Infection and Drug Resistance

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REVIEW

Current Aspects in the Biology, Pathogeny, and Treatment of *Candida krusei*, a Neglected Fungal Pathogen

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Abstract: Fungal infections represent a constant and growing menace to human health, because of the emergence of new species as causative agents of diseases and the increment of antifungal drug resistance. Candidiasis is one of the most common fungal infections in humans and is associated with a high mortality rate when the fungi infect deep-seated organs. *Candida krusei* belongs to the group of candidiasis etiological agents, and although it is not isolated as frequently as other *Candida* species, the infections caused by this organism are of special relevance in the clinical setting because of its intrinsic resistance to fluconazole. Here, we offer a thorough revision of the current literature dealing with this organism and the caused disease, focusing on its biological aspects, the host-fungus interaction, the diagnosis, and the infection treatment. Of particular relevance, we provide the most recent genomic information, including the gene prediction of some putative virulence factors, like proteases, adhesins, regulators of biofilm formation and dimorphism. Moreover, *C. krusei* veterinary aspects and the exploration of natural products with anti-*C. krusei* activity are also included.

Keywords: virulence, candidiasis, host-fungus interplay, antifungal drug, immune sensing

Introduction

Candidiasis is the infection caused by members of the fungal genus *Candida*, which can be a superficial or a deep-seated disease. The latter is often associated with high morbidity and mortality rates, in particular in hospitalized or immunosuppressed patients. A rate of 3–28 patients out of 1000 intensive care unit admissions in European hospitals develop candidemia;[1] and in the United States of America the scenario is similar, as the Transplant-Associated Infection Surveillance Network reported that 3.8% of solid organ transplant recipients developed invasive candidiasis.[2] Studies that have enrolled cancer patients admitted into hospitals placed in Europe or the Middle East showed a 36–39% mortality rate after one month of hospitalization,[3,4] and these figures suffer minor modifications when the mortality associated to systemic candidiasis among intensive care unit patients is analyzed, which has been calculated in 48%.[5] This rate though can scale to figures in the range of 63–75%, depending on the hospital and the patient's staying ward.[6] Thus, there is no doubt that candidemia represents a global healthcare problem and a significant burden on patients.

Candida albicans is the most frequent etiological agent of candidiasis, although other *Candida* species are also relevant in the clinical setting, causing about 35–65%

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of candidemia cases.[7,8] These include *Candida tropicalis, Candida parapsilosis, Candida glabrata, Candida guilliermondii, Candida dubliniensis, Candida auris*, and *Candida krusei*.[6,9] Collectively, these species are the causative agents of non-*albicans* candidiasis and infections by *C. krusei* are characterized because of their high mortality rate (40–58%) and poor response to standard antifungal therapies.[10,12]

Due to the clinical relevance of this organism and the significant amount of information generated in recent years; here, we provide a literature revision on the *C. krusei* basic and clinical aspects.

Biological and Fungal Aspects

C. krusei produces cylindrical yeast cells that may have up 25 µm of length (Figure 1). They usually resemble long-grain rice, which contrasts with the spheric or ovoid shape of other *Candida* species.[13] Like *C. albicans, C. krusei* shows thermodimorphism, producing hyphae when growing at 37°C and blastoconidia and pseudohyphae when incubated at lower temperatures (Figure 1).[14,15] The colony morphology is the typical one of other *Candida* species, with no obvious features that could provide a hint about the species: round, creamy, and smooth whitish colonies of about 5–8 mm diameter when grown at 25–28°C in rich culturing media, such as malt yeast extract glucose agar, yeast extract peptone glucose agar, or Sabouraud agar (Figure 1). Even though colony morphology switching has been reported,[13] no systematic attempt to classify the morphological variations has been reported, like those in *C. parapsilosis, C. tropicalis*, and *C. albicans*.[16–18] One interesting contrast with other medically relevant *Candida* species is the presence of sexual reproduction in *C. krusei*, being *Issatchenkia orientalis* the teleomorph.[13]

Like other fungal cells, a cell wall, intracellular vesicles, endoplasmic reticulum, mitochondria, ribosomes, and intracellular glycogen-like granules have been described when cells are inspected under transmission electron microscopy.[19] Importantly, the microscopical examination indicates that these are mononuclear cells.[19] Thus far, the study of the *C. krusei* organelles has not been reported in detail, with the exception of the cell wall. This bias in the study of *C. krusei* components is likely to be related to the relevance of this structure during the interaction with the host and because it is a target of some antifungal drugs, as revised in the following sections. The early study of the *C. krusei* cell wall by transmission electron microscopy showed the presence of three major layers: the outermost is an electron-dense layer that includes flocculent material surrounding the cell, followed by an electron-transparent layer in the middle with the appearance to be composed of fluffy material and scatter granules, and an innermost electron-dense layer closer to the plasma membrane.[19] Recently, our group characterized the basic components of the *C. krusei* cell wall and found that contains the same polysaccharides found in the *C. albicans* wall: chitin, β-glucan, and mannans.[20] Even though both species have similar levels of cell wall β-glucan, the chitin content is a 4.1-fold higher in *C. krusei* than in *C. albicans*, and mannan is 34% less abundant in *C. krusei* when

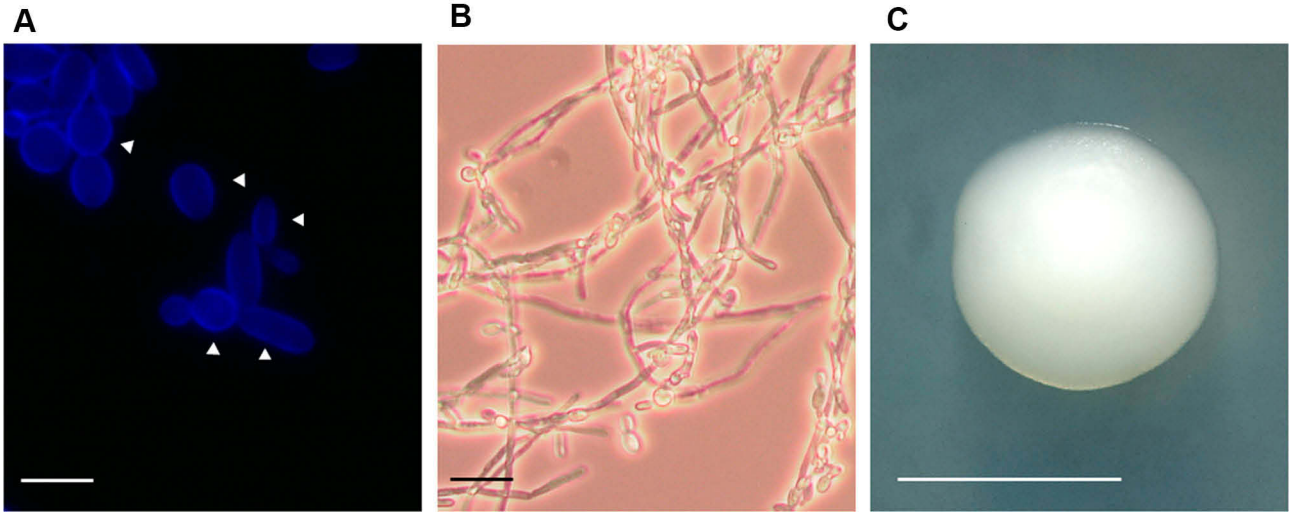

Figure 1 *Candida krusei* cell and colony morphology. (**A**) Yeast cells were grown in YPD broth until reach the exponential phase and then stained with calcofluor white, to label chitin. Scale bar = 10 µm. The arrowheads indicate the mother cells. (**B**) Cell filamentation was stimulated in RPMI medium incubated at 37°C. Scale bar = 20 µm. (**C**) A *C. krusei* colony grown on a YPD plate. Scale bar = 5.0 mm. Images from panels A and B were taken with a Zeiss Axioscope-40 microscope and an Axiocam MRc camera.

compared to the *C. albicans* mannan content.[20] In agreement with this observation, the cell wall protein content, and *O*-linked and *N*-linked mannans decorating the *C. krusei* wall glycoproteins were lower than those found in *C. albicans*.[20] The structural study of the *C. krusei N*-linked mannans indicated that the outer chain is short and lightly branched with α-1,2-mannose units,[21] which supports our observations and contrasts with the structure of the *C. albicans N*-linked mannans, where the outer chain is highly branched with α-1,2-mannose units and capped with either α-1,3-mannose or β-1,2-mannose residues.[22] In regard to the *C. krusei O*-linked mannans, these are oligosaccharides composed of α-1,2-mannose units that can contain from two to four sugar residues,[23] which again contrasts with the *O*-linked mannans found on the *C. albicans* surface, that may contain up to seven α-1,2-mannose residues.[24] Like other *Candida* species, the *C. krusei* mannans are modified with mannose residues bound via phosphodiester links, named phosphomannan, although the content of this is about the half of the phosphomannan found in the *C. albicans* cell wall.[20]

Like in other *Candida* species, the *C. krusei* structural polysaccharides chitin and β-1,3-glucan are localized underneath other cell wall components, and this impairs the proper sensing of these polysaccharides by the host immunity.[20]

Thus far, only one report dealing with the *C. krusei* cell wall proteome has been reported, but this was performed with walls from cells growing in the presence of oxidative stressors.[25] Interestingly, only moonlighting proteins were identified, which could be a result of contaminants from intracellular compartments, since cells were disrupted with an ultrasonic homogenizer.[25] Nonetheless, the presence and abundance of canonical cell wall proteins found in other *Candida* species remain to be established.

The metabolism of this fungal species is another aspect poorly studied to date. This yeast cell is capable of using exclusively glucose as carbon source,[13] which is a trait exploited in its identification by zymograms and chromogenic culturing media.[26] This has also been taken in advantage to produce and accumulate glycerol in fermentative processes with potential industrial applications,[27] and to prepare traditional meals and alcoholic beverages used by some African communities.[28,29] The fermentation process involving *C. krusei* is positively affected by the presence of lactic acid bacteria, which promote tolerance to short-term changes in the extracellular pH.[30] Interestingly, and contrary to this restricted carbohydrate assimilation, *C. krusei* has been isolated from the decaying wood from *Ficus religiosa*; suggesting this could be an environmental niche of this fungal species.[31] Even though arabinitol is produced by many yeast-like cells and the presence of this metabolite has been reported in serum from patients with invasive candidiasis, *C. krusei* is incapable of producing this five-carbon polyol.[32]

In regard to the *C. krusei* genome, it has been reported this species does not belong to the CUG clade of the *Candida* genus, it is a diploid and heterozygous organism, with the genetic information distributed in five chromosomes.[33–35] The *C. krusei* genome sequence of a clinical isolate (strain 81-B-5) showed that the nuclear genome sequence size is 10.9 Mbp, the mitochondrial genome contains 51.3 kbp, the single nucleotide polymorphism rate was calculated in 1/340 bases, being higher than that reported for *C. albicans* isolates.[36] Moreover, it was reported a GC content of 38.42%, a 2.15% of repeat content with no significant similarity to the repeat sequences found in *C. albicans*, and a total of 4949 protein-encoding genes.[33,35] The number of intron-containing genes in the coding regions was calculated in 205.[35] Different from other *Candida* species where high variability in the mating loci has been reported,[36] these subtelomeric loci are complete in *C. krusei*.[33] A recent study reported the sequencing of 32 strains of *C. krusei, Pichia kudriavzevii, Issatchenkia orientalis*, and *Candida glycerinogenes* and found that they are the same species with more of 99% identical DNA sequences.[35] Since the analysis of single nucleotide polymorphisms could not segregate between clinical and environmental strains, it was suggested that infections by *C. krusei* are acquired from the environment.[35] Finally, the analysis of the genome sequences supported the re-classification of these organisms in the *Pichia* genus, being a distant relative of the *Candida* species.[35]

Recent Understanding of the Host-Pathogen Interaction

Virulence Factors

Since *C. krusei* and *C. albicans* belong to the same taxonomical genus, it has been assumed they share biological traits that help them to interact with the host, a wrong rationale that applies not only to *C. krusei* but other medically relevant non-*albicans Candida* species.[20,37-39] Therefore, the study of *C. krusei* virulence factors is a research area with limited information, if compared

with the vast amount of reports dealing with *C. albicans* virulence. In this section, we will provide the most relevant information about *C. krusei* virulence factors and a genomic comparison to predict putative orthologs of well-known factors already described in *C. albicans.*

Virulence is classically determined by the ability to damage cells, tissues, organs, or a whole organism, and in mycology, both the in vitro and in vivo systems are thoroughly used. In the murine model of systemic candidiasis, *C. krusei* was incapable of killing both female or male mice, contrasting with the high mortality rate associated with *C. albicans*.[40,41] When the colony-forming units of these organisms were analyzed in infected spleen, liver, kidneys, and lungs, a gradual reduction in the *C. krusei* burden was observed during the observation period, with a fungal clearance at day 21 post-infection, contrasting again with the fungal loads in *C. albicans*-infected organs, which were constants or slightly reduced during the same observation period.[40] Therefore, *C. krusei* displays lower virulence than *C. albicans* in the murine model of systemic candidiasis. This in vivo system is regarded as the gold standard to assess the virulence of *Candida* species and isolates, but in recent years, logistical issues to include large numbers of animals per experimental condition and the increased ethical concerns about the use of these animals in basic research have limited their inclusion in the experimental design and stimulated the search for alternative models to study fungal virulence. *Caenorhabditis elegans* is an invertebrate model that has been used as an alternative for studying the *Candida* species virulence.[42] Upon administration of fungal cells by feeding, *C. krusei* and *C. albicans* showed similar ability to kill *C. elegans* and were ranked as the most lethal species in this experimental setting.[42] Like *C. albicans, C. krusei* was capable of producing aspartyl proteinases, phospholipase, hemolysins, and to develop biofilms, providing a possible explanation to the lethal behavior in the *C. elegans* system.[42] Moreover, this invertebrate model has been useful in establishing the effect of the antibacterial drugs cefepime, imipenem, meropenem, and vancomycin on *Candida* spp. virulence. *C. albicans, C. parapsilosis, C. krusei*, and *C. tropicalis* incremented the proteolytic activity and killing of *C. elegans* upon incubation with these drugs, whereas amoxicillin potentiated the virulence of *C. krusei* and *C. tropicalis*.[43] The wax moth *Galleria mellonella* has been proved as a good model to study infections caused by *C. krusei*. Upon injection into the hemocele, fungal cells decreased hemocyte density, induced melanization and animal dead.[44] The virulence in this host is similar to that observed in the murine model, with *C. krusei* showing low to moderate ability to kill *G. mellonella* (median survival of larvae was 7 days), which contrast with the high mortality associated with *C. albicans* infection (median survival of larvae was 2 days)[45] In addition, these larvae have helped to propose that *Lactobacillus paracasei, Lactobacillus fermentum*, and *Lactobacillus rhamnosus*, acid bacteria used as probiotics, have a prophylactic effect on the larvae, increasing the animal survival upon administration of a *C. krusei* lethal dose.[46] *Drosophila melanogaster* is another invertebrate model that has been used to evaluate the *C. krusei* virulence. Adult flies with mutations in the toll signaling pathway were highly susceptible to infection with either *C. albicans* or *C. krusei*, demonstrating this model is useful for virulence assessment.[47] Moreover, these data strongly suggest that *C. krusei* lethality depends on the immunological status of the host, as in immunocompetent animals this *Candida* species was not capable of killing the host.[40,41]

Cell and tissue adhesion are part of the early stage of the *Candida*-host interaction and will lead to the establishment of both commensalism and pathogenesis. Adhesion is paramount to establish colonization and tissue invasion in the oral epithelium, as this tissue is in constant contact with saliva, which cleans the epithelial surface. *C. krusei* binds to human buccal epithelial cells but not as efficient as *C. albicans* and *C. tropicalis*.[48] Accordingly, exposure of the epithelial cells to the minimum inhibitory concentration of nystatin affected the *Candida* species adhesive properties, with *C. krusei* showing a 64% reduction of adhesion to epithelial cells, a value higher to that found in *C. albicans* (54%).[48] Similar to epithelial cells, endothelial cells are also a surface where *C. krusei* can adhere, but not as efficiently as *C. albicans*.[49] In agreement with these observations, *C. krusei* showed 11-fold lower colonization potential of the rat oral surface than *C. albicans*.[50] However, *C. krusei* adheres in great numbers to acrylic surfaces.[50] Interestingly, when the *C. krusei* phenotypical switching was induced with phloxine B, a 30-fold increment in adhesion to saliva-coated glass surface was observed.[51] The Eap1, Iff4, Mp65, Phr1, Int1, Ecm33, and *ALS* gene family members are the major *C. albicans* adhesins.[52–58] The putative functional orthologs of the genes encoding for Phr1 and Int1 were identified within the *C. krusei* genome but no those encoding for Eap1, Iff4, Ecm3 or any of the Als family members

(Table 1). Interestingly, three putative orthologs of *C. albicans* Mp65 were identified (Table 1).

The cell wall hydrophobicity is an important aspect of interaction with the host components. A study that involved 20 *C. krusei* isolates showed these had higher wall hydrophobicity than *C. albicans* cells, and there was a correlation between hydrophobicity and adhesion to HeLa cells, but not to acrylic surfaces.[59]

Several studies have demonstrated that *C. krusei*, like other *Candida* species, is capable of secreting hydrolytic enzymes that could degrade host macromolecules, contributing to nutrient acquisition, to degrade immune effectors, or in the dissemination within the host tissues. In two independent studies, using clinical isolates, *C. krusei* did not show phospholipase activity, contrasting with *C. albicans* strains that had a strong presence of this enzyme activity.[60] However, other studies have shown that this enzyme activity is found in *C. krusei*.[61–63] Whether this discrepancy is due to different methodologies used to measure phospholipase activity or reflects the phenotypical plasticity of the species remains to be addressed. Nonetheless, the *C. krusei* genome contains two putative orthologs of the *PLB* gene family that encodes for the major *C. albicans* secreted phospholipase activity (Table 1).[64,65] Interestingly though, no putative orthologs of the genes encoding for secreted lipases belonging to the *C. albicans LIP* gene family[66] were found within the *C. krusei* genome (Table 1).

Other hydrolytic activities like proteinase, hemolytic factors, and DNase have been reported in *C. krusei*.[60,67,68] A study that included clinical isolates from Turkish patients diagnosed with candidiasis, found that about half of the *C. krusei* isolates formed biofilms, 22% showed coagulase activity and all the isolates were capable of hemolyzing red blood cells.[69] In *C. albicans*, most of the secreted

Table 1 Prediction of Some Virulence Factors in *Candida krusei*

Virulence Factor	*C. albicans* Gene	*C. krusei* Gene*	E value**	Similarity (%)**
Adhesins	*EAP1*	No found	–	–
	IFF4	No found	–	–
	MP65	ONH75632	$5e^{-118}$	66
		ONH70941	$1e^{-68}$	55
		ONH73292	$8e^{-42}$	55
	PHR1	ONH72606	0.0	72
	INT1	ONH72359	$2e^{-57}$	53
	ALS	No found	–	–
	ECM33	No found	–	–
Secreted hydrolases	*PLB1-PLB5*	ONH77577	$1e^{-166}$	61
		ONH74522	$2e^{-88}$	51
	LIP5 or *LIP8*	No found	–	–
	SAP1 – SAP5	ONH77652	$2e^{-39}$	47
		ONH72963	$4e^{-22}$	45
		ONH70287	$2e^{-22}$	42
		ONH77630	$5e^{-20}$	46
		ONH77640	$7e^{-10}$	55
Biofilms	*HSP90*	ONH74083	$4e^{-123}$	92
	BCR1	ONH74628	$8e^{-29}$	70
	EFG1	ONH73730	$9e^{-66}$	92
	ROB1	No found	–	–
	BRG1	OUT23966	$9e^{-21}$	67
	ZAP1	OUT21350	$2e^{-59}$	52
Dimorphism	*HGC1*	AWU73609	$4e^{-16}$	45
	NRG1	ONH70717	$1e^{-27}$	64
	TUP1	ONH77322	0.0	67
	CPH1	OUT20780	$2e^{-79}$	59

Notes: *Gene nomenclature corresponds to accession codes of the GeneBank database (https://www.ncbi.nlm.nih.gov/genbank/). **When comparing the encoded protein of *C. krusei* gene with the putative ortholog in *Candida albicans*.

proteolytic activity is associated with members of the *SAP* gene family.[70,71] A search for putative orthologs of members of this gene family in *C. krusei* identified *SAP1-SAP5*, but not *SAP6-SAP10* (Table 1). These genes are likely to account for the secreted proteolytic activity reported in *C. krusei* clinical isolates.

C. krusei is capable of forming biofilms on polyethylene, polyvinylchloride, and glass.[67,72] These fungal biofilms are particularly sensitive to fluconazole when generated on polystyrene surfaces.[73] This represents a promising observation that remains to be confirmed in vivo. In agreement with the reported ability to generate biofilms, the *C. krusei* genome contains key genes involved in the establishment of these multicellular communities. The chaperone Hsp90, besides contributing to the establishment of apical growth during the *C. albicans* dimorphism, is required to neutralize the cellular stress generated during biofilm formation;[74] while Efg1, Brg1, Zap1, and Bcr1 are transcriptional factors required for biofilm formation in both in vitro and in vivo conditions.[75–77] Putative orthologs for these genes were found within the *C. krusei* genome (Table 1). However, for the case of Rob1, a transcriptional factor required for biofilm formation in *C. albicans*,[76] no putative ortholog was found within the *C. krusei* genome (Table 1), suggesting that the regulatory network of biofilm formation in both organisms could share master regulators but their contribution might be species-specific.

As mentioned, *C. krusei* belongs to the members of the genus capable of forming true hyphae. The *C. albicans* dimorphism has been associated with tissue invasion and the expression of several virulence factors that are morphology specific.[78] The Hgc1 is a hypha-specific G1 cyclin essential for the establishment of the apical growth and is negatively regulated by the transcriptional repressors Nrg1 and Tup1; while Cph1 and Efg1 are transcriptional factors required to sustain hyphal growth.[79–81] The *C. krusei* genome contains putative orthologs of these genes (Table 1), suggesting the central regulatory network that controls dimorphism is similar in both species.

In this regard, it is noteworthy to mention that *C. krusei* is capable of inhibiting the *C. albicans* filamentation and biofilm formation.[82] Even though there is no formal explanation yet for these observations, the production of signaling molecules by *C. krusei* to avoid polarized growth in *C. albicans*, competition for nutrients, adherent surfaces, and space between the biofilms are the main hypotheses currently under study.[82]

Gliotoxin is an immunosuppressive compound from the fungal metabolism and has been suggested as a fungal virulence factor, including in *C. albicans*.[83] However, a chemical analysis based on highly sensitive HPLC and tandem mass spectrometry of 100 clinical isolates of *Candida* spp, including *C. albicans* and *C. krusei* did not detect intracellular or extracellular gliotoxin production, suggesting this compound does not participate in the pathogenesis of *Candida* spp.[83] In the same line, the *C. albicans* cytolytic peptide toxin named candidalysin, which is essential for mucosal infection and encoded by *ECE1*,[84] has no putative ortholog within the *C. krusei* genome.

It is noteworthy to mention that *C. krusei* has been isolated from bat feces in a Brazilian urban region, and showed the ability to secrete proteases, to form biofilms, and kill laboratory animals, suggesting the animal depositions could be an environmental source for *C. krusei* infections.[85] Similarly, *C. krusei* has been isolated from droppings of healthy breeding rheas, chickens and hens,[86,87] and from the vestibule and vagina of healthy female horses.[88]

The *C. krusei*-Immune System Interaction

Similar to other pathogens, the *C. krusei* recognition and interaction with components of the host immunity is required to establish a response that could protect against the infection. Both the innate and adaptive branches of immunity are essential to control fungal pathogens, including *C. albicans* and other causative agents of candidiasis.[89,90]

As part of the humoral factors that belong to the innate immunity, some cells produce antimicrobial peptides that show antifungal properties. The human β-defensin 2 is produced by epithelial cells, while the human neutrophil peptides 1–3 are α-defensins synthesized by circulating white blood cells.[91] Both kinds of antimicrobial peptides were stimulated by *C. albicans* and *C. krusei*, being the former a stronger inductor than *C. krusei* cells.[91] With no doubt, this study showed the ability of *C. krusei* to stimulate both local and systemic responses against this pathogen. Similarly, it was reported that *C. krusei* is 1.4 times more sensitive to lactoferrin, a secreted antimicrobial protein, than *C. albicans* cells; and this difference has been suggested to be relevant to modulate the fungal oral carriage.[92]

The peripheral blood mononuclear cells (PBMCs) are a heterogeneous group of immune cells with the ability to produce cytokines upon the interaction between pathogen-associated molecular patterns and their pattern recognition receptors.

Different to the human PBMCs-*C. albicans* interaction, where low levels of TNFα, IL-6, IL-1β, or IL-10 were stimulated, the immune cells interacting with *C. krusei*

produced higher levels of these four cytokines.[20] Both *C. albicans* and *C. krusei* heat-killed cells, which expose inner wall components like β-1,3-glucan and chitin at the cell surface, stimulated higher levels of TNFα, IL-6, IL-1β, or IL-10, when compared to live cells.[20] Interestingly, a difference between *C. krusei* and *C. albicans* was observed when *O*-linked mannans were removed from the cell wall: *C. albicans* recognition by PBMCs was not affected, indicating this wall component is dispensable for cytokine stimulation; while in *C. krusei*, cells with no *O*-linked mannans on the surface were capable of stimulating higher cytokine levels, most likely because of the unmasking of β-1,3-glucan and increased recognition via dectin-1.[20] Like in *C. albicans*, this immune receptor is essential to control *C. krusei* infections, as dectin-1 knock out mice are more susceptible to *C. krusei* and showed poor ability to establish a protective anti-*C. krusei* immunity.[93]

In contrast with *C. albicans* cells, *C. krusei* yeast cells induced lower levels of complement components C3 and factor B, and the granulocyte-macrophage colony-stimulating factor, but a significant amount of IL-12 (p70).[94,95] This differential ability to stimulate IL-12 (p70) could be part of the *C. albicans* strategies to avoid the establishment of an effective type I immune response against this pathogen, a situation likely to occur fo the case of *C. krusei*.[94]

Contrary to this observation though, *C. albicans* is more readily phagocytosed by neutrophils than *C. krusei* cells (37% vs 9%),[96] and more susceptible to the neutrophil-expressed antimicrobial protein S100A12 than *C. krusei*,[97] underscorings that the differences in pathogenicity and control by the innate immune system are difficult to be reduced to the analysis of a handful of biological parameters.

Like other pathogens, *C. krusei* is capable of interacting with macrophages, but interestingly the outcome is variable. Rat alveolar macrophages phagocytosed *C. glabrata* and *C. albicans* in a similar rate, but this was significantly slower for the case of *C. krusei*, due to reduced attachment.[98] This differential recognition was abrogated though when the fungal uptake was performed with opsonized cells.[98] For the case of primary human PBMC-derived macrophages, the results are the opposite: *C. krusei* is more readily phagocytosed than *C. albicans, C. auris, C. tropicalis*, and *C. guilliermondii*.[20] In mice, both a macrophage-like cell line and primary macrophages are capable of uptaking *C. krusei* yeast cells, with around 10 to 20% of the immune cells ingesting yeasts after 2 h, without opsonization.[99] However, *C. krusei* was capable of surviving and undergoing filamentation inside the phagocytic cells, induced defects in the phagolysosome maturation, yeast transfer between infected macrophages, macrophage fusion, and death of the immune cells.[99] These data clearly show that the origin of the immune cells has to be taken in to account before drawing general conclusions.

Pyroptosis, an inflammasome-mediated macrophage death process, is activated upon interaction with *C. albicans* cells.[100] This caspase-1-, ASC-, and NLRP3-dependent pathway is triggered in lower levels by *C. krusei* cells and does not restrict the fungal replication.[100]

The interaction between *C. krusei* with dendritic cells has particular outcomes too. The *C. krusei* mannan but not the cell wall component isolated from *C. albicans, C. tropicalis* or *C. glabrata* induced strong cytokine production by these immune cells and led to apoptosis.[101] These effects on dendritic cells were mediated by TLR2 and activation of a MyD88-dependent pathway, which controlled the production of the polarizing cytokines IL-12 and IL-6, and thus the Th1/Th17 switching.[101]

Interestingly, human PBMCs tend to proliferate in the presence of either voriconazole or caspofungin and produce increased levels of IL-2, IFN-γ, and IL-6 when stimulated with either *C. albicans* or *C. krusei*, with no effect on the stimulation of TGF-β and IL-10.[102] These data suggest that antifungal therapy has a positive immunomodulatory effect on human PBMCs, an observation that should be further explored and taken into account during the treatment of candidiasis and other fungal infections.

