

ORAL *Candida* spp. COLONIZATION IN HUMAN IMMUNODEFICIENCY VIRUS- INFECTED INDIVIDUALS

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ABSTRACT: Several yeast species of *Candida* genus can colonize the skin as well as the mucous membrane of the vagina and the digestive tract for short or long periods. Depending on the host's immunological state and the yeast's virulence, colonization can become an infection, invading the colonized tissues and also disseminating. AIDS is characterized by the host's intensive and progressive immunodepression which manifests as diverse symptoms, mainly lesions in the mouth. Oral candidiasis is the most prevalent opportunistic infection in individuals infected with human immunodeficiency virus (HIV) and is an important indicator of the disease progress and the immunosuppression increase. The factors involved in the equilibrium between *Candida* spp. and HIV-infected subjects are sometimes contradictory and were evaluated in the present study specially for colonization.

KEY WORDS: *Candida* spp., colonization, human immunodeficiency virus-infected individuals.

CONFLICTS OF INTEREST: There is no conflict.

CORRESPONDENCE TO:

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INTRODUCTION

The genus *Candida* was created during the IX International Botanical Congress, held in Canada in 1959, and substituted the term *Monilia*, used until then (58).

Candidiasis is an infection caused by a yeast species of the genus *Candida*, which belongs to the family Cryptococcaceae. This genus included 196 species with physiological affinity for both basidiomycetes and ascomycetes (73). Then, species that have affinity for basidiomycetes were excluded, but the genus continued heterogeneous, presenting 163 species, which makes difficult its taxonomical characterization (56).

As dimorphic fungi, *Candida* yeasts have globose, ellipsoidal, cylindroidal, or elongate, occasionally ogival, triangular or lunate cells. Reproduction is by holoblastic budding. Pseudohyphae and septate hyphae may be formed. Arthroconidia and ballistoconidia are not formed (73,117).

In general, yeasts have asexual reproduction, by budding or fission, and each asexual reproductive unity is called blastoconidium. Some yeasts produce chains of blastoconidia that do not separate, forming a pseudohypha. The capacity of forming filaments, or filamentation, is strictly related to tissue invasion and, consequently, to pathogenicity (45, 57, 68).

Berkhout (7) was concerned about *Candida* yeasts existing in nature as well as about species found in men as saprophytic or parasites that were isolated from the skin, oral cavity, intestines and urogenital system. *Candida* species are confined to human and warm-blooded animal reservoirs; however, they can also be recovered from soil, food, water and, sometimes, air (5). The human gastrointestinal system is considered the biggest habitat for *Candida* spp. which are most frequently isolated from distal segments (23).

Mucous membrane surfaces constitute the largest interface between the host and the environment and develop several defense mechanisms to combat microorganisms, particularly pathogenic ones (67, 119). *Candida* yeasts can be the agents of local or systemic opportunistic infections, mainly in hospitalized patients, especially those under intensive treatment (1). Infections caused by opportunistic agents including *Candida* spp. are frequent in diverse pathological states that induce immunodeficiency such as neutropenia, neoplasia, decompensated diabetes mellitus, malnutrition, organ transplantation, and AIDS (61).

PATHOGENICITY AND PATHOGENY

Virulence Factors of *Candida* Genus

There are differences in pathogenicity among *Candida* spp. isolates. Some properties related to *Candida albicans* cells give them the capacity to cause disease. Adherence to cell surface, germ tube formation with consequent development of the filamentous form, phenotypic variability, and production of toxins and extracellular enzymes constitute important factors for the emergence of infections by *Candida* (13, 14, 30).

Ghannoum & Abu-Elteen (36) proposed an infection model in which the sequence was initiated by the yeast adherence to epithelial cells of the skin and mucous membrane, followed by cell multiplication and latter formation of germ tube and filaments. The enzymes produced then, especially proteinase and phospholipase, allowed the yeast penetration into the cells, inducing inflammatory response with injury of adjacent tissues.

Depending on the host's immunological state and on the microorganism's virulence, the latter can invade tissues causing infection which may disseminate. Enzymes destroy, alter or damage the integrity of the host's cell membrane, leading to dysfunction or interruption of its activities. Lipids and proteins of eukaryotic cell membranes constitute a target for such enzymatic attack. Thus, pathogenicity of *Candida* spp. is attributed to their different properties such as production of exoenzymes like phospholipases (47) and proteinases (46).

1. Aspartyl proteinases (SAPs)

The role of SAPs produced by *C. albicans* has been one of the main aims of physiological and biochemical studies on such yeast. These enzymes have proteolytic activity at low pH values (2.0–4.0) with a highly broad action including keratin, collagen, albumin, hemoglobin, immunoglobulin heavy-chain and extracellular matrix proteins (21). SAPs can be studied in culture medium containing bovine albumin as the only source of nitrogen by verifying quantitative differences among *Candida* species (22, 95, 96).

MacDonalds & Odds (64) observed a direct correlation between pathogenesis and proteolytic activity of *Candida* spp. isolates, showing that *C. albicans* mutants, proteinase-deficient, had lower virulence. These isolates were more easily phagocyted by human and mouse polymorphonuclear leukocytes.

Pepstatin A, which is an inhibitor of SAPs, was used in the treatment of mice infected with *C. albicans* and produced a protective effect characterized by reduction in proteinase activity and microorganism virulence – findings that are probably correlated (54, 94).

In a study about infection of the human oral epithelium by *Candida* spp., the inhibitory effect of pepstatin A on SAPs determined a great reduction in lesions, indicating that the proteinase activity contributed to tissue injury (102).

