CHAPTER III

REVIEW LITERATURE

Biology

Candida belong to the class Fungi Imperfecti, the order Moniliales, and the family Cryptococcaceae [1].

Family: Cryptococcaceae

Genus 1: *Cryptococcus*; unicellular budding cells only; reproduce by blastoconidia pinched off the mother cell. Most are urease-positive. Cell surrounded by a heteropolysaccharide capsule and produces starchlike compounds; carotenoid pigments are usually lacking.
Example: *Cryptococcus neoformans* (caused cryptococcal menigitis)

Genus 2: *Torulopsis*; some as above, but do not have capsules or produce iodine positive, starchlike substance; urease negative, and there is no assimilation of inositol.
Example: *Torulopsis glabrata* (caused torulopsosis)

Genus 3: *Malassezia*; Mostly unicellular budding cells which reproduce by blastoconidia that develop from a reduced phialide. Cells may adhere, forming short hyphal strands. Growth stimulated by lipids. There is no fermentative ability.
Example: *Malassezia furfur* (caused pityriasis versicolor)

Genus 4: *Rhodotorula*; Unicellular budding forms that rarely produce pseudomycelium, are generally encapsulated, but do not produce starchlike substance. They do not assimilate inositol or ferment sugars. Carotenoid pigments are produced.
Example: *Rhodotorula rubra* (caused rare pulmonary and systemic infections)

Genus 5: *Candida*; Reproduction is by pinched blastoconidia. They may form pseudomycelium or true mycelium; urease is generally negative; capsules are not formed; strach or carotenoid pigments are not produced; inositol is not assimilated. In Table 3 was shown the list of some pathogenic species of this genus.

Genus 6: *Trichosporon*; Reproduction is by blastoconidia and arthroconidia. Mycelium and pseudomycelium are formed.
Example: *Trichosporon beigelii* (caused white piedra and systemic infections)

Genus 7: *Geotrichum*; Reproduction is by arthroconidia only. A true mycelium is formed.
Example: *Geotrichum candidum* (caused rare pulmonary geotrichosis)

The genus *Candida* is composed of an extremely heterogenous group of organism that grow as yeast (Fig. 2A). Most member of genus also produce a filamentous type of growth (Fig. 2B and Table 4.), but *C. albicans* and *C. dubliniensis* also from true hyphae (Fig. 2C) in addition to pseudohyphae. Therefore, both species are considered to be polymorphism. The distinction that is made between hyphae and pseudohyphae is related to the way in which they are formed. Pseudohyphae form from yeast cells or from hyphae by budding, but the new cell remains attached to the parent cell and elongates, resulting in filaments with constrictions at the cell-cell junctions of filaments. In comparison, true hyphae form from yeast cells or branches of existing hyphae. Outgrowths of the yeast cell (germ tube) grow by apical extension and cross walls (Fig. 2C), the latter of which are perpendicular to the main axis of the hyphae. The appearance of hyphae differs somewhat when grown in serum, where hyphal branching occurs and budding is delayed. The pseudohyphae appears to be an intermediate growth form of yeast and hyphal morphologies. Because *C. albicans* and *C. dubliniensis* produce hyphae form yeast cells, these species are also referred to as being germ tube positive (Table 4) [1, 57, 58].

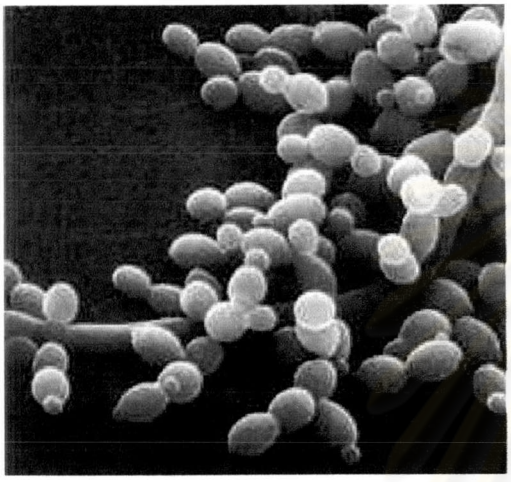
Candida is normally inhabits our digestive system: the mouth, throat, intestines and genitourinary tract. *Candida* is a normal part of the bowel flora (the organisms that naturally live inside our intestines, and are not parasitic). It has many functions inside our digestive tract, one of them to recognize and destroy harmful bacteria. Healthy person can have a millions of *Candida albicans*. Our immune system is supposed to keep it under control, together with "friendly" bacteria. If the number of friendly bacteria is decreased in

relation to a number of *Candida*, the immune systems is weakened or other conditions for yeast proliferation occur. *Candida* is a conditionally pathogenic fungus. It causes infection when antibiotics or other factors reduce our natural resistance to its overgrowth. Most *Candida* infections are superficial, limited to mucous membranes [1, 57, 58].

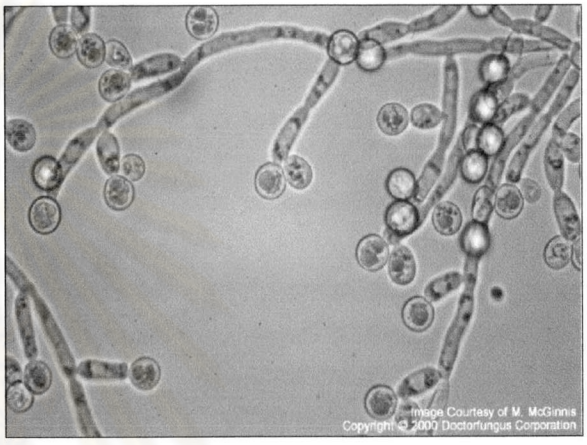
Morphology [57]

Candida appears as a gram positive oval budding yeast and/or pseudohyphae, measuring 2 – 3 x 4 – 6 μm (Table 4). On Sabouraud's agar incubated at room temperature within 2 or 3 days, soft, cream – colored colonies with a yeasty odor develop. The surface growth consists of oval budding cells. The submerged growth consists of pseudomycelium, composed of blastoconidia at the nodes and sometimes chlamydoconidia (Figure 2B). Blastoconidia of *Candida* spp. vary in shape, from round to oval to elongate. Asexual reproduction is by multilateral budding, and mycelia may be present. If sexual reproduction occurs, the yeasts are classified by their teleomorphic state. Growth on fungal media can be detected as early as 24 hrs: however, colonies usually are visible in 48 -- 72 hrs. as white cream colored or tan. They are creamy and may become more membranous and convoluted with age. Most *Candida* grows well aerobically at 25 – 30 °C and may with grow at 37 °C or above. Carbohydrate assimilation and occasionally fermentation studies are needed to differentiate the species. Production of pseudohyphae is one of the major differentiating factors separating *Candida* from *Torulopsis* spp. Observation of germ tubes, only the budding cells of 24 hrs. old cultures of *C. albicans* and *C. dubliniensis* and not of other species will form germ tubes (Fig. 2 and Table 4) in 2 – 3 hrs. when placed in serum at 37 °C and chlamydoconidia is helpful in identifying *C. albicans* [1, 57-59].