Another promising study on new immunomodulatory approaches for the treatment of candidiasis caused by *C. krusei* involves chromogranin A, a mammalian-expressed soluble protein of the adrenal medullary chromaffin granules and neurons. Chromogranin A N-46, a 46 amino acid portion of the chromogranin A N terminal has shown to have antifungal properties.[103] In line with this observation, treatment with this peptide (60 mg/kg/day) had positive effects on mice infected with *C. krusei*. Treated animals showed increments in the body weight and survival, along with higher counts of circulating monocytes, lymphocytes, and neutrophils.[103]

Candidiasis Caused by *C. krusei*

The list of the etiological agents of candidiasis is vast and new species have been added in recent years; however, most of the cases are caused only by five species, named

C. albicans, C. glabrata, C. tropicalis, C. parapsilosis, and *C. krusei*.[104] Even though *C. albicans* is the most common cause of candidemia worldwide, infections caused by *C. krusei* are an emergent public health threat. Data from the ARTEMIS DISK registry gathered from 1997 to 2007 indicated that the frequency of *C. krusei*-associated infections was stable, ranging from 1.7 to 3.2%.[104]

A report from a tertiary care academic hospital in Montreal, which included 190 cases of candidemia, found that *C. krusei* was the causative agent in 7% of the patients, but this increased up to 13% in the period of 2003–2006. [105] The most likely explanation for this observation in a specific period of time was the systematic prophylaxis with fluconazole in risk groups to develop candidemia.[105] Similarly, a Cancer Center of Texas, USA, and a tertiary health care center localized in Haryana, India reported that 8% and 9% of candidemia cases were associated with *C. krusei*, respectively, but in these cases, the studies were conducted in a pediatric population.[106,107] Another study from the USA, but in this case performed in Ihowa, reported that 3.4% of candidemia cases were due to infection with *C. krusei*, and these showed the lowest 90-day survival rate.[108] A study that enrolled 26 short-stay university hospitals in the Paris area reported that the candidemia incidence caused by *C. krusei* in patients not admitted to ICU was 5.2% in patients with hematological diseases, 3.7% in patients with an oncological condition, and 1.2% in patients with no malignancy diagnosed; while in patients in ICU, the figures were 5.1%, 4.4%, and 2.3% for patients with a hematological disease, oncological conditions, and no malignancy, respectively. [109] Similarly, studies conducted in the Republic of Korea, Australia, Spain, the USA, India, Taiwan, Saudi Arabia, and Portugal reported that 2.6%, 4%, 6%, 5%. 3.3%, 4%, 6%, and 5% of candidemia cases were caused by *C. krusei*, respectively, with 25% of the Portuguese isolates resistant to posaconazole.[110–118] Contrary to these figures though, a study based on a tertiary care hospital in North China found that only 0.9% of the candidemia cases were caused by *C. krusei*.[110–118] Similar to this Chinese study, reports from two Greek, one Brazilian, one Swiss consortium of hospitals, and one Mexican tertiary hospital found that 1.8%, 0.2%, 1%, 2%, and 2.2% of candidemia cases were caused by *C. krusei*, respectively,[119–123] and a study conducted in neonates admitted to ICU, participating in the National Nosocomial Infection Surveillance system from 1995 to 2004 in the USA reported only 0.15% of candidemia cases associated to *C. krusei*.[124]

As an outlier report, a study carried out in the University of Texas M. D. Anderson Cancer Center with clinical records of patients admitted from 1993 to 2003 found that *C. krusei* was the causative agent of 24% and 2% candidemia cases in patients with hematological malignancies and solid tumors, respectively.[125] The authors of this study proposed that this prevalence in patients with hematological conditions is due to the prevalent use of fluconazole as a prophylactic antifungal agent, especially in patients with hematological malignancies and recipients of bone marrow transplantation.[125] The figures above reported were similar in the period of 2001 to 2007 in the same cancer center, where 17% of candidemia cases were caused by *C. krusei*.[126]

The risk factors for fungemia due to *C. krusei* include the recent surgery report (< 30 days), artificial implants, splenectomy, neutropenia, the presence of oncological conditions such as solid tumors, acute leukemia, or lymphoma as an underlying disease; stem cell transplantation, preexposure to fluconazole, echinocandins or antibacterial agents, specifically vancomycin or piperacillin-tazobactam.[104,109,110,125,127,128] At first glance, it is difficult to relate the use of antibacterial agents with the risk to acquire an infection caused by *C. krusei* or other *Candida* species. It has been proposed that vancomycin can alter the ecology of the normal skin microbiota, promoting colonization by *Candida* species and thus increasing the potential to develop a systemic infection; while anti-anaerobic antibacterial agents such piperacillin-tazobactam, may promote overpopulation of yeast species and colonization of the gastrointestinal tract.[128] In neonatal patients, among the risk factors associated with *C. krusei* fungemia are parenteral nutrition, recent fluconazole exposure, use of broad-spectrum antimicrobials, and the presence of a percutaneous inserted central catheter.[129,130]

Besides the systemic disease, *C. krusei* is also associated with superficial infections. This organism can cause bronchopneumonia and vulvovaginal candidiasis but is a rare etiological agent in the latter, being isolated only in 0.1% of cases and has a good response to nystatin.[131,132] *C. krusei* has been also found infecting the tonsils, where only surgical removal of the organ offered a permanent cure, causing septic arthritis, ulcers, urinary tract infections, and vasculitis[133–136]

In veterinary, this organism can also cause infections and deteriorate the health conditions in animals. *C. krusei*

was reported as the causative agent of bovine bronchopneumonia and mastitis in Japan, China, Turkey, Algeria, Canada, Polony, and the United Kingdon;[137–143] while in cats could be responsible for the failure of long-term gastrostomy tubes.[144] For the case of bovine mastitis, it has been suggested that wheat silage, rather than unappropriated milking is the source of the *C. krusei* cells affecting the udder.[145] Despite it is part of the normal microbiota of birds, C. *krusei* has been associated with gastrointestinal diseases in white-crowned parrots (*Pionus senilis*),[146] and acute necrotizing ventriculitis in Eclectus parrot (*Eclectus roratus*). [147]

C. krusei Identification

Since *C. krusei* belongs to a fungal genus that contributes with several species as etiological agents of candidiasis, the methods for identification of *C. krusei* have been originally developed to discriminate *C. albicans* from other species. Nonetheless, the following strategies have been refined to identify other *Candida* species, including *C. krusei*.

Biochemical reactions are the most common strategies for speciation of *Candida* isolates, and these have been taken into account to develop chromogenic media that easily differentiate *Candida* species, depending on the color and morphology of the colonies growing on the plates. *C. krusei* generates purple fuzzy, large rough colonies with flat pale edges when grown on HiCrome Candida; while the color change to pink fuzzy when cultured on CHROMagar Candida (CHROMagar) or CHROMagar-Pal's plates.[148] In Brilliance™ Candida Agar (formerly Oxoid Chromogenic Candida Agar, OCCA) this species grows like dry, irregular pink-brown colonies.[149] Even though this colony color could be informative for species identification, it could be mistaken with the one generated by other species that develop pinkish colonies, such as *C. parapsilosis, Candida kefyr*, and *Candida haemulonii*.[148,149] Another medium for quick detection of *Candida* species is Candi*Select*™ 4 (BioRad), where *C. krusei* generates large turquoise-blue colonies with a characteristically rough morphotype, a dry appearance, and an irregular outline. However, *C. tropicalis* and *C. glabrata* also grow like smooth, turquoise colonies, making the species identification troublesome.[150] Due to these limitations, these media are used for preliminary species identification, and additional phenotypic or genotypic tests should be included for the proper identification of *C. krusei* and other *Candida* species. The API ID32C method is currently the gold standard for phenotypic characterization of these organisms, but another alternative is Micronaut-*Candida* (Bornheim), a microplate-based system that contains 21 biochemical reactions, and 14 carbohydrate assimilation tests (melibiose, D-xylose, L-rhamnose, gentibiose, D-glucose, inositol, cellobiose, saccharose, trehalose, galactose, maltose, lactose, raffinose, and a control reaction), and urease test with its control. Results are generated in 24 h and interpretation assisted by the Micronaut software.[151] This strategy proved to be as good as the API ID32C method for the *C. krusei* identification.[151] The Vitek 2 system (bioMérieux) is an automated alternative for *C. krusei* identification based also on biochemical reactions and has 100% specificity to identify this species.[152]

Another alternative for *Candida* identification based on phenotypic traits is the analysis of volatiles using offline gas chromatography and mass spectrometry. The p-xylene, 2-octanone, 2-heptanone, and n-butyl acetate are signature volatiles of the *C. krusei* presence in in vitro analyses.[153]

Among the molecular strategies, PCR is one of the techniques thoroughly analyzed and applied for the identification of *C. krusei* and other *Candida* species. It was reported that a single primer pair aiming to amplify a fragment of *L1A1* gene, which encodes for a cytochrome P-450 lanosterol-14α-demethylase, was capable of detecting fungal DNA in clinical specimens with a sensitivity of 200 fg of DNA but the amplicon size was not useful for *Candida* species discrimination, as this was in the range of 336 to 350 bp for all the analyzed species.[154] However, combined with restriction enzyme analysis using *Hinc*II, *Nsi*I, and *Sau*3A it was generated a species-specific pattern of restriction fragments.[154] Similarly, the amplification of the gene coding for the small ribosomal subunit 18S-rRNA and restriction with *Alu*I, *Ban*I, *Bbs*I, *Dra*II, *Eco*147I, and *Nhe*I generated a species-specific profile able to discriminate *C. krusei* from other *Candida* species.[155] The PCR-RFLP method amplifying the ITS1-5.8S-ITS2 rDNA region followed by restriction with *Msp*I was reported as another alternative to identify this organism.[156]

Alternatively, a PCR method using a primer pair that amplifies the polymorphic species-specific repetitive sequence *C. krusei* repeated sequence 1 (CKRS-1) of the non-transcribed intergenic regions of rRNA genes showed 100% specificity and a sensitivity to detect 10 to 100 fg of purified DNA.[157] Another alternative for *C. krusei*

identification by PCR is the amplification of part of the gene encoding for topoisomerase II. A nested PCR reaction using a combination of degenerate and specific primers was reported to amplify a characteristic 227 bp DNA fragment from the *C. krusei* genome, with 100% specificity and a sensitivity of 40 fg of genomic DNA.[158]

The real-time PCR has been also adapted for *C. krusei* identification. The amplification of the 5.8 rRNA gene demonstrated that was possible to obtain positive reactions with a detection limit of 10 CFU/mL blood, with 100% specificity.[159]

More recently, it has been developed the CanTub-simplex PCR based on the amplification of the gene encoding for β-tubulin in a real-time platform, where the amplicons melting temperature is species-specific.[160] Following the same rationale, amplification of the internally transcribed spacer region 2 and analysis of melting peaks and curves, allowed the discrimination of *C. krusei* from the other 15 *Candida* species included in the study.[161]

The current molecular alternatives developed for *C. krusei* identification also include microarrays. Based on the nucleotide sequences of the internal transcribed spacer regions (ITS1 and ITS2) of the rRNA gene a chip to identify 32 fungal pathogens was recently reported.[162] Even though the results of the microarray and the automated system Vitek 2 were concordant in 96.7% of cases for all the pathogens tested, for the case of *C. krusei* the specificity was of 100%.[162]

The multianalyte profiling system has also been adapted for the identification of *Candida* species. This consists of mixed polystyrene beads covalently linked to specific DNA probes for *C. albicans, C. glabrata, C. tropicalis, C. parapsilosis, C. krusei*, and *C. dubliniensis*, which are incubated with amplicons containing the ITS2 region of *Candida* species rRNA gene previously amplified with universal fungal primers. The beads conjugated with the corresponding amplicons are analyzed in the multianalyte profiling system flow cytometer that measures the fluorescence produced by the different pairs of amplicons and beads.[163] This approach was 100% specific and showed a sensitivity limit of 0.5 pg of DNA.[163]

The strategies for *C. krusei* identification also include immunological tests, although these are not as specific as the molecular methods. The Krusei color test (Fumouze) is a latex beads agglutination assay performed with red particles coated with a monoclonal antibody that specifically reacts with a *C. krusei* antigen found on the cell surface. Although all the *C. krusei* strains used in the study agglutinated the latex beads, false-positive reactions were observed with *C. famata, C. glabrata, C. guilliermondii, C. kefyr, C. parapsilosis*, and *C. tropicalis*.[164]

Therapy

The treatment of *Candida* infections includes the use of several kinds of family compounds, named polyenes, azoles, echinocandins, nucleoside analogs, and allylamines. Fluconazole is one of the most common antifungal drugs used for empirical treatment of candidiasis; however, *C. krusei* is a species intrinsically resistant to this drug, with more than 95% of clinical and veterinary isolates being fluconazole-resistant.[141,165] The mechanisms behind this observation are not fully understood yet, but the flux pump activity of the ATP-binding cassette transporter Abc1 and reduced fluconazole affinity to Erg11 have been associated with this phenotypic trait.[165,166] It has been also proposed that both proteins could be part of the resistance mechanisms observed in some itraconazole-resistant strains.[165] The in vitro acquisition of resistance to voriconazole has been reported after exposing daily *C. krusei* to 1 μg/mL of the drug. In these cells, drug resistance was associated with increased expression of the *ABC1* gene and point mutations within *ERG11*.[167]

Several studies conducted with clinical samples have shown that most of the *C. krusei* strains are susceptible to voriconazole, itraconazole, posaconazole, anidulafungin, micafungin, 5-flucytosine, and amphotericin B; but intermediate resistance to caspofungin has been reported in some isolates.[168–170] Although *C. krusei* is a rare etiological agent of vaginitis, the use of local clotrimazole, ciclopirox olamine, terconazole, and boric acid is recommended.[171,172]

In veterinary infections though, isolates resistant to fluorocytosine, itraconazole, ketoconazole, and amphotericin B have been reported.[141] The pharmacological alternatives to treat candidiasis caused by *C. krusei* in animals include sulphamethoxypyridazine in cases of bovine mastitis.[173]

There is a vast amount of studies addressing the search and design of compounds with antifungal activity, as well as the use of herbal derivatives with anti-*Candida* activity, with the potential of being explored as new alternatives to control candidiasis. Among the most relevant new alternatives are VT-1161 and VT-1129, a new generation of CYP51 inhibitors, a lanosterol 14-α-demethylase that belongs to the cytochrome P450 family and has a role in ergosterol biosynthesis, which showed the inhibition of *C. krusei* growth at concentrations of ≤ 2 μg/mL after 24 h of incubation.[174] Another alternative that is currently under investigation is the use of nanoparticles to deliver antifungal drugs into the fungal cells. It has been recently

demonstrated that lipid core nanocapsules containing fluconazole were capable of reducing the effective dose of this antifungal drug and reverted the resistance to fluconazole observed in several *C. krusei* strains.[175] The use of palmatine, in combination with either fluconazole or itraconazole, has shown antifungal synergism, in a mechanism that inhibits the efflux pumps, with the consequent increment in the intracellular azole content.[176]

A trypsin inhibitor from *Tecoma stans* (yellow elder) leaves has been isolated, characterized, and shown to have anti-*Candida* activity.[177] The minimal inhibitory concentration for this compound against *C. krusei* cells was 100 µg/mL, whereas the minimal fungicidal concentration was 200 µg/mL. ATP depletion and lipid peroxidation are thought to be the mechanisms behind its antifungal activity. In addition, it showed no cytotoxicity against human PBMCs,[177] being a promising candidate to move forward in the search for new antifungal compounds to treat candidiasis. Flavonoid and tannic fractions from *Psidium guajava L*. contain high levels of phenolic compounds and showed anti-*C. krusei* activity that synergizes with fluconazole and affects the morphological transition.[178] Similarly, flavonoids from *Plinia cauliflora* leaves, which mainly contain glycosylated quercetin and myricetin showed inhibitory activity against *C. krusei* (minimal inhibitory concentration of 19 µg/mL) and low cytotoxicity effect on human cells.[179] The water-insoluble fraction from *Uncaria tomentosa* (cat's claw) showed a synergistic effect with either terbinafine or fluconazole in a mechanism that involves the action of the plant proanthocyanidins on the fungal cell wall.[180]

These studies show that traditional medicine, mainly based on herbology, could be a source of a new generation of antifungal drugs.

Animals are also a source of molecules with antifungal activity. The 2-lysophosphatidylcholines isolated from deer antler extracts showed fungistatic activity, suppressing the morphological transition in *C. albicans, C. krusei, C. guilliermondii*, and *C. parapsilosis*, in a mechanism mediated through the mitogen-activated protein kinase pathway.[181]

Concluding Remarks

In recent years, there is a significant amount of information gathered about *C. krusei* biological and clinical aspects, underscoring the relevance of this organism as an emergent species, most likely because of its intrinsic resistance to fluconazole. The *C. krusei* genomic sequencing has opened new doors for basic research in this organism that could be translated into clinical applications. The genes prediction, along with their organization within the genome, the proteomic, transcriptomic, and metabolomic analyses could unveil species-specific genes related to virulence or drug resistance, information that could be later exploited in the diagnosis or treatment of the infection. The isolation of this organism from vegetal material and animal dropping points out to the environment as the source of candidiasis caused by *C. krusei* and provides the background to expand this kind of analysis to get a closer panorama of the *C. krusei* ecological distribution. The further expansion of our current knowledge on the *C. krusei*-host interaction would discover singularities in this species, which might be exploited for the design of alternative strategies to control the disease caused by this and other *Candida* species. This is of particular interest because thus far no vaccine is currently available to prevent candidiasis.[182] However, new immunotherapeutic approaches, and the ultimate development of a vaccine against *Candida* species will rely on the deep knowledge of the immunity against these organisms.

Even though there are phenotypical and molecular strategies to identify *C. krusei* available in the clinical setting, faster, cheaper and more accurate alternatives are desirable for the early diagnosis of *C. krusei* and other *Candida* species. We provided some examples of natural products that could have antifungal activity, and these efforts should be replicated and look into the mechanisms behind the antifungal effect, as these compounds could be part of a new generation of drugs to treat candidiasis and other fungal infections. Finally, the search for the mechanisms behind the resistance to fluconazole in this species would provide useful information for the design of new treatment alternatives.

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Disclosure

The authors declare no conflicts of interest in this work.

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