Ollerte *et al.* (83) verified that the proteolytic activity of *C. albicans* isolates from AIDS patients was higher than that of isolates from subjects not infected with HIV, highlighting thus the importance of such enzyme in the pathogenesis of oral candidiasis in HIV-infected individuals. They also observed a direct correlation between proteinase activity and resistance to antifungals. *Candida albicans* isolates causing oral candidiasis and producing high proteinase levels were less susceptible to azole antifungals than *C. albicans* isolates from HIV-negative subjects.

Research on proteinase production by *C. albicans* isolates is generally carried out using the method of Ruchel *et al.* (95).

Candida albicans isolates are seeded at equidistant points on the culture medium and the plates are incubated at 37°C for seven days. A translucent halo of protein degradation formed around the yeast colony indicates enzymatic activity. The enzymatic activity (Pz) is defined as the ratio between colony diameter (Dc) and colony diameter plus degradation zone diameter (Dc + Dz), as follows:

$$Pz = Dc / Dc + Dz$$

Thus, Pz=1.00 indicates absence of proteolytic activity, whereas Pz<0.64, for example, means that the *Candida* spp. isolate is producing high levels of SAPs.

Some authors (92) proposed a classification for proteolytic activity, according to its intensity, at the ranges presented in Table 1.

Table 1. Characterization of proteolytic activity (Pz), as a function of the ratio between colony diameter (Dc) and colony diameter plus degradation zone diameter (Dc + Dz)*.

Pz Limits	Enzymatic Production
>0.49	Negative
[0.41–0.49]	Low
[0.31–0.40]	Intermediate
[0.21–0.30]	High
≤0.20	Very High

* Ribeiro *et al.*, 2004 (92).

2. Phospholipases

Phospholipases are hydrolytic enzymes capable of degrading phospholipids. Phospholipase B is the dominant fraction, being responsible for 90% of the extracellular activity of phospholipase produced by *Candida* spp. (97). The lipolytic activity of phospholipase is concentrated at the yeast cell poles, facilitating tissue adhesion and acting on lipidic constituents of the host's cell membrane, as demonstrated for phospholipase A and lipophospholipase (90).

Evaluation of the activity of phospholipase produced by *C. albicans* was standardized by Price *et al.* (89) using egg yolk which is rich in phospholipids, mainly phosphatidylcholine and phosphatidylethanolamine that serve as substrates for the enzyme. Phospholipase activity is assessed by measuring the precipitation halo represented by a calcium complex and fatty acids released due to the enzymatic activity.

Phospholipase activity (Pz) is defined as the ratio between colony diameter and colony diameter plus precipitation zone diameter, as follows:

$$Pz = Dc / Dc + Dz$$

where Dc is colony diameter and Dz is precipitation zone diameter.

Thus, $P_z=1.00$ indicates absence of phospholipase activity, whereas $P_z=0.63$, for example, means that the *Candida* spp. isolate is releasing high levels of phospholipase.

Candida spp. isolates from different sites and organic fluids of patients with disseminated candidiasis were noticed to have the same phospholipase activity. Such finding suggests that phospholipase production is not influenced by the infection site and that the presence of *C. albicans* in different organs and/or organic fluids of the same patient is indicative of systemic infection. Thus, phospholipase activity can be used for characterizing strains (82, 122).

Samaranayake *et al.* (97) evaluated the influence of incubation period, pH and sugar concentration in the culture medium on phospholipase activity. They demonstrated a direct correlation between phospholipase activity and incubation period until the fourth day, when enzymatic activity stabilized. Similarly, phospholipase activity was higher at pH 4.4 than at pH 3.3, but not at pH 5.1 or 6.3, although there was a great fungal growth at higher pH values. There was also a direct correlation between phospholipase activity and galactose or sucrose concentration in the culture medium. Contrastingly, increased glucose concentration (above 100mM) results in the complete cessation of phospholipase activity.

It is important to mention that the works of Price *et al.* (89) and Samaranayake *et al.* (97) demonstrated that: variation in the inoculum size does not interfere with phospholipase activity; P_z value of each isolate is relatively constant; and phospholipase-negative *C. albicans* isolates keep negative independently of the culture conditions.

A study with *C. albicans* in mice revealed a direct correlation between pathogenicity and phospholipase activity; 90% of *C. albicans* isolates showing high phospholipase production were pathogenic to mice (52).

Clancy *et al.* (22) showed that *Candida* species other than *C. albicans* also produced extracellular phospholipases, however, at lower levels than those secreted by *C. albicans*. Such difference can be attributed to variations in the isolates or differences in the medium preparation or in the substrate employed for phospholipase detection. Ghannoum (40), in an excellent review about the role of phospholipases on virulence and pathogeny of several fungus genera including *Candida*, suggested that fungal phospholipases can be evaluated as possible therapeutic effects.

Research on phospholipase production by *Candida* spp. isolates is commonly carried out using the method of Price *et al.* (89), with modifications suggested by the results of Samaranayake *et al.* (97).

Candida albicans isolates are seeded at equidistant points on the culture medium and the plates should be incubated at 37°C for four days. A turbid precipitation zone formed around the colony indicates the presence of the enzyme.

Some authors (92) proposed the classification of phospholipase activity, according to its intensity, at the ranges presented in Table 2.

Table 2. Characterization of phospholipase activity (Pz), as a function of the ratio between colony diameter (Dc) and colony diameter plus precipitation zone diameter (Dc + Dz)*.

Pz Limits	Enzymatic Production
>0.80	Negative
[0.71–0.80]	Low
[0.61–0.70]	Intermediate
[0.51–0.60]	High
≤0.50	Very High

* Ribeiro *et al.*, 2004 (92).

Pathogeny

The process by which *Candida* spp. colonizes and penetrates the epithelium of the digestive tube can be analyzed in four stages: initial adhesion to the epithelium; replication and colonization; formation of hyphae; and epithelial lesion and penetration (14, 45). Genes INT1 and PLB1 have been related to colonization (6, 60). Mechanical barriers, inflammatory cells and cellular immunity restrict *Candida* spp. to non-sterile superficial sites. Besides, the resident bacterial microbiota generally limits the number of fungal cells, blocks their adhesion to epithelial cells, competes with them for nutrients, and prevents the fungus conversion into its most invasive form, the filamentous form. When one or more of these defenses become deficient, infections by *Candida* spp. can develop.

Mucosal surfaces have a well regulated defense system, which is still little known. The mucous immune system has both a localized and a diffused portion. In the former, foreigner antigens are taken to highly structured sites, where the immune response begins. The diffused portion is composed of effector cells such as B and T lymphocytes, differentiated plasmatic cells, macrophages and other antigen-presenting cells, eosinophils, basophils, and mastocytes. Together, these two portions induce mucosal response, antibody production, cellular immune response or even tolerance, characterized by anergy.

A mucosal surface can include a huge area like the human gastrointestinal tract, with more than 300m², which requires a significant participation of lymphoid cells and effector molecules related to cellular immunity (124).

The pathogenicity of *Candida* spp. depends on the composition of their cell wall as the external surface is responsible for their interface with the host.

A study on *C. albicans* interaction with macrophages revealed a rapid phagocytosis of the fungus which undergoes morphological transformation, forming elongated germ tubes that penetrate the macrophage membrane, destroying it. This way, the fungal cell goes back to its environment. Such study demonstrated the importance of polymorphism, as mutants that did not form filaments showed to be avirulent (62).

Candida spp. antigens stimulate specific cellular and humoral immune response. The interest in studying antibody response in *Candida* spp.-infected animals has been renewed. The mannoprotein of 58 kDa found at *C. albicans* surface is highly immunogenic; it is expressed by all *C. albicans* isolates and stimulates intense antibody response during candidemias (16). The gene that codifies it is FBP1/PRH1. However, the role of humoral immunity in the defense against fungal infections, including those caused by *Candida* spp., is still highly controversial (44).

With regard to innate immunity, macrophages and neutrophils seem not to have a relevant role.

The Th1 branch of cell-mediated immune response is considered fundamental for such defense. Thus, when the number of CD4⁺ T lymphocytes is below a protective level, like in AIDS patients, other defense mechanisms are needed to protect against oral candidiasis.

Regulation of cytokines related to Th1 and Th2 branches plays an important role in the host response to deep infections caused by *Candida* spp. Interferon- γ , tumor necrosis factor- α (TNF- α), interleukin (IL)-12 and IL-15 present a direct action

increasing phagocytosis, superoxide production, and antifungal activity of mononuclear cells and neutrophils. Contrastingly, Th2 branch-related cytokines, IL-4 and IL-10, present immunosuppressive effect by compromising the last phase of monocytes response against *C. albicans*, i.e. microbicidal activity and hyphal lesions are reduced, whereas phagocytosis is not affected or even increased by the action of IL-10 (121). Transforming growth factor- β (TGF- β), a Th2-type cytokine, is expressed at high concentrations by hepatocytes and mononuclear cells of granulomatous lesions in chronic disseminated candidiasis. *Candida albicans* also induces, *in vitro*, the expression of TGF- β by circulating blood monocytes.

Besides, human and animal epithelial cells present antifungal activity. *In vitro* coculture of epithelial cells with *Candida* spp. reveals inhibition of fungal growth by human epithelial cells within only nine hours (33, 79, 109, 110). The inhibitory effect of oral epithelial cells on fungal growth is much higher than that of vaginal epithelial cells. However, such epithelial cells cannot destroy fungal cells. Thus, epithelial cells may represent an important mechanism of innate resistance against mucous candidiasis as they inhibit fungal cell growth, which is attributed to a portion constituted of carbohydrates (still not determined but existent) at the cells surface. On the other hand, *C. albicans* wild-type strains incubated with a layer of CaCO₂ epithelial cells in a Transwell^R system reduced transepithelial resistance after 7.5 hours. Such reduction was specific for *C. albicans* and required the transcription of factors CPH1 and EFG1 as well as proteinases SAP 4–6 (63).

Imam *et al.* (48) demonstrated that severe vaginal candidiasis can affect HIV-infected patients with low or no degree of immunosuppression of CD4⁺ T lymphocytes or CD4⁺ T/CD8⁺ T cell ratio. As immunosuppression increases, oral candidiasis can be observed, and in more immunocompromised patients, esophageal candidiasis is noticed. Thus, those authors suggested a hierarchy of mucosal infections caused by *Candida* spp. in HIV-infected patients.

IDENTIFICATION OF *Candida* SPECIES

There are several available procedures to identify *Candida* spp., most of which consist in the association of morphological and biochemical traits like the form and size of blastoconidia, the production of chlamydoconidia, pseudohyphae and true hyphae, and the capacity to assimilate carbohydrate and nitrogen and to ferment different sugars (56).

In Sabouraud agar and blood agar, *Candida* spp. grow within 24h–48h at 37°C in the form of marble-white, humid, circular, convex colonies. The colonies composed of blastoconidia can produce pseudohyphae. Chlamydoconidia and true hyphae can be formed under special nutrient and oxygen conditions. True hyphae are formed from yeast-like cells through development of the germ tube. Germ tubes are formed within three hours at 37°C, in the presence of albumin, only in *C. albicans* (56) and *C. dubliniensis* cultures (112, 114). Such characteristic is used in routine laboratory for identification.

About 95% of *C. albicans* isolates produce germ tube. In its absence, biochemical tests must be carried out. The difference between germ tube and pseudohypha is that the former does not present septa but parallel walls and has no constriction at the mother cell neck (91).

Chlamydoconidium or chlamydoconidium is a resistance structure formed by thick cell wall and condensed cytoplasm. It is generally produced when the yeast is under unfavorable growth conditions. Microculture, i.e. culture in slides, is carried out to verify the micromorphology of *Candida* spp., the existence or not of filamentation, the production of chlamydoconidia and blastoconidia, as well as the layout of such elements at the mycelium. *Candida albicans* and *C. dubliniensis* produce round chlamydoconidia at intercalary or terminal position to the pseudohypha (29).

Assimilation test, also called auxanogram, evaluates the capability of certain yeasts to utilize several compounds as the only source of carbon or nitrogen in the presence of oxygen. Each species has its own assimilation pattern. Such tests are highly sensitive and useful for yeast identification and are conditioned to permeability factors, enzymatic systems that catalyze carbon hydrate degradation, and the reductase system which intervenes in nitrate reduction.

The capability of a yeast species to ferment certain sugars (which is determined by the fermentation test or zymogram) depends on the presence of a transportation system that allows sugar absorption under low O₂ tension and on the existence of an enzymatic system that acts on sugar glycolytic degradation with formation of ethanol and carbon anhydride. The fermentation of a certain sugar contributes to distinguish between species (53)

In the recent past, manual and automated boards were commercialized for yeast identification by evaluating the assimilative capacity of biochemical and enzymatic substrates, which are easier to apply and allow faster interpretation than the

conventional methods auxanogram and zymogram (100, 104, 106). The systems API 20C AUX and ID32 BioMeriux (St. Louis, Mo), which contain dehydrated substrates for assimilation tests, are examples of semi-automated methods. Positive and negative results correspond to presence and absence of turbidity, respectively, in the wells of each substrate. Reading of these reactions is carried out by comparison with growth controls. Hypha or pseudohypha formation is also evaluated and, after reading, the biochemical profile is converted into a numerical profile composed of a seven-digit code. Interpretation is based on the analytical catalogue API 20 C AUX, in which the found numerical profile is compared with a database of 44 taxa. Such taxa can be a genus, a species, a biotype within a species or multiple species, i.e. a group of different species that cannot be distinguished by the performed tests. The identification is interpreted as: excellent (%id \geq 99.0 and T \geq 0.75), very good (%id \geq 99.0 and T \geq 0.5), acceptable (%id \geq 90.0 and T \geq 0.25) or unacceptable (%id \geq 80.0 and T \geq 0), in which %id is the percentage of identification or an estimate of how closely the profile corresponds to the taxon, relative to all other taxa in the database, and the index T is an estimate of how closely the profile corresponds to the most typical set of reactions for each taxon.

The Vitek system, bioMérieux Laboratories, is an automated process for the identification of several microorganisms, among which are *Candida* yeasts. The Vitek Yeast Biochemical Card (YBC) contains thirty tests, 26 substrates and four controls. Carbohydrate assimilation, urea hydrolysis, resistance to cycloheximide, and nitrate and nitrite reduction are evaluated (72).

It must be emphasized that the morphological and biochemical tests and the manual and automated boards for yeast identification do not allow differentiation between *C. albicans* and *C. dubliniensis*.

Recently, different chromogenic culture media capable of distinguishing *C. albicans* from other yeasts of clinical interest have been commercialized. Such media are based on the color alteration yielded by the colonies, which is measured by using indicators of pH and fermentation of specific compounds or chromogenic substrates for presumptive identification of *C. albicans*, *C. tropicalis* and *C. krusei*. β -glucosaminidase is used as substrate and yeasts are differentiated according to the morphology and color of colonies. The utilization of these media facilitates detection

and identification of the above-mentioned yeasts, providing presumptive results in less time than conventional methods and confirming the isolates purity (34, 35, 85). It must also be highlighted that chromogenic media do not allow a safe differentiation between *C. albicans* and *C. dubliniensis*.

MOLECULAR BIOLOGY

The molecular methods used for the study of candidiasis are mostly designed to verify the genetic similarity between isolates of the same species (107). Such methods can also be used to confirm phenotypic identification, once each species presents characteristic biochemical profile and morphological aspects.

Molecular typing methods based on different principles have been developed and applicable for various *Candida* species. Among several techniques are those based on pulsed field gel electrophoresis (PFGE) and polymerase chain reaction (PCR). PFGE karyotyping and PFGE of fragments generated by cutting restriction endonucleases were highly reproducible but expensive, laborious and time-consuming. These techniques were already applied for several *Candida* species (21, 87, 105).

Among the PCR-based methods, randomly amplified polymorphic DNA (RAPD) is the most used. It is easy and rapid but presents reproducibility problems inter and intra laboratories (107). There are reports about its application for several *Candida* species (86, 116). Recently, two new techniques arose: amplified fragment length polymorphism (AFLP) (3) and multilocus sequence typing (MLST) (12, 32, 115).

Ideally, more than one method or two or more random primers (in the case of RAPD) should be used in order to verify which of them has the best discriminating power for that species.

Because of the high degree of similarity between phenotypic characteristics of *C. albicans* and *C. dubliniensis*, various molecular techniques have been successfully proposed to identify *C. dubliniensis* (20, 65, 74).

COLONIZATION AND INFECTION BY *Candida* spp.

Candida spp. constitute the normal microbiota of the mouth of 25%–50% healthy individuals; this condition is called colonization (81). *Candida albicans* is the most prevalent species among colonizers; however, species other than *C. albicans* are also isolated (111).

Several yeast species of *Candida* genus, as well as some other microorganisms, can colonize mucosa, skin and digestive tract, transitorily or for long periods. However, when they are present at different sites and when the culture repeatedly reveals the same type of biological isolate, microbiota alteration is suggested. Excessive growth of *Candida* spp. at those colonization sites may facilitate tissue invasion, mainly in predisposed hosts (8).

About 70% of the healthy population has the gastrointestinal tract colonized by *Candida* spp., which can precede fungemia due to alteration in the resident microbiota and translocation of the pathogen through the gastrointestinal mucosa. The vast majority of candidemias follow colonization by the same yeast species. Genotyping methods for *Candida* spp. showed similarity between colonizing and infecting strains, indicating a probable endogenous origin of most infections (24, 80).

Any factor causing microbiota disequilibrium or mucosa lesion can be considered an agent that facilitates *Candida* spp. translocation to mesenteric capillaries. Factors that increase intestinal colonization by *Candida* spp. (such as antibiotic use and intestinal occlusion) or those that cause mucosa atrophy or lesion (such as prolonged fasting, total parenteral nutrition, arterial hypotension and chemotherapy) can reinforce the translocation phenomenon across the intestinal epithelium (1).

Rodero *et al.* (93) suggested that candidiasis begins with an endogenous commensal agent which converts into a pathogen in an immunocompromised host. The most frequent microorganism in these infections is again *C. albicans*, especially because it is the predominant species in the gastrointestinal tract. Candidiasis presents varied clinical manifestation. Cutaneous candidosis involves intertriginous areas of skin in the hands, groins and armpits; mucocutaneous candidiasis affects tissues of the oral and vaginal mucosa. The latter infects individuals with or without predisposing factors; however, its chronic form is one of the most severe forms of the disease, affecting patients with genetic or metabolic defects. Systemic candidiasis develops after hematogenic dissemination in susceptible hosts with predisposing factors, especially neutropenic patients. In such cases, tissue invasion may occur, leading to pulmonary candidiasis, nephritis, suppurative phlebitis, arthritis, osteomyelitis, endophthalmitis, balanitis, SNC lesions, myocarditis, pericarditis and endocarditis, in which obstruction of cardiac valves and embolism may also occur due to the large number of blastoconidia and pseudohyphae (13).

In the last years, the number of infections caused by species other than *C. albicans* has increased. In 1963, there were five *Candida* species capable of causing disease to human beings, *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. stellatoidea* and *C. guilliermondii*. Nowadays, about 20 species are known to cause infections in humans (25, 31).

Candida albicans, *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. guilliermondii* and *C. lusitaniae* are the main species of medical interest. However, cases of superficial or invasive diseases related to emerging *Candida* species have been reported including *C. kefyr*, *C. rugosa*, *C. famata*, *C. utilis*, *C. lipolytica*, *C. norvegensis*, *C. inconspicua*, *C. dubliniensis* (25) and *C. pelliculosa* (*Pichia anomala*) (66).

Candida albicans is the species most frequently isolated as colonizer or superficial and invasive infection agent at different anatomical sites all over the world. It has a well-known pathogenic potential and its main pathogenicity and virulence factors are: capacity to adhere to different mucosae and epithelia; dimorphism, with production of pseudohyphae helping tissue invasion; thermotolerance; and production of exoenzymes like proteinases and phospholipases (31). This species is naturally susceptible to all systemic-use drugs; however, cases of developed resistance to azoles have been described, mainly in subjects that had been exposed to such antifungals for a long time. Resistance to amphotericin B is rare (101).

Candida tropicalis can behave as an opportunistic agent when the host is neutropenic, when there is bacterial flora suppression due to antimicrobial use or when there is gastrointestinal mucosa injury. This species is responsible for candidemias in neoplastic patients and is more frequent in leukemia than in solid tumors (123).

Clinical isolates of *C. tropicalis* are susceptible to amphotericin B and, most of the times, to triazoles. In Latin American countries, especially Brazil, it is frequent even in non-cancer patients and constitutes the second or third most important cause of candidemia (26, 37, 38).

Since the 1980s, *Candida parapsilosis* has been an important cause of fungemia, being responsible for 7%–15% of candidemias in the United States (84) and Europe (120). It proliferates in solutions containing glucose, produces biofilm and frequently colonizes the skin. Fungemia by *C. parapsilosis* is associated with the utilization of venous catheter (84). Clinical isolates of such species are susceptible to

amphotericin B and triazoles (101). This species has been recognized as the second main cause of invasive infection in Latin American countries (26, 37, 38).

The epidemiology of *C. glabrata* and *C. krusei* infections is different from that of other species. These infections mostly begin after prophylactic use of antifungals (e.g. fluconazole) in patients with hematological diseases and are not associated with catheter use or previous antibiotic therapy. *Candida krusei* presents the highest lethality rate, followed by *C. glabrata* (84). It has intrinsic resistance to fluconazole, which may explain its increase in neutropenic patients exposed to this antifungal (123). *Candida glabrata* can develop resistance with exposure to fluconazole (84). Consequently, an increase in the colonization or infection rate by *C. glabrata* has been observed in different groups of patients subjected to prolonged exposure to fluconazole (84). Lower susceptibility to amphotericin B has also been recorded for this species (120).

Candida dubliniensis, described in Ireland in 1995 by Sullivan *et al.* (114), presents biochemical and morphological traits very similar to those of *C. albicans*, which requires molecular methods to distinguish between them. This new species was isolated from the oral cavity of AIDS/HIV-infected patients, among which 17%–35% are colonized or infected with *C. dubliniensis* (113). The risk factors for the presence of such species are not well established, but its virulence seems to be similar to that of *C. albicans*.

Proteinase activity is known to be higher in *Candida dubliniensis* than in *C. albicans*. The former also presents higher adherence to the oral mucosa but slower hypha formation, suggesting less invasive capacity (113). This new *Candida* species had probably been present in the community for a long time mistakenly identified as *C. albicans*.

Cases of systemic diseases related to this new species are still rare, and most of them are associated with oral mucosa infection. Such emerging species seems to be less pathogenic than *C. albicans* but more capable of developing resistance to azoles (55).

Candida lusitanae is a yeast species less frequently isolated as a causative agent of invasive diseases but has been reported as cause of candidemia in immunocompromised patients. Clinical isolates of *C. lusitanae* generally show natural resistance to amphotericin B or rapidly develop it, but are susceptible to triazoles (70).

Candida guilliermondii has been recognized as an emerging agent, although its invasive infections are not frequent yet. There are reports about *in vitro* resistance of clinical isolates to amphotericin B and reduced clinical response of patients treated with such polyene (43).

HIV infection is associated with an increase in the rate of subjects colonized by *Candida* spp., which can be observed even before the establishment of evident immunosuppression. The frequency of isolation of *Candida* spp. from the oral cavity of such patients increases with the cell immune compromising progress (88).

AIDS-CANDIDIASIS COINFECTION

AIDS is characterized by the host's intensive and progressive immunodepression, which is manifested by diverse symptoms. Oral alterations in AIDS patients include more than 40 types of lesions, which very frequently constitute the main manifestations of the disease (59, 71, 78, 108).

Oral candidiasis occurs in 90% AIDS patients, being the most prevalent opportunistic infection and an important indicator of the disease progress and the immunosuppression increase (71).

In a study about the oral manifestations observed in 100 AIDS or HIV-infected patients in Natal, Rio Grande do Norte State, Brazil, oral candidiasis was the most common manifestation (99 patients), presenting pseudomembranous (the most frequent form), followed by erythematous, hyperplastic and angular cheilitis forms (71), which confirms the findings of other authors (59).

HIV-infected women with low CD4⁺ T cell counts presented higher risk of oral *C. albicans* colonization and infection than those with high CD4⁺ T cell counts (118).

Following the introduction of highly active antiretroviral therapy (HAART), there was a reduction in occurrence of opportunistic infections, prevalence of oral manifestations and oral candidiasis (18). Since then, the frequency of candidiasis has decreased, even in the presence of viral resistance to the treatment or in the absence of noticeable CD4⁺ T cells recovery (77).

Arribas *et al.* (2) suggested that the reduction in the frequency of oral candidiasis was only related to immunological improvement after introduction of antiretroviral therapy including protease inhibitor (PI), which increases the number of CD4⁺ T cells.

However, some HIV-positive patients with relatively high CD4⁺ T cell counts developed oral candidiasis (49). On the other hand, Hoegh *et al.* (44) suggested that inhibitors of HIV protease inhibited the prevalence of oral candidiasis in HIV-infected patients by their direct action on the secretion of SAPs by *Candida* spp., which are considered to belong to the same class of HIV aspartic proteases. Thus, the success of antiretroviral therapy could be attributed to several factors such as inhibition of one or more *C. albicans* virulence factors and recovery of the immunological state of infected patients (2, 18, 41, 42, 46, 49, 77). SAPs by *C. albicans* was evaluated in 18 isolates from HIV-infected subjects with CD4⁺ cell counts inferior to 400 cells/mm³ and in other 18 isolates from HIV-seronegative subjects, which was the control group (125). The authors observed that all *C. albicans* isolates from HIV-positive patients secreted proteinase whereas only 56% of those from the control group secreted it. Proteolytic activity was higher in isolates obtained from the HIV-infected group. They also evaluated the inhibitory effect of polyene and imidazole antifungals, at levels below the minimal inhibitory concentration – MIC (1/4 and 1/16), on proteinase production in seven isolates from each group. There was a less intense inhibitory effect on proteinase production by isolates from HIV-infected patients, compared with that by isolates from the control group and, in general, there was a dose-dependent effect (125).

***Candida* spp. COLONIZATION IN HIV-POSITIVE PATIENTS**

In 1998, Schuman *et al.* (103) conducted a multicenter study in which they evaluated oral mucosa colonization by *Candida* spp. in 518 HIV-infected women, who had not developed AIDS, and in 207 at-risk HIV-seronegative women.

Colonization was more prevalent ($p < 0.001$) among HIV-infected (60.4%) than among seronegative women (47.5%). However, the prevalence of *C. albicans* colonization did not differ between groups, being 87.3% in the HIV-infected and 85.8% in the seronegative group. The prevalence of *Candida* species did not vary among seropositive women, according to CD4⁺ T lymphocyte counts. However, CD4⁺ T lymphocyte count was lower ($p < 0.004$) in colonized (363 cells/mm³) than in non-colonized seropositive women (405 cells/mm³).

Previous use of antifungals was associated with higher colonization by species other than *Candida albicans* ($p = 0.012$).

Among seropositive women, multivariate logistic regression analysis revealed an association of *Candida* spp. colonization with recent cigarette smoking and intravenous drug use. More than one *Candida* species could be isolated from the same sample in 11% cultures; this prevalence did not vary between groups.

Gottfredsson *et al.* (39) verified the intensity of oropharyngeal *Candida* spp. colonization and correlated the findings with viral load, CD4⁺ T lymphocyte counts, prophylaxis against pneumonia by *P. jiroveci* prior to fungal infection, and number of administered antiretroviral drugs. The authors revealed that 70% out of 63 studied patients were colonized, 56 out of 78 patients presented one single *Candida* species, and 92% of these species were *C. albicans*. The only factor related to colonization, assessed by multivariable regression analysis, was viral load.

However, Klein *et al.* (50) suggested that, besides the factors demonstrated by Gottfredsson *et al.* (39), the direct action of HIV on *Candida* spp. as well as the direct effect of PIs on *Candida* SAPs should also be taken into consideration. The direct action of HIV on *C. albicans* was revealed by Gruber *et al.* (42), in 1997, while showing that HIV glycoproteins increase the fungus virulence. Finally, the direct effect of PIs on *Candida* SAPs (which favors mucosal invasion) was demonstrated by Cassone *et al.* (18) and Blanco *et al.* (9). Several studies have proved that PIs are not only directed against HIV proteases but also against the production of *C. albicans* aspartyl proteinases, which belong to the same class of viral proteases (11, 17-19, 41, 42, 51, 75).

In 2002, Barchiesi *et al.* (4) studied *Candida* sp. colonization in 102 HIV-infected patients, observing 67% prevalence and isolating *C. albicans* from 63 out of 68 colonized patients. The prevalence of colonization was evaluated as a function of viral load, CD4⁺ T lymphocyte count, adopted antiretroviral regimen and previous oropharyngeal candidiasis. The only factor that influenced colonization was prior oropharyngeal candidiasis (p=0.009). The authors also demonstrated that 93% of the isolates were susceptible to fluconazole and 7% were classified as dose-dependent susceptible isolates.

Ribeiro *et al.* (92) studied the prevalence of *Candida* spp. in the oral mucosa of 332 women infected or colonized by this fungus; out of them, 127 were also infected with HIV (group I), whereas the remaining 205 were seronegative for such virus (group II). The authors observed that among HIV-infected women, 68% were colonized by different species of *Candida*, which could be isolated in all cases (100%) of oral

candidiasis. Among HIV-seronegative women, these values were 32% and 80%, respectively. There was high predominance of *C. albicans* among the species isolated from the oral mucosa in both groups: 79% in the HIV-negative and 78% in the HIV-positive group. At a decreasing frequency rate, *C. tropicalis* and *C. parapsilosis* were observed in group I, and *C. tropicalis*, *C. parapsilosis* and *C. guilliermondii* in group II. Isolates from HIV-infected patients had higher enzymatic activity than those from patients not infected with HIV. Patients under HAART treatment including PI presented *C. albicans* isolates with lower enzymatic activity which was equivalent to that observed in HIV-negative individuals.

Moris (76) assessed the prevalence of *Candida* spp. colonization in HIV-infected subjects as a function of species, T CD4⁺ and T CD8⁺ lymphocyte counts, plasma HIV viral load, use of viral PIs in antiretroviral treatment, and enzymatic activity of *C. albicans* isolates. A prevalence-period study was carried out in 156 HIV-infected subjects and 92 healthy subjects (control). The prevalence of colonization was higher in HIV-infected (84.0%) than in healthy individuals (28.25%). *Candida albicans* was more prevalent than non-*albicans Candida* both in HIV-infected (82.1% vs 17.9%) and in healthy individuals (85.2% vs 14.8%, all *C. parapsilosis*). The prevalence of *Candida* sp. colonization, according to CD4⁺ T lymphocyte count, independently of the HIV infection stage, tended to be higher in patients with counts <200 cells/mm³ (0.05 < p < 0.10). *Candida albicans* isolates showing low proteinase activity were prevalent, especially in HIV-infected non-AIDS individuals without the use of PIs. The prevalence of colonization by *Candida* spp., mainly *C. albicans*, showed a direct association with plasma HIV viral load. *Candida albicans* isolates presenting low proteolytic activity were more prevalent in AIDS patients who had undetectable viral load.

As it could be observed, few works have evaluated oral *Candida* spp. colonization in HIV-infected patients, especially in Brazil.

Campisi *et al.* (15) studied oral *Candida* spp. colonization in 42 HIV-infected individuals and 41 volunteers that were not at risk for AIDS by using the concentrated oral rinse technique. Results revealed that the carriage rate was higher in HIV-infected (61.9%) than in healthy individuals (29.3%) [p=0.003] and that the density carriage was also higher in the HIV-positive group [p=0.0002].

Carriage rate and density were not associated with HIV-1 viral load, TCD4⁺ cell count, gender, route of HIV infection, or antiretroviral therapy. On the contrary, oral

Candida carriage was influenced by Plaque Index [$p=0.009$]. Among HIV-positive patients, after adjustment for TCD4⁺ counts and viral loads, cigarette smoking correlated with carriage rate [$p=0.011$] but not with density carriage, which was not associated with number of cigarettes per day.

Species identification, performed only for isolates from the HIV-positive group, showed predominance of *C. albicans* (73.1%) and only one *C. glabrata* (3.8%); candidal combinations always included *C. albicans* with one or more non-*C. albicans* species (seven carriers, 19.2%).

The authors suggested that smoking and bad oral hygiene status might act synergistically with HIV-related predisposing factors, such as saliva pH, composition and flow rate as well as mucosal immune dysfunction, to elicit increased *Candida* colonization.

Costa *et al.* (27) collected swabs from 99 HIV-infected patients, 62 of them were colonized by *Candida* spp. *Candida albicans* presented the highest frequency (50%) whereas non-*C. albicans* species were represented by *C. tropicalis* (20.9%), *C. parapsilosis* (19.3%), *C. guilliermondii* (4.8%), *C. lusitaniae* (1.6%), *C. krusei* (1.6%), and *C. kefyr* (1.6%). Association between *Candida* species and use of HAART was not observed.

Resistance to fluconazole was observed in 8.1% of *Candida* isolates, 8.2% of which were obtained from 49 patients under HAART. Considering the break points for resistance determined in NCCLS A27-A2 document for itraconazole ($\geq 1.0\mu\text{g/ml}$) and fluconazole ($\geq 64\mu\text{g/ml}$), the values suggested for amphotericin B ($> 1.0\mu\text{g/ml}$) and voriconazole ($\geq 1.0\mu\text{g/ml}$) and the observed MIC₉₀, susceptibility to voriconazole was higher than that to fluconazole. All isolates were susceptible to amphotericin B.

Using the same material, Costa *et al.* (28) also demonstrated no correlation between *Candida* spp. colonization and TCD4⁺ cell count or HIV-1 viral load.

Menezes *et al.* (69) studied the prevalence of *Candida* spp. in the oral cavity of 100 patients from Fortaleza (Ceará State, Brazil). Swabs were collected from patients with or without lesions characteristic of oral candidiasis. Among *Candida* spp. isolates from 80 patients, 65% were *C. albicans*, 27.5% *C. tropicalis*, 2.5% *C. glabrata*, 2.5% *C. krusei* and 2.5% *C. guilliermondii*. Assessment of the enzymatic activity of all 52 *C. albicans* isolates indicated intermediate proteolytic activity in 69.2% and low or intermediate phospholipase activity in 73.0% isolates. Although

such findings correspond to colonization plus infection and were not compared with those of a control group from the same region, the positivity rate seems to be high.

In 2006, Yang *et al.* (126) published the results of a prospective study which evaluated the effects of HAART on HIV-infected patients. Increase in TCD4⁺ count from 232.5 to 316.0 cells/mm³ [p=0.0003], decrease in the frequency of patients with TCD4⁺ count lower than 200 cells/mm³ from 50.0% to 28.9% [p=0.0003], decrease in the prevalence of oral candidiasis from 10.6% to 2.1% [p=0.0004] and a tendency towards decrease in *Candida* spp. colonization from 57.8% to 46.5% [p=0.06] were observed. There was also a decrease in the percentage of patients who were hospitalized or received antifungal compounds and antibiotics – risk factors for *Candida* spp. acquisition. These findings suggest that most patients under HAART continue being colonized by *Candida* spp. but do not develop oral candidiasis.

Blignaut (10) studied colonization and infection by *Candida* spp. in the oral mucous membrane of 87 children from orphanages and 330 not-hospitalized, AIDS or HIV-infected adults in Gauteng (South Africa). Clinical material was collected, using swabs, from the dorsal surface of the tongue. Prevalence of colonization or infection was 41.7% among children, predominating non-*C. albicans* species, and 91.5% among adults, clearly predominating *C. albicans*. The frequency of *C. dubliniensis* isolation was high; however, it must be emphasized that such species was identified based only on phenotypic methods. None of the children or adults was receiving antiretroviral treatment at the time of the study.

Candida spp. colonization and infection were evaluated in Mexico by Sánchez-Vargas *et al.* (98, 99) in 111 HIV-positive patients (51 adults and 60 children) and in 201 healthy controls (109 adults and 92 children). Swabs were taken from oral lesions (when present), oral mucous membranes and dorsal surface of the tongue. Yeasts were identified to species level by standard methods. *Candida albicans* isolates were serotyped by an indirect immunofluorescent assay (IIA) with the IgM monoclonal antibody B9E. Presumptive diagnosis of *C. dubliniensis* was based on the growth of all *C. albicans* isolates at 45°C for 48h in Sabouraud dextrose agar, on chlamydoconidia production in Casein agar, and on an IIA with a polyclonal antiserum against this species. Antifungal susceptibility tests were carried out using standardized methods.

Yeast isolation was higher in adults both with and without HIV infection (74.5% and 61.5%, respectively) than in children HIV-infected or not (60.0% and 40.2%,

respectively). Oral carriage in HIV-positive patients was not associated with TCD4⁺ cell count (623 cells/ μ l in colonized and 643 cells/ μ l in non-colonized patients), or viral load (52,275 copies/ml in colonized and 55,173 copies/ml in non-colonized patients). In addition, the antiretroviral regimen was not associated with the colonization/infection status – 69.4% of patients undergoing HAART and 59.5% of patients without this regimen. Oral candidiasis in HIV/AIDS patients was not associated with TCD4⁺ cell count (775 cells/ μ l in patients with oral candidosis and 643 cells/ μ l in those without candidosis), or viral load (60,995 and 55,173 copies/ml in patients with and without candidiasis, respectively). However, candidiasis was less frequent in patients undergoing HAART (36.1%) than in patients without antiretroviral therapy (45.9%) [$p < 0.05$]. This finding was also observed in children with TCD4⁺ cell count lower than 500 cells/ μ l, when compared with children not treated with antiretrovirals.

Candida albicans was the most prevalent species in colonized and infected patients as well as in healthy individuals; serotype A predominated in all four groups. Non-*albicans* species, mainly *C. glabrata* and *C. tropicalis*, were isolated from 16.5% colonized patients and from 38.5% candidiasis patients. Isolation of multiple species was observed in only nine episodes of infection or colonization, seven of which included *C. albicans* and non-*C. albicans*, and two showed association of two non-*albicans* species.

According to resistance and intermediate susceptibility, the following results were obtained, respectively: itraconazole (ITZ) – 10.7% and 28.9%; ketoconazole (KTZ) – 10.2% and 20.8%; miconazole (MCZ) – 2.7% and 17.6%; fluconazole (FCZ) – 3.2% and 11.2%; amphotericin B (AMB) – 0.0 and 2.1%; 5-fluorcytosine (5-FC) – 0.0 and 0.0%. The most resistant isolates were *C. glabrata*, from HIV-negative individuals, and *C. albicans*, from HIV-positive patients with pseudomembranous candidiasis. Results of the evaluation of *C. albicans* isolates for resistance and intermediate susceptibility were, respectively, 10.3% and 28.7% to KTZ; 8.1% and 21.3% to ITZ; 0.7% and 28.7% to MCZ; and 2.2% and 5.9% to FCZ. Identification of serotype B in 5 out of 14 ketoconazole-resistant *C. albicans* was significant.

Results of the analysis of factors involved in colonization are many times contradictory, demonstrating how the pathogeny of candidiasis is little known, which indicates that further studies are needed to better understand colonization and infection by *Candida* spp. in HIV-infected patients.

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