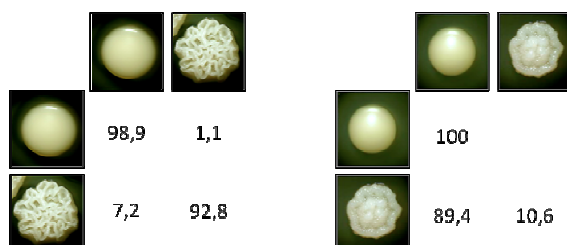


Figure 26. Switching in BC014S (a) and BC014R_{PSC} strains (b). The proportion (in percentage) of colony variants emanating from a single non-sectored colony of the indicated morphology are shown in vertical columns. Top bar from left to right: (a) smooth and crepe; (b) smooth and concentric. The results represent the average results from ten plates.

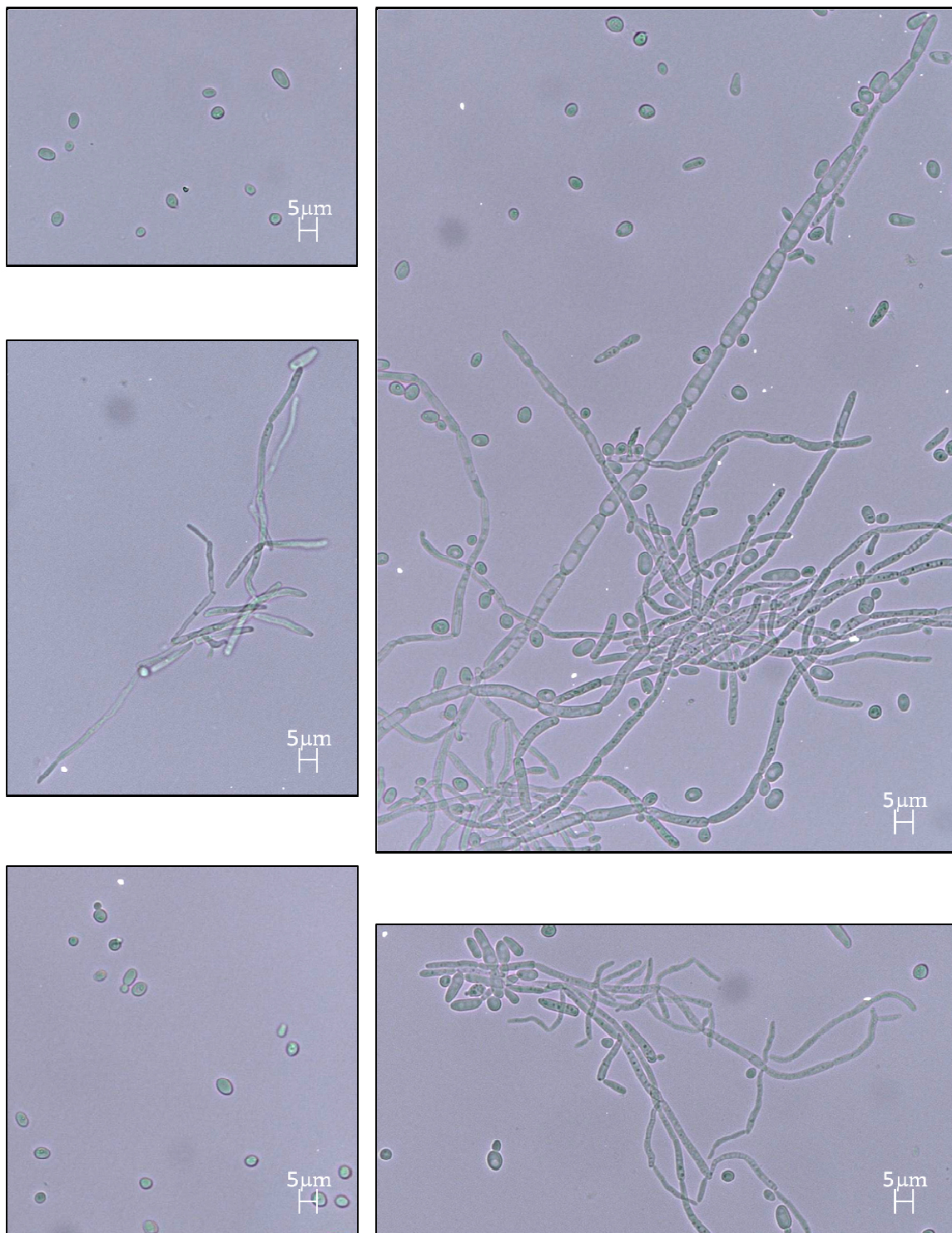


Figure 25. Cells from different colony morphotypes of BC014S and BC014R_{pSC} strains, photographed under the same magnification (40 X). (a) Smooth phenotype of BC014S strain. (b) Crepe phenotype of BC014S strain. (c) Smooth phenotype of BC014R_{pSC} strain. (d) Concentric phenotype of BC014R_{pSC} strain. (e) Cells from opaque colonies of BC014R_{pSC} strain.

AGAR INVASION

The effect of phenotype upon agar invasion ability by BC014S and BC014R_{pSC} strains was determined. The colonies were photographed and then washed off the plate with running water (Fig. 27). The mass remaining after washing is an indication of the amount of invasion of the agar. The crepe phenotype of BC014S strain is the greatest invader, leaving a larger mass of cells after washing in the agar; then the concentric phenotype of BC014R_{pSC} and at last the smooth phenotype of both strains, which invades in a much lesser extent.

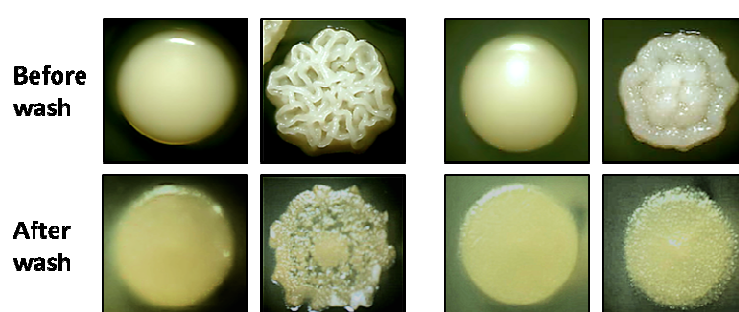


Figure 27. Agar invasion by the morphotypes of BC014S (a) and BC014R_{pSC} (b) strains. Equal volumes and cell numbers from stationary-phase cultures of the four phenotypes were spotted onto YEPD agar plates and incubated at 30°C for 30 days. Colonies were photographed before and after removing cells on the surface by washing under running water.

PSEUDOHYPHAL DEVELOPMENT

Experiments were carried out to assess the capacity of each strain to produce pseudohyphae *in vitro*. Figure 28 shows that resistant strain (BC014R_{pSC}) was capable of producing more numerous and longer pseudohyphal filaments in rich serum-based medium than the susceptible parent strain (BC014S).

RESULTS

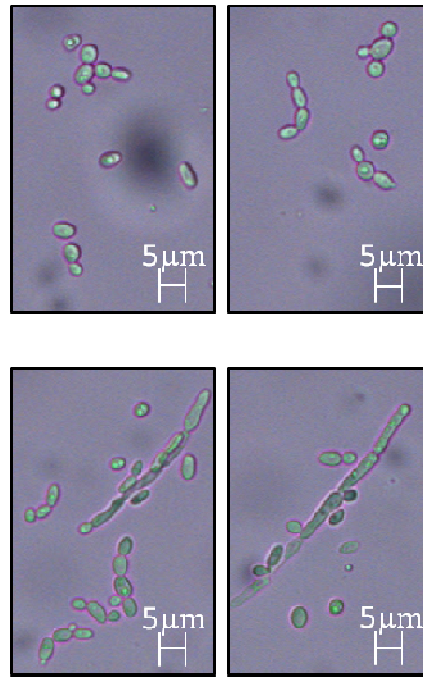


Figure 28. Pseudohyphal development of BC014S (a) and BC014R_{PSC} (b) strains photographed under the same magnification (40 X). Cells were induced to form pseudohyphae by 24 h incubation at 37°C in RPMI 1640 medium containing 10% fetal calf medium. The photographs are representative of the whole-mounted microscopic plates.

ADHERENCE

Flow cytometric results regarding adherence are shown in Fig. 29. Adherence of BC014R_{PSC} strain was higher than BC014S strain. Similar results were obtained with the classic methodology (data not shown).

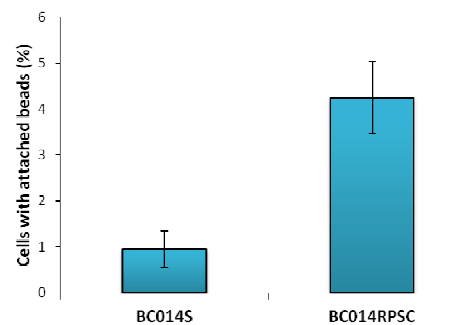


Figure 29. Adhesion ability (%) displayed by BC014S and BC014R_{PSC} strains to polisterene beads assessed by flow cytometric assay. Each result is representative of three experiments.

BIOFILM FORMATION

We determined the ability of the BC014S and BC014R_{PSC} strains to form biofilm. BC014S strain produced more biofilm when compared with BC014R_{PSC} (Fig. 30). Both the CV and XTT assays showed similar results.

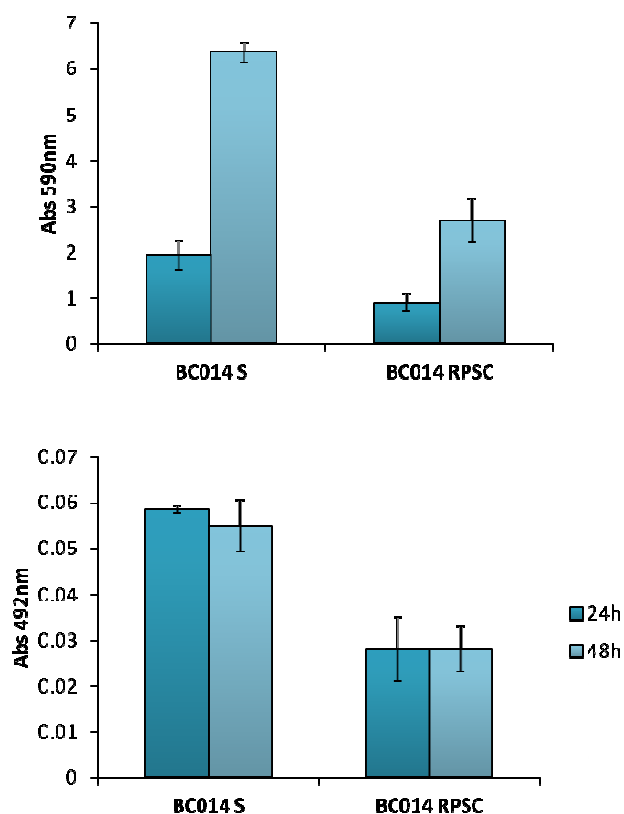


Figure 30. Biofilm formation by BC014S and BC014R_{PSC} strains. The biofilm mass was measured by CV assay (a) and the metabolic activity by a XTT assay (b). The values represent the mean $\pm$ standard deviation absorbance values, from three independent biofilms.

ENZYMES EXPRESSION

Coagulase production was assessed by the classical tube test for BC014S and BC014R_{PSC} strains. Both strains were coagulase negative (data not shown).

Catalase production for BC014S and BC014R_{PSC} strains was assessed by growth inhibition in the presence of hydrogen peroxide 25 mM (Fig. 31). BC014R_{PSC} strain growth is higher than the growth observed for BC014S strain, therefore BC014R_{PSC} strain supports better the oxidative stress.

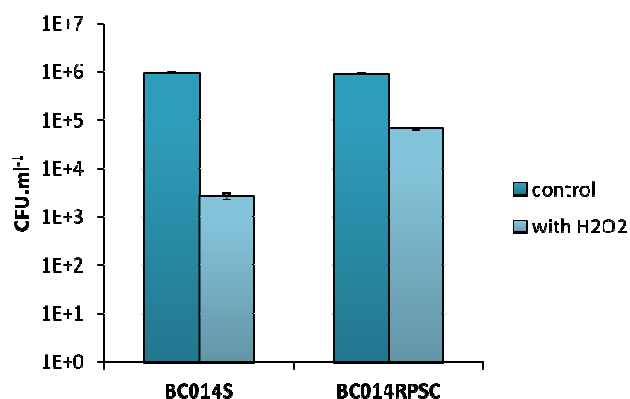


Figure 31. Growth inhibition of BC014S and BC014R_{PSC} strains in the presence of hydrogen peroxide 25 mM. Each result is representative of three experiments.

MICROARRAYS (2): TRANSCRIPTION PROFILING

The microarrays platform was used to elucidate the antifungal resistance mechanisms, as already described (Antifungal Resistance Mechanisms section). Information regarding virulence attributes was also extracted from the microarray results.

Microarray analysis identified 598 genes (341 up-regulated and 257 down-regulated) with differential expression (FC >2, $p < 0.05$) in BC014R_{PSC} strain. The full gene list is available in Table S3 and the confirmation of expression of certain genes by RT-qPCR was previously mentioned (Antifungal Resistance Mechanisms section).

Groups of genes (filamentous growth, lipid metabolic process, hyphal growth, pathogenesis, response to stress, interaction with host, pseudohyphal growth, cell budding, cell adhesion, biofilm formation and protein catabolic process) associated with fungal virulence were found to be overrepresented in the resistant strain. Table 15 details such findings (full list is available in Table S4).

Table 15. GO terms overrepresent in BC014R_{PSC} strain.

Cluster frequency ^a	Background frequency ^a	p-value	GO term
50/409	310/5085	7.20E-4	Response to drug
56/409	393/5058	8.57E-3	Filamentous growth
70/409	504/5085	1.45E-3	Response to chemical stimulus
31/409	177/5085	1.93E-2	Pathogenesis
57/409	413/5085	1.88E-2	Growth

^aCluster frequency: fraction of genes that are differentially regulated with homologs identified in *C. albicans* assigned to GO term; Background frequency: fraction of entire dataset with homologs identified in *C. albicans* assigned to GO term.

CHITIN CONTENT

CFW RESISTANCE

Susceptible profiles of *C. parapsilosis* strains included in this study are represented in Table 16. These strains were screened against CFW to determine its sensitivity to this cell-wall-damaging agent (Fig. 32). Increased resistance to CFW is indicative of a reduction in cell-wall chitin content. Overall, *C. parapsilosis* strains displayed different sensitivity levels to CFW, meaning different amounts in chitin. The sensitivity to CFW is unrelated to the antifungal susceptible profile to echinocandins. *In vitro* induction of echinocandin resistance with anidulafungin increased cells chitin content (BC041S *versus* BC041NS).

Table 16. MIC value ($\mu\text{g.ml}^{-1}$) and susceptibility phenotype displayed by *C. parapsilosis* strains to echinocandins.

Strain	MIC/Phenotype ^a		
	CSF	ANF	MCF
BC011	4/NS	1/S	2/S
BC017	4/NS	8/NS	2/S
BC022	4/NS	8/NS	8/NS
BC002	2/S	1/S	0.5/S
BC021	0.5/S	2/S	1/S
BC034	2/S	0.5/S	0.5/S

^aS, susceptible (MIC $\leq 2 \mu\text{g.ml}^{-1}$); NS, nonsusceptible.

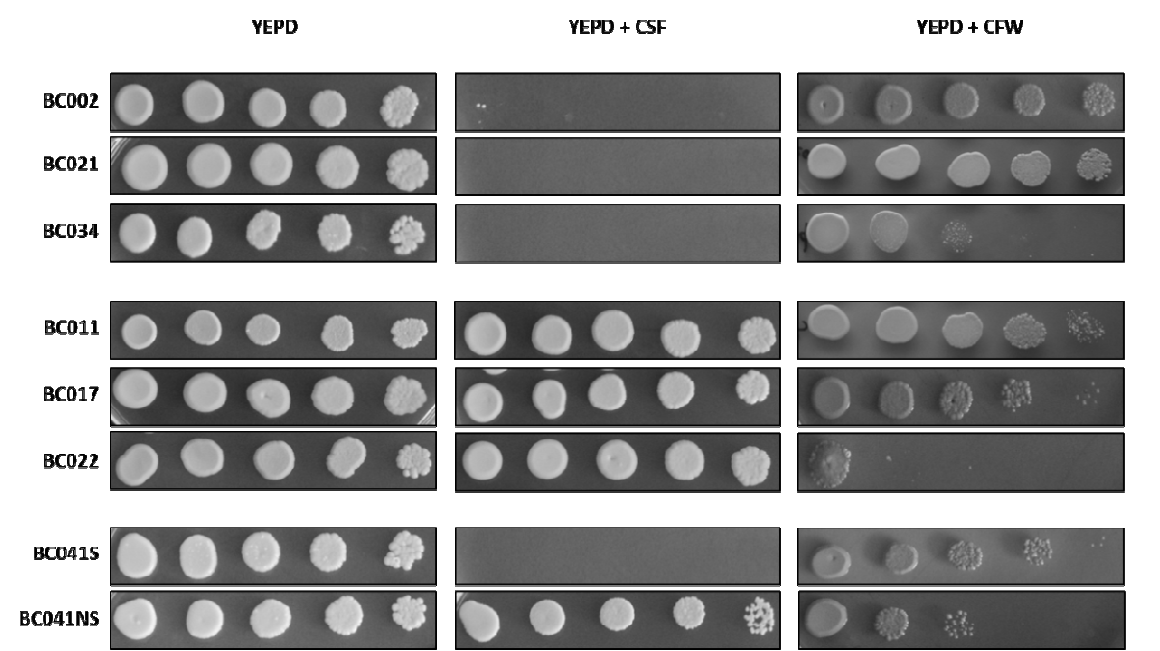


Figure 32. Sensitivity of *Candida parapsilosis* strains to Calcofluor White (CFW). *C. parapsilosis* strains were grown in YEPD overnight; serial dilutions corresponding to 10^6 - 10^2 yeast cells were spotted in solid medium containing caspofungin (CSF) $4\ \mu\text{g.ml}^{-1}$ or CFW $50\ \mu\text{g.ml}^{-1}$ and plates were incubated at 30°C for 3 days. BC002, BC021 and BC034 are echinocandin susceptible strains and BC011, BC017 and BC022 are echinocandin nonsusceptible strains (see Table 16); BC041S and BC041NS are strains from the *in vitro* induction assay.

FLOW CYTOMETRY

The results obtained by flow cytometry are detailed in Fig. 33a. This method revealed the discrepancy in chitin content of control strains, SC5314 and Δchs3 , which have different chitin content levels measured by a classic methodology as already described [146]. *C. parapsilosis* strains tested exhibited different chitin contents, as demonstrated previously with CFW. Interestingly, the strain with higher chitin level is nonsusceptible and the *in vitro* induced nonsusceptible strain (BC041NS) exhibited higher chitin level than the parent strain. The results were consistent with epifluorescence microscopy observations (representative images are represented in Fig. 33b). Overall, the quantification of chitin content of all strains did not allow establishing a relation between chitin levels and NS phenotype to echinocandins.

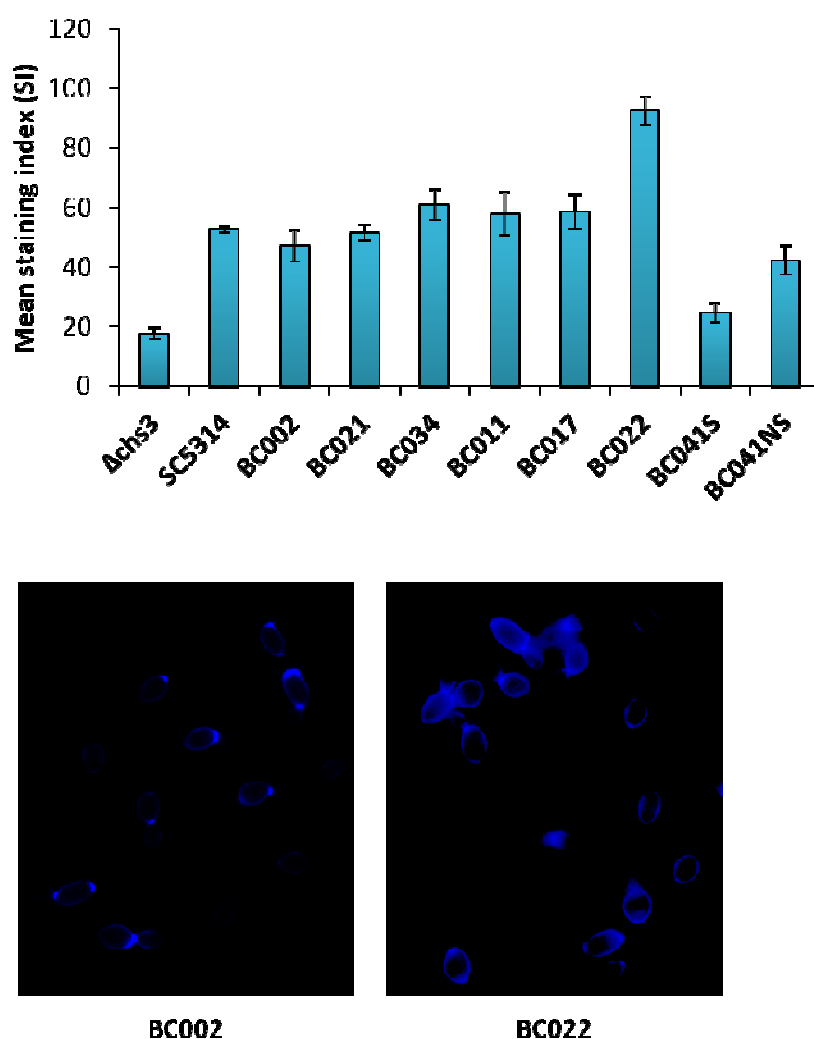


Figure 33. Cell wall chitin content of *Candida parapsilosis* strains. (a) Flow cytometry results. Yeast cells were stained with $2.5 \mu\text{g}.\text{ml}^{-1}$ CFW and fluorescence emitted was quantified. (b) Epifluorescence microscopy results. Cells were photographed after $25 \mu\text{g}.\text{ml}^{-1}$ CFW staining. SC5314 (wild type strain) and Δchs3 (chitinase deleted strain) are control strains;; BC002, BC021 and BC034 are echinocandin susceptible strains and BC011, BC017 and BC022 are echinocandin nonsusceptible strains (see Table 16); BC041S and BC041NS are strains from the *in vitro* induction assay.

Other species (*C. glabrata*, *C. tropicalis*, *C. albicans*, *C. krusei* and *Cryptococcus neoformans*) were also evaluated in this study (Fig. 34). *In vitro* antifungal susceptibility and paradoxical effect of CFS against these clinical isolates can be consulted in manuscript VIII in Publications section. The comparison between species revealed that *C. parapsilosis*, *C. tropicalis* and *C. albicans* strains showed higher CFW staining, and therefore higher chitin content in the cell wall comparing to *C. glabrata*, *C. krusei* and *C. neoformans*. Curiously, the strain with high chitin level exhibited a paradoxical growth effect.

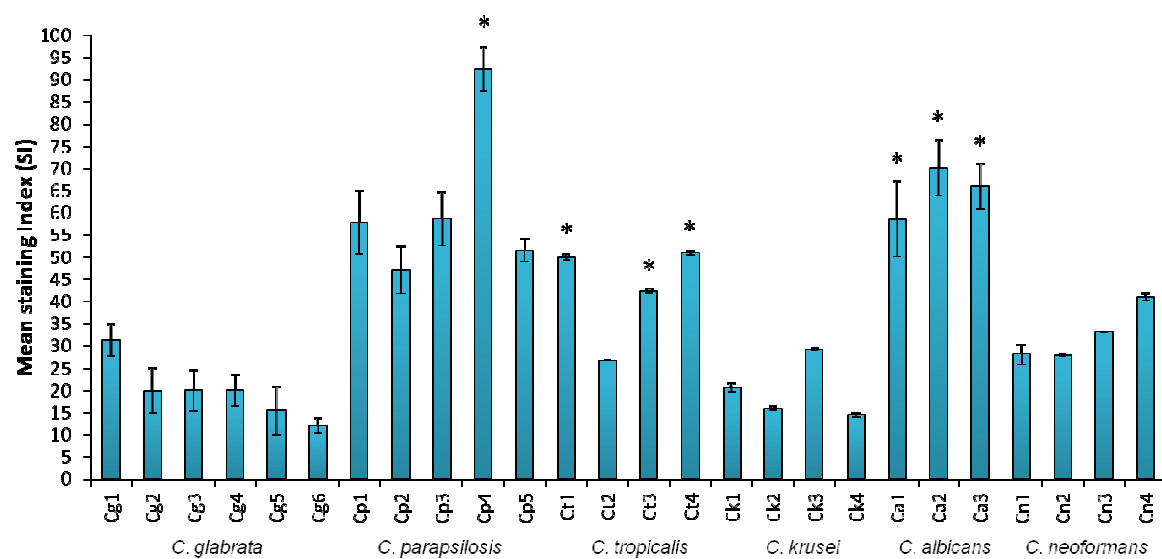


Figure 34. Cell wall chitin content of *Candida* spp. and *Cryptococcus neoformans* clinical isolates. Yeast cells were stained with $2.5 \mu\text{g}.\text{ml}^{-1}$ CFW and fluorescence emitted was quantified by flow cytometry. A higher staining index (SI) were observed in strains that showed paradoxical growth (*) in the presence of CSF. Cp1: BC011 strain, Cp2: BC002 strain, Cp3: BC017, Cp4: BC022 strain and Cp5: BC021 strain.

DISCUSSION

INCIDENCE AND DISTRIBUTION OF *C. PARAPSILOSIS*, *C. ORTHOSILOSIS* AND *C. METAPSILOSIS*

Three distinct species within the *C. parapsilosis* groups (I, II and III) were previously recognized on a molecular basis. Subsequent studies focusing on the level of heterozygosity and on the linear and circular forms of the mitochondrial genome of *C. parapsilosis* support the existence of these distinct species [14]. In 2005, after sequencing analysis of several genes, groups II and III were reclassified as two new distinct species from *C. parapsilosis* and were named *C. orthopsilosis* and *C. metapsilosis*, respectively [84].

After molecular analysis of a large set of *C. parapsilosis* isolates (n=175) from a large university hospital (Hospital S. João), 91.4% were found to correspond to *C. parapsilosis* sensu stricto, while 2.3% and 2.9% corresponded to *C. orthopsilosis* and *C. metapsilosis*, respectively. Discrepancies between the results of biochemical identification and molecular analysis were found since six isolates (3.4%) corresponded to other distinct species. This fact highlights the relevance of complementing traditional biochemistry-based identification methods with DNA-based molecular assays such as PCR, since such tests provide a much higher discriminatory powers. These results improve the knowledge about the national distribution of *C. parapsilosis* sensu stricto, *C. orthopsilosis*, and *C. metapsilosis*. Lockhart *et al.* [87] had identified among 67 Portuguese yeast isolates 1 *C. orthopsilosis* isolate and not a single *C. metapsilosis* isolate. In another study in a hospital in the centre of Portugal involving 50 isolates identified biochemically as *C. parapsilosis*, 5 (10%) were re-identified as *C. metapsilosis* and 6 (12%) as *C. orthopsilosis* [218]. The values found in our study are in accordance with data reported from international studies and including isolates from different parts of the world, namely, 91.3% *C. parapsilosis* sensu stricto isolates, 6.1% *C. orthopsilosis* isolates, and 1.8% *C. metapsilosis* isolates [87]. Similar prevalence rates were reported by other surveillance studies, namely, in Brazil, Spain, Italia and Taiwan [124, 162, 175, 177], the majority of isolates corresponding to *C. parapsilosis* sensu stricto and the minority to *C. metapsilosis*.

Our study reinforces the assumption that *C. parapsilosis* sensu stricto is the predominant among clinical isolates, namely, isolates from patients with hematogenous infections. Interestingly, *C. orthopsilosis* and *C. metapsilosis* isolates were recovered from other biological products, like urine, respiratory tract samples, and mucosal surfaces. The higher prevalence of *C. parapsilosis* sensu stricto may be related to its ubiquitous nature, since it commonly inhabits different environmental sources. Such niches may represent potential routes for nosocomial transmission to patients [25, 26] and/or may be related with a wider set of virulence attributes, compared to the other two species [96, 106, 122]. In fact, the strains isolated from the hospital

air and patient food corresponded to *C. parapsilosis* sensu stricto, which supports the first assumption. Pertaining the last assumption, the literature describes *C. parapsilosis* sensu stricto as the most virulent species and *C. metapsilosis* as the least virulent within the *C. parapsilosis* groups [122, 126, 219].

GENETIC HOMOGENETY OF *C. PARAPSILOSIS* ISOLATES

Identification of *Candida* spp. from clinical specimens is important to facilitate optimal antifungal therapy and patient management [220, 221]. Molecular techniques are excellent tools for identification and strain typing of yeasts. In this study the genetic heterogeneity of *C. parapsilosis* clinical isolates (n=160) were investigated. RAPD analysis using a single primer, *RPO2*, was used to provide a screening of the genetic heterogeneity of the isolates. The RAPD profiles generated were stable. Only 3 RAPD types were recognized, RAPD type A1 being the most frequent. Not surprisingly, the environmental isolates were RAPD type A1. The genomic homogeneity among *C. parapsilosis* isolates observed by RAPD analysis in this study has been demonstrated previously by molecular techniques [78, 84, 86]. However, a recent investigation of the size polymorphisms in loci harboring microsatellite repeat sequences of *C. parapsilosis* group I isolates [92] demonstrated 30 different microsatellite genotypes from 42 isolates. Due to the low discriminatory power of RAPD analysis, in contrast with microsatellite analysis, this technique should not be used for *C. parapsilosis* outbreak and epidemiological investigations.

The existing studies that also used RAPD analysis [33, 84, 86, 91] only described one single profile for *C. parapsilosis* isolates. According to their results, the advantage of this technique is the power to discriminate between the three *C. parapsilosis* groups (*C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis*). Whenever RFLP analysis was also applied [33], more profiles were found for *C. parapsilosis*. Our study revealed two additional profiles (RAPD type A2 and RAPD type A3), probably due to the higher amount of isolates.

Our typing results were reproduced by another technique, namely restriction endonuclease analysis of the mitochondrial DNA. REA for *Candida* species was first described by Scherer and Stevens [222] who considered this method to be an extremely valuable tool for epidemiological studies. Fujita et al. [223] described *HinfI* restriction patterns as exhibiting a superior discriminatory power among distinct *Candida* isolates compared to patterns obtained with other enzymes such as *EcoRI* or *MspI*. Additionally, Sancak et al. [224] established a correspondence of almost 100% between the results obtained with *HinfI* restriction endonuclease-based analysis and PCR methodologies. Recently, in a study, undertook by our group, involving 22 *C. krusei* isolates and using REA analysis [225], a long-lasting colonization by *C. krusei* was confirmed.

ANTIFUNGAL SUSCEPTIBILITY PROFILES OF *C. PARAPSILOSIS*, *C. ORTHOSILOSIS* AND *C. METAPSILOSIS*

We evaluated the *in vitro* activity of fluconazole, voriconazole, posaconazole, caspofungin, anidulafungin, and amphotericin B against *C. parapsilosis* sensu stricto, *C. orthopsilosis*, and *C. metapsilosis*. The definition of the susceptibility profiles provides information relevant to the development of recommendations for prophylactic, empirical, or targeted antifungal therapy [226-228], particularly at a national level. In this evaluation, statistical comparison of the antifungal susceptibility patterns of the three species were seriously compromised due to the reduced number of strains of *C. orthopsilosis* and *C. metapsilosis* tested.

Although the MIC values of echinocandins were high for all three species, *C. parapsilosis* sensu stricto isolates were less susceptible to CSF and ANF than *C. orthopsilosis* and *C. metapsilosis* isolates. Other studies corroborate our finding, demonstrating that all echinocandins (CSF, ANF and MCF) exhibit lower MIC values for *C. metapsilosis* and *C. orthopsilosis* than for *C. parapsilosis* sensu stricto [124, 175, 229]. Not surprisingly, MIC values of CSF were higher than MIC values of ANF for all the three species. Previous studies by our team [19] had already reported high CSF MIC values for *C. parapsilosis* groups (for all *C. parapsilosis* isolates, the CSF MIC values were $\geq 2 \mu\text{g}.\text{ml}^{-1}$). It has repeatedly been shown that echinocandin MIC values are consistently higher for the *C. parapsilosis* groups than for *C. albicans* or *C. glabrata*, when assessed by broth microdilution methods [19, 38, 228, 230-232], thus suggesting that the *C. parapsilosis* groups are less susceptible to this class of antifungal agents. Recently, new clinical breakpoints were appointed for these antifungals regarding *C. parapsilosis*: S phenotype for a MIC value $\leq 2 \mu\text{g}.\text{ml}^{-1}$, S-DD for a MIC value $4 \mu\text{g}.\text{ml}^{-1}$ and R for a MIC value $\geq 8 \mu\text{g}.\text{ml}^{-1}$ [233]. The reduced susceptibility of *C. parapsilosis* groups to echinocandins has been related to a naturally occurring polymorphism at the edge of HS1 region of *FSK1* gene [144, 234, 235]. van Asbeck et al. [148] suggested that interspecies variation in CSF susceptibility resulted from the combination of structural differences in the cell wall components, from a reduced affinity for the glucan synthase protein complex or from variations in the regulatory network of this complex.

Susceptibility phenotype was established according to the CLSI protocol [190, 191] described in Material and Methods section. However, Pfaller et al [236, 237] revised those clinical breakpoints considering MIC value $\geq 4 \mu\text{g}.\text{ml}^{-1}$ for fluconazole and MIC value $\geq 2 \mu\text{g}.\text{ml}^{-1}$ for voriconazole and posaconazole the resistant breakpoints. Azole resistance was detected only amongst *C. parapsilosis* sensu stricto isolates but not *C. orthopsilosis* or *C. metapsilosis* isolates. In contrast, other studies reported higher MIC values to both FLC and VRC for *C. metapsilosis*

and *C. orthopsilosis* than those for *C. parapsilosis* sensu stricto [124, 175]. The differences in FLC susceptibility among the three species might reflect the distinct affinity of azoles for the key ergosterol-synthesizing enzyme, lanosterol 14 α -demethylase, or other enzymes involved in this metabolic pathway [148].

Interestingly in our study, two *C. parapsilosis* sensu stricto isolates were resistant to AMB, an uncommon finding.

In summary, we detected a multiechinocandin-, multiazole-resistant phenotype of *C. parapsilosis* sensu stricto, as reported by Moudgal et al. [238] and Ghannoum et al. [239]. The development and subsequent nosocomial expansion of echinocandin- and azole-resistant *C. parapsilosis* sensu stricto strains may have important clinical implications. Continuous monitoring for the emergence of this multidrug-resistant phenotype of *C. parapsilosis* sensu stricto should represent an important component of the effective prevention of nosocomial infections. Although FLC showed good activity against *C. parapsilosis* sensu stricto, *C. orthopsilosis*, and *C. metapsilosis*, local routine monitoring of the susceptibility profiles of these organisms may be highly advisable. The combination of decreased susceptibility to FLC and the ability to form extensive biofilm at the surface of central venous catheters and other medical indwelling devices may become problematic [25, 95, 100, 240, 241]. Recently, our group characterized the microorganisms that colonized central venous catheters in intensive care patients admitted in Hospital S. João during 12 months [241]; 104 (7%) of isolates correspond to *Candida* spp., however no antifungal resistance was detected among the low number (n=35) of tested isolates. In over 50% of the episodes of *C. parapsilosis* candidaemia, the source is a vascular catheter, and such infections usually develop in patients who had previously received antifungal treatment [18]. Thus, an optimal response to FLC or to any other antifungal may not be fully obtained. The administration of the antifungal should be coupled with the prompt removal of the catheter to ensure a successful response [234].

***IN VITRO* ACQUISITION OF AZOLE RESISTANCE**

The acquisition of azole resistance, particularly after prolonged exposure, as it happens with prophylactic overuse, is a well-known phenomenon in fungi [242-245]. The widespread use of azole antifungals, especially fluconazole, resulted in a growing incidence of *Candida* species in which resistance is easily induced, such as *C. glabrata* [246], or species that show intrinsic resistance, such as *C. krusei* [247]. Previous studies with *C. albicans* [136], *C. dubliniensis* [248] and *C. tropicalis* [249] demonstrated that resistance to fluconazole can be promoted following repeated *in vitro* exposure to the drug. The ability of a drug to induce *in vitro* resistance suggests that similar mechanisms may also occur *in vivo*, which may thus become problematic, for instance during prophylactic regimens.

Although *C. parapsilosis* is not considered to be particularly prone to the development of antifungal resistance [36, 95, 147], several recent reports suggested that its decreased susceptibility to azoles and echinocandins might become a cause for clinical concern [148-151]. Scarce data is yet available regarding resistance amongst *C. parapsilosis*. Therefore in order to assess and characterize its *in vitro* acquisition of resistance to different antifungals, studies of induction were undertaken. The results stress several relevant characteristics of the *in vitro* acquisition of resistance by *C. parapsilosis* after prolonged exposure to antifungals.

Following the incubation of *C. parapsilosis* with therapeutic fluconazole concentrations (aiming to reproduce *in vivo* antifungal therapy), azole resistance quickly developed; similar results were obtained with a lower concentration of the same antifungal, showing that of the three azoles tested, fluconazole is an easy and fast inducer of resistance. The two different approaches used for induction of resistance corroborate this conclusion. The resistant phenotype obtained was stable after exposure was stopped and population analysis clearly demonstrated the homogeneous nature of the population, excluding the possibility of a mixture of susceptible and resistant clones being present in the final cultures. Our findings stress the need to review therapeutic protocols. Both voriconazole and posaconazole can induce resistance at concentrations of 2 and 1 $\mu\text{g}\cdot\text{ml}^{-1}$, respectively, but at lower concentrations the susceptibility pattern did not change drastically. As expected, incubation with any of the three azoles did not induce resistance to AMB and CSF, as the azole targets are distinct and selective. Our data strongly suggest that the dynamics of *in vitro* development of azole resistance in *C. parapsilosis* is different from that described for *C. tropicalis* and *C. albicans* [194, 249, 250]. For *C. albicans*, the time required to develop fluconazole resistance is longer and such phenotype could be fully reverted to baseline when the antifungal exposure was stopped [194].

***IN VITRO* ACQUISITION OF ECHINOCANDIN RESISTANCE**

Following the incubation of *C. parapsilosis* with anidulafungin, a stable echinocandin resistance pattern quickly developed and cross-resistance between all three echinocandins was observed. Echinocandins are non-competitive inhibitors of the fungal 1,3- β -D-glucan synthase complex, which is responsible for the biosynthesis of the major structural component of the cell wall (glucan), by targeting the putative catalytic subunit Fks1 [251, 252]. Since they have a common target, it is understandable that the resistance mechanism developed affected all three compounds. Resistance to these antifungal drugs is more often associated with mutations in two highly conserved regions of Fks1p, known as hot spot 1 and hot spot 2 [144, 253]. However, other mechanisms could be involved (discussed below).

INDUCED AZOLE RESISTANCE MECHANISMS

IN VITRO INDUCED

The mechanisms of azole resistance due to antifungal exposure have been extensively investigated in clinical strains of *C. albicans* [137, 138, 141, 142, 254, 255]. Other studies have also elucidated the mechanisms of azole resistance in *C. glabrata*, *C. krusei* and *C. tropicalis* [256-258]. However, very few studies have addressed the mechanisms of azole resistance by *C. parapsilosis*. Throughout our study, we shed some light on drug resistance mechanisms developed by *C. parapsilosis* after exposure to fluconazole, voriconazole and posaconazole.

Candida albicans isolates that became resistant, *in vitro* and *in vivo* induced, showed an increase of mRNA specific for a CDR ATP-binding cassette transporter efflux pump [136, 194]. The *in vitro* induction of resistance in *C. tropicalis* has been associated with increased expression of *CDR1* and *MDR1* genes [249], coding for a specific fluconazole transporter. Distinct antifungal resistance mechanisms have been described among *Candida* spp., active efflux being the most relevant mechanism of azole resistance; its molecular base is relatively well known for *C. albicans* [141]. Overexpression of these efflux proteins confers resistance to most azoles but in the same degree. Whereas, ketoconazole, fluconazole, itraconazole and voriconazole are transported readily, posaconazole is transported in a much lesser extent [259]. Apparently, this compound is not the ideal substrate for these transporters. Our study expectations were similar to studies involving *C. albicans* and *C. tropicalis*; therefore the presence of efflux pumps was phenotypically checked. If the resistance mechanism induced after antifungal exposure was overexpression of ATP-binding cassette efflux pumps, we would expect that in the presence of efflux blockers, like verapamil and ibuprofen, MIC values would decrease and that Rh-6G staining in resistant cells would be lower when compared with nonexposed cells. Moreover, a synergistic effect between azole and FK506 should be observed. According to our results, efflux seems to play a minor role in the induced resistance; however, the hypothesis of the existence of specific *C. parapsilosis* efflux pumps blocked by different modulators or evidenced with fluorescent substrates other than Rh-6G cannot be excluded. However, the hypothesis that induced resistance in *C. parapsilosis* may be due to mutation in genes that codify enzymes involved in the ergosterol biosynthesis pathway gains considerable strength. This mutation is probably induced by the antifungal and does not result from selection of an initial resistant clone. This last assumption was supported by the results of population studies, as both the susceptible population (without antifungal exposure) as well as the resistant population obtained following induction, were shown to be homogeneous. The azoles bind in the vicinity of

the haeme group of lanosterol-14 α -demethylase. Although there are silent mutations scattered throughout the gene, at least a dozen different mutations in the active region may create a lower affinity for azoles, resulting in a greater influence upon the antimicrobial activity of fluconazole and voriconazole than of posaconazole and itraconazole [25]. These two drugs are able to bind to an additional domain of the enzyme by means of their long side chain [260, 261], meaning that these two antifungals can still inhibit the target at a point when fluconazole and voriconazole are no longer active [262]. Accordingly, such mutations should result in a lower impact upon the susceptibility profile to posaconazole than to fluconazole or voriconazole. Because of the two different binding sites of posaconazole, the occurrence of large-spectrum cross-resistance is highly unlikely. Based upon phenotypic experiments (antifungal susceptibility in the presence of efflux pumps blockers and flow cytometric assay with Rh-6G), different assumptions support the occurrence of drug induced mutation: the rapidity of development of the resistant phenotype; the fact that the three azoles are affected, although in a different extent; the insignificant role of efflux; and the stability of resistance following prolonged absence of drug. Spontaneous mutations are very infrequent, occurring at frequencies of 10^{-6} - 10^{-8} per gene. The constancy of the MIC values along time when strains were incubated in drug-free medium strongly supports the unlikely occurrence of spontaneous mutation. Diminished sensitivity of the target is probably the main resistance mechanism to azoles in *C. parapsilosis*. Likewise, phenotypically stable resistance to azole antifungals in *C. albicans* can result from mutations in genes involved in the ergosterol synthesis pathway (including the target enzyme lanosterol-14 α -demethylase) [260, 263-265].

At this point, *C. parapsilosis* azole resistance mechanisms were not clarified. In the attempt to obtain additional information, the global constitutive changes in gene expression of resistant strains obtained after exposure to different azoles (BC014R_{FLC}, BC014R_{VRC} and BC014R_{PSC} strains) were assessed using a microarray platform.

Microarray analysis identified differential expression of 1128, 210 and 598 genes in BC014R_{FLC}, BC014R_{VRC} and BC014R_{PSC} strains, respectively. Alterations in expression levels of a large number of genes have also been reported in azole resistant *C. albicans* strains [266-268]. Thus, it is clear that the presence of azoles induces a reformulation of the transcriptome of *Candida* species, possibly as a mechanism of adapting to stress response.

Gene Ontology analysis clearly demonstrated that azoles impaired the expression of genes involved in multiple cellular processes, resulting in a pleiotropic effect upon gene expression.

Expression of *MDR1* and other members of the major facilitator superfamily of multidrug transporters are up-regulated in the BC014R_{FLC} and BC014R_{VRC} strains, whereas its expression in

BC014R_{PSC} strain remained unchanged. Overexpression of *MDR1* is also associated with resistance to fluconazole in clinical *C. albicans* isolates and with reduced susceptibility to voriconazole [214, 269]. Overexpression of *MDR* genes renders yeast cells less susceptible to voriconazole [269]. In *C. albicans*, expression of *MDR1* is regulated by the transcription factors *MRR1*, *CAP1* and *MCM1* [214, 270, 271]. Mrr1p appears to play a central role in the development of drug resistance in *C. albicans*. Using a genome-wide gene expression analysis approach, Morschhäuser et al. [214] identified mutations in the *MRR1* gene in drug-resistant clinical isolates of *C. albicans* which resulted in up-regulation, and were associated with a coordinate up-regulation of *MDR1* [272, 273]. Interestingly, expression of *MRR1* was also up-regulated in *C. parapsilosis* BC014R_{FLC} and BC014R_{VR}C strains; we therefore determined the sequence of the *MRR1* gene in such strains. A single nucleotide exchange was detected in both (but it was different from each other) when compared with the *MRR1* sequence of the susceptible strain (BC014S). In *C. albicans* several mutations in the *MRR1* gene in fluconazole resistant clinical isolates have been described [214, 272]; thus, it is likely that mutations described herein may also play a key role in *MDR1* overexpression in *C. parapsilosis*. However, this hypothesis remains to be validated experimentally.

Overexpression of *MDR1* in *C. albicans* is also correlated with increased expression of aldo-keto reductases and other genes associated with oxidative stress response, which may protect from cell damage caused by toxic molecules produced in the presence of azoles, and also contribute to antifungal drug resistance [274]. The expression of these genes is also regulated by Mrr1p transcription factor in *C. albicans* [214]. We observed a similar response in *C. parapsilosis*. Expression of several putative aldo-keto reductases and NADPH oxidoreductases is increased in both BC014R_{FLC} and BC014R_{VR}C strains. As a representative example, it is the case of stress response gene *GRP2*, whose up-regulation in *C. albicans* is correlated with increased expression of *MDR1* [214] and is associated with the stepwise development of resistance to azoles [267, 275]. However, *GRP2* expression is not only triggered by azoles but it is also regulated positively by certain compounds like fluphenazine, benomyl [216] and farnesol [201].

The comparison of the expression patterns of *C. parapsilosis* (BC014R_{FLC} and BC014R_{VR}C strains) with *C. albicans* azole resistant isolates [214, 272] reveals that the alterations in the transcriptome underlying azole resistance are similar in both species. We therefore suggest that resistance to fluconazole and voriconazole in *C. parapsilosis*, in particular in BC014R_{FLC} and BC014R_{VR}C strains, is conferred by the increased expression of *MRR1* transcription factor, which results in a concomitant overexpression of *MDR1* and possibly other members of MFS family, and of aldo-keto reductases. As described with *C. albicans* [214, 272], *C. parapsilosis* gain-of-

function mutations in *MRR1* results in overexpression. Mutations in *MRR1* are unlikely to cause resistance to posaconazole, which is a poor substrate for Mdr1p [260, 276]. The decreased susceptibility to posaconazole observed with BC014R_{FLC} and BC014R_{VR}C strains is likely to involve another yet unidentified mechanism. We did observe the up-regulation of the transcription factor *NDT80* in BC014R_{FLC}, which may result in overexpression of the *CDR1* drug efflux pump; however, this was not verified. In *C. albicans* Ndt80p modulates azole tolerance by controlling the expression of the gene *CDR1* [215].

Several genes linked to ergosterol synthesis, including *ERG11* gene, are down-regulated in both BC014R_{FLC} and BC014R_{VR}C strains. Since azoles target the cytochrome P450 sterol 14 α -demethylase enzyme, encoded by *ERG11* gene, the reduction in ergosterol metabolism is likely to be a direct consequence of fungal cell exposure to azoles during induction of resistance *in vitro*. The decreased expression of sterol genes we found in BC014R_{FLC} and BC014R_{VR}C strains is unlikely to be related to the azole resistance phenotypes; in contrast, we would expect resistant isolates to demonstrate an increased expression of sterol synthesis genes. However, we have not directly tested the role of the ergosterol pathway in these strains.

Gene expression of BC014R_{PSC} strain was substantially different from the other two azole-resistant isolates analyzed in this study. Although 46 of the differentially expressed genes were shared with the other two resistant strains (BC014R_{FLC} and BC014R_{VR}C), none of them plays a known role in conferring drug resistance. BC014R_{PSC} strain displayed an overexpression of thirteen genes directly linked to ergosterol biosynthesis, which strongly indicates that an increase in ergosterol content is the most probable cellular mechanism conferring reduced susceptibility pattern to all azoles in this strain. In *C. albicans*, exposure to ketoconazole, also increases the expression of genes involved in lipid, fatty acid, and sterol metabolism, including *ERG11* [277]. We also observed increased expression of the transcription factors *NDT80* and *UPC2* in BC014R_{PSC}. Ndt80p was recently identified in *C. albicans* [215] as one of transcription factors that binds to the promoters of ergosterol biosynthesis genes, including the azole target *ERG11* gene. Like *NDT80*, the zinc cluster transcriptional factor *UPC2* also mediates the up-regulation of ergosterol biosynthesis genes in *C. albicans* [139], and gain-of-function mutations result in overexpression of ergosterol biosynthesis genes [140]. Using genome-wide gene expression profiling, Dunkel et al. [272] found that *UPC2* and other genes involved in ergosterol biosynthesis are coordinately up-regulated with *ERG11* in a fluconazole-resistant, clinical isolate compared with a matched susceptible isolate from the same patient. Expression of *UPC2* gene is up-regulated in *C. parapsilosis* BC014R_{PSC}. We therefore propose that, similarly to what happens with *C. albicans*, resistance to azoles in *C. parapsilosis* can be partly conferred via induction of

NDT80 or *UPC2*, resulting in increased ergosterol production. To determine whether the overexpression of the transcription factors *NDT80* and *UPC2* is associated with point mutations, the genes were sequenced both in BC014R_{PSC} and BC014S strains. No difference in the nucleotide sequence was detected between the susceptible and the BC014R_{PSC} strains. Expression of *SOD4* gene was also up-regulated in the BC014R_{PSC} strain. Superoxide dismutases are enzymes that convert superoxide radicals into less damaging hydrogen peroxide and are required for the protection against oxidative stress and the establishment of full virulence [278].

High-level of azole resistance in clinical isolates of *C. albicans* and *C. glabrata* is often associated with overexpression of plasma membrane efflux pumps [279-281]. There are two main families of efflux proteins, the ATP-binding cassette (ABC) pumps and the major facilitator superfamily (MFS) transporters [276]. Expression of the ABC transporters *CDR1* and *CDR2* is increased in some azole-resistant isolates of *C. albicans* [282]. *CDR* genes expression is regulated by the Tac1p transcription factor and its induction occurs independently from the MFS family expression levels [282, 283].

The *C. parapsilosis* genome encodes at least 14 members of the ABC transporter family [75]. The expression of one these transporters, CPAG_0151, is up-regulated in the BC014R_{PSC} strain. This gene is a syntenic homolog of *C. albicans CDR3* [205]. Expression of CPAG_02192, another ABC transporter, is up-regulated both in BC014R_{FLC} and BC014R_{VR} strains. *C. albicans* ABC transporters most frequently involved in azole resistance are the Cdr1p and Cdr2p [137, 280]. Interestingly, in *C. parapsilosis* the expression of *CDR1* ortholog is not altered between susceptible and resistant strains and the *CDR2* ortholog was not identified. However, expression of CPAG_03665, an ortholog of *C. albicans PDR16*, is up-regulated in BC014R_{FLC}, BC014R_{VR} and BC014R_{PSC} strains. *PDR16* encodes a phosphatidylinositol transfer protein, which modifies the passive diffusion of hydrophobic drugs across the cell plasma membrane, thereby modulating multi-drug resistance in *Saccharomyces cerevisiae* [284]. *PDR16* is co-expressed with *CDR1* and *CDR2* in azole resistant clinical isolates of *C. albicans* [285]. BC014R_{FLC} and BC014R_{VR} strains displayed a reduced expression of CPAG_03819. This gene is an ortholog of *C. albicans* orf19.3902, and its reduced expression was correlated with over-expression of *CDR1* and *CDR2* in *C. albicans* azole resistant isolates [216]. It is therefore possible that azole resistance in *C. parapsilosis* is also associated with increased expression of ABC transporters. Nevertheless, our results suggest that such transporters play minor role; the expression of CPAG_02274 (*SNQ2*) was downregulated in BC014R_{FLC} and BC014R_{VR} strains, while the expression of the other ABC transporter family members remained unchanged.

The expression levels of several genes involved in copper and iron transportation were found to be differently altered in all three azole resistant *C. parapsilosis* strains. Due to this variability among resistant strains, it is difficult to effectively evaluate the impact of gene expression changes on the overall transport of metals. However, it is clear that azoles interfere with transport of iron and copper, and consequently its metabolism and management by *C. parapsilosis*. Similar phenotypes have been described in drug resistant isolates of *C. albicans* [266, 268]. Copper is essential for enzymes involved in a multitude of biological processes, such as signaling of transcription, protein trafficking machinery, oxidative phosphorylation, iron mobilization, neuropeptide maturation, and ultimately normal cell development [286, 287]. The acquisition of iron is essential for growth, survival and virulence of *C. albicans* [288]. Moreover, recent studies suggest that iron depletion increases membrane fluidity, which in turn leads to enhanced passive diffusion of drugs, resulting in increased drug susceptibility [289]. Expression of two genes, *FLC1* and *FLC2*, involved in heme uptake [217] is increased in the BC014R_{PSC} strain. Heme is a nutritional iron source required for the activity of a variety of enzymes including cytochrome P450 enzymes and iron-sulfur clusters. Azoles inhibit the synthesis of ergosterol by binding to the heme cofactor located in the active site of lanosterol 14 α -demethylase. Increasing heme transport may contribute to enhanced lanosterol 14 α -demethylase production, and thus contribute to the azole resistant phenotype of BC014R_{PSC}.

Other resistance mechanisms apart from those already addressed (after phenotypic and genotypic studies), such as mutations in the target enzymes might be implicated; however they were not covered by transcriptome analysis. In fact, as already discussed, for all resistant strains obtained, a mutation in the target enzyme may correlate with the resistant profile observed. Azole drugs bind to the area surrounding the heme group of lanosterol-14 α -demethylase. Fluconazole and voriconazole bind only to one single domain, while posaconazole, by means of its long side chain, attaches to two binding sites in the enzyme [260, 261]. Thus, a point mutation in one domain of the enzyme might explain resistance to fluconazole and voriconazole. Regarding acquisition of resistance to posaconazole, mutation in both domains of the enzyme would have to occur in order to explain azole cross-resistance.

To our knowledge, our study is the first to use cDNA microarray analysis to investigate whole genome level gene expression in sets of azole-resistant *versus* susceptible *C. parapsilosis* strains. Microarray gives an overview of genes that are differently expressed, from which important information should be extracted and studied in more detail using other molecular tools, in order to comprehensively systematize the molecular mechanisms underlying *C. parapsilosis* drug resistance.

In summary, the two different approaches undertaken to provide insight about azole resistance mechanisms in *C. parapsilosis* showed to be coherent; they did not attribute a major role to ATP-binding cassette transporter efflux pumps and both hypothesized the acquisition of a mutation during the induction assay, which was confirmed for BC014R_{FLC} and BC014R_{VRC} strains.

In Vivo INDUCED

For the three *in vivo* cases reported here, there was a rapid decrease in azole susceptibility, the isolates exhibiting azole cross resistance in one case. In order to get on insight about the resistance mechanisms developed, phenotypic and genotypic studies were carried out with isolates from patient A (last isolate was resistant to all azoles tested). Antifungal susceptibility testing with an efflux blocker and flow cytometry with Rh-6G revealed the scarce role of ATP-dependent efflux pumps. Several studies support the opposite, that ATP-dependent efflux pumps play a major role in antifungal resistance. For instance, clinical resistant *C. albicans* isolates exhibited *CDR1* and *CDR2* overexpression in a study carried out by Ricardo et al. [290]. In other study [281], azole-resistant clinical *C. glabrata* isolates showed upregulation of *CDR1* compared to susceptible strains, while *ERG11* expression was minimally changed.

As already mentioned, regarding *C. albicans*, several studies described the presence of point mutations in the *ERG11* gene, whereas some isolates contained more than one single mutation, as the fluconazole resistance mechanism [263-265]. Li et al. [260] sequenced the *ERG11* genes from seven sequential *C. albicans* isolates from an AIDS patient and concluded that multiple mutations in *ERG11* gene are required to confer simultaneously decreased susceptibility to several azoles, including posaconazole. According to our results, the *ERG11* gene of the last *C. parapsilosis* isolate did not show alterations.

Similar to *in vitro* strains, the gene expression profile for *in vivo* strains also was evaluated. The microarray quality was not very high, probably because we are working with strains obtained directly from the patient and not with *in vitro* strains obtained after successive subcultures in the presence of the antifungal in stable environmental conditions. Nevertheless, relevant information could be extracted. The genes involved in lipid metabolism process were increased, which included *ERG* genes responsible for ergosterol biosynthesis. Azoles are responsible for the shortage of this lipid in yeast cell membrane. Through quantitative real-time PCR we could confirm the increase in *ERG11* and *ERG6* genes. Moreover, we could also observe a slight increase in transcription factors genes *NDT80* and *UPC2*. Its role in increasing the expression of *ERG* genes was already discussed; however the search for point mutations was not performed. Constitutive *ERG11* overexpression may contribute to fluconazole resistance in

clinical *C. albicans* isolates. Goldman et al. [264] found a few resistant *C. albicans* clinical isolates that showed increased mRNA expression of the *ERG11* gene. Morschhauser et al. [291] found *ERG11* overexpression in many fluconazole-resistant *C. albicans* isolates compared with matched susceptible isolates. However, White et al. [254] had reported, from a collection of fluconazole-resistant and -susceptible isolates, considerable variation in the levels of *ERG11* mRNA expression; such expression did not seem to be related to azole resistance.

In summary, and although the results were not conclusive, we can say that most probably the increase in membrane ergosterol content accounts for the cross-resistance profile observed in the *in vivo* induced resistant strain.

Comparing the *in vitro* with the *in vivo* induction of azole resistance similarities and discrepancies can be appointed. In the *in vitro* assays, when the antifungal fluconazole was used to induce resistance, this phenotype developed very fast and ATP-dependent efflux pumps were not important for the resistance phenotype. The same happened in the *in vivo* case. Susceptibility against the different azoles is affected differentially by fluconazole. For instance, in the *in vivo* induced strain the susceptibility pattern to posaconazole was resistant while in the *in vitro* induced strains kept susceptible. The major facilitators (like MDR1 efflux pump) seem to be important in the *in vitro* induced strain whilst in the *in vivo* induced strain are *ERG* genes, which enclose the *in vivo* case with the *in vitro* induction of resistance with posaconazole. In fact, in both cases a cross-resistance profile was achieved.

According to our results, the *in vitro* induction of resistance model did not match exactly with the findings in an *in vivo* induction case. In the human body, the physiologic conditions interfere in the fitness of microorganisms; therefore different susceptibility phenotypes or virulence attributes can be expressed. The clinical outcome is dependent on factors that include host defenses, the virulence of the fungal pathogen, and drug efficacy/toxicity, as well as drug interactions [292, 293]. Additional factors, such as age, gender, underlying disease status, and concomitant therapies, can account for the drug efficacy, but host genetics probably play important roles in both drug efficacy and toxicity [294]. Meletiadiis et al. [295] accomplished all the human factors that might be implicated in antifungal therapy, which are not taken accounted in the *in vitro* studies. Notably that the strain used in the *in vitro* assays was not the same isolate from the *in vivo* case; even if it was the same strain, the different environmental conditions could lead to different phenotypes for the same genotype.

The findings from the *in vitro* induction assay and from the *in vivo* induction case of resistance characterized in this study demonstrated that different azole resistance mechanisms

DISCUSSION

can be developed by *C. parapsilosis* and that they are quite similar to the findings with *C. albicans*. It should be viewed as examples and not as definitive rules.

ECHINOCANDIN RESISTANCE MECHANISMS

Echinocandins inhibit β -1,3-D-glucan synthase, which catalyzes the biosynthesis of β -1,3-D-glucan, the major glucan component of *Candida* cell walls [296, 297]. Glucan synthase is an enzyme complex with at least two subunits, Fksp and Rho1p. Fksp, encoded by three related genes, *FKS1*, *FKS2*, and *FKS3*, contains the active site, which catalyzes the transfer of sugar moieties from activated donor molecules to specific acceptor molecules forming glycosidic bonds [298-300]. The inhibition of glucan synthase by echinocandin drugs disrupts the structure of the growing cell wall, resulting in osmotic instability and the death of susceptible yeast cells [301, 302].

Echinocandin resistance in species such as *C. albicans*, *C. tropicalis*, and *C. krusei* has been associated with aminoacid substitutions in Fks1p [144]. Likewise, it is now understood that aminoacid substitutions in Fks1p and Fks2p are responsible for clinical echinocandin resistance in *C. glabrata* [303-307]. Our group recently described the acquisition of resistance by *C. glabrata* after anidulafungin treatment [308]. The resistant isolate exhibited a Ser663Pro mutation in HS1 region of *FKS2* gene.

Pfaller et al. [233] evaluated several isolates from candidiasis cases, refractory to echinocandins, with *FKS* mutations and corresponding to MIC values in the susceptible category using the breakpoint $\leq 2 \mu\text{g}.\text{ml}^{-1}$. In these cases there was a relationship between high or increasing echinocandin MIC values and a poor clinical outcome. News species specific cutoffs were proposed. Regarding *C. parapsilosis*, the values $\leq 2 \mu\text{g}.\text{ml}^{-1}$, $4 \mu\text{g}.\text{ml}^{-1}$ and $\geq 8 \mu\text{g}.\text{ml}^{-1}$ were established for susceptible, susceptible-dose dependent and resistant phenotypes, respectively.

The clinical *C. parapsilosis* strains studied exhibited high echinocandin MIC values, being considered as nonsusceptible, at least for one antifungal, according to the older breakpoints [190, 191]. However, at present some of them are considered susceptible. After sequencing the portion HS1 from *FKS1* gene, no differences in the corresponding amino acid sequences were observed when compared to GenBank nucleotide sequence database. In fact *C. parapsilosis* strains are predisposed to exhibit high MIC values of echinocandins. A naturally occurring amino acid polymorphism in the highly conserved Fks1p HS1 region of *C. parapsilosis*, relatively to other *Candida* species, has been suggested to be responsible for the reduced echinocandin susceptibility of these species [144, 234, 235]. *C. parapsilosis* clinical isolates exhibited different MIC values concerning the three echinocandins tested, which stressed the need to check whether mutations accounted for the differences in the antifungal susceptibility profile of *C. parapsilosis* isolates; this hypothesis was not confirmed. Ghannoum et al. [239] study corroborates our results; they showed that *C. parapsilosis* isolates which were susceptible to

anidulafungin but not to caspofungin and micafungin did not exhibit mutations in 493-bp region of *FKS1* gene.

Regarding the *in vitro* induction assay, the resistant strain obtained exhibited MIC value $\geq 8 \mu\text{g}.\text{ml}^{-1}$ for all three echinocandins. The pattern of decreased enzyme sensitivity to inhibition extends across all three of the echinocandins. In contrast to our expectation, no mutation in the HS1 region of *FKS1* gene was found when compared the sequences for the induced and the parent strains.

In summary, none of our *C. parapsilosis* strains had characteristic mutations or exhibited polymorphism on HS1 region of *FKS1* gene.

This study has some limitations. The entire sequence of *FKS1* gene should have been checked and the sequencing of other genes, like *FKS2* and *FKS3*, addressed. However, for other *Candida* species, namely *C. dubliniensis* and *C. krusei*, mutations outside the hot-spot regions did not confer echinocandin nonsusceptibility [233].

The existence of other echinocandin resistance mechanisms should not be excluded, like for instance, the expression level of *FKS* gene, that codifies the echinocandins target enzyme (similarly to what happens with azole resistance mechanism). As well as the role of chitin cell wall content (discussed below).

SUSCEPTIBILITY TO PHYSICAL AND CHEMICAL AGENTS

It is a crude reality the fact that candidaemia can also be exogenous in origin in the hospital setting and that acquisition of yeasts by indirect contact is important in the context of hospital infections. Regarding *C. parapsilosis*, the carriage on the skin and hands of health care personal and its consequent direct and indirect transmission have been identified as responsible for outbreaks of catheter-related *C. parapsilosis* infections in Intensive Care Units [309, 310]. Contamination of medical devices and surfaces from the patient's own colonization or from hands of the health care workers is of at most importance concerning nosocomial infections [311].

Therefore it is important to take appropriate prophylactic measures to avoid dissemination and transmission of *C. parapsilosis* and other *Candida* species. Russell et al. [312] noted a general lack of information about the antifungal activity of chemical germicides relevant for hospital settings. Much of the available data has been generated using test protocols that bear little resemblance to the field situation [313]. We assessed the effectiveness of chemical and physical agents against *C. parapsilosis*, in particular addressing the concentration and the exposure time required to inactivate this species.

Different studies demonstrated the relative resistance of *C. parapsilosis* to certain categories of germicides. Traoré et al. [186] demonstrated that the reduction of *C. parapsilosis* by hydrogen peroxide is low comparing to *C. albicans* and *C. tropicalis*. Silverman et al. [185] took into account the range of exposure times and potential dilutions recommended by manufacturers of different commercial antiseptics for washing skin against different *Candida* species. The study demonstrated that some products failed to kill some *Candida* species, even at undiluted concentration. In our study the chemical agents were tested independently, for determination of the lethal concentration, which is important to develop products that combine different substances, improving the effectiveness against *C. parapsilosis*. Furthermore, it included environmental strains, collected from air and hospital food. In addition, it also provides information about the potential of physical agents to disinfect hospital environment.

Chemical agents are very important for surface, medical devices and hand cleaning. According to the results obtained, chlorhexidine revealed to be effective at a very low concentration (approximately 0.06%), considering that commercial disinfectants contain 1% chlorhexidine. Ethanol and sodium hypochlorite were also effective at low concentrations (10% and 2.5%, respectively). Hydrogen peroxide (0.06%), potassium permanganate (0.1%) and acid boric (1%) was equally effective.

When commercial solutions were tested some discrepancies with pure compound testing were observed. Surprisingly some solutions (Hydrogen Peroxide 3% and Acid Boric 3%) were not effective as expected, despite its concentration was higher than the MLC value determined. This can be due to the differences in both methodologies, especially in cell concentration and contact time. Probably for commercial solutions the time needed to kill cells is higher than 30 min.

Physical agents are very important to disinfect surface and medical devices and moreover to sterilize water and food, which may be very relevant, especially with immunocompromised patients. We can conclude that a short microwave irradiation time is sufficient to inactivate yeast cells present in water or food, being the easier and faster methodology to apply in hospital for food or water. Cell membrane lesion was observed with microwave irradiation and heating at 60°C treatments by flow cytometry. The same was not observed for UV irradiation, which needs further studies.

Overall, little is known about the mechanism of antifungal effects of chemical agents. Potential targets include cell membranes, metabolic pathways and enzymes driving those pathways and DNA integrity or replication and gene expression. The results from the cytometric studies indicate that the effect of heating at 60°C and microwave irradiation results in a lethal primary membrane lesion, which is an irreversible phenomenon. For chemical agents (commercial solutions), only for Hydrogen Peroxide physical membrane damage appears to be the primary cause of the antifungal effect, since as the percentage of death increased, PI staining also increased after Hydrogen Peroxide exposure. Indeed, Hydrogen Peroxide provokes oxidation that leads to membrane rupture. Regarding the other chemical agents, further studies should be addressed involving other fluorescence substrates in order to put in evidence the mechanism of action.

Generally, the sensitivity of *C. parapsilosis* clinical and *C. parapsilosis* environmental strains to the chemical and physical agents did not vary much; however differences were found regarding ultraviolet irradiation and heating at 60°C, since the reduction among environmental strains was lower than the reduction among clinical strains, which means that this type of strains are more tolerant to these treatments.

With this study we look forward to establish effective measures and protocols for hospital surface disinfection and hand washing. We hope to provide a valuable contribution to the reduction of the incidence of candidaemia by *C. parapsilosis*, by limiting its presence in environmental niches.

It is widely recognized that routine cleaning of high-touch surfaces does not completely kill microbial pathogens. Therefore, improved disinfecting methods addressing air and surface decontamination are needed. Preliminary studies suggest that hydrogen peroxide disinfection (vapor and aerosol) is a promising technology for environmental decontamination of the hospital settings [314]. In our study, we used the new Glosair™ 400® system which provides aerosolised hydrogen peroxide/silver cations dry-mist. The antimicrobial efficacy of this technology was assessed both *in situ* and *in vitro* (manuscript VII in Publications section); susceptibility testing of several microorganisms to the disinfectant solution (Glosair™ 400® Cartridge) was also performed. Regarding the antimicrobial activity against *C. parapsilosis*, the dry-mist system resulted in a 5 *log* reduction of growth. According to the susceptibility testing results, the disinfectant solution exhibited a potent antimicrobial activity even following dilution. Despite this classic approach does not mimic “real life” situation, *in vitro* susceptibility testing is very helpful to optimize novel disinfection cycles and disinfectant concentrations, as could be the case with shorter duration cycles, an interesting option for the disinfection of high turnover facilities like operation theatres.

The antimicrobial properties of the product are the result of the oxidative action of hydrogen peroxide on the lipid membrane, which results in modification in ribosomes and DNA, and the effect of silver cations, which reverse membrane polarity and inhibit protein synthesis and cytoplasmic enzyme activity [315]. Flow cytometry technology is considered a very reliable methodology to evaluate the mechanism of action of antimicrobial drugs. Our results confirmed the cell membrane lesion in a short period of time by the hydrogen peroxide/silver cations mist, meaning its microbicidal effect.

Thus, our results strongly support the use of the hydrogen peroxide/silver cations dry-mist system as an excellent alternative to manual disinfection of hospital facilities due to its broad microbicidal activity and high efficacy.

VIRULENCE ATTRIBUTES

AZOLE RESISTANT AND SUSCEPTIBLE STRAINS

Overall, antifungal drug resistance and fungal virulence are critical issues for the host-parasite relationship during a fungal infection. There is a growing concern about the rise of antifungal drug resistance, but scarce knowledge is yet available regarding the biological behaviour and pathogenicity of drug-resistant fungal strains. Due to the growing medical importance of *C. parapsilosis*, we decided to study the virulence attributes of two strains with opposite susceptibility profiles; the resistant strain derived from prolonged exposure to the antifungal *in vitro* and the susceptible parent strain. We compared both phenotypic and genotypic attributes of virulence.

Our findings have extended the observations of Lott et al. [77] and Enger et al. [83] that phenotypic switching occurs in *C. parapsilosis*. Additionally, the results demonstrated that switching is unrelated with the antifungal susceptibility profile since it occurs in both strains, although in different extent. We identified for each strain two phenotypes: smooth and crepe for the susceptible strain and smooth and concentric for the resistant strain. The phenotypes were similar to those described by Laffey et al. [109], who also found that the majority of *C. parapsilosis* isolates switched between at least two different morphologies. Interestingly, we identified only in the resistant strain the white-opaque transition and the sectoring phenomenon. It is the first time that white-opaque transition is described for *C. parapsilosis* species. For *C. albicans* it was described that white and opaque cells harbor identical genomes yet they differ in cellular morphologies, gene expression profiles, mating abilities, and virulence properties [316-322]. Regarding virulence, the opaque phase is considered less virulent. Phenotypic switching has been associated with promoted adherence of *C. albicans* to mammalian cells. In the well-studied white-opaque transition, *C. albicans* cells expressing the white phenotype display increased adhesion to buccal epithelia than cells expressing the opaque phenotype [323]. Vargas et al. [324] showed that in a different *C. albicans* switching system with four phenotypes, there is a hierarchy of adhesion to buccal epithelia and stratum corneum from pigs; the phenotype o-smooth is the most adhesive, followed by irregular wrinkle, revertant smooth and star. Our results demonstrated that *C. parapsilosis* resistant strain exhibited increased adherence, also being the strain displaying higher frequency of smooth phenotype. Potential *in vivo* inducible virulence factors of *C. parapsilosis* are considered to include adherence and slime production, an attribute of special importance for adhesion to

plastic and therefore for catheter-related candidaemia [76] or infection of other medical indwelling devices.

The propensity for biofilm formation has recently been shown to have an impact upon both pathogenicity and drug resistance [325]. Angiolella et al. [326] observed a marked increase in the propensity for biofilm formation by *C. albicans* when the fungus acquired antifungal drug resistance following exposure to the drug *in vitro*. In contrast, for *C. parapsilosis* species, our results demonstrated that the resistant strain produces less biofilm. We believe that this is related with phenotypic switching rather than the antifungal resistant trait itself. Laffey et al. [109] found that the concentric phenotype formed the greatest amount of biofilm, whilst the smooth phenotype formed less biofilm. Despite the resistant strain displaying the concentric phenotype, the frequency of the smooth phenotype is much higher.

Regarding *C. albicans* there is substantial agreement that both adherence properties and the capacity to switch from yeast to mycelium - which are possibly interrelated - play a substantial role in host tissue invasion [327]. Filamentation in *C. parapsilosis* strains studied occurred in more extension in the resistant strain. The higher filamentation should relate with higher biofilm production, since most biofilm is composed of filamentous cells. However, this was not observed due the frequency of the smooth phenotype; this is the phenotype that predominantly grows as budding yeasts and produces less biofilm and does not invade agar. Nevertheless, we believe that filamentation and biofilm formation in *C. parapsilosis* are closely correlated like in *C. albicans*.

According to Laffey et al. [109] the smooth phenotype grows more rapidly. Indeed, the cell number for the resistant *C. parapsilosis* strain is higher, at all points of the growth curve.

Although secreted enzymes are putative virulence factors for all yeast species, the patterns of expression vary considerably across species. Herein, we studied two enzymes, coagulase and catalase, the last being more active in the resistant strain. Studies with secreted aspartyl proteases (SAPs) and phospholipases could have also been addressed. However, its importance as virulence factors for *C. parapsilosis* is somehow controversial. Although SAPs may play a role in *C. albicans* adhesion [328], they are not important for the primary mode of invasion (through the gut mucosa) since knockout strains do not display attenuated virulence [116]. However, SAPs may be important for pathogenesis at other sites or stages of infection. Regarding *C. parapsilosis*, De Bernardis et al. [96] found an inverse relationship with invasiveness. Kuhn's findings also did not support the concept that SAP expression is a critical virulence factor for *C. parapsilosis* [95]. Phospholipases are important virulence factors for *C. albicans* [115]. Kuhn

et al [95] did not find correlation between phospholipase activity and the site of infection or other virulence factors in *C. parapsilosis*.

The assumption that antifungal drug resistance clearly influences fungal virulence was not fully and definitively confirmed; the differences observed could be linked to the presence of the different phenotypes. Nevertheless, several studies described similar findings for *C. albicans*. Previous studies described the correlation between expression of multiple virulence factors with antifungal resistance for *C. albicans* [326].

The definitive analysis of the role of virulence attributes would require further genetic analysis and studies in animal models, examining the behavior of knockout mutants of *C. parapsilosis*.

A first step was taken by us with microarray analysis, which revealed that several groups of genes associated with fungal virulence were overrepresented in the resistant strain. Genes that regulate adherence to plastic and biofilm development in *C. albicans* are often also involved in cell adherence and invasion. *EFG1* is a well characterized gene, which apart from being required for filamentation and biofilm development [329, 330], is also necessary for adherence and invasion of reconstructed human epithelia [331]. Members of the ALS adhesin family are also required for adhesion to human cells [332, 333] and are expressed differentially in biofilms [334]. *C. parapsilosis* microarray analysis showed that *EFG1* and *ALS1* genes were both up-regulated in resistant strain. This finding correlates closely with the adherence assay results but not with biofilm development, which possible results from phenotypic switching in resistant strain. Anyway, this first genetic approach supports the correlation between azole resistance and a promoted expression of virulence attributes.

ECHINOCANDIN NONSUSCEPTIBLE AND SUSCEPTIBLE STRAINS

Chitin is an essential part of the carbohydrate skeleton of the fungal cell wall and is a molecule that is not represented in humans or other vertebrates. Complex regulatory mechanisms enable chitin to be positioned at specific sites throughout the cell cycle to maintain the overall strength of the wall, also enabling rapid and life-saving modifications to be made under cell wall stress conditions. Chitin has also recently emerged as a significant player in the activation and attenuation of immune responses to fungi and other chitin-containing parasites [213]. Recently, the increase in the production of chitin has been related to reduced efficacy of echinocandins: treatment of *C. albicans in vitro* with sub-MIC caspofungin activates chitin synthesis, and reciprocally cells that have higher cell wall chitin are less susceptible to caspofungin [335, 336].

With the aim to study the chitin role and its contribution to decreased susceptibility to echinocandins by *C. parapsilosis*, the cell wall chitin content was compared between susceptible and nonsusceptible strains both by CFW resistance test and a novel flow cytometric protocol. This last revealed a higher discriminatory power than the classic methodology. Additionally, a nonsusceptible strain generated *in vitro* following exposure to anidulafungin was studied. In fact, for some nonsusceptible clinical strains and for the nonsusceptible *in vitro* induced strain, the cell wall chitin content was higher than for the susceptible strains. However this was not a constant pattern since other susceptible strains also had high chitin levels. Overall the results did not allow to correlated echinocandin nonsusceptibility to cell wall chitin content; nonetheless this is a hypothesis that cannot be excluded in future echinocandin resistance mechanism studies. It was shown that *C. parapsilosis* displays high levels of chitin when compared to other *Candida* species. The possibility that it can rise in the presence of the antifungal or that it can contribute to the intrinsic lower susceptibility to echinocandins displayed by *C. parapsilosis* deserves to be confirmed. In fact, our group already evaluated by flow cytometry the chitin level in different *Candida* species in the absence and the presence of caspofungin, independently the susceptibility phenotype. We observed that caspofungin triggered the level of chitin, including in *C. parapsilosis*, after caspofungin exposure for a short period of time. The same effect could occur during induction of resistance by echinocandins as is the case of BC041NS nonsusceptible strain obtained after cultured for 30 days the BC041S susceptible strain in the presence of anidulafungin.

Elevated chitin content appears to represent an adaptive growth response in the presence of echinocandins, in an attempt to maintain cell wall integrity and subsequent cell growth. This may occur either by selection of a sub-population of naturally occurring chitin-rich cells, and/or by induction of cell wall compensatory mechanisms that activate chitin synthesis. This increase in chitin content happens also whenever *Candida* strains display a paradoxical growth at high levels of drugs, despite exhibiting a normal susceptibility pattern in presence of a lower minimal inhibitory concentration value [337]. van Asbeck et al. [148] demonstrated that occurrence of the paradoxical effect with caspofungin is higher in *C. parapsilosis* than with *C. albicans*. The paradoxical growth effect described was observed with one single *C. parapsilosis* strain tested for chitin level.

Studies concerning the role of individual chitin synthase genes should also be addressed in the future. Munro et al. [146] described that treatment of *C. albicans* with low levels of echinocandins stimulated chitin synthase (CHS) gene expression, increased Chs activity, elevated chitin content and reduced efficacy of these drugs. Elevation of chitin synthesis was mediated

via the PKC, HOG, and Ca(2+)-calcineurin signalling pathways. Stimulation of Chs2p and Chs8p by activators of these pathways enabled cells to survive otherwise lethal concentrations of echinocandins, even in the absence of Chs3p and the normally essential Chs1p, which regulate the synthesis of the chitinous septal ring and primary septum of the fungus. Under such conditions, a novel proximal offset septum was synthesized, which restored the capacity for cell division, sustained the viability of the cell, and abrogated morphological and growth defects associated with echinocandin treatment and the Chs mutations.

Another limitation of our study is the fact that it should have included more *C. parapsilosis* strains and although the strains had no mutation in the HS1 region of *FKS1* gene, as previous discussed, mutations in other locations should have been looked for.

CONCLUSIONS

MOLECULAR DISTINCTION BETWEEN *C. PARAPSILOSIS*, *C. ORTHOSILOSIS* AND *C. METAPSILOSIS*

- In a Portuguese university hospital, *C. parapsilosis* sensu stricto was predominant among clinical isolates, whilst the incidence of *C. orthopsilosis* and *C. metapsilosis* was low, especially regarding isolates from blood cultures.

GENOTYPING OF *C. PARAPSILOSIS*

- *C. parapsilosis* isolates (clinical and environmental) were genomic homogeneous.

ANTIFUNGAL SUSCEPTIBILITY TESTING

- *C. parapsilosis* groups displayed low susceptibility against echinocandins.
- *C. parapsilosis* groups displayed high susceptibility against amphotericin B.
- Azoles resistance was detected only among *C. parapsilosis* sensu stricto isolates.
- A multi-echinocandin and multi-azole resistant phenotype was observed among *C. parapsilosis* sensu stricto isolates.
- The differences in antifungal susceptibility described for different species (*C. parapsilosis* sensu stricto, *C. orthopsilosis* and *C. metapsilosis*) were not sufficiently distinct to warrant discrimination of the species in the clinical laboratory.

IN VITRO INDUCTION OF ANTIFUNGAL RESISTANCE

- The development of *in vitro* azole resistance in *C. parapsilosis* after growth in the presence of azole antifungals was rapid and stable.
- *In vitro* induction assays of resistance revealed that fluconazole was the fastest inducer while posaconazole was the slowest.
- The development of echinocandins resistance in *C. parapsilosis* after growth in the presence of anidulafungin was stable.

***IN VIVO* INDUCTION OF AZOLE ANTIFUNGAL RESISTANCE**

- Fluconazole rapidly induced a decreased susceptibility to itself and other azoles; the resistant phenotype acquired was stable.
- Post-treatment isolates with reduced susceptibility were genetically identical to susceptible pre-treatment isolates, confirming the acquisition of azole resistance.

ANTIFUNGAL RESISTANCE MECHANISMS (*IN VITRO* AND *IN VIVO* ACQUIRED)

- ATP-dependent efflux pumps played a minor role in azole resistance in *C. parapsilosis*.
- Azoles impaired the expression of genes involved in multiple cellular processes, resulting in a pleiotropic effect upon gene expression.
- Resistance displayed by *C. parapsilosis* strains was associated with increased expression of MDR1p and other drug efflux pumps members, namely MFS and with ergosterol production.
- *MDR1* overexpression was correlated with increased expression of the *MRR1* transcription factor caused by single gain-of-function mutations.
- Overexpression in ergosterol production was not associated with mutations in transcription factors *NDT80* and *UPC2*.
- Cross-resistance between different azoles occurred due to increase in lipid metabolism.
- Different findings were obtained for *in vitro* and *in vivo* induced resistance with fluconazole.
- Similar mechanisms confer resistance to azole drugs in *C. parapsilosis* and *C. albicans*.

SUSCEPTIBILITY TO PHYSICAL AND CHEMICAL AGENTS

- Heating at 60°C, microwave irradiation, chlorhexidine, ethanol and sodium hypochlorite seem to be very effective against *C. parapsilosis* in a short period of time.

- The hydrogen peroxide/silver cations dry-mist system was a good microbicidal effect, namely against *C. parapsilosis*.

VIRULENCE ATTRIBUTES

- A relationship between virulence and resistance for *C. parapsilosis* could not be established, although some attributes were enhanced for resistant strain (growth capacity, phenotypes diversity, pseudohyphal development, adherence and catalase expression).
- Microarray analysis evidenced that some especially groups of genes correlated with pathogenicity were found to be overexpressed in *C. parapsilosis* resistant strain.
- Nonsusceptibility to echinocandins exhibited by *C. parapsilosis* clinical isolates was not associated with mutation in HS1 region of the *FKS1* gene.
- Cell wall chitin levels differed within *C. parapsilosis* species although it was not possible to establish a relation between chitin levels and echinocandins high MIC values displayed by *C. parapsilosis* isolates.
- Long time *in vitro* exposure to anidulafungin increase cell wall chitin levels.
- An association between a paradoxical growth effect and a high chitin content was established.

FUTURE PERSPECTIVES

The results of the different topics addressed in our study provide a useful insight into several aspects of *C. parapsilosis*, such as epidemiology, virulence and drug susceptibility, leading to further research. Some aspects that should be taken into consideration for future investigations are:

1. To evaluate the impact of point mutations in the transcription factor *MRR1* upon *C. parapsilosis* azole resistance; to achieve this goal, a double deleted *C. parapsilosis* strain for the mentioned transcription factor must be constructed and the susceptibility profile and gene expression evaluated.
2. To continue the surveillance study in Portuguese university hospitals in order to characterize the susceptibility profile of *C. parapsilosis* clinical isolates; the resistant isolates identified should be screened for point mutations in transcription factors, *MRR1*, *UPC2* and *NDT80* and its role in azole resistance assessed.
3. The followed up of *in vivo* infection cases by *C. parapsilosis*: the isolates from patients that developed candidaemia and are receiving azole treatment should be evaluated; the goal is to establish a parallelism with our *in vitro* findings regarding the resistance mechanisms developed following prolonged exposition to different classes of antifungals.

SUPPLEMENTAL MATERIAL

Table S1. Genes with differential expression in BC014R_{FLC}.

Table S2. Genes with differential expression in BC014R_{VRC}.

Table S3. Genes with differential expression in BC014R_{PSC}.

Table S4. GO terms overexpressed in BC014R_{VRC} and BC014R_{PSC} strains.

Table S5. Genes differentially regulated in BC014R_{FLC} and BC014R_{VRC} strains.

Supplementary Tables S1, S2, S3, S4 and S5 are available at Supplemental Material section of the manuscript “Transcriptional profiling of azole-resistant *C. parapsilosis* strains” (manuscript VI in Publications section) in the Antimicrobial Agents Chemotherapy journal internet homepage <http://aac.asm.org/cgi/content/full/55/7/3546/DC1>.

Table S6. Genes with differential expression in BCA₃ strain.

Cpar.ID	C.a.Orthologue	log2FC _l oligo1	log2FC _l oligo2	P_value ₁ l _{oligo1}	P_value _l oligo2	Gene.Name	Description
cpar1995	orf19.1406	-129.848.462.616.646	-0.803655524442245	0.118889469075455	0.315926026090364		Predicted ORF in Assemblies 19, 20 and 21
cpar1019	orf19.2023	-208.681.919.176.618	-198.339.402.110.611	0.168422850557742	0.203884150033615	HGT7	Putative glucose transporter, major facilitator superfamily; glucose-, fluconazole-, Snf3p-induced, expressed at high glucose; upregulated in biofilm; C. albicans glucose transporter family comprises 20 members; 12 TM regions predicted
cpar2226	orf19.2259	-109.812.221.253.864	-0.229801973550969	0.1590878412121263	0.615201371335854		Predicted ORF in Assemblies 19, 20 and 21; possibly spurious ORF (Annotation Working Group prediction)
cpar3013	orf19.2877	-252.457.404.091.387	-256.204.154.229.564	0.173549553274852	0.184243584388577	PDC11	Protein similar to pyruvate decarboxylase; antigenic; at hyphal cell surface, not yeast-form cells; soluble in hyphae; regulated by Gcn4p, Efg1p, Efh1p; fluconazole-, farnesol-, biofilm-induced; repressed upon amino acid starvation
cpar2008	orf19.2879	-1.408.388.435.316	-0.95112049605935	0.113684589481628	0.29387897540467	IFF5	Putative GPI-anchored protein of unknown function
cpar2919	orf19.3089	-0.96685065998711	-114.238.391.964.187	0.168422850557742	0.168422850557742		Predicted ORF in Assemblies 19, 20 and 21
cpar4678	orf19.3159	-139.307.284.382.967	-0.868214068148786	0.115085094881521	0.509703582864182	UTP20	Protein likely to be essential for growth; similar to S. cerevisiae Utp20p, which is a putative snoRNA-binding protein; downregulated in core stress response; mutation confers resistance to 5-fluorocytosine (5-FC)
cpar2571	orf19.3220	-0.924609057874373	-101.024.858.924.456	0.135031917608232	0.150472294925116		Predicted ORF in Assemblies 19, 20 and 21
cpar5497	orf19.3303	-123.597.126.255.186	-0.0261968890658263	0.115085094881521	0.984570838663422		Predicted ORF in Assemblies 19, 20 and 21
cpar2651	orf19.3307	-171.312.109.018.534	-0.973357910598634	0.166845897159102	0.447668055461013		Predicted ORF in Assemblies 19, 20 and 21
cpar3341	orf19.3364	-149.366.406.193.974	-0.691838134071811	0.116681069316788	0.326347388052806		Predicted ORF in Assemblies 19, 20 and 21; possibly spurious ORF (Annotation Working Group prediction)
cpar3649	orf19.3470	-111.325.033.867.753	-0.723881395702415	0.164801209130932	0.403305306646575		Putative flavodoxin; similar to S. cerevisiae Ypl207p; predicted Kex2p substrate
cpar685	orf19.3559	-108.651.415.875.393	-107.086.072.684.391	0.163158994725551	0.184749346626205		Predicted ORF in Assemblies 19, 20 and 21
cpar2424	orf19.382	-118.951.965.208.955	-0.553512740686964	0.150472294925116	0.650976319472361	TEF2	Translation elongation factor 1-alpha; genes encoding ribosomal subunits; translation factors, and tRNA synthetases are downregulated upon phagocytosis by murine macrophage
cpar2481	orf19.3914	-124.083.398.479.148	-108.732.728.809.534	0.170975793680534	0.236568627073898		Predicted ORF in Assemblies 19, 20 and 21
cpar5639	orf19.3938	-147.401.077.720.855	-0.945103653395126	0.162895879610040	0.496936707868275		Predicted ORF in Assemblies 19, 20 and 21; mutation confers hypersensitivity to 5-fluorocytosine (5-FC), 5-fluorouracil (5-FU), and tubercidin (7-deazaadenosine)
cpar519	orf19.397	-101.427.604.342.712	-105.810.238.542.044	0.141579140830077	0.150472294925116		Predicted ORF in Assemblies 19, 20 and 21
cpar439	orf19.4000	-113.622.696.089.941	-110.165.359.578.049	0.113684589481628	0.113684589481628		Putative homeodomain transcription factor; predicted ORF in

cpar2006	orf19.4072	-110.392.637.074.991	-0.869109928866887	0.164801209130932	0.304629632350125	IFF6	Assemblies 19, 20 and 21
cpar5085	orf19.4398	-117.745.480.838.293	-0.59103535209286	0.152882379390760	0.604038430044196		Putative GPI-anchored protein of unknown function; opaque-specific transcription; macrophage-induced gene
cpar5525	orf19.4599	-114.348.042.319.759	-0.0263385145823501	0.141579140830077	0.987874523953426	PHO89	Predicted ORF in Assemblies 19, 20 and 21
cpar94	orf19.4774	-104.453.412.224.675	-0.90879081433176	0.168422850557742	0.209599338296351	AOX1	Putative phosphate permease; expression is regulated upon white-opaque switching; alkaline upregulated by Rim101p; induced upon biofilm formation; possibly adherence-induced
cpar3515	orf19.5305	-116.076.625.977.950	-0.64402327957662	0.168422850557742	0.584752850739337	RHD3	Alternative oxidase; low abundance; constitutively expressed; one of two isoforms (Aox1p and Aox2p); involved in a cyanide-resistant respiratory pathway present in plants, protists, and some fungi, although absent from <i>S. cerevisiae</i>
cpar1821	orf19.5867	-107.292.672.375.471	-0.779441351805829	0.141579140830077	0.357073018715095	WSC1	Putative GPI-anchored protein that localizes to the cell wall; transcription is decreased upon yeast-hyphal switch; transcriptionally regulated by iron; expression greater in high iron; clade-associated gene expression
cpar837	orf19.6199	-133.474.247.567.601	-138.671.433.069.514	0.153925841537802	0.157808324913626		Protein described as a cell wall component; increased mRNA abundance observed in <i>cyr1</i> homozygous null mutant (yeast-form or hyphal cells)
cpar240	orf19.6643	-123.918.753.187.167	-111.134.944.905.182	0.146451372361865	0.167329309882749	MCT1	Predicted ORF in Assemblies 19, 20 and 21; intron in 5'-UTR
cpar2247	orf19.6873.1	-103.693.840.817.237	-0.710819952715596	0.152882379390760	0.431980468107784		Putative malonyl-CoA acyl carrier protein acyltransferase
cpar396	orf19.6879	-103.862.626.796.340	-0.972203936588795	0.173108241298162	0.1749333180701379		ORF Predicted by Annotation Working Group
cpar5722	orf19.6919	-105.882.575.587.725	-0.422403614218905	0.121110746060842	0.78709167525693		Predicted ORF in Assemblies 19, 20 and 21
cpar1605	orf19.7463	-126.482.415.698.285	-110.990.166.096.962	0.150472294925116	0.256740819704904		Predicted ORF in Assemblies 19, 20 and 21
cpar1498	orf19.7561	-103.524.525.969.257	-0.700866076396272	0.141579140830077	0.450609272645031	DEF1	Protein required for filamentous growth and for escape from epithelial cells and dissemination in an RHE model; transcription induced in oral candidiasis clinical isolates; induced by fluconazole, high cell density; hyphally regulated
cpar3603	orf19.7595	-157.667.225.205.263	-140.067.037.808.502	0.113684589481628	0.1739968639873865		Predicted ORF in Assemblies 19, 20 and 21; possibly spurious ORF (Annotation Working Group prediction)
cpar5511		-124.912.506.622.687	-0.706946822161488	0.113684589481628	0.189117298499006	NA	NA
cpar1239		-162.787.418.200.686	-0.952191930624375	0.128319236290678	0.459482926886255	NA	NA
cpar5370		-151.005.523.143.012	-126.052.363.250.399	0.129314843364686	0.150472294925116	NA	NA
cpar1299		-158.393.606.967.242	-125.790.639.483.921	0.13079836932517	0.456198525771049	NA	NA
cpar3574		-136.795.595.977.652	-101.913.786.781.044	0.139383155994552	0.424775682775721	NA	NA

cpar4612	-0.996237867152487	-106.604.362.607.409	0.118889469075455	0.139383155994552	NA	NA
cpar3884	-114.813.690.520.605	-111.466.833.014.937	0.141579140830077	0.188923986373931	NA	NA
cpar2678	-136.201.553.387.650	-0.193494142311092	0.141579140830077	0.79663641990539	NA	NA
cpar4384	-103.161.453.133.521	-111.601.485.633.008	0.150472294925116	0.150472294925116	NA	NA
cpar5250	-0.868383149710646	-104.778.954.928.058	0.135031917608232	0.159087841221263	NA	NA
cpmt12	-133.960.143.714.398	-113.253.044.392.833	0.162895879610040	0.304169453961649	NA	NA
cpar4749	-132.460.482.242.225	-0.524542770509344	0.163158994725551	0.506156003882823	NA	NA
cpar4242	-111.628.782.225.742	-0.974559128801264	0.165058766612597	0.465259720769405	NA	NA
cpar2491	-117.334.060.435.774	-110.547.297.368.973	0.168422850557742	0.191085887568539	NA	NA
cpar1915	-106.053.151.690.252	-102.113.793.223.478	0.173414822250465	0.182703519298771	NA	NA
cpara5839_part	-101.187.667.557.711	-0.934052716539512	0.174933180701379	0.192821758867636	NA	NA
cpar3675	orf19.1089	100.541.692.363.093	0.141579140830077	0.188859278256094	PEX11	Putative protein involved in fatty acid oxidation; expression is Tac1p-dependent
cpar1478	orf19.1212	111.605.219.067.689	0.141579140830077	0.174933180701379		Predicted ORF in Assemblies 19, 20 and 21
cpar2961	orf19.1239	141.975.224.365.255	0.113684589481628	0.153925841537802		Predicted ORF in Assemblies 19, 20 and 21; exogenously expressed protein is a substrate for Kex2p processing in vitro; fluconazole-downregulated; possibly spurious ORF (Annotation Working Group prediction)
cpar472	orf19.1288	180.298.666.239.916	0.113684589481628	0.118889469075455	FOX2	Predicted 3-hydroxyacyl-CoA epimerase, required for fatty acid beta-oxidation; upregulated upon phagocytosis; transcription is regulated by Mig1p; expression is regulated upon white-opaque switching
cpar170	orf19.1313	127.361.727.221.673	0.113684589481628	0.113684589481628	CDR3	Transporter of the Pdrp/Cdrp family of the ATP-binding cassette (ABC) superfamily; transports phospholipids in an out-to-in direction; expressed in opaque-phase cells; induced by macrophage interaction; fluconazole-downregulated
cpar176	orf19.1324	14.748.119.244.729	0.625404362315534	0.190094064922648	RAD2	Ortholog of <i>S. cerevisiae</i> RAD2, nucleotide excision repair nuclease; null mutant is extremely sensitive to UV irradiation; transcription is upregulated upon interaction with macrophages
cpar902	orf19.1344	134.360.721.195.315	0.113684589481628	0.188923986373931		Predicted ORF in Assemblies 19, 20 and 21; fluconazole-induced; possibly spurious ORF (Annotation Working Group prediction)
cpar4307	orf19.1655	131.362.942.197.217	0.118889469075455	0.118889469075455	PXP2	Putative acyl-CoA oxidase; enzyme of fatty acid beta-oxidation; induced during macrophage infection; expression is opaque specific; has putative peroxisome targeting signal
cpar486	orf19.1671	112.916.857.176.958	0.163145602541302	0.440957508922585	UTR2	Putative glycosidase; cell wall; role in adhesion; required for hyphal growth on Spider (not serum) medium; chitin-binding, glycosyl hydrolase, GPI anchor domains; induced during cell wall

cpar4458	orf19.1684	135.700.488.849.138	0.497261258821386	0.128319236290678	0.258586648258297		regeneration; mRNA detected in yeast-form cells
cpar4464	orf19.1704	134.312.830.706.516	145.567.126.246.569	0.168422850557742	FOX3		Predicted ORF in Assemblies 19, 20 and 21
cpar3794	orf19.1760	131.535.755.671.526	125.567.783.519.818	0.118889469075455	RAS1		Putative peroxisomal 3-oxoacyl CoA thiolase; expression is regulated upon white-opaque switching
cpar3006	orf19.1801	109.262.590.763.011	0.246405689063002	0.127770380594641			RAS signal transduction GTPase; regulates cAMP and MAP kinase pathways; role in hyphal induction, virulence, apoptosis, heat-shock sensitivity; nonessential; glutamine-rich C terminus; complements viability of <i>S. cerevisiae</i> ras1 ras2 mutant
cpar1693	orf19.1901	128.269.946.043.326	0.528663637251353	0.145446738950241	MCM3		Predicted ORF in Assemblies 19, 20 and 21; periodic mRNA expression, peak at cell-cycle M/G1 phase
cpar1060	orf19.2078	133.363.705.853.807	-0.0177666518985552	0.140854496147095			Predicted ORF in Assemblies 19, 20 and 21
cpar1815	orf19.2220	243.572.089.700.814	191.425.578.585.756	0.129466785652842			Predicted ORF in Assemblies 19, 20 and 21; induced by Rgt1p
cpar5215	orf19.2227	135.693.901.279.406	0.396183093607269	0.141579140830077			Predicted ORF in Assemblies 19, 20 and 21; repressed by nitric oxide
cpar2230	orf19.2253	109.879.929.003.289	134.843.559.829.524	0.121798291335418			Predicted ORF in Assemblies 19, 20 and 21; expression is regulated upon white-opaque switching; transcription is repressed in response to alpha pheromone in SpiderM medium
cpar5086	orf19.2397.3	122.620.585.793.055	181.397.294.130.370	0.113684589481628			ORF Predicted by Annotation Working Group
cpar4955	orf19.2460	119.805.201.258.528	136.382.632.067.720	0.113684589481628			Predicted ORF in Assemblies 19, 20 and 21; exogenously expressed protein is a substrate for Kex2p processing in vitro; transcription is repressed in response to alpha pheromone in SpiderM medium
cpar4968	orf19.2480.1	128.828.273.461.531	166.498.359.402.344	0.113684589481628	AUT7		ORF Predicted by Annotation Working Group; macrophage/pseudohyphal-repressed; intron in 5'-UTR
cpar461	orf19.2499	106.278.538.884.471	0.438931873757049	0.150472294925116			Predicted ORF in Assemblies 19, 20 and 21
cpar5822	orf19.2633	161.016.743.731.380	173.406.870.415.838	0.118889469075455	HGT14		Putative glucose transporter of the major facilitator superfamily; the <i>C. albicans</i> glucose transporter family comprises 20 members; 12 probable membrane-spanning segments; expressed in rich medium with 2% glucose
cpar3460	orf19.2693	123.934.878.199.729	0.98307557963911	0.118889469075455			Increased transcription is observed upon benomyl treatment transcription and in populations of cells exposed to fluconazole over multiple generations; regulated by Nrg1p, Tup1p; induced by nitric oxide
cpar4736	orf19.272	118.445.253.235.981	113.020.783.488.583	0.121110746060842	FAA21		Predicted acyl CoA synthetase; upregulated upon phagocytosis; transcription is regulated by Nrg1p and Mig1p
cpar2018	orf19.2747	132.797.225.804.351	0.402309740260914	0.131235411369423	RGT1		Transcriptional repressor involved in the regulation of glucose transporter genes; has zinc-cluster; similar to <i>S. cerevisiae</i> Rgt1p
cpar2017	orf19.2748	117.080.338.768.053	0.899579273534549	0.141579140830077			Predicted zinc-finger protein of unknown function; clade-associated gene expression; similarity to <i>S. cerevisiae</i> Arg81p, which is a transcription factor involved in the regulation of arginine-responsive

								genes
cpar826	orf19.2787	129.463.414.703.296	154.261.187.904.155	0.118889469075455	0.16730827800205	PRY1		Transcription is opaque specific; transcription is repressed in response to alpha pheromone in SpiderM medium
cpar2665	orf19.2809	100.025.774.085.499	106.048.096.331.833	0.153925841537802	0.164910811732469	CTN3		Predicted peroxisomal carnitine acetyl transferase; Ura+ deletion strain has no obvious metabolic, hyphal, or virulence defects; transcription induced by macrophage engulfment, hyphal growth, starvation, or nonfermentable carbon sources
cpar3881	orf19.2843	118.643.238.994.621	0.527007896528811	0.150472294925116	0.225051606987859	RHO1		Small GTPase of Rho family; regulates beta-1,3-glucan synthesis activity and binds Gsc1p; essential; expected to be geranylgeranylated by geranylgeranyltransferase type I
cpar4872	orf19.2941	154.842.177.196.551	176.723.272.498.714	0.113684589481628	0.13463368376311	SCW4		Putative cell wall protein; exogenously expressed protein is a substrate for Kex2p processing in vitro; expression is regulated upon white-opaque switching; alkaline downregulated
cpar780	orf19.3029	108.074.049.965.789	135.381.510.589.750	0.150472294925116	0.212869545632114			Predicted ORF in Assemblies 19, 20 and 21
cpar2807	orf19.305	129.473.234.635.742	134.932.449.495.773	0.113684589481628	0.113684589481628			Predicted ORF in Assemblies 19, 20 and 21; greater mRNA abundance observed in a <i>cyr1</i> homozygous null mutant than in wild type
cpar2805	orf19.307	111.760.674.703.986	0.374888166210256	0.141579140830077	0.313177650884735			Predicted ORF in Assemblies 19, 20 and 21
cpar5225	orf19.3152	110.166.511.284.606	12.240.389.482.704	0.118889469075455	0.216108710052902	AMO2		Protein similar to <i>A. niger</i> predicted peroxisomal copper amino oxidase; mutation confers hypersensitivity to toxic ergosterol analog; induced upon biofilm formation
cpar5074	orf19.3232	162.518.221.284.057	138.842.788.072.008	0.113684589481628	0.113684589481628			Putative transporter; mutation confers hypersensitivity to toxic ergosterol analog; fungal-specific (no human or murine homolog)
cpar3216	orf19.3353	108.885.072.831.008	130.728.043.743.736	0.118889469075455	0.247032123656604			Protein described as similar to a mitochondrial complex I intermediate-associated protein; fluconazole-downregulated
cpar1224	orf19.3405	111.783.197.982.510	0.609302884015753	0.145136852837564	0.194365145457278			Putative transcription factor with zinc finger DNA-binding motif; heterozygous null mutant displays sensitivity to virgineone; possibly spurious ORF (Annotation Working Group prediction)
cpar3395	orf19.342	120.065.972.281.322	0.852822955256202	0.158923870649283	0.443313305080928	BMT7		Putative beta-mannosyltransferase, member of a 9-gene family that includes characterized genes BMT1, BMT2, BMT3, and BMT4 with roles in beta-1,2-mannosylation of cell wall phosphopeptidomannan; downregulated in an azole-resistant strain
cpar1185	orf19.3434	155.705.326.353.378	163.671.994.287.001	0.168422850557742	0.194365145457278			Predicted ORF in Assemblies 19, 20 and 21
cpar698	orf19.3548.1	155.124.647.132.339	186.079.415.062.240	0.164801209130932	0.195494274437383	WH11		Cytoplasmic protein expressed specifically in white phase yeast-form cells; expression in opaque cells increases virulence and frequency of opaque-to-white switching; null mutant shows wild-type switching; similar to <i>S. cerevisiae</i> Hsp12p
cpar2239	orf19.3615	107.848.257.352.869	0.968597287446255	0.141579140830077	0.150472294925116			Predicted ORF in Assemblies 19, 20 and 21; induced in core caspofungin response; increased expression observed in an <i>ssr1</i> homozygous null mutant; induced by nitric oxide in <i>yhb1</i> mutant
cpar2613	orf19.3829	113.896.377.117.921	155.267.459.739.321	0.129466785652842	0.207140293703005	PHR1		Glycosidase of cell surface; may act on cell-wall beta-1,3-glucan prior to beta-1,6-glucan linkage; E169 and E270 at active site; role in

									systemic but not vaginal virulence (neutral, not low pH); high pH or filamentation induced
cpar3508	orf19.3833	126.267.394.398.914	0.188063781230896	0.118889469075455	0.500058533462697				Putative DNA-binding transcription factor; decreased transcription is observed upon fluphenazine treatment
cpar277	orf19.3854	103.404.066.024.744	123.421.937.884.540	0.162895879610040	0.223941466378254				Protein similar to <i>S. cerevisiae</i> Sat4p; amphoterin B induced; clade-associated gene expression
cpar415	orf19.3902	147.246.695.260.280	144.307.728.052.565	0.141579140830077	0.141579140830077				Predicted ORF in Assemblies 19, 20 and 21; decreased transcription is observed upon fluphenazine treatment or in an azole-resistant strain that overexpresses CDR1 and CDR2
cpar2490	orf19.3931	117.857.237.119.107	125.783.330.549.268	0.113684589481628	0.141579140830077	SFC1			Alkaline upregulated
cpar5486	orf19.3981	167.680.969.872.461	172.915.080.126.468	0.141579140830077	0.157169887772819	MAL31			Putative protein of unknown function, transcription is upregulated in clinical isolates from HIV+ patients with oral candidiasis; alkaline upregulated
cpar5485	orf19.3982	119.365.777.761.849	161.515.207.442.841	0.118889469075455	0.192821758867636				Maltase; induced during growth on sucrose; transcription is induced in response to alpha pheromone in SpiderM medium
cpar5574	orf19.4041	111.048.439.740.592	102.520.880.908.173	0.113684589481628	0.113684589481628				Predicted ORF in Assemblies 19, 20 and 21
cpar5024	orf19.4063	111.429.702.930.806	143.180.306.259.454	0.141579140830077	0.239116583918972	GPT1			GABA/polyamine transporter; 9 to 11 membrane spanning segments; complements GABA uptake defect of an <i>S. cerevisiae</i> uga4 put4 gap1 triple mutant; complements growth of an <i>S. cerevisiae</i> spe1 mutant under polyamine limitation
cpar1828	orf19.4066	139.044.012.045.450	175.810.982.654.530	0.118889469075455	0.162895879610040				Predicted ORF in Assemblies 19, 20 and 21; Hog1p-downregulated
cpar5606	orf19.4229	101.247.620.006.074	0.468026867147086	0.162895879610040	0.685357138088234				Predicted ORF in Assemblies 19, 20 and 21
cpar2447	orf19.4317	166.872.425.170.788	115.539.222.596.674	0.113684589481628	0.118889469075455	GRE3			Similar to D-xylose reductases; antigenic in murine systemic infection; soluble protein in hyphae; transcriptionally induced by macrophage interaction; transcriptionally induced by Mnl1p under weak acid stress; farnesol-induced
cpar4185	orf19.4355	231.645.278.140.619	169.769.984.519.189	0.141579140830077	0.1734148222250465				Predicted ORF in Assemblies 19, 20 and 21
cpar5102	orf19.4384	152.371.701.216.639	171.537.117.420.602	0.113684589481628	0.1734148222250465	HXT5			Protein described as a sugar transporter; transcription is upregulated in response to treatment with ciclopirox olamine; Snf3p-induced; alkaline downregulated; shows colony morphology-related gene regulation by Ssn6p
cpar5167	orf19.4504	112.557.042.485.144	161.559.099.602.546	0.141579140830077	0.188867864038972				Predicted ORF in Assemblies 19, 20 and 21
cpar1011	orf19.4520	162.358.798.171.802	190.366.515.967.805	0.141579140830077	0.167329309882749				Predicted ORF in Assemblies 19, 20 and 21
cpar749	orf19.4527	122.640.810.516.774	101.922.903.083.994	0.13767067633346	0.189117298499006	HGT1			High-affinity glucose transporter, member of major facilitator superfamily; transcription induced by progesterone and by drugs including chloramphenicol and benomyl; likely essential for growth, based on an insertional mutagenesis strategy
cpar3565	orf19.4531	136.240.661.519.903	0.519552630054771	0.113684589481628	0.192821758867636				Putative PDR-subfamily ABC transporter

cpar3757	orf19.4551	157.837.980.960.958	199.203.012.644.790	0.128231279547375	0.178865961487306	CTN1	Predicted carnitine acetyl transferase; required for growth on nonfermentable carbon sources but not for hyphal growth or virulence in mouse systemic model; transcription induced in macrophage; macrophage/pseudohyphal-repressed after 16 hr
cpar3459	orf19.4682	224.145.666.125.088	184.654.172.409.633	0.113684589481628	0.145446738950241	HGT17	Putative glucose transporter of the major facilitator superfamily; the C. albicans glucose transporter family comprises 20 members; 12 probable membrane-spanning segments; induced at low (0.2%, compared to 2%) glucose in rich media
cpar4635	orf19.4744	112.514.553.853.974	0.399436784494625	0.150472294925116	0.369182148048191		Predicted ORF in Assemblies 19, 20 and 21; transcription is repressed in response to alpha pheromone in SpiderM medium
cpar348	orf19.4789	144.302.050.322.201	0.486346913429651	0.162895879610040	0.469333458769361		Predicted ORF in Assemblies 19, 20 and 21
cpar1547	orf19.4997	109.452.117.075.212	100.149.265.126.335	0.119306232442655	0.127770380594641	KIS2	Scaffold protein of Snf1p complex; similar to S. cerevisiae Gal83p and Sip2p; interacts with Snf4p; interaction with Snf1p complex is regulated by carbon source, decreased on ethanol; N-terminal myristoylation; Hog1p-downregulated
cpar1555	orf19.5000	198.625.239.991.167	151.454.073.722.573	0.118867694847168	0.141579140830077	CYB2	Protein described as precursor protein of cytochrome b2; transcriptionally regulated by iron; expression greater in high iron; alkaline downregulated; shows colony morphology-related gene regulation by Ssn6p
cpar950	orf19.5169	148.374.522.138.947	145.993.768.908.200	0.118889469075455	0.118889469075455		Predicted ORF in Assemblies 19, 20 and 21
cpar2258	orf19.5215	121.254.819.394.424	176.975.736.639.953	0.113684589481628	0.192821758867636	TES15	Predicted ORF in Assemblies 19, 20 and 21
cpar99	orf19.5234	102.944.867.820.403	0.850749811373377	0.141579140830077	0.376836828793016	RBD1	Rhomboid-like protein; similar to putative intramembrane serine proteases; involved in filamentous growth; Gcn4p-regulated; caspofungin repressed
cpar103	orf19.5238	101.989.656.541.741	0.918268781979994	0.150472294925116	0.476851558609565		Predicted ORF in Assemblies 19, 20 and 21
cpar667	orf19.5274	134.729.274.529.744	111.159.716.028.495	0.131235411369423	0.577586130807058		Predicted ORF in Assemblies 19, 20 and 21
cpar4080	orf19.5282	141.820.254.096.557	171.473.845.007.838	0.118889469075455	0.193818200678424		Predicted ORF in Assemblies 19, 20 and 21; decreased expression in hyphae compared to yeast-form cells; regulated by Efg1p and Efh1p; intron in 5'-UTR; transcriptionally activated by Mnl1p under weak acid stress
cpar3518	orf19.5307	208.219.389.564.207	197.083.495.909.585	0.172320611639595	0.189117298499006	JEN2	Protein described as carboxylic acid transporter; induced by Rgt1p
cpar298	orf19.532	123.055.522.375.351	108.673.713.101.407	0.121110746060842	0.141579140830077	RBR2	Cell wall protein; expression is repressed by Rim101p; expression is regulated upon white-opaque switching; transcription is repressed in response to alpha pheromone in SpiderM medium; macrophage-induced gene
cpar3845	orf19.5339	127.408.408.679.544	0.703442549040105	0.118889469075455	0.163884880962957		Predicted ORF in Assemblies 19, 20 and 21
cpar4483	orf19.5392	159.678.691.142.476	125.255.564.028.535	0.113684589481628	0.115085094881521	NGT1	N-acetylglucosamine (GlcNAc)-specific transporter; role in GlcNAc (but not serum) induced hyphal growth; localizes to plasma membrane; induced by GlcNAc, macrophage engulfment; 12

cpar4489	orf19.5431	149.902.844.978.514	106.468.111.935.626	0.150472294925116	0.530970253203355			transmembrane, major facilitator superfamily
cpar4503	orf19.5447	162.951.785.692.086	157.771.575.451.002	0.113684589481628	0.113684589481628	HGT19		Predicted ORF in Assemblies 19, 20 and 21
cpar4504	orf19.5449	157.407.044.319.041	162.677.552.127.486	0.113684589481628	0.113684589481628			Putative glucose/myo-inositol transporter of major facilitator superfamily; 12 transmembrane segments, extended N terminus; expressed in rich medium; 2% glucose; phagocytosis-induced; C. albicans glucose transporter family has 20 members
cpar3312	orf19.5536	154.139.273.701.715	206.087.089.727.513	0.141579140830077	0.168422850557742			Predicted ORF in Assemblies 19, 20 and 21
cpar3669	orf19.5565	140.177.927.961.753	165.457.116.351.247	0.113684589481628	0.190966342481824			Predicted ORF in Assemblies 19, 20 and 21
cpar3583	orf19.5611	110.574.137.279.596	138.365.861.700.735	0.121110746060842	0.172565992117270			Predicted ORF in Assemblies 19, 20 and 21
cpar1345	orf19.5640	0.892630237105151	111.230.075.734.619	0.140854496147095	0.141579140830077	PEX5		Protein of the Pex5p family; required for PTS1-mediated peroxisomal protein import; fatty acid beta-oxidation; similar to S. cerevisiae Pas10p peroxisomal targeting receptor; macrophage/pseudohyphal-repressed
cpar1441	orf19.5711	106.432.177.606.304	100.822.197.258.706	0.113684589481628	0.118889469075455			Predicted ORF in Assemblies 19, 20 and 21
cpar1467	orf19.5728	139.484.063.061.597	158.686.190.955.684	0.113684589481628	0.141579140830077			Predicted ORF in Assemblies 19, 20 and 21
cpar805	orf19.5753	0.647040683659456	133.583.403.005.602	0.153925841537802	0.162895879610040	HGT10		Glycerol permease involved in glycerol uptake; member of the major facilitator superfamily; induced by osmotic stress, at low glucose in rich media, during cell wall regeneration; 12 membrane spans
cpar3176	orf19.5806	205.972.487.333.849	177.822.625.002.226	0.163653700504823	0.188923986373931	ALD5		Protein described as an aldehyde dehydrogenase; decreased expression in fluconazole-resistant isolate, or in hyphae; biofilm induced; fluconazole-downregulated; protein abundance is affected by URA3 expression in the CAI-4 strain background
cpar1822	orf19.5869	111.339.237.493.611	0.80509811463345	0.169964006000173	0.46710637170607			Predicted ORF in Assemblies 19, 20 and 21
cpar1829	orf19.5917	130.291.707.090.356	133.918.321.135.204	0.150472294925116	0.233148234172390	STP3		Transcription factor; regulates SAP2, OPT1 expression and thereby protein catabolism for nitrogen source; activated via amino-acid-induced proteolytic processing; macrophage/pseudohyphal-repressed
cpar2109	orf19.5975	160.296.222.116.459	171.454.486.688.412	0.113684589481628	0.141579140830077			Putative transcription factor with zinc finger DNA-binding motif; fluconazole-downregulated; expression regulated during planktonic growth; increased expression observed in an ssr1 homozygous null mutant
cpar4336	orf19.6005	109.486.048.604.736	132.648.916.870.318	0.150472294925116	0.215977518208061	HGT5		Putative glucose transporter of the major facilitator superfamily; the C. albicans glucose transporter family comprises 20 members; 12 probable membrane-spanning segments, extended N terminus; expressed in rich medium with 2% glucose
cpar5473	orf19.6017	101.850.093.601.303	131.408.050.637.952	0.119221800209380	0.310410656528566			Predicted ORF in Assemblies 19, 20 and 21

cpar4559	orf19.6070	143.333.824.047.517	153.891.853.125.160	0.118889469075455	0.150472294925116	ENA2	Protein described as sodium transporter; transcription is upregulated in response to ciclopirox olamine; alkaline upregulated by Rim101p; repressed upon high-level peroxide stress; upregulated in oral candidiasis clinical isolates
cpar3192	orf19.6084	153.049.347.831.148	214.859.018.490.319	0.118889469075455	0.219655408762192		Predicted ORF in Assemblies 19, 20 and 21
cpar2900	orf19.6167	100.006.591.192.443	0.93346729454823	0.118889469075455	0.123710904529043	AVR1	Protein described as putative oxidoreductase; transcriptionally induced by interaction with macrophage
cpar849	orf19.6214	110.961.981.117.131	0.729937040555767	0.127078471847030	0.534059197131604	ATC1	Cell wall acid trehalase; catalyzes hydrolysis of the disaccharide trehalose; similar to <i>S. cerevisiae</i> vacuolar acid trehalase (Ath1p)
cpar2390	orf19.6229	160.790.308.316.079	171.733.382.912.970	0.153925841537802	0.295666857468395	CAT1	Catalase; role in resistance to oxidative stress, neutrophils, peroxide; role in murine systemic virulence; regulated by iron, ciclopirox olamine, fluconazole, growth, carbon source, pH, Rim101p, Ssn6p, Hog1p, farnesol, core stress response
cpar5317	orf19.6323	189.912.692.555.246	177.759.161.040.612	0.169503017674369	0.179967093685447		Predicted ORF in Assemblies 19, 20 and 21
cpar5311	orf19.6336	117.062.966.556.528	126.335.471.379.952	0.150472294925116	0.153925841537802	PGA25	Putative GPI-anchored protein of unknown function; fluconazole-downregulated
cpar1539	orf19.6343	112.015.816.577.301	118.684.234.445.209	0.129466785652842	0.248870594310554		Predicted ORF in Assemblies 19, 20 and 21
cpar3440	orf19.6459	104.012.601.673.929	0.753730954020977	0.173108241298162	0.601146246237651	DPP3	Protein similar to <i>S. cerevisiae</i> pyrophosphate phosphatase Dpp1p; required for farnesol biosynthesis; downregulated in response to 17-beta-estradiol, ethynyl estradiol
cpar3441	orf19.6460	110.964.003.154.064	133.735.446.572.182	0.127136414910064	0.195723834246427		Predicted ORF in Assemblies 19, 20 and 21
cpar3913	orf19.652	122.600.803.954.845	0.418503805198983	0.135737969772094	0.33043642542301		Predicted ORF in Assemblies 19, 20 and 21
cpar114	orf19.6591	177.535.205.843.351	120.442.295.270.769	0.113684589481628	0.227500241544515		Predicted ORF in Assemblies 19, 20 and 21
cpar305	orf19.6637	145.075.671.001.291	167.669.632.707.134	0.113684589481628	0.141579140830077		Predicted ORF in Assemblies 19, 20 and 21
cpar3117	orf19.6654	102.319.818.258.521	0.479125428694817	0.174933180701379	0.540085244186950		Predicted membrane transporter, member of the L-amino acid transporter-3 (LAT3) family, major facilitator superfamily (MFS)
cpar857	orf19.677	111.473.827.312.018	122.555.158.948.203	0.119221800209380	0.153925841537802	CHO1	Putative phosphatidylserine synthase; similar to <i>S. cerevisiae</i> Cho1p, which is involved in phosphatidylserine biosynthesis; transposon mutation affects filamentous growth; regulated by Nrg1p, Tup1p; no human or murine homolog
cpar1650	orf19.6838	134.184.307.614.397	159.146.785.374.437	0.113684589481628	0.212058206022502		Putative protein of unknown function, transcription is upregulated in clinical isolates from HIV+ patients with oral candidiasis; predicted ORF in Assemblies 19, 20 and 21
cpar4281	orf19.6917	143.082.083.849.465	0.344705029732048	0.171701835969910	0.400543119816932		Predicted ORF in Assemblies 19, 20 and 21
cpar4449	orf19.6993	169.966.585.541.461	189.719.685.719.275	0.164910811732469	0.188859278256094	GAP2	Protein similar to amino acid permeases; ketoconazole, flucytosine repressed; induced by histidine, and induction requires Ssy1p; regulated by Nrg1p, Tup1p; shows colony morphology-related gene regulation by Ssn6p

cpar3640	orf19.7001	108.553.497.089.535	0.492984653580489	0.155907327655574	0.219363766922362	YCK2	Protein similar to <i>S. cerevisiae</i> Yck2p, which is a plasma membrane-bound casein kinase I isoform; detected at yeast-form cell plasma membrane by mass spectrometry; transcriptionally activated by Mnl1p in weak acid stress
cpar3263	orf19.7093	185.214.454.173.459	189.398.644.521.165	0.113684589481628	0.113684589481628	HGT13	Predicted sugar transporter, involved in glycerol utilization; member of the major facilitator superfamily; 12 transmembrane; gene has intron; oxidative stress-induced via Cap1p; expressed in rich medium, 2% glucose
cpar2870	orf19.7118	103.058.383.274.602	0.221179767156741	0.150472294925116	0.509703582864182		Predicted ORF in Assemblies 19, 20 and 21
cpar46	orf19.7244	126.390.649.389.131	0.782105475161473	0.141579140830077	0.273086049772560		Predicted ORF in Assemblies 19, 20 and 21; induced by nitric oxide in yhb1 mutant
cpar1523	orf19.7282	100.469.262.305.948	0.887293480189909	0.118889469075455	0.346748363631391	PEX13	Protein required for peroxisomal protein import mediated by PTS1 and PTS2 targeting sequences; transcription is upregulated in an RHE model of oral candidiasis
cpar1968	orf19.7296	170.851.486.693.822	166.036.276.014.695	0.141579140830077	0.150472294925116		Predicted ORF in Assemblies 19, 20 and 21; similar to stomatin mechanoreception protein; induced by Rgt1p
cpar1966	orf19.7298	107.909.594.366.397	110.380.459.363.544	0.174933180701379	0.180163703697246	CHS2	Chitin synthase; nonessential; required for wild-type chitin deposition in hyphae; transcription is regulated during dimorphic transition; Chs1p and Chs2p, but not Chs3p, are inhibited by the protuberberine HWY-289; fungal-specific
cpar4348	orf19.7365	106.096.909.817.717	0.458791942493246	0.168422850557742	0.216855616151878		Predicted ORF in Assemblies 19, 20 and 21
cpar4343	orf19.7379	202.788.438.657.375	238.958.436.742.098	0.113684589481628	0.123495546854116	FAA2	Predicted acyl CoA synthetase; expression is regulated upon white-opaque switching
cpar1858	orf19.7447	200.138.257.214.382	235.467.751.404.241	0.113684589481628	0.121110746060842	JEN1	Lactate transporter; transcriptionally induced by lactic acid; activated by Cat8p; alkaline downregulated by Rim101p; localizes to plasma membrane; similar to <i>S. cerevisiae</i> Jen1p
cpar3863	orf19.7520	116.073.737.508.239	107.976.405.295.398	0.113684589481628	0.113684589481628	POT1	Putative peroxisomal 3-oxoacyl CoA thiolase; transcription is regulated by Nrg1p and Mig1p; farnesol regulated
cpar5337	orf19.86	120.879.296.432.774	0.210631631002669	0.113684589481628	0.457049369483292		Protein described as glutathione peroxidase; macrophage-downregulated gene
cpar1250	orf19.893	103.170.251.075.321	138.528.160.702.500	0.127770380594641	0.238372306471201	PGA17	Putative GPI-anchored protein of unknown function; exogenously expressed protein is a substrate for Kex2p processing in vitro; transcription is repressed in response to alpha pheromone in SpiderM medium; macrophage-induced gene
cpar1243	orf19.899	147.433.865.656.905	159.672.889.865.371	0.160514925603551	0.228427717568708		Predicted ORF in Assemblies 19, 20 and 21
cpar2284	orf19.906	109.742.004.242.943	0.565109568300137	0.163653700504823	0.502932469754111	ROM2	Predicted ORF in Assemblies 19, 20 and 21
cpar1069		203.511.779.973.866	278.282.176.835.499	0.113684589481628	0.113684589481628	NA	NA
cpar1925		159.658.495.981.194	197.851.807.705.477	0.113684589481628	0.150472294925116	NA	NA
cpar3027		15.027.087.471.356	168.688.853.255.663	0.113684589481628	0.159087841221263	NA	NA

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cpar1924	113.682.488.463.546	101.075.083.881.679	0.113684589481628	0.121110746060842	NA	NA
cpar2989	116.704.461.243.426	151.640.075.277.985	0.113684589481628	0.289720305616276	NA	NA
cpar4077	210.979.623.574.081	154.130.630.799.748	0.113684589481628	0.162895879610040	NA	NA
cpar4924	119.726.622.148.968	113.548.657.856.610	0.113684589481628	0.118889469075455	NA	NA
cpar2427	169.499.625.845.283	115.333.289.790.282	0.118889469075455	0.139383155994552	NA	NA
cpar4891	135.498.695.879.401	169.135.631.434.161	0.118889469075455	0.157169887772819	NA	NA
cpar2969	145.539.005.353.466	0.698707933855286	0.129314843364686	0.162895879610040	NA	NA
cpar181	107.174.587.327.574	156.723.354.844.223	0.131235411369423	0.248870594310554	NA	NA
cpar5432	126.000.902.238.176	0.711613887414284	0.136842877428105	0.310908241037115	NA	NA
cpar746	212.854.253.920.017	220.259.780.351.616	0.139383155994552	0.141579140830077	NA	NA
cpar1506	111.918.140.577.202	141.731.633.056.076	0.140854496147095	0.21492638613483	NA	NA
cpar182	197.826.812.967.796	200.310.714.762.026	0.141579140830077	0.146885116558105	NA	NA
cpar806	167.368.329.168.638	152.877.005.802.895	0.141579140830077	0.164801209130932	NA	NA
cpar2945	122.505.129.782.249	138.579.567.182.461	0.141579140830077	0.225709279439969	NA	NA
cpar2231	134.135.967.044.922	123.516.783.432.606	0.146451372361865	0.162895879610040	NA	NA
cpar403	168.565.377.341.284	195.357.580.962.182	0.153352265732680	0.153925841537802	NA	NA
cpar2449	162.272.861.297.384	161.323.561.476.812	0.153946886142007	0.21020255087436	NA	NA
cpar449	105.009.512.849.223	0.181302500863345	0.162895879610040	0.676197416165812	NA	NA
cpar4716	146.650.243.493.768	139.578.396.197.856	0.164910811732469	0.174933180701379	NA	NA
cpar1159	177.016.199.171.350	243.070.126.321.129	0.166051009618301	0.197411565998529	NA	NA
cpar148	181.608.959.824.949	199.477.906.194.606	0.168422850557742	0.173968639873865	NA	NA
cpar2022	0.872254175024708	150.417.788.594.668	0.131235411369423	0.168422850557742	NA	NA

Column descriptions:

Cpar.ID: Gene designations are derived from an in-house annotation

C.a.Orthologs: The *C. albicans* orf19 orthologs were obtained from the Candida Gene Order Browser (<http://cgob.ucd.ie/>).

log2FC.oligo1: log fold change of the first oligo.

log2FC.oligo2: log fold change of the second oligo.

P_value.oligo1: p_value associated with oligo 1.

P_value.oligo2: p_value associated with oligo 2.

Gene.Name: Gene name of the *Candida albicans* ortholog.

Description: Description of the *Candida albicans* ortholog.

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SUMÁRIO

As espécies do género *Candida* representam uma importante causa de infeções nosocomiais. A espécie *Candida parapsilosis* é atualmente o segundo isolado mais frequente de hemoculturas na Europa e tem sido descrita como sendo responsável por uma grande variedade de manifestações clínicas, que ocorrem geralmente em indivíduos imunocomprometidos.

A espécie *C. parapsilosis* representava anteriormente um complexo de três grupos geneticamente distintos (grupo I, II e III). Atualmente, estes grupos foram renomeados espécies distintas: *C. parapsilosis sensu stricto*, *C. orthopsilosis*, e *C. metapsilosis*.

Apesar de ser considerada um membro da população microbiana endógena em indivíduos saudáveis, *C. parapsilosis* é também isolada de diferentes fontes ambientais e das mãos do pessoal prestador de cuidados de saúde, sugerindo assim outras vias de transmissão nosocomial.

Esta espécie apresenta várias características biológicas, algumas relacionadas com a sua virulência. *C. parapsilosis* forma biofilmes em dispositivos médicos implantados, o que aumenta a sua adesão a tais equipamentos, produz enzimas extracelulares, expressa diferentes fenótipos e é capaz de proliferar na presença de concentrações elevadas de glucose e lipídios.

De um modo geral, as estirpes de *C. parapsilosis* têm sido descritas como sendo suscetíveis aos antifúngicos; contudo, estirpes resistentes têm sido descritas, em particular aos azóis e às equinocandinas. Poucos estudos de vigilância da atividade destes agentes sobre isolados de hemoculturas de *C. parapsilosis* têm sido conduzidos e os mecanismos de resistência aos antifúngicos são ainda desconhecidos. Acresce ainda que há pouca informação disponível acerca da eficácia de desinfetantes e antisépticos assim como de agentes físicos como radiação U.V. e micro-ondas, de uso corrente nos hospitais, sobre este patógeno.

Procedemos ao estudo de isolados de *C. parapsilosis* de doentes admitidos num hospital universitário Português e isolados ambientais do mesmo hospital. Descrevemos a incidência e a distribuição de *C. parapsilosis sensu stricto*, *C. orthopsilosis* e *C. metapsilosis* entre os 175 isolados clínicos e ambientais previamente identificados como *C. parapsilosis* por métodos convencionais. Adicionalmente, avaliamos a suscetibilidade *in vitro* dos isolados ao fluconazole, voriconazole, posaconazole, anfotericina B, caspofungina e anidulafungina. Dos 175 isolados estudados, 160 (91.4%) foram identificados como *C. parapsilosis sensu stricto*, 4 (2.3%) como *C. orthopsilosis*, e 5 (2.9%) como *C. metapsilosis*. Seis isolados correspondem a outras espécies de *Candida* que não pertencem ao grupo da *C. parapsilosis*. A avaliação do perfil de suscetibilidade demonstrou que apenas 9 (5.6%) isolados de *C. parapsilosis sensu stricto* eram resistentes ou suscetível-dose dependente ao fluconazole, e apenas um isolado apresentava um fenótipo multi-azolerresistente; 2 (1.3%) isolados de *C. parapsilosis sensu stricto* eram resistentes à anfotericina B. Todos os isolados de *C. orthopsilosis* e *C. metapsilosis* eram

suscetíveis aos azoles e à anfotericina B. Um número elevado de estirpes de *C. parapsilosis* sensu stricto era não-suscetível às equinocandinas.

Com o objetivo de explorar a diversidade genética entre os isolados de *C. parapsilosis* (um total de 160 isolados clínicos e ambientais) obtidos dos doentes e do ambiente hospitalar, duas técnicas moleculares distintas foram aplicadas: *random amplified polymorphic DNA analysis* e *mitochondrial DNA restriction endonuclease analysis*. Os resultados obtidos vão de encontro a outros estudos que referem que a diversidade genética para *C. parapsilosis* é mínima, tendo sido encontrado um perfil maioritário.

No sentido de caracterizar o desenvolvimento da resistência antifúngica em *C. parapsilosis*, 4 estirpes clínicas suscetíveis e uma estirpe da *American Type Culture Collection* foram cultivadas repetidamente na presença de fluconazole, voriconazole e posaconazole em diferentes concentrações. Os isolados desenvolveram diferentes graus de resistência aos azoles de acordo com o antifúngico utilizado. O fluconazole foi o indutor mais rápido e o posaconazole foi o mais lento. O fluconazole e o voriconazole induzem resistência a eles próprios e um ao outro, mas não ao posaconazole. O posaconazole induz resistência a todos os azoles. A resistência desenvolvida é estável; esse facto foi confirmado após cultivo em meio sem antifúngico. Os isolados resistentes aos azoles revelaram uma população homogénea. O efeito dos bloqueadores das bombas de efluxo (verapamil e ibuprofeno) é mínimo na resistência após avaliação por microdiluição e citometria de fluxo.

Relativamente à classe de antifúngicos equinocandinas, a indução da resistência foi igualmente realizada com uma estirpe clínica de *C. parapsilosis* com a anidulafungina. A resistência desenvolvida é estável e não está associada a mutações na região *HS1* do gene *FKS1*. A associação a níveis elevados de quitina na parede celular não está confirmada.

Para esclarecer os mecanismos moleculares de resistência aos azoles, hipotéticas alterações no perfil de expressão de genes da *C. parapsilosis* associadas à aquisição experimental de resistência aos azoles foram analisadas. As estirpes resistentes obtidas após exposição do fluconazole e do voriconazole demonstraram um aumento de expressão do fator de transcrição *MRR1*, do transportador *MDR1*, e de diversas redutases e oxidoredutases. Curiosamente, e semelhante ao que está descrito para *C. albicans*, a sobreexpressão de *MRR1* e de *MDR1* está relacionada com mutações pontuais no gene *MRR1* nas estirpes resistentes. A estirpe resistente obtida após exposição ao posaconazole tem a expressão de dois fatores de transcrição (*UPC2* e *NDT80*) e de 13 genes envolvidos na biossíntese do ergosterol aumentada. Este estudo é o primeiro que aborda a base molecular dos mecanismos de resistência aos azoles

em *C. parapsilosis*; os resultados sugerem que de modo semelhante a *C. albicans*, a tolerância aos azoles envolve a ativação de bombas de efluxo e/ou o aumento da síntese do ergosterol.

Na tentativa de estabelecer um paralelismo entre a indução de resistência *in vitro* e *in vivo*, três doentes com infecções sistêmicas por *C. parapsilosis* tratadas com fluconazole foram acompanhados; os isolados foram recolhidos antes, durante e/ou após tratamento. O teste de suscetibilidade aos antifúngicos demonstrou um aumento na concentração mínima inibitória de vários azoles para os isolados obtidos após tratamento com fluconazole. A tipagem molecular dos isolados demonstrou que os isolados pós-tratamento com reduzida suscetibilidade eram geneticamente idênticos aos isolados pré-tratamento suscetíveis, sugerindo a aquisição *in vivo* da resistência aos azoles. Os fenótipos de suscetibilidade apresentados pelos isolados eram estáveis *in vitro*. O papel do efluxo ativo dos antifúngicos na resistência foi avaliado por citometria de fluxo; a marcação nos isolados pós-tratamento e nos isolados pré-tratamento é semelhante. Uma análise mais profunda nas alterações a nível de expressão de genes foi realizada recorrendo a tecnologia de *microarrays*. Verificou-se que a expressão de genes envolvidos no metabolismo lipídico estava aumentada.

O potencial de diferentes agentes físicos (aquecimento a 60°C, micro-ondas e radiação UV) e agentes químicos (clorohexidina, etanol, hipoclorito de sódio, peróxido de hidrogénio, permanganato de potássio e ácido bórico) de uso corrente para inativar *C. parapsilosis* foi avaliado. Para os agentes químicos, a atividade antifúngica foi determinada recorrendo ao método da microdiluição e a ensaios do *time-kill*. Relativamente aos agentes físicos, a cinética de sobrevivência das células durante o tratamento foi determinada. Adicionalmente, a ocorrência de lesão de membrana provocada por cada tratamento foi avaliada por citometria de fluxo com iodeto de propídeo. As concentrações 0.06% de clorohexidina, 10% de etanol, 2.5% de hipoclorito de sódio, 0.06% de peróxido de hidrogénio, 0.1% de permanganato de potássio e 1% de ácido bórico são capazes de matar todas as células das estirpes de *C. parapsilosis* estudadas. Relativamente aos agentes físicos, após aquecimento a 60°C durante 5 min, radiação micro-ondas durante 60 s e radiação UV durante 10 min, foi confirmada uma redução de pelo menos 3 *log* no crescimento de *C. parapsilosis*. A marcação com iodeto de propídeo demonstrou que o aquecimento a 60°C e a radiação micro-ondas, assim como o peróxido de hidrogénio provocam lesões primárias graves na membrana. Adicionalmente, estudamos a atividade antimicrobiana de um novo sistema de desinfecção hospitalar; envolvendo a produção de um aerossol de peróxido de hidrogénio com catiões de prata. A atividade *in vitro* sobre suspensões de bactérias, leveduras e fungos filamentosos foi avaliada. A citometria de fluxo foi utilizada para confirmar a lesão de membrana das células microbianas após exposição ao desinfetante. Uma redução no

crescimento de pelo menos 5 *log* foi observada *in vitro* com todos os micro-organismos testados, sendo a solução desinfetante bastante ativa mesmo em baixa concentração. A citometria de fluxo evidenciou a ocorrência de lesão primária de membrana.

Com o objetivo de estudar a relação entre a resistência aos antifúngicos e os atributos de virulência, comparamos, pela primeira vez, o perfil de expressão de genes da estirpe resistente com a estirpe suscetível de *C. parapsilosis* através da análise de *microarrays*. Alguns grupos especiais de genes relacionados com patogenicidade estavam sobreexpressos na estirpe resistente: filamentação, metabolismo de lípidos, formação de hifas, patogénese, resposta ao *stress*, interação com o hospedeiro, gemulação, adesão celular, formação de biofilme e catabolismo de proteínas. Diversos estudos fenotípicos foram realizados, nomeadamente avaliação da capacidade de adesão, diversidade fenotípica, capacidade de crescimento, desenvolvimento de pseudohifas e expressão da catalase, cujos resultados corroboram os achados anteriores.

O conteúdo em quitina da parede celular (considerado atributo de virulência) dos isolados clínicos de *C. parapsilosis* com diferentes perfis de suscetibilidade às equinocandinas foi avaliado por microscopia de epifluorescência e por citometria de fluxo. Apesar do papel conhecido da quitina na tolerância aos antifúngicos, nenhuma relação entre o conteúdo em quitina e a resistência aos antifúngicos foi estabelecida; todavia parece existir uma possível relação com o efeito de crescimento paradoxal na presença de caspofungina. A citometria de fluxo representou uma eficiente e rápida abordagem para quantificar o conteúdo em quitina das células leveduriformes; através da marcação com *calcofluor white* desenvolvemos um protocolo citométrico que avalia o conteúdo em quitina das leveduras.

Esforços consideráveis são ainda necessários para elucidar acerca das fontes de *C. parapsilosis* no ambiente hospitalar, a relação entre isolados clínicos e ambientais, seus atributos de virulência e perfil de suscetibilidade, no sentido de reduzir a morbilidade e mortalidade e os custos inerentes.

Em suma, realizamos um estudo compreensivo acerca da génese da infeção sistémica por *C. parapsilosis*, uma espécie frequentemente isolada entre isolados clínicos hospitalares, nomeadamente no Hospital de S. João, um grande hospital universitário do Norte do país.

ABSTRACT

Candida species play a relevant role as agents of nosocomial infections. *Candida parapsilosis* is nowadays the second most common isolate from blood cultures in Europe and has been reported to be responsible for a broad variety of clinical manifestations that generally occur in individuals with impaired immunity.

C. parapsilosis was previously made up of a complex of three genetically distinct groups (groups I, II, and III). Recently, the *C. parapsilosis* groups have been renamed as distinct species: *C. parapsilosis sensu stricto*, *C. orthopsilosis*, and *C. metapsilosis*.

Although being sometimes considered a member of the indigenous flora in healthy individuals, *C. parapsilosis* has also been isolated from different environmental sources and from the hands of health care workers, thus suggesting potential routes for nosocomial transmission.

This species displays many interesting biological characteristics, some of them presumed to be related to its virulence. *C. parapsilosis* tends to form biofilms on implanted medical devices, is capable of slime production and adhesion to indwelling devices, production of extracellular enzymes, expression of phenotype switching; it is also able to proliferate in the presence of high glucose and lipids concentration.

Overall *C. parapsilosis* has been described to be susceptible to antifungal drugs, however resistant strains have been reported, in particular to azoles and echinocandins. Few prospective surveillance studies regarding the activity of these agents against *C. parapsilosis* bloodstream isolates are available and the antifungal resistance mechanisms expressed by *C. parapsilosis* are not yet described. Additionally, there is very limited information about the effectiveness of disinfectants and antiseptics as well as physical agents, e.g. U.V. and microwave radiation, current used in hospital, against this pathogen.

We studied *C. parapsilosis* isolates from patients admitted at a Portuguese university hospital and environmental isolates isolated from the same hospital. The incidence and distribution of *C. parapsilosis sensu stricto*, *C. orthopsilosis*, and *C. metapsilosis* among 175 clinical and environmental isolates previously identified by conventional methods as *C. parapsilosis* were established. Furthermore, the *in vitro* susceptibility patterns to fluconazole, voriconazole, posaconazole, amphotericin B, caspofungin and anidulafungin were assessed. Of the 175 isolates tested, 160 (91.4%) were identified as *C. parapsilosis sensu stricto*, 4 (2.3%) as *C. orthopsilosis*, and 5 (2.9%) as *C. metapsilosis*. Six isolates corresponded to *Candida* species other than the *C. parapsilosis* group. The susceptibility profile showed that only nine (5.6%) *C. parapsilosis sensu stricto* strains were resistant or susceptible-dose dependent to fluconazole, while a single strain displayed a multi-azole-resistant phenotype; two (1.3%) *C. parapsilosis sensu stricto* strains were amphotericin B resistant. All *C. orthopsilosis* and *C. metapsilosis*

isolates were susceptible to azoles and amphotericin B. A high number of *C. parapsilosis* sensu stricto strains were nonsusceptible to the echinocandins.

In order to assess about genetic diversity among *C. parapsilosis* isolates recovered from patients and hospital environment (a total of 160 isolates) two different molecular techniques were applied: random amplified polymorphic DNA and mitochondrial DNA restriction endonuclease analysis. Our results corroborate previous studies that showed that the genetic diversity for *C. parapsilosis* is scarce, being one genetic profile dominant.

In order to characterize the development of antifungal resistance by *C. parapsilosis*, four azole-susceptible clinical strains and one type strain from the American Type Culture Collection were repeatedly cultured in the presence of fluconazole, voriconazole and posaconazole at different concentrations. The isolates developed variable degree of azole resistance according to the antifungal used. Fluconazole was the fastest inducer while posaconazole was the slowest. Fluconazole and voriconazole induced resistance to themselves and each other, but not to posaconazole. Posaconazole induced resistance to all three azoles. Developed resistance was stable; it could be confirmed after repeated subcultured in drug-free medium. Azole-resistant isolates revealed a homogeneous population structure; the role of azole transporter efflux pumps (ATP-dependent) was minor after evaluation by microdilution and flow cytometric assays with efflux pump blockers (verapamil and ibuprofen).

Regarding the echinocandin antifungal class, induction of resistance was also performed with one clinical *C. parapsilosis* strain incubated with anidulafungin. The developed resistance was stable and was not associated with mutation in the HS1 region of the *FKS1* gene. The correlation with high chitin level was not confirmed.

To highlight the molecular azole resistance mechanisms, the changes in gene expression profile of *C. parapsilosis* associated with the acquisition of experimentally-induced resistance to azole antifungal drugs were also described. The resistant strains obtained after exposure to fluconazole and to voriconazole displayed increased expression of the transcription factor *MRR1*, of the major facilitator transporter *MDR1*, and of several reductases and oxidoreductases. Interestingly, and similarly to what has been described in *C. albicans*, up-regulation of the *MRR1* and *MDR1* correlated with point mutations in *MRR1* in the resistant strains. The resistant strain obtained after exposure to posaconazole displayed up-regulation of two transcription factors (*UPC2* and *NDT80*) and increased expression of 13 genes involved in ergosterol biosynthesis. This was the first study addressing global molecular mechanisms underlying azole resistance by *C. parapsilosis*; the results suggest that similarly to *C. albicans*, tolerance to azoles involves the activation of efflux pumps and/or increased ergosterol synthesis.

In an attempt to compare *in vitro* and *in vivo* induction of resistance, isolates from three patients with *C. parapsilosis* fungaemia were serially recovered before, during and/or after treatment with fluconazole. A microbiological study was performed to characterize these isolates. *In vitro* antifungal susceptibility testing showed the increase for several azoles of the minimal inhibitory concentration values in isolates recovered after fluconazole treatment. Molecular typing of isolates showed that the post-treatment isolates with reduced susceptibility were identical to susceptible pre-treatment isolates, confirming the *in vivo* acquisition of azole resistance. The susceptibility phenotypes displayed by isolates were confirmed to be stable *in vitro*. The role of active efflux of drugs in resistance was evaluated by flow cytometry; the staining profiles in post-treatment isolates and pre-treatment isolates were similar. A deep analysis into genomic changes by microarray technology showed that the expression of genes involved in lipid metabolism process was increased among the resistant isolate.

The potential of different physical (heating at 60°C, microwave and ultraviolet irradiation) and chemicals agents (chlorhexidine, ethanol, sodium hypochlorite, hydrogen peroxide, potassium permanganate and acid boric) to inactivate *C. parapsilosis* was determined. For chemical agents the antifungal activity was determined using the microdilution method and a time-kill assay. Regarding physical agents, the kinetics of survival of the cells during treatment were assessed. Additionally, the membrane lesion provoked by each treatment was evaluated using a flow cytometric assay with propidium iodide. Concentrations of 0.06% of chlorhexidine, 10% of ethanol, 2.5% of sodium hypochlorite, 0.06% of hydrogen peroxide, 0.1% of potassium permanganate and 1% of acid boric were able to kill all *C. parapsilosis*. Regarding physical agents, after heating at 60°C for 5 min, microwave irradiation after 60 s and ultraviolet irradiation for 10 min, a reduction of at least 3 log in *C. parapsilosis* growth was achieved. Propidium iodide staining demonstrated that heating at 60°C, microwave irradiation and hydrogen peroxide causes serious primary membrane lesions. We also report on the antimicrobial activity of a new disinfection system: aerosolized hydrogen peroxide in combination with silver cations dry-mist. The *in vitro* activity against suspensions of pathogenic bacteria, yeasts and moulds was assessed. Additionally, flow cytometry was used to confirm membrane lesion of microbial cells following exposure to the disinfectant. A growth reduction of at least 5 log was observed *in vitro* with all tested microorganisms, the disinfectant solution being highly active even at very low concentration; flow cytometry evidenced cell membrane lesion.

In order to study the correlation between drug resistance and pathogenicity attributes, we compared, for the first time, large-scale gene expression profile between susceptible and

resistant strains using microarray analysis. Some particular groups of genes usually correlated with pathogenicity were found to be differentially expressed in the resistant strain: filamentous growth, lipid metabolic process, hyphal growth, pathogenesis, response to stress, interaction with host, pseudohyphal growth, cell budding, cell adhesion, biofilm formation and protein catabolic process. Several phenotypic studies, namely adherence ability, phenotype diversity, growth capacity, pseudohyphal development and catalase expression, corroborated such results.

Cell wall chitin content, considered a virulence attribute, of *C. parapsilosis* clinical isolates with different echinocandin susceptibility profiles was evaluated by epifluorescence microscopy and flow cytometry. Despite the chitin role in drug tolerance, no relation between the chitin amount and antifungal resistance was established; although a possible relation with paradoxical growth effect of echinocandins could be established. Flow cytometry represents an efficient and fast approach in analysis of cell phenotypes; using calcofluor white staining, we developed a flow cytometric assay to quantitatively measure chitin content in yeasts.

Considerable efforts are needed to provide elucidation regarding the sources of *C. parapsilosis* in the hospital milieu, the relation between environmental isolates and the clinical isolates, its virulence attributes and its respective antifungal susceptibility profile, in order to reduce morbidity and mortality in a cost-effective manner.

Herein we performed a comprehensive evaluation of many of such topics involved in the genesis of bloodstream infection by *C. parapsilosis*, which is a common isolate in hospitals in Portugal, namely in S. João Hospital, a large University Hospital in the North of country.

PUBLICATIONS



RESEARCH ARTICLE

Dynamics of *in vitro* acquisition of resistance by *Candida parapsilosis* to different azoles

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Keywords

Candida parapsilosis; antifungal resistance; azoles; inducible resistance.

Abstract

Candida parapsilosis is a common isolate from clinical fungal infectious episodes. Resistance of *C. parapsilosis* to azoles has been increasingly reported. To analyse the development of resistance in *C. parapsilosis*, four azole-susceptible clinical strains and one American Type Culture Collection type strain were cultured in the presence of fluconazole, voriconazole and posaconazole at different concentrations. The isolates developed variable degrees of azole resistance according to the antifungal used. Fluconazole was the fastest inducer while posaconazole was the slowest. Fluconazole and voriconazole induced resistance to themselves and each other, but not to posaconazole. Posaconazole induced resistance to all azoles. Developed resistance was stable; it could be confirmed after 30 days of subculture in drug-free medium. Azole-resistant isolates revealed a homogeneous population structure; the role of azole transporter efflux pumps was minor after evaluation by microdilution and cytometric assays with efflux pump blockers (verapamil, ibuprofen and carbonyl cyanide 3-chloro-phenylhydrazone). We conclude that the rapid development of azole resistance occurs by a mechanism that might involve mutation of genes responsible for ergosterol biosynthesis pathway, stressed by exposure to antifungals.

Introduction

During the last decade *Candida* species have emerged as major opportunistic pathogens, mainly due to the increase in the number of immunocompromised patients (Pfaffer & Diekema, 2007). Among *Candida* species causing nosocomial infections, the opportunistic yeast *Candida parapsilosis* is at present the second or third most common blood culture fungal isolate in Europe, Canada and Latin America, outranking *Candida albicans* in some European hospitals (Pfaffer & Diekema, 2007).

Although sometimes considered a member of the indigenous microbial population in healthy individuals, *C. parapsilosis* can be also recovered from distinct environmental sources and from the hands of health care workers, thus suggesting its role as a potential route for nosocomial transmission (Levin *et al.*, 1998; Kojic & Darouiche, 2004).

Unfortunately, fungal pathogens acquire resistance to azoles, particularly after prolonged exposure, as is the case with its prophylactic overuse. The widespread use of antifungal molecules, especially fluconazole, has selected *Candida* species with easily inducible resistance, such as *Candida glabrata* (Wingard *et al.*, 1993) and *Candida tropicalis* (Barchiesi *et al.*, 2000) or species that show intrinsic resistance, such as *Candida krusei* (Wingard *et al.*, 1991). *Candida albicans* resistance to fluconazole was promoted following incubation with subinhibitory concentrations of the drug (Marr *et al.*, 2001). In the case of an antifungal drug being more prone to induce *in vitro* resistance in comparison with others, this might represent a hypothetical drawback for its use *in vivo*, at least for prophylactic treatment.

Three distinct mechanisms of azole resistance have been described so far in *C. albicans*: (1) failure to accumulate the drug intracellularly, which may be caused by the lack of drug

penetration due to changes in membrane lipids or sterols (Hitchcock *et al.*, 1986) or (2) by active efflux of drugs resulting from overexpression of genes *CDR1*, *CDR2* and *MDR1* (Albertson *et al.*, 1996; Sanglard *et al.*, 1996; White, 1997; Marr *et al.*, 1998); and (3) increased production of the azole target enzyme and point mutations in genes that codify for this enzyme, the products of which have reduced affinity to azoles (Sanglard *et al.*, 1998). Lanosterol-14 α -demethylase is a common target for all azole antifungals. This enzyme belongs biochemically to the group of cytochrome P450 enzymes and is involved in the synthesis of ergosterol, which is a major and essential lipid constituent of the cell membrane of fungi (Akins, 2005). All azoles, including posaconazole (Munayyer *et al.*, 2003; Hof, 2006), inhibit the production of ergosterol, causing a depletion of this compound. Mutations in other enzymes involved in the synthesis of ergosterol may also contribute to azole resistance.

Although several resistance mechanisms may operate in fungal pathogens, efflux-mediated drug tolerance is the major factor responsible for clinical resistance (Kontoyianis & Lewis, 2002; Sanglard & Odds, 2002; White *et al.*, 2002). Previous studies demonstrated that resistance related to overexpression of efflux pumps can be reverted by modulators, particularly verapamil or sex hormones (Ford & Hait, 1990) and more recently by ibuprofen (Pina-Vaz *et al.*, 2005). Inhibition of the efflux pumps that are H⁺-dependent in yeasts by carbonyl cyanide 3-chlorophenylhydrazone (CCCP) has also been demonstrated (Prudêncio *et al.*, 2000; Guinea *et al.*, 2006).

Surprisingly, very few data are yet available regarding the mechanisms of azole resistance in *C. parapsilosis*.

The initial aim of our study was to assess and characterize the *in vitro* induction of resistance by distinct azole antifungals at different concentrations in four blood culture isolates and one American Type Culture Collection (ATCC) type strain of *C. parapsilosis*. Additional aims of this study were to elucidate whether such induced resistance is stable and to assess the involvement of efflux pumps.

Materials and methods

Strains

Four blood culture isolates of *C. parapsilosis* (BC014, BC011, BC237 and BC190) isolated from patients admitted to Hospital S. João in Porto, Portugal, were used in this study. They were characterized by Vitek YBC identification cards (BioMérieux, Paris, France). Until testing, the strains were stored in Brain–Heart broth (Difco) with 5% glycerol at -70°C . For each experiment, each strain was subcultured twice on Sabouraud agar (Difco) at 35°C for 48 h. *Candida parapsilosis* type strain ATCC 22019 was included. To study the role of efflux pumps on antifungal-induced resistance,

two *C. albicans* strains with well-characterized mechanisms of resistance were also included as controls: *C. albicans* strain 95-68, with overexpression of *CDR1* and *CDR2* genes (ATP-dependent efflux pumps), and *C. albicans* strain 2-76, showing lower levels of resistance gene expression (a kind gift from Dr Ted White).

Chemicals and culture media

Standard powders of fluconazole (Pfizer, Groton, CT), voriconazole (Pfizer, New York, NY), posaconazole (Schering-Plough, Kenilworth, NJ), amphotericin B (AMB; Bristol Myers Squibb, New York, NY) and caspofungin (CAS; Merck, Rahway, NJ) were obtained from the respective manufacturers. A stock solution of fluconazole and CAS were prepared in distilled water; voriconazole, posaconazole and AMB were prepared with dimethyl sulfoxide. Antifungal drugs were diluted afterwards with Roswell Park Memorial Institute 1640 medium (RPMI 1640; Sigma, St. Louis, MO) buffered to pH 7.0 with 0.165 M morpholine propanesulfonic acid buffer (MOPS; Sigma) and stored at -70°C until use. Verapamil, ibuprofen and CCCP were purchased from Sigma. The culture medium used for induction assays of resistance and evaluation of its stability was RPMI 1640 with 0.165 M MOPS, pH 7.0. Rhodamine 6G (Rh-6G) used in flow cytometric assays was purchased from Sigma.

Strategy for induction of antifungal resistance and for assessing its stability

A single, randomly selected colony from each *C. parapsilosis* strain was incubated in 10 mL of RPMI 1640 overnight in a rotating drum at 150 r.p.m., 35°C . An aliquot of this culture, containing 10^6 blastoconidia, was transferred to different vials, each containing 10 mL of culture medium with or without an antifungal drug (fluconazole, voriconazole or posaconazole), and incubated overnight as described above. The following day, aliquots from each culture containing 10^6 blastoconidia were again transferred into fresh medium containing the same antifungal and reincubated as described. Each day, for the 30 days of the assay, a 1-mL aliquot from each subculture was mixed with 0.5 mL of 50% glycerol and frozen at -70°C for later testing. In this type of experiment two different approaches were taken: (1) incubation for 30 days with constant concentrations of fluconazole ($16\text{ }\mu\text{g mL}^{-1}$), voriconazole ($2\text{ }\mu\text{g mL}^{-1}$) or posaconazole ($1\text{ }\mu\text{g mL}^{-1}$); these concentrations correspond to therapeutic serum levels obtained during antifungal treatment (Azanza *et al.*, 2007) and (2) incubation with fluconazole, voriconazole and posaconazole at twice the concentration of the minimal inhibitory concentration (MIC) value, for 2 weeks, and afterwards at four times the concentration until the 30th day.

To assess resistance stability, the resistant isolates obtained were subcultured daily in the absence of the drug for 30 days. A single colony from each isolate was incubated in 10 mL drug-free RPMI 1640 at 35 °C, 150 r.p.m.. The following day, aliquots were transferred into fresh medium. At each subculture, a 1-mL aliquot of the suspension was mixed with 0.5 mL of 50% glycerol, and frozen at –70 °C for later testing.

Antifungal susceptibility testing

The MIC values of each antifungal drug were determined according to the Clinical Laboratory Standards Institute (CLSI, 2008) M27-A3 protocol. MIC was registered after 24 and 48 h. Interpretative criteria for fluconazole and voriconazole were those of the CLSI: for fluconazole: susceptible (S)-MIC $\leq 8 \mu\text{g mL}^{-1}$, susceptible-dose dependent (S-DD)-MIC $16\text{--}32 \mu\text{g mL}^{-1}$, and resistance (R)-MIC $\geq 64 \mu\text{g mL}^{-1}$; for voriconazole, S-MIC $\leq 1 \mu\text{g mL}^{-1}$ and R-MIC $\geq 4 \mu\text{g mL}^{-1}$. The interpretative criteria for CAS were those recently assigned by the CLSI (June 2007): S $\leq 2 \mu\text{g mL}^{-1}$. Although susceptibility breakpoints have not yet been established for posaconazole and AMB, strains inhibited by $\leq 1 \mu\text{g mL}^{-1}$ of each were considered to be susceptible (Pfaller *et al.*, 2001, 2003). Every 5 days of incubation, with or without antifungal, MIC values were redetermined for the five antifungals tested. *Candida parapsilosis* type strain ATCC 22019 was used in each testing assay, as recommended.

Population analysis

This assay was performed as described previously by Marr *et al.* (2001). A single colony of the initially susceptible and of the final resistant isolates were cultured overnight in YEPD broth (1% yeast extract, 2% peptone, 2% glucose) at 35 °C; a suspension containing 10^3 blastoconidia mL^{-1} was prepared in RPMI 1640 and plated in Sabouraud agar with and without fluconazole (1, 4, 16 and $64 \mu\text{g mL}^{-1}$), voriconazole (0.125, 0.5, 2 and $8 \mu\text{g mL}^{-1}$) and posaconazole (0.25, 1, 4 and $16 \mu\text{g mL}^{-1}$). Growth was quantified after 48 h of incubation at 35 °C. The number of colonies growing in the presence of the drug at each concentration, relative to the number growing in its absence, was calculated and plotted.

Study of efflux pumps

The role of efflux pumps in the resistance mechanism developed was evaluated using two different approaches:

(1) Antifungal MIC values were determined according to the CLSI M27-A3 protocol (CLSI, 2008) in the presence of different drugs described as efflux blockers: verapamil 100 μM , ibuprofen 100 mg L^{-1} (Pina-Vaz *et al.*, 2005) and CCCP 0.5 $\mu\text{g mL}^{-1}$ (Guinea *et al.*, 2006).

(2) Flow cytometric analysis using Rh-6G, a specific substrate of efflux pumps was performed as described previously (Sanglard *et al.*, 1999; Posteraro *et al.*, 2003), to compare the induced resistant strains with susceptible strains (incubated in drug-free medium) and the induced resistant strains with and without incubation for 90 min with verapamil 100 μM , as described by Pina-Vaz *et al.* (2005). Briefly, yeast cells were grown to logarithmic phase in 5 mL of YEPD medium at 35 °C under constant agitation. Labelling of cells was conducted in 1 mL of YEPD, containing 10^7 blastoconidia and 10 μM Rh-6G for 30 min at 35 °C, after which the reaction was stopped by placing the tubes on ice. The mixture was then diluted 40-fold with cold sterile 0.1 M phosphate-buffered saline (Sigma) at pH 7.0 and analysed using flow cytometry in a FACSCalibur (BD Biosciences, Sydney, Australia) equipped with a 15-nm argon laser. From each yeast suspension, 30 000–50 000 blastoconidia were analysed; cell fluorescence was determined at FL1 (515 nm). All experiments were performed in duplicate.

Results

Development of resistance

Table 1 shows the MIC values determined for fluconazole, voriconazole and posaconazole before and after induction with different azoles for all tested strains. After exposure to therapeutic serum concentrations of the different azoles, distinct patterns of resistance were observed. Following 15 days of antifungal exposure, only the fluconazole susceptibility phenotype changed. This finding was observed with most tested strains exposed to fluconazole and with a few strains exposed to voriconazole; following posaconazole exposure, only minor MIC variations were registered after this short period of time. Fluconazole and voriconazole increased MIC values to themselves and to each other, the susceptibility phenotype changing to R after 30 days, but not to posaconazole, which maintained the S phenotype. Posaconazole induced resistance to all azoles only at the very end of incubation time. Therefore, the activity of fluconazole was the most impaired and it was the fastest inducer of resistance.

In a different strategy to compare the ability of the same azole drugs to induce resistance, incubation with MIC values two- and fourfold that of the antifungals were assayed. A representative example (type strain ATCC 22019) of the development of resistance in such instances is detailed in Fig. 1. The lower concentration of fluconazole was sufficient to induce resistance to itself and to voriconazole but not to posaconazole; the exposure of the yeast cells to the lower concentration of voriconazole only changed the fluconazole phenotype (S strain turned to S-DD), as only minor variations on MIC values were observed with

Table 1. MIC values ($\mu\text{g mL}^{-1}$) and phenotypes (Phen) of *Candida parapsilosis* type strain (ATCC 22019) and BC014, BC011, BC237 and BC190 clinical strains before and after induction with fluconazole ($16 \mu\text{g mL}^{-1}$), voriconazole ($2 \mu\text{g mL}^{-1}$) and posaconazole ($1 \mu\text{g mL}^{-1}$)

Induction with	Strains	MIC/Phen								
		Fluconazole			Voriconazole			Posaconazole		
		Day 0	Day 15	Day 30	Day 0	Day 15	Day 30	Day 0	Day 15	Day 30
Fluconazole	ATCC	0.5/S	16/S-DD	64/R	0.015/S	0.25/S	2/S-DD	0.06/S	0.06/S	0.125/S
	BC014	1.0/S	16/S-DD	128/R	0.03/S	0.5/S	4/R	0.03/S	0.25/S	0.5/S
	BC011	1.0/S	8/S	128/R	0.03/S	0.25/S	2/S-DD	0.03/S	0.125/S	0.5/S
	BC237	1.0/S	32/S-DD	128/R	0.03/S	0.25/S	4/R	0.06/S	0.125/S	0.5/S
	BC190	0.5/S	16/S-DD	128/R	0.03/S	0.125/S	4/R	0.06/S	0.125/S	0.5/S
Voriconazole	ATCC	0.5/S	16/S-DD	64/R	0.015/S	0.06/S	2/R	0.06/S	0.06/S	0.125/S
	BC014	1.0/S	32/S-DD	128/R	0.03/S	1/S	4/R	0.03/S	0.125/S	0.5/S
	BC011	1.0/S	4/S	64/R	0.03/S	0.25/S	2/S-DD	0.03/S	0.125/S	0.5/S
	BC237	1.0/S	4/S	64/R	0.03/S	0.125/S	2/S-DD	0.06/S	0.125/S	0.5/S
	BC190	0.5/S	2/S	64/R	0.03/S	0.125/S	4/R	0.06/S	0.125/S	0.5/S
Posaconazole	ATCC	0.5/S	1/S	64/R	0.015/S	0.03/S	8/R	0.06/S	1/S	16/R
	BC014	1.0/S	1/S	128/R	0.03/S	0.03/S	16/R	0.03/S	0.125/S	32/R
	BC011	1.0/S	1/S	128/R	0.03/S	0.03/S	16/R	0.03/S	0.06/S	32/R
	BC237	1.0/S	1/S	64/R	0.03/S	0.03/S	2/S-DD	0.06/S	0.06/S	8/R
	BC190	0.5/S	1/S	128/R	0.03/S	0.06/S	16/R	0.06/S	0.125/S	32/R

the other tested azoles. Posaconazole had the least effect on the MIC values of all azoles in such experimental conditions.

Incubation with these three azoles, independently on the tested concentration, did not induce resistance to AMB and CAS.

Regarding the strains successively incubated without antifungals, the MIC values of the five antifungals tested remained constant throughout the study period.

Stability of azole resistance *in vitro*

Regarding the stability of the developed resistance by resistant organisms subcultured in drug-free medium for 30 days, the resistance pattern remained stable; no decreases greater than one dilution were observed in MIC values of all azoles tested.

Analysis of cellular populations

To determine whether the induced azole resistance in *C. parapsilosis* isolates was associated with selection of a resistant subpopulation, analysis of resistance within the cellular population was performed with susceptible and induced resistance strains. Population analysis clearly demonstrated the existence of a homogeneous population both for the resistant-induced strains and for the susceptibility strain. A representative example of these studies is shown for BC014 strain in Fig. 2.

Activity of efflux pumps

Effect of efflux blockers on MIC value of azoles

After incubation with ibuprofen, verapamil and CCCP, a decrease of MIC values of no more than one dilution

was registered, and the resistance pattern remained stable. Regarding the control strains, *C. albicans* 95-68 in the presence of verapamil and ibuprofen, MIC values of fluconazole, voriconazole and posaconazole decreased drastically; as expected for strain *C. albicans* 2-76, the MIC values of the three antifungals did not change.

Rh-6G staining

The steady state regarding Rh-6G fluorescence of the susceptible phenotype (strains incubated in drug-free medium) and of resistant phenotypes obtained after 30 days of antifungal exposure (fluconazole $16 \mu\text{g mL}^{-1}$, voriconazole $2 \mu\text{g mL}^{-1}$ and posaconazole $1 \mu\text{g mL}^{-1}$) were compared. Similar intensity of fluorescence was obtained with induced resistant strains and susceptible strains (Fig. 3a). This intensity did not increase in the presence of the efflux blocker verapamil (data not shown). *Candida albicans* strain 95-68, with overexpression of *CDR1* and *CDR2* resistance genes, behaved as expected, showing an increase in Rh-6G staining when incubated with verapamil (Fig. 3b), whereas *C. albicans* strain 2-76 did not (data not shown), which validates the assays.

Discussion

Mechanisms of azole resistance due to antifungal exposure have been extensively investigated in clinical strains of *C. albicans* (Sanglard *et al.*, 1995, 1996, 1998; White, 1997; Franz *et al.*, 1998). Other studies have also elucidated the mechanisms of azole resistance in *C. glabrata*, *C. krusei* and *C. tropicalis* (Marichal *et al.*, 1997; Barchiesi *et al.*, 1998; Orozco

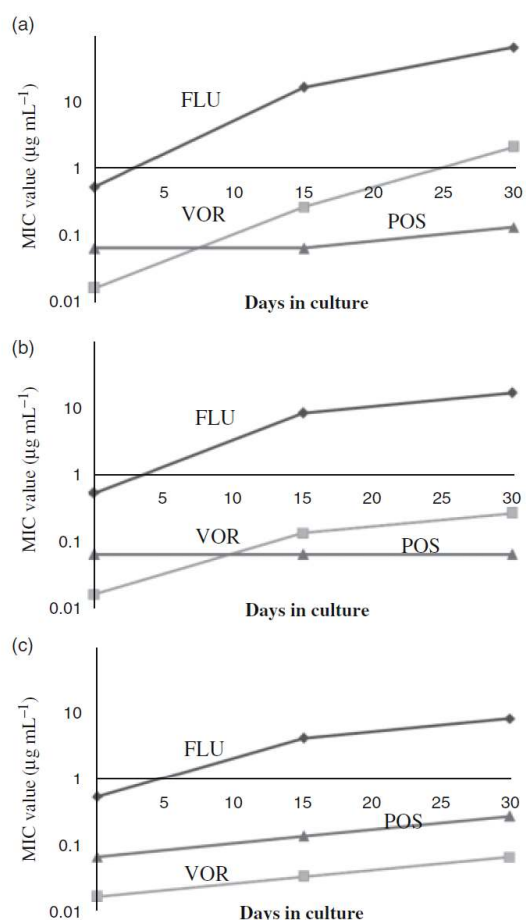


Fig. 1. Variation of fluconazole (FLU), voriconazole (VOR) and posaconazole (POS) MIC values for *Candida parapsilosis* type strain (ATCC 22019) cultured in medium containing two- and fourfold MIC values of FLU (a), VOR (b) and POS (c) for 30 days. —◆—, MIC of FLU; —■—, MIC of VOR; —▲—, MIC of POS.

et al., 1998). However, very few data are yet available regarding azole resistance in *C. parapsilosis*. This *Candida* species is an opportunistic yeast responsible for nosocomial infections, especially in immunocompromised or debilitated patients.

Resistance to fluconazole among *Candida* spp. has been reported to emerge whenever this drug is used extensively (Richardson, 2005). The results of our study stress several relevant characteristics of the *in vitro* acquisition of resistance by *C. parapsilosis* after prolonged exposure to antifungals. When incubated with a therapeutic fluconazole concentration (aiming to reproduce *in vivo* antifungal treatment), azole resistance quickly developed; similar results were obtained with a lower concentration of the same antifungal, showing that of the three azoles tested, fluconazole is an easy and fast inducer of resistance. The two

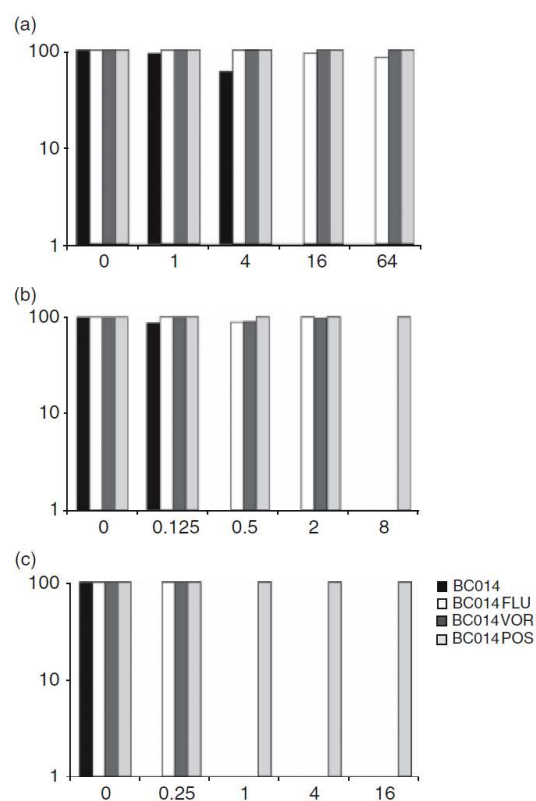


Fig. 2. Population analysis (a representative example) of a susceptible (BC014) and the respective resistant-induced strains following 30 days of exposure to azoles (BC014 FLU, BC014 VOR and BC014 POS). The percentage of resistant cells (y-axis, log scale) is defined as the ratio of colonies with growth on plates containing the indicated concentration of fluconazole (FLU) (a), voriconazole (VOR) (b) and posaconazole (POS) (c) relative to growth in the absence of drug.

different approaches used for induction of resistance corroborate this conclusion. This finding underlines the need to review therapeutic protocols. Both voriconazole and posaconazole induce resistance at concentrations of 2 and 1 µg mL⁻¹, respectively, but at lower concentrations the susceptibility pattern did not change drastically. As expected, incubation with any of the three azoles did not induce resistance to AMB and CAS, as azole targets are distinct and selective.

Our data strongly suggest that the dynamics of *in vitro* development of azole resistance in *C. parapsilosis* is quite different from that described for *C. tropicalis* and *C. albicans* (Calvet *et al.*, 1997; Barchiesi *et al.*, 2000; Marr *et al.*, 2001). For *C. albicans*, the time required to develop fluconazole resistance is longer and it could be fully reverted to baseline when the antifungal exposure stopped. *Candida albicans* isolates that became resistant showed an increase of mRNA

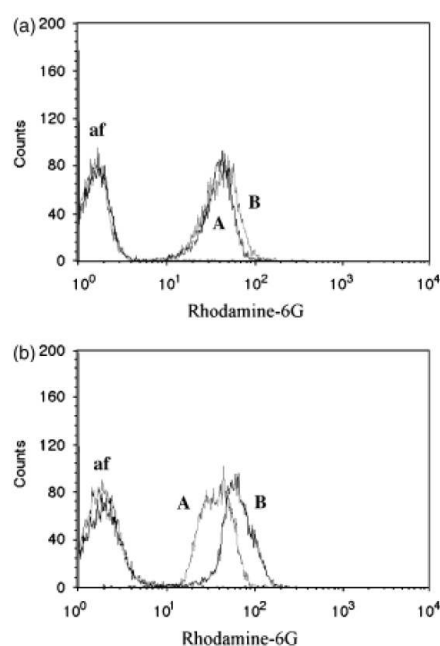


Fig. 3. Representative example of flow cytometric analysis of yeast blastoconidia after Rh-6G staining, an efflux pump substrate. (a) Histogram obtained by flow cytometric analysis at FL1 (515 nm) of BC014-susceptible strain incubated in drug-free medium (A) and BC014-resistant strain after incubation during 30 days with $16 \mu\text{g mL}^{-1}$ of fluconazole (B). (b) Histogram obtained by flow cytometric analysis at FL1 (515 nm) of resistant *Candida albicans* 95-68 strain (overexpression of genes that codify to efflux pumps) without (A) and with (B) the efflux blocker verapamil. af, autofluorescence of yeast blastoconidia.

specific for a CDR ATP-binding cassette transporter efflux pump (Marr *et al.*, 1998, 2001). The *in vitro* induction of resistance in *C. tropicalis* has been documented with associated increased expression of *CDR1* and *MDR1* genes (Barchiesi *et al.*, 2000), coding for a specific fluconazole transporter.

Distinct antifungal resistance mechanisms have been described among *Candida* spp., active efflux being the most relevant mechanism of azole resistance; its molecular base is relatively well known for *C. albicans* (Sanglard & Odds, 2002). Overexpression of these efflux proteins confers resistance to most azoles but in the same degree. Whereas ketoconazole, fluconazole, itraconazole and voriconazole are transported readily, posaconazole is transported to a much lesser extent (Chau *et al.*, 2004). Apparently, this compound is not the ideal substrate for these transporters.

If the underlying resistance mechanism induced after antifungal exposure were overexpression of efflux pumps, we would expect that in the presence of both types of efflux blockers MIC values would show a decrease and Rh-6G staining in resistant cells exposed to antifungal would be lower when compared with nonexposed cells. According to

these results, the efflux seems to play a minor role on the resistance induced; however, the hypothesis of the existence of specific *C. parapsilosis* efflux pumps blocked by different modulators or with fluorescence substrate other than Rh-6G cannot be excluded.

In this study, the hypothesis that induced resistance in *C. parapsilosis* may be due to mutation in genes that codify enzymes of the ergosterol biosynthesis pathway gains considerable strength. This mutation is probably induced by the antifungal and does not result from selection of an initial resistant clone. This last hypothesis was not supported by the results from our population studies, as both the susceptible population (without antifungal exposure) as well as the resistant population, obtained following induction, were shown to be homogeneous.

The azoles bind in the vicinity of the haeme group of lanosterol-14 α -demethylase. Although there are silent mutations scattered throughout the gene, at least a dozen different mutations in the active region result in a lower affinity for azoles, resulting in a greater influence on the antimicrobial activity of fluconazole and voriconazole than of posaconazole and itraconazole (Levin *et al.*, 1998). Moreover, the latter two drugs are able to bind to an additional domain of the enzyme by means of their long side chain (Li *et al.*, 2004; Akins, 2005), meaning that these two antifungals can still inhibit the target at a point when fluconazole and voriconazole are no longer active (Sabatelli *et al.*, 2006). Accordingly, such mutation should result in a lower impact on the susceptibility profile to posaconazole than to fluconazole or voriconazole. Because of the two different binding sites of posaconazole, the occurrence of large-spectrum cross-resistance is highly unlikely.

Different assumptions support the occurrence of drug-induced mutation during the described assays: the rapidity of development of the resistant phenotype; the fact that the three azoles are affected, although to a different extent; the insignificant role of efflux; and the stability of resistance following prolonged absence of drug.

Spontaneous mutations are very infrequent, occurring at frequencies of $c. 10^{-6}$ – 10^{-8} per gene. The constancy of the MIC values in time when strains were incubated in drug-free medium strongly supports the assumption that the occurrence of spontaneous mutations is uncommon.

Diminished sensitivity of the target is probably the main resistance mechanism to azoles in *C. parapsilosis*. Likewise, phenotypically stable resistance to azole antifungals in *C. albicans* can result from mutations in genes involved in the ergosterol synthesis pathway (including the target enzyme 14 α -demethylase). Regarding *C. parapsilosis*, the *ERG* sequences of the biosynthetic pathway of ergosterol need to be determined in resistant strains and compared with susceptible strains to clarify whether mutations are associated with azole resistance, as well as to assess changes in membrane

lipid content. Studies involving a large number of isolates of *C. parapsilosis*, aiming to characterize the cellular and molecular factors conferring resistance are being pursued.

In summary, we described the rapid and stable development of azole resistance in *C. parapsilosis* after growth in the presence of azole antifungals, fluconazole being the fastest inducer while posaconazole was the slowest.

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Prevalence, Distribution, and Antifungal Susceptibility Profiles of *Candida parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* in a Tertiary Care Hospital[▽]

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Candida parapsilosis, an emergent agent of nosocomial infections, was previously made up of a complex of three genetically distinct groups (groups I, II, and III). Recently, the *C. parapsilosis* groups have been renamed as distinct species: *C. parapsilosis sensu stricto*, *C. orthopsilosis*, and *C. metapsilosis*. In Portugal, no data pertaining to the distribution and antifungal susceptibility of these *Candida* species are yet available. In the present report, we describe the incidence and distribution of *C. parapsilosis sensu stricto*, *C. orthopsilosis*, and *C. metapsilosis* among 175 clinical and environmental isolates previously identified by conventional methods as *C. parapsilosis*. We also evaluated the in vitro susceptibilities of the isolates to fluconazole, voriconazole, posaconazole, amphotericin B, and two echinocandins, caspofungin and anidulafungin. Of the 175 isolates tested, 160 (91.4%) were identified as *C. parapsilosis sensu stricto*, 4 (2.3%) were identified as *C. orthopsilosis*, and 5 (2.9%) were identified as *C. metapsilosis*. Six isolates corresponded to species other than the *C. parapsilosis* group. Interestingly, all isolates from blood cultures corresponded to *C. parapsilosis sensu stricto*. Evaluation of the antifungal susceptibility profile showed that only nine (5.6%) *C. parapsilosis sensu stricto* strains were susceptible-dose dependent or resistant to fluconazole, and a single strain displayed a multiazole-resistant phenotype; two (1.3%) *C. parapsilosis sensu stricto* strains were amphotericin B resistant. All *C. orthopsilosis* and *C. metapsilosis* isolates were susceptible to azoles and amphotericin B. A high number of strains were nonsusceptible to the echinocandins (caspofungin and anidulafungin).

At present, *Candida parapsilosis* stands out as the second most common yeast species isolated from patients with bloodstream infections in Latin America and Asia (38, 44), but it is also commonly found in European surveys (1, 32, 44). Similar results were found in Portugal, according to a prospective, observational study of bloodstream fungal infections (6).

This pathogenic fungus is responsible for a broad variety of clinical manifestations that generally occur in individuals with impaired immune systems, neutropenia, or burns and patients admitted to neonatal or surgical intensive care units (32) and that also commonly occur in pediatric units (11, 39).

Isolates of *C. parapsilosis* are phenotypically indistinguishable but are genetically heterogeneous. On the basis of randomly amplified polymorphic DNA analysis (20), multilocus enzyme electrophoresis (22), analysis of the internal transcribed spacer sequences of DNA encoding ribosomes (22), DNA relatedness analysis (41), morphotyping (2), analysis of mitochondrial DNA sequence differences (25), DNA topoisomerase II gene sequence analysis (14), and analysis with an oligonucleotide probe used for fingerprinting of *C. parapsilosis* isolates (9), it was found that *C. parapsilosis* forms a complex composed of three genetically distinct groups (groups I, II, and III). Tavanti et al. (47) proposed the replacement of the exist-

ing designations of *C. parapsilosis* group II and group III by *C. orthopsilosis* and *C. metapsilosis*, respectively, and retention of the designation *C. parapsilosis* for the former group I isolates. In order to distinguish between the three species, Tavanti et al. (47) proposed the analysis of the restriction polymorphism of the *SADH* gene, which encodes a secondary alcohol deshydrogenase and which is common to all three species.

Group I, or *C. parapsilosis sensu stricto*, represents the predominant species among clinical isolates, which may partially be explained by its enhanced ability to form biofilms (47). Recent studies have shown that some clinical isolates formerly identified as *C. parapsilosis* in fact correspond to isolates of the closely related species *C. orthopsilosis* and *C. metapsilosis* (15, 22, 23, 47) and account for about 10% of the total number of former *C. parapsilosis* isolates (23). These two new species may inhabit important niches in certain patient populations, thus stressing the need for continued surveillance in each country to monitor the prevalence and distribution of these three species.

Although *C. parapsilosis* is not considered to be particularly prone to the development of antifungal resistance (19, 31, 34, 48), several recent reports suggested that its decreased susceptibility to azoles and echinocandins might become a cause for clinical concern (3, 34, 36, 45, 48). The emergence of *C. parapsilosis* strains that have decreased susceptibility to fluconazole (FLU) and that may be transmitted throughout the hospital environment may be related to the extensive use of FLU in combination with suboptimal hand hygiene and central venous catheter care in a population of seriously ill patients (1).

Few studies have yet addressed the global epidemiology and

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TABLE 1. Distribution of *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis*

Site of isolation	No. (%) of isolates			Total
	<i>C. parapsilosis</i> sensu stricto	<i>C.</i> <i>orthopsilosis</i>	<i>C.</i> <i>metapsilosis</i>	
Urine	9	4	0	13
Respiratory tract	2	0	1	3
Central venous catheter	6	0	0	6
Blood	60	0	0	64
Mucosal surface	65	0	4	71
Peritoneal fluid	1	0	0	1
Bile fluid	2	0	0	2
Spinal fluid	1	0	0	1
Hospital environment	8	0	0	8
Unknown	6	0	0	6
Total	160 (91.4)	4 (2.3)	5 (2.9)	175 ^a (100)

^a The total number of isolates is higher than the sum of the numbers of isolates of *C. parapsilosis* sensu stricto, *C. orthopsilosis*, and *C. metapsilosis* since the molecular analysis of six isolates showed that they corresponded to distinct *Candida* species.

antifungal susceptibility profile of *C. parapsilosis* (1, 34, 49). Induction studies showed an increase in the level of resistance after exposure to azoles (46). This fact, in association with the lower susceptibility of *C. parapsilosis* to echinocandins, urged the collection of all possible additional information regarding this opportunistic fungal pathogen. Moreover, the definition of the antifungal susceptibility profile of the new species *C. orthopsilosis* and *C. metapsilosis* is of clinical relevance. The differences in susceptibility between these three species may influence the therapeutic choices that are made.

Due to the medical importance of *C. parapsilosis* in Portugal (6) and the lack of availability of local epidemiological data, we aimed to provide insight into the incidence and distribution of *C. parapsilosis* sensu stricto, *C. metapsilosis*, and *C. orthopsilosis* in a large (1,350-bed) university hospital in the north of Portugal. Additionally, we evaluated the susceptibilities of such isolates to six commonly used antifungal agents, namely, FLU, voriconazole (VOR), posaconazole (POS), amphotericin B (AMB), and the echinocandins caspofungin (CAS) and anidulafungin (ANI).

MATERIALS AND METHODS

Strains. One hundred sixty-seven strains were collected from patients admitted to different care units of the Hospital S. João over a 4-year period; the assumed phenotypic and genetic diversity of this set of strains is based on the distinct date and anatomic site of isolation. Isolates obtained from environmental studies were also included: five isolates were collected from the air by filtration with a MAS-100 Eco instrument (Merck Eurolab, Switzerland), and three isolates were collected from food administered to patients and isolated in Cooke Rose Bengal agar (Difco Laboratories, Sparks, MD). All isolates were initially identified as *C. parapsilosis* by the use of Vitek YBC identification cards (bio-Mérieux, Paris, France). Type strains ATCC 22019, ATCC 96139, and ATCC 96144, from the American Type Culture Collection, were used as controls for *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis*, respectively. Details regarding the 175 *C. parapsilosis* isolates are depicted in Table 1.

DNA extraction. Yeast genomic DNA was extracted as described by Hoffman and Winston (13), with some adaptations. Briefly, yeast cells grown overnight in YPD medium (2% glucose, 2% peptone, 1% yeast extract) at 30°C with agitation were harvested and lysed by vortexing for 10 min with 0.3 g of glass beads (diameter, 0.45 to 0.52 mm; Sigma, St. Louis, MO), 200 µl of lysis buffer (100

mM Tris-HCl, pH 8.0, 2% Triton X-100, 1% sodium dodecyl sulfate, 1 mM EDTA), and 200 µl of phenol-chloroform-isoamyl alcohol (25:24:1; Fluka Biochemika). After the mixture was vortexed, 200 µl of TE (1 mM EDTA, 10 mM Tris-HCl, pH 8.0) was added to the lysate, the mixture was microcentrifuged at 20,000 × g for 10 min, and the aqueous phase was transferred to a new vial. DNA was precipitated following the addition of 1 ml of ethanol and afterwards was treated with RNase (10 mg/ml; Sigma) for 1 h at 37°C; the DNA was reprecipitated and resuspended in 100 µl of TE.

***SADH* gene restriction profile.** Amplification of the *SADH* gene was performed by PCR with the primers previously described by Tavanti et al. (47). The amplification conditions were as follows: a first cycle of 2 min at 94°C, followed by 35 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s and a final step of 10 min at 72°C. PCRs were performed in a Realplex Mastercycler instrument (Eppendorf, Madrid, Spain). The PCR product was then digested with the BanI enzyme (New England Biolabs, Hitchin, United Kingdom) in a 40-µl volume containing 20 µl of the PCR product and 40 U of BanI. The digestion products were separated on a 2% agarose gel. The clinical isolates were discriminated as *C. parapsilosis* sensu stricto, *C. orthopsilosis*, and *C. metapsilosis* according to the *SADH* restriction profile. Furthermore, the amplified fragments of the *C. orthopsilosis* strains were sequenced to exclude the hypothesis that a point mutation exists in the restriction site of *C. parapsilosis* sensu stricto.

Antifungals. Standard antifungal powder of FLU (Pfizer, Groton, CT), VOR (Pfizer), POS (Schering-Plough, Kenilworth, NJ), AMB (Bristol-Myers Squibb, New York, NY), CAS (Merck, Rahway, NJ), and ANI (Pfizer) were obtained from the respective manufacturers. Stock solutions of FLU and CAS were initially prepared in distilled water; VOR, AMB, POS, and ANI were prepared in dimethyl sulfoxide. All antifungals were kept frozen at -70°C until use. The antifungal agents were then diluted with RPMI 1640 medium (Sigma) and buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid buffer (Sigma).

Antifungal susceptibility testing. The MICs of each antifungal drug were determined according to the M27-A3 protocol and the M27-S3 supplement of the Clinical and Laboratory Standards Institute (CLSI) (4, 5). The MICs were registered after 24 h of incubation for the echinocandins and after 48 h for all the azoles and AMB. The susceptibility breakpoints were those of the CLSI (4). For FLU, the MIC for susceptibility was ≤8 µg/ml, the MIC for susceptible-dose dependent was 16 to 32 µg/ml, and the MIC for resistance was ≥64 µg/ml; for VOR, the MIC for susceptibility was ≤1 µg/ml and the MIC for resistance was ≥4 µg/ml. The interpretive criteria for the echinocandins (CAS and ANI) were based upon the MIC-2 (the MIC-2 is the lowest concentration at which a visual reduction in growth of approximately 50% was registered according to the M27-A3 protocol of the CLSI [4]), and susceptibility was considered an MIC of ≤2 µg/ml. Standard MIC₅₀s and MIC₉₀s were calculated as MIC-2's. For POS and AMB, strains inhibited by ≤1 µg/ml of each antifungal were considered to be susceptible. The *C. parapsilosis* ATCC 22019 type strain was used for quality control of antifungal susceptibility testing.

RESULTS

Reidentification of *C. parapsilosis*. Amplification of the *SADH* gene fragment was performed by using the genomic DNA of the isolates as the template. Isolates displaying a fragment with a size other than 716 bp were excluded from this study. Six strains (3.4%), corresponding to four blood isolates and two mucosal isolates, were not confirmed to belong to *C. parapsilosis* groups (Fig. 1a, lane 3). For the *C. parapsilosis* groups, a fragment of the *SADH* gene (716 bp) was amplified by PCR (Fig. 1a, lanes 1, 2, 4, 5, and 6). According to the BanI restriction pattern of the fragment described above, isolates were molecularly discriminated as *C. parapsilosis* sensu stricto, *C. orthopsilosis*, and *C. metapsilosis* (Fig. 1b) as follows: *C. parapsilosis* sensu stricto has one BanI restriction site (at position 196), *C. orthopsilosis* has no restriction site, and *C. metapsilosis* has three BanI restriction sites (at positions 96, 469, and 529). The *SADH* fragments of the previously identified *C. orthopsilosis* isolates were sequenced; all four sequenced isolates were in fact *C. orthopsilosis*. Therefore, the possibility of misidentification due to a possible point mutation in *C. parapsilosis* sensu stricto was excluded (data not shown).

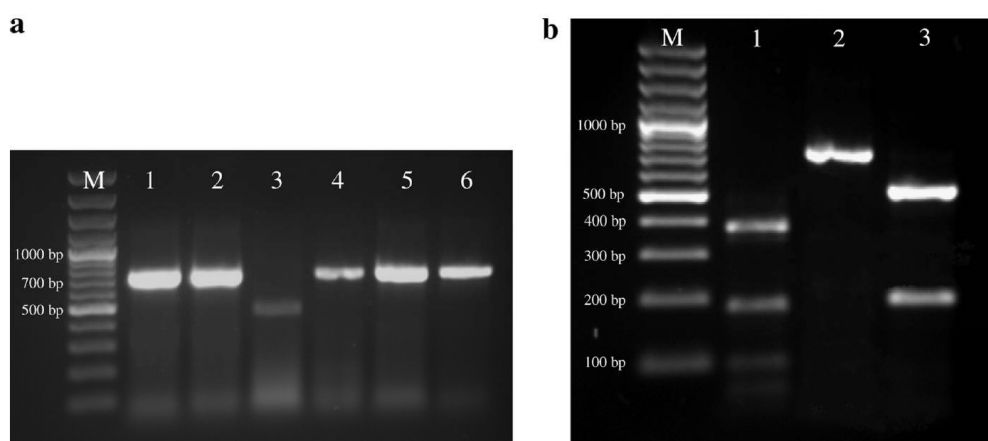


FIG. 1. Discrimination of *C. parapsilosis* (group I), *C. orthopsilosis*, and *C. metapsilosis* isolates according to their *SADH* gene restriction profiles. (a) Amplification of a 716-bp *SADH* gene fragment from the genomic DNA of isolates provided genetic confirmation of the *C. parapsilosis* groups (lanes 1, 2, 4, 5, and 6); the amplification of smaller *SADH* gene fragments corresponds to misidentified *C. parapsilosis* species (lane 3). (b) Representative *SADH* gene restriction profile for *C. metapsilosis* (lane 1), *C. orthopsilosis* (lane 2), and *C. parapsilosis* (lane 3) type strains. Lanes M, 100-bp ladder.

Incidence and distribution of *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis*. The incidence and distribution of the three species are detailed in Table 1; 160 isolates (91.4%) were identified as *C. parapsilosis sensu stricto*, 4 (2.3%) were identified as *C. orthopsilosis*, and 5 (2.9%) were identified as *C. metapsilosis*; 6 isolates corresponded to other *Candida* species. *C. orthopsilosis* and *C. metapsilosis* were found in biological products other than blood; *C. parapsilosis sensu stricto* species were found in all types of biological samples tested. All eight environmental strains were identified as *C. parapsilosis sensu stricto*.

Antifungal susceptibility profile. The profiles of susceptibility to the azoles and AMB are summarized in Table 2. The vast majority of the *C. parapsilosis sensu stricto* isolates (94.4%)

were susceptible to FLU; 8 isolates (5%) were susceptible-dose dependent to FLU, and a single (0.6%) isolate was resistant to FLU. No FLU-resistant or FLU-susceptible-dose-dependent strains were detected among the *C. orthopsilosis* and *C. metapsilosis* isolates; the highest MIC was 4 µg/ml. A single isolate of *C. parapsilosis sensu stricto* was resistant to VOR and POS. All isolates of *C. orthopsilosis* and *C. metapsilosis* were susceptible to VOR and POS. The AMB MICs were equal to or less than 1 µg/ml for all isolates tested, with the exception of two *C. parapsilosis sensu stricto* isolates, which were resistant. The AMB MICs for *C. orthopsilosis* and *C. metapsilosis* were lower than the AMB MICs for *C. parapsilosis sensu stricto*. The patterns of susceptibility to the echinocandins are detailed in Table 3. A high number of *C. parapsilosis sensu stricto* isolates nonsusceptible to CAS (38%), as well as a wide range of MICs (4 to 32 µg/ml), were found, while all *C. orthopsilosis* and *C. metapsilosis* isolates were susceptible to CAS. Similar findings were registered for ANI: all *C. orthopsilosis* and *C. metapsilosis* isolates were susceptible to ANI, while 29% of the *C. parapsilosis*

TABLE 2. Susceptibilities of *Candida parapsilosis* (group I), *C. orthopsilosis*, and *C. metapsilosis* to the tested azoles and AMB

Species (no. of isolates)	Antifungal	MIC range (µg/ml)	% of isolates ^a	
			S	S-DD
<i>C. parapsilosis</i> (160)	FLU	0.125–64	94.4	5
	VOR	≤0.015–8	99.4	
	POS	≤0.03–2	99.4	
	AMB	0.06–2	98.8	
<i>C. orthopsilosis</i> (4)	FLU	0.5–1	100	0
	VOR	≤0.015–0.03	100	
	POS	≤0.03	100	
	AMB	0.5	100	
<i>C. metapsilosis</i> (5)	FLU	1–4	100	0
	VOR	≤0.015–0.125	100	
	POS	<0.03–0.125	100	
	AMB	0.125–1	100	

^a S, susceptible; S-DD, susceptible-dose dependent.

TABLE 3. Susceptibilities of *Candida parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* to echinocandins

Species (no. of isolates)	Antifungal	Phenotype (% of isolates) ^a	MIC-2 range (µg/ml)
<i>C. parapsilosis</i> (160)	CAS	S (62)	0.06–2
	CAS	NS (38)	4–32
	ANI	S (71)	0.125–2
	ANI	NS (29)	4–16
<i>C. orthopsilosis</i> (4)	CAS	S (100)	1–2
	ANI	S (100)	1
<i>C. metapsilosis</i> (5)	CAS	S (100)	1–2
	ANI	S (100)	0.5–1

^a S, susceptible (MIC ≤ 2 µg/ml); NS, nonsusceptible.

silosis sensu stricto isolates were nonsusceptible to ANI but the ANI MICs (4 to 16 µg/ml) were less than those of CAS. For *C. parapsilosis* sensu stricto, the standard MIC₅₀ and MIC₉₀ of both echinocandins were 2 and 4 µg/ml, respectively. For *C. orthopsilosis*, the standard MIC₅₀ of CAS was 2 µg/ml and that of ANI was 1 µg/ml. For *C. metapsilosis*, the standard MIC₅₀s of both echinocandins were 1 µg/ml. The MIC₉₀ was not calculated because the number of *C. orthopsilosis* and *C. metapsilosis* isolates was less than 10.

DISCUSSION

Three distinct groups of *C. parapsilosis* (groups I, II, and III) have previously been recognized on a molecular basis. Subsequent studies focusing on the levels of heterozygosity and on the linear and circular forms of the mitochondrial genome in *C. parapsilosis* support the existence of these distinct species (43). In 2005, after sequencing analysis of several genes, groups II and III were reclassified as two new species separate from *C. parapsilosis* and were named *C. orthopsilosis* and *C. metapsilosis*, respectively (47).

After molecular analysis of a large set of *C. parapsilosis* isolates from a national university hospital, 91.4% were found to be *C. parapsilosis* sensu stricto, while 2.3% and 2.9% corresponded to *C. orthopsilosis* and *C. metapsilosis*, respectively. Discrepancies between the results of biochemical identification and molecular analysis were found since six isolates (3.4%) corresponded to other distinct *Candida* species. This fact highlights the relevance of complementing traditional biochemistry-based identification methods with DNA-based molecular assays such as PCR, since such tests provide higher discriminatory powers. These results improve the knowledge about the national distribution of *C. parapsilosis* sensu stricto, *C. orthopsilosis*, and *C. metapsilosis*. Lockhart et al. (23) identified among 67 Portuguese yeast isolates 1 *C. orthopsilosis* isolate and not a single *C. metapsilosis* isolate. The values found in our study are in accordance with data reported from international studies, namely, 91.3% *C. parapsilosis* sensu stricto isolates, 6.1% *C. orthopsilosis* isolates, and 1.8% *C. metapsilosis* isolates (23).

This study reinforces the assumption that *C. parapsilosis* sensu stricto is predominant among clinical isolates, namely, isolates from patients with hematogenous infections. Interestingly, *C. orthopsilosis* and *C. metapsilosis* isolates were obtained from other biological products, like urine, respiratory tract samples, and mucosal surfaces. The higher prevalence of *C. parapsilosis* sensu stricto may be related to its ubiquitous nature, since it is commonly isolated from different environmental sources. Those sources may represent potential routes for nosocomial transmission to patients (16, 21) and/or may be related to a larger set of virulence attributes compared to the amounts of virulence attributes of the other two species (7, 10, 42). However, the last assumption still needs to be fully confirmed. In fact, the strains isolated from the hospital air and patient food corresponded to *C. parapsilosis* sensu stricto, which supports the first assumption.

We evaluated the in vitro activities of FLU, VOR, POS, CAS, ANI, and AMB against *C. parapsilosis* sensu stricto, *C. orthopsilosis*, and *C. metapsilosis*. The establishment of susceptibility profiles provides information relevant to the develop-

ment of recommendations for prophylactic, empirical, or targeted antifungal therapeutic strategies (17, 30, 35), particularly at the local level.

Valid statistical comparisons of the antifungal susceptibility patterns of the three species were seriously compromised due to the reduced number of strains of *C. orthopsilosis* and *C. metapsilosis* tested. Although the MICs of the echinocandins were high for all three species, the *C. parapsilosis* sensu stricto isolates were less susceptible to CAS and ANI in vitro than the *C. orthopsilosis* and *C. metapsilosis* isolates. Previous studies by our team (6) had already described high CAS MICs (for all *C. parapsilosis* isolates, the CAS MICs were ≥2 µg/ml). The profiles of susceptibility to both echinocandins (CAS and ANI) of the *C. parapsilosis* isolates from our hospital are quite different from the ones described in other surveys, like the study of Pfaller et al. (37). This finding deserves further attention. It has repeatedly been shown that echinocandin MICs are consistently higher for the *C. parapsilosis* group than for *C. albicans* or *C. glabrata* when they are assessed by broth microdilution methods (6, 26, 33, 36, 37, 40), thus suggesting that the *C. parapsilosis* group is less susceptible to this class of antifungal agents. The reduced susceptibility of *C. parapsilosis* to echinocandins (ANI, CAS, and micafungin) has been related to polymorphisms in the *FSK1* gene (27, 28, 29). van Asbeck et al. (48) suggested that interspecies differences in CAS susceptibility result from the combination of structural differences in the cell wall components, a reduced affinity for the glucan synthase protein complex, or variations in the regulatory network of this complex. Azole resistance was detected only among *C. parapsilosis* sensu stricto isolates and not among *C. orthopsilosis* or *C. metapsilosis* isolates. The differences in FLU susceptibility among the three species reflect the distinct affinity of azoles for the key ergosterol-synthesizing enzyme, 14-demethylase, or other enzymes involved in this metabolic pathway (48). Only two *C. parapsilosis* sensu stricto isolates were resistant to AMB. The levels of nonsusceptibility were similar between isolates from different biological products (data not shown).

We detected a multiechinocandin-, multiazole-resistant phenotype of *C. parapsilosis* sensu stricto, as reported by Moudgal et al. (24) and Vazquez and colleagues (12). The development and subsequent nosocomial expansion of echinocandin- and azole-resistant *C. parapsilosis* sensu stricto have important clinical implications. Continuous monitoring for the emergence of this multidrug-resistant phenotype of *C. parapsilosis* sensu stricto represents an important component of the effective prevention of nosocomial infections.

Although FLU showed good activity against *C. parapsilosis* sensu stricto, *C. orthopsilosis*, and *C. metapsilosis*, local routine monitoring of the susceptibility profiles of these organisms may be highly advisable. The reduced echinocandin susceptibility profile reported in this study also represents a problematic issue, at least in Portugal. The combination of decreased susceptibility to FLU and the ability to form extensive biofilms on central venous catheters and other medical indwelling devices may become problematic (8, 18, 19, 21). In over 50% of the episodes of *C. parapsilosis* fungemia, the source is a vascular catheter, and such infections commonly develop in patients who had previously received antifungal treatment (1). Thus, an adequate response to FLU or to any other antifungal may not be fully obtained. The administration of the antifungal should

be coupled with the prompt removal of the catheter to ensure an optimal response (27).

The data reported here represent the most comprehensive data from a study of the incidence and antifungal susceptibility profiles of *C. parapsilosis* sensu stricto, *C. orthopsilosis*, and *C. metapsilosis* conducted to date in Portugal.

We demonstrated the low incidence of *C. orthopsilosis* and *C. metapsilosis* among clinical isolates, especially isolates from blood cultures. The differences in antifungal susceptibility described here are not sufficiently distinct to warrant discrimination of the species in the clinical laboratory.

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Disclaimer: The views expressed in this letter are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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Colonization of central venous catheters in intensive care patients: A 1-year survey in a Portuguese university hospital

To the Editor:

Patients admitted at intensive care units (ICUs) often require central venous catheterization. Catheter colonization may lead to bacteremia and, eventually, end up as a catheter-related bloodstream infection.¹ To review the etiology and antimicrobial susceptibility pattern of central venous catheter (CVC) isolates from ICU patients, a retrospective study of the clinical database of Hospital of S. João, in Porto, was performed during a 12-month period. All positive results from the culture of distal tips of CVCs removed from ICU patients and antimicrobial susceptibility pattern of isolates were reviewed.

From 1482 cultures of CVC distal tips performed during the study period, 647 positive results (43.7%) were found. As expected,² coagulase-negative

staphylococci (54.7%), mainly *Staphylococcus epidermidis* (40.2%), were the most common isolates; *S aureus* (12.8%), Enterobacteriaceae (8.6%), *Candida* spp (7.0%), *Pseudomonas aeruginosa* (6.2%), enterococci (4.8%) and *Acinetobacter baumannii* (2.8%) followed in the ranking. Of major concern was the resistance rate of *S aureus* to methicillin (80%), of *Enterococcus faecium* to vancomycin (85.7%), of *P aeruginosa* to meropenem (34.8%) and imipenem (65.2%) and of *A baumannii* to both carbapenems (94.4%).³⁻⁷ Interestingly, no resistance to azole antifungals (fluconazole, itraconazole, voriconazole, posaconazole) was detected among the low number of tested *Candida* isolates (n = 35), in accordance with previous findings.⁸ This fact might result from the limited exposure to antifungal agents in this hospital because no antifungal prophylactic treatment is routinely prescribed to such patients.

Globally, the etiology of CVC colonization in ICU patients was similar to international available data. However, higher levels of antimicrobial resistance were found for *S aureus*, *E faecium*, *P aeruginosa* and *A baumannii* isolates. Routine surveillance for resistance patterns and the prospective evaluation of new therapeutic protocols might be highly advisable in such setting.

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Candida krusei reservoir in a neutropaenia unit: molecular evidence of a foe?

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Abstract

Candida krusei has been documented as an emerging pathogen causing nosocomial outbreaks. The consecutive isolation of *C. krusei* strains in three patients admitted to the same hospital department within 2 months lead us to consider the possibility of an outbreak. Additionally, *C. krusei* isolates were collected from the room surfaces, whereas another isolate had been recovered from the blood of one patient 2 years before. *HinfI* DNA restriction endonuclease-based analysis of all *C. krusei* isolates was performed and restriction profiles were compared. Surprisingly, isolates from different patients were unrelated, whereas isolates from biological products of the same patient showed indistinguishable *HinfI* restriction patterns and were similar to those obtained from the surrounding environment of the respective patients. The study approach revealed the endogenous origin of the *C. krusei* infectious episodes observed and demonstrated that, subsequent to colonizing a patient, *C. krusei* can be involved in infectious episodes distant in time. The hypothesis of an outbreak was excluded, although we believe that the methodology employed in the present study represents a valuable tool for diagnostic and epidemiological surveys.

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Introduction

In the last two decades, invasive fungal infections in hospitalized patients have increased significantly worldwide. According to data obtained from the USA and Europe, *Candida* species represent, respectively, the fourth and sixth most frequent cause of invasive healthcare-related infections [1,2], accounting for 8–15% of all episodes of sepsis acquired in hospital settings [3]. Inherent to these types of infection are the extremely high morbidity and mortality rates, particularly among immunocompromised patients [4,5].

Fluconazole is one of the antifungal agents mostly used in both prophylactic and therapeutic protocols. Fluconazole prophylaxis has been associated with a decrease in the prevalence of *Candida* species such as *Candida tropicalis* and

Candida albicans, and to an increase in that of *Candida krusei* and *Candida glabrata* [3]. *C. krusei* presents intrinsic resistance to fluconazole and, to some extent, reduced susceptibility to amphotericin B [6].

Infectious outbreaks in hospitals, especially in intensive care units, represent a serious health problem and are mainly due to *Candida lusitanae* [7], *C. albicans* [8], *Candida parapsilosis* [9], and *C. krusei* [10,11]. Many factors may account for their occurrence (e.g. barrier loss, lack of proper infection control measures by health care workers when managing patients, resistance to prescribed antifungal drugs, as well as insufficient drug levels).

Molecular methods represent a powerful tool to clarify transmission pathways in health care facilities (i.e. to investigate the occurrence of possible outbreaks). Techniques such as karyotyping, restriction fragment length polymorphism analysis by pulsed-field gel electrophoresis, southern blot hybridization, PCR fingerprinting and randomly amplified polymorphic DNA fingerprinting have been extensively used for *Candida* typing [12–16]. Restriction endonuclease analysis (REA) has been described in the last decade as a valuable tool for *Candida* spp. characterization. REA of the mitochondrial

DNA (mtDNA) was first applied in the biotechnology industry in order to characterize yeast strains used for wine fermentation [17,18] and, more recently, to discriminate between *Candida* clinical strains [19–22]. The data obtained demonstrate the relevance of using molecular genetic methods in many different areas, including taxonomic, ecological and clinical surveys.

Recently, we were challenged by a hypothetical outbreak as a result of *C. krusei* in the neutropaenia unit of the Haematology Department of Hospital S. João, Porto, Portugal. Within a short period of time, several *C. krusei* isolates were cultured from biological products of three patients. In addition, *C. krusei* was found in the surrounding environment of the patients. All isolates were compared using mtDNA REA, which is a convenient and powerful tool that allows valid comparisons between isolates of the same yeast species.

Patients and Methods

Patients

Patient A was a 41-year-old male with acute lymphoblastic leukaemia (T/NK) diagnosed in July 2006. In August 2008, the patient was initially treated with amoxicillin and ciprofloxacin because of undetermined fever. When symptoms remained unchanged, a myelogram was performed; a first relapse of the haematological disease was diagnosed. He was admitted to the neutropaenia unit for salvage intensive chemotherapy with fludarabine, idarubicin and ara-C, followed by growth factor (G-CSF), administered through a central venous catheter. Upon the onset of the aplastic period (28 August), he received antimicrobial treatment for 2 weeks [ciprofloxacin, amoxicillin-clavulanic acid, imipenem, acyclovir and fluconazole (200 mg/day)]. At the time of fungaemia detection, he displayed the following haematologic parameters: white blood cells 0.03×10^9 cells/L, haemoglobin level 8.5 g/dL and blood platelets 11×10^9 cells/L. As soon as *C. krusei* was identified, the patient was started on caspofungin for 12 days without improvement; the treatment was then changed to amphotericin B.

This patient had previously developed a fungaemia episode as a result of *C. krusei* during chemotherapy in 2006 and a corresponding isolate had been stored at -70°C .

The patient remained in room number 1 from August to October 2008.

Patient B was a 53-year-old male with non-Hodgkin's lymphoma, who was admitted to room number 2 for 3 weeks in September 2008. He was administered cyclophosphamide, doxorubicin, vincristine, and prednisone, followed by G-CSF.

On 12 September 2008, he was started on caspofungin but, as a result of sustained fever on day 5, treatment was changed to voriconazole, completing 14 days of antifungal therapy. He never received fluconazole. At that fungaemia episode, he displayed the following haematologic parameters: white blood cells 2.41×10^9 cells/L, haemoglobin 9.3 g/dL and blood platelets 73×10^9 cells/L.

Patient C was a 60-year-old male with acute myeloid leukaemia secondary to myelodysplastic syndrome; he was admitted to room number 2 for 3 weeks in October 2008, subsequent to patient B. He received Ara-C, etoposide and doxorubicin for 5 days, followed by another 5 days of Ara-C; he was also prescribed amoxicillin, ciprofloxacin and allopurinol. In addition, prophylactic treatment included imipenem, vancomycin, fluconazole and acyclovir from 29 September to 20 October 2008. On that latest date, he displayed the following haematologic parameters: white blood cells 0.6×10^9 cells/L, haemoglobin 9.8 g/dL and blood platelets 24×10^9 cells/L.

Patient D served as a typing control; one single *C. krusei* isolate was recovered from the patient's bronchial secretions in September 2008 upon his admission to the Internal Medicine Department.

Clinical strains

Eighteen *C. krusei* isolates were collected from different clinical specimens (blood, urine, stools and bronchial secretions) of the above mentioned patients admitted to the neutropaenia unit within a 2-month period. Additionally, a previous *C. krusei* isolate (2006) from a blood culture of patient A was included in the study, as well as a *C. krusei* isolate obtained from the control patient D. All isolates were identified using the automatic system Vitek2 YBC identification cards (BioMérieux, Paris, France), stored at -70°C in Brain Heart infusion (Merck KGaA, Darmstadt, Germany) with 10% glycerol and sub-cultured twice on Sabouraud agar (Merck KGaA) to ensure purity prior to experimental assays.

Environmental strains

Several environmental samples were collected from the patients' rooms (1 and 2) using Sabouraud agar contact plates (Merck KGaA); two *C. krusei* isolates were cultured: one from the bedside table of patient A (room 1) and another from the bed of patient C (room 2). The isolates were characterized and stored as described above for clinical samples. Air samples were collected by filtration with a MAS-100 Eco instrument (Merck Eurolab, Dietlikon, Switzerland), containing Sabouraud agar plates (Merck KGaA); no *C. krusei* isolates were obtained.

Antifungal susceptibility testing

Voriconazole (Pfizer, New York, NY, USA), posaconazole (Schering-Plough; Kenilworth, NJ, USA), amphotericin B (Bristol Meyers Squibb, New York, NY, USA), caspofungin (Merck, Rahway, NJ, USA) and anidulafungin (Pfizer) stock solutions were prepared according to CLSI protocols (M27-A3) [23] and maintained at -70°C until use. Minimal inhibitory concentration (MIC) of each antifungal drug was determined according to CLSI protocol M27-A3 [23].

Total genomic DNA extraction

Yeast cells were cultured overnight at 30°C in 10 mL of YPD liquid medium, with continuous orbital shaking at 180 r.p.m., and subsequently collection by centrifugation. Total DNA was extracted using phenol:chloroform:isoamyl alcohol 25:24:1, precipitated using 100% ice-cold ethanol and redissolved in 200 μL of TE buffer. The DNA was treated with 20 μg of RNase (Applichem, Darmstadt, Germany), incubated at 37°C for 30 min to 1 h. For final precipitation, 20 μL of 4 M ammonium acetate, pH 4.8 (Sigma-Aldrich, Munich, Germany) and 600 μL of ice-cold 100% ethanol (Applichem) were added and samples were incubated overnight at -20°C . The DNA was re-dissolved in TE buffer 1x, assessed in a biophotometer 6131 (Eppendorf, Hamburg, Germany) and adjusted to a final concentration of 2.0–2.5 $\mu\text{g}/\mu\text{L}$. To assay DNA integrity, approximately 3–5 μg of DNA was run in agarose gel (1%, w/v) (Sigma-Aldrich) in TBE buffer 1x and stained with ethidium bromide (0.5 mg/mL) (Applichem). DNA samples were stored at -20°C for subsequent use.

REA mt DNA

For each sample, a reaction mixture was prepared containing 1x *Hinf*I enzyme reaction buffer (Metabion, Planegg, Germany), 1 $\mu\text{g}/\mu\text{L}$ RNase, 0.5 U/ μL *Hinf*I restriction enzyme (Metabion), approximately 25–30 μg of total DNA and DNase-RNase free water up to 20 μL final volume; reaction tubes were incubated overnight at 37°C . Restriction was ended upon *Hinf*I inactivation by incubating for 20 min at 80°C . The total reaction mixture was run on a 1% agarose gel (20 cm $\times$ 24 cm) at 120 mV for 3–5 h, stained with ethidium bromide (0.5 mg/mL) and the DNA visualized under UV light.

Restriction patterns were analyzed using the UVIDOC 12.6 software for Windows (Topac Inc., Cohasset, MA, USA) and the resulting groups of strains were compared.

Results and Discussion

There have been an increasing numbers of reports describing non-*albicans* *Candida* hospital outbreaks. Given that *C. krusei* is

not the main pathogen causing nosocomial infections, the detection of simultaneous episodes at the neutropaenia unit of our hospital lead us to consider the possibility of an outbreak.

The *C. krusei* isolates were all susceptible to all the antifungals assayed (fluconazole was not tested because *C. krusei* presents intrinsic resistance to this agent). Variations in the antifungal MICs for different isolates were not significant. The MICs of amphotericin B were from 0.06 to 1 mg/L; for caspofungin from 0.125 to 1 mg/L; for anidulafungin 0.06 mg/L; for voriconazole from 0.25 to 2 mg/L; and for posaconazole from 0.03 to 0.5 mg/L.

The routine biochemical identification protocols or antifungal susceptibility profiles are usually not sufficient to either corroborate or exclude an outbreak hypothesis. REA for *Candida* species was first described by Scherer and Stevens [24] who considered this method to be an extremely valuable tool for epidemiological studies. Fujita et al. [21] described *Hinf*I restriction patterns as exhibiting a superior discriminatory power among distinct *Candida* isolates compared to patterns obtained with other enzymes such as *Eco*RI or *Msp*I. Additionally, Sancak et al. [22] established a correspondence of almost 100% between the results obtained with *Hinf*I restriction endonuclease-based analysis and PCR methodologies. In the present study, a total of 22 *C. krusei* isolates (20 clinical, two environmental) were analyzed using REA. A high number of *C. krusei* isolates was obtained from different biological products of patient A in distinct periods of time. All of them showed the same restriction pattern, including the isolate recovered in 2006 from a blood culture (A_{BC1}) (Fig. 1, lanes A_{BC1} , A_{BC2} and A_{CV1}), indicating that they are the same strain. Most certainly, this patient harbors a reservoir of *C. krusei* and was colonized throughout a long period of time, as described similarly for *Acinetobacter* [25] and *Pseudomonas* [26]. This is the first report, to our knowledge, describing a long-lasting colonization by *C. krusei*. These results have implications in terms of prophylactic measures (i.e. fluconazole is not recommended in a patient with previous isolation of *C. krusei*). Other antifungals, such voriconazole or amphotericin B, are more likely to be efficient in these cases [27,28].

The *C. krusei* isolates from each patient yielded distinct *Hinf*I restriction patterns, suggesting that the isolates were different strains (Fig. 1, lanes A_{BC2} , B_{BS} , C_5), at the same time discarding the hypothesis of an outbreak in the neutropaenia unit where the patients were admitted. The *C. krusei* strain isolated from patient D showed a pattern distinct from those isolated from the remaining patients (Fig. 1, lane D_{BS}), as expected.

The two environmental *C. krusei* isolates collected from the surfaces of the rooms where patient A (A_{E9}) (room 1) and patients B and C (C_E) (room 2) had stayed were

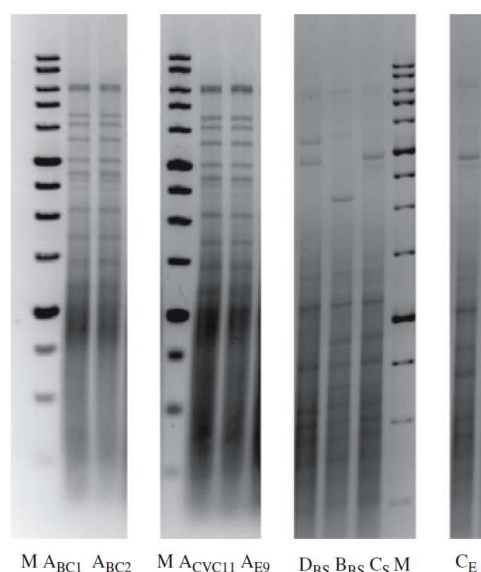


FIG. 1. Restriction endonuclease patterns of *Hinfi*-digested DNA obtained after agarose gel electrophoresis. Each pattern corresponds to *Candida krusei* isolates from patients A, B, C and D (upper case letters), from different biological products (BC, blood cultures; CVC, central venous catheter; BS, bronchial secretions; S, stools), and to *C. krusei* isolates from the room environment of patients A and C (A_E and C_E , respectively); M, molecular weight marker (1 kb DNA ladder; Metabion).

different, as depicted in Fig. 1. This excludes the possibility of different *C. krusei* strains being transmitted as a result of patient handling by health care workers. However, both the environmental and the clinical *C. krusei* isolates associated with the same patient displayed an undistinguishable restriction pattern, as shown in Fig. 1 (lanes A_{CVC11} and A_{E9} vs. C_S and C_E), suggesting a putative environmental reservoir and the possibility of subsequent transmission from it to other patients. Our results emphasize the need to enhance preventive infection control measures, both when handling the patients directly and when cleaning patients' facilities, particularly those admitting neutropenic patients. Indeed, a study by Berrouane *et al.* [29] described the relatedness among *C. krusei* clinical isolates obtained from different biological products from both patients and health care workers.

As described in the present study, we were able to exclude the hypothesis of an outbreak occurring in the neutropaenia unit throughout the time period considered, supporting the usefulness and suitability of the REA methodology. Moreover, the present study provided very useful information concerning *C. krusei* reservoirs existing in patients and their surrounding environment.

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Transparency Declaration

The authors declare that there is no source of funding and no potential conflicts of interest.

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FKS2 Mutations Associated with Decreased Echinocandin Susceptibility of *Candida glabrata* following Anidulafungin Therapy[∇]

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This is the first case report of *Candida glabrata*-disseminated candidiasis describing the acquisition of echinocandin resistance following anidulafungin treatment. The initial isolates recovered were susceptible to echinocandins. However, during 27 days of anidulafungin treatment, two resistant strains were isolated (from the blood and peritoneal fluid). The resistant peritoneal fluid isolate exhibited a Ser663Pro mutation in position 1987 of *FKS2* HS1 (hot spot 1), whereas the resistant blood isolate displayed a phenylalanine deletion (Phe659).

Candida glabrata infections represent a serious clinical problem due to patient comorbidities, the inherent high mortality rate, and the predisposition to rapidly develop azole resistance (6, 11, 20). Therefore, efforts were made to develop new therapeutic alternatives. A new class of antifungal agents has arisen, including caspofungin (CSF), micafungin (MCF), and more recently, anidulafungin (ANF), which inhibit β (1,3)-glucan synthase activity. This enzyme, including the regulatory subunit Rho-1p, is responsible for producing β (1,3)-glucan, a key component of the fungal cell wall, and is encoded by *FKS* genes.

Due to their excellent clinical effectiveness and safety profile, these antifungals became the first-line therapy in many hospitals for the treatment of invasive candidiasis (17, 18, 26). Despite the wide use of CSF, reports describing primary or secondary echinocandin resistance in candidiasis are rare. Reduced susceptibility to echinocandins has been linked to mutations in hot spot regions of *FKS* genes (19). We report for the first time the *in vivo* acquisition of echinocandin resistance following ANF therapy in a patient with *C. glabrata* invasive candidiasis.

A 71-year-old female patient was admitted to the intensive care unit (ICU) with a diagnosis of acute pancreatitis that developed after a laparoscopic cholecystectomy. The patient's medical history included hypertension and dyslipidemia. An exploratory laparoscopy was performed on the second day after admission to the ICU. The surgical findings were peritoneal necrosis, necroses of the ileum and jejunum, and pancre-

atic necrosis. During the hospital stay, several antibacterial drugs were administered to the patient, namely tazobactam, piperacillin, meropenem, tigecycline, and vancomycin. She had no previous antifungal exposure. Her medical condition was further complicated by multiple *Candida* infections, including disseminated candidiasis and central venous catheter-related infections. Starting on day 6, she received a 200-mg load of ANF, followed by 100 mg ANF/day for 27 days. Cultures of multiple sites, including blood, exudates, central venous catheter (CVC), and urine, were positive for *C. albicans* and *C. glabrata*. While *C. albicans* fungemia cleared following 24 h of ANF therapy, *C. glabrata* persisted. On day 17, the patient received noradrenaline support. Subsequently, she was submitted to a second exploratory laparoscopic surgery on day 30. The procedure revealed extensive cytosteatonecrosis. Amphotericin B was then initiated, and 2 days later, the patient developed multiorgan failure. The patient died 34 days after admission. No autopsy was performed.

Twenty-one isolates were recovered from the patient's blood and other biological samples throughout ANF treatment (Fig. 1). The assessment of the MICs of azoles, amphotericin B, and echinocandins for all clinical isolates was performed in RPMI 1640 (Sigma), according to the CLSI M27-A3 protocol (4, 5).

From the set of *C. glabrata* isolates, two isolates were resistant to echinocandins, one recovered from the blood (isolate 18-1) and the other from the peritoneal fluid (isolate 30-2) (isolated 18 and 30 days after ICU admission, respectively) (Fig. 1 and Table 1). The acquisition of ANF resistance was associated with an increase in the CSF and MCF MIC values, suggesting the development of cross-resistance among these three compounds. Although MIC values are usually considered predictors of clinical response to antimicrobial therapy in invasive fungal infections, no strong correlation has yet been

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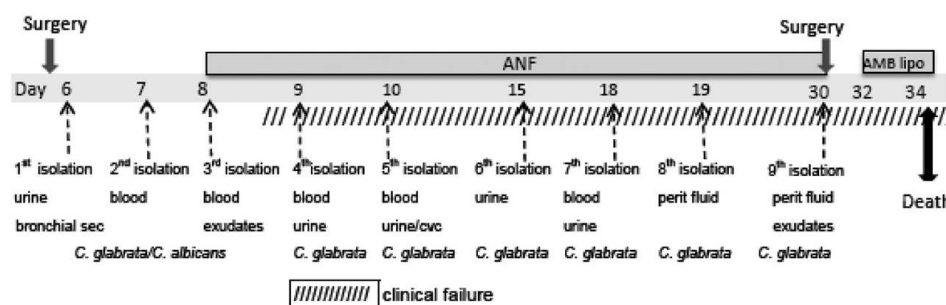


FIG. 1. Time line of antifungal therapy administered to the patient. The number of days after admission and fungal strains isolated from biological samples are also shown. ANF, anidulafungin; AMB lipo, amphotericin B lipid complex.

found between *in vitro* susceptibility results and clinical outcome (6, 12, 13, 15, 16, 21, 22).

In the clinical case described herein, a correlation between the *in vitro* results and the *in vivo* lack of efficacy of the drug was found. The MIC values obtained (4 µg/ml) from the blood (strain 18-1) and peritoneal fluid (strain 30-2) isolates were above the susceptibility breakpoint.

Random amplification of polymorphic DNA (RAPD) was carried out using isolates obtained from different biological samples to determine their isogenicity. Using the primers OPE-18 (5' GGACTGCAGA 3') and OPA-18 (5' AGCTGA CCGT 3') (2), identical band profiles were displayed by all isolates, with the exception of strain 30-2, which was recovered from the peritoneal fluid prior to patient death (Fig. 2). Considering the surgical interventions, the evidence of CVC infection, and the long stay in the ICU (34 days), where nosocomial infections are likely to occur, it is plausible that this strain had an exogenous source.

Mutations in *FKS* genes have been shown to be responsible for echinocandin resistance during caspofungin treatment, namely of *C. glabrata* (3, 9, 10, 14, 24). To determine whether ANF resistance was associated with mutations in the target genes, genomic DNA of *C. glabrata* isolates was extracted and, using the hot spot 1 (HS1) regions of the *FKS1* and *FKS2* genes, were amplified with specific primers (*FKS1* HS1F2 [5'-CTTATGTTTGATTTTTCGA-3']) (8, 24). DNA products were sequenced in an ABI Prism 3130 genetic analyzer (Applied Biosystems). The coding sequences of the *Candida glabrata* *FKS1* and *FKS2* genes (GenBank accession numbers XM_446406 and XM_448401, respectively) were aligned with those obtained from the clinical isolates. No sequence alter-

ations were observed in HS1 of the *FKS1* gene; however, several point mutations were found in HS1 of the *FKS2* gene, with most of them corresponding to synonymous substitutions that did not result in nucleotide changes. Furthermore, the isolate 30-2, which was recovered following a long period of ANF exposure, displayed a C-T mutation at position 1987. This mutation leads to a replacement of serine 663 by proline in HS1 of *FKS2* (Table 1). In the same HS, a deletion of 3 nucleotides was found in the blood isolate 18-1, which results in the deletion of one of the two consecutive phenylalanines at positions 658 and 659 (Table 1). These findings are consistent with previous reports describing increased resistance to echinocandins associated with mutations, namely, the S663P substitution and Phe659 deletion in the *FKS2* gene (9, 10). The highest frequency of resistance-associated mutations is found within HS1 (1). HS1 is a highly conserved region among the Fks family; hence, the amino acid changes in this region implicate a modification of the echinocandin target and a reduced susceptibility phenotype.

The significant increase in the chitin content following *in vitro* echinocandin exposure has been suggested as an escape or salvage mechanism for echinocandins (7, 23, 25). In order to unveil the role of chitin in the resistance displayed by isolates 18-1 and 30-2, the cell wall chitin content was measured initially and after 30 days of subculturing in drug-free medium. While the resistant phenotype remained, the chitin content decreased (data not shown). Attending to such results, we were compelled to conclude that the deletion and the mutation detected in the *FKS2* gene confer echinocandin resistance to the isolates 18-1 and 30-2, respectively.

Overall, our findings suggest that structural alterations in the

TABLE 1. *In vitro* antifungal susceptibility of *C. glabrata* isolates to CSF, ANF, and MCF^a

Strain	Isolation (no. of days after admission)	Sample type	MIC ₅₀ (µg/ml)			<i>FKS2</i> HS1 SNP	AA profile
			CSF	ANF	MCF		
10-1	10	Blood	0.125	≤0.06	≤0.06	Positions 1987-1989, TCT	Ser
18-1	18	Blood	>32	4	4	Positions 1977-1979, CTT deletion	ΔPhe659
19-1	19	Peritoneal fluid	≤0.06	≤0.06	≤0.06	Positions 1987-1989, TCT	Ser
30-2	30	Peritoneal fluid	32	4	8	T1987C	Ser663Pro

^a Determined according to the CLSI M27-A3 protocol (4). MICs were determined by considering a prominent inhibition, corresponding to 50%, as the endpoint. SNP, single nucleotide polymorphism; AA, amino acid.

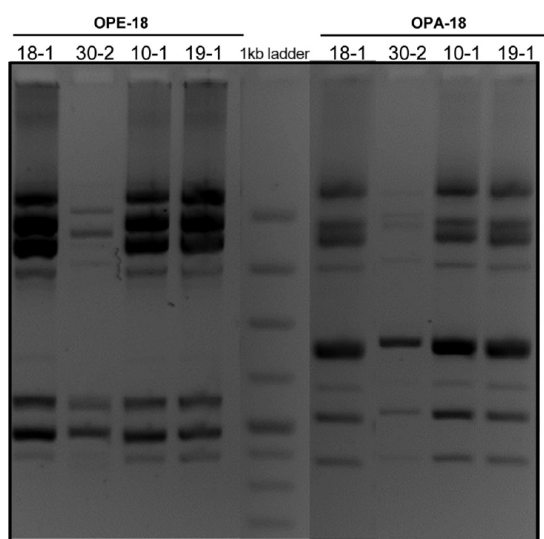


FIG. 2. Random amplification of polymorphic DNA gel patterns of *C. glabrata* isolates 18-1, 30-2, 10-1, and 19-1, resistant and susceptible to echinocandins, obtained with primers OPE-18 and OPA-18.

HS1 of the *FKS2* molecule due to the Ser663Pro substitution and Phe659 deletion lead to a dramatic decrease in echinocandin efficacy. Although just a few cases describing the development of echinocandin resistance have been reported so far, our case report emphasizes the crucial need for antifungal susceptibility surveillance in patients under extended echinocandin therapy.

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Transcriptional Profiling of Azole-Resistant *Candida parapsilosis* Strains^{∇†}

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Herein we describe the changes in the gene expression profile of *Candida parapsilosis* associated with the acquisition of experimentally induced resistance to azole antifungal drugs. Three resistant strains of *C. parapsilosis* were obtained following prolonged *in vitro* exposure of a susceptible clinical isolate to constant concentrations of fluconazole, voriconazole, or posaconazole. We found that after incubation with fluconazole or voriconazole, strains became resistant to both azoles but not to posaconazole, although susceptibility to this azole decreased, whereas the strain incubated with posaconazole displayed resistance to the three azoles. The resistant strains obtained after exposure to fluconazole and to voriconazole have increased expression of the transcription factor *MRR1*, the major facilitator transporter *MDR1*, and several reductases and oxidoreductases. Interestingly, and similarly to what has been described in *C. albicans*, upregulation of *MRR1* and *MDR1* is correlated with point mutations in *MRR1* in the resistant strains. The resistant strain obtained after exposure to posaconazole shows upregulation of two transcription factors (*UPC2* and *NDT80*) and increased expression of 13 genes involved in ergosterol biosynthesis. This is the first study addressing global molecular mechanisms underlying azole resistance in *C. parapsilosis*; the results suggest that similarly to *C. albicans*, tolerance to azoles involves the activation of efflux pumps and/or increased ergosterol synthesis.

Candida parapsilosis is the second most common *Candida* species isolated from patients with bloodstream infections in Latin America and Asia (46, 60), and it is also commonly found in European surveys (4, 17, 43, 67). It is responsible for a broad variety of clinical manifestations that generally occur in individuals with impaired immune systems, in neutropenic or burn patients, as well as in patients admitted to medical or surgical intensive care units (43), especially pediatric units (26, 48).

Azoles are the most commonly used drugs for the treatment of *Candida* infections (13). They target lanosterol 14 α -demethylase, a member of the cytochrome P450 enzymes, which is required for the synthesis of ergosterol (1, 76). Ergosterol is a major and essential lipid constituent of the fungal cell membrane (1). The acquisition of azole resistance, particularly after prolonged exposure, as happens with prophylactic overuse, is a well-known phenomenon in fungi (5, 6, 29). The widespread use of azole antifungals, especially fluconazole (FLC), resulted in a growing incidence of *Candida* species in which resistance is easily induced, such as *Candida glabrata* (75), or species that show intrinsic resistance, such as *C. krusei* (74). Previous studies with *C. albicans* (38), *C. dubliniensis* (59), and *C. tropicalis* (9) demonstrated that resistance to fluconazole can be pro-

moted following repeated *in vitro* exposure to the drug. The ability of a drug to induce *in vitro* resistance suggests that similar mechanisms may also occur *in vivo*, which may thus become problematic, for instance, in prophylactic regimens.

Although *C. parapsilosis* is not considered particularly prone to the development of antifungal resistance (34, 44, 45, 69), recent reports suggest that its decreased susceptibility to azoles and echinocandins might become a cause for clinical concern (14, 42, 45, 64, 69). We previously described the *in vitro* development of stable azole resistance in *C. parapsilosis*, which was induced rapidly following fluconazole exposure or more slowly when it was exposed to posaconazole (PSC) (47).

Three distinct mechanisms of azole resistance have so far been described in *Candida* species: (i) failure to accumulate the drug intracellularly, which may be caused by the lack of drug penetration, due to changes in membrane lipids and sterols (28), or by active efflux of drugs, resulting particularly from overexpression of the *CDR1*, *CDR2*, and *MDR1* genes (3, 38, 62, 73); (ii) increased production of the azole target enzyme (23, 27, 63); or (iii) point mutations in genes coding for the target enzyme, reducing azole target affinity (61). Overexpression of efflux proteins and associated increased activity are considered to be the most relevant mechanism of azole resistance, conferring cross-resistance to several azoles. The molecular basis underlying azole resistance is relatively well established for *C. albicans* (63), and the use of DNA microarray technology has played an important role in identifying the cellular players involved (10, 19, 77, 78). However, there are still scarce data regarding mechanisms of azole resistance in *C. parapsilosis*.

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In the present study, we shed some light on drug resistance mechanisms developed by *C. parapsilosis* after exposure to fluconazole, voriconazole (VRC), and posaconazole. A set of resistant strains was generated following successive *in vitro* subculturing of an azole-susceptible *C. parapsilosis* clinical isolate in the presence of constant concentrations of each of the three antifungal drugs. The global constitutive changes in gene expression were then determined using a microarray platform. Our results suggest that, similar to *C. albicans*, *C. parapsilosis* acquires resistance to azoles either through upregulation of the *MDR1* multidrug transporter family or via increased expression of the sterol biosynthetic pathway. These comparative analyses may help in the design of future strategies for antifungal therapy.

MATERIALS AND METHODS

***C. parapsilosis* strains.** A *C. parapsilosis* clinical isolate (BC014) susceptible to fluconazole, voriconazole, and posaconazole was cultured daily in the presence of each of the three different azoles, namely, fluconazole, voriconazole, and posaconazole. Yeast suspensions containing 10^6 cells in 10 ml of RPMI 1640 medium (RPMI 1640; Sigma, St. Louis, MO) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma) were prepared; to each suspension, each azole drug was added to a final concentration of 16 $\mu\text{g}/\text{ml}$ of FLC (Pfizer, Groton, CT), 2 $\mu\text{g}/\text{ml}$ of VRC (Pfizer), and 1 $\mu\text{g}/\text{ml}$ of PSC (Schering-Plough, NJ). These concentrations are similar to therapeutic blood levels achieved during antifungal therapy (8). Yeast suspensions were grown overnight at 35°C with agitation (150 rpm). On the following day and from each culture, aliquots containing 10^6 cells were transferred into fresh medium (total volume, 10 ml) containing the same antifungal drug in the same concentration and reincubated as described. This procedure was followed for 60 days. MIC values of each of the three antifungals were determined for the parent strain and the final subcultured strains. In summary, three resistant strains were generated from the initial susceptible strain, strain BC014S, which were designated BC014R_{FLC}, BC014R_{VRC}, and BC014R_{PSC}. Aliquots of the parent strain and the final resistant strains were stored at -70°C in 40% glycerol. Population analysis studies of the BC014R_{FLC}, BC014R_{VRC}, and BC014R_{PSC} strains were performed as described previously by Marr et al. (39). Briefly, a single colony of the strain was incubated overnight in YEPD (1% yeast extract, 2% peptone, 2% glucose) broth at 150 rpm and 35°C; a suspension containing 10^3 cells/ml was prepared in RPMI 1640 and plated in Sabouraud agar (Merck, Darmstadt, Germany) without and with fluconazole (1, 4, 16, and 64 mg/ml), voriconazole (0.125, 0.5, 2, and 8 mg/ml), and posaconazole (0.25, 1, 4, and 16 mg/ml). Growth was assessed after 48 h of incubation at 35°C. The percentage of colonies growing in the presence of the drug, at each concentration, in relation to the total number of colonies growing in its absence was calculated.

Determination of stability of antifungal-resistant phenotype. The stability of the resistant phenotypes was assessed by subculturing the resistant strains (BC014R_{FLC}, BC014R_{VRC}, and BC014R_{PSC}) in 10 ml of RPMI 1640 medium buffered to pH 7.0 with 0.165 MOPS buffer without any antifungal drug for a total of 60 days. Daily, an aliquot of 10^6 cells of the previous day's culture was inoculated into fresh medium (total volume, 10 ml), while another aliquot was taken and frozen as described. Afterwards the strains were thawed, MIC values to each of the three antifungals were redetermined.

Susceptibility testing of *C. parapsilosis* isolates. MIC values of FLC, VRC, and PSC were determined according to the M27-A3 protocol and the M27-S3 supplement of the Clinical and Laboratory Standards Institute (CLSI) (15, 16). MIC values were registered following 48 h of incubation, for all the azoles. The susceptibility breakpoints applied were those established by the CLSI (15). For FLC, the MIC for susceptibility was ≤ 8 $\mu\text{g}/\text{ml}$, the MIC for susceptible-dose dependent was 16 to 32 $\mu\text{g}/\text{ml}$, and the MIC for resistance was ≥ 64 $\mu\text{g}/\text{ml}$. For VRC, the MIC for susceptibility was ≤ 1 $\mu\text{g}/\text{ml}$, the MIC for susceptible-dose dependent was 2 $\mu\text{g}/\text{ml}$, and the MIC for resistance was ≥ 4 $\mu\text{g}/\text{ml}$. For PSC, strains inhibited by ≤ 1 $\mu\text{g}/\text{ml}$ were considered to be susceptible. The *C. parapsilosis* ATCC 22019 type strain from the American Type Culture Collection was used for quality control of antifungal susceptibility testing, as recommended (15, 16).

RNA isolation. RNA was extracted from a 50-ml volume of a *C. parapsilosis* culture that had been grown overnight in YEPD broth (without antifungal drugs) at 150 rpm and 35°C to an optical density at 600 nm of approximately 1.0. RNA

was extracted using a RiboPure yeast kit (Ambion, Austin, TX), according to the manufacturer's instructions. Concentration, quality, and integrity levels of RNA samples were determined with an Agilent Technologies 2100 bioanalyzer (Santa Clara, CA), according to the manufacturer's instructions. Only samples yielding 28S rRNA/18S rRNA ratios ranging from 1.6 to 2.2 and showing the absence of degradation were used in subsequent analyses.

Microarray. All the experiments were carried out using *C. parapsilosis* microarrays manufactured by Agilent Technologies. The microarray platform is described on the NCBI Gene Expression Omnibus (GEO) Database with the identifier GPL13192. The oligonucleotides were designed with eArray from Agilent Technologies using an in-house annotation of 5,834 putative open reading frames. Each gene is represented by two sets of probes, both spotted in duplicate. Probes are randomly distributed. Four copies of each array were printed on a single slide (4x44K) and hybridized individually (57).

Probe preparation and microarray hybridization. Twenty-four micrograms of total RNA was incubated with 200 pmol of anchored oligonucleotide deoxyribosylthymine (Invitrogen, Carlsbad, CA) for 10 min at 70°C. Then, first-strand buffer (1 $\times$; Invitrogen); 1.5 mM dATP, dTTP, and dGTP; 25 μM dCTP; 10 mM dithiothreitol; 2 μl Superscript II reverse transcriptase (Invitrogen); and 37.5 μM Cy3-dCTP or 37.5 μM Cy5-dCTP (GE Healthcare Europe GmbH, Freiburg, Germany) were mixed in a final volume of 40 μl . The mixture was incubated at 42°C for 2 h. An additional 1 μl of Superscript II was added to the mixture, and incubation was continued for 1 h at 42°C. Residual RNA was degraded after RNase treatment, which consisted of treatment with 1 μl of RNase A (Qiagen, Hilden, Germany) at 50 $\mu\text{g}/\text{ml}$ and 1 μl of RNase H (Invitrogen) at 0.05 unit/ μl for 30 min at 37°C. Labeled cDNAs were purified using a QIAquick PCR purification kit (Qiagen), according to the manufacturer's instructions, and were afterwards pooled in 45 μl of hybridization buffer (supplied with the microarrays). This mixture was denatured at 95°C for 2 min, immediately cooled in ice, applied to the DNA microarray, and covered with a 24- by 60-mm coverslip. Slides were placed in a hybridization chamber (Corning, Palo Alto, CA) and incubated at 42°C for approximately 16 h. Slides were washed twice with oligonucleotide aCGH wash buffer 1, oligonucleotide aCGH wash buffer 2, and with stabilization and drying solution from Agilent. The microarray analysis of BC014R_{FLC}, BC014R_{VRC}, and BC014R_{PSC} strains was performed in distinct experiments; comparisons were made with the BC014S strain. In each experiment, four biological replicates (including two dye swaps) were prepared.

Data analysis. The microarrays were scanned with a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA) at 10- μm resolution. Data were acquired with GenePix Pro (version 5.0) software (Molecular Devices, Sunnyvale, CA), and low-quality spots were automatically flagged. In addition, saturated spots or spots without background-corrected intensities greater than 20 in the Cy3 or the Cy5 channel were flagged. In each experiment, four biological replicates were performed. In order to estimate and adjust any probe-specific dye effect, two arrays were dye swapped in each experiment. Data were analyzed using the Limma package (68) from the Bioconductor Project (<http://bioconductor.org>). Three data sets were preprocessed using Loess normalization and no background correction, as suggested by Zahurak et al. (79). Duplicate probes within each array were considered technical replicates. This assumption allows full advantage of the platform design to be taken by analyzing the within-array replicate spots using a pooled correlation method. Quality controls were performed in order to assess the good quality of the arrays and to verify the presence of any chromosomal bias expression (7) (data not shown). One of the arrays in the posaconazole experiment did not pass our quality control and it was therefore discarded as an outlier. Only probes with a fold change (FC) of greater than 2 and an adjusted *P* value of less than 0.05 were used in analyses.

GO analysis. A total of 5,091 *C. parapsilosis* orthologs of *C. albicans* genes (87.3% of the *C. albicans* genome) were extracted from the Candida Gene Order Browser (25). Gene ontology (GO) enrichment analysis was performed using the web tool GoTermFinder, available on the website of the Candida Genome Database (<http://www.candidagenome.org/cgi-bin/GO/goTermFinder>).

Clustering analysis. The three data sets were preprocessed using the Limma package from the Bioconductor project (<http://bioconductor.org>). Loess normalization was used to normalize the ratio of the intensities between the two channels for each array separately (within-array normalization), followed by quantile normalization to normalize the ratio of the intensities to be compared across arrays. Hierarchical clustering of the normalized data sets was performed using Euclidean distance and complete linkage (21). Clustering approaches to identify gene expression patterns from DNA microarray data were performed (21).

cDNA synthesis and RT-qPCR. First-strand cDNA was synthesized from 100 ng of total RNA in a 20- μl reaction volume using a Superscript III reverse transcriptase kit (Invitrogen) according to the manufacturer's instructions. For

TABLE 1. Sequences of primers used in RT-qPCR

Primer name	Primer sequence
ALD5-F	5'-ACTTACAAACAACCAACGGG-3'
ALD5-R	5'-ATCTTCCTCCAATGCTTCA-3'
ERG6-F	5'-GAAACAAAGAGATTGAGCGACT-3'
ERG6-R	5'-GAAAGAAGAACCCAGCC-3'
ERG11-F	5'-GGTTTACTTGTGTTTGTCTCT-3'
ERG11-R	5'-GTCCATAAGATACGGCTGAAC-3'
GRP2-F	5'-GTTGTATTCATCAGTGGAGCC-3'
GRP2-R	5'-GACCAGCAGACTTGAGCA-3'
MDR1-F	5'-TTCTGTGATAGTTTTGGTGGTAG-3'
MDR1-R	5'-TGAACCTGGAGTGAATCTTGT-3'
MRR1-F	5'-ACAATGGTCTGAGCAATGAA-3'
MRR1-R	5'-GGCAATACTGGTATGGA-3'
NDT80-F	5'-CTCTGGTGGTGCTAATGATG-3'
NDT80-R	5'-ATGTTGTTGAGGGAGTGAAGA-3'
RPL82-F	5'-TTGCTTCTGCTGATGAGG-3'
RPL82-R	5'-CCATAATACCACACCCC-3'
SOD4-F	5'-TCAGGAAGACACGGTAAGATT-3'
SOD4-R	5'-GCAAAGTGGATAACAATGGA-3'
TUB4-F	5'-TGTATTCCACAATGATGCCT-3'
TUB4-R	5'-TGCCTTGAAACGAAGTAG-3'
UPC2-F	5'-ATTGGAGTGTGGGTATCTTCAT-3'
UPC2-R	5'-CCTTCGCCTTCTTCAGTTC-3'

each real-time quantitative PCR (RT-qPCR), five replicates for each strain were integrated. The PCR regimen used included a 1-min hot start at 95°C, followed by a 35-cycle program composed of a 15-s denaturation step at 95°C, a 30-s annealing step at 60°C, and a 30-s extension step at 60°C, followed by a 5-min final extension step at 60°C. PCRs were performed using PerfeCTa SYBR green Fast Mix (Quanta Biosciences) on a Realplex Mastercycler instrument (Eppendorf, Madrid, Spain). The oligonucleotides used were designed using the Oligo Explorer program and are listed in Table 1. The signal obtained for each gene was normalized with the *TUB4* signal.

Sequencing and data analysis of *MRR1*, *UPC2*, and *NDT80* genes. Specific primers (Table 2) were used for PCR amplification of *MRR1* (for BC014S, BC014R_{FLC}, and BC014R_{VRC} strains), *UPC2* (for BC014S and BC014R_{PSC} strains), and *NDT80* (for BC014S and BC014R_{PSC} strains) genes. Genomic DNA was extracted as described by Hoffman and Winston (30). PCRs were performed using Phusion High-Fidelity PCR master mix with HF buffer (New England Biolabs, Ipswich, MA) on a Realplex Mastercycler instrument using specific amplification conditions defined for each gene: denaturation step of 2 min at 94°C, followed by 30 cycles of 30 s at 94°C; 30 s at 60°C for *MRR1*, 58°C for *UPC2* or 62°C for *NDT80*, and 50 s at 72°C; and a final extension step of 7 min at 72°C.

PCR products were treated with ExoSAP-IT (USB Corporation, Cleveland, OH) and used as template for the sequencing reactions, performed with a BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Carlsbad, CA). DNA products were purified with Sephadex G-50 Fine (GE Healthcare, Buckinghamshire, United Kingdom) and sequenced in an ABI Prism 3130 genetic analyzer (Applied Biosystems). Results were analyzed with Sequencing Analysis (version 5.2) software from Applied Biosystems.

The coding sequences of the BC014S *MRR1*, *UPC2*, and *NDT80* genes were aligned with those obtained for the resistant strains (BC014R_{FLC}, BC014R_{VRC}, and BC014R_{PSC}) using MUSCLE software (24). Alignments were analyzed in BioEdit software (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>).

Microarray data accession number. Raw microarray data and the description of the array have been deposited in the Gene Expression Omnibus Database

TABLE 2. Sequences of primers used in genes sequencing

Primer name	Primer sequence
MRR1-F	5'-CCCTTTCTCCGCAGATTTC-3'
MRR1-R	5'-CGTTGTAAAGATGGCGTGGT-3'
NDT80-F	5'-TCTCGTGCCGATATCTTTG-3'
NDT80-R	5'-TCTCGCATACAGTCTCTC-3'
UPC2-F	5'-TTTGCCGGTAAACCATCC-3'
UPC2-R	5'-TTTCCTCCACCCCTATTGTAG-3'

TABLE 3. MIC values and susceptibility phenotypes of *C. parapsilosis* strains after incubation with FLC, VRC, and PSC

Strain ^a	MIC (μg/ml)/phenotype		
	Fluconazole	Voriconazole	Posaconazole
BC014S	1/S	0.03/S	0.03/S
BC014R _{FLC}	128/R	4/R	0.5/S
BC014R _{VRC}	128/R	4/R	0.5/S
BC014R _{PSC}	128/R	16/R	32/R

^a Susceptible (S) and resistant (R) *C. parapsilosis* strains were obtained after incubation of S strain with 16 μg/ml of fluconazole (BC014R_{FLC}), 2 μg/ml of voriconazole (BC014R_{VRC}), or 1 μg/ml of posaconazole (BC014R_{PSC}) for 60 days.

under accession number GSE27409 (superseries). The link is <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE27409>.

RESULTS AND DISCUSSION

Resistant strains. Three *C. parapsilosis* resistant strains (BC014R_{FLC}, BC014R_{VRC}, and BC014R_{PSC}) were generated *in vitro* as previously described. Population analysis clearly demonstrated the homogeneous nature of the population for all strains (data not shown), excluding the possibility that a mixture of susceptible and resistant clones is present in the final cultures. The MIC values of the three tested antifungals for the parent strain as well as for the corresponding drug-resistant derivative strains are shown in Table 3. Strains obtained after exposure to either fluconazole (BC014R_{FLC}) or voriconazole (BC014R_{VRC}) acquired resistance to both drugs, but not to posaconazole, although the posaconazole MIC value increased 4 dilutions. In addition, the strain obtained after exposure to posaconazole (BC014R_{PSC}) developed resistance to all three azoles, with high MIC values.

All three induced resistant strains maintained a stable resistance pattern after they were subcultured in the absence of antifungal drugs for 60 days. No decrease greater than 1 dilution was found in MIC values (data not shown).

Transcriptional profiling. Microarray analysis identified differential expression ($FC > 2$, $P < 0.05$) of 1,128 (595 upregulated and 533 downregulated), 210 (138 upregulated and 72 downregulated), and 598 (341 upregulated and 257 downregulated) genes in BC014R_{FLC}, BC014R_{VRC}, and BC014R_{PSC} strains, respectively (Fig. 1; all data are available in Tables S1 to S3 in the supplemental material). Alterations in expression levels of a large number of genes have also been reported in azole-resistant *C. albicans* strains (10, 56, 77). Thus, it is clear that the presence of azoles induces a reformulation of the transcriptome of *Candida* species, possibly as a mechanism of adapting to stress response.

The change in expression of specific genes was confirmed by RT-qPCR and is discussed below.

Gene ontology analysis. GO analysis was used to identify similarities and differences between the treatments. Azoles impaired the expression of genes involved in multiple cellular processes, resulting in a pleiotropic effect upon gene expression. The large number of genes affected makes it difficult to identify overrepresented categories. However, in the BC014R_{FLC} strain, up to 13% of the upregulated genes were associated with RNA metabolic processes and ribosome bio-

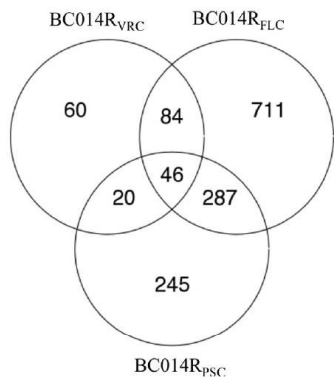


FIG. 1. Distribution of genes with differential expression in BC014R_{FLC}, BC014R_{VRC}, and BC014R_{PSC} strains.

genesis and 10% were associated with translation (Fig. 2; see Table S1 in the supplemental material). In BC014R_{VRC}, the GO term associated with response to chemical stimulus is overrepresented, whereas in the BC014R_{PSC} strain, GO terms associated with sterol biosynthesis, response to chemical synthesis, and response to drugs are overrepresented (Table 4; see Table S4 in the supplemental material).

Gene expression profile of BC014R_{FLC} and BC014R_{VRC} strains. Because BC014R_{FLC} and BC014R_{VRC} strains displayed similar resistance profiles, the genes that were differentially regulated in both were identified (130 genes, representing 62% of total genes in BC014R_{VRC}). Figure 3 shows that these genes are distributed into 5 clusters distinguished by

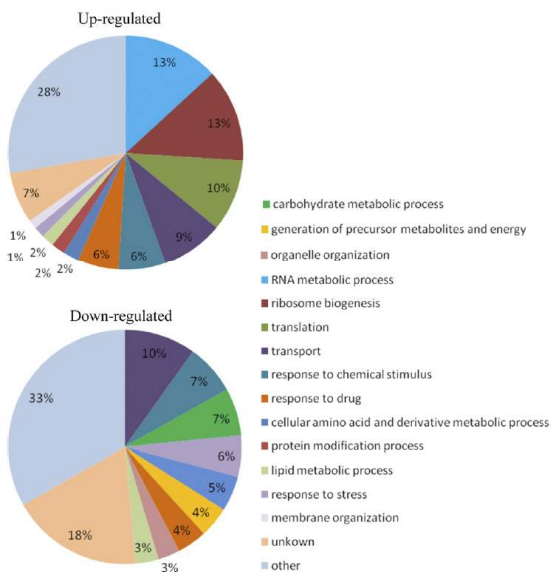


FIG. 2. Selected process GO terms overrepresented in BC014R_{FLC} strain. The full list is available in Table S1 in the supplemental material.

TABLE 4. GO terms overrepresented in BC014R_{VRC} and BC014R_{PSC} strains

Cluster frequency ^a	Background frequency ^b	Corrected <i>P</i> value	GO term
BC014R _{VRC} 31/155	504/5,085	0.0322	Response to chemical stimulus
BC014R _{PSC} 19/409	32/5,085	1.17E-10	Sterol biosynthesis
70/409	504/5,085	0.0014	Response to chemical stimulus
50/409	310/5,085	0.0007	Response to drugs

^a Cluster frequency is the fraction of genes that are differentially regulated with homologs identified in *C. albicans* assigned to a GO term.

^b Background frequency is the fraction of entire data set with homologs identified in *C. albicans* assigned to GO term.

the level of gene expression (all members of the clusters are listed in Table S5 in the supplemental material following the same order depicted in Fig. 3). Clusters 1, 2, and 3 contain genes with increased expression in BC014R_{FLC} and BC014R_{VRC} strains; cluster 3 showed the greatest changes in gene expression (Table 5).

Cluster 2 contains *MDR1* and *CPAG_03790* genes, both members of the major facilitator superfamily (MFS) of multidrug transporters. Expression of a third member of the MFS family (*CPAG_01932*) was also increased, albeit to a lesser extent (cluster 1; Fig. 3; Table 5). Expression of *MDR1* is upregulated by 19.43- and 40.22-fold (confirmed by RT-qPCR; Fig. 4) in BC014R_{FLC} and BC014R_{VRC} strains, whereas its expression in the BC014R_{PSC} strain remained unchanged. Overexpression of *MDR1* is also associated with resistance to fluconazole in clinical *C. albicans* isolates and with reduced susceptibility to voriconazole (41, 72). Overexpression of *MDR* genes renders yeast cells less susceptible to voriconazole (72).

In *C. albicans*, expression of *MDR1* is regulated by the transcription factors *MRR1*, *CAP1*, and *MCMI* (2, 41, 55). Mrr1p appears to play a central role in the development of drug resistance in *C. albicans*. Using a genome-wide gene expression analysis approach, Morschhauser et al. (41) identified mutations in the *MRR1* gene in drug-resistant clinical isolates of *C. albicans* which resulted in upregulation and were associated with a coordinate upregulation of *MDR1* (22, 65). Interestingly, expression of *MRR1* is also upregulated in *C. parapsilosis* BC014R_{FLC} and BC014R_{VRC} strains (cluster 1; Fig. 3 and Table 5; the result was confirmed by RT-qPCR [Fig. 4]). We therefore determined the sequence of the *MRR1* gene in BC014R_{FLC} and BC014R_{VRC}. A single nucleotide exchange was detected in both, compared with the *MRR1* sequence of the susceptible strain (BC014S). A substitution of G1747A in the BC014R_{FLC} strain results in an amino acid exchange, G583R (glycine to arginine). In the BC014R_{VRC} strain, a different mutation, A2619C, which results in the replacement of a lysine by an asparagine (K873N) was found. In *C. albicans*, several mutations in the *MRR1* gene associated with fluconazole-resistant clinical isolates have been described (22, 41); thus, it is likely that mutations described herein may also play a key role in *MDR1* overexpression in *C. parapsilosis*. However, this hypothesis remains to be tested experimentally.

Overexpression of *MDR* in *C. albicans* is also correlated with increased expression of aldo-keto reductases and other genes

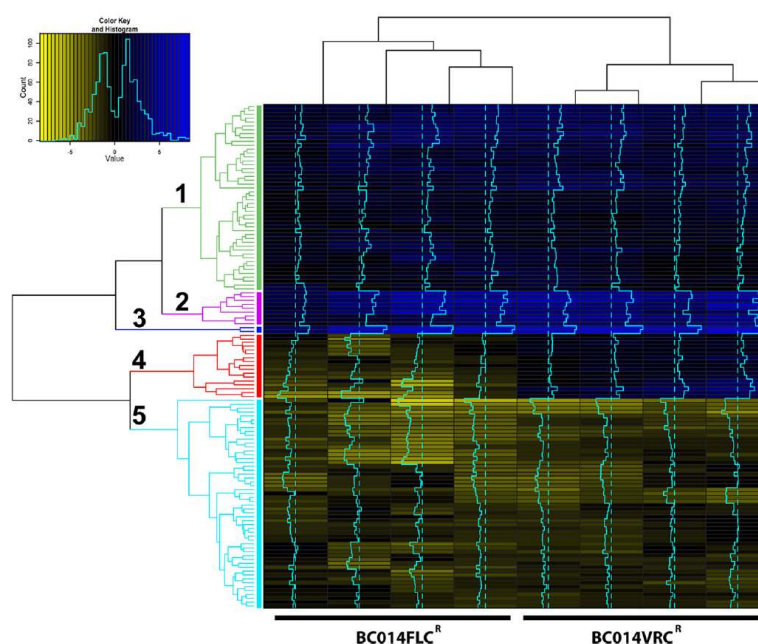


FIG. 3. Hierarchical cluster analysis of gene expression changes in BC014R_{FLC} and BC014R_{VRC}. Cluster analysis of 130 genes that are differentially expressed in both BC014R_{FLC} and BC014R_{VRC} strains. Each cluster is represented by a different number, and the members of each cluster are listed in Table S5 in the supplemental material. The genes identified to be *C. albicans* orthologs are shown. Genes with decreased and increased expression are marked in yellow and blue, respectively. Genes in black were found with unchanged expression. The histogram in the upper left shows the distribution of the differentially expressed genes on the basis of their log FC levels. The x axis represents the log FC levels in the heat map. The y axis (labeled count) shows how many genes have a specific value or differential expression level.

associated with the oxidative stress response, which may protect cells from damage caused by toxic molecules produced in the presence of azoles and also contribute to antifungal drug resistance (35). The expression of these genes is also regulated by Mrr1p transcription factor in *C. albicans* (41). We observed a similar response in *C. parapsilosis*. Expression of several putative aldo-keto reductases and NADPH oxidoreductases (orthologs of *orf19.3442* and *OYE32*) is increased in both BC014R_{FLC} and BC014R_{VRC} (clusters 1 and 2; Fig. 3; Table 5). Cluster 3 contains the stress response gene *GRP2*, which is upregulated 107.63- and 207.94-fold in BC014R_{FLC} and BC014R_{VRC} strains, respectively; these findings were confirmed by RT-qPCR (Fig. 4). The second member of cluster 3, the *CPAG_02538* gene, has a high sequence identity to *GRP2*, and therefore, it is likely to encode a protein in the same family. Upregulation of *GRP2* in *C. albicans* is correlated with increased expression of *MDR1* (41) and is associated with the stepwise development of resistance to azoles (31, 56). However, *GRP2* expression not only is triggered by azoles but also is regulated positively by certain compounds like fluphenazine, benomyl (33), and farnesol (58).

Comparing the expression patterns of *C. parapsilosis* (BC014R_{FLC} and BC014R_{VRC} strains) with *C. albicans* azole-resistant isolates (22, 41) reveals that the alterations in the transcriptome underlying azole resistance are similar in both species. We therefore suggest that resistance to fluconazole and voriconazole in *C. parapsilosis*, in particular, in

BC014R_{FLC} and BC014R_{VRC} strains, is conferred by the increased expression of the *MRR1* transcription factor, which results in a concomitant overexpression of *MDR1* and possibly other members of the MFS family and of aldo-keto reductases. As described in *C. albicans* (22, 41), *C. parapsilosis* gain-of-function mutations in *MRR1* result in overexpression.

Mutations in *MRR1* are unlikely to cause resistance to posaconazole, which is a poor substrate for Mdr1p (12, 36). The decreased susceptibility to posaconazole observed in BC014R_{FLC} and BC014R_{VRC} strains is likely to involve another as yet unidentified mechanism. We did observe upregulation of the transcription factor *NDT80* in BC014R_{FLC}, which may result in overexpression of the *CDR1* drug efflux pump; however, this was not verified. In *C. albicans*, for example, Ndt80p modulates azole tolerance by controlling the expression of the gene *CDR1* (66).

Genes in cluster 4 are differentially regulated in BC014R_{FLC} and BC014R_{VRC} strains and are unlikely to play a role in azole resistance. Genes in cluster 5 are downregulated in both isolates. These include several genes linked to ergosterol synthesis (Fig. 5). The expression of *ERG1* and *ERG11* is reduced in BC014R_{FLC} (2.60- and 2.69-fold; see Table S1 in the supplemental material) and BC014R_{VRC} (2.28- and 2.01-fold; see Table S2 in the supplemental material). Additionally, expression of *ERG3* and *ERG2* is reduced in BC014R_{FLC} (4.86- and 2.01-fold; see Table S1 in the supplemental material). The levels of expression of the *ERG11* gene for both strains were

TABLE 5. Genes with increased expression in BC014R_{FLC} and BC014R_{VRC} from Fig. 3

CPAG identifier ^a	<i>C. albicans</i> homolog ^b	Gene name ^c	Description ^c	Avg FC in gene expression		
				BC014R _{FLC}	BC014R _{VRC}	
Cluster 1						
CPAG_00168	orf19.7306	MRR1	Aldo-keto reductase family	5.62	8.51	
CPAG_00971	orf19.2446		Uncharacterized	5.78	7.73	
CPAG_02192	orf19.6000*		Multidrug transporter of ABC superfamily	5.94	9.58	
cpar447	orf19.6943*		Uncharacterized	4.69	7.11	
CPAG_02875	orf19.7372		Regulator of <i>MDR1</i> transcription	5.24	5.70	
CPAG_01174	orf19.345			9.19	9.00	
CPAG_04671				9.71	6.82	
CPAG_04730	orf19.6586		Uncharacterized	6.77	10.93	
CPAG_02752	orf19.5517*		Alcohol dehydrogenases	6.82	8.57	
CPAG_01932	orf19.5604*		Member of the MDR family of major facilitator transporter superfamily	7.41	5.06	
CPAG_01173	orf19.5860*	ALD5	Uncharacterized	6.54	4.63	
CPAG_01317	orf19.5806		Aldehyde dehydrogenase	3.12	4.72	
CPAG_03039	orf19.6348		Not essential for viability	3.16	4.50	
CPAG_03040	orf19.6349		Uncharacterized	3.63	3.92	
CPAG_00536	orf19.909		Putative transcription factor	3.34	4.66	
CPAG_01908	orf19.4907		Uncharacterized	3.51	3.68	
CPAG_03665	orf19.1027		Phosphatidylinositol transfer protein	5.17	3.68	
CPAG_04263	orf19.7235		Uncharacterized	3.92	3.76	
CPAG_04761	orf19.7166		Uncharacterized	2.97	6.77	
CPAG_03215	orf19.4609		Uncharacterized	3.48	5.13	
CPAG_05176	orf19.3131	OYE32	NAD(P)H oxidoreductase	2.69	8.75	
CPAG_03284	orf19.4886	LAP4	Uncharacterized	3.76	7.46	
CPAG_02183	orf19.6671		Similar to aminopeptidase I	2.55	3.12	
CPAG_00234	orf19.3661		Uncharacterized	2.60	2.89	
CPAG_00905	orf19.3045		Uncharacterized	2.68	2.55	
CPAG_03631	orf19.6838		Uncharacterized	2.25	3.14	
CPAG_00668	orf19.2726		Uncharacterized	2.13	2.62	
CPAG_02710	orf19.3442		Uncharacterized	1.90	2.91	
CPAG_03888	orf19.1663		Alpha-1,2-mannosyltransferase	2.44	2.16	
CPAG_03148	orf19.4592		UDP-glucose:ceramide glucosyltransferase	2.25	1.99	
CPAG_02044	orf19.1139		Uncharacterized	2.10	2.41	
CPAG_00123	orf19.5611	HSX11	Uncharacterized	1.99	2.17	
CPAG_00563				1.93	2.36	
CPAG_04323	orf19.6594		Secreted phospholipase B	3.89	2.53	
CPAG_05296	orf19.1240		Uncharacterized	2.83	2.66	
cpar744	orf19.7372*		Zn finger transcription factor, related to Mrr1 (partial)	3.46	2.00	
CPAG_02639	orf19.1839		Uncharacterized	5.13	2.16	
CPAG_02927	orf19.4393		RPA190			
CPAG_03981	orf19.4688		CIT1	Citrate synthase	3.16	2.68
CPAG_05645	orf19.4377		DAG7	Uncharacterized	3.56	2.85
CPAG_04458	orf19.2829		KRE1	Predicted GPI ^d anchor, role in 1,6-beta-D-glucan biosynthesis	3.76	2.17
CPAG_03615	orf19.756	SAP7	Uncharacterized	2.79	2.16	
CPAG_00669	orf19.2724		Secreted aspartyl proteinase	2.55	2.16	
CPAG_00730	orf19.655*		Uncharacterized	2.10	2.26	
CPAG_02152	orf19.932		High-affinity phosphate transporters	2.51	2.17	
CPAG_04056	orf19.1430		Uncharacterized	2.55	3.43	
CPAG_04294	orf19.4765		Uncharacterized	1.88	3.39	
CPAG_03868	orf19.5383		PAG6	Putative GPI-anchored cell wall protein	2.25	2.07
			PMA1	Plasma membrane H ⁺ -ATPase; fluconazole induced	2.19	2.07
Cluster 2						
CPAG_02122	orf19.3544		MDR1	Uncharacterized	32.22	35.02
CPAG_03963	orf19.5604	Member of the MDR family of major facilitator transporter superfamily		19.43	40.22	
CPAG_00506	orf19.5517	Alcohol dehydrogenases		22.63	29.45	
CPAG_00978	orf19.320	Uncharacterized		18.38	24.42	
CPAG_00478	orf19.2812*	Member of orf19.308 family		11.71	21.86	
CPAG_03790	orf19.5604*	Member of the MDR family of major facilitator transporter superfamily		21.70	9.58	
CPAG_03994	orf19.2693*	GST2/URE2 family		6.19	30.91	
CPAG_03636	orf19.771	LPG20		Aldo-keto reductase family	6.50	22.63
CPAG_04322	orf19.6594	Secreted phospholipase B		3.92	15.89	
Cluster 3						
CPAG_02182	orf19.4309	GRP2	Similar to <i>S. cerevisiae</i> Gre2p (methylglyoxal reductase)	107.63	207.94	
CPAG_02538	orf19.4309*		Similar to <i>S. cerevisiae</i> Gre2p (methylglyoxal reductase)	71.51	148.06	

^a CPAG identifier from genome annotation of *C. parapsilosis* (11). Where no CPAG gene has been annotated, the cpar annotation is used (see <http://cgob.ucd.ie>).

Genes are listed in the order shown in Fig. 3.

^b *C. albicans* orthologs are identified where possible from <http://cgob.ucd.ie>. *, genes are homologs but not orthologs.

^c *C. albicans* gene names and descriptions are taken from the Candida Genome Database.

^d GPI, glycosylphosphatidylinositol.

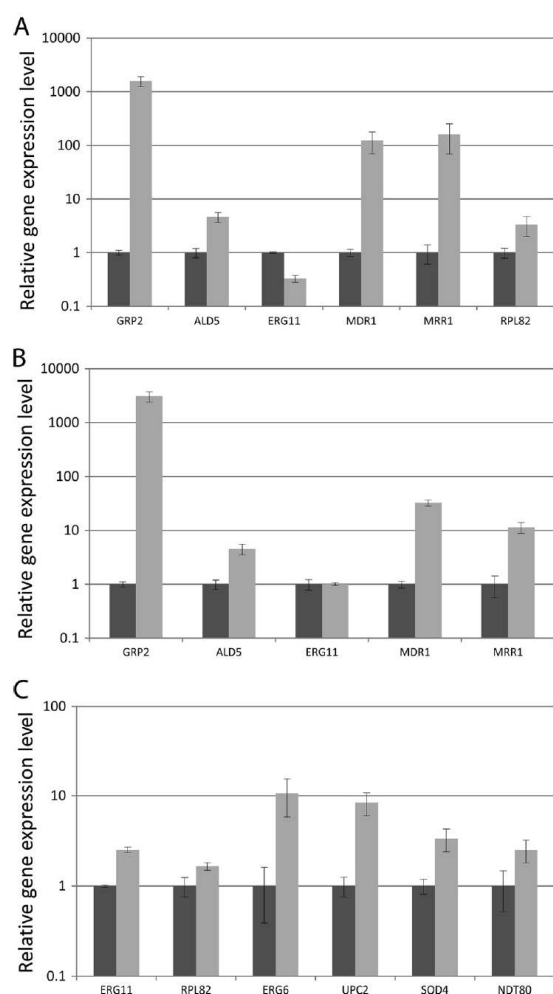


FIG. 4. RT-qPCR analysis of genes whose expression was found to be altered in microarray analysis and that have been implicated in antifungal resistance (*ERG5*, *ERG11*, *MDR1*, *MRR1*, *NTD80*), stress response (*GRP2*), and metabolism (*ALD5*, *RPL82*). Black bars, susceptible strain BC014S; gray bars, BC014R_{FLC} (A), BC014R_{VRC} (B), and BC014R_{PSC} (C) strains. In panels A and B, y-axis scales are logarithmic due to the large variance in gene expression levels. The expression level shown for each gene represents the variation in gene expression relative to the susceptible strain and is expressed as the average with standard deviation of five independent experiments. Each mean value was normalized to the amplification efficiency and to the amount of *TUB4* (endogenous reference gene). All genes displayed levels of expression similar to the microarray results.

confirmed by RT-qPCR (Fig. 4). Since azoles target the cytochrome P450 sterol 14 α -demethylase enzyme, encoded by the *ERG11* gene, the reduction in ergosterol metabolism is likely to be a direct consequence of fungal cell exposure to azoles during induction of resistance *in vitro*. The decreased expression of sterol genes that we observe in BC014R_{FLC} and BC014R_{VRC} is unlikely to be related to the azole resistance

phenotypes; in contrast, we would expect resistant isolates to demonstrate increased expression of sterol synthesis genes. However, we have not directly tested the role of the ergosterol pathway in these strains.

Gene expression profile of BC014R_{PSC} strain. Gene expression of the BC014R_{PSC} strain was substantially different from that of the other two azole-resistant isolates analyzed in this study. Although 46 of the differentially expressed genes were shared with the other two resistant strains (BC014R_{FLC} and BC014R_{VRC}), none of these have a known role in conferring drug resistance (Fig. 1).

In contrast, the BC014R_{PSC} strain displayed an overexpression of 13 genes directly linked to ergosterol biosynthesis (Table 6; Fig. 5). Expression of the *ERG6* (5.24-fold) and *ERG11* (2.89-fold) genes was confirmed by RT-qPCR (Fig. 4). This result strongly indicates that an increase in ergosterol content is the most probable cellular mechanism conferring a pattern of reduced susceptibility to all azoles in this strain. In *C. albicans*, exposure to ketoconazole, an azole antifungal, also increases expression of genes involved in lipid, fatty acid, and sterol metabolism, including *ERG11* (37).

We also observed increased expression of the transcription factors *NDT80* and *UPC2* in BC014R_{PSC} (Table 6). Ndt80p was recently identified in *C. albicans* (66) as one of the transcription factors that binds to the promoters of ergosterol biosynthesis genes, including the azole target *ERG11* gene. Like *NDT80*, the zinc cluster transcriptional factor *UPC2* also mediates the upregulation of ergosterol biosynthesis genes in *C. albicans* (23), and gain-of-function mutations result in overexpression of ergosterol biosynthesis genes (27). Using genome-wide gene expression profiling, Dunkel et al. (22) found that *UPC2* and other genes involved in ergosterol biosynthesis are coordinately upregulated with *ERG11* in a fluconazole-resistant, clinical isolate compared with a matched susceptible isolate from the same patient. Expression of the *UPC2* gene is upregulated (1.93-fold; confirmed by RT-qPCR [Fig. 4]) in *C. parapsilosis* BC014R_{PSC}. We therefore propose that, similar to what happens with *C. albicans*, resistance to azoles in *C. parapsilosis* can be partly conferred via induction of *NDT80* or *UPC2*, resulting in increased ergosterol production. To determine whether the overexpression of the transcription factors *NDT80* and *UPC2* is associated with point mutations, the genes were sequenced from both BC014R_{POS} and BC014S strains. No difference in the nucleotide sequence was detected between the susceptible and BC014R_{PSC} strains (data not shown).

Expression of the *SOD4* gene was also upregulated in the BC014R_{PSC} strain (2.68-fold; confirmed by RT-qPCR [Fig. 4]). Superoxide dismutases are enzymes that convert superoxide radicals into less damaging hydrogen peroxide and are required for the protection against oxidative stress and the establishment of full virulence (32).

Antifungal transporter genes. High-level azole resistance in clinical isolates of *C. albicans* and *C. glabrata* is often associated with overexpression of plasma membrane efflux pumps (40, 49, 71). There are two main families of efflux proteins: the ATP-binding cassette (ABC) pumps and the MFS transporters, described above (12). Expression of the ABC transporters *CDR1* and *CDR2* is increased in some azole-resistant isolates of *C. albicans* (18). *CDR* gene expression is regulated by the

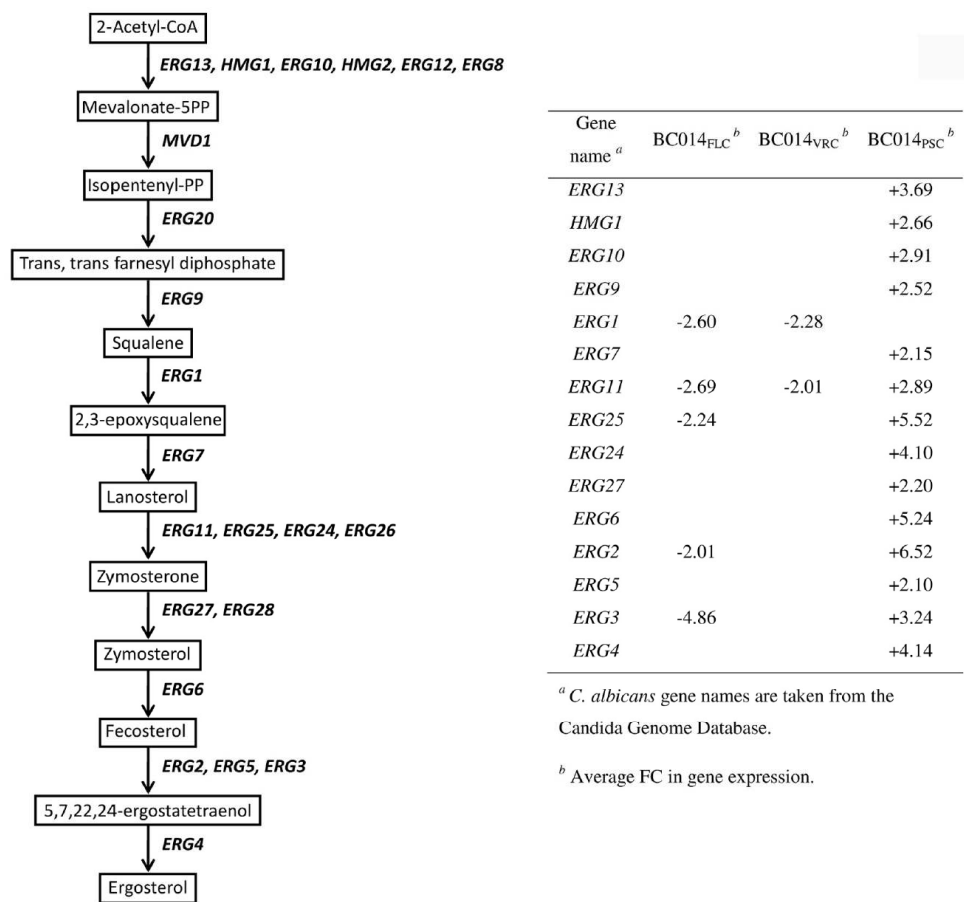


FIG. 5. Simplified ergosterol metabolic pathway, as described for *S. cerevisiae* (<http://www.yeastgenome.org/>). Genes upregulated or down-regulated in BC014_{FLC}, BC014_{VR}, and BC014_{PSC} strains are indicated in the table. Acetyl-CoA, acetyl coenzyme A.

Tac1p transcription factor, and its induction occurs independently from the MFS expression levels (18, 80).

The *C. parapsilosis* genome encodes at least 14 members of the ABC transporter family (11). The expression of one these transporters, *CPAG_0151*, is upregulated (2.30-fold) in the BC014_{PSC} strain (Table 6). This gene is a syntenic homolog of *C. albicans CDR3* (25). Expression of *CPAG_02192*, another ABC transporter, is upregulated in both BC014_{FLC} and BC014_{VR} strains (Table 5). The *C. albicans* ABC transporters most frequently involved in azole resistance are Cdr1p and Cdr2p (49, 62). Interestingly, in *C. parapsilosis* the expression of the *CDR1* ortholog is not altered between susceptible and resistant strains and the *CDR2* ortholog was not identified. However, expression of *CPAG_03665*, an ortholog of *C. albicans PDR16*, is upregulated 5.17-, 3.68-, and 2.93-fold in BC014_{FLC}, BC014_{VR}, and BC014_{PSC} strains, respectively (Table 5 and Table 6). *PDR16* encodes a phosphatidylinositol transfer protein, which modifies the passive diffusion of hydrophobic drugs across the cell plasma membrane,

thereby modulating multidrug resistance in *Saccharomyces cerevisiae* (70). *PDR16* is coexpressed with *CDR1* and *CDR2* in azole-resistant clinical isolates of *C. albicans* (20).

BC014_{FLC} and BC014_{VR} strains displayed decreased expression of *CPAG_03819* (Fig. 3; see Table S5 in the supplemental material). This gene is an ortholog of *C. albicans orf19.3902*, and its reduced expression is correlated with overexpression of *CDR1* and *CDR2* in *C. albicans* azole-resistant isolates (33). It is therefore possible that azole resistance in *C. parapsilosis* is also associated with increased expression of ABC transporters. Nevertheless, our results suggest that they play a minor role; the expression of *CPAG_02274 (SNQ2)* was down-regulated in BC014_{FLC} and BC014_{VR} strains (Fig. 3; see Table S5 in the supplemental material), while the expression of the other ABC transporter family members remained unchanged.

Copper transport- and iron mobilization-related genes. The expression levels of several genes involved in copper and iron transportation were found to be altered in all three azole-

TABLE 6. Genes with increased expression in BC014R_{PSC}

CPAG identifier ^a	<i>C. albicans</i> homolog ^b	Gene name ^c	Description ^c	Avg FC in gene expression in BC014R _{PSC}
CPAG_00389	orf19.7077*		Protein with similarity to ferric reductase	10.27
CPAG_00124			Uncharacterized	6.68
CPAG_01721	orf19.3829	PHR1	Glycosidase of cell surface	5.78
CPAG_01381	orf19.5753*	HGT10	Glycerol permease involved in glycerol uptake	5.94
CPAG_05645	orf19.4377	KRE1	Predicted GPI ^d anchor, role in 1,6-beta-D-glucan biosynthesis	3.76
CPAG_01409	orf19.1031	HMG1	HMG-CoA ^e reductase enzyme of sterol pathway	2.66
CPAG_01467	orf19.2311	RPL82	Predicted ribosomal protein	2.10
CPAG_02523	orf19.4336	RPS5	Predicted ribosomal protein	2.87
CPAG_03665	orf19.1027	PDR16	Phosphatidylinositol transfer protein	2.93
CPAG_01511	orf19.1313	CDR3	Transporter of the Pdrp/Cdrp family of the ABC superfamily	2.99
CPAG_02597	orf19.2501	FLC1	Protein involved in heme uptake	2.28
CPAG_05474	orf19.1813	FLC2	Protein involved in heme uptake	2.03
CPAG_04555	orf19.2062	SOD4	Copper- and zinc- containing superoxide dismutase	2.68
CPAG_03048	orf19.6026*	ERG2	C-8 sterol isomerase; enzyme of ergosterol biosynthesis pathway	6.50
CPAG_05078	orf19.1631	ERG6	Delta(24)-sterol C-methyltransferase; enzyme of ergosterol biosynthesis pathway	5.24
CPAG_04483	orf19.3732	ERG25	Putative C-4 methyl sterol oxidase; enzyme of ergosterol biosynthesis pathway	5.50
CPAG_03871	orf19.5379	ERG4	Protein described as similar to sterol C-24 reductase; enzyme of ergosterol biosynthesis pathway	4.14
CPAG_05162	orf19.1598	ERG24	C-14 sterol reductase; enzyme of ergosterol biosynthesis pathway	4.08
CPAG_00173	orf19.7312*	ERG13	Protein similar to <i>S. cerevisiae</i> Erg13p; enzyme of ergosterol biosynthesis pathway	3.68
CPAG_04530	orf19.1591	ERG10	Protein similar to acetyl-CoA acetyltransferase; enzyme of ergosterol biosynthesis pathway	2.91
CPAG_03624	orf19.767	ERG3	C-5 sterol desaturase; enzyme of ergosterol biosynthesis pathway	3.25
CPAG_03310	orf19.922	ERG11	Lanosterol 14-alpha-demethylase; enzyme of ergosterol biosynthesis pathway	2.89
CPAG_05249	orf19.3616	ERG9	Putative farnesyl diphosphate farnesyltransferase; enzyme of ergosterol biosynthesis pathway	2.53
CPAG_03443	orf19.5178	ERG5	Putative C-22 sterol desaturase; enzyme of ergosterol biosynthesis pathway	2.08
CPAG_02967	orf19.3240	ERG27	3-Keto sterol reductase of ergosterol biosynthesis; enzyme of ergosterol biosynthesis	2.20
CPAG_03183	orf19.4631	ERG251	Uncharacterized	2.14
CPAG_04608	orf19.2119	NDT80	Activator of <i>CDR1</i> induction by antifungal drugs and transcriptional regulator of ergosterol biosynthesis genes	2.53
CPAG_01906	orf19.391	UPC2	Transcriptional regulator of ergosterol biosynthesis genes and sterol uptake	1.93

^a CPAG identifier from genome annotation of *C. parapsilosis* (11). Where no CPAG gene has been annotated, the cpar annotation is used (see <http://cgob.ucd.ie>).

^b *C. albicans* orthologs are identified where possible from <http://cgob.ucd.ie>. *, genes are homologs but not orthologs.

^c *C. albicans* gene names and descriptions are taken from the Candida Genome Database.

^d GPI, glycosylphosphatidylinositol.

^e HMG-CoA, 3-hydroxy-3-methyl-glutaryl coenzyme A.

resistant *C. parapsilosis* strains: *CTR1* (copper transporter; 4.53-fold) and *FRE10* (ferric reductase; 2.08-fold) are upregulated, while *CRP1* (copper transporter; 30.70-fold) is downregulated in the BC014R_{FLC} strain; *FTR1* (iron membrane permease; 2.25-fold) is downregulated in the BC014R_{VRC} strain; *CTR1* (6.32-fold) is upregulated, while *CRP1* (28.25-fold) is downregulated in the BC014R_{PSC} strain (see Tables S1 to S3 in the supplemental material). Due to this variability among resistant strains, it is difficult to effectively evaluate the impact of gene expression changes on the overall transport of metals. However, it is clear that azoles interfere with transport of iron and copper and, consequently, their metabolism by *C. parapsilosis*. Similar phenotypes have been described in drug-resistant isolates of *C. albicans* (10, 77). Copper is essential for enzymes involved in a multitude of biological processes, such as signaling of transcription, protein trafficking machinery, oxidative phosphorylation, iron mobilization, neuropeptide mat-

uration, and ultimately, normal cell development (53, 54). The acquisition of iron is essential for growth, survival, and virulence of *C. albicans* (52). Moreover, recent studies suggest that iron depletion increases membrane fluidity, which in turn leads to enhanced passive diffusion of drugs, resulting in increased drug susceptibility (50).

Expression of two genes, *FLC1* and *FLC2*, involved in heme uptake (51) is increased 2.28- and 2.03-fold, respectively, in the BC014R_{PSC} strain (Table 6). Heme is a nutritional iron source required for the activity of a variety of enzymes, including cytochrome P450 enzymes and iron-sulfur clusters. Azoles inhibit the synthesis of ergosterol by binding to the heme cofactor located in the active site of lanosterol 14 α -demethylase. Increasing heme transport may contribute to an enhancement in lanosterol 14 α -demethylase production and thus contribute to the azole-resistant phenotype of BC014R_{PSC}.

In conclusion, we used microarray analysis to investigate

global changes in gene expression occurring in azole-resistant strains of *C. parapsilosis* obtained after prolonged *in vitro* exposure to fluconazole, voriconazole, and posaconazole. Resistance to azole compounds involves a variety of mechanisms, some of which are yet poorly understood. These data shed a new insight into the molecular basis underlying azole resistance in *C. parapsilosis*. Our results strongly suggest that resistance displayed by BC014R_{FLC} and BC014R_{VRC} strains is associated with increased expression of *MDR1* and other drug efflux pump members, namely, MFS. *MDR1* overexpression is correlated with increased expression of the *MRR1* transcription factor. As reported for *C. albicans*, upregulation of *MRR1* may be caused by single gain-of-function mutations (22, 41). We identified mutations (G1747A and A2619C) in the *MRR1* coding sequence of azole-resistant *C. parapsilosis* isolates BC014R_{FLC} and BC014R_{VRC} that resulted in an amino acid exchange of G583R and K873N.

Conversely, ergosterol production seems to be the primary cause of posaconazole resistance (ergosterol biosynthesis genes were overexpressed in the BC014R_{PSC} strain), with drug efflux playing a minor role in resistance to this azole. Overexpression of *ERG* genes is correlated with increased expression of the transcription factors *UPC2* and *NDT80*.

Other resistance mechanisms apart from those already described, such as mutations in the target enzymes, might be implicated; however, they were not covered by transcriptome analysis. In fact, for all resistant strains obtained, a mutation in the target enzyme may correlate with the resistance profile observed. Azole drugs bind to the area surrounding the heme group of lanosterol 14 α -demethylase. Fluconazole and voriconazole bind only to a single domain, while posaconazole, by means of its long side chain, attaches to two binding sites in the enzyme (1, 36). Thus, a point mutation in one domain of the enzyme might explain resistance to fluconazole and voriconazole. Regarding acquisition of resistance to posaconazole, mutations in both domains of the enzyme have to occur in order to explain azole cross-resistance.

To our knowledge, our study is the first to use cDNA microarray analysis to investigate whole-genome-level gene expression in sets of azole-resistant versus azole-susceptible *C. parapsilosis* strains. Microarray analysis gives an overview of genes that are differently expressed, and important information should be extracted from that analysis and studied in more detail using other molecular tools, in order to comprehensively systematize the molecular mechanisms underlying *C. parapsilosis* drug resistance.

In summary, our results demonstrate that similar mechanisms confer resistance to azole drugs in *C. parapsilosis* and *C. albicans*.

ACKNOWLEDGMENTS

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MANUSCRIPT VII

*Manuscript

**Aerosolized hydrogen peroxide dry-mist system for hospital environmental
disinfection: assessment of *in vitro* and *in situ* efficacy**

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Abstract

Objective: In order to bring safety to patients and healthcare workers, thus improving health quality services, effective environmental decontamination strategies need to be urgently addressed. We report on the antimicrobial activity of aerosolized hydrogen peroxide in combination with silver cations dry-mist diffused by Glosair™ 400® system.

Design: We conducted *in vitro* and *in situ* studies with Glosair™ 400® system.

Setting: The *in situ* tests were conducted in a Microbiology research laboratory and in patient rooms in a Burn Unit.

Methods: Two distinct approaches were used: (1) surfaces and air decontamination (patient rooms in a Burn Unit and a research laboratory room at the Microbiology Department); (2) *in vitro* activity against suspensions of pathogenic bacteria, yeasts and moulds, including the human pathogen *M. tuberculosis*. Additionally, flow cytometry was used to confirm membrane lesion of microbial cells following exposure to the disinfectant.

Results: No surface and air contamination was detected in Burn Unit after disinfection process. The air and surface contamination levels of the research laboratory also decreased drastically despite no previous cleaning. A growth reduction of at least 6 *log* was observed *in vitro* with all tested microorganisms, the disinfectant solution being highly active even at very low concentration. Flow cytometry evidenced cell membrane lesion.

Conclusions: The hydrogen peroxide/silver cations combination exhibited an extensive microbicidal effect and hence Glosair™ 400® system may represent a valuable tool for decontamination of patient rooms, medical equipment and laboratory facilities.

Introduction

Antimicrobial resistance is a growing problem among agents of healthcare-associated infections (HCAI) and it has been attributed to past and present inadequate protocols of antimicrobial drug prescription¹. The unique nature of the intensive care unit (ICU) environment makes it a focus for the emergence and spread of multi-resistant pathogens within the hospital setting but also to the community². Critical care patients are commonly exposed to broad-spectrum antimicrobial drugs while opportunities for cross-transmission from patient-to-patient abound. In most instances, the main reservoirs of resistant microorganisms are colonized or infected patients, although the hospital environment is by itself an important piece in the HCAI puzzle. These resistant microorganisms, which co-exist with environmental and endogenous patient flora, are positively selected in result of antimicrobial pressure. Not surprisingly, resistance rates are high for most pathogens associated with HCAI, a finding which causes serious concern among physicians and health care authorities given the growing number of immunocompromised and transplanted patients and of aging population.

Rational antimicrobial use combined with microbial surveillance, improvement of hand washing compliance, use of barrier precautions such as gloving (especially if impregnated with antiseptic substances) and the adoption of isolation measures are currently guidelines followed by healthcare institutions to reduce microbial transmission. Notwithstanding, these measures are not enough when treating neutropenic or severely immunocompromised patients or patients colonized or infected with multidrug resistant (MDR) pathogens. Inadequate terminal disinfection of areas

that have been previously occupied by infected patients promotes significantly the risk for new incoming patients. Although the main route of cross-transmission is via the transiently contaminated hands of healthcare workers (HCW), such contamination result from either direct patient contact or indirectly from touching contaminated environmental surfaces. In fact, environmental surfaces have frequently been described as the source for outbreaks of nosocomial infections since most healthcare-associated pathogens are able to persist on inanimate surfaces for weeks or even months, which represent in such instances a continuous focus for transmission if no appropriate surface disinfection procedure is adopted³. Improved surface decontamination has been shown to decrease hospital contamination by methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *enterococci* (VRE), *Acinetobacter* spp., *Pseudomonas aeruginosa*, *Clostridium difficile*, and *Candida* spp.. However, conventional manual cleaning with disinfectants has been reported to allow the remaining of 10% of high-touch surfaces contaminated due to obvious difficulties to achieve the correct disinfectant concentration, its throughout application to all areas but also sufficient contact time⁴. Moreover, manual procedures are only applied to surface decontamination; meanwhile, aerosol and airborne transmission represent important routes for the dissemination and spread of nosocomial pathogens⁵, such as MRSA, *Acinetobacter* spp., *Legionella* spp., *Aspergillus* spp. and *Mycobacterium tuberculosis*. These evidences suggest that both surface as air decontamination should be addressed in order to reduce the risk of environmental transmission.

Regarding the poor performance of manual decontamination, no touch disinfection methods such as the hydrogen peroxide aerosolization technology are highly recommended and preferred to avoid HCW safety. The use of hydrogen peroxide as a disinfectant has a long history in infection control and prevention since this substance

1 was found to be effective against a wide range of microorganisms. However, the
2 application of hydrogen peroxide as vapor or aerosol to disinfect a sealed area
3 represents a novel approach. Preliminary studies suggested hydrogen peroxide
4 vapor/aerosol decontamination to be a promising technology⁶. In contrast with
5 formaldehyde fumigation or chlorine dioxide vapors, hydrogen peroxide is catalytically
6 converted to water and oxygen at the end of preprogrammed cycle, two no harmful by-
7 products to humans.
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10 The present study evaluated the efficacy of the hydrogen peroxide/silver cations dry-
11 mist system (GlosairTM 400[®], Advanced Sterilization Products) for air and surface
12 decontamination in burn patient rooms (clean area) and in a microbiology research
13 laboratory (unclean area). The contamination level was compared in both areas to
14 uncover the contribution of previous cleaning to GlosairTM 400[®] efficacy. *In vitro*
15 antimicrobial activity was also addressed, involving the exposure of pathogenic
16 microorganisms to dry-mist disinfection. Moreover, the disinfectant solution itself
17 (GlosairTM 400[®] Cartridge) was tested by determining the minimal inhibitory
18 concentration against several microorganisms; for this approach, bacterial and fungal
19 species known to inhabit environmental reservoirs and being frequently responsible for
20 nosocomial infections were chosen. Aerosolised hydrogen peroxide dry-mist technology
21 was previously described to be effective against *Clostridium difficile* spores⁷, *M.*
22 *tuberculosis*⁸ and against MDR microorganisms such as MRSA^{9,10} and *A.*
23 *baumannii*^{10,11}. Flow cytometry was used in order to clarify the disinfectant ability to
24 induce cell membrane lesion.
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Methods

Dry-mist system and disinfectant. Glosair™ 400® system (formerly Sterinis® system) is a programmable dry-mist generator which combines with the disinfectant Glosair™ 400® Cartridge, diffusing hydrogen peroxide and silver cations as small size particles (approximately 10 µm in diameter). These droplets circulate freely in the air as a dry aerosol. The Glosair™ 400® Cartridge is composed of hydrogen peroxide 5%, phosphoric acid <50 ppm, silver cations <50 ppm, gum arabica <1 ppm and bi-osmotic water 95%. The spraying times and the volume of disinfectant were used as recommended by the manufacturer. Diffusion times are calculated according to the room size in order to reach the target concentrations. After the diffusion time there is a time of exposure/contact before the room is accessible; the complete cycle does not take more than 2 hours.

Air and surface decontamination. The *in situ* trials were conducted at two distinct settings: patient rooms at the Burn Unit of Hospital S. João of Porto and one fungal research laboratory at the Microbiology Department of the Faculty of Medicine of Porto. The decontamination of the patient rooms with Glosair™ 400® system was performed whenever a patient was discharged and immediately after the routine standard disinfection procedure: i) room and equipment surfaces were disinfected with undiluted ANIOS D.D.S.H.® (Quaternary ammonia propionate; Laboratoires ANIOS, France) and ii) floor was disinfected with 0.25% diluted SURFANIOS CITRON® (N-(3-aminopropyl)-N-dodecylpropane-1,3-diamine; Laboratoires ANIOS). In the microbiology laboratory no previous cleaning was performed. No equipment was

1 removed either from patient or laboratory rooms. Each burn patient room has its own
2 laminar air flow; the ventilation systems, including the laminar air flow system were not
3
4 shut down. The Glosair™ 400® system was placed in a room corner and one single
5
6 disinfection cycle was conducted. The test rooms were closed throughout the
7
8 decontamination process and no persons were present. The air and surfaces were
9
10 sampled before and after the disinfection Glosair™ 400® system. Five hundred l of air
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12 from each patient room and 100 l from the laboratory were filtered with MAS-100 Eco
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14 air sampler (Merck, Eurolab, Switzerland), the particles being deposited on agar
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16 medium surface. In the patient rooms, the walls, inner door, window surface, floor, bed,
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18 bathroom, television and monitoring equipment (vital sign monitor and ventilator)
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20 surfaces were sampled using contact plates. In the laboratory, the floor, washbasin, one
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22 shelf, the workbench, the trash container, the refrigerator and the incubator surfaces
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24 were sampled also using contact plates. Brain-Heart agar (BH; Difco Laboratories,
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26 Sparks, MD) and Dichloran-Glycerol 18 agar (DG18; Difco Laboratories) culture
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28 medium plates were used both for air and surface sampling; DG18 medium was used to
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30 limit the confluent growth of filamentous fungi. The agar plates were incubated both at
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32 25°C and 35°C for 5 days and afterwards the number of Colony Forming Units (CFU)
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34 was evaluated. Experiments were performed in duplicate at different periods. To ensure
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36 that the residual disinfectant itself was not detrimental to microbial cell viability BH and
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38 DG18 contact plates were pressed into the surface and afterwards inoculated with the
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40 studied microorganisms. The growth in such plates was compared to the growth in
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42 control contact plates (inoculated and incubated in the same conditions but without
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44 contact with disinfectant).
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57 **Antimicrobial activity against pathogenic microorganisms.** Reference strains from
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Acinetobacter baumannii ATCC 25393, *Escherichia coli* ATCC 32218, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 2912, *Candida albicans* ATCC 90028, *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258, *Aspergillus fumigatus* ATCC 204305 and *Penicillium marneffei* ATCC 18224 and one clinical isolate of each of the above mentioned species were included on this study. McFarland 0.5 suspensions of fungi (containing approximately 10^6 cells/ml) and of bacteria (containing approximately 10^8 cells/ml) were prepared in phosphate buffered saline pH 7.4 (PBS; Sigma, St. Louis, MO); serial dilutions ranging from 10^6 cells/ml or 10^8 cells/ml to 10^1 cells/ml were prepared and 100 μ l from each dilution were spread on agar medium (BH for bacteria and yeasts and on DG18 for moulds). These plates were then placed open in the microbiology laboratory room in different positions and heights, and exposed to one single cycle of dry-mist decontamination. Afterwards, the plates were removed and incubated at 25°C and at 35°C for 5 days. CFU were quantified and compared to control plates (not exposed to decontamination with Glosair™ 400® system but incubated in similar conditions). The reduction of growth for each species was calculated. All experiments were performed in duplicate. To ensure that the proprieties of agar medium were not altered during the exposure to hydrogen peroxide/silver cations, control plates (not inoculated with microbial suspensions) were exposed to the disinfection cycle during the laboratory room decontamination and were afterwards inoculated with the studied microorganisms. Growth was assessed by CFU enumeration.

Determination of minimal inhibitory concentration. The determination of minimal inhibitory concentration (MIC) value of the disinfectant (Glosair™ 400® Cartridge) was performed according to the CLSI protocols for susceptibility testing (M100-S20¹² for bacteria, M27-A3¹³ for yeasts and M38-A2¹⁴ for moulds) regarding the ATCC strains previously described. The disinfectant was successively diluted (1:2) in Muller-Hinton

broth (Difco Laboratories), in RPMI 1640 medium (Sigma) or in PBS, according to susceptibility testing protocols; ten concentrations were tested, ranging from 5% to 0.01% of hydrogen peroxide and <50 ppm to <0.1 ppm silver cations. The MIC values were established by visual reading following 48 hours of incubation. MIC value was defined as the lowest concentration at which a visual drastical reduction in growth ($\geq 90\%$) was registered.

Activity against *Mycobacterium tuberculosis*. Three clinical isolates and ATCC 27294 type strain of *Mycobacterium tuberculosis* were cultured according to the standard procedures of the BD BACTEC MGIT 960 system (Becton Dickinson, NJ, USA) using Mycobacteria Growth Indicator Tubes (MGIT). Each set of MGIT included a positive and a negative growth control, and four decreasing concentrations of the disinfectant solution (Glosair™ 400® Cartridge): 1:70, 1:140, 1:280 and 1:560. MGIT tubes were incubated in the BACTEC 960 apparatus; growth was assessed automatically, based upon the emission of fluorescence, and compared with the positive control. All tubes were kept in the Bactec incubator at 35°C until becoming positive or until 40 days of incubation. After positive detection, *M. tuberculosis* growth was confirmed by Ziehl-Neelsen staining and by Lowenstein-Jensen culture; the same procedure was used for tubes showing growth inhibition.

Evidence of cell membrane damage. Suspensions with a final concentration of 10^6 cells/ml of *E. faecalis* or of *C. albicans* were prepared in 1 ml of disinfectant (Glosair™ 400® Cartridge). After 5 min of incubation at room temperature, microbial cells were washed and re-suspended in PBS with propidium iodide (PI) at a final concentration of 1 µg/ml. PI is a fluorescence probe that only stains cells with serious cytoplasmic membrane damage, i.e., dead cells¹⁵. About 30.000-50.000 cells were analysed in a

FACSCalibur flow cytometer (BD Biosciences, Sydney, Australia) equipped with a 15
nm argon laser; cell fluorescence was evaluated at FL3 (650 nm).

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Results

Regarding the patient rooms, few bacteria and moulds were recovered from some surfaces before the Glosair™ 400® system disinfection process (figure 1A). No yeasts were found in all surfaces sampled. No bacteria were recovered from inner door, surface window and wall. No moulds were recovered from inner door, surface window, wall and television. Notably, after disinfection with Glosair™ 400® system no growth was observed. Interestingly, in one single patient room the floor was contaminated with *Acinetobacter baumannii*, which was not recovered after the disinfection procedure with Glosair™ 400® system (data not shown). Figure 1B illustrates the CFU number of bacteria and moulds cultured from the fungal research laboratory surfaces before and after the disinfection process with Glosair™ 400® system. No yeasts were found in all surfaces sampled. The most contaminated surfaces with bacteria included the floor, trash container, workbench and shelf; moulds were mostly recovered from the incubator, workbench and shelf. In the refrigerator and incubator no microorganisms were found. The disinfection process reduced drastically, in most instances to zero, surface contamination with bacteria and moulds. In patient and laboratory rooms, similar results were obtained with BH and DG18 agar medium plates. The microbial cell viability is not affected by eventual residual disinfectant.

The air study results from the patient rooms and research fungal laboratory are detailed in figure 2. Once again, no yeasts were recovered from the air while bacteria were a common finding. Despite filtering a higher volume in the burn patient rooms, the air contamination levels before the disinfection process with Glosair™ 400® system were

lower than in the laboratory room, as might be expected, but were reduced to zero after the disinfection process. In the laboratory room, after the disinfection process, air contamination was drastically reduced. The results obtained with BH agar medium were similar to DG18 agar medium.

Regarding the *in vitro* antimicrobial efficacy against specific microorganisms, a reduction in growth of 8 *log* for bacteria and 6 *log* for fungi was observed following exposure to Glosair™ 400® system; such results were obtained with all ATCC and clinical strains. The possibility that the dry-mist could deposit into agar medium and interfere with microorganism growth was discharged since no growth inhibition was found (data not shown).

The MIC value obtained for each microorganism is detailed in table I. Although bacteria seem to be slightly less susceptible than fungi, the disinfectant, even at a very low concentration, showed a potent antimicrobial activity.

Regarding *M. tuberculosis*, the susceptibility results were determined only after 12-15 days, the time needed for detection of growth in the positive control. Only the higher dilution tested (1:560) was unable to inhibit growth. All negative tubes were afterwards double confirmed (stain and culture).

The microbicidal activity of Glosair™ 400® Cartridge was characterized by flow cytometry. Figure 3 represents the PI staining intensity of *E. faecalis* cells untreated and treated during 5 min with the disinfectant; this cell population was significantly stained, meaning to be dead due to membrane lesion induced by exposure to the disinfectant.

Similar findings were found to occur with *C. albicans* yeasts cells.

Discussion

Routine cleaning of high-touch surfaces does not completely kill microbial pathogens. Therefore, improved disinfecting methods addressing air and surface decontamination are needed. Preliminary studies suggest that hydrogen peroxide disinfection (vapor and aerosol) is a promising technology for environmental decontamination of the hospital settings^{16,17}. In our study, we used the Glosair™ 400® system which provides aerosolised hydrogen peroxide/silver cations dry-mist. The antimicrobial efficacy of this technology was hereby assessed both *in situ* and *in vitro*. For the first time, the air decontamination efficacy of aerosolized hydrogen peroxide/silver cations was evaluated. Susceptibility testing of several microorganisms to Glosair™ 400® Cartridge was also performed. Our *in vitro* study included *M. tuberculosis* which is a major cause of morbidity and mortality worldwide. Successful airborne infection control strategies are mandatory given the high transmission rate of HIV infection-associated tuberculosis with a MDR phenotype.

Our results showed that one single disinfection cycle was enough to reduce drastically (more than 90%) the number of surface and airborne bacteria and moulds, both in the patient rooms and the fungal research laboratory. Thus, previous surface cleaning is not a mandatory or a limiting step of dry-mist decontamination performance. Despite of indoor air levels of pathogens being greatly reduced by air filtration systems such as high-efficiency particulate air (HEPA) filters, patient room air is still contaminated because such filters saturate rapidly and, most importantly, they do not kill

microorganisms^{18,19}. Strikingly, successful air decontamination was achieved following one single GlosairTM 400[®] standard cycle.

The applicability of dry-mist generated hydrogen peroxide/silver cations to disinfect the surfaces of medical equipment, which are important transmission vehicles, was also investigated in this study and its efficacy demonstrated. Contamination of equipment that are extraordinarily difficult to clean, such as ventilators, monitors, and tangles of tubing and electronic cables, is particularly problematic when new patients are admitted to rooms previously occupied by colonized or infected patients. Recent studies using the Hydrogen Peroxide Vapor (HPV) technology from Bioquell did not report any damage or malfunction of the medical equipment exposed to HPV decontamination⁴. Therefore, no equipment was removed before dry-mist disinfection; vital signs and ventilator monitors were sampled. No microorganisms were recovered from it after disinfection procedure.

Regarding the antimicrobial activity against the nosocomial microorganisms tested, the GlosairTM 400[®] system was able to reduce the growth of all bacteria and fungi, 8 *log* and 6 *log* respectively. Indeed, our results demonstrate for the first time the high efficacy of the GlosairTM 400[®] system against moulds and yeasts. In addition, our results corroborate previous studies⁸, by demonstrating the efficacy of the disinfectant against *M. tuberculosis*. In contrast, Andersen et al.²⁰ had described the inefficacy of hydrogen peroxide 5% to kill *M. tuberculosis*; however the experimental conditions were quite different from ours. According to the susceptibility testing results, the disinfectant solution (GlosairTM 400[®] Cartridge) exhibited a potent antimicrobial activity even when diluted. Despite this approach does not mimic “real life”, *in vitro* susceptibility testing is very helpful to optimize novel disinfection cycles, namely to select disinfectant concentrations. Thus, our results suggest that one single cycle with shorter exposure

time could be enough to decontaminate patient areas where the level of environmental contamination might be low. This short cycle would imply shorter waiting times, during which important critical areas such as operation theatres are closed, making possible its use for daily routine disinfection, thus promoting patient safety as in the case in particular with immunosuppressed patients.

The antimicrobial properties of the product was described as the result of the oxidative action of hydrogen peroxide on the lipid membrane, which leads to modification in ribosomes and DNA, and the cationic effect of silver cations, which reverses membrane polarity and inhibits protein synthesis and cytoplasmic enzyme activity⁷. Flow cytometry technology is a reliable methodology to evaluate the mechanism of action of antimicrobial drugs. Our results confirm the occurrence of cell membrane lesion following a short period of time by exposure to hydrogen peroxide/silver cations mist, meaning its microbicidal effect.

In summary, our results support the use of the GlosairTM 400[®] as an adjunct to manual disinfection of patient facilities due to its broad microbial activity, high efficacy and easiness of use.

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Potential conflicts of interest

All authors report no conflicts of interest relevant to this article.

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Table I. Results of susceptibility testing of Glosair™ 400® Cartridge against selected bacterial and fungal species. For each strain is appointed the dilution and the respective concentration of the disinfectant that inhibited growth corresponding to the MIC value.

Strain	Dilution	Concentration (hydrogen peroxide % and sylver cations ppm)
<i>A. baumannii</i>	1:128	0.04 and <0.4
<i>E. coli</i>	1:128	0.04 and <0.4
<i>S. aureus</i>	1:128	0.04 and <0.4
<i>P. aeruginosa</i>	1:128	0.04 and <0.4
<i>E. faecalis</i>	1:128	0.04 and <0.4
<i>C. krusei</i>	1:512	0.01 and <0.1
<i>C. parapsilosis</i>	1:256	0.02 and <0.2
<i>C. albicans</i>	1:256	0.02 and <0.2
<i>A. fumigatus</i>	1:256	0.02 and <0.2
<i>P. marneffe</i>	1:256	0.02 and <0.2
<i>M. tuberculosis</i>	1:280	0.02 and <0.2

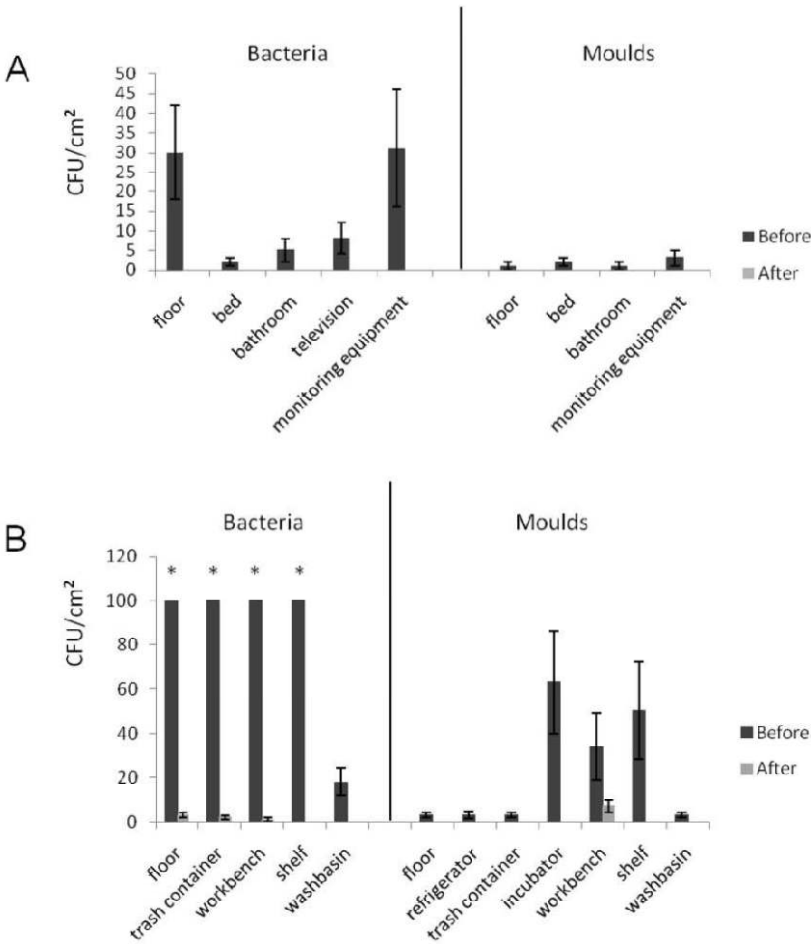
Figure legends

Figure 1. Results of culture of surfaces from four patient rooms (A) and the fungal research laboratory (B), represented as number of CFU per cm², before and after disinfection process with Glosair™ 400® system. Samples were obtained by pressing gently BH agar medium contact plates against surfaces. Experiments were performed in duplicate.

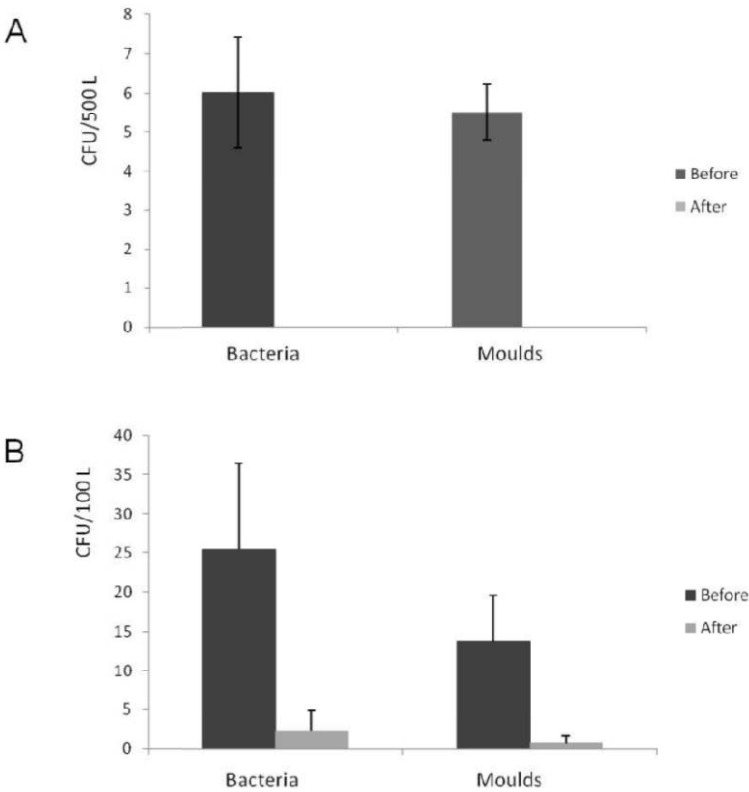
Figure 2. Results of air contamination levels in four patient rooms (A) and the fungal research laboratory (B), expressed as the number of CFU before and after disinfection process with Glosair™ 400® system. Samples were obtained after filtering 500 l (A) or 100 l (B) of air and culture in BH agar medium. Experiments were performed in duplicate.

Figure 3. Histogram obtained after flow cytometric analysis at FL3 of *E. faecalis* stained with PI, following 5 min of treatment with Glosair™ 400® solution. A- autofluorescence; B-control live cells; C-control dead cells and D-cells treated with Glosair™ 400® Cartridge.

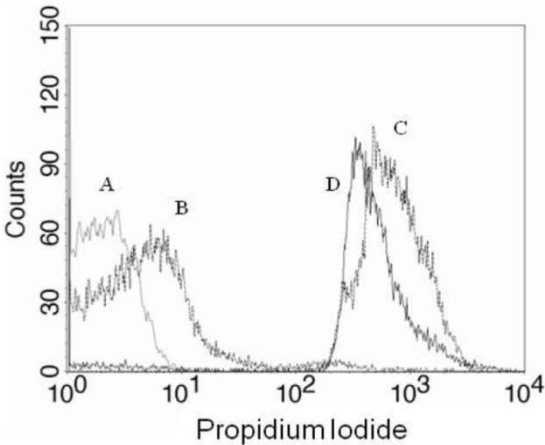
Figure_1



Figure_2



Figure_3



MANUSCRIPT VIII

1 Title: A new approach that determines fungal cell wall chitin content by flow cytometry

2

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17 Running title: Flow cytometry analysis of chitin

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50 **Abstract**

51 Chitin is an essential part of the fungal cell wall and its synthesis is considered a
52 promising target for antifungal action. The conventional methods used to evaluate chitin
53 content in fungi, such as biochemical assessment of glucosamine release after acid
54 hydrolysis or epifluorescence microscopy, are low throughput, laborious, time-
55 consuming and cannot evaluate a large number of cells. Flow cytometry is an efficient
56 and fast technology for the analysis of cell phenotypes. We developed a flow cytometric
57 assay based upon Calcofluor White (CFW) staining to quantitatively measure chitin
58 content in yeast cells. CFW is a fluorescent dye that binds specifically to chitin. A
59 staining index was defined and its value was directly related to chitin amount. Twenty
60 two *Candida* spp and 4 *Cryptococcus neoformans* clinical isolates with distinct
61 susceptibility profiles to caspofungin, were evaluated. *C. albicans* clinical isolate
62 SC5314 and an isogenic chitin synthase deleted strain (*chs3Δ/chs3Δ*), were used as
63 controls. As expected, the wild type strain displayed a significantly higher staining
64 index ($p<0.001$) than the *chs3Δ/chs3Δ* strain. A variable CFW staining index was found
65 within strains belonging to the same species, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*
66 whereas *C. neoformans* had less intra-species variation. Although no relationship
67 between chitin content and antifungal susceptibility phenotype was found, an
68 association was established between paradoxical growth in the presence of high
69 caspofungin concentrations and chitin content.

70 This novel flow cytometry protocol is a simple and reliable assay to accurately quantify
71 cell wall chitin of yeasts.

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75 Introduction

76 Chitin is a β -1,4-homopolymer of *N*-acetylglucosamine that is synthesized by chitin
77 synthase enzymes (13). This polysaccharide is present in most fungi and together with
78 β -1,3-glucan, plays a fundamental role in maintaining fungal cell integrity and
79 conferring structural rigidity during growth and morphogenesis (8, 13, 17). Mutations in
80 glucan synthase genes reduce glucan levels in the cell wall while stimulating salvage
81 pathways leading to increased chitin synthesis. This pathway restores the strength of the
82 cell wall matrix and prevents antifungal action (22). Since chitin is not present in human
83 cells, inhibition of chitin synthesis has been proposed as a potential, selective antifungal
84 target.

85 The assessment of cell wall chitin content based upon glucosamine release through acid
86 hydrolysis has been used extensively; however this method is very laborious and time
87 consuming (12, 18, 24). Epifluorescence microscopy has also been widely used to
88 quantify chitin levels in fungi stained by Calcofluor White (CFW), a specific chitin dye
89 (10, 11, 14, 16, 24, 25). However, with this approach, only a limited number of yeast
90 cells can be analyzed and the quantification of the fluorescence emitted cannot be
91 performed accurately (1, 6, 24). Flow cytometry represents an efficient and fast
92 approach for the analysis of cell architecture and functional phenotypes, with
93 considerable advantages over conventional methods (7, 19, 20, 21).

94 Here we describe a fast and reliable protocol to measure cell wall chitin content in
95 yeasts cells based upon flow cytometric analysis after CFW staining.

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99 **Material and methods**

100 Strains

101 Twenty two *Candida spp* and 4 *Cryptococcus neoformans* clinical isolates with well
 102 characterized susceptibility profiles to caspofungin, (antifungal chosen as representative
 103 of echinocandin class), were used in this study (detailed in Table 1). SC5314 (9) with
 104 wild type chitin levels and *chsΔ3/chs3Δ* mutant with low chitin content (2) were used as
 105 control strains.

106 Measurement of cell wall chitin content

107 SC5314 and *chs3Δ/chs3Δ* yeast cells were grown in YPD broth medium at 35°C, 150
 108 rpm, until late logarithmic phase, and used to optimize the flow cytometric protocol. A
 109 10⁶ yeast cells/ml suspension in sterilize distilled water was stained with 0
 110 (autofluorescence), 2.5, 6.25, 12.5 and 25 µg/ml of CFW (Fluka, St. Louis, USA), a
 111 specific chitin dye (excitation at 365 nm and emission at 430 nm), for 15 minutes at
 112 room temperature. Afterwards, the yeast cells were washed twice and blue fluorescence
 113 (Pacific blue channel) emitted by 50000 cells was quantified, using a BD FACSCanto™
 114 II (Becton Dickinson, Madrid, Spain) flow cytometer. BD FACSCanto™ II system
 115 consists of an excitation source with three lasers: blue (488-nm, air-cooled, 20-mW
 116 solid state), red (633-nm, 17-mW HeNe), and violet (405-nm, 30-mW solid state). The
 117 mean intensity of fluorescence (obtained from three independent experiments) emitted
 118 from stained (positive population) and non stained (autofluorescence or negative
 119 population) yeast cells was analyzed and processed with FACSDiva software. In each
 120 experiment a staining index (SI) was calculated as follows: (mean intensity of
 121 fluorescence of positive population – mean intensity of fluorescence of negative
 122 population)/ 2 x standard deviation of the mean intensity of fluorescence of negative

123 population (15). The chitin content of the 27 clinical isolates was assessed according to
124 the described protocol, after staining with 2.5 µg/ml of CFW; the SI was calculated as
125 described.

126 Epifluorescence microscopy

127 In order to confirm the flow cytometry results, epifluorescence microscopy analysis was
128 performed. Yeasts cells were grown and prepared as described for flow cytometric
129 assays and stained with 25 µg/ml of CFW for 15 minutes. Following staining, 30 µl of
130 the cell suspension were placed on a glass slide and overlapped with vectashield
131 fluorescence mounting medium (Vector Laboratories, Peterborough, UK) and observed
132 under an epifluorescence microscope (400X) imager Z Apotome (Zeiss, Barcelona,
133 Spain).

134 Paradoxical effect of caspofungin

135 The ability of the clinical isolates to grow in the presence of high caspofungin (Merck,
136 Rahway, NJ, USA) levels, termed paradoxical growth was tested over a range of
137 concentrations varying from 0.03 to 256 µg/ml and MICs were determined using
138 prominent inhibition as an endpoint corresponding to 50% (MIC₅₀) (3, 4, 5). The
139 paradoxical effect was defined as a progressive increase in cell growth occurring at least
140 two drug dilutions above the MIC, following 48 hours incubation (3).

141 Data Analysis

142 The SI mean values displayed by the different isolates after CFW staining were
143 compared using the Student's *t*-test. Significant effects were accepted at $p < 0.05$. The
144 SPSS Statistics 17.0 Software for Windows was used to perform the statistical analysis.
145 All experiments were performed in triplicate.

146

147 **Results and discussion**

148 The optimization of the cytometric protocol involved two strains with known
149 differences in chitin contents: SC5314 and the *chs3Δ/chs3Δ* mutant. A range of CFW
150 concentrations was tested and 2.5 µg/ml CFW was revealed to be the most adequate to
151 differentiate the chitin content of the two strains used as controls. Significantly
152 different SI values ($p < 0.001$) were obtained with this CFW concentration (Figure 1a),
153 concordant with the chitin determinations previously described by others (2, 24). The
154 results obtained by flow cytometry were consistent with epifluorescence microscopy
155 observations (Figure 1b and 1c). The flow cytometric protocol for chitin quantification
156 is considerably less laborious and more accurate in comparison with the previously
157 described methods since a large amount of cells (50000) are randomly evaluated,
158 without operator interference.

159 Given the variation in yeast morphology such as cell shape and size, different species
160 may emit different levels of autofluorescence, which arises from endogenous
161 fluorophores. This autofluorescence emission analyzed under epifluorescence
162 microscopy or flow cytometry results in a background “noise” which may interfere with
163 the quantification of fluorescence emitted by stained cells (15). To avoid
164 autofluorescence interference, especially when fluorescence emitted by cells from
165 different species is compared, normalization of data is mandatory (15). This was
166 achieved through the calculation of a staining index which provides sensitivity and
167 reliability to the output data and enables comparison of the fluorescence emitted by cells
168 with distinct morphologies.

169 The relationship between the CFW staining index and the caspofungin susceptibility
170 phenotype displayed by clinical strains is detailed in Table 1 and Figure 2. Among the
171 distinct species included in this study, *C. parapsilosis*, *C. tropicalis* and *C. albicans*

172 clinical isolates showed a higher CFW staining index, and therefore a higher cell wall
173 chitin content comparing to *C. glabrata*, *C. krusei* and *C. neoformans* (Figure 2). As
174 shown in Figure 2, a direct relationship between chitin levels and MIC values $>4\mu\text{g/ml}$
175 (NS phenotype) was found.

176 Notably, several *C. parapsilosis* and *C. tropicalis* (Cp4, Ct1, Ct3 and Ct4) isolates
177 showed a significant increase in chitin level ($p<0.001$) in comparison with other isolates
178 from the same species (figure 2). Interestingly, these strains, along with all *C. albicans*
179 isolates tested, exhibited a paradoxical growth in the presence of high caspofungin
180 concentrations. The ability to grow at high caspofungin concentrations has been
181 frequently described among *C. albicans*, *C. parapsilosis* and *C. tropicalis* (3) and has
182 been suggested to relate to a compensatory increase in cell wall chitin (23, 24). This
183 salvage mechanism strengthens cell wall damaged by exposure to echinocandins.
184 Regarding these yeast species, chitin quantification by flow cytometry was revealed to
185 be a highly sensitive method. It enabled the sensitive detection of different chitin levels
186 which allowed us to validate the association between a higher amount of cell wall chitin
187 and paradoxical growth in the presence of caspofungin concentrations well above the
188 MIC. The clinical relevance of this *in vivo* effect is yet uncertain. Although unrelated to
189 resistance, the paradoxical effect may represent a drug tolerance mechanism and an
190 adaptive response to the presence of caspofungin. In contrast, *C. glabrata*, *C. krusei* and
191 *Cryptococcus neoformans* strains showed an absence of paradoxical growth, displaying
192 the lowest chitin levels. Interestingly, the accuracy of this novel methodology can
193 predict the occurrence of this paradoxical effect within a few minutes, representing a
194 valuable tool for detection of an antifungal compensatory mechanism in the presence of
195 high antifungal concentrations, namely echinocandins.

196 The flow cytometry protocol described herein may constitute a useful tool to evaluate
197 the inhibition of chitin synthesis by new drugs presently under development.

198

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310 **Figure legends**

311 Figure 1- Cell wall chitin content of SC5314 and *chs3Δ/chs3Δ* strains. (a) A suspension
312 of 10^6 cells/ml was stained with 2.5 µg/ml CFW and the intensity of fluorescence was
313 quantified by flow cytometry. The SI mean values displayed by the different strains
314 were determined after three independent experiments. Strain SC5314 and double
315 deleted *chs3* strain had significantly different ($p<0.001$) SI mean values, revealing
316 diverse chitin levels in the cell wall. This result was confirmed by epifluorescence
317 microscopy after CFW staining of both strains, SC5314 (b) and *chs3Δ/chs3Δ* (c).

318

319 Figure 2- Cell wall chitin content of *Candida* spp and *Cryptococcus neoformans* clinical
320 isolates. Yeast cells were stained with 2.5 µg/ml CFW and fluorescence emitted was
321 quantified by flow cytometry. Higher staining index (SI) values were observed in strains
322 that showed a paradoxical growth (*) in the presence of caspofungin.

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331 Table 1- *In vitro* antifungal susceptibility and paradoxical effect of caspofungin (CFS)
 332 against *Candida* spp and *Cryptococcus neoformans* clinical isolates. Minimal inhibitory
 333 concentrations (MIC; µg/ml) were determined using prominent inhibition as an end
 334 point, corresponding to 50% (MIC₅₀), according to CLSI protocol.

Yeast	Strain code	Source	CFS MIC ₅₀ (µg/ml) / Phenotype	Paradoxical growth (mean values) Start point/ end point (µg/ml)
<i>C. glabrata</i>	Cg1	Blood	>32/ NS	NF
	Cg2	Blood	0.125/ S	NF
	Cg3	Peritoneal fluid	32/ NS	NF
	Cg4	Fecal	0.125/ S	NF
	Cg5	Peritoneal fluid	0.5/ S	NF
	Cg6	Blood	0.25/ S	NF
<i>C. parapsilosis</i>	Cp1	Peritoneal fluid	4/ NS	NF
	Cp2	Blood	2/ S	NF
	Cp3	Blood	4/ NS	NF
	Cp4	Blood	4/ NS	16/64
	Cp5	Blood	0.5/ S	NF
<i>C. tropicalis</i>	Ct1	Blood	0.5/ S	8/16
	Ct2	Peritoneal fluid	0.5/ S	NF

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	Ct3	Pus	4/ NS	16
	Ct4	Pus	4/ NS	16
<i>C. krusei</i>	Ck1	Urine	1/ S	NF
	Ck2	Blood	1/ S	NF
	Ck3	Bronchial secretions	1/ S	NF
	Ck4	Bronchial secretions	1/ S	NF
<i>C. albicans</i>	Ca1	Blood	0.5/ S	16/32
	Ca2	Blood	0.5/ S	16/32
	Ca3	Blood	0.5/ S	16/32
<i>C. neoformans</i>	Cn1	Blood	16/ NS	NF
	Cn2	Blood	16/ NS	NF
	Cn3	Blood	16/ NS	NF
	Cn4	Blood	32/ NS	NF

335 NS non susceptible phenotype; S susceptible phenotype; NF not found

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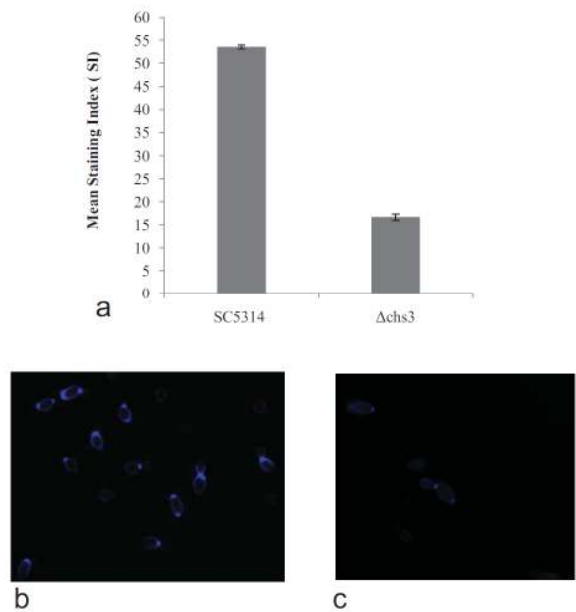
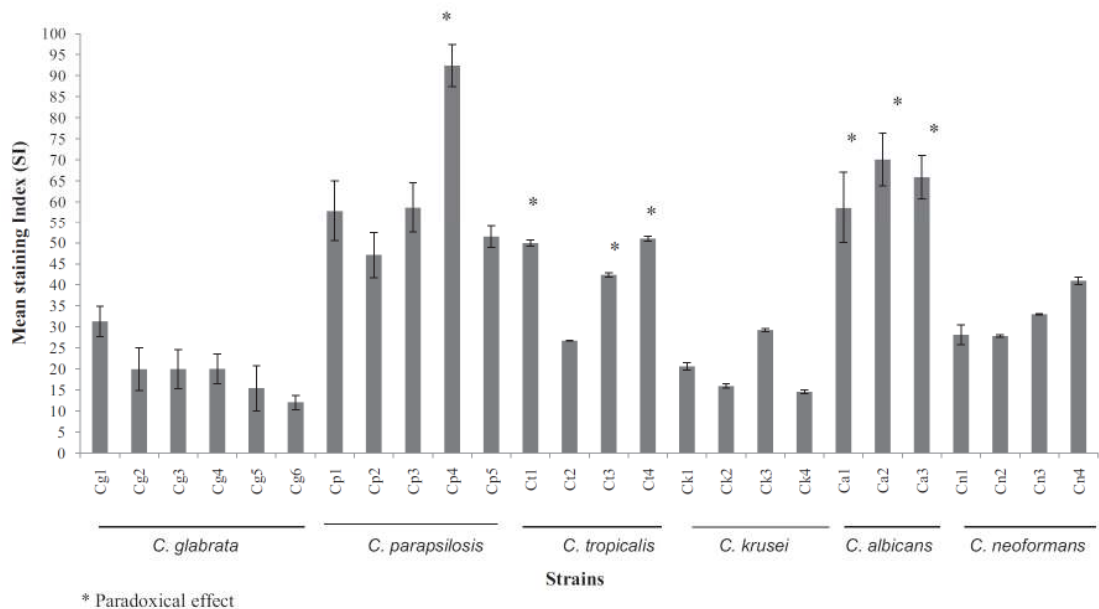


Figure 1- Cell wall chitin content of SC5314 and *chs3* Δ /*chs3* Δ strains. (a) A suspension of 10^6 cells/ml was stained with 2.5 μ g/ml CFW and the intensity of fluorescence was quantified by flow cytometry. The SI mean values displayed by the different strains were determined after three independent experiments. Strain SC5314 and double deleted *chs3* strain had significantly different ($p<0.001$) SI mean values, revealing diverse chitin levels in the cell wall. This result was confirmed by epifluorescence microscopy after CFW staining of both strains, SC5314 (b) and *chs3* Δ /*chs3* Δ (c).



* Paradoxical effect

Figure 2- Cell wall chitin content of *Candida* spp and *Cryptococcus neoformans* clinical isolates. Yeast cells were stained with 2.5 μ g/ml CFW and fluorescence emitted was quantified by flow cytometry. Higher staining index (SI) values were observed in strains that showed a paradoxical growth (*) in the presence of caspofungin.

ABSTRACT I

M-1083

Transcriptional Profile Highlight the Increased Expression of Virulence Attributes in Azole Resistant *C. parapsilosis*

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Background: *C. parapsilosis* is a common yeast, particularly isolated from invasive infections. To get insight whether there is an effective relation between drug resistance and virulence, we compared large-scale gene expression profile of susceptible and resistant strains using microarray analysis. **Methods:** Resistant strain of *C. parapsilosis* was obtained following successive subcultures of a susceptible strain (S) with posaconazole, that conferred cross-resistance to all azoles. Total RNA were extracted from S and R strains and cDNAs was synthesized and labeled with Cy3 and Cy5. After RNase treatment, labeled cDNAs were purified, denaturated and mixed with hybridization buffer, and applied to *C. parapsilosis* DNA microarrays from Agilent Technologies. The microarrays were scanned with an Axon 4000B scanner and data acquired with GenePix Pro 5.0 software and statistically analyzed using an R programming environment. In parallel, phenotypic characterization (biofilm formation, growth kinetic, phenotypic switching and pseudohyphal development) of S and R strains was carried out. **Results:** Microarray analysis identified 410 genes with differential expression in the R strain. Groups of genes (filamentous growth, lipid metabolic process, hyphal growth, pathogenesis, response to stress, interaction with host, pseudohyphal growth, cell budding, cell adhesion, biofilm formation and protein catabolic process) associated with fungal virulence were found to be overexpressed in R strain. Phenotypic studies corroborated microarrays results, while adherence ability, biofilm and pseudohyphal formation and phenotypic switching occurred extensively among R strain. **Conclusions:** Our results demonstrated that virulence factors are enhanced in *C. parapsilosis* resistant strain, which suggests increased pathogenic ability.

ABSTRACT II

P08.03

Antifungal activity of chemical agents against *Candida albicans*

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Hospital reservoirs of *Candida* organisms may include not only human endogenous sources, but also environmental and the hands of health care personnel. The activity of several chemical germicides commonly used to disinfect surfaces, medical devices, as well as hands or wound lesions was assessed regarding *Candida* species.

Ten strains of each *C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, *C. krusei* and *C. guilliermondii* were used. Chlorhexidine (C), ethanol (E), sodium hypochlorite (SH), hydrogen peroxide (HP), potassium permanganate (PP), boric acid (BA) and cerium nitrate (CN) were studied. The minimal inhibitory concentration (MIC) and minimal lethal concentration (MLC) of each chemical agent was assessed according to the CLSI M27-A3 protocol. Additionally, the growth inhibition potential of each agent (commercial solutions) was determined following time killing assays. Flow cytometry analysis after staining the cells with propidium iodide was performed.

For all *Candida* strains, MIC and MLC values ranged between 0.004–0.06% for C, 1.25–10% for E, 3.125–25 µl/ml for SH, 0.03–0.12% for HP, 0.008–2 g/l for PP, 0.78–25 mg/ml for BA and 0.75–12.5 mM for CN. C, E and SH inhibited 100% the growth soon after 1 min of contact. HP, PP and CN were able to inhibit 100% the growth, but only after 30 min for the first two agents and 10 min for the last. BA inhibited the growth at a maximum of 66.7%. Flow cytometry confirmed the fungicidal activity of the different chemical agents.

Under the experimental conditions of the study, all solutions studied showed antifungal activity; however chlorhexidine, ethanol and sodium hypochlorite revealed to be the most effective, thus supporting its use for disinfection procedures.

ABSTRACT III

P08.05**Efficacy of a hydrogen peroxide dry-mist disinfection system for hospital environment disinfection**

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In order to control the spread of pathogens within hospital environments, the adoption of hygienic routines are mandatory. In this study we evaluated the microbicidal effect of the mixture of hydrogen peroxide (5%) and silver cations (<50 ppm) diffused by the dry mist generator Sterinis® (Gloster Santé Europe, Labège Cedex, France) upon several species. Three distinct approaches were followed: (1) Sterinis® was tested for the decontamination of two patient rooms of the burn unit of Hospital S. João and in a fungal research laboratory; the surfaces and air were sampled before and after the disinfection procedure. (2) Suspensions of 10⁶ cells of several microorganisms (*Acinetobacter* spp., *E. coli*, *S. aureus*, *P. aeruginosa*, *E. faecalis*, *C. krusei*, *C. parapsilosis*, *C. albicans*, *A. fumigatus* and *Penicillium* spp.) were plated on agar medium and afterwards exposed to the dry-mist; the number of colony forming units was determined and compared with control suspensions (not exposed). (3) Susceptibility of *M. tuberculosis* was determined with the BACTEC MGIT 960 (BD, Sparks, MD, USA); variable amounts of the disinfectant solution were incubated with cells and the growth signaled by the equipment. Flow cytometry was performed in order to confirmed membrane lesion of cells after 5 min of exposure to the disinfectant.

The contamination levels of the research laboratory decreased drastically after Sterinis® disinfection. The environment quality of the burn unit rooms improved significantly when Sterinis® was included in the disinfection protocols. A reduction in growth of at least two log were observed in the in vitro studies with all microorganisms. *M. tuberculosis* did not grow in the presence of the disinfectant. Flow cytometry demonstrated an evident cell membrane lesion.

The hydrogen peroxide dry-mist exhibited an excellent bactericidal and fungicidal effect. Sterinis® may thus represent a valuable tool for decontamination of patient rooms and laboratory facilities.

ABSTRACT IV

P1940 Correlation between azole resistance and virulence attributes displayed by *C. parapsilosis*

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(Porto, PT)

Objectives: *C. parapsilosis* is common yeast, particularly isolated from invasive infections. There is a growing concern about the rise of antifungal drug resistance, although short knowledge is yet available regarding phenotypic characterization of drug-resistant fungal strains. In order to study the correlation between drug resistance and pathogenicity attributes, we induced resistance in one susceptible *C. parapsilosis* clinical strain by sub-culturing it in the presence of increasing concentrations of fluconazole, voriconazole and posaconazole. The set of strains obtained displayed variable degrees of resistance and was characterized regarding their virulence attributes.

Methods: Growth Kinetic. Strains were incubated at 30°C under agitation for 48 h using an initial inoculum density of 0.1 in liquid YPD medium; growth was assessed by measuring O.D. along time. Adherence Assay. From a overnight culture, a PBS yeast suspension (106 blastoconidia/mL); a stock solution of 105 microspheres/mL in PBS was prepared; equal volumes of both suspension were mixed; the percentage of yeast cells attached to microspheres was assessed by flow cytometry. Biofilm Formation. A initial 5 x 10⁷ blastoconidia/mL suspension was incubated at 37°C in YPD medium in 12 wells polystyrene plates; after 24 and 48 hours biofilm mass was determined by crystal violet method. Phenotypic Switching. Blastoconidia were plated onto YPD and incubated at 30°C for 96 h; the presence of distinct colony phenotypes was scored and the frequency of switching was determined. Pseudohyphal Development. Blastoconidia were incubated in RPMI 1640 medium with 10% fetal calf serum for 24 h at 37°C; pseudohyphal development was checked microscopically.

Results: Overall, specific growth rates and adherence ability displayed by resistant strains were higher compared to the susceptible strain; however, resistant strains were less capable to form biofilm. phenotypic switching and pseudohyphal formation occurred extensively among resistant strains.

Conclusion: Our results demonstrate that *in vitro* induced resistance to azoles alters growth rate, adherence, phenotypic switching and pseudohyphal development of *C. parapsilosis*, considered important attributes favouring invasive fungal infection.

ABSTRACT V

P835 Fungaemia by *Candida parapsilosis*: *in vivo* induction of azole resistance due to prolonged therapeutic exposure

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A.G. Rodrigues (Porto, PT)

Objectives: A patient with *C. parapsilosis* fungaemia was submitted to fluconazole during eleven days, after which he died. Several *C. parapsilosis* isolates had been collected before, during and after initiation of fluconazole therapeutics. A microbiological characterization of the first and the last isolate was performed.

Methods: *In vitro* antifungal susceptibility testing to four azoles (fluconazole, itraconazole, voriconazole and posaconazole) was performed according to CLSI protocols. Molecular typing of the *C. parapsilosis* isolates was carried out by RAPD. The stability of resistant phenotype (acquired *in vivo*) was assessed *in vitro* through repeated incubation of the isolate without antifungal. The role of active efflux pumps in drug resistance was evaluated using flow cytometric analysis with rhodamine 6G (Rh-6G), a specific substrate for efflux pumps.

Results: The initial isolate was susceptible to all azoles, while the last recovered isolate was resistant to all four tested azoles, displaying high MIC values. Molecular typing of isolates demonstrated that the last isolate and pre-treatment isolate were isogenic. Therefore, we report, the *in vivo C. parapsilosis* acquisition of antifungal resistance, which maintained stable after incubation without antifungal. No difference in Rh-6G staining was found between the susceptible and the resistant isolate, suggesting that *in vivo* azole resistance did not involve increase in efflux pumps activity.

Conclusion: We report a clinical case of fungaemia by *C. parapsilosis* which acquired resistance to azoles after exposure to fluconazole. The resistance was characterized by an increase of fluconazole, itraconazole, voriconazole and posaconazole MIC values, which kept stable. Interestingly, azole resistance due to overexpression of efflux pumps was not confirmed. Continued fluconazole pressure was associated with the development of multi-resistance.

ABSTRACT VI

P281

Easy quantification of yeast chitin cell wall content by flow cytometry

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Flow cytometry represents an efficient and fast approach for the analysis of cell architecture and functional phenotypes, with considerable advantages over conventional methods. Taking advantage of the specific chitin dye calcofluor white (CFW) that has already been used in epifluorescence microscopy we developed a flow cytometric assay to quantitatively measure cell wall chitin content in the yeast cell wall.

Twenty two *Candida* spp and 4 *Cryptococcus neoformans* clinical isolates with distinct susceptibility profile to caspofungin, were included in this study. The *C. albicans* SC5314 wild type and the Δ chs3/ Δ chs3 strain (with low chitin content) were used as control strains for optimization of the flow cytometric protocol. Cells were grown in YPD broth medium at 35 °C, 150 rpm, until late logarithmic phase. A 10⁶ yeast cells/ml suspension in distilled water was stained with 0 (autofluorescence), 2.5, 6.25, 12.5 and 25 µg ml⁻¹ of CFW for 15 min at room temperature. Afterwards, yeast cells were washed twice and the blue fluorescence emitted by 50000 cells was quantified using a BD FACSCanto[™] II (Becton Dickinson, Madrid, Spain) flow cytometer. The mean intensity of fluorescence emitted from stained (positive population) and the nonstained (autofluorescence or negative) yeast cell population (obtained from three independent experiments) was analyzed and processed with FACSDiva software. For each experiment a staining index (SI) was calculated as follows: (mean intensity of fluorescence of positive population - mean intensity of fluorescence of negative population) / 2 × standard deviation of the mean intensity of fluorescence of negative population. The chitin content of 27 clinical isolates was assessed according the described protocol, after staining with 2.5 µg ml⁻¹ of CFW. Next the isolates grown for 48 h in the presence of a range of caspofungin concentrations varying from 0.03 to 256 µg ml⁻¹ were tested. MICs were also determined using prominent inhibition as an endpoint corresponding to 50% (MIC₅₀). The aim was to establish a relationship between fungal chitin content and the occurrence of the paradoxical growth phenomenon registered among clinical isolates in the presence of high caspofungin levels. The best CFW concentration to differentiate chitin content among the two control strains was 2.5 µg ml⁻¹. As expected, the wild type strain displayed a significantly higher staining index ($P < 0.001$) than the chs3/chs3 strain. Variable CFW staining index values were found among strains belonging to the same species, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. neoformans* had less intra-species variation. Although no relationship between chitin content and the caspofungin susceptibility pattern was found, an association between paradoxical growth in the presence of caspofungin and chitin content was established.

This novel flow cytometry protocol is a simple and reliable assay to accurately quantify cell wall chitin in yeast cells. The accuracy of this novel methodology can predict the occurrence of the paradoxical effect, representing a valuable tool for detection of antifungal compensatory mechanisms in the presence of high echinocandin concentrations.