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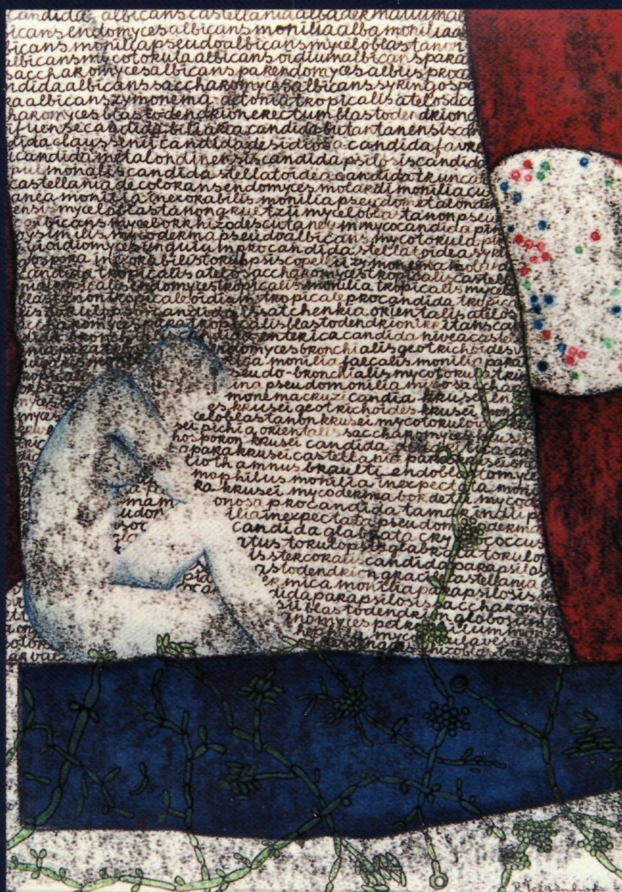
Epidemiology of systemic *Candida* infections



Andreas Voss

Epidemiology of systemic *Candida* infections:

Studies to pursue the molecular and clinical epidemiology.

Andreas Voss

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1997

Drukkerij Ponsen & Looijen Wageningen

Epidemiology of systemic *Candida* infections:

Studies to pursue the molecular and clinical epidemiology.

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van de Medische Wetenschappen

Proefschrift

ter verkrijging van de graad van doctor

aan de Katholieke Universiteit Nijmegen,

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We have no dreams at all or interesting ones.
We should learn to be awake the same way -
not at all or in an interesting manner.

Friedrich Nietzsche

Aan Angela, Timothy en Allegra

Epidemiology of systemic *Candida* infections

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I. Introduction

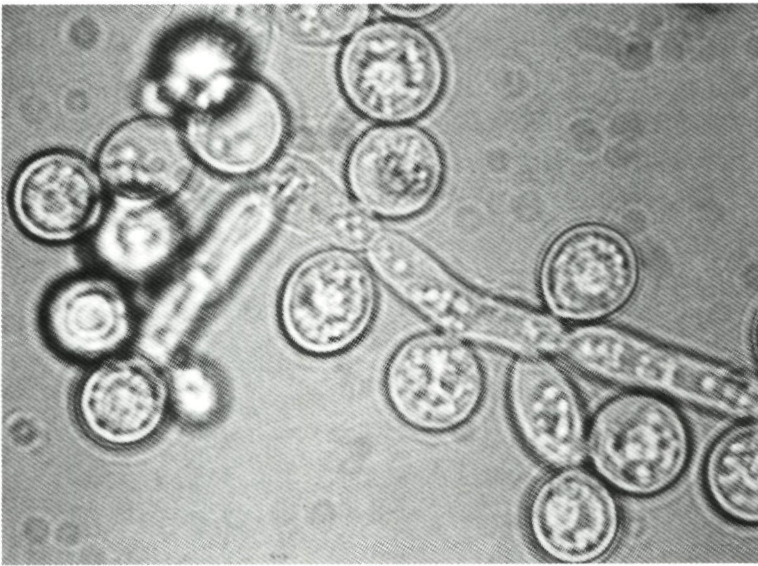

Figure 1. Yeast cells with typical budding (blastoconidia) and pseudohyphae

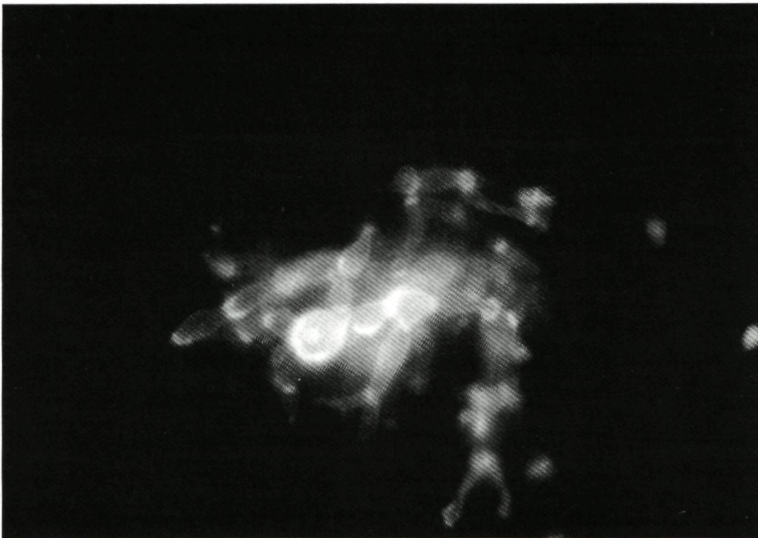

Figure 2. Yeasts stained with calcofluor white

Chapter 1

Candidosis

adapted from:

Andreas Voss, Paul E. Verweij, Jacques F.G.M Meis

submitted

and

Bart Jan Kullberg and Andreas Voss

Nederlands Tijdschrift voor Geneeskunde, 1996;140:148-151

The term candidosis (mostly named candidiasis in the USA) is used to refer to infections due to organisms belonging to the genus *Candida*. In addition to mucosal and cutaneous infections, these opportunistic pathogens can cause deep-seated infections, which may be restricted to one organ or disseminate throughout the human host.

The organism

Species of the genus *Candida* are anamorphic (asexual) yeasts classified as Fungi Imperfecti. Most members of the genus are dimorphic, in the sense that they form round or oval yeast cells with typical budding (blastoconidia) or generate pseudohyphae (Figure 1). *C. albicans* can also form true hyphae, whereas *C. glabrata* (formerly *Torulopsis glabrata*) never forms hyphae or pseudohyphae. Despite the fact that the presence of hyphae in tissue has been frequently postulated to be associated with true infection or invasion, and the presence of budding as colonization, respectively, these criteria are not firmly established [174].

Taking taxonomic changes into account, approximately 200 *Candida* species are known [10], of which about 10% are recognized to cause infections in humans (Table 1). So far, *Candida albicans* is the single, most common fungal species causing (nosocomial) infections. In National Nosocomial Infections Surveillance (NNIS) hospitals, *C. albicans* accounted for 76% of 24,227 *Candida* infections during 1980-1990 [8]. In the Netherlands, 73% of all *Candida* infections were shown to be caused by *C. albicans* (Table 2) [210]. *C. tropicalis* is the second most frequent *Candida* species to cause infections in humans and may even be predominately isolated among children with leukemia and bone marrow transplant patients [106 221]

Table 1: Common and rare *Candida* species known to cause human infections

Species	Clinical manifestation
Common	
C. albicans	skin/nail infections, vaginitis, thrush, esophagitis, enteritis, cystitis, disseminated infections such as: arthritis, pyelonephritis, pneumonia, endocarditis, meningitis, brain abscess, candidemia, and chronic disseminated candidosis
C. glabrata	candidemia and systemic candidosis, urinary tract infections
C. krusei	candidemia, endopthalmitis, diarrhea in infants [110,176]
C. parapsilosis	candidemia, endocarditis, septic arthritis, and peritonitis especially associated with prosthetic or indwelling devices. Epidemic infections due to contaminated devices and solutions [20,90,232]
C. tropicalis	candidemia and systemic candidosis especially among leukemic patients and iv drug abusers [47,106,221]
Rare	
C. catenulata	onychomycosis
C. ciferrii	onychomycosis [35]
C. dubliniensis	oral candidosis in HIV-infected patients [208]
C. guilliermondii	systemic candidosis, endocarditis in iv drug abusers
C. haemulonii	candidemia, skin infections [54]
C. kefyr (syn. *C. pseudotropicalis*)	systemic candidosis
C. lipolytica	catheter-related candidemia [228]
C. lusitaniae	candidemia and disseminated infections such as pneumonia and pyelonephritis [64]
C. norvegensis	candidemia in patients after kidney transpantation [126]
C. pulcherrima	invasive candidosis in the compromised host [152,230]
C. rugosa	catheter-related candidemia [161]
C. utilis	single case of catheter-related candidemia [4]
C. viswanathii	meningitis [179]
C. zeylanoides	candidemia, arthritis [15,101]

Table 2: Distribution of *Candida* species causing nosocomial infections in USA hospitals (1980 - 1990) and in the Netherlands (1989)

Candida species	% of isolates (USA)*	% of isolates (NL)**
C. albicans	69.3	73
C. non-albicans		
C. glabrata	8.8	9
C. tropicalis	6.4	3
C. parapsilosis	2.3	<1
C. krusei	<1	<1
C. guilliermondii	<1	--
C. stellatoidea	<1	--
C. pseudotropicalis	<1	<1
Unspecified *Candida* species	12	13

*Barnerjee et al .[8], ** Tan et al. [210]

Pathogenesis

Members of the genus *Candida* are common inhabitants of the human gastrointestinal, and genitourinary tract. Mucosal surfaces may be colonized with *C. albicans* in up to 80% of hospitalized patients and tends to be significantly lower (2-37%) in healthy individuals [60,127]. Despite the fact that mere isolation from these sites is no proof of infection, adherence and persistence of *Candida* spp. on mucosal surfaces is the initial step in the development of candidosis. The strongest adherence capability is consequently found in the most virulent species, *C. albicans* and *C. tropicalis*, the latter showing an especially high affinity for plastic polymers [120,171].

Candida spp. produce a variety of virulence factors, such as proteinases and lipases that assist invasion of host tissue, but in general a combination of host and organism factors, rather than a single virulence factor, seems to favor the development of diseases [128]. Infections are probably initiated by changes in the host defense that mutilate the host-pathogen equilibrium. Whereas infections of the skin and mucosa are mainly due to changes of hydration, pH, nutrient concentrations, or the microbial environment of the skin and mucosa, disseminated candidosis is usually associated with insufficiencies in host defense that commonly occur in hospitalized cancer and intensive care patients, receiving chemotherapy

and broad-spectrum antimicrobial treatment [98,191,198,211,248]. Furthermore, in patients with systemic infections, the phagocytic cell defense systems seems to be severely altered [37,97,99]. Additional determinants of candidal pathogenesis are listed in Table 3.

Table 3: Pathogenetic determinants of *Candida* species

- Adherence (mannoproteins)
- Candidal load
- Enzymes (lipase, proteinase)
- Formation of true- and pseudohyphae
- Impediment of host immune defense
- Persorption
- Phenotypic variability ("Switching")
- Synergism with bacterial and viral pathogens

adapted from 55,128,171

Invasion of mucosal barriers and subsequent systemic infections may furthermore be related to the candidal load (magnitude of colonization), as shown by an "healthy" investigator who "consumed" a suspension with 10^{12} *C. albicans* cells which lead to persorption with subsequent candidemia and a Lancet publication [82].

Epidemiology

Recently, a world-wide increase of mucocutaneous and deep-seated fungal infections was observed. The total number of fungal infection in hospitals participating in the NNIS-system (National Nosocomial Infections Surveillance System) increased from 6% in 1980 to 10.4% in 1990 [13]. Nearly 80% of these infections were caused by *Candida* species. An increasing incidence was observed for all clinical manifestations including oropharyngeal infections, postoperative site infections, and urinary tract infections, but was especially dramatic for candidemia [13].

The rising incidence of *Candida* infections and candidemia can be attributed to a variety of (mostly) endogenous and exogenous factors.

- Granulocytopenia is the most important risk factor for the development of systemic candidosis in patients with cancer [19,63]. The improved and intensified treatment of patients with cancer induced significantly prolonged granulocytopenia, thereby increasing the chance of fungal infections. Damage of the oropharyngeal mucosa through the use of ,

aggressive cytostatic drugs, such as cytarabine, facilitate *Candida* colonization and subsequent invasion [19].

- The escalating use of broad-spectrum antibiotics in hematology- and intensive care patients has been described as an independent risk factor for the development of *Candida* infections [23,238]. Especially orally administered antibiotics or those with high entero-hepatic circulation, will enhance proliferation of *Candida* species colonizing the gastrointestinal tract (Table 4). In parallel, increased colonization or infections with non-albicans *Candida* species are associated with the use of azole antifungals [193,244].

- Histamine (H2) antagonists presumably effect the gastric flora either by elevation of the gastric pH or changes of mucosal attachment, allowing for increased *Candida* colonization [196,204].

Table 4: Risk factors of systemic candidosis (according to multivariate analysis)

- Age
- Azotemia
- Central venous catheters
- Chemotherapy
- Colonization
- Damaged mucosal barrier
- Graft versus host disease
- Hemodialysis
- Hyperglycemia
- Long-term use of broad-spectrum antibiotics
- Neutropenia
- Parenteral nutrition
- Steroids
- Surgery (multiple, extended, abdominal)

adapted from 23,59,81,234,237

<u>Portal of entry</u> The portal of entry depends on the individual patient's condition and probably specific site preference of certain *Candida* species. In patients with candidemia, intravenous lines were the single most common (39%) portal of entry [81]. In general most candidemias were due to *C. albicans*, but 38% of the *Candida* isolates from bloodstream infections originating from the gastrointestinal tract were *C. tropicalis* [81]. In patients receiving parenteral hyperalimentation, fungemia was predominantly due to *C. parapsilosis* [117,67]. The role of iv devices is controversially discussed by different authors [59,75,190], but given the high incidence of candidemia among patients with central venous catheters found by Curry and Quie [34], the fact that 50% of these individuals had invasive infection at autopsy, and the

results of other studies, using multivariant analysis [23,238] we believe that central venous lines are an important risk factor for candidemia.

<u>*Candida* typing</u> Since the first description of the two *C. albicans* serotypes by Hasenclever et al in 1961 [66] *Candida* strain typing has progressed through various phenotypic and genotypic approaches (Table 5). DNA based methods such as restriction enzyme analysis using conventional or pulsed-field gel electrophoresis [41,184,217,218,222,225], electrophoretic karyotyping [77,104,143,159,222], arbitrarily primed PCR fingerprinting [95,189], and Southern hybridization analysis using midrepeat sequence probes [186,187] have been proven to be the most helpful and are presently used as diagnostic tools or are applied to population studies. At present, a combination of phenotypic markers such as antifungal susceptibilities or biochemical profiles and a DNA-based typing method, or (in the research setting) two different DNA-based methods, should be used to characterize *Candida* strains for clinical and epidemiological ("molecular epidemiology") purposes, respectively. Standardized methodologies and optimal methods to analyze typing results are still lacking, but a recently founded European working group on epidemiological markers may solve these problems in the near future.

Table 5: *Candida* strain typing

Phenotyping	serotyping	(66)
	morphotyping	(146)
	resistotyping	(229)
	biotyping	(130)
	killer factor typing	(151)
Genotyping	karyotyping	(104)
	restriction fragment length polymorphism	(184)
	Southern bloting	(187)
	Random amplified polymorphic DNA typing	(214)

<u>Molecular epidemiology</u> Molecular typing was used to evaluate the pathogenesis of endogenous *Candida* infections, as well as to prove exogenous transmission.

Evaluating the sequence of *Candida* colonization and candidemia it was proven that in about 90% of the neutropenic patients [158,214] and in 75-80% of the non-neutropenic patients [222] the subsequently infecting strains were identical with the previous colonizing isolate. Furthermore, most patients were shown to develop infections with their own distinct *Candida* strain, thereby confirming the concept of endogenous infection [112,137,140,158,222]. On the other hand, classical concepts of endogenous pathogenesis such as that of gastrointestinal colonization and recurrent vulvovaginitis may become questionable. Stein et al. [205]

demonstrated that vaginal and rectal *Candida* strains from the same women with recurrent vulvovaginitis were usually different, whereas sequential vaginal isolates were identical, therefore suggesting "relapse" instead of autoinfection.

Although the majority of *Candida* infections are probably of endogenous origin, exogenous sources and cross-infection have been proven using molecular typing methods. Among others, pigeon guano, contaminated devices (syringes [re-]used for total parenteral nutrition), and contaminated lemon juice have been described as exogenous sources [14 61 192]. The first outbreak of candidemia due to cross-infections via hands was described using sero- and biotyping, but later outbreaks were confirmed using DNA-based methods [16 25 27 39 41].

Diagnosis

Since clinical manifestations of localized and deep-seeded candidosis are not specific for *Candida* species, a laboratory identification of this microorganism is essential to establish a definitive diagnosis. Standard approaches to laboratory diagnosis include:

a) direct microscopy
b) culture
c) histopathology

ad a) Direct microscopy of freshly obtained specimens provides information about the relative amount of *Candida* species and their morphologic features, and the cellular composition of the inflammatory response. Direct examination of specimens may provide a rapid diagnosis of involvement by *Candida* species. The detection of fungal elements in tissue biopsy specimens, such as liver biopsy, is of great importance, since culture of the same material often remains negative. Special fungal stains, including potassium hydroxide (KOH) and calcofluor white (Figure 2), are available to facilitate the detection of fungal elements.

ad b) *Candida* species generally grow well on common mycological and bacteriological media, including Sabouraud glucose agar, malt extract agar and sheep blood agar. Using these media *Candida* grows as smooth to wrinkled textured colonies of white or beige color. Identification of *Candida* species should be conducted to species level for all organisms isolated from normally sterile body fluids and from most cultures of mucosal surfaces. The identification of different *Candida* species carries both prognostic and therapeutic significance. Although the germ tube test permits the distinction between *C. albicans* (germ tube-positive) and non-albicans *Candida* species (germ tube-negative), the detection of multiple yeast species in culture may be difficult and often time consuming. Differential and selective media have been developed by incorporating chromogenic or fluorogenic substrates directly into the growth medium (e.g. CHROMagar [131], Sabouraud TTC [226]). The formation of colored colonies in which the coloration is species specific allows the direct (presumptive) identification of different *Candida* species on primary isolation.

ad c) The demonstration of organisms morphologically consistent with *Candida* in tissue sections is the gold standard of demonstrating deep tissue *Candida* infection With appropriate special stains such as periodic acid-Schiff (PAS) and Gomori methenamine silver (GMS) yeast-like bodies with pseudohyphae can be seen Histopathology allows at best a generic identification and culture of the same material may provide an identification to species level Furthermore the differentiation of *Candida* organisms from other opportunistic fungi such as *Aspergillus Histoplasma* or *Trichosporon cutaneum* based on histologic examination alone may be difficult Since invasive procedures are required to obtain appropriate tissue specimens the use of histopathology is often precluded in patients with severe thrombocytopenia

Detection of *Candida* species in the bloodstream is a key clinical laboratory strategy for establishing a diagnosis of invasive candidosis Single positive blood culture or blood cultures from a vascular catheter growing *Candida* species should be considered significant until proved otherwise However the diagnostic yield of blood cultures for detection of fungemia is only 15-50% [138] The recovery of *Candida* species from blood cultures may be improved by the use of biphasic media by venting the blood culture bottle or the increase of cultured volume of blood A major advance was constituted by use of a lysis centrifugation blood culture system This system was superior to other systems with respect to both the overall rate of recovery and the time to recovery Although data to evaluate the recovery of *Candida* from blood specimens with fully automated blood culturing systems (e g BactAlert Bactec 9240) are scarce there are data that support an increased recovery and earlier detection of yeast cells by automated systems when compared to conventional culturing methods [240] However invasive candidosis remains difficult to diagnose and the above mentioned diagnostic methods often lack sensitivity in early recognition of this infection Investigational non-cultural diagnostic systems are being developed to facilitate diagnosis more rapidly and improve therapeutic monitoring Detection of antigens genomic fragments and metabolites are now under investigation although no single system will supplant the standard diagnostic tools Investigational strategies for detection of antigenic markers include the detection of cell wall mannan by radioimmunoassay or latex agglutination *Candida* enolase by immunoassay [227] d-arabinitol by gas-liquid chromatography [57] or 1 3-ß-D-glucan by amebocyte lysate assay [121] Technical complexity poor reproducibility or positive results in patients only colonized with *Candida* species are problems which have to be overcome and are currently undergoing more extensive investigation At present no firm recommendation about any of the antigen tests can be given for the routine diagnostic laboratory Another approach involves the detection of *Candida* DNA fragments by polymerase chain reaction (PCR) Circulating *Candida* genome was detected by PCR in blood-specimens collected from mice with invasive candidosis while those from mice only colonized in the gastrointestinal tract with *Candida* remained negative [215] PCR promises to be more sensitive than blood cultures and may be an adequate technique in the early detection of invasive candidosis although the true relevance

and utility of this test remains to be established. Improved assay's may be useful indicators of deep infections in neutropenic patients complementing the diagnostic utility of blood cultures.

Susceptibility testing

Until recently, "rolling dices" was probably equally accurate (but much cheaper) in order to determine clinically useful in vitro susceptibility data for triazole antifungals. MIC results of different antifungal susceptibility testing methods were shown to vary as much as 50,000-fold [53]. The major sources of variation in susceptibility testing have been attributed to the choice of medium and pH, inoculum preparation and size, incubation temperature and time, and end-point criteria. In 1992, after nearly 10 years of development, the National Committee for Clinical Laboratory Standards (NCCLS) published a tentative version of their reference method for broth dilution susceptibility testing for yeasts [52 165]. While not without flaws, and being depending on individual host factors, this standardized, reproducible method allows multiple investigators to generate data, based on correlation between antifungal MIC and clinical outcome. Detailed review of the NCCLS guideline and of the current status of the correlation of in vitro data with clinical outcome is given in the above mentioned publications, and a minireview by Ghannoum et al. [56]. Despite the recent advances in antifungal susceptibility testing no definitive correlation between the results of in vitro susceptibility testing and in vivo response could be established. In the present situation routine antifungal susceptibility testing of yeasts should therefore be discouraged, with the exception of isolates from blood cultures.

Clinical manifestations

In general *Candida* infections are classified as localized or deep-seeded (Table 6). The terminology applied to describe "hematogenous" *Candida* infections has been used with great inconsistency. Bodey et al.[18] suggested a standard terminology dividing hematogenous infections into three groups:

A <u>Candidemia</u> is the occurrence of any *Candida* species in one or more blood cultures. An association with clinical symptoms or intravascular catheters should be specified.

B. <u>Acute disseminated candidosis</u> is defined as a proven infection of multiple non-contiguous organs due to hematogenous seeding, including candidemia with skin lesions or endophthalmitis

C <u>Chronic disseminated</u> candidosis, formerly hepatosplenic candidosis, in patients during periods of prolonged neutropenia.

If not separately stated, the description of clinical manifestations are derived from different standard text books, including those of Odds[128], Dupont[42], and Bodey[17].

Table 6: Clinical manifestation of *Candida* infections

Localized, muco-cutaneous infections

- skin and nail infections
- thrush/oropharyngeal infections
- chronic mucocutaneous candidosis (CMC)
- vulvovaginitis and balanitis
- esophagitis
- gastro-intestinal candidosis

Deep-seated candidosis

- peritonitis and intra-abdominal abscess
- meningitis
- endocarditis
- urinary tract infection
- osteoarticular candidosis
- ocular candidosis
- candidemia
- chronic-disseminated candidosis

Cutaneous candidosis

Cutaneous candidosis includes intertrigo (Figure 3), diaper candidosis (Figure 4), paronychia and onychomycosis (Figure 5). Predisposing factors of this common candidal infection are occluded moisture (diaper, bandages, wet bathing suits), heat, disruption of the epidermal barrier (friction/maceration), hormonal changes, obesity, diabetes mellitus, antibiotic treatment and immunosuppression. Common location are the moist folds of skin such as axillae, groin, intergluteal, or sub-mammary folds, and the interdigital spaces of fingers and toes. The lesions consist of a moist, macular erythematous rash with typical "satellites" on the surrounding healthy skin.

Paronychia of the finger nails may be seen as an occupational hazard in persons whose hands are subject to constant wetting or consistent occlusion (gloves), leading to maceration of the cuticle (nail fold). Lesions are characterized by mild to moderate erythema of the nail fold, associated with moderate discomfort. In chronic cases the infection may progress to cause onychomycosis, characterized by a complete destruction of the nail tissue. The last is more or less exclusively seen in patients with chronic muco-cutaneous candidosis.

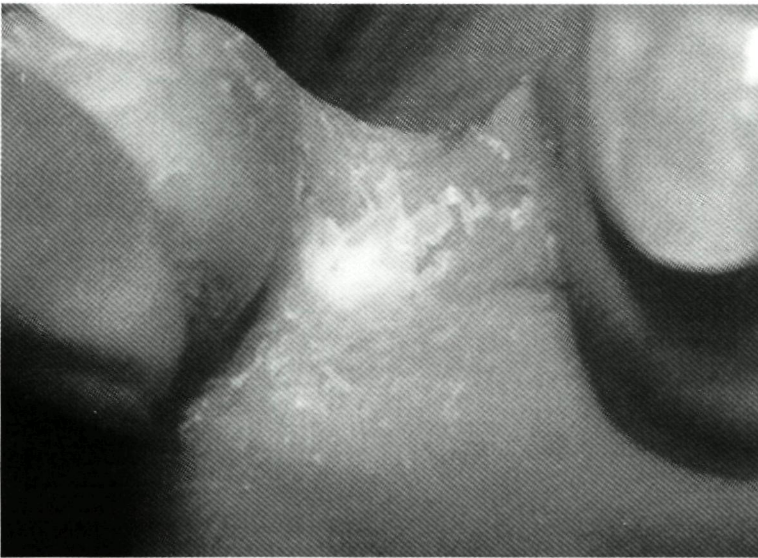

Figure 3. Intertrigo

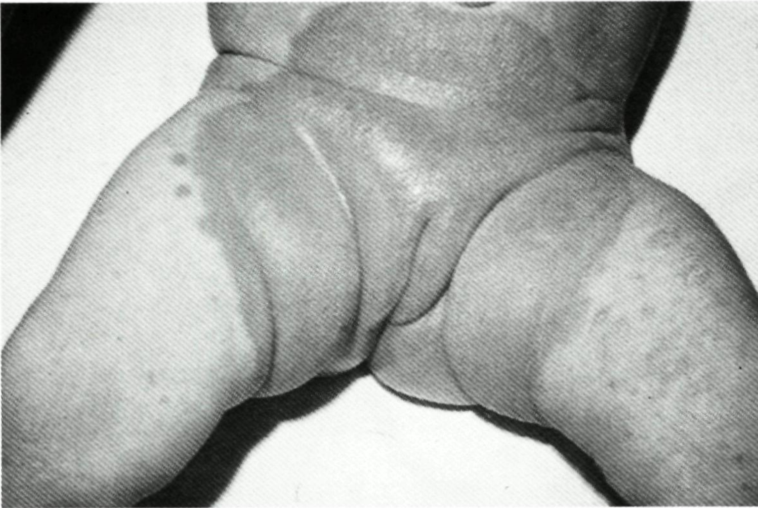

Figure 4: Diaper candidosis

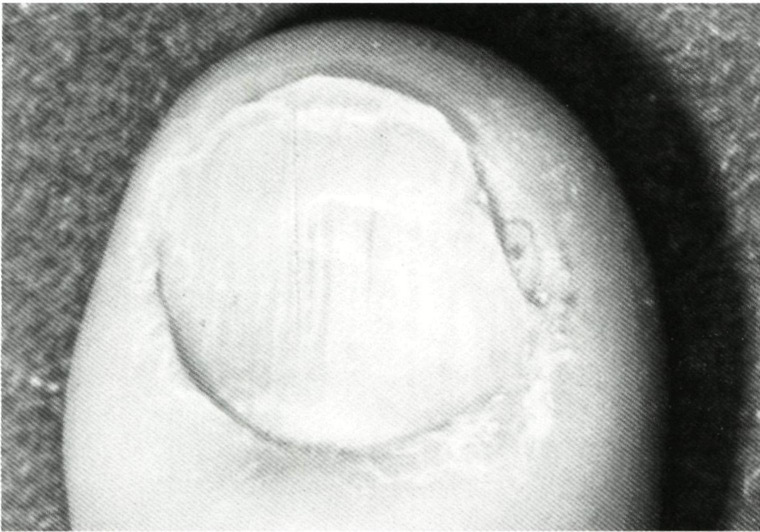

Figure 5: Onychomycosis

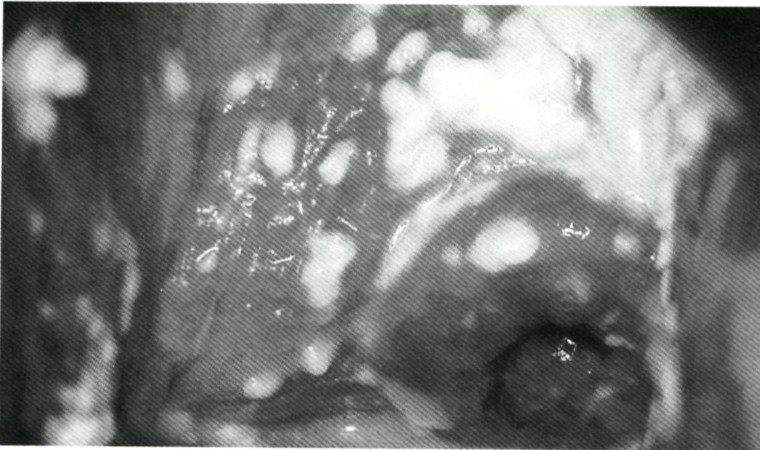

Figure 6: Vulvovaginal candidosis

The treatment of cutaneous candidosis depends on the extent and location of disease and the host's defense Topical therapy such as clotrimazole miconazole or ketoconazole may be given to add systemic treatment or alone in less extended infections The selection of the form (powder or ointment) depends on the local skin condition Extensive infections especially in compromised hosts should be treated systemically Classically oral ketoconazole was used but newer oral or parenteral azoles (fluconazole itraconazole) are advantageous In general antifungal treatment should be accompanied by restoring the epidermal barrier by keeping the infected area dry and cool

Chronic mucocutaneous candidosis

Chronic mucocutaneous candidosis is a form of persistent and recurrent *Candida* infection of the skin nails and mucous membranes practically exclusively caused by *C albicans* The clinical features include oral esophageal and vaginal candidosis as well as infections of the skin and nails Skin lesions are painless crusted with mild inflammation but the extent of cutaneous involvement varies *Candida* granuloma is the most severe localized form with hyperkeratotic granulomatous lesions (horn formation) Chronic mucocutaneous candidosis is mainly seen in young children and AIDS patients with impaired T lymphocyte function The occurrence of this disorder should alert physicians to the possibility of a defect immune system

Topical treatment may initially be used in patients with limited cutaneous infections but in general systemic antifungal therapy with orally administered azoles is necessary Whereas cutaneous and mucous lesions improve already after 1-2 weeks of systemic antifungal therapy most nail infections need to be treated for several months Additionally attempts should be undertaken to correct the patient's immune deficiency

Vulvovaginal candidosis and balanitis

Candida infections of the genital tract are mainly seen in pregnant women (last trimester) during or after broad spectrum antibiotic treatment and patients with diabetes mellitus Other predisposing factors may be low vaginal pH tight synthetic clothing sexual activity IUDs oral contraception with high estrogen-content dietary practices and specific immunologic defects [160] Clinical symptoms include vulval pruritus vaginal discharge and dyspareunia and may increase shortly before onset of menstruation Typically the discharge is creamy white and curd-like (Figure 6) The erythematous inflammation may be limited to the genital mucosa but may extend to include the perineum and the entire inguinal area In HIV /AIDS patients the infection may be chronic and refractory to treatment

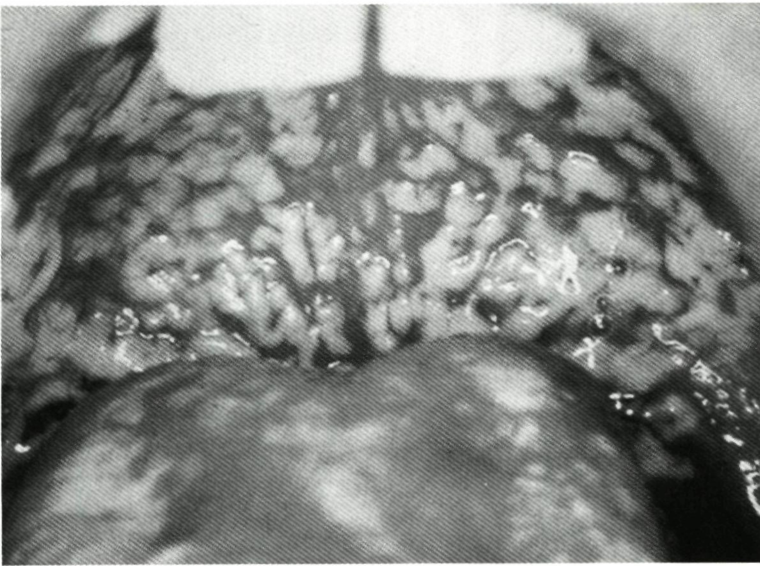

Figure 7: Trush

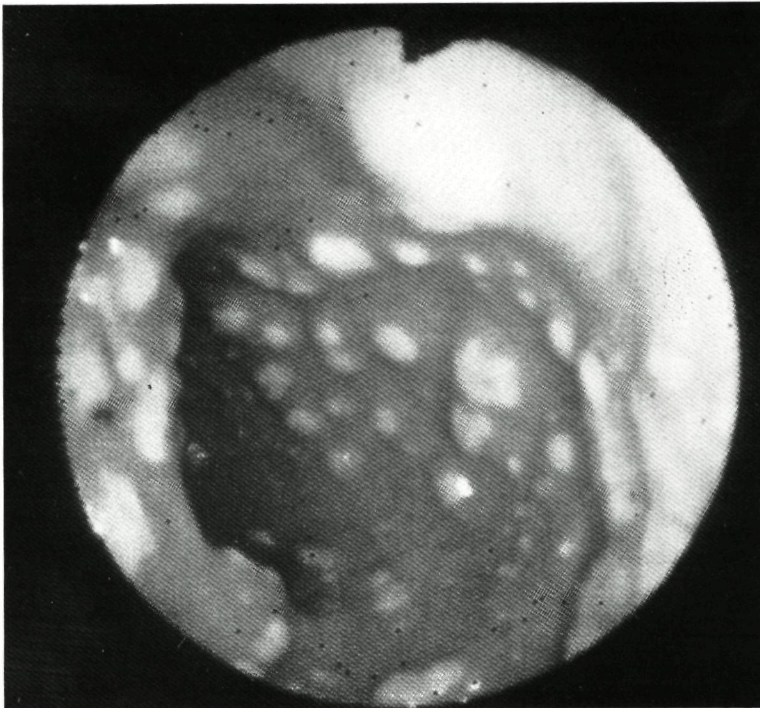

Figure 8: *Candida* esophagitis

Candida balanitis is characterized by pruritus, erythema, and gray-white pustules on the glans penis or preputium Despite the fact that it is still unknown to which extend sexual transmission occurs, the sexual partner should be investigated for vulvovaginitis.
Candida vaginitis can be treated with topical polyene and azole compounds in a variety of formulations. The clinical efficacy is not influenced whether lotions, creams, vaginal tablets, or suppositories are used. Short or single-dose oral treatment is possible, due to the use of higher doses of mainly azole antifungals. In women with recurrent and chronic infections, initially predisposing factors should be investigated and if possible eradicated. After initial topical or oral therapy, maintenance treatment with low dose oral azole compounds is recommended.

Oropharyngeal candidosis

The clinical features of oropharyngeal candidosis includes pseudomembranous candidosis (= thrush, Figure 7), glossitis, stomatitis, and perleche (angular cheilitis). The infection is associated with different endogenous and exogenous predisposing factors, such as immunologic impairment due to age (neonates), diabetes mellitus, leukemia, malignancy, neutropenia, HIV infection, and the use of histamine antagonists. The combined use of broad-spectrum antibiotics and steroids was demonstrated to cause *Candida* laryngotracheitis in otherwise healthy patients [28]. In patients with thrush the oral mucosal surface is covered with whitish, creamy pseudomembranes, consisting of hyphae, yeast cells, bacteria, and cell debris. Removal of plaques will expose a erythematous, bleeding, and possible painful mucosa Spreading of the infection may cause glossitis. Stridor or hoarseness of unknown cause in an immunocompromised patient may be caused by *Candida* epiglottitis [7].
Stomatitis occurs in patients with dentures and may lead to generalized hyperemia and granular inflammation of the predominately maxillary prosthesis. The infection may involve the angles of the mouth, causing localized fissures (perleche).
Despite the fact, that fluconazole (p.o. 100 mg/d) can be easily administered and is highly effective its use should primarily be avoided to reduce the chance of resistance development Instead, non-absorbable, topical antifungals such as nystatin suspension or clotrimazole troches should be initially used.

Esophagitis

Esophagitis often develops in AIDS patients or patients treated for leukemia and solid tumors. Patients suffer from painful dysphagia with retrosternal chest pain and burning sensation. Vomiting and nausea may occur. Although 30% of the patients have oral candidosis, the presence or absence if either one of the infections (thrush and esophagitis) is independent

from each other. To diagnose the infection, endoscopy (including brush specimen or biopsy) seems superior to (barium contrast) radiographs. It is still discussed whether primarily the response to empiric antifungal treatment should be assessed in order to differentiate between candidal and non-candidal esophagitis, before other diagnostic measurements are taken. Endoscopic findings show creamy whitish pseudomembranes, intense inflammation, and superficial ulcers (Figure 8). In general, clinical, radiographic, and endoscopic findings must be differentiated from other causes of esophagitis, especially viral infections (Herpes simplex - and cytomegalovirus), and non-infectious conditions such as esophageal Kaposi's sarcoma, stenosis, idiopathic ulcers, and peritracheal non-Hodgkin lymphoma [133,134].
Despite isolated reports of successful topical treatment, systemic therapy (eg. 100-200 mg p.o. or i.v. fluconazole) is recommended. Commonly the intravenous formulation is advantageous since patients cannot tolerate oral treatment. The exact duration of treatment is unknown, but should be continued for at least 10 days after resolution of symptoms [200]. Especially in AIDS patients with recurrent esophagitis, maintenance treatment is suggested.

Table 7: Endogenous and exogenous factors predisposing to gastrointestinal candidosis

Endogenous

- old and young age
- diabetes mellitus and other endocrinopathies
- mucocutaneous candidosis
- AIDS
- defect phagocytic function
- chemotherapy (via disruption of mucosal barier)

Exogenous

- Broad-spectrum antibiotics
- Corticosteroids and other immunosupressive agents
- Antacids
- Duration of hospitalization
- Drains

Gastrointestinal candidosis, peritonitis and intra-abdominal abscess

Superficial candidosis of gastrointestinal mucosal surfaces is commonly seen in patients with malignancies or surgical intensive care patients. Other predisposing factors are listed in table 7. The infection usually is asymptomatic during life, only diagnosed at autopsy. Lesions can be found at any site in the gastrointestinal tract. Progression and perforation may lead to hematogenous spread into organs, or cause peritonitis.

Candida peritonitis can furthermore result from other perforations due to ulcers, colitis, neoplasma, or surgery, and may also originate from colonized CAPD catheters Unless patients are severely immunocompromised, the infection remains localized to the abdominal cavity Occasionally, *Candida* may cause, pancreatic abscess, cholangitis, and biliary tract infection

One of the most controversial aspects in the diagnosis of *Candida* infections is probably the clinical significance of *Candida* isolation from intra-abdominal sources In general, patients undergoing surgical treatment have a low risk of disseminated infection, even if *Candida* is isolated from their peritoneal fluid or an intra-abdominal abscess [173] *Candida* should only be considered as clinically important, when isolated from intra-abdominal foci of "high-risk" patients, who received immuno-supressive therapy or had prior episodes of sepsis [3 58] In addition to antimicrobial treatment of bacterial co-pathogens, and adequate drainage, these patients should receive 400 mg once daily fluconazole

Ocular candidosis

Candida endophthalmitis implies hematogenous dissemination to multiple organs in patients with candidemia or catheter-associated infection Rarely, exogenous infection may occur following ocular trauma or surgery The infection is commonly caused by *C albicans* In a review based on the English-literature between 1965 and 1989 only 6 well documented cases were found [74] The incidence of non-albicans endophthalmitis due to *C parapsilosis* and *C tropicalis* might be increasing [31 45]

Lesions are commonly located near the macula leading to impaired (cloudy) vision In general ophthalmologic consultation is recommended to establish the diagnosis, to assess the clinical response, or to detect complications of hematogenous *Candida* infections

The standard treatment consists of iv amphotericin B in combination with flucytosine [188] Recently, systemic treatment with fluconazole (with or without intraviteal amphotericin B) was shown to be effective [45 182 219], but still, partial vitrectomy may be indicated to prevent loss of sight

Osteoarticular candidosis

Candida joint infections may occur after direct inoculation during implant surgery or intra-articular corticosteroid injections or by hematogenous dissemination The last is mainly seen as late complication in low birth-weight neonates neutropenic patients or patients with pre-damaged joints (prosthetic or rheumatoid) Osteoarticular infections of the vertebra and sternoclavicular joints are frequently seen in iv drug abusers

The symptoms of *Candida* arthritis include fever indolent pain and effusion In infections due to hematogenous spread usually more than one joint is involved

Pulmonary candidosis

Pulmonary candidosis may follow aspiration from the colonized upper respiratory tract, resulting in a local or diffuse bronchopneumonia, or may result from seeding of the lung during hematogenous spread, resulting in nonspecific, patchy infiltrates affecting both longs. Contrary to previous studies in cancer patients with *Candida* pneumonia Haron et al. [65] found only a small proportion of cases with severe neutropenia and a surprising lack of other organ involvement or candidemia. Aspiration of oropharyngeal content was suspected as the main route of infection in these patients. The major clinical presentations were fever and tachypnoe. Since the isolation of yeasts from respiratory specimens (including BAL) and the radiologic and clinical presentation is nonspecific, most patients are diagnosed at autopsy.

Central nervous system (CNS) candidosis

Among this very uncommon presentation of *Candida* infections, meningitis is the most frequent. The infection primarily occurs in low-birth-weight infants with hematogenous candidosis and in patients with intracerebral devices, such as ventriculoperitoneal shunts. Symptoms include hydrocephalus, fever, and meningeal irritation, but the course of disease may be indolent with minimal fever [177]. In shunt infections the recommended treatment is replacement of the shunt and iv (and intrathecally) amphotericin B.
Other forms of CNS candidosis include brain abscess and metastatic encephalitis, all of which are mainly diagnosed at autopsy.

Cardiac candidosis

Particulary in patients with prosthetic or previously damaged heart valves hematogenous spread of *Candida* may lead to the establishment of endocarditis, the most common form of cardiac candidosis. Other predisposing factors are central venous catheters and iv drug abuse. Clinical symptoms are similar to those of bacterial endocarditis, but embolic phenomena may even be more frequent in fungal infections.
The treatment consists of antifungal therapy and surgical repair/replacement of the valves. Regarding the antifungal treatment, amphotericin B in combination with flucytosine is recommended, followed by long-term fluconazole maintenance [219 246].

Urinary tract candidosis

Due to the lack of well-established guidelines, fungal urinary tract infections (UTI) present a diagnostic and treatment dilemma [62]. The recovery of *Candida* species from urine cultures may be consistent with asymptomatic colonization, local infection, or might be the first symptom of systemic fungal infection. The clinical significance largely depends on the patient's clinical condition.

Despite apparent problems as to which candidal colony count to use as a threshold, accurate diagnostic is needed to ensure necessary treatment and prevent avoidable adverse events in patients without need of toxic antifungal treatment.

Most lower UTIs result from a local spread of yeasts from the genital- and gastrointestinal tract. *Candida* cystitis or bladder colonization is usually seen in patients with prolonged catheterization in combination with antibiotic treatment, diabetes mellitus, or by mechanical obstructions that lead to incomplete bladder emptying (anatomical abnormalities, prostatic hypertrophy, cervical cancer).

Candida pyelonephritis is usually a result of hematogenous dissemination, but may furthermore occur due to ascending infection. Infection may be complicated by development of "fungus balls" in the renal pelvis.

Amphotericin B bladder irrigation is a common mode of therapy for uncomplicated candiduria, although no specific guidelines exists as to the optimal dose and best manner of administration. Traditionally 50 mg/l amphotericin B was used, but recently doses as low as 5-10 mg/l were recommended [180]. According to a recent literature review the therapy of choice might be oral fluconazole (50-200 mg/d) which appears to be of clinical value in the treatment of both uncomplicated and complicated candiduria [224].

Candidemia

Candidemia used to be a rare disease limited to patients with burn wounds and other severe traumas. *Candida* spp. were therefore classified as harmless commensals. During the past decades, the enlarging proportion of patients in need of chemotherapy, transplant surgery and intensive care, was paralleled by a steady increase in the incidence of nosocomial *Candida* infections [13 72]. In US hospitals, the incidence of candidemia increased by two- to fivefold and one- to fourfold in teaching and non-teaching hospitals, respectively [8 183], with *Candida* species being the fourth or even third most common pathogen isolated from blood cultures [144 183].

Despite antifungal treatment, the mortality of candidemia is still as high as 60%, with an attributable mortality of 38% (CI_{95}: 26-49) [237]. In those patients surviving candidemia the average hospital stay is prolonged by 30 days, compared to patients without *Candida*

infections [237] Candidemia therefore is not only a life-threatening yeast-infection but furthermore significantly increases the costs of health care
Candidemia remains a difficult entity to treat The need to adopt treatment to the individual patients complicates the development of uniform therapeutic recommendations Regarding the frequency of hematogenous dissemination its high mortality and the difficulties to establish accurate bedside diagnosis all patients with candidemia regardless of its source or duration should be treated [22 50 87 200 237] Terms as 'benign' candidemia should be abandoned The question remains which antifungal to use in which doses for how long and if it should be given alone or in combination In patients with catheter related candidemia the removal of the indwelling device seems essential to eradicate the yeasts [43 87] Failure to perform catheter exchange was strongly associated with the persistence of candidemia in a study by Rex et al [162]

Chronic disseminated candidosis

Chronic disseminated candidosis formerly referred to as hepatosplenic candidosis is seen in patients with severe neutropenia particularly due to acute leukemia Histology is characterized by diffuse necrotic lesions or abscesses in the spleen and/or liver Typically the infection begins during a period of prolonged neutropenia and presents as fever with liver enzyme disturbance after hematologic remission and recovery of neutrophil count Clinically abdominal pain and enlargement of the liver and/or spleen may be detected Diagnosis may be hindered by the inability to receive diagnostic material (biopsy) and the fact that blood and biopsy cultures remain negative despite visible (pseudo-)hyphae in the lesions Numerous small radiolucent lesions in the liver or spleen may be detected by CT scans or ultrasound
Classically amphotericin B was used for the treatment of patients with chronic disseminated candidosis Recent studies suggest that fluconazole therapy may be at least equally effective and better tolerated [5 36]

Antifungals used in the treatment of candidemia and deep-seated infections

Until recently there have been very few systemic antifungals and amphotericin B was considered as the golden standard However clinicians were reluctant to use the drug since they were appalled by its adverse effects Due to its ease of administration and safety fluconazole seemed promising but until confirmed by Rex et al [163] it was unknown that amphotericin B and fluconazole are similarly effective in the treatment of non neutropenic patients with primarily catheter-related candidemia Fluconazole was furthermore proven to be effective for various candidal infections including renal disease hepatosplenic candidosis and deep organ candidosis in organ transplant recipients and patients with

hematological malignancies [5 36 76 118 135]. In surgical patients the combination of amphotericin B (0.5 mg/kg) and flucytosine (4 x 25 m/kg) lead to an earlier microbiological response, but cure rates were identical to those of fluconazole monotherapy [83]. With the probable exception of acute hematogenous candidosis in neutropenic patients all (tri)azoles can be effective alternatives to amphotericin B (± flucytosine) for selected systemic candidal infections.

Amphotericin B

The polyenes include three main compounds: natamycin, nystatin, and amphotericin B, of which only the last can be used parenterally in the treatment of systemic fungal infections. Amphotericin B is a naturally occurring compound produced by *Streptomyces nodosus*. It became the treatment of choice in patients with candidemia and deep-seated infections, aspergillosis, and zygomycosis right after its introduction in the late 1950. The clinical effect of amphotericin B is due to its irreversible binding to ergosterol of the fungal cell membrane. The major disadvantage of the conventional formulation of amphotericin B is the high incidence of adverse reactions, often leading to early termination of treatment (Table 8). The total dose of conventional amphotericin B is limited to 4g since renal tubular damage is predictable in patients exceeding this cumulative dose. Meanwhile some of the early adverse reactions might be prevented by slow built up of dosage, or co-administration of antihistamines, the major problem of nephrotoxicity remains almost unchanged. Considerable effort has been expanded in the quest for less toxic drug analogues and different formulations. The various lipid formulations of amphotericin B were shown to significantly reduce the toxicity and be equally effective, using a dosage of up to five-times the one of the conventional amphotericin B.

Flucytosine

Flucytosine inhibits the protein synthesis of yeasts. The penetration of the drug into body cavities, including the cerebrospinal fluid is good, but it's clinical use is limited by the common occurrence of primary resistance and development of resistance during treatment. Furthermore, flucytosine can suppress the bone marrow, especially limiting its use in AIDS patients. Flucytosine in combination with (reduced dosages) amphotericin B was shown to be effective in patients with persistent candidemia and/or deep-seeded candidosis involving the liver, spleen, bones, CNS, or the heart [49].

Azoles

The azoles are divided into the older imidazoles (miconazole and ketoconazole) introduced in the early 1980's, and the newer triazoles, fluconazole and itraconazole, introduced in 1990 and 1992, respectively. The main antifungal effect of the azoles is through inhibition of the

ergosterol synthesis, thereby interacting with the main sterol in the fungal cell membrane, leading to an increased permeability and obstruction of cell growth.

Table 8: Adverse effects of antifungal agents used in the treatment of systemic candidosis

(Organ) system		Amphotericin B	Flucytosine	Ketoconazole	Fluconazole	Itraconazole
GI tract	nausea	+	5%	<10%	<5%	<10%
	vomiting	+	5%	<10%	<5%	<10%
	anorexia	+		+		
	diarrhea		+			
	abdominal pain		+	+		
Skin	rash	+	+	+		+
	pruritus		+	+		+
	Stevens-Johnson-syndrom				+	
Liver	elevation plasma aminotransferase		7%	2-10%	1-7%	1-5%
	hepatitis		rare	+	rare	rare
Bone marrow	anemia	+	less common			
	leukopenia		+			
	thrombocytopenia		+			
Kidney	azotemia	80%				
	tubular acidosis	+				
	hypokalemia	+				+
	hypomagnesemia	+				
Endocrine system	adrenal insufficiency			rare		
	decreased libido			+		
	impotence			+		rare
	gynecomastia			+		
Other	(thrombo)phlebitis	+				
	headache	+	+	+	+	+
	fever & chills	+		+		
	confusion		+			+
	hypertension					+
	edema					
	photophobia			+		
	seizure				+	
	dizziness					+

adapted from Como and Dismukes [32]

Table 9: Characteristics of antifungal agents used in the treatment of systemic candidosis

Characteristics	Amphotericin B	Flucytosine	Ketoconazole	Fluconazole	Itraconazole
Trade name	Fungizone®	Ancotil®	Nizoral®	Diflucan®	Trisporal®
Formulation	po or iv	po or iv	po	po* or iv	po* (iv under investigation)
Oral bio-availibility	--	++	+ pH dependent	++	+ pH dependent
Protein binding	++	--	++	--	++
Unchanged drug in urine	--	++	--	++	--
CSF concentration	--	++	--	++	--
Peak plasma concentration (µg/ml)	1.2-2.0	30-45	1.5-3.1	10.2	0.2-0.4
Terminal elimination half-life	20-24 h (end-phase 15 d)	3-6 hr	7-10 hr	22-31 hr	24-42 hr
Dialysable	HD - PD -	HD + PD +	HD - PD -	HD + PD ?	HD PD
Renal insufficiency	no adjustment necessary	extend interval	no adjustment necessary	CrCl < 40 ml/ min 200 mg, CrCl <20 ml/min 100 mg/24h	
Supplemental dose after HD	not required	37.5 mg/kg q6h	not required	200 mg	not required
Contraindication	potential renal failure severe hepatic failure	pregnancy, neonates	pregnancy, lactation	pregnancy, lactation	pregnancy, lactation
Increases plasma concentration of:			cyclosporine cumarines phenytoin	cyclosporine cumarines phenytoin	cyclosporine cumarines (phenytoin) (digoxin)
Decreasing azole plasma concentration			antacids H_2-antagonist isoniazid phenytoin rifampin	rifampin	antacids H_2-antagonist phenytoin rifampin
Resistance	*C. lusitaniae*	up to 50% primarily resistant	*C. krusei* *C. glabrata*	*C. krusei* *C. glabrata*	
Dosage Adults	0.6-1 mg/kg/d q24h (daily increase of 0.1-0.25 mg/kg, maximum total dose: 5g)	37.5 mg/kg q6h	200-400 mg q24h	200-400 mg q24h, initially once 400 mg	200 q12h
Dosage Children	0.6-1 mg/kg/d	37.5 mg/kg q6h	3.3 - 6.6 mg/kg	6-12 mg/kg q24h	
Comments	bladder irrigation 10-50 mg/l	use in combination with other antifungals	antagonism with AmB	penetrates well into CSF	
	intrathecal: 0.2-0.5 mg q48-72h (diluted with CSF or glucose)		not recommended for systemic infection		
	add heparin to reduce thrombophlebitis				

* tablets and suspension

-- no/very low, - low, +/- middel, + = high, ++ very high, HD = hemodialysis PD = peritoneal dialysis

Table 10: New and future antifungal agents

Generic	Trade name	Comment
Liposomal amphotericin B	AmBisome	licensed , unilamellar liposomal vesicle
Amphotericin B Lipid Complex	Abelcet	licensed in USA sheets
Amphotericin B colloidal dispersion	Amphocil	micelle, licensed
Long-circulating pegylated AmB liposomes		tested in vitro
Liposomal nystatin i.v.	Nyotran	clinical phase IIa studies, III underway
Terbinafine	Lamisil	old po drug new indications ?
Itraconazole i.v.	Trisporal	triazole, under clinical investigation
Fluconazole - high dose	Diflucan	under clinical investigation 800-1600 mg/d
ER-30346		triazole, tested in vitro
Sch 510448		azole, tested in vitro
D 807		triazole, tested in vitro (?)
D 8581		triazole, tested in vitro
UK-109.496 (voriconazole)		triazole, under clinical investigation
UR-9746 and UR-9751		triazole, tested in-vitro
LY 121.019 (cilofungin)		
LY 303366		echinocandin B antifungal, tested in-vitro
L-733560		pneumocandin antifungal, tested in-vitro
BMS-18118		pradimicin, tested in-vitro
Syringomycin E		lipodepsipeptide, tested in vitro

Azoles (cont.)

In contrast to fluconazole, the bioavailability and absorption of oral ketoconazole and itraconazole is influenced by the stomach pH, thereby altered by the presence of food or gastric acid [175] Ketoconazole and itraconazole are almost exclusively excreted in feces and urine, after metabolisation in the liver The proportion of the drugs excreted unchanged via urine is so little that doses need not to be adjusted in patients with renal impairment In contrast, fluconazole doses must be reduced in patients with a glomerular filtration rate of < 50 ml/minute, since 80% of the administered dose is excreted unchanged in the urine The most important drug interactions are listed in table 9 Since ketoconazole, and rarely the triazoles, may cause clinically important, even fatal hepatitis, azole therapy always should be discontinued in patients with symptoms of laboratory evidence of hepatic dysfunction [102 124 213] In general, the azoles, especially the triazoles, are a safe and effective alternative to amphotericin B for the treatment of systemic *Candida* infections In patients with ketoconazole refractory candidosis, in vitro cross resistance to triazole antifungal's and in one case clinical treatment failures were demonstrated [194] Potential problematic, might be the development of resistance and/or selection of primary resistant *Candida* species during treatment with azole antifungals

In case of the newest treatment options (Table 10) such as the lipid or liposomal preparations of amphotericin B not only the clinical effect must be determined, but furthermore the economic implications since these preparations are extremely expensive Next to liposomal amphotericin B a huge variety of new azole and triazole compounds, liposomal formulation of other antifungals, and new dosages of clinically used antifungals (1600 mg fluconazole) are in development or are clinically evaluated

Emergence of non-*Candida albicans* species and antifungal resistance

As mentioned above *C albicans* used to be - and to a lesser degree still is - the *Candida* species predominantly isolated from clinically important sites Recently, infections are increasingly caused by other *Candida* species, such as *C tropicalis* *C parapsilosis*, *C krusei* and *C glabrata* [84 242] In some institutions the proportion of non-albicans *Candida* isolates actually exceeded that of *C albicans* [236] At the University Hospital Nijmegen the proportion of non-albicans *Candida* species increased from 12% in 1990 to 23% in 1995 (Table 11)

With the exception of *C tropicalis* the virulence of non-albicans species seems to be comparable to that of *C albicans* [81] The increase of *C tropicalis* infection in patients with leukemia is probably due to a higher virulence of this species in this patient group in whom the gastrointestinal mucosal "barrier" is damaged through the use of cytarabine [243] Despite its high virulence (colonization with *C tropicalis* frequently leads to invasive infections) the

mortality of *C. tropicalis* candidemia was shown to be lower than that of *C. albicans* [116]. Certainly, more studies are needed to further evaluate the pathogenicity of *C. tropicalis* and the other non-albicans species. Presently, all *Candida* species isolated from clinical relevant material should be classified as possible pathogen.

Table 11: Frequency of *Candida* species isolated from clinical material at the University Hospital Nijmegen, 1990 to 1995

***Candida* spp.**	**1990**	**1991**	**1992**	**1993**	**1994**	**1995**
total	1604	2258	2659	2456	2393	2579
albicans	1406 87.7%	1867 85.3%	2190 82.2%	1966 80.0%	1895 79.2%	1979 76.7%
glabrata	2 0.1%	56	146	180	228	263 10.2%
krusei	2 0.1%	24	20	39	42	119 4.6%
tropicalis	1 0.05%	55	37	68	72	92 3.7%
parapsilosis	23 1.5%	53	39	45	61	54 2.1%
lusitaniae	0 0.0%	4	7	8	6	20 0.7%
others	49 3.1%	44	35	62	47	48 1.9%
not-speciated non-albicans	121 7.5%	155	185	88	42	4 0.1%
non-albicans	198 12.3%	391 14.7%	469 17.8%	490 20.0%	498 20.8%	600 23.3%

With the recent clinical use of new azole compounds not only a shift among the *Candida* species involved in nosocomial infections is discussed, but furthermore the development of resistance [244]. Next to the extensive use of new antifungals, and the changing patient population, the application of standardized antifungal susceptibility tests and the use of molecular typing methods may be other reasons to explain the current epidemiological trend. Not only, that the use of this method allow us to better understand the epidemiology of candidosis, but we are furthermore able to prove the development of resistance in sequential isolates of individual patients receiving antifungal treatment.

Resistance of *Candida* spp. to amphotericin B appears to be extremely uncommon. Immunocompromised patients with candidemia due to isolates with amphotericin B MIC of > 0.8 mg/l, were shown to have a significant higher mortality, compared to those whose *Candida* blood-culture isolates had an MIC of less than 0.8 mg/l (154). Aside from these

reports, primarily resistance and development of resistance to amphotericin B is well documented in *C. lusitaniae* [64 132]. Amphotericin B-resistant *C. lusitaniae* are increasingly isolated from hematology patients in the US, where amphotericin B is heavily used in the prophylaxis of this patient group [242] Recently, the development and spread of amphotericin B (and 5-fluorocytosine-resistant) *C. lusitaniae* strains within the hospital environment were reported [16 79 142 178].

Meanwhile amphotericin B, due to its toxicity and cumbersome administration was restricted to patients with severe fungal infections, fluconazole is widely used for the (early) treatment of candidosis, possibly leading to resistance development or a shift towards non-albicans species. Furthermore, it is applied over long periods or even continuously in AIDS patients with remittent oropharyngeal candidosis. Subsequently, fluconazole resistance was not only first reported in this setting [9 44 119 125 129 159], but is still hardly ever seen outside this group [166]. Most of these cases were advanced AIDS patients who had notable prior exposure to fluconazole. Resistant isolates generally had an elevated MIC for fluconazole and were genotypically identical with the initial isolate, although reinfection during treatment with a genotypically different isolate may occur [9 11]. In those cases where development of fluconazole resistance occurred patients suffered from long lasting neutropenia, infections with intrinsically resistant strains, such as *C. krusei*, failure to remove a potentially infected device, or received low dose (100-200 mg per day) treatment [1 2 164 166]. In a randomized multicenter trail of fluconazole versus amphotericin B as treatment of non-neutropenic patients no significant increase in MIC among serial isolates from individuals with persistent *Candida* infections could be demonstrated [164]. Therefore, in patients, others than those (AIDS patients) with recurrent oropharyngeal candidosis, who receive shorter courses of therapy the chances of resistance development are very low [164]. A good clinical response to fluconazole treatment can still be expected, especially if an appropriate dose (≥400 mg per day) is given, and intrinsically resistant species, such as *C. krusei*, are ruled out.

In general, available data illustrate the possibility of resistance development during fluconazole treatment and selection of inherently resistant strains [166]. It seems that the shift from albicans to non-albicans species respectively from susceptible to less susceptible or resistant *Candida* strains is limited to a small group of severely immunocompromised patients, and patients treated for extended periods, or receiving long term prophylaxis with less than 200 mg/d. Furthermore, other factors influence this shift, since some investigators could not demonstrate any association between the use of azole antifungals and the emergence of non-albicans species [85 86]. Despite the growing perception of azole-resistance and shifting proportions of *Candida* species as clinical relevant pathogens, the true incidence of the problem and strategies to prevent it are still unclear.

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Antifungal prophylaxis in patients with neutropenia

To prevent hematogenous fungal disease in patients with neutropenia, antifungal agents are prophylactically used. Recently, antifungal prophylaxis has focused on the azoles, especially fluconazole, which was shown to reduce the frequency of hematogenous candidosis, and fungal-related death [58,170,245]. The use of fluconazole as prophylactic anti-fungal increases the selection of resistant or less susceptible *Candida* species (*C. krusei, C. glabrata*) and other primarily resistant fungi, such as *Aspergillus* species, Zygomycetes, and *Fusarium* species.

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II. Comparison of typing systems for *Candida* species

Comparison of pulsed-field gel electrophoresis with isoenzyme profiles as a typing system for *Candida tropicalis*

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Abstract

***Candida* species are important nosocomial pathogens, particularly in immunocompromised and critically ill patients. A variety of methods have been used to differentiate strains, but an optimal system has not been established. We compared methods for typing a panel of nine related isolates of *Candida tropicalis* from an outbreak of sternal wound infections as well as four unrelated control isolates of this species. (The genetic relationships of the nine isolates in the panel had been confirmed previously by restriction fragment analysis). Typing was undertaken without knowledge of an isolate's origin. Karyotyping by contour-clamped homogenous electric field (CHEF) gel electrophoresis failed to distinguish between outbreak and control isolates. However, when chromosome-sized DNA was digested with *SfiI*, *EagI*, *SacII*, or *NaeI* and the fragments were separated by CHEF electrophoresis, the outbreak isolates were readily identified. The isoenzyme profiles of the outbreak isolates were identical and were distinctly different from those of the control isolates. While both isoenzyme profiles and the modified CHEF procedure were discriminatory, the latter is recommended as a relatively convenient and reproducible technique for comparison of types of *C. tropicalis*.**

Introduction

The *Candida* species have emerged as common pathogens in a variety of infections, including arthritis, osteomyelitis, endophthalmitis, myocarditis, meningitis, peritonitis, and vaginitis [128,160] Recent data from the National Nosocomial Infections Surveillance System demonstrate that the overall frequency of nosocomial candidemia has increased by 219%-487% in teaching hospitals and by 75%-370% in non-teaching hospitals over the past decade [8,183] Currently the *Candida* species are responsible for 8% of nosocomial bloodstream infections in the United States These species are etiologic agents of primary bloodstream infections which occur at a rate of 0 28-0 61/1 000 patients discharged from small and large hospitals [8,183]

A number of nosocomial outbreaks of candidiasis have been reported, yet the significance of person-to-person transmission of *Candida* species has not been well documented in most situations. Outbreaks of infection due to *Candida albicans* have been reported in association with parenteral nutrition [123] and in both adult and neonatal intensive care units [27 145 206 216] *Candida parapsilosis* has been reported as the etiologic agent in outbreaks of fungal endophthalmitis [109] and systemic infection related to parenteral nutrition [202 233], and an outbreak of *Candida glabrata* in a unit treating adult leukemic patients has been described [89].

Recently, Isenberg and colleagues reported the first nosocomial outbreak of infection with *Candida tropicalis*; the fungus caused sternal wound infections in eight patients who had undergone coronary artery bypass surgery [71]. An epidemiological investigation implicated a single scrub nurse, cultures of whose nasopharynx and fingertips yielded *C. tropicalis*, as the probable source of the fungus. When we typed the outbreak isolates by restriction fragment analysis (RFA), using conventional electrophoresis in association with a computerized image analysis system, we were able to discriminate between outbreak and control strains [39]. However, RFA may not be performed easily in all laboratories, and other procedures for typing yeasts may be more appropriate in different settings. In particular, typing procedures involving isoenzymes [114 209] and electrophoretic karyotypes [77 105 113 114 148 218] have been used extensively. Although distinct types of *C. tropicalis* can be detected by isoenzyme analysis [209], the value of isoenzymes in discriminating between epidemiologically related and unrelated isolates has not been assessed. Previous studies of this species with the use of contour-clamped homogenous electric field (CHEF) gel electrophoresis have been limited, and simple karyotypes may not be useful in epidemiological investigations of *C. tropicalis* outbreaks. Indeed, in one study, the electrophoretic karyotype of two different strains appeared identical [148].

The outbreak described by Isenberg et al. [71] provides an excellent opportunity to evaluate the epidemiological utility of various typing methods when applied to a well-defined collection of *C. tropicalis* isolates from a point-source outbreak. Here we report on these isolates in terms of their isoenzyme profiles, their electrophoretic karyotypes, and their CHEF-RFA types - i.e., the pattern of DNA fragments produced when CHEF gel electrophoresis is applied to the resolution of pieces of nucleic acid cleaved from chromosome-sized DNA by "rare-cutting" restriction endonucleases. We contrast our findings with those obtained for the same isolates by RFA alone [39].

Materials and Methods

Candida isolates. Outbreak isolates included strains of *C. tropicalis* from the sternal wounds of seven patients (isolates E3-E9) and from the nasopharynx and fingertips of the

epidemiologically linked scrub nurse (isolates E1 and E2) from the cluster described by Isenberg et al. [71]. Isolates from the remaining patient in the epidemic were not available for further study. Control isolates included four strains of *C. tropicalis* from epidemiologically unrelated, hospitalized patients. All yeasts were stored as suspensions in sterile water at ambient temperature until typed. The isolates were identified as *C. tropicalis* from their morphology on cornmeal agar and from their carbohydrate assimilation profile, which was determined with the API 20C system (Analytab Products, Plainview, NY). Each isolate was assigned a code to allow typing without knowledge of its origin.

CHEF electrophoresis. Two colonies (> 1 mm) of each yeast isolate were incubated overnight in 10 mL of YEPD broth (yeast extract, 10 g/L; peptone, 10 g/L; and D-glucose, 20 g/L). The cells were packed by centrifugation (1,000g, 5 min) and washed twice with 50 mM sodium EDTA (pH 8.0); a 130-µL aliquot of packed cells was then suspended in 580 µL of 50 mM sodium EDTA (pH 8.0). The suspension of cells was evenly mixed with 100 µL of yeast cell wall-degrading enzymes (Lyticase, L5263, partially purified grade; 1,250 U/mL in 50% glycerol [vol/vol.], 0.01 M $NaPO_4$, pH 7.5; Sigma Chemical, St. Louis) and 0.9 mL of 1% low-melting-point agarose (SeaPlaque GTG, FMC BioProducts, Rockland, ME) at 58°C. Aliquots of 400 µL of the yeast-agarose suspension were placed in individual molds (Bio-Rad, Richmond, CA) to form agarose plugs and were incubated for 2 hours at 37°C. Individual agarose inserts were removed from the molds and incubated at 50°C for 15 hours in 2 ml of buffer (10 mM Tris-HCl, pH 7.5; 400 mM EDTA; 1% Sarkosyl) containing proteinase K (1 mg/mL; Protease type XXVIII, 20 U/mg; Sigma). Agarose inserts were washed twice with 50 mM sodium EDTA (pH 8.0), incubated overnight at 25°C, washed twice more, and then stored at 5°C

- The chromosome-sized pieces of DNA were resolved on 0.7% agarose gels (SeaKem GTG, FMC BioProducts) by pulsed-field electrophoresis under conditions similar to those described by Monod et al.[122] *Saccharomyces cerevisae* chromosome/DNA size standards (Bio-Rad) and DNA prepared from *C. albicans* were included in each gel as standards. In all instances electrophoresis was performed with a CHEF-DRII pulsed-field electrophoretic system (Bio-Rad). The conditions used for CHEF electrophoresis were modified in an attempt to better resolve the chromosome-sized DNA. Agarose gel concentrations ranged from 0.6% to 1.0% (wt/vol.), and voltages varied from 50 V to 150 V, with switch times ramped from 10 seconds to 360 seconds. The duration of electrophoresis varied from 3 days to 6 6 days. The electrophoretic conditions that gave the best separation of the chromosomes were a 0.7% agarose gel (SeaKem GTG) at 150 V and 13°C. The pulse interval was 120 seconds for 24 hours followed by 240 seconds for 36 hours. The use of additional electrophoretic conditions to further differentiate among large chromosomes was unsuccessful. After electrophoresis, gels were stained with ethidium bromide, illuminated under ultraviolet light, and photographed with Polaroid 107C film (Polaroid, Cambridge, MA).

CHEF-RFA For restriction endonuclease digestion, agarose plugs containing chromosome-sized DNA were prepared as described above and placed into 100 mM Tris-HCl (pH 8 0) containing 5 mM magnesium chloride After two washes, the agarose inserts were placed in 100 µL of buffer containing 20 U of a "low frequency-cutting" restriction endonuclease Restriction endonucleases *SfiI, EagI, SacII,* and *NaeI* were obtained from New England Biolabs (Beverly, MA) Overnight digestion was performed as directed by the endonuclease manufacturer CHEF pulsed-field electrophoresis was performed at 13°C for 24 hours at 200 V in a 1% agarose gel (SeaPlaque GTG) Pulse times were ramped from 10 seconds to 90 seconds throughout the 24-hour period Photographs of ethidium bromide-stained agarose gels were inspected visually Each major and minor band was identified, and the distance from the origin of the gel relative to the lambda molecular weight standard was measured Isolates were considered different if any readily detectable band did not match In addition the coefficient of similarity [39] was calculated for each isolate relative to an outbreak isolate obtained from the index nurse A coefficient of similarity of >0 95 was considered to represent a match in banding patterns

Isoenzyme typing Crude extracts of *C tropicalis* yeasts were obtained by methods similar to those used previously [92] In brief, isolates were grown with shaking (150 rpm 72 hours, 28°C) in 250 mL Erlenmeyer flasks containing 100 mL of broth (yeast nitrogen base without amino acids, 100 mM sucrose, 30 mM mannitol) The yeasts were harvested by centrifugation and washed, they were then broken by beating with glass beads in the presence of 2 0 mM phenylmethylsulfonyl fluoride (Sigma) an inhibitor of proteinases [156] Isoenzyme profiles were determined after native PAGE of the crude extracts in a Tris-HCl-glycine discontinuous buffer system where protein separation took place at pH 9 5 [92] After overnight electrophoresis, each gel was washed twice with the buffer used for detection of enzyme activity and was then stained for the appropriate activity with the chromogenic reagents described previously (unless stated otherwise) [78,114] Staining patterns for each enzyme activity were read, and the different patterns were assigned a code (a, b, etc) The enzyme activities, together with the Enzyme Commission identifier, the amount of protein loaded per well, and the precentage (wt/vol) of polyacrylamide used in the resolving gel, were esterase (EC 3 1 1 1 350 µg, 7 5% [chromogenic substrate, 4-methylumbelliferyl acetate]), α-glucosidase (EC 3 2 1 20, 350 µg 7 5%) β-glucosidase (EC 3 2 1 21, 350 mg, 5 0%) 5 0%) α -mannosidase (EC 3 2 1 24 350 µg, 5 0% [chromogenic reagent, 4-methylumbelliferyl-α-D-mannopyranoside (Sigma, 67 mg/mL) in 100 mM sodium acetate buffer, pH 5 5]) alkaline phosphatase (EC 3 1 3 1, 350 µg 5 0%) acid phosphatase (EC 3 1 3 2, 350 µg, 7 5%), glucose-6-phosphate dehydrogenase (EC 1 1 1 49, 350µg 5 0%), malate dehydrogenase (EC 1 1 1 37, 350 µg, 7 5%) mannitol dehydrogenase (EC 1 1 1 67, 350 µg, 7 5%) super-oxide dismutase (EC 1 15 1 1, 100 µg, 7 5%) and catalase (EC 1 11 1 6, 100 µg, 5 0%)

Results

Typically, the CHEF-defined karyotype of the outbreak and control isolates of *C. tropicalis* was composed of three chromosome bands (figure 1, table 1). There were two exceptions in which four bands were seen; isolate E2, an outbreak isolate, had a faint large-sized extra band; and isolate U4, an epidemiologically unrelated isolate, showed two small-sized bands (figure 1). Regardless of the different conditions used for electrophoresis, the basic three-band pattern remained for the majority of *C. tropicalis* isolates. The largest band of all of the isolates was not well resolved and may have contained a variety of pieces of DNA; indeed, there is the suggestion of an extra high-molecular-weight molecule in isolate E6, another isolate from the outbreak (figure 1). The karyotype of the *C. albicans* control, with eight bands resolved, differed greatly from that of *C. tropicalis*.

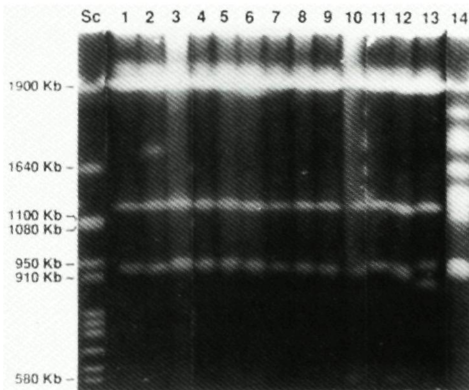

Figure 1. CHEF electrophoresis of *C. tropicalis*. Lanes 1–9 show outbreak isolates; lanes 10–13, epidemiologically unrelated isolates; lane 14, *C. albicans;* and lane Sc, *S. cerevisiae* chromosome/DNA size standards. Note the similarity of the control and outbreak isolates.

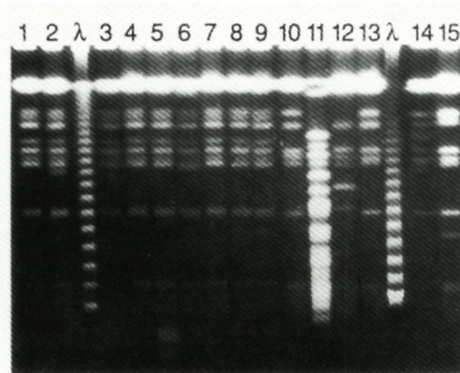

Figure 2. CHEF-RFA electrophoresis of *C. tropicalis* isolates after digestion with *Sfi*I. Outbreak isolates are shown in lanes 1–9 and unrelated control isolates in lanes 10 and 12–14. Lane 11 shows the *C. albicans* control, and lane 15 shows a *C. tropicalis* control (ATCC 13803). λ = lambda-phage DNA ladders (interval = 48.5 kilobase pairs) as controls. Note the different banding patterns of the control isolates and the similarity of the outbreak isolates.

Far greater heterogeneity was found for the DNA fragment patterns obtained by the CHEF-RFA technique when chromosome-sized DNA was digested with *SfiI* before CHEF gel electrophoresis. Each of the control isolates had a unique pattern that differed distinctly from the single pattern of fragments characterizing all of the outbreak isolates (figure 2, table I).

Table 1. Comparison of RFA, CHEF gel electrophoresis, CHEF-RFA, and isoenzyme patterns in the molecular typing of *C. tropicalis*.

Isolate*	RFA¥ pattern	CHEF pattern	CHEF-RFA pattern	α-GLU	β-Glu	EST	SOD	ACP	GDH	Isoenzyme type
E1	A1	a	A	a	a	a	a	a	a	Aa
E2	A1	a	A	a	a	a	a	a	a	Aa
E3	A1	a	A	a	a	a	a	a	a	Aa
E4	A1	a	A	a	a	a	a	a	a	Aa
E5	A1	a	A	a	a	a	a	a	a	Aa
E6	A1	a	A	a	a	a	a	a	a	Aa
E7	A1	a	A	a	a	a	a	a	a	Aa
E8	A1	a	A	a	a	a	a	a	a	Aa
E9	A1	a	A	a	a	a	a	a	a	Aa
U1	B1	a	B	b	a	b	a	b	b	Bb
U2	C1	a	C	a	b	b	a	a	a	Cc
U3	D1	a	D	b	b	c	b	a	a	Dd
U4	E1	b	E	b	c	d/a$	a	a	a	Ee

Note. Patterns observed on gels and isoenzymes types are shown, for example, in the form A1, a, A, a, and Aa, where these letters refer to the patterns associated with isolates from the outbreak. Patterns distinctly different from that observed among outbreak isolates are referred to with consecutive letters, i.e., B1, C1, D1, E1, or B, C, D, E.
* Isolates E1-E9 were obtained from the outbreak; isolates U1-U4 were epidemiologically unrelated
¥ Data are from (39)
α-GLU=α-glucosidase, β-Glu= β-glucosidase, EST=esterase, SOD=superoxide dismutase, ACP=acid phosphatase, and GDH=glucose-6-phosphate dehydrogenase
$ d/a = either pattern a or pattern d

When *EagI* was used as the restriction endonuclease in the CHEF-RFA procedure, all outbreak isolates again shared a DNA fragment pattern that differed from the patterns of the control isolates (data not shown). With *SfiI*, the coefficient of similarity ranged from 0.95 to 1.0 for all outbreak isolates and from 0.30 to 0.89 for unrelated controls. With *EagI*, the coefficient of similarity was 1.0 for all outbreak isolates and ranged from 0.00 to 0.92 for unrelated controls. Results were similar with *SacII* and *NaeI*, although the differences were not as easily visualized. Isoenzyme typing discriminated between the two groups of *C. tropicalis* isolates in the same manner as did CHEF-RFA (figure 3). Of the enzymes tested, several showed no heterogeneity. Both acid and alkaline phosphatase activities had the same patterns on gels, but the former gave better staining results. The isoenzyme profile used for determining the type was based on the patterns of enzymes that demonstrated heterogeneity; these were the α-glucosidases, ß-glucosidases, esterases, superoxide dismutases, acid phosphatases, and glucose-6-phosphate dehydrogenases (table 1).

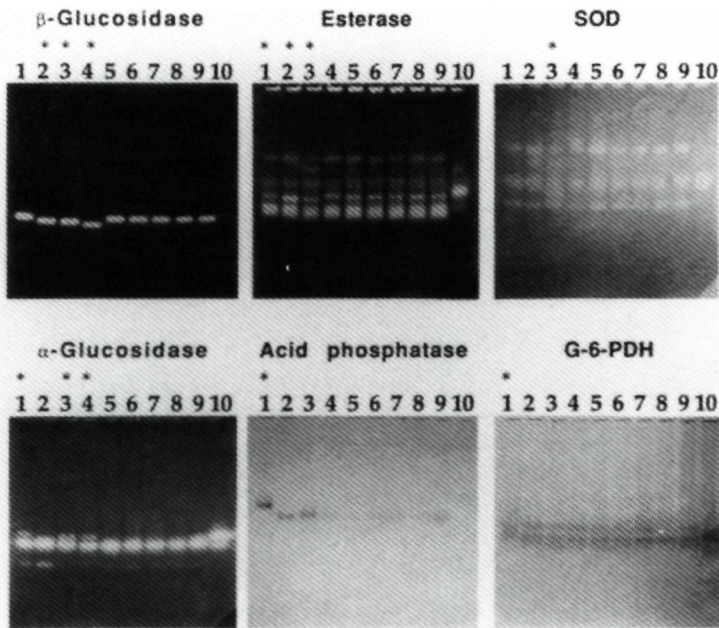

Figure 3. Isoenzymes of outbreak and unrelated control isolates of *C. tropicalis*. Lanes 1–4 show *C. tropicalis* control isolates U4, U1, U2, and U3, respectively; lanes 5–9, outbreak isolates E4–E8; and lane 10, *C. albicans* control. G-6-PDH = glucose-6-phosphate dehydrogenase; SOD = superoxide dismutase. Asterisks indicate lanes where enzyme migration of a *C. tropicalis* control isolate differs clearly from that of the outbreak isolates.

Discussion

C. tropicalis is becoming increasingly common as an etiologic agent of nosocomial infections in neutropenic patients, patients undergoing surgery, and patients with indwelling catheters [81,128]. For an assessment of the importance of features such as person-to-person transmission among patients and medical and nursing staff, it is necessary to develop reliable and highly discriminatory typing procedures suitable for use with this species. Although the authors of some early studies reported strain heterogeneity determined by RFA [184] or isoenzyme profiles [209], they did not analyze a panel of epidmiologically characterized isolates: thus, the usefulness of methods for evaluating outbreaks has not been determined. Here we have used CHEF gel electroporetic karyotyping, CHEF-RFA, and isoenzyme analysis to establish molecular types for *C. tropicalis*, and we have compared our findings with those reported recently when RFA, coupled with computerized image analysis, was applied to the same panel of isolates [39]. While conventional CHEF gel electrophoresis did not prove to be a useful molecular typing procedure, the types defined by both isoenzyme profile and CHEF-RFA are in concordance with the types obtained by RFA. These data further confirm the epidemiological link (reported by Isenberg et al.[71]) between isolates from the scrub nurse and those from the patients' wounds.

Karyotypes established by CHEF-gel electrophoresis seem to constitute a reliable molecular typing tool for discrimination among strains of *C. albicans* [105,113,122,148,218]. Similarly, it seems that

karyotyping will be useful for discriminating among strains of other species, including *C. guilliermondii*, *C. kefyr* (*Candida pseudotropicalis*), *C. glabrata* (*Torulopsis glabrata*), and *C lusitaniae* [77 114 148]. In an extensive survey of clinical isolates of yeasts, Pittet and associates showed the value of electrophoretic karyotypes in the analysis of infections caused by *C. albicans* and many other species [148]; notably, however, there appears to be only limited heterogeneity associated with the electrophoretic karyotypes of *C. tropicalis* [122 148].

In the current study, we were unable to discriminate between epidemiologically related and unrelated isolates of *C. tropicalis* by standard CHEF gel electrophoresis alone. Coupled with the results of Pittet and colleagues [148], our findings indicate that karyotyping by conventional CHEF gel electrophoresis is inadequate as a procedure for delineating strains of *C. tropicalis*. Only with very long and complex pulsed-field gel electrophoresis protocols have the chromosomes of *C. tropicalis* been resolved to a reasonable extent [51 209]. For a single alkane-utilizing strain of *C. tropicalis*, electrophoresis had to be performed for 100-166 hours, and even then the larger chromosomes were not very well resolved. Compared with such lengthy electrophoretic runs, the 24-hour separation of chromosome fragments with CHEF-RFA was extremely convenient. Heterogeneities in CHEF-RFA patterns allowed us to differentiate among strains of *C tropicalis* easily. As a result, the epidemiologically related isolates could be grouped. The advantages of CHEF-RFA over conventional RFA lie in its reproducibility and the ready interpretation of the more limited pattern of bands. The limits to the discriminatory power of this procedure have not been fully explored, and the resolution of DNA fragments by CHEF-RFA is likely to be improved as a result of the use of different conditions during electrophoresis and possibly the use of other restriction endonucleases.

There was complete concordance between the types observed by RFA [39] and the types defined by CHEF-RFA and isoenzyme profiles However, other researchers have reported differences in discriminatory power between electrophoretic karyotyping and other methods used for typing *Candida* species. Merz et al described a greater discriminatory power of electrophoretic karyotypes than of isoenzyme profiles in the molecular typing of *C. lusitaniae* [114]. Similarly, Vazquez and co-workers, who evaluated 35 isolates of *C. albicans* from 20 patients, claimed that karyotyping by CHEF gel electrophoresis had a greater discriminatory power than RFA; they found 23 distinct electrophoretic karyotypes but only 17 distinct RFA types [218]. The epidemiological significance of the types described by Vazquez et al. is far from clear, however. We consider it advisable, whenever possible, to compare typing procedures in tests with isolates whose genetic and epidemiological relationships have been well defined. Such a panel of isolates was provided by the outbreak of *C. tropicalis* infection described by Isenberg et al. [71], and the genetic relationships of the isolates in this panel have been confirmed by RFA coupled to computerized image analysis [39]. Therefore, we are confident that both the isoenzyme profile and the CHEF-RFA pattern could be used to delineate strains in epidemiological investigations of *C. tropicalis*

While none of the three typing methods for *C. tropicalis* - RFA, CHEF-RFA, and isoenzyme analysis - can be considered routine for the clinical laboratory, each is fairly easy to perform. Isoenzymes are somewhat inconvenient in requiring both a large quantity of yeast for culture and several different chromogenic reagents, buffers, and concentrations of acrylamide in the polyacrylamide gels. In addition, a certain amount of run-to-run variation can lead to difficulties in the comparison of isoenzyme patterns from different gels. Furthermore, while isoenzyme profiles seem excellent markers that allow discrimination among species [94 209], they are not as discriminatory as either electrophoretic karyotypes [114] or RFA pattterns [96 103] for delineating strains of *Candida* species other than *C. tropicalis*. In contrast to isoenzyme analysis, both RFA and CHEF-RFA require few reagents, and the type of any isolate is based on a pattern of DNA fragments resolved within a single gel. Zhang et al. [250] have cited the investigation of *Mycobacterium tuberculosis* isolates by pulsed-field gel electrophoresis in combination with chromosome digestion by rare-cutting restriction enzymes; likewise, CHEF-RFA provides a convenient tool for epidemiological studies on yeasts. Once conditions have been established for investigating a species, the procedure is simple to perform. The clear resolution of DNA fragments may make CHEF-RFA preferable to RFA when the interpretation of fragment patterns is easiest by means of a computerized image analysis unit [39]. Regardless of the method chosen, meaningful epidemiological studies of *C. tropicalis* should prove feasible.

Chapter 2.2

Epidemiologic genotyping of *Candida* species by pulsed-field gel electrophoresis and PCR-fingerprinting

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submitted

Abstract

Molecular typing methods are increasingly applied for studies where the interpretation of data essentially relies on the typing results rather than epidemiological data. In this situation, the discriminatory power (ability to identify differences among epidemiologically unrelated strains) of the typing method is important in allowing and drawing valid conclusions. By applying PCR-fingerprinting, electrophoretic karyotyping, and restriction fragment endonuclease analysis using standard restriction enzymes and primers proven to be useful in previous studies, we evaluated whether the use of multiple genotyping methods is sufficient to delineate known unrelated strains among seven *Candida* species. All three methods identified individual genotypes for each of the seven *Candida* species studied, however optimal strain delineation required the combined use of all three typing methods and was observed only with the C. *albicans and* C. *tropicalis* isolates tested.

Introduction

The increasing incidence of nosocomial candidosis has become a serious clinical problem [13 144 150]. This trend is paralleled by an increasing need to define the pathogenesis and the mode of transmission of invasive candidosis, in order to direct preventive measurements.
Epidemiological typing of microorganisms is frequently used to supplement a careful epidemiological investigation, such as the investigation of outbreaks of infection due to different *Candida* species [12 14 26 27 39 46 73 136 168 186 192 225 249]. Recently, genotyping methods have been applied to studies on pathogenesis [158 214 222], or antifungal resistance development [11 119 159 172] where the interpretation of the data relies heavily on the typing results In this situation investigators must be sure that the typing method employed, either singly or in combination, has the ability (discriminative power) to identify differences among epidemiologically unrelated strains Most of these so-called "molecular epidemiology" studies depend upon DNA-based typing techniques [68 69 112 137 139]; and under most circum-

stances the rationale for the epidemiological typing boils down to "whether two or more strains of a given species are the same or different" [141].

Various molecular epidemiologic typing methods such as restriction endonuclease analysis of genomic DNA [158 183 216], PCR-fingerprinting [95 214], Southern hybridization analysis [4 48 108 199], electrophoretic karyotyping using pulsed-field gel electrophoresis (PFGE) [104 222], and immunoblot fingerprinting [26 88] have been applied to studies of *Candida* species. Furthermore, polyacrylamide gel electrophoresis (PAGE) [88 216], and multilocus enzyme electrophoresis [93] have been used successfully in *Candida* typing. Applied to specific situations, each of these methods has advantages and disadvantages. Two methods, PCR-fingerprinting and restriction endonuclease analysis of genomic-DNA (REAG) using PFGE, are frequently used to type *Candida* spp., due primarily to ease of performance and discriminatory power, respectively. Previous studies using PCR-fingerprinting and REAG to identify strains of *C. albicans* and *C. tropicalis*, respectively, documented a high level of discrimination [41 214]. For future epidemiological investigations it is likely that these methods will be applied to other species of *Candida*, possibly without re-evaluating the discriminatory power.

We evaluated whether these methods are sufficient to discriminate among epidemiologically unrelated isolates of other *Candida* species, or if detailed information on the primers/enzymes used for PCR-fingerprinting and PFGE are necessary to achieve a sufficiently high discriminative power.

Materials and Methods

Isolates. We chose four epidemiologically unrelated isolates from each of seven *Candida* species, namely *C. albicans, C. tropicalis, C. krusei, C. glabrata, C. parapsilosis, C pseudotropicalis,* and *C. guillermondii.* All of these (reference) strains originated from the Centraal Bureau voor Schimmelcultures (CBS), Baarn, The Netherlands. Isolates were coded and were sent as *Candida* spp. for typing in laboratories that were blinded to their origin and identification.

PFGE *Molecular typing and DNA preparation.* *Candida* strains were karyotyped by contour-clamped homogeneous electric field (CHEF) electrophoresis of genomic DNA [39], and also typed by restriction endonuclease analysis of genomic DNA (REAG) after digestion with the restriction endonuclease *BssHII* [41]. The DNA for the molecular typing was prepared as described earlier [222].

CHEF karyotyping. Electrophoresis was performed with a CHEF-DRII pulsed-field electrophoretic system (BioRad) in 0.7% agarose gel (SeaKem GTG) - 0.5 x 100mM Tris (pH 9.5), 100 mM boric acid, and 1.0 mM EDTA at 150V and 13°C. The pulse interval was 120 seconds for 24 hours followed by 240 seconds for 36 hours. *Saccharomyces cerevisiae*

chromosome/DNA molecular weight markers (BioRad) were included in each gel as standard After electrophoresis, the gels were stained with ethidium bromide, illuminated under ultraviolet light, and photographed with Polaroid 107C film (Polaroid, Cambridge, MA,USA)

REAG For the restriction endonuclease analysis of genomic DNA (REAG), agarose inserts containing chromosome-sized DNA were prepared as described above and placed into 100 mM Tris-HCl (pH 8 0) containing 5 mM magnesium chloride Agarose inserts were washed twice and placed in 100 µL of buffer containing 20 U of *BssH* II restriction endonuclease (New England Biolabs, Beverly, MA,USA) Overnight digestion was performed as described by the manufacturer

Electrophoresis was performed with a CHEF-DRII pulsed-field electrophoretic system (BioRad) in 1 0% agarose gel (SeaKem GTG) - 0 5 x TBE buffer at 200V and 13°C The pulse interval was ramped from 10 to 90 seconds over 24 hours The 48 5-kb lambda DNA ladder (BioRad) was included in each gel as molecular weight standard After electrophoresis, gels were stained with ethidium bromide, illuminated under ultraviolet light, and photographed with Polaroid 107C film (Polaroid)

PCR *DNA extraction* All *Candida* isolates were grown in Sabauroud glucose broth Genomic DNA for RAPD was prepared as follows cultures were centrifuged and washed twice in phosphate buffered saline, resuspended in 250 µl STET buffer (233 mM sucrose, 50 mM TRIS-HCl pH 8 0 20 mM EDTA, 5% Triton X-100) Lysozyme was added to a final concentration of 1 7 mg/ml The suspension was incubated at room temp for 5 min, heated at 100°C for 1 min and put on ice for another 2 min In succession sodium dodecyl sulphate and proteinase K was added to the solution to a final concentration of 0 3% and 0 5 mg/ml respectively, which was then incubated at 55°C for 2 hours Following extraction with phenol 0 03 mg/ml RNAse A was added and the mixture was incubated at 37°C for 20 min The solution was extracted successively with phenol/ chlorophorm/isoamyl-alcohol (25 24 1) and chlorophorm/isoamyl-alcohol (24 1) DNA was precipitated overnight and resuspended in 100 µl of distilled water An alliquot was electrophoresed in a 1% agarose gel containing 0 1 µg/ml ethidium bromide to estimate the DNA yield and verify DNA integrity

RAPD analysis PCR fingerprinting of *Candida* DNA (50 ng) was performed in a 50 µl reaction volume containing 75 mM Tris-HCl (pH 9 0), 2 5 mM $MgCl_2$, 20 mM $(NH4)_2SO_4$ 0 01% Tween-20, 0 2 mM dNTPs each 50 pmol of primer 1283 (5'-GCGATCCCCA-3') and 0 2 U of Taq DNA polymerase (Thermoperfectplus DNA polymerase,Integro, Zaandam, The Netherlands) A Perkin-Elmer 9600 thermocycler was used for amplification The cycling program run was 4 cycles of 94 °C for 5 min 36 °C for 5 min 72°C for 5 min followed by 30 cycles of 94°C for 1 min 36°C for 1min 72 °C for 2 min and a 10 min incubation at 72 °C Amplified DNA (5 µl) was separated by electrophoresis in 1 5 % agarose gels and visualized by ethidium bromide staining (0 1 µg/ml) A molecular size marker (100 bp ladder Pharmacia, Woerden, The Netherlands) was used for reference

Analysis Photographs of ethidium bromide-stained gels were analyzed to detect similarities and differences in banding patterns. Isolates were considered "identical" when all of the bands matched; "similar" when ≥ 95% but less than 100% of the bands matched, and "different" with less than 95% matching bands.

Results

Genotyping results as determined by PFGE karyotyping, REAG, and PCR fingerprinting are described in table 1.

Table 1: Genotypes of seven different *Candida* species determined by karyotyping and *BssH* II macro restriction analysis with pulsed-field gel electrophoresis and PCR fingerprinting.

Strain No.	Species	Reference	Genotype according to*:			
			Karyotype	REAG	PCR	overall
1	*C. albicans*	CBS 562	A	1	I	a
2		ATCC 10231	B	2	II	b
3		ATCC 24433	C	3	I	c
4		AZN 3982	D	4	III	d
5	*C. krusei*	CBS 6891	E	5	IV	e
6		ATCC 62069	F	6	IV	f
7		CBS 573	E	5	IV'	e
8		AZN 3962	E	7	IV'	g
9	*C. glabrata*	CBS 1518	G	8	V	h
10		CBS 860	G'	8	V'	h
11		CBS 7307	H	8'	V'	i
12		AZN 3944	I	9	V	j
13	*C. tropicalis*	CBS 8072	J	10	VI	k
14		CBS 6320	J	11	VI	l
15		CBS 6957	K	12	VII	m
16		AZN 3672	J	13	VIII	n
17	*C. parapsilosis*	CBS 1954	L	14	IX	o
18		CBS 6318	L'	14	IX	o
19		CBS 2194	L''	14'	IX	o'
20		AZN 3526	L	14'	IX'	o'
21	*C. pseudotropicalis*	CBS 2234	M	15	X	p
22		CBS 1561	N	16	X'	q
23		CBS 2231	M	15'	X	p
24		AZN 402	N'	16'	X	q
25	*C. guillermondii*	CBS 6316	P	17	XI	r
26		CBS 2021	P'	17'	XI	r
27		CBS 7099	P'	17'	XI	r
28		AZN 2102	P	17	XI	r

CBS strains = Reference strains from the Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands
AZN strains = Clinical strains from the University Hospital Nijmegen, strain identity confirmed by CBS
REAG = restriction endonuclease analysis of genomic-DNA pattern
PCR = PCR-fingerprinting pattern using *BssH* II
* similar but not identical pattern (share 95-99% of bands) are denoted as ' or "

Overall 18 different genotypes were determined among the 28 unique *Candida* strains; 16 by karyotyping, 17 by REAG, and 11 by PCR-fingerprinting, respectively. All typing methods showed individual genotypes for each of the seven *Candida* species evaluated and were able to identify the four different *C. albicans* strains tested. *C. tropicalis* was the only other *Candida* species in which the use of one or more typing methods was able to identify all four strains. Combining the results of all three methods resulted in the identification of three of the four unique strains of *C. krusei* and *C. glabrata*, and two of the four strains of *C. pseudotropicalis*. None of the three typing methods alone or in combination was able to discriminate among the *C. parapsilosis* and *C. guillermondii* reference strains. Of the three typing methods, PCR-fingerprinting was the least discriminative method.

Discussion

In general, epidemiologic typing systems are used to study microbial population genetics, the pathogenesis or surveillance of infectious diseases, and the investigation of outbreaks. Until recently no common definitions and guidelines on performance criteria, such as typeability, reproducibility, stability, discriminatory power, test population, and epidemiologic concordance were in place. The lack of such performance criteria made a standardized interpretation of epidemiological typing results difficult and may have contributed to the suggestion, that no single typing method is sufficient to fulfill the above mentioned tasks, and that most epidemiologic investigations may require more than one method for optimal strain delineation.

Recently, a European consensus guideline for appropriate use and evaluation of microbial epidemiologic typing systems was published [207]. The authors suggest that the discriminatory power should be >0.95, thereby complying with the conventional 5% level of acceptable probability of type I error. We hypothesized that these standards will not be matched by "today's practice", the use of two independent DNA-based methods. This practice might be insufficient to delineate without fail epidemiologically related and unrelated strains of *Candida* species. To comply with the above mentioned standards, additional validation of the primers/enzymes used are probably needed, for each species of *Candida* tested.

Various molecular epidemiologic typing methods have been used to delineate strains within *Candida* species [4,26,48,95,104,108,158,183,199,214,216,222]. Karyotyping by PFGE was shown to be a useful molecular typing tool to identify different *C. albicans* strains [113,122,148,218]. Similarly, karyotyping was shown to provide some discrimination among strains of *C. pseudotropicalis*, *C. glabrata*, and *C. lusitaniae* [114,148], an observation we could not confirm in the present study. Among the seven *Candida* species evaluated, karyotyping was only able to discriminate all four *C. albicans* strains and three of the four *C. glabrata* reference strains tested. The inability of this typing method to reliably delineate strains among all species of *Candida* was shown earlier for *C. tropicalis* [41].

Our study shows that with the exception of *C. albicans* and *C. tropicalis* even the use of several different genotyping methods in combination may indicate identity among epidemiologically unrelated strains of non-*C. albicans* species. The fact that several of the isolates showed an identical typing pattern clearly increases the chance for invalid epidemiological conclusions.

In order to evaluate the resolution of their PCR assay, van Belkum et al. [214] reviewed genotyping studies on a panel of 21 *C. albicans* reference isolates. Despite the use of PFGE karyotyping, RFLP, Southern-blot hybridization, and 8 different PCR assays, only 13 different types were found. The general inability of all typing systems to delineate among different epidemiologically unrelated strains of *C. albicans* was not further discussed. The inability of similar typing methods in the present study to reliably identify unrelated strains of non-*C. albicans* species might be due to a limited genetic diversity of some *Candida* species, or may just be an expression of the fact that molecular epidemiological studies done so far do not reach the discriminative ability that is asked for in the recent standard [207]. Furthermore, this observation is an indication that the number of isolates used to evaluate the discriminative power is of utmost importance.

Typing assays that are able to delineate a certain *Candida* species, may not be used blindly for other species of that genus. In addition to the recent standardized definitions and performance criteria, and the urge to use at least two different (geno)typing systems, species-specific restriction enzymes and primers, which were validated using a large number of different strains, should be used. Using the normal statistical threshold of 0.05 we should expect that one or more typing assays used to answer important epidemiologic and pathogenetic questions, should delineate 19 of 20 reference strains. This level of discrimination certainly has not been reached in a fair proportion of the published studies using molecular typing. Despite recent progress to standardize and validate typing systems, the available typing methods still need further attention, especially to increase the discriminative power

III. Molecular epidemiology and pathogenesis

Investigation of the sequence of colonization and candidemia in nonneutropenic patients

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Abstract

Among neutropenic patients with hematologic malignancies, candidemia has been shown to arise typically from autoinfection after colonization. In patients without neutropenia, we examined the similarities of strains colonizing or infecting various body sites and those subsequently causing *Candida* bloodstream infections. Strain similarity was examined by karyotyping and restriction endonuclease analysis of genomic DNA (REAG) by using two restriction enzymes (*SfiI* and *BssHII*). The banding patterns of 42 isolates from 19 patients were independently evaluated in a blinded fashion by three observers. The interobserver reliability measured with a generalized kappa statistic was 0.59 for karyotyping, 0.48 for REAG with *SfiI*, and 0.88 for REAG with *BssHII* ($P < 0.001$ for each). REAG classified the initial colonizing or infecting isolate and subsequent blood isolates as identical in 16 patients (84%). The mean duration of colonization or infection prior to a positive blood culture was 5 and 23 days in patients infected with related and unrelated isolates, respectively ($P = 0.14$; 95% confidence interval = -14.5 to 50.5). Karyotyping results matched the REAG results for isolates from 14 of the 19 patients (74%). In patients infected with identical isolates, the initial isolate was most frequently recovered from the urine ($n = 5$) or vascular catheter tips ($n = 4$). In the five subjects with organisms showing disparate results between the methods, karyotyping revealed different banding patterns, whereas REAG suggested that the isolates were identical. *Candida* colonization or infection with an identical strain frequently precedes bloodstream infection in nonneutropenic patients. Future studies should evaluate whether patients at high risk for candidemia and who have vascular catheter or urine samples that are positive for a *Candida* on culture should be treated empirically.

Introduction

In recent years, *Candida* spp. have emerged as important nosocomial pathogens. Between 1978 and 1984, the incidence of *Candida* bloodstream infections increased by 3- to 10-fold [30,48] Over the past decade, the incidence of *Candida* bloodstream infections increased by

two- to fivefold in U.S. teaching hospitals and by one- to fourfold in nonteaching hospitals [8 183]. Candidemia occurs most frequently in immunocompromised patients with an underlying malignancy or hematologic disorder [115 117], severely ill burn patients, and patients in surgical and neonatal intensive care units [6 23 29 155 197 203]. Therefore, the incidence of candidemia is highest in tertiary-care referral hospitals. At our center, the incidence was 8.5/10.000 admissions in 1992. Candidemia is associated with a 57% crude and a 38% attributable (direct) mortality [237]

The clinical importance of *Candida* colonization at different body sites has been controversial. For example, some have argued whether strains colonizing the urine or stool subsequently cause candidemia. Among hematologic malignancy and bone marrow transplantation patients, Reagan et al. [158] demonstrated that colonizing and subsequently infecting strains of *Candida* spp. were identical in 94% of patients In a carefully conducted case-control study, four independent risk factors for candidemia were identified after accounting for underlying disease. colonization by *Candida* species at various body sites, prior exposure to hemodialysis, central intravascular catheters, and previous exposure to antibiotics [238]. Relatively little, however, is known about the molecular epidemiology of antecedent colonization and candidemia in nonneutropenic patients.

Since May 1987, our laboratory has routinely banked all *Candida* isolates from the bloodstream as well as any available *Candida* isolate from other clinical sites in the same patient. The purpose of the study described here was to evaluate the similarity of *Candida* bloodstream isolates and prior colonization or infecting isolates in nonneutropenic patients. Fingerprinting of the organisms was performed by karyotyping and restriction endonuclease analysis of genomic DNA (REAG) by using contour-clamped homogenous electrophoretic field (CHEF) electrophoresis

Materials and Methods

Hospitals and patients. The University of Iowa Hospitals and Clinics is a 900-bed teaching hospital and tertiary-care referral center, with approximately 200 beds designated for intensive care. Patients with *Candida* infections were identified retrospectively by reviewing the microbiology reports for isolates banked from May 1987 to July 1992. Neutropenic hematology-oncology and bone marrow transplant patients and those without a previous sample from another site that was positive for *Candida* species on culture before their blood sample was positive on culture were excluded from the study. During the study period, all *Candida* isolates were stored in sterile distilled water at ambient temperature Isolates from patients included in the current study were retrieved from this isolate bank and were identified to the species level by using the API 20C system (Analytab Products, Plainview, N Y)

Molecular typing and DNA preparation. Ten colonies (> 1 mm) of each *Candida* isolate from 48-h cultures on Sabouraud agar plates were incubated overnight at 37°C in 10 ml of YEPD broth (yeast extract, 10 g/liter; peptone, 10 g/liter; and D-glucose, 20 g/liter). The cells were packed by centrifugation (1,000 x g for 15 min), washed in sterile distilled water, and introduced into preweighed Eppendorf tubes. The pellets were suspended in 1 volume of 50 mM sodium EDTA (50E; pH 8.0). The cell titer was normalized by adding the volume (in microliters) equal to the weight of the cells (in milligrams). A 173-ml of lyticase (L5263; Sigma Chemical, St. Louis, Mo.), and 295 ml of 2% agarose (SeaPlaque GTG; FMC BioProducts, Rockland, Maine) that was previously melted and kept liquid at 56°C. Aliquots were placed in forms (Bio-Rad, Hercules, Calif.) that were incubated for 2 h at 37°C. The inserts were removed from the forms and were placed in 2 ml of a buffer of 10 mM Tris-HCl (pH 7.5), 400 mM EDTA, 1% Sarkosyl, 0.750 mg of proteinase K per ml (protease type XXVII; Sigma) and incubated overnight at 50°C. The inserts were washed six times in 50E and were stored at 5°C until use.

CHEF karyotyping. Electrophoresis was performed with a CHEF-DRII pulsed-field electrophoretic system (Bio-Rad) in 0.7% agarose (SeaKem GTG agarose; FMC BioProducts)-0 5x 100 mM Tris (pH 8.5)-100 mM boric acid-1.0 mM EDTA (TBE buffer) at 13°C and 150 V. The pulse interval was 120 s for 24 h and then 240 s for 36 h. *Saccharomyces cerevisiae* chromosome DNA molecular weight markers (Bio-Rad) were included in each gel as standards. After electrophoresis, the gels were stained with ethidium bromide, illuminated with UV light, and photographed.

REAG. For restriction endonuclease digestion, agarose inserts containing chromosome-sized DNA were prepared as described above and were placed into 100 mM Tris-HCl (pH 8.0) containing 5 mM magnesium chloride. After two washes with the buffer, the agarose inserts were digested overnight with 20 U of *SfiI* or *BssHII* (New England Biolabs, Beverly, Mass), as recommended by the manufacturer.

Electrophoresis was performed with a CHEF-DRII pulsed field electrophoretic system (Bio-Rad). Electrophoresis was performed in 1.0% agarose gel (SeaKem GTG)-0.5 x TBE at 13°C and 200 V. The pulse interval was ramped from 5 to 90 s over 24 h. The 48.5-kb bacteriophage lambda DNA ladders (Bio-Rad) were included in each gel as molecular size standards. After electrophoresis, the gels were stained with ethidium bromide, illuminated under UV light, and photographed.

Analysis. Three observers, blinded to the origin of the isolate and the results of the other observers, examined the photographs of the ethidium bromide-stained gels to detect similarities and differences in banding patterns. All bands had to match exactly to classify isolates as identical; any difference in a major or a minor band was considered important. Banding patterns with >95% but less than 100% of the bands matching were termed "similar." Isolates with less than 95% of bands matching were considered different.

Interobserver reliability was calculated with the generalized kappa statistic for measurement of agreement [247]. Means were compared with a t-test for independent samples after evaluation for equality of the variances by Levene's test. Alpha was set at 0.05, and all P values are two-tailed

Results

During the study period, 107 patients whose blood was positive for *Candida* spp. on culture were identified; 19 patients (17.8%) met the inclusion criteria. The other patients were neutropenic and had a hematologic malignancy or were undergoing bone marrow transplantation (40.2%), did not have positive cultures of samples from another site before their positive blood culture (34.6%), or had different fungal species isolated from their blood and another body site previously (7.5%). Therefore, 42 *Candida* strains (*C. albicans* [n = 35], *C. tropicalis* [n = 5], *C. parapsilosis* [n = 2]) from 19 patients (Table 1) were available for molecular typing. The 19 patients represented 30% of the total number of nonneutropenic patients with candidemia during the study period.

The underlying diseases of the 19 patients included 6 (31%) with solid tumors, 1 (5%) with acute lymphocytic leukemia prior to treatment, and 11 (58%) with prior surgery involving the gastrointestinal tract. Diabetes mellitus or prior antibiotic treatment were present in 16 and 89% of the study patients, respectively. None of the patients included in the study were neutropenic either prior to or at the time of candidemia. The interval between the isolation of *Candida* spp. from a blood culture and another body site previously ranged from 1 to 31 days, with a mean of 7.8 days (median, 5 days). In patients in whom the isolate from a blood culture and isolates from other sites were identical by REAG, the interval between the isolations ranged from 1 to 11 days, with a mean of 5 days (median, 4 days). By contrast, nonidentical isolates from colonized or infected body sites occurred from 8 to 31 days earlier than the bloodstream isolate, with a mean of 23 days (median 30 days). This mean difference in time from positivity for a prior *Candida* isolate was not statistically significant (P = 0.14; 95% confidence interval about the difference = -14.5 to 50.5), although the power to detect a difference was low, given the relatively few subjects with nonidentical isolate pairs. In the 16 patients with identical isolates from a blood culture and another site, the urine (n = 5) and catheter tips (n = 4) were the most frequent prior sites. Specimens from the respiratory tract (n = 1) and remote (> 30 days) tissue biopsy specimens (n = 2) were the sources of the initial isolates among the three patients with nonidentical isolate pairs by REAG.

Table 1: Clinical characteristics of study patients

Patient code	Age (yr)	Sex[a]	Underlying disease[b]	Species
A	36	M	Pancreas and kidney transplant, DM	*C. albicans*
B	4	F	Acute lymphocytic leukemia	*C. albicans*
C	27	M	Motor vehicle accident, burn injury (52% BSA)	*C. albicans*
D	68	F	Transoral odontoid resection and tracheostomy	*C. albicans*
E	87	M	Motor vehicle accident, intra-ventricular hemorrhage	*C. albicans*
F	58	F	Ovarian neoplasma, DM	*C. albicans*
G	52	F	Cervical carcinoma, post radiation cystitis and enteritis	*C. albicans*
H	76	M	Head and neck cancer, intraspinal abscess	*C. albicans*
I	78	F	Amyloidosis, stroke	*C. albicans*
J	27	M	Burn injury (90% BSA)	*C. albicans*
K	56	F	Biliary tract obstruction, pancreatitis	*C. albicans*
L	61	M	Pancreatitis	*C. albicans*
M	73	M	Aortic valve stenosis, CABG, DM	*C. albicans*
N	46	F	GI surgery, urinary diversion	*C. albicans*
O	52	F	Status post-GI surgery, myocardial infarction	*C. albicans*
P	70	M	Coronary artery disease	*C. albicans*
Q	80	M	Bladder carcinoma, cystectomy, with ileal conduit	*C. tropicalis*
R	60	F	Cervical carcinoma, status post-pelvic exoneration	*C. parapsilosis*
S	69	M	Status post-oropharyngeal carcinoma, dysphagia	*C. albicans*

[a] M: males, F; females

[b] BSA: body surface area, DM: diabetes mellitus, CABG: coronary artery bypass graft, GI: gastrointestinal

In the analysis of the fingerprint patterns of the isolates, a high interobserver reliability was observed; the generalized kappa statistic was 0.59 for karyotyping, 0.84 for REAG with *SfiI*, and 0.88 for REAG with *BssHII* (< 0.001 for each). The results of the overall interpretations (consensus call of the three observers) are given in Table 2. Overall, the method revealed 20 unique karyotypes. The karyotype banding patterns of initial site and subsequent bloodstream isolates (Fig. 1) were identical in 11 of 19 (57.9%) patients. In eight patients infected with either *C. albicans* (n = 7) or *C. parapsilosis* (n = 1), the karyotypes of the paired isolates did not match.

Table 2: Results of karyotyping and REAG of isolates from a blood culture and other body sites

Patient code	Isolate lane no.	Origin	Interval (days)	Result by karyotyping	Result by REAG with *Sfi* I[a]	Result by REAG with *BssH* II[a]
A	1	Blood		a	1	1
	2	Urine	8	a	1	1
B	3	Blood		b	2	2
	4	Stool	4	b	2	2
C	5	Blood		a	3	3
	6	Tracheal	11	a	3	3
	7	Catheter	1	a	3	3
D	8	Blood		c	4	4
	9	Tissue	30	d	5	5
E	10	Blood		e	6	6
	11	Catheter	1	f	6	6
F	12	Blood		g	7	7
	13	Urine	6	g	7	7
	14	Urine	1	g	7	7
G	15	Blood		h	3	3
	16	Tracheal	8	i	8	8
H	17	Blood		j	9	9
	18	Tissue	31	k	10	10
I	19	Blood		k	11a	11
	20	Sputum	3	k	11a	11
J	21	Blood		l	11a	12
	22	Urine	1	k	11a	12
K	23	Blood		k	11a	11
	24	Urine	11	m	11a	11
L	25	Blood		n	12	13
	26	Urine	5	n	12	13
	27	Bile	4	n	12	13
M	28	Blood		o	13	14
	29	Pleura	8	p	13	14
N	30	Blood		k	14	15
	31	Tracheal	4	k	14	15
O	32	Blood		k	11b	16a
	33	Catheter	2	k	11b	16a
P	34	Blood		k	11a	16b
	35	Catheter	1	k	11a	16b
Q	36	Blood		q	15	17
	37	Ascites	8	q	15	17
	38	Ascites	8	q	15	17
R	39	Blood		r	14	18
	40	Ascites	3	s	14	18
S	41	Blood		t	16	19
	42	Ascites	4	t	16	19

[a] The difference denoted a or b are insufficient to classify the isolates as different (sharing <95% of bands) rather than similar (sharing ≥95% but < 100% of bands)

The relationships among isolates within a given patient determined by REAG (Fig. 2 and 3) were identical regardless of the restriction enzyme employed. The only difference observed in the REAG with the two different endonucleases was in the interpatient evaluation e.g., whether a specific banding pattern was classified as unique or shared with another patient. By the REAG method, isolates from the initial site and blood cultures were shown to be identical in 16 patients (84.2%). In three patients with *C. albicans* infections, the paired isolates were different. Isolates from three patients (patients J, O, and P) were classified as identical with *SfiI*, whereas their banding patterns after digestion with *BssHII* classified them as different. Thus, the REAG method identified 16 unique patterns after digestion with *SfiI* and 19 after digestion with *BssHII*.

Despite a similar number of unique karyotype patterns, the results matched the overall REAG results for only 14 of the 19 patients (74%). The isolates from five patients whose initial site and blood isolates had different karyotypes appeared to be identical when REAG was done with both enzymes (see Fig. 1 to 3, patients J and K).

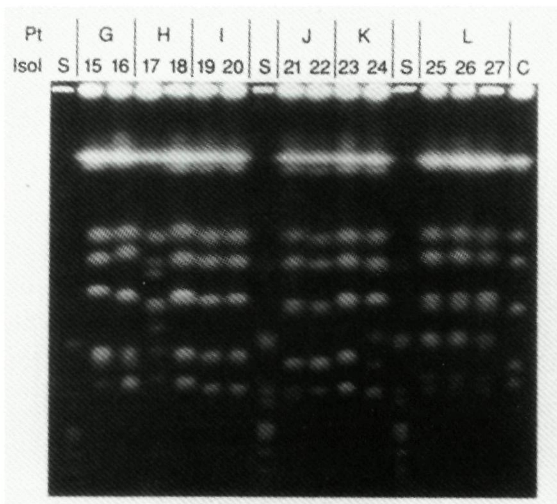

FIG. 1. CHEF karyotype. Shown are the blood culture and other site isolates from patients G to L. Note that the patterns of isolates from patients I and L appear to be identical. The karyotype pattern denoted k (see Table 2) is seen in isolates 18, 19, 20, 22, and 23 of patients H, I, J, and K, respectively. Note that isolates 21 and 22 (patient J) appeared to be different because of the spacing of the banding patterns. Abbreviations: Pt, patient; Isol, isolate number; S, molecular size standard; C, *C. albicans* control strain.

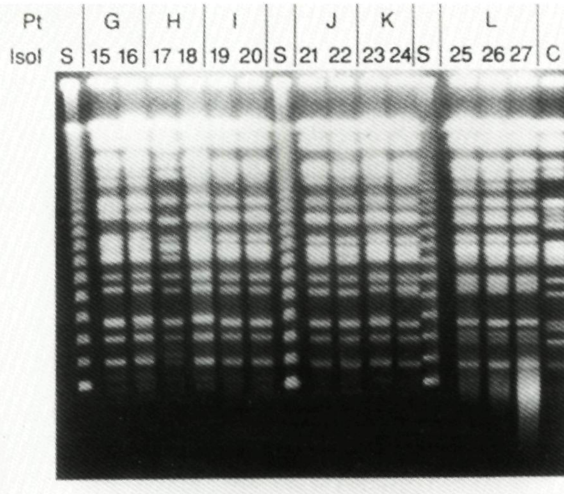

FIG. 2. REAG with *Sfi*I. Shown are blood culture and other site isolates from patients G to L. Banding patterns of paired isolates of patients I to L appeared to be identical, including isolates from patients J and K with karyotype patterns that appeared to be different (see Fig. 1). The REAG pattern with *Sfi*I denoted 11a (see Table 2) was shared by patients I, J, and K. Abbreviations: Pt, patient; Isol, isolate number; S, molecular size standard; C, *C. albicans* control strain.

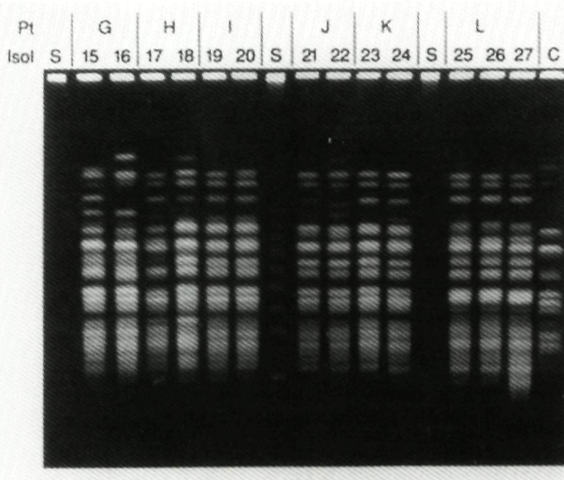

FIG. 3. REAG with *Bss*HII. Shown are blood culture and other site isolates from patients G to L. Banding patterns of paired isolates of subjects I to L appeared to be identical, as demonstrated previously by REAG with *Sfi*I. The overall (within-subject) evaluation was identical for REAG patterns with *Sfi*I and *Bss*HII. A difference between the two methods was seen only in the between-subject evaluation. Note that the banding patterns of isolates 21 and 22 (from patient J) do not match the banding patterns of isolates from patients J and K, as seen by REAG with *Sfi*I. Abbreviations: Pt, patient; Isol, isolate number; S, molecular size standard; C, *C. albicans* control strain.

Discussion

Rates of nosocomial bloodstream infections have increased markedly over the last decade, particularly because of the increased number of infections caused by coagulase-negative staphylococci, *Candida* spp., *Staphylococcus aureus*, and enterococci. In 1975, *Candida* spp. accounted for less than 3% of nosocomial bloodstream infections [147] but are currently responsible for approximately 10% [237]. Risk factors involved in the pathogenesis of candidemia have been identified in numerous studies [6 27 29 75 81 100 155 167 197 203 238]. Case-control studies that use multivariate analysis to control for underlying diseases have convincingly demonstrated that colonization by *Candida* spp. at various body sites, prior exposure to hemodialysis, central intravascular catheters, previous exposure to antibiotics, and azotemia are independent risk factors for candidemia [27 75 81 238]. Until recently, it was suggested that candidemia arises more or less exclusively from autoinfection caused by colonizing organisms in the intestine. The availability of molecular typing methods has allowed investigators to confirm this hypothesis [158 185]. Furthermore, unique strains colonize the patient, persist, and are frequently responsible for subsequent infection or relapse [48 205]. Additionally, a number of documented outbreaks of *Candida* infections have highlighted the importance of transmission of *Candida* spp. from an exogenous source to the patient [27 39 71 123 192 225 235 239].

Our study, in which we used the REAG method for fingerprinting, demonstrated identical colonizing or infecting and blood culture isolates in the majority (84%) of the patients analyzed. Reagan et al. [158] found a slightly higher frequency of identical colonizing and infecting strains among hematologic malignancy and bone marrow transplant patients than we found in the current study. Importantly, the isolates compared by Reagan et al. [158] were routinely obtained as part of an ongoing surveillance program in severely immunocompromised patients. In contrast, most of the isolates from nonneutropenic patients in the present study were from cultures of clinical samples taken during sporadic epidemiologic surveillance or when an infection was suspected. However, our study population represented one-third of all nonneutropenic patients with candidemia during the study period. Furthermore, Reagan and colleagues [158] reported that matching strains could be found even after an interval of up to 432 days. In our patients, the interval between a positive culture of a sample from another site an a positive blood sample culture was inversely related to the likelihood of finding identical colonizing and blood culture isolates. The fact that most of the isolates in our study were not obtained during ongoing surveillance may have created a selection bias that precluded a widespread generalization to all nonneutropenic patients. However, the high proportion of study patients with identical colonizing or infecting isolates prior to bloodstream infection supports the clinical view that the bloodstream infection frequently arises endogenously.

The difference in interval (days) between the previous isolation of *Candida* species from a body site and a blood culture in patients in whom the blood culture and other site isolates were identical was approximately one-fifth of that in patients in whom the isolates were different. However, given the relatively small number of subjects, this difference did not reach statistical significance. The two patients whose tissue biopsy specimens were infected with *C. albicans* were among three patients colonized or infected with a different strain prior to the development of candidemia. The extended time interval was long enough to allow recolonization and infection with a second strain, or the patients had been colonized with strains that were not isolated in the current study. Isolation of *Candida* species from tracheal secretions alone did not predict a subsequent bloodstream infection with the same strain. Among the patients with matching *Candida* strain pairs, the organisms were isolated initially most frequently from urine or vascular catheter tips. Since the isolates were not routinely obtained as part of an ongoing surveillance program, it is difficult to draw firm conclusions about this time interval. The observed differences in the interval between colonization or infection and subsequent bloodstream infection and initial isolation of *Candida* from a body site emphasize the fact that colonization of certain body sites (respiratory tract and remote tissue biopsy specimens) may infrequently result in candidemia with that same strain, despite long periods of follow-up. However, the presence of a *Candida* species at other sites (urine or catheter tip) may be associated with subsequent candidemia within relatively short periods of time.

Patients with vascular catheters are at a significantly increased risk for fungal infections [100 238]. This may be due to the unique surface properties of *Candida* species, which allow it to invade the host by attaching to plastic surfaces and evade the host immune system [80 169]. Some investigators argue that patients with catheter tip cultures positive for a *Candida* species require antifungal therapy, although this point is controversial. The safety and efficacy of early antifungal treatment of patients with a positive urine or vascular catheter tip culture and suspected fungal infections in preventing *Candida* species bloodstream infections could be evaluated prospectively.

With four exceptions, each patient was infected by his or her own unique *Candida* strain, suggesting no clustering or transmission of a predominant strain The discriminative value of standard CHEF gel electrophoresis of *C. albicans* has been demonstrated [122 148]. However, data from two prior studies [41 122] suggest that karyotyping may be of limited value for the discrimination of *C. tropicalis* isolates. The three typing methods used in the current study showed the identity of the *C. tropicalis* strains in patients Q and S. Compared with the more easily interpretable banding patterns of REAG, the interpretation of standard CHEF gel electrophoresis may be difficult because of the fewer number of bands. In our study, the initial site and bloodstream isolates from five patients had different patterns by karyotyping, yet they had identