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Genetic determinants of virulence – *Candida parapsilosis*

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ABSTRACT

The global epidemiology of fungal infections is changing. While overall, *Candida albicans* remains the most common pathogen; several institutions in Europe, Asia and South America have reported the rapid emergence to predominance of *Candida parapsilosis.* This mini-review examines the impact of gene deletions achieved in *C. parapsilosis* that have been published to date. The molecular approaches to gene disruption in *C. parapsilosis* and the molecularly characterized genes to date are reviewed. Similar to *C. albicans*, factors influencing virulence in *C. parapsilosis* include adherence, biofilm formation, lipid metabolism, and secretion of hydrolytic enzymes such as lipases, phospholipases and secreted aspartyl proteinases. Development of a targeted gene deletion method has enabled the identification of several unique aspects of *C. parapsilosis* genes that play a role in host–pathogen interactions – CpLIP1, CpLIP2, SAPP1a, SAPP1b, BCR1, RBT1, CpFAS2, OLE1, FIT-2.

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Determinantes genéticos de la virulencia de *Candida parapsilosis*

Palabras clave:
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RESUMEN

La epidemiología mundial de las infecciones fúngicas está cambiando. Aunque *Candida albicans* sigue siendo el patógeno más común, varios centros en Europa, Asia y Sudamérica han descrito la rápida emergencia de *Candida parapsilosis*, que ha terminado por predominar. La presente revisión examina la influencia de las deleciones genéticas producidas en *C. parapsilosis* que se han publicado hasta la fecha. Se revisan las estrategias moleculares de la alteración de genes de *C. parapsilosis* y los genes caracterizados molecularmente hasta la fecha. Al igual que en *C. albicans*, los factores que influyen en la virulencia de *C. parapsilosis* incluyen la adherencia, formación de biopelículas, el metabolismo de lípidos y la secreción de enzimas hidrolíticas, como lipasas, fosfolipasas y aspartilproteinasas. El desarrollo de un método de deleción génica dirigido ha permitido la identificación de varios aspectos exclusivos de los genes de *C. parapsilosis* que participan en las interacciones huésped-patógeno-CpLIP1, CpLIP2, SAPP1a, SAPP1b, BCR1, RBT1, CpFAS2, OLE1, FIT-2.

Este artículo forma parte de una serie de estudios presentados en el «V International Workshop: Molecular genetic approaches to the study of human pathogenic fungi» (Oaxaca, México, 2012).

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Since the late 1970s, fungal infections have increasingly become a significant cause of morbidity and mortality especially among hospitalized and immunosuppressed patients.[69] *Candida* species are the fourth most frequent causative agent of blood-stream infections, constituting 8–15% of hospital-acquired infections.[87] In the United States, *Candida albicans* is the most common pathogen followed by *Candida parapsilosis* or *Candida glabrata*, depending on the study. However, *C. parapsilosis* has become the leading causative agent in some institutions located in Europe, Asia and South America, and it is the *Candida* species with the largest increase in incidence since 1990.[1,6,10–12,20,36,48,52,54,70,74,81,87] Of all candidal isolates, *C. parapsilosis* accounts for 15.5% in North America, 16.3% in Europe and 23.4% in Latin America.[81] In the US, it has been the third most common cause of neonatal sepsis.[66]

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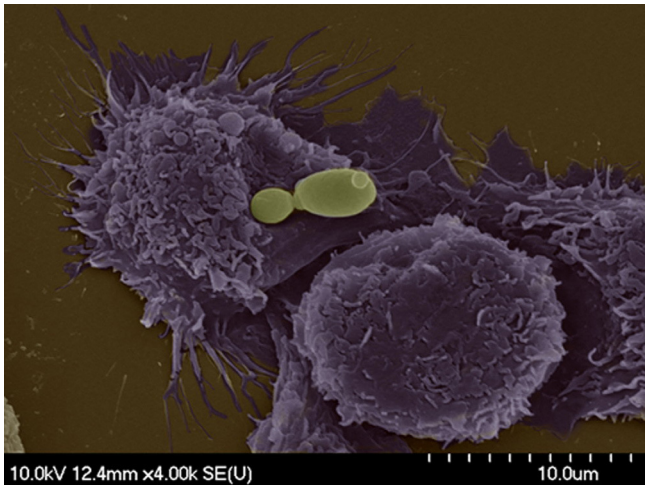

Fig. 1. Scanning electron microscopy of *C. parapsilosis* interacting with murine macrophages (photo credit to T. Németh, T. Petkovits, and A. Gácser).

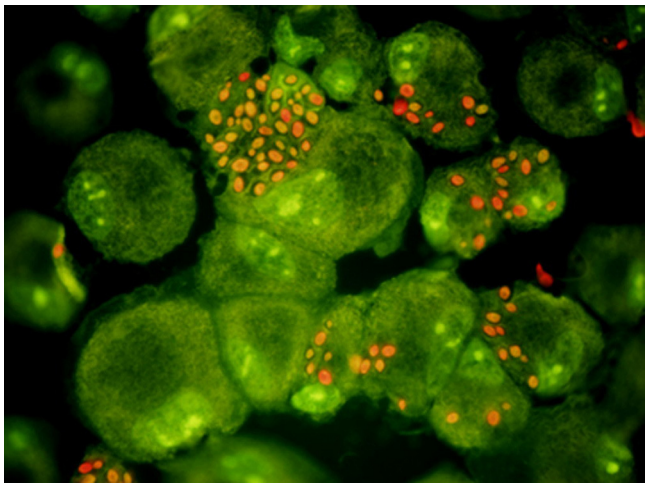

Fig. 2. Immunofluorescence microscopy of *C. parapsilosis* and human macrophages. Red yeast are dead (photo credit to C. Papp and A. Gácser).

First isolated in 1928 from a stool specimen and thought to be non-pathogenic,[2,86] *C. parapsilosis* is now recognized as being fairly ubiquitous as it can be isolated from humans as a normal skin commensal as well as from domestic animals, insects, soil and marine environments.[19,81,85] It is now especially well documented as a pathogen that arises from exogenous sources of infection in intravenous drug users and via medical instrumentation (e.g. catheters and hyperalimentation solutions).[34,81] In particular, *C. parapsilosis* is recognized for its ability to cause invasive disease in patients without prior evidence of colonization via horizontal transmission through medical devices including catheters, parenteral nutrition solutions, and the hands of healthcare workers.[81] Invasive disease occurs more often in immunocompromised patients, such as individuals with AIDS, cancer and in patients undergoing gastrointestinal surgical procedures.[81] Other risk factors reported in studies include transplant receipt, antibiotic exposure, ophthalmic irrigating solutions and, especially, low birth weight in premature neonates.[1,44,78,85,86] Clinical manifestations of *C. parapsilosis* include endocarditis, meningitis, peritonitis, arthritis, endophthalmitis, keratitis, otomycosis, onychomycosis, vulvovaginitis and urinary tract infections.[81] Mortality rates attributed to *C. parapsilosis* range from 4% to 45%, with an average mortality rate of 28.5%.[6,11,28,81] Biofilm producing isolates are associated with outbreaks[38] and significantly higher mortality rates.[82]

Determinants of virulence for candidal disease include adhesion capability to host surface, ability to switch morphology between yeast and filamentous growth, biofilm formation and secretion of extracellular hydrolytic enzymes such as lipases, phospholipases or secreted aspartyl proteinases.[51,83] Conflicting data exist regarding phospholipase activity, with some studies demonstrating their presence in clinical isolates and others their absence.[14,16,26,47] Nevertheless, its role in virulence bears consideration. The development of gene disruption methods to produce mutants has been a pivotal achievement in our capacity to gain insights into the interactions of *C. parapsilosis* with hosts and host effector cells (Figs. 1 and 2). This review will focus on the observations made on specific genes that have so far been characterized and shown to significantly influence these virulence traits.

Gene disruption

C. parapsilosis has a diploid genome and does not have a described sexual cycle. Genetic analysis was initially limited by the availability of appropriate study tools. The first targeted gene disruption method was developed in 2007[23] based on previously established gene disruption protocols in *C. albicans*. The first targeted deletion in *C. parapsilosis* was the disruption of secreted lipases. This efficient gene deletion system was developed utilizing the repeated use of the dominant nourseoethricin marker (caSAT1) and subsequent deletion by FLP-mediated, site-specific recombination.[23] Applying this technique, the lipase locus in *C. parapsilosis* containing the adjacent lipase genes *CpLIP1*, *CpLIP2* were deleted and *CPLIP2* reconstructed providing an understanding of the role of lipase activity in virulence.[23]

Lipases

Microbial extracellular lipases are virulence factors in a broad range of bacteria and fungi, including *Candida* species.[81] So far, 10 lipase genes have been identified in *C. albicans*,[32] and disruption, such as the deletion of *LIP8*, can significantly affect virulence.[22] Only two lipase genes have been elucidated in *C. parapsilosis* – *CpLIP1* and *CpLIP2*, of which only the latter has been demonstrated to code for an active protein.[7,53] Utilizing the described targeted gene deletion method, disruption of these lipase genes provides evidence that they play a role in pathogenesis (i) by the decreased tissue damage seen in the presence of lipase inhibitors, (ii) the decreased ability of *CpLIP1-CpLIP2* homozygous mutants to form complex biofilms, (iii) requirement for lipid-rich media, (iv) increased susceptibility to phagocytosis by macrophage-like cells, and (v) decreased virulence in comparison to wild-type *C. parapsilosis* yeast in infections of human oral epithelium or during murine intraperitoneal challenges.[21,23] These observations hold significant clinical relevance since *C. parapsilosis* infections are particularly more commonly seen in patients receiving lipid-rich total parenteral nutrition and thus lipases may be a potential target for future antifungal agent development.[81]

Secreted aspartyl proteinase (Sap)

Secreted aspartyl proteinase (Sap) genes have been demonstrated in most pathogenic *Candida* including *C. albicans, Candida dubliniensis*, *Candida tropicalis* and *C. parapsilosis*.[25,45,49,89] However, they are notably absent in many non-pathogenic yeasts (e.g. *Saccharomyces cerevisiae*) suggesting their possible role in virulence.[30] Sap isoenzymes have several functions such as (i) digestion of host proteins for provision of nitrogen sources, (ii) degradation of host cell surface structure and intracellular substances promoting tissue adhesion and invasion, and (iii)

destruction of cells and molecules of the host immune system such as immunoglobulin G heavy chains, 2-macroglobulin, C3 protein, lactoglobulin, lactoperoxidase, collagen, and fibronectin[71,77] thereby enabling evasion of antimicrobial activity.[31] For example, vulvovaginal and skin *C. albicans* isolates exhibit higher *in vitro* Sap activity compared to blood isolates, which possibly explains the earlier clearance of fungemia in comparison to sustained skin infection in infection models.[8,14,15,88] To date, ten *SAP* genes (Sap1p-Sap10p) have been identified in *C. albicans*.[51] Their role in *C. parapsilosis* was unclear until the recent identification of 2 *SAPP1* genes: *SAPP1a* and *SAPP1b*.[30]

The *C. parapsilosis* genome database (www.sanger.ac.uk/sequencing/candida/parapsilosis) was used to perform *in silico* analysis of the *SAPP1* genes. A 2871 base pair-duplicated upstream (*SAPP1a*) and downstream (*SAPP1b*) regions were defined in the genome.[30] All 4 alleles of the *SAPP1* gene were deleted from wild-type (WT) *C. parapsilosis*.[30] Separate homozygous *ΔΔsapp1a*, *ΔΔsapp1b* and a double-homozygous mutant *ΔΔsapp1a-ΔΔsapp1b* were generated.[30] Subsequent experiments revealed that in an inducer medium, Sapp1p production was reduced by about 50% in the *ΔΔsapp1a* and *ΔΔsapp1b* mutants, but a similar effect was not observed for the *SAPP2* gene. On the other hand, Sapp2p production was greater in the *ΔΔsapp1a-ΔΔsapp1b* mutant compared to wild-type yeast. This mutant was also hyper-susceptible to human serum, had decreased proteolytic activity, and decreased capacity to damage host-effector cells. Additionally, there was also greater phagocytosis and killing of the *ΔΔsapp1a-ΔΔsapp1b* yeasts by both human peripheral blood mononuclear cells (PBMCs) and PBMC-derived macrophages (PBMC-DM). After phagocytosis, the *ΔΔsapp1a-ΔΔsapp1b* yeasts induced more frequent phagolysosomal fusion, suggesting a role for Sapp1p in promoting intracellular survival.[30] Both the lipase and proteinase genes play a major role in biofilm formation which is the hallmark of both bacterial and fungal organisms involved in device related infection. While the basic characteristics for biofilm formation may be similar among biofilm producing organisms, unique differences exist as well.

Biofilm formation

Colonization and infection due to *C. parapsilosis* are initiated by the organism's ability to adhere to host cells and tissues followed by the formation of biofilm on medical devices,[81] which is accomplished in part by cell surface hydrophobicity[67] and slime production.[4] Compared to *C. albicans*, *C. parapsilosis* has a 20.6% greater avidity to buccal epithelial cells and 143.7% increased adhesiveness to acrylic material,[67] although smaller studies have shown less significant differences.[81] After adherence, biofilm formation is initiated by the establishment of cellular layers via cell-to-cell contact.[75] In *C. albicans*, after adherence to tissues, the cells transform from yeast to hyphal forms, which appear to be a requirement for a structured biofilm.[3,73] Once the mature biofilm is formed it is covered by an extracellular matrix that renders it less susceptible to antifungal medications. The transcriptional changes that occur during this process have been well studied in *C. albicans*, which reveal significant increases in the expression of genes that participate in glycolysis, amino acid and lipid metabolism.[24,50] *C. parapsilosis* and *C. albicans* differ in the nature of the biofilm formed. *C. parapsilosis* forms smaller and less complex structures,[29,37] possibly because they do not produce hyphae. *C. parapsilosis* biofilms consist of yeast and pseudohyphal cells.[17,23,37] Of note, pseudohyphal phenotypes can generate more biofilm and exhibit greater invasiveness into agar than strains in yeast forms.[42] Similarities exist as well between the two species: biofilms of both are inhibited by exogenous farnesol[42,76] and Bcr1 (Biofilm and Cell wall Regulator 1) is a major regulator for both species.[17,60,61]

BCR1

In vivo rat catheter models have been utilized to describe the role of *BCR1* in the development of biofilms.[18] *BCR1* has been demonstrated to be a required fungal transcription factor for the formation of biofilms in both *C. albicans* and *C. parapsilosis*.[17,60,61] Deletion of *BCR1* in either species impairs the ability for biofilm formation.[17,61] In *C. albicans*, *BCR1* encodes genes targeting adhesins and cell-wall proteins (*ALS1*, *ALS3*, *HWP1* and *RBT5*) indicating its role in the early adhesive stages of biofilm formation.[60–63] The effect of Bcr1 on biofilm formation is also thought to be in part secondary to its influence on the expression of CFEM (Common in Fungal Extracellular Membranes) family of proteins. CFEMs were initially identified in *Magnaportha grisea*. They are similar to epidermal growth factor (EGF) domains that are found in extracellular membrane regions and contain an eight-cysteine domain.[39,40] The role of CFEM is likely to act as cell surface receptors (adhesins).[39] At least 5 CFEM members have been identified in *C. albicans*: *PGA7*, *PGA10*, *RBT5*, *CSA1* and *CSA2*. Three of them, *PGA10*, *RBT5* and *CSA1*, have been identified as vital for biofilm development.[68]

C. parapsilosis has seven CFEM members (*CFEM1-CFEM7*) including tandem duplicates of orthologs of *C. albicans RBT5*, *PGA10* and *CSA1*.[18] *CFEM1-CFEM4* are in tandem and syntenic with *RBT5* and *PGA-7*, which in *C. parapsilosis* is postulated to have single gene duplications thereby forming *CFEM1/CFEM2* and *CFEM3/CFEM4*. *CFEM5-6* are orthologous with *CSA1*. An ortholog of *CFEM7* has not been identified in the *Candida* clade and may possibly be specific to *C. parapsilosis*.[18] Bcr1 exerts a different influence on the *CFEM* family in *C. parapsilosis*. In Bcr1d mutants, one member of each orthologous pair of *CFEM2*, *CFEM3* and *CFEM6* are down regulated, whereas expression of *CFEM1*, *CFEM4*, *CFEM5* and *CFEM7* is not affected.[18] Interestingly, *CFEM 2, CFEM3* or *CFEM6* do not appear to be a requirement for the formation of biofilms in *C. parapsilosis*. *CFEM2* and *CFEM3* are necessary and *CFEM6* is partially required for the heme utilization. Global transcriptional profiling of cells in an iron-depleted environment has shown the increased expression of 59 genes and decrease in 89 genes. Increased expression occurred in genes linked with cellular iron ion hemostasis and iron ion transport (*FTH1*, *FRE9* and *FRE10*). Decreased expression was seen in heme containing and iron sulfur proteins (*YHB1*, *SDH2* and *ISA1*) and all mitochondrial genes.[18]

RBT1

When *C. parapsilosis* produces biofilms there is an upregulation of the genes involved in the glycolysis, fatty acid metabolism and ergosterol synthesis.[75] These changes are similar to the ones observed when *C. albicans* cells are grown under hypoxic conditions.[79] Although *C. parapsilosis* does not produce true hyphae, the genome includes a member of the hyphae producing gene family, Hwp1.[76] One of the members in this family, the gene *RBT1* is induced during the production of biofilms and under hypoxia.[76] In *C. albicans, RBT1* is induced during filamentation and mutants have been demonstrated to have decreased virulence in rabbit and mouse cornea models.[5,33] In *C. parapsilosis, RBT1* knockout isolates produce structurally much thinner biofilms than wild type while the heterozygous strains produce an intermediate thickness biofilm. This suggests that for full and appropriate biofilm development both of the *RBT1* alleles are required.[75]

Lipid metabolism

Fatty acid formation is vital for the functioning of organisms in all kingdoms. They are building blocks of cell membranes that are products of cellular biosynthesis and require a complex enzyme

system.[58] So far, three major fatty acid synthesis systems have been identified. Eukaryotes and advanced prokaryotes (*Mycobacterium*, *Nocardia* and *Corynebacterium*) utilize Type I Fatty Acid synthesis system (*FAS1*), most bacteria utilize Type II, and parasites such as *Trypanosma* and *Leishmania* use *FAS3*.[43] While similar enzymes are found in the three *FAS* systems, the organization of the encoding genes may significantly vary.[58] In fungal organisms, production of essential fatty acids such as saturated and unsaturated fatty acids is critical for the generation and maintenance of cell membranes. Fatty acid synthase (*FAS*) and fatty acid desaturase (*OLE*) are important enzymes in this pathway.[55]

Fatty acid synthase

Fungal FAS enzymes initiate the formation of a 2,6-MD heterodacemeric complex including subunits that are encoded by *FAS1* and *FAS2*.[58] These enzymes are therefore critical for normal yeast growth, and disruption of even a single gene can significantly alter the organisms' physiological phenotype and virulence. Fas2 inhibition has been demonstrated to attenuate pathogenicity of *Cryptococcus neoformans*, *C. parapsilosis* and *C. albicans*.[9,90,91]

C. parapsilosis Fas2 (encoded by *CpFAS2*) heterozygous, homozygous and reconstituted Fas2 mutants have been generated.[58] *CpFAS2* is required for growth in standard medium and gene expression was repressed in the presence of fatty acids.[58] Further, up-regulation of *CpFAS2* occurred when only glucose was made available as the carbon source. In comparison to a wild type yeast, *CpFAS2* disruptants had a significant decrease in the concentration of unsaturated fatty acids.[58] *CpFAS2* genes are also necessary for normal biofilm formation. Microarray studies demonstrate that *CpFAS2* is upregulated during *in vitro* biofilm formation under hypoxic stress. *Δfas2* strains were shown to have decreased capability for biofilm formation on polystyrene and silicone surfaces.[58] *CpFAS2* also appears to play a vital role in helping the organism survive the fungicidal activity of monocytes such as neutrophils and macrophages. Intracellular survival of *CpFAS2* disruptants was reduced by 40% when compared with wild type and heterozygous strains with a single *FAS2* gene.[58] This decrease in survival could likely be due to diminished membrane stability, thereby enhancing susceptibility to reactive oxygen species secreted by the macrophages. The *Δfas2* strains demonstrate a leaky phenotype when grown under stress conditions. This action, in combination with defective fatty acid production, alters intracellular viability and proliferation of strains with *CpFAS2* deletion.[58]

The Fas2 enzyme also appears to be critical for survival of *C. parapsilosis* in serum.[55] This is a vital process in fungal pathogenesis as exposure to serum influences virulence traits such as filamentation and biofilm formation.[59] Efficacy of antifungal drugs is decreased in serum, making the eradication of systemic infections difficult.[65] *CpFAS2* disruptants are hypersensitive to serum and induce cell death.[55] Cell survival also appears to be influenced by the presence of glucose, which causes mitochondria-dependent cell death. Mechanisms of glucose toxicity in *Δfas2* strains are yet unclear, as glucose is usually a preferred carbon source for normal yeast growth. Toxicity may potentially be secondary to uncontrolled metabolism of glucose by the mutant cells, thereby creating an imbalance of cellular contents and subsequent triggering of a cell death response,[55] especially via energy-requiring apoptosis pathways, overproduction of ROS, nuclear fragmentation and cell shrinkage.[27,84]

Fatty acid desaturase (OLE1)

In addition to glucotoxicity, elevated lipid content is thought to be detrimental to normal yeast growth.[57] In *C. parapsilosis*, exposure to high glucose induces formation of lipid droplets (LD); it is considered a possible mechanism through which yeast cells survive gluco- and lipo-toxicity.[57] Both the *CpFAS2* and fatty acid desaturase genes (*OLE1*) appear to play a role. Disruption of either of these genes inhibits LD formation from glucose.[57] Moreover, *OLE1* inhibition resulted in gluco/lipotoxicity of log phage yeast cells. While survival of wild type yeasts was decreased after 2 days of incubation, *Δole1* mutants died within a few hours of incubation, indicating its role in glucose detoxification.[57] In addition to glucose, *OLE1* deletion strains are also hypersusceptible to fructose, galactose, and mannose.

FIT2

Lipid droplets (LDs) are cytoplasmic compartments that contain triacylglycerides (TAGs) that serve as fatty acid reservoirs[46] and lipid precursors of fatty acids (FA) and diacyl glycerol (DAG) for membrane lipids such as glycerophospholipids and sphingolipids.[13,41] TAGs and steryl esters are major components of LDs and utilize fatty acyl CoA as a common substrate.[72] Akin to mammalian cells, LDs may significantly influence the functioning of pathogenic fungi. In *C. parapsilosis*, formation of LDs is necessary for normal cell growth and virulence.[56] A family of proteins involved in LD formation – fat storage-inducing transmembrane proteins 1 and 2 (*FIT1* and *FIT2*) – has recently been demonstrated in both humans and mice.[35,56] In mammalian cells, *FIT2* orthologs enable compartmentalization of TAG in LDs and depleting *FIT2* transcripts in adipocytes decreased LD formation.[35] The *FIT2* proteins are primarily localized to the endoplasmic reticulum and are responsible for lipid partitioning rather than lipid synthesis.[35] Effects of *FIT2* on LD accumulation are therefore likely downstream in comparison to the DGA1 pathway.[64,80]

In *C. parapsilosis*, disruption of *FIT2* impairs LD formation, alters the lipidome and attenuates virulence.[56] Both *in vitro* and *in vivo* studies demonstrated that heterozygous strains with deletion of one *FIT2* allele did not decrease TAG formation or LD size. However, deletion of both *FIT2* alleles significantly decreased TAG content (39%) and LD accumulation.[56] Alterations were also noted in other lipid species with *FIT2* deletion. While a 25% reduction was observed in fatty acids (FFA), unsaturated fatty oleic (4.63%) and linoleic acids (12.31%), elevations were noted in steryl esters (135%), diacylglycerols/sterols (96%) and phospholipids (112%), palmitic (1.5%) and stearic acids (15%).[56] However, *FIT2* deletion did not alter susceptibility to standard antifungal drugs.

Conclusions

The epidemiology of microorganisms that impact human morbidity and mortality is constantly evolving. Globally, there is increased life expectancy, improved and more frequent access to the healthcare system, rising use of immunosuppressants, immunomodulators and biologics, and increased utilization of indwelling devices such as catheters and cardiovascular devices. Microbes that have the ability to evade the host immune system, produce biofilms and develop resistance to available treatment options have and will significantly impact morbidity and mortality. This is well exemplified by the globally increasing prevalence of *C. parapsilosis* infections. A significant work has been accomplished in identifying the genetic factors that determine the organism's virulence, and increased efforts are urgently needed to counter this global threat.

Conflict of interests

The authors have no conflicts of interest.

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