Fig. 2. The growth forms of *Candida* species. (A) Blastoconodida and pseudohyphae , (B) Chlamydoconidia (*C. albicans* and *C. dubliniensis*), and (C) Germ tube (*C. albicans* and *C. dubliniensis*)



A



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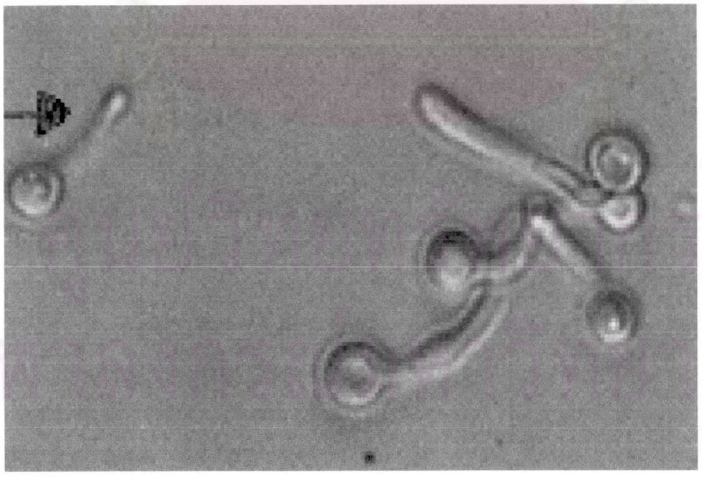


C

Table 3. A partial list of pathogenic species of *Candida* [1]

Reported by 1988	Current list
C. albicans	*C. albicans*
C. tropicalis	*C. tropicalis*
C. glabrata	*C. glabrata*
C. parapsilosis	*C. parapsilosis*
C. krusei	*C. krusei*
C. guilliermondii	*C. guilliermondii*
C. kefyr	*C. kefyr*
C. viswanathii	*C. viswanathii*
	C. lusitaniae
	C. dubliniensis
	C. fumata
	C. inconspicua
	C. utilis

Table 4. Morphological features of selected pathogenic species of *Candida* [1]

Species	Chlamydospore	Germ tube	Pseudohyphae	Size of yeast (μm)
C. albicans	+	+	+	4-6 x 6-10
C. tropicalis	±	-	+	4-6 x 5-11
C. glabrata	-	-	-	1-4
C. parapsilosis	-	-	+	2.5-4 x 2.5-9
C. krusei	-	-	±	3-5 x 6-20
C. dubliniensis	+	+	+	3-7 x 3-14
C. guilliermondii	-	-	+	2-5 x 3-7
C. lusitaniae	-	-	Variable	2-6 x 3-10
C. kefyr	-	-	+	2.5-5 x 5-10
C. inconspicua	-	-	+	1.5-3 x 3-5
C. fumata	-	-	+	3.7-5 x 2.7-4.7

Candida albicans

C. albicans strains produce white to cream colored, pasty, smooth colonies on Sabouraud dextrose agar. On cornmeal tween 80 agar and at 25 °C for 72 hrs., *C. albicans* produces clusters of round blastoconidia along the hyphae and particularly at points of septa. Pseudohyphae and true hyphae are also observed. *C. albicans*, together with *C. dubliniensis*, are the two *Candida* spp., which produce a typical asexual spore, chlamydoconidium. Chlamydoconidia are round, large, thick-walled and usually terminal. *C. albicans* produces no surface growth when inoculated in Sabouraud broth. Germ tube production is the other typical characteristic of this species. Germ tube is the beginning of true hyphae and observed by microscopic examination upon inoculation of the strain in serum. Similar to the chlamydoconidia production, the two *Candida* spp. that produce germ tube are *C. albicans* and *C. dubliniensis*, investigations on simple methods, as well as molecular studies are on the way to differentiate these two species. In the interim, in routine laboratory work, a strain that produces germ tube and chlamydoconidia is defined as *C. albicans* [60, 61].

Candida stellatoidea, once accepted to be a separate *Candida* species, is now classified as a subspecies of *C. albicans* which differs from *C. albicans* by not assimilating sucrose and not being resistant to cycloheximide [60].

Candida tropicalis

On Sabouraud dextrose agar, *C. tropicalis* colonies are cream-colored with a slightly mycelial border. It may produce a thin surface film and bubbles when grown in Sabouraud broth. On cornmeal tween 80 agar and at 25 °C after 72 hrs., it produces oval blastospores which are located along the long pseudohyphae. The blastospores may appear singly or in clusters. The pseudohyphae branch abundantly *C. tropicalis* may also produce true hyphae [60, 61].

Candida glabrata

This species, once classified in genus *Torulopsis* is now accepted to be included in genus *Candida*. *C. glabrata* colonies are small, pasty, white to cream in color and glistening. *Candida glabrata* does not grow on the surface when inoculated into Sabouraud

broth. On cornmeal tween 80 agar and at 25 °C after 72 hrs., it produces clusters of tiny, oval, budding blastospores. The absence of pseudohyphae is very typical [60, 61].

Candida parapsilosis

C. parapsilosis colonies are white, creamy, shiny, and smooth or wrinkled on Sabouraud dextrose agar. It does not grow on the surface when inoculated into Sabouraud broth. On cornmeal tween 80 agar and at 25 °C after 72 hrs., it produces blastospores which are located along the pseudohyphae. Typically, the pseudohyphae may be curved and large mycelial (hyphal) elements which are called "giant cells" may be observed [60, 61].

Candida krusei

C. krusei colonies differ from those of several *Candida* spp. They are typically dry, dull and often have a mycelial border on Sabouraud dextrose agar. The colonies are cream colored and their shape is often lenticular. *C. krusei* produces a prominent surface growth film when inoculated into Sabouraud broth. On cornmeal tween 80 agar and at 25 °C after 72 hrs., it produces blastospores which are typically elongate and have a tree-like crossing appearance. *Candida krusei* produces plenty of and branching pseudohyphae [60, 61].

Candida guilliermondii

C. guilliermondii colonies are flat, moist, smooth, and cream to yellow in color on Sabouraud dextrose agar. It does not grow on the surface when inoculated into Sabouraud broth. On cornmeal tween 80 agar and at 25 °C after 72 h, it produces clusters of small blastospores along the pseudohyphae and particularly at septal points. Pseudohyphae are short and few in number [60, 61].

Candida dubliniensis

C. dubliniensis is a novel species defined in genus *Candida*. In addition to its many other comparable characteristics to *C. albicans*, its colony morphology is also similar to that of *C. albicans* and appears creamy and smooth [30]. On cornmeal tween 80 agar and at

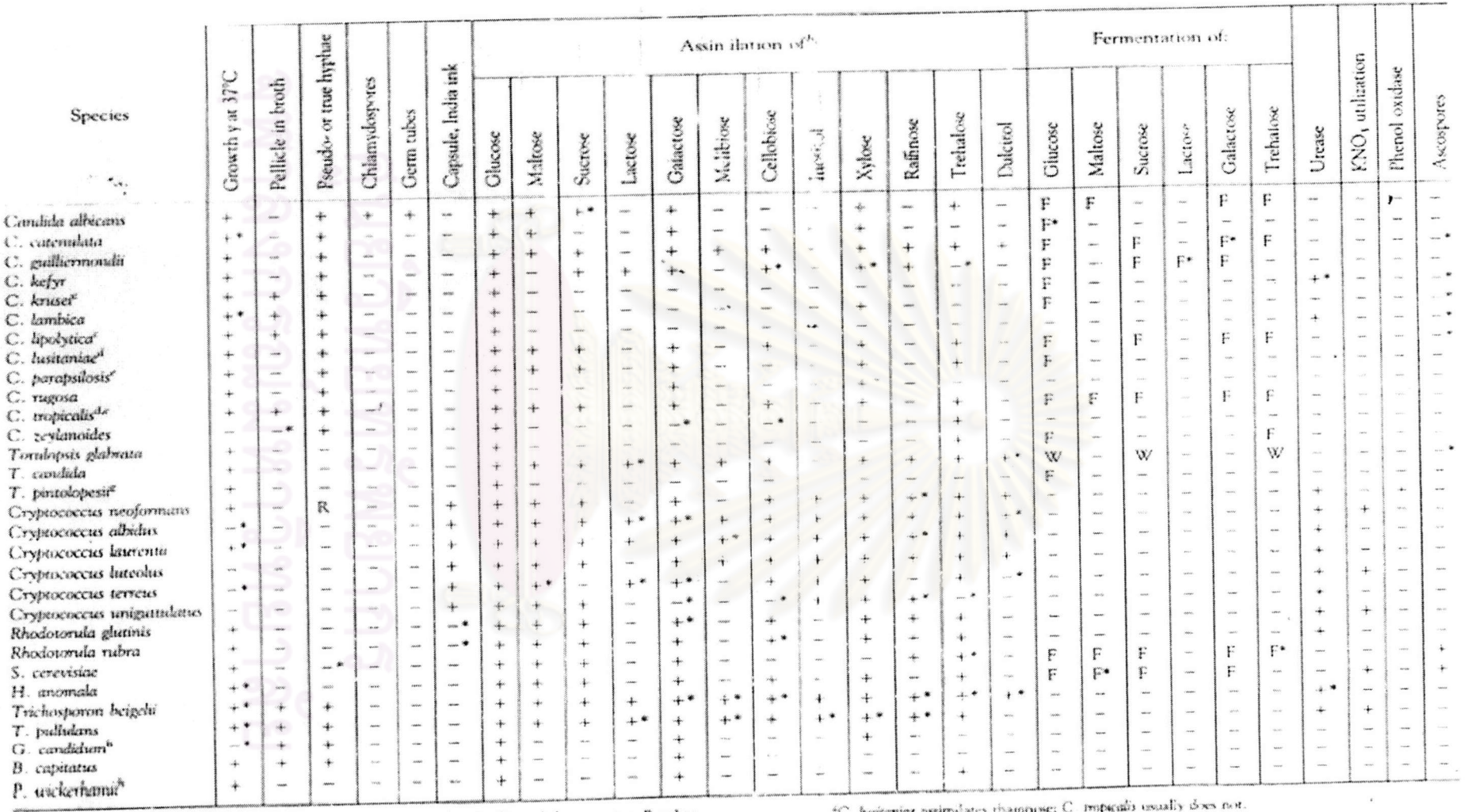
25 °C after 72 hrs., it produces blastospores, pseudohyphae and abundant chlamydospores. The numerous number of chlamydospores and their tendency to appear in pairs and triplets are typical but not sufficiently distinctive for definitive differentiation of the species from *C. albicans*. Similar to *C. albicans*, *C. dubliniensis* also produces germ tubes when inoculated in serum.

C. dubliniensis is most often isolated from the mouth in patients with AIDS [26, 30, 62]. One may inquire whether it is really necessary to differentiate *C. dubliniensis* from *C. albicans*. In routine practice, the common approach is to identify a strain as *C. albicans* if it produces germ tube or chlamydospores. However, fluconazole resistance has been detected in a number of *C. dubliniensis* isolates [63]. These isolates usually remained susceptible to other drugs such as amphotericin B, flucytosine, voriconazole, and caspofungin.

Physiology

Physiological properties need to be examined in conjunction with morphological criteria. Carbohydrate assimilation profiles for *Candida* species (Table 5) can be obtained by examining zones of candidal growth around dises or wells impregnated with various sugar on basal agars [64]. In contrast, fermentation tests are generally performed in liquid media and are based on demonstration of acid or gas production. Individually these physiological tests are time-consuming and laborious to perform, particularly in routine diagnostic laboratories where there may be a high throughput of specimens.

Table 5. Cultural and biochemical characteristic of yeasts frequently isolated from clinical specimens [57]



Species	Growth at 37°C	Pellicle in broth	Pseudo- or true hyphae	Chlamydospores	Germ tubes	Capsule, India ink	Assimilation of[b]: Glucose	Maltose	Sucrose	Lactose	Galactose	Maltiose	Cellobiose	Inositol	Xylose	Raffinose	Trehalose	Dulcitol	Fermentation of: Glucose	Maltose	Sucrose	Lactose	Galactose	Trehalose	Urease	KNO_3 utilization	Phenol oxidase	Ascospores
Candida albicans	+	−	+	+	+	−	+	+	+*	−	+	−	−	−	+	−	+	−	F	F	−	−	F	F	−	−	−	−
C. catenulata	+*	−	+	−	+	−	+	+	+	−	+	−	−	−	+	−	+	−	F*	−	−	−	−	−	+	−	−	−
C. guilliermondii	+	−	+	−	+	−	+	−	+	+	+	+	+	−	+	+	−	−	F	−	F	−	F*	F	−	−	−	−
C. kefyr	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	+	−	−	F	−	F	F*	F	−	−	−	−	−
C. krusei[c]	+	−	+	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
C. lambica	+*	−	+	−	+	−	+	−	+	−	+	−	+	−	+	+	+	−	F	−	F	−	F	F	+	−	−	−
C. lipolytica[f]	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	+	+	−	F	−	F	−	F	F	−	−	−	−
C. lusitaniae[d]	+	−	+	−	+	−	+	+	+	−	+	−	+	−	+	−	+	−	F	−	−	−	−	−	−	−	−	−
C. parapsilosis[e]	+	−	+	−	+	−	+	+	+	−	+	−	+	−	+	−	+	−	F	−	F	−	F	F	−	−	−	−
C. rugosa	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	F	−	−	−	−	−	−	−	−	−
C. tropicalis[d,e]	+	−	+	−	+	−	+	−	+	−	−	−	−	−	+	−	+	−	F	−	W	−	F	F	−	−	−	−
C. zeylanoides	−	−	−	−	−	−	+	−	+	−	+	−	+	−	+	−	+	−	W	−	W	−	W	W	−	−	−	−
Torulopsis glabrata	+	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	F	−	−	−	−	−	+	−	−	−
T. candida	+	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	W	−	−	−	−	−	+	−	−	−
T. pintolopesii[g]	+	−	R	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	F	−	−	−	−	−	+	−	−	−
Cryptococcus neoformans	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+	+	−	−
Cryptococcus albidus	+*	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−
Cryptococcus laurentii	−	−	−	−	−	+	+	−	+	−	+	−	+	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−
Cryptococcus luteolus	−	−	−	−	−	+	+	−	+	+	+	+	+	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−
Cryptococcus terreus	−	−	−	−	−	+	+	−	+	−	+	−	+	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−
Cryptococcus unigattulatus	−	−	−	−	−	+	+	−	+	−	+	−	+	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−
Rhodotorula glutinis	+	−	−	−	−	+	+	−	+	−	+	−	+	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−
Rhodotorula rubra	+	−	−	−	−	+	+	−	+	−	+	−	+	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−
S. cerevisiae	+	−	−	−	−	−	+	+	+	−	+	−	+	+	+	+	+	+	F	F	F	F	F	F*	−	−	−	−
H. anomala	+*	−	−	−	−	−	+	+	+	−	+	−	+	−	+	+	+	+	F	F*	F	−	−	−	+	−	−	−
Trichosporon beigelii	+*	+	+	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−
T. pullulans	+*	+	+	−	−	−	+	+	+	−	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+	−	−	−
G. candidum[h]	−*	+	+	−	−	−	+	+	+	−	+	−	+	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−
B. capitatus	+	+	+	−	−	−	+	+	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
P. wickerhamii[h]	+	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

*, Strain variation; R, rare; F, sugar is fermented (i.e., gas is produced); W, weak fermentation. Based on data from Barnett et al. (6) and Kreger-van Rij (44).

[a] In these columns, + indicates growth greater than that of the negative control.

[b] C. lipolytica assimilates erythritol; C. krusei does not. Maximum growth temperatures are 43 to 45°C for C. krusei and 33 to 37°C for C. lipolytica.

[c] C. lusitaniae assimilates rhamnose; C. tropicalis usually does not.

[d] C. parapsilosis assimilates L-arabinose; C. tropicalis usually does not.

[e] Rare strains of C. tropicalis produce teardrop-shaped chlamydospores.

[f] Torulopsis pintolopesii is a thermophilic yeast capable of growth at 40 to 42°C.

[g] Not yeasts but may be confused with several yeast genera.

Epidemiology

Candida species are ubiquitous organisms [65]. An increasing incidence of fungal infections with *Candida* species has been noted in immunocompromised patients such as intensive care, post surgical, neutropenic patients, AIDS [15, 66-68]. *Candida* species are most frequently isolated from the oral cavity and are detected in approximately 31 to 55% of healthy individuals. Colonization rates increase with severity of illness and duration of hospitalization [65]. Historically, *C. albicans* accounted for 70 to 80% of the isolates recovered from infected patients. *C. glabrata* and *C. tropicalis* each accounted for approximately 5 to 8% of isolates, while other non-*albicans Candida* species occur only rarely [66, 69]. However, more recent epidemiological data reveal a mycological shift from *C. albicans* to the non-*albicans Candida* species such as *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei* [19, 20, 66, 70]. The *Candida* species listed in Table 3 should probably be expanded as more recent surveillance studies have included *C. fumata, C. inconspicua, C. utilitaniae,* and other species from blood and other sites of patients with candidiasis.

Candida albicans

C. albicans is the most commonly isolated yeast species in the oral cavity both in health and disease [65]. It accounts for about 47% to 75% of the oral yeast isolates, while other medically important yeast pathogens, *C. tropicalis, C. parapsilosis, C. krusei, C. kefyr, C. glabrata* and *C. guilliermondii* each represent less than 10% of isolates [65]. According to recent reports yeast species other than *C. albicans* cause increasing number of infections, including arthritis, osteomyelitis, endocarditis, endopthalmitis, meningitis, and fungemia [6].

Candida tropicalis

C. tropicalis is one of the three most commomly ioslated NAC species(the other being *C. parapsilosis* and *C. krusei*). *C. tropicalis* accounts for 4 to 25% of all *Candida* isolates and a higher proportion (20-45%) of NAC isolated from blood

culture [71]. In terms of the patient group affected, *C. topicalis* is seen more frequently in cancer patients. The proportion of infection in children due to this species is lower than in adults (0-8%) [72-75]. *C. tropicalis* is less frequently observed in surgical and ITU patients (4-18%) [76-79]. In Walsh *et al.*'s review of fungaemia in HIV-positive children, 8% of infections were due to *C. tropicalis* [74]. The occurrence of *C. tropicalis* infection in cancer patients varies according to the underlying diagnosis and treatment modality, with an incidence of 11-25% in BMT patients, 18% in haematologic malignancies and 4-9% in solid tumor patients [79-83].

Candida parapsilosis

C. parapsilosis is probably the only species to show an overall increasing incidence since 1990 in most studies of fungaemia [80, 81, 84]. Recently published series from North America and Europe show that *C. parapsilosis* is now the predominant cause of candidaemia in some centers [85]. *C. krusei* and *C. glabrata* are observed more frequently in cancer patients, but *C. parapsilosis* occurs mainly in non-cancer patients, particularly in children [76, 85-87]. *C. parapsilosis* causes 17-50% of fungaemia in chlidren compared with 2.5-12% in adult surgical or ITU populations [76, 78, 79]. The incidence in cancer patients varied from 6 to 38 % in the USA and 12-15% in Europe centres, and was higher in BMT and leukaemic patients (35%) than in patients with other malignancies or solid tumours [81,88-90]. The highest proportion has been observed in children, varying from 27 to 50% of total longitudinal studies gave compared the yearly incidence of *C. parapsilosis* over the last 8-10 years; there has been a sustained increase, in the USA from 7 to 37% and in Europe from 0-21% [71]. Before 1991, *C. parapsilosis* appeared to cause around 10% of cases.

Candida grabrata

C. grabrata represents 8-37 % of all *Candida* blood culture isolates [78, 91]. Some centers report an increasing proportion of infections due to *C. glabrata* over the last 10 years. The incidence is higher in adults (5.4-37%) than in children (0-6.6%), and is lowest in neonates (0-2%). With regard to predisposing factors and underling

disease, the incidence is lower in cancer patients (4.5-13%) than in the general patient population (15-21%), or ITU and surgical patients (20-25%) [75, 80, 81, 91].

Candida krusei

C. krusei is an emerging NAC species. With a particular predilection for neutropenic adult cancer patients [84]. Akin to *C. albicans*, the gastrointestinal tract is probably its origin. *C. krusei* causes 2-25% of fungaemias; the proportion is lower in ITU and surgical patients (2-4%) than in cancer patients (13%), and also lower in children (1-3%) than adults [71, 81, 84, 92]. The highest proportion is seen in patients with leukaemia (13-25%) and the lowest in neonates [73, 80, 93, 94]. As illustrates, the variation from center to center and hospital to hospital is very high, suggesting that features of local epidemiology underlies the wide variation seen [95-97]. In some centers, the proportion of infection due to *C. krusei* appears to be increasing in at least seven reports, an association with fluconazole usage was observed [70, 98, 99]. A somewhat different picture is gained from three studies of fungal isolates obtained from surveillance cultures, which compared the proportion of *C. krusei* seen in 1989-1991 with the period 1993-1996 [82, 95].

In all three studies, the observed increase or decrease was not statistically significant and there was no correlation with fluconazole consumption [100]. Two longitudinal studies on fungaemias provide further information on the possible link between *C. krusei* and azole use. The study spanning the time period 1989-1998 showed no increase in *C. krusei* infections despite the widespread use of fluconazole prophylaxis after 1991 [71, 101]. The second did show an increase in *C. krusei* fungaemia, from 9% in 1991 to 37% in 1996 [101].

Candida guilliermondii

C. guilliermondii causes infection in humans only rarely. It was previously considered as an animal saprophyte, but this situation has changed [80]. In the 40 years preceding 1990, only 10 cases of infection in man were documented, but a further 33 cases of fungaemia have been reported since 1990 [80, 81, 84, 86, 102]. In Wingard's review of fungaemia (1960-1990), 10 cases of *C. guilliermondii* infection were seen (0.7% of the total). Three of those patients died, giving a 30 % mortality.

Abi Said's single cancer centre review of candidaemias includes three cases (0.7%), all of whom survived [80]. Finally the multicentre European study included 12 cases (5.5%), of whom two survived [81]. Of the 140 cases of fungaemias reported from a single center, only three (2.1%) were caused by *C. guilliermondii* and one died [71]. In a nationwide Dutch study, nine cases were seen among 671 fungaemias (1.1%) [78]. In a series of 148 cases of fungaemia in BMT patients, one case (0.7%) was identified; this patient died despite therapy with amphotericin B colloidal dispersion (ABCD). To summarize the curretly published data, the overall proportion of *C. guilliermondii* has increased (from 0-0.7% in 1950-1990 to 0.7-5.5% in 1991-1998). It appears to affect patients with cancer (who constituted 31 of 43 cases), ITU and surgical patients.

Candida dubliniensis

Isolates of *C. dubliniensis* were first identified in oral specimens from HIV-infected and AIDS patients with recurrent oral candidiasis attending the Dublin Dental Hospital [103, 104]. Since then *C. dubliniensis* has been recovered in a wide variety of clinical settings from individuals on all five continents [30, 31, 36, 105-107]. The vast majority of *C. dubliniensis* isolates have been recovered from the oral cavities of HIV-infected individuals or, more frequently, from patients with AIDS [30, 31]. However, *C. dubliniensis* has also been found as an oral carriage organism and has been implicated as an agent of oral candidosis in HIV-negative individuals, including normal healthy individuals, including normal healthy individuals and diabetics [30, 31, 36]. A limited number of *C. dubliniensis* isolates have also been recovered from the vaginal tracts of HIV negative women with vaginitis [108]. Similarly, *C. dubliniensis* isolates have been recovered from including faces, sputum, urine, wounds and the respiratory tract [26, 106].

Pathogenesis

C. albicans is a human commensal so that the infectious sources are primarily endogenous and secondary exogenous.

1. Endogenous : The gastrointestinal tract is considered a major reservior for *C. albicans*, e.g. in vaginitis or diaper rash. Moreover, the fungus can invade the

bloodstream from gastrointestinal tract after damage to the gastrointestinal mucosa, such as that induced by anti-cancer treatment (irradiation or chemotherapy) or major surgery; it can then spread heamatogenously into various organs, causing deep-seated /disseminated infections.

2. Exogenous : *C. albicans* can, however, be introduced from exogenous sources as well. These may include introduction through various catheters and lines, or other dwelling/prosthetic medical devices [109]. This route is of particular importance in the development of deep-seated and systemic candidiasis because most of these therapeutic modalities are used primarily in compromised hosts whose defence systems are unable to combat the introduced pathogen. Person-to-person transmission is not a predominant mechanism of pathogenesis in candidiasis. It is seen primarily in the oral thrush of those newborns whose mothers have vaginal infections, acquiring them during birth, or is noted in the sexual transmission from patients with vaginitis to their sexual partners.

Clinical disease [1, 57, 58]

- **Generalized cutaneous candidiasis**: This is an unusual form of cutaneous candidiasis that manifests as a diffuse eruption over the trunk, thorax, and extremities. The patient has a history of generalized pruritus, with increased severity in the genitocrural folds, anal region, axillae, hands, and feet. Physical examination reveals a widespread rash that begins as individual vesicles that spread into large confluent areas.
- ***Candida* folliculitis**: The infection is found predominantly in the hair follicles and rarely can become extensive.
- **Paronychia and onychomycosis**: Frequently, paronychia and onychomycosis are associated with immersion of the hands in water and with diabetes mellitus. The patient has a history of a painful and erythematous area around and underneath the nail and nail bed. Physical examination reveals an area of inflammation that becomes warm, glistening, tense, and erythematous and may extend extensively under the nail. It is associated with secondary nail thickening, ridging, discoloration, and occasional nail loss.

Chronic mucocutaneous candidiasis

- Chronic mucocutaneous candidiasis describes a group of *Candida* infections of the skin, hair, nails, and mucus membranes that tend to have a protracted and persistent course.
    - Chronic mucocutaneous candidiasis frequently is associated with endocrinopathies, such as the following:
        - Hypoparathyroidism
        - Addison disease
        - Hypothyroidism
        - Diabetes mellitus
        - Autoimmune antibodies to adrenal, thyroid, and gastric tissues (approximately 50%)
        - Thymomas
        - Dental dysplasia
        - Polyglandular autoimmune disease
        - Antibodies to melanin-producing cells

Gastrointestinal tract candidiasis

- **Oropharyngeal candidiasis**

The patient has a frequent history of using broad-spectrum antibiotics or inhaled steroids or having HIV infection, chemotherapy, dentures, or diabetes mellitus. Variable symptoms include the following:

- Asymptomatic
- Sore and painful mouth
- Burning mouth or tongue
- Dysphagia
- Whitish thick patches on the oral mucosa.

- **Esophageal candidiasis**

The patient's history usually includes the use of broad-spectrum antibiotics or inhaled steroids or the presence of HIV infection, chemotherapy, or hematologic or solid organ malignancy. Variable symptoms that include the following:

- Asymptomatic
- No oral disease (>50% of patients)
- Dysphagia
- Odynophagia
- Retrosternal pain
- Epigastric pain
- Nausea and vomiting

Respiratory tract candidiasis

The respiratory tract frequently is colonized with *Candida* species, especially in hospitalized patients. In ambulatory patients, 20-25% of the population is colonized by *Candida* species.

- **Laryngeal candidiasis**: This is very unusual but may be a source for disseminated candidiasis. Laryngeal candidiasis primarily is observed in hematologic malignancies. The patient may present with a sore throat and hoarseness. Physical examination generally is unremarkable, and the diagnosis is made by direct or indirect laryngoscopy.
- ***Candida* pneumonia**: It does not exist alone and occurs only rarely as part of disseminated candidiasis. The most common form is multiple abscesses due to hematogenous dissemination of *Candida* species. The high degree of colonization and isolation of *Candida* species from the respiratory tract makes it difficult to make a diagnosis. The patient's history reveals similar risk factors for disseminated candidiasis, and patients complain of shortness of breath, cough, and respiratory distress. Physical examination reveals fever, dyspnea, and variable breath sounds, from clear to rhonchi to scattered rales.

Genitourinary tract candidiasis

- **Vulvovaginal candidiasis (VVC)**: This is the second most common cause of vaginitis.The patient's history includes vulvar pruritus, vaginal discharge, dysuria, and dyspareunia. Approximately 10% of women experience repeated attacks of VVC without precipitating risk factors. Physical examination includes a vagina and labia that usually are erythematous, a thick curdlike discharge, and a normal cervix on speculum examination.

- ***Candida* balanitis**: Patients complain of itchiness of the penis. Lesions and whitish patches are present. *Candida* balanitis is acquired through sexual intercourse with a partner who has VVC. Physical examination reveals vesicles on the penis that develop later into patches resembling thrush. The rash may spread to the thighs, gluteal folds, buttocks, and scrotum.

- **Asymptomatic candiduria**: Most catheterized patients with persistent candiduria are asymptomatic, as noncatheterized patients may be. The majority of patients with candiduria have easily identifiable risk factors for *Candida* colonization. Thus, the distinction between invasive disease and colonization cannot be made solely on culture results because approximately 5-10% of all urine cultures may be positive for *Candida.*

Systemic candidiasis

Systemic candidiasis can be divided into 2 major categories, which are candidemia and disseminated candidiasis (organ infection by *Candida* species). Deep organ infections due to *Candida* species generally are observed as part of the disseminated candidiasis syndromes, which may be associated with either single or multiorgan involvement.

- **Candidemia**

Candida species currently are the fourth most commonly isolated organism in blood cultures, and *Candida* infection generally is considered a nosocomially acquired infection. The patient's history commonly will reveal the following:

- Fever for several days unresponsive to broad-spectrum antimicrobials, frequently the only marker of infection
- Prolonged intravenous catheterization
- Possibly associated with multiorgan infection

- **Other causes of candidemia without invasive disease**

include the following:

- **Intravascular catheter-related candidiasis**: This entity usually responds promptly to catheter removal and antifungal treatment.
- **Endocarditis**: The frequency of endocarditis has increased in the past few years. Endocarditis is the most common cause of fungal endocarditis and is primarily due to *Candida albicans* (>60% of cases). The most common valves involved are the aortic and mitral. Two different forms of endocarditis exist- exogenous, which is secondary to direct infection during surgery, and endogenous, which is due to secondary spread during candidemia and disseminated candidiasis. Endocarditis frequently is associated with 4 main risk factors, which are the following: (1) intravenous heroin use, which frequently is associated with infection due to *C. parapsilosis,* (2) chemotherapy, (3) prosthetic valves (approximately 50%), and (4) prolonged use of central venous catheters.
- **Disseminated candidiasis**: This frequently is associated with multiple deep organ infections or may involve single organ infection. Unfortunately, of patients with disseminated candidiasis, as many as 40-60% may have blood cultures negative for *Candida* species. The history of a patient with presumptive disseminated candidiasis reveals a fever unresponsive to broad-spectrum antimicrobials and negative results on blood culture. Physical examination reveals fever (may be the only symptom) with an unknown source and sepsis and septic shock.

Virulence factor

Virulence in *Candida albicans* includes host recognition. Binding of the organism to host cells, host cell proteins or microbial competitors (co–aggregation) more than likely prevents or at least reduces the extent of clearance by the host. *C. albicans*, expresses several virulence factors that contribute to pathogenesis. These factors include host recognition biomolecules (adhesins), secreted aspartyl proteases (SAP) and phospholipases (PL), phenotypic switching, and hyphae [110].

Manoproteins

Mannoproteins are the major component of the cell wall of *C. albicans* an include several "adhesins " involved in the recognition of and adherence to host cells [111]. Adherence to mucosal epithelial cells or blood vessel endothelial cells is probably essential to the yeast being able to survive and establish itself in the host, and a prerequisite to invasive infection. The host cell components recognized by these adhesins (receptors or "ligands') include epithelial cell carbohydrates (alpha-fucosyl residues and asialo-GM1), endothelial cell matrix proteins (such as collage and C3d) and lipids [112]. An example is the 66-kDa adhesin, MP66, which is the major structural component of the fimbrial processes which have been seen on *C. albicans* yeast cells [111]. Antibodies raised against a fimbrial extract containing MP66 inhibited the binding of yeast cells to human buccal epithelial cells [111]. Antibodies to the mannoprotein MP60, an adhesin recognized by component of complement, have been described in the sera of a patient with mucocutaneous candidiasis [111]. A monoclonal antibody to MP60 blocked binding of the yeast to C3d-conjugated erythrocytes. Several different models of murine candidiasis have suggested that antimannan antibodies are protective and therefore, by implication, that mannoproteins play a significant role in the disease process. Han and Cutler [113] found that an antimannan serum and monoclonal antibody (B6.1) protected both native immunocompetent mice and SCID (severe combined immunodeficiency) mice from intravenous challenge with *C. albicans*.

From the experimental observations, it appears that mannoproteins are important not only in the adherence of the yeast to host epithelial and endothelial cells, but also in the interaction of the yeast with components of the host defense system such as complement and neutrophils.

The secreted aspartyl proteinases (SAP)

The SAP family comprise of nine proteins [114]. SAPs are not limited to *C. albicans* and their presence has been demonstrated in *Candida tropicalis* as well as *Candida parapsilosis* and *Candida guilliermondii* [112]. The open reading frames (ORFs) of SAP1-9 range in size from ~1170 to 1780 bp. In guinea pig and murine models of invasive disease, deletions in SAP1-6 attenuated virulence[115, 116]. The contribution of Saps was also studied in cultured human oral tissue and human oral lesions using strains of *C. albicans* deleted individually of Saps or with triple deletions (SAP1-3, SAP4-6) [117]. In this study, SAP1-3 were deemed important and SAP4-6 were not required. An *in vitro* human epidermis model thus, it would appear that SAP1-6 are required for invasive disease. Expression was correlated with invasion of the tissue, that is, early invasion (SAP1, 2), extensive penetration (SAP8) and extensive hyphal growth (SAP6). In vaginitis models, although all SAPs have not been evaluated, SAP2 is required for disease development [117].

Phospholipases (PL)

The Phospholipases family comprise of PLA, PLB, PLC and PLD, only PLB1 has been shown to be required for virulence in an animal model of candidiasis; a gene-deleted strain produced less phospholipase *in vitro* and was less virulent (40% killing versus 100% by wild-type cells). Plbip activity has recently been detected at hyphal tips during tissue invasion. Plbip is an 84-kDa glycoprotein that has both hydrolase and a lysophospholipase-transacylase activity.

Phenotypic switching

Candida is capable of high-frequency, reversible phenotypic switching, which could generate phenotypes beneficially adapted to different host environments [111]. Phenotypic traits affected by this switching include cell morphology, lipid and sterol content, adhesion to buccal epithelium, levels of protease secretion, antigen expression and susceptibility to antifungal agents [111]. Freshly isolated strains from vaginitis or systemically infected patients have higher frequencies of switching [118]. This observation could be crucial in establishing a direct association of switching with disease development or might only signify genome variation, of which switching could be one component. Of interest in the white–opaque switch system, opaque cells colonize the skin in a cutaneous model more so than white-phase cells, but are less virulent in a systemic animal model [119]. Thus, a repertoire of factors (including colony morphology) is expressed by *Candida* that is important in its adaptation of the organism to specific anatomical sites.

Hyphae

Several investigators suggest that the hyphal form of *C. albicans* is associated with its invasiveness [112, 120-122]. It has been demonstrated that there is a strong correlation between germ tube formation and increased adhesion of *C. albicans* to buccal epithelium cell [122]. The study showed that germ tubes of *C. albicans* exhibited enhanced adhesion to human mucosal cells, which it was suggested, could be one of the mechanisms related to virulence in candida species. It is thought that proteinases are produced during hyphal formation which help to disrupt the integrity of the oral mucosa [123, 124]. Odds [65] considers that both morphological forms of *C.albicans*, blastospore and hyphae, appear to initiate and sustain pathological responses in mammalian hosts. However, it seems that one form may be better adapted than the other to survive under specific ecological conditions.

Antifungal drug susceptibility

The biological diversity of the organisms classified in the genus *Candida* has other implications for the management of infections caused by these species and for the development of antifungal agents. The ideal antifungal agents need to be effective against a broad spectrum of organisms, including all of the medically important yeast of the genus *Candida* [125]. Developing such agents has become increasingly challenging due to the multitude of fungi that are now recognized to cause human disease. The most important classes of antifungal agents effective against *Candida* species are the azole and the polyene antifungal agents [1, 126-128]. The first generation of drugs in the 1970s, when *C. albicans* was responsible for most fungal infections. Although most of the medically significant NAC species are susceptible to these agents in vitro, concern has arisen due to the emergence of organisms with intrinsic (primary) resistance and to the emergence of organisms that have a tendency to develop (secondary) resistance to these agents [129, 130]. In recent years, the widespread use of antifungal drugs has increased both therapeutically and prophyloactically, due mainly to the general increased incidence of candidiasis. Evidence has been produced that suggests that this has led to changes in the epidemiology of candidiasis and the emergence of *Candida* species that are less susceptible to these agents [1, 131].

Fluconazole is now one of the most commonly used azole antifungal agents and has proven efficacious in the treatment of both superficial and systemic fungal disease [1, 127]. However, a number of NAC species are now recognized to exhibit intrinsic resistance to this drug. It is now generally accepted that *C. krusei* is intrinsically resistant to fluconazole *in vitro* and that fluconazole therapy is totally inappropriate for treatment of infections caused by this species [132]. In addition, *C. inconspicua* and *C. norvegenis*, two species that have only recently been associated with human disease, also exhibit intrinsic resistance to fluconazole [133, 134]. In contrast, *C. glabrata* possesses the ability to rapidly develop resistance to fluconazole [135]. In comparison with most *Candida* species, which are diploid, *C. glabrata* is haploid and this may partly explain why mutations that lead to the development of fluconazole resistance arise at high frequency in this organism. Developed resistance to azole drugs has been described in other NAC species including isolates of *C. dubliniensis*, *C. guilliermondii*, and *C. tropicalis*, usually recovered from HIV-

infected patients following prolonged azole therapy [136, 137]. Both *C. dubliniensis* and *C. tropicalis* have been shown to acquire resistance to fluconazole following exposure to this drug *in vitro* [138]. Since the introduction and widespread use of fluconazole in the 1990s there have been changes in the epidemiology of candidiasis, with many studies reporting an increase in the incidence of *C. glabrata* infection and the emergence of *C. krusei* [1, 20]. Although not proven conclusively, many studies have suggested that the introduction and widespread use of the newer azole drugs itraconazole and fluconazole may have contributed significantly to the selection and emergence of strains and species of *Candida* with reduced susceptibility to these agents [20, 131].

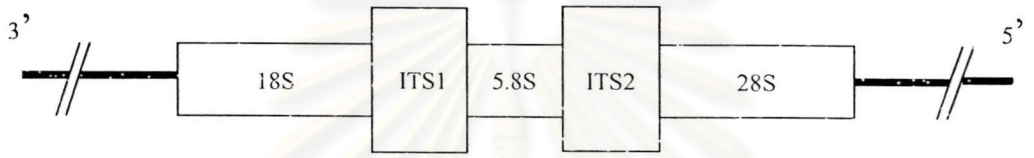
The polyene agent amphotericin B has been in use since the 1950s and has a low incidence of developed resistance in the clinical setting [1]. *C. albicans* isolates that exhibit resistance to amphoterin B have been described only rarely [129, 130]. However, recent evidence suggests that a significant proportion of *C. glabrata* and *C. krusei* isolates may exhibit resistance to this agent [1, 139]. Resistance to amphotericin B is most often associated with *C. lusitaniae*, a relatively uncommon pathogen. Although this organism is usually susceptible to amphotericin B, several reports have described the recovery of amphotericin B-resistant isolates from blood culture [1].

The ribosomal DNA (rDNA) structure

The overall rDNA gene complex includes (from 5' to 3' orientation) the intergenic spacer region (IGS), which contains the external transcribed spacer (ETS) 1 region (also called the nontranscribed spacer) on the 5' end and the ETS 2 region on the 3' end, along with a variable positioning of the 5S rDNA gene, the 18S rDNA gene, the ITS1 region, the 5.8S rDNA gene, the ITS2 region, and the 28S rDNA gene [140] (Fig. 3). During early transcription in the cell nucleous, the initial precursor in the formation of RNA contains nucleotides for the 5'-ETS1/18S/ITS1/5.8S/ITS2 /28S/ETS2-3' subunit, referred to as the 35S to 45S rDNA transcription unit. This genomic unit, which contains the genes necessary for the formation of ribosomal RNA (rRNA), has been designated the rDNA gene complex of the fungal genome [140]. As the 5S r DNA gene is processed during transcription in a step distinct from

the other rDNA gene products, it is not included as part of the precursor RNA transcript. Therefore, the 5S gene will be excluded from the discussion on the rDNA complex, although it is vitally important for the formation of ribosomes [140].

Figure 3. Representation of the rDNA gene complex in fungi denoting gene order and position of the ITS region [140]



rDNA complex genes

All three genes within the rDNA complex have been used in studies on the molecular evaluation of fungi. The 18S gene region is about 1800 bp in size with both conserved and variable domain sequences. Sequence variations within this region have been used to assess the taxonomic relationships of the major groups of living organisms and to separate genera and species based on sequence polymorphism [140, 141]. However, the drawback to using this region for the identification of species is the relative sequence homology among fungal species and the need to sequence a large number of based in order to do comparative analysis. The 5.8S region on the other hand is only about 160 bp long and highly conserved within major organism groups. Owing to this small size and conserved nature, it is not appropriate for phylogenetic studies to classify fungal species. However, this conserved sequence within major groups of microorganisms has been useful as an attachment site for universal primers to amplify flanking spacer regions within the eukaryotic genome [140]. Finally, the 28S rDNA gene, which is around 3400 bp in size within fungi, also contains both conserved and variable nucleotide sequence regions. The variable

domains of this large ribosomal subunit have also been used to allow comparisons from high taxonomic levels to the species level [140].

rDNA complex spacer regions [140]

Four transcribed spacer regions are located within the rDNA gene complex of fungi; two external to the 18S and 28S rDNA genes in the IGS noncoding region (ETS 1 and ETS2) and two internal, flanking the 5.8S r DNA gene (ITS1 and ITS2) (Fig. 3). Both ETS regions contain conserved sequences among fungal cells and although their biological role is not fully understood, both appear vital for early rRNA processing during transcription.

The ITS1 and ITS2 regions on the other hand, which flank the 5.8S rDNA gene, show extensive sequence diversity among major groups of eukaryotic microorganisms and even within species of the same organism group. It was shown that these DNA spacer regions are also important for early transcription during rRNA processing.

Biology of the ITS regions [140]

The biological role of the ITS regions during processing of the rRNAs remains an intriguing puzzle, even though a number of functions have been suggested. During the initiation of transcription, the primary 35S to 45S rRNA transcription unit is formed. This precursor transcript undergoes enzymatic removal of the four spacer sequences to become a primary rRNA transcript that is modified through RNA methylation and other base conversions and assembled in to an 80S nucleolar ribonucleoprotein particle (RNP). The 80S RNP particle undergoes cleavage to form the 18S, 5.8S and 28S mature rRNAs ultimate combine with proteins to become functional ribosomes. These functional ribosomes bind to mRNA and tRNA leading to protein production during translation.

Studies have shown that removal of one or both ITS regions prior to the initiation of transcription has a deleterious effect on the formation of mature rRNAs. Musters *et al.* [142] reported that removal of ITS1 prevented the formation of mature 18S rRNA and hence protein production during translation. Additionally, vander Sande *et al.* [143] showed that removal of ITS2 sequence prevented the maturation of

5.8S and 28S rRNAs. Further work by Good *et al.* [144] confirmed the important of the ITS2 sequence in the formation of functional ribosomes. They suggested that during rRNA maturation, there appeared to be an interdependence in the role of the ITS region as quality control mechanism to ensure that only functional rRNA was incorporated into the ribosomes. As DNA of ITS regions is removed and it is not part of the mature RNA molecule, they are considered noncoding regions of the genome. However, they are not normally identified as introns, but as intervening non-coding sequence or pseudo-introns because of their importance as precursors in protein manufacturing [140].

Laboratory identification of *Candida* species

Conventional method

Traditionally, methods for the identification of *Candida* species rely on a combination of morphological and biochemical characteristics

Mophology tests [1]

- Appearance and color of colonies on primary isolation media (Sabourauds, CHROMagar)
- Size and shape of cells
- Production of hyphae and/ or pseudohyphae
- Ability to produce chlamydoconidia (chlamydospores)
- Ability to produce germ tubes

A. Germ tubes test [57, 59]

A germ tube is defined as a filamentous extension from a yeast cell that is about one half the width and three to four times the length of the cell (Fig.1C). The true germ tube of *C. albicans* and *C. dubliniensis* have no constriction at the point of origin, early pseudohyphae of *C. tropicalis* may be confused but characteristically show a point of constriction adjacent to the mother cell. A constricted germ tube represents a pseudohyphae formation derived from a budding process of the

blastoconidia. In this experiment, both constricted and nonconstricted "germ tubes" may be seen in the germ tube test for *C. albicans* and *C.dubliniensis*; however, if the preparation appears to contain only constricted germ tubes, one should seriously consider the possibility of *C. tropicalis* or other *Candida* species. In addition to using a known culture of *C. albicans* as a positive test control, negative controls using *C. tropicalis* and *C. glabrata* should also be included. The preparation should be incubated longer than 3 hrs., as other hypha-producing yeasts will begin to germinate after this time. In cases in which the differentiation may be clinically significant, carbohydrate assimilation studies should be performed.

B. Cornmeal agar morphology [57-59]

The cornmeal agar preparations should be examined for the presence of hyphae, blastoconidia, chlamydoconidia, or arthrocinidia. In the past, cornmeal agar morphology was used successfully for the detection of characteristic chlamydospores produced by *C. albicans*. This method is currently satisfactory for the definitive identification of *C. albicans* and *C. dubliniensis* when the germ-tube test is negative. In other instances, microscopic morphologic features on cornmeal agar differentiate the *Candida, Geotrichum,* and *Trichosporon*. Previously, it was believed that the morphologic features of the common species of *Candida* were distinct enough to provide a presumptive identification. This can be accomplished for *C. albicans, C. glabrata, C. krusei, C. parapsilosis,* and *C. tropicalis* if one keeps in mind that there are numerous other species, uncommonly recovered in the clinical laboratory, that might resemble microscopically any of the previously mentioned species. In general, this method performs well since the previously mentioned genera and species are more commonly seen in clinical laboratories. For the uncommonly encountered isolates, cornmeal agar morphology will have less value.

Biochemical tests

A. Carbohydrate assimilation tests

B. Carbohydrate fermentation tests

A. Carbohydrate assimilation tests [58, 59]

The mainstay of yeast identification to the species level is the carbohydrate assimilation test, which measures the ability of a yeast cell to utilize a specific carbohydrate as the sole source of carbon in the presence of oxygen. Once the carbohydrate utilization profile is obtained, reactions may be compared to those listed in Tables 1. In most instances, carbohydrate assimilation tests provide the definitive identification of an organism.

B. Carbohydrate fermentation tests [58, 59]

Carbohydrate fermentation tests are preferred by some laboratories and are simply performed using purple broth containing different carbohydrate substrates. Fermentative yeasts recovered from clinical specimens produce carbon dioxide and alcohol; therefore, production of gas rather than a pH shift is indicative of fermentation. This test is most helpful in differentiating the various species of *Candida, Cryptococcus* and *Rhodotorula* spp. are nonfermentative.

Commercial kits [1]

For laboratories where the identification of yeasts constitutes a small part of the routine workload, the use of commercially produced auxanographic kits is particularly attractive. All of these kits will identify *Candida* species within 24 to 36 hrs., and many of them will provide an identification profile that can be used for epidemiological strain typing purposes.

The following products have found a useful place in the routine laboratory, although the list is not exhaustive: API ID 32C, API 20C AUX (commonly used in the United States), Fungicrom, Auxacolor, Candifast, and Fungitest. The development of commercial kits has provided the routine laboratory

with the opportunity to standardize the identification of *Candida* spp. However, for many laboratories, the costs involved may exclude the use of kits, but this decision must be weighed against the efforts in preparing media and reagents.

Molecular Identification methods for *Candida* species

General approach

Various molecular techniques using the ITS regions as molecular targets for the identification of fungi have been evaluated (Table 6). These methods include direct sequence analysis of amplified DNA, utilization of genus- or species- specific primer and oligonucleotide probes, and rDNA restriction fragment length polymorphism (RFLP) analysis. Most studies have shown that enough sequence variability exists among fungi within either the ITS1 or ITS2 region to allow for species identification. However, in rare instances the nucleotide sequence of one or both of the spacers is >99 % similar between species (interspecies similarity), making it difficult to separate species by sequence information alone [140]. Additionally, strains within some single species have not shown enough variability (intraspecies variation), making it difficult to assign species based totally on sequence information because of this diversity [140]. This sequence difference among fungal strains and sequence similarity among species may in some cases represent a misidentification based solely on phenotypic characteristics or a mislabeling of the taxonomic positioning of the microorganism. Likewise, however, genotypic differences within the ITS regions of sinle species has also proven to be useful for typing [145]. Numerous examples will be discussed describing the reliability of using the ITS regions as molecular targets to identify and characterize fungi to the genus and species level.

Table 6. *Candida* species identification by molecular technique

Study	Detection method	Target gene	Primer	Target fungus
Williams *et al.* [146]	PCR – RFLP	ITS1, 5.8S rDNA, ITS2	ITS1, ITS4	*Candida* spp.
Baere *et al.* [147]	Sequencing analysis	ITS2	ITS86, ITS4	*Candida* spp.
Elie *et al.* [148]	Candida-specific probes	ITS2	ITS3, ITS4	*Candida* spp.
Guiver *et al.* [149]	Sequencing analysis	ITS2	ITS3, ITS4	*Candida* spp.
Ferrer *et al.* [150]	Seminested PCR and Sequencing analysis	ITS1, 5.8S rDNA, ITS2	ITS1, ITS4 and ITS86, ITS4	*Candida* spp.
Shin *et al.* [151]	Candida-specific probes and PCR-ELISA	5.8S rDNA, ITS2, 28S	ITS3, ITS4	*Candida* spp.
Wahyuningsih *et al.* [146]	Candida-specific probes and PCR-ELISA	5.8S rDNA, ITS2, 28S	ITS3, ITS4	*C. albicans*
Morace *et al.* [152]	PCR-REA (Species-specific)	LIA1	$P450_1$, $P450_2$	*Candida* spp.
Fujita *et al.*[153]	PCR-ELISA	5.8S rDNA, ITS2, 28S	ITS3, ITS4	*C. albicans*
Kan *et al.* [154]	Southern blot	Actin	CA1, CA2	*C. albicans*

Direct sequence analysis

The utilization of direct sequence analysis was an important technical development in the field of amplification-based genotypic pathogen identification [140]. The rDNA complex was shown to be a useful target because of the preservation of conserved gene sequences (18S, 5.8S and 28S rDNA) that flank highly variable signature sequences (ITS1 and ITS2 regions). The direct sequence analysis of ITS regions was useful for the identification of both yeast and filamentous fungi.

The complete nucleotide sequence of the *Candida albicans* 5.8S rRNA coding gene and the flanking ITS regions was reported by Mercure *et al.* in 1993 [155]. Lott *et al.*[156] subsequently performed work using the ITS2 regions of the rRNA gene to identify *Candida* species based on sequence variations. They evaluated multiple strains of *C. albicans*, as well as a single strain of *C. parapsilosis*. Their results showed that *C. albicans* displayed no intraspecies variation and that the ITS2 region sequences alone appeared species-specific to allow for the differentiation of *C. albicans* from *C. parapsilosis*. Botelho and Planta [157] later carried out sequence analysis of both the ITS1 and ITS2 regions of *C. albicans* and *C. tropicalis*. They found that both spacer regions contained sufficient sequence diversity to make either of them suitable as specific target sites for the identification of *C. albicans*. These researchers later developed two ITS2 oligonucleotide probes that showed promise for the identification of *C. albicans*. In another study, intraspecies sequence heterogeneity within *Candida* species was reported to be common in the ITS1 region with the ITS2 region showing stability for identification purposes [145]. Lott *et al.* [158] later determined the nucleotide sequences of the ITS2 region for 13 additional species from those reported earlier within the genus *Candida*. They showed that no two *Candida* species had identical sequences within the ITS2 region and that the base-pair sizes of this region varied fourfold among the yeast studied. To expand on this observation, Chen *et al.* [159] conducted a study to evaluate 34 yeast species from multiple genera for ITS2 sequence polymorphism. They also determined that enough variation existed within this region to develop a database composed of the ITS2 length and sequence polymorphism for these isolates. Million *et al.* [160] in 2002 showed that the sequence analysis of the PCR amplified ITS region of rDNA using ITS1 and ITS4

primer showed six variable sites in the base order yielding 10 types of sequence from the 39 *C. albicans* isolates. In Table 7 showed the position of nucleotide changes and deletion in Million's study.

Table. 7 The positions for nucleotide changes and deletions [160]

	Exchanges site					
	90	**106**	**130'**	**135**	**423**	**428**
SE1	a	c	*	g	a	c
SE3	a	c	*	g	a	t
SE5	a	c	t	g	a	t
SE7	a	c	t	t	a	c
SE9	a	t	t	g	a	c
SE10	g	c	*	g	a	t
SE2D	a	c	t	g	a	c
	a	c	t	g	a	t
SE4D	a	c	*	g	a	t
	a	c	t	g	g	t
SE6D	a	c	t	g	a	c
	a	t	t	g	a	c
SE8D	a	c	*	g	a	c
	a	t	t	g	a	t

***= Deletion, SE = Sequence, D = Double**

Genus/species-specific primers and oligonucleotide probes

Once it was demonstrated that ITS sequences could be used for the identification of fungal species, additional studies focused on the recognition of ITS signature sequences for the development of specific primers and probes. Most of the work to date has been in the creation of probes to identify *Candida* species. Fujita *et al.* [153] constructed probes from the ITS2 region for the identification of *C. albicans, C. tropicalis, C. parapsilosis, C. krusei and C. glabrata*. They used a micro-titer plate enzyme immunoassay (EIA) with a digoxigenin-labeled probe method and showed that probes designed from this spacer region were useful for the identification of these *Candida* species (Table 5). They also showed that this EIA assay was highly sensitive and was able to detect amplified DNA from as few as two *C. albicans* cells per 0.2 ml of blood. To expand on this highly sensitive methodology, Martin *et al.* [151] recently developed a reverse-hybridization line probe assay. This assay, combined with PCR amplification of the ITS region, detected and identified a number of clinically significant fungal species, with a detection limit of 2-10 cells ml^{-1}.

The EIA approach developed by Fujita *et al.* [153] was larger applied to the identification of *Candida* species in blood cultures. Elie *et al.* [151] constructed probes from an additional 13 *Candida* species pathogenic for humans. These species included *C. guilliermondii, C. kefyr, C. lambica, C. lusitaniae, C. pelliculosa, C. rugosa, C. zeylanoides, C. haemulonii, C. norvegica, C. norvegensis, C. utilis, C. viswanathii and C. dubliniensis* (Table 5). These workers concluded that the probes designed from the ITS2 region were reliable for the molecular identification of *Candida* species (Table 5). Kan *et al.* [154] published a PCR method in which primers were designed to amplify a 158 bp segment of the actin gene using known cultures as well as blood 'spiked' with *Candida* spp.

Restriction fragment length polymorphism analysis

Amplified DNA can be cleaved with a restriction endonuclease and evaluated electrophoretically in a process referred to as post-amplification restriction fragment length polymorphism (PCR-RFLP) analysis. Variations in the number and size of the restriction fragments have been used for the identification of

microorganisms. The PCR-RFLP analyses when applied to amplified products of the 5.8S ribosomal DNA-intervening ITS regions has shown promise in distinguishing between different fungal species. In combination with morphological analysis, the results of PCR-RFLP have been useful for the characterization of isolates in both clinical and commercial applications [140]. Described in the following section are some of the clinical applications of this technology using the ITS region targets.

Identification of *Candida* species using RFLP analysis of the rDNA repeat has also been achieved [52, 55]. Williams *et al.* [55] evaluated eight species of *Candida* by PCR-RFLP analysis of the ITS regions. They applied their genotypic method to the identification of *Candida* species from a range of sources and felt that this was a reliable method to differentiate *Candida* species.

The LIA1-based assay has since been refined by making it species-specific using REA [152] (Table 5). This assay was found that can be separate *Candida* species.

Comparison of PCR and culture-based methods

Theoretically, PCR offers great potential for providing the basis of a rapid and sensitive diagnostic test for systemic candidiasis. In conventional PCR tests in which human and rabbit blood samples have been deliberately inoculated or spiked with defined numbers of *Candida* cells, the sensitivity observed in most studies was in the region of 100 CFU per ml. In similar experiments using enhanced PCR product detection methods (e.g. nested PCR, hybridization, and EIA) the sensitivity can be increased by at least a factor of 10 [1]. In some studies as few as one to two cells or femtogram amounts of DNA per ml were detected [161]. In another study in which patients deemed to be at risk of candidemia were assessed, four out of nine patients who were culture-negative yielded positive PCR results. One of these PCR-positive patients subsequently became culture-positive 7 days later, thus in dicating the potential of PCR to allow early diagnosis in individuals at risk [146]. One complication, however, is that PCR also has the ability to detect *Candida* DNA or nonviable cells in blood, thus leading to positive test results that would prove negative on culture. Most studies have also demonstrated that PCR is an excellent means of correctly identifying particular species, with most studies reporting a specificity of

100%. Similar levels of specificity and sensitivity can be attained using conventional diagnostic methods; however, the main advantage of PCR over this method is the speed with which results can be obtained. Most culture-based methods take a minimum of 48 hrs. to allow detection of *Candida* in samples; however, species identification can take at least another 48 hrs. (often longer in the case of slow-growing species). In contrast, PCR tests, even with additional adaptations to improve sensitivity and specificity, can be performed in as little as 8 hrs. [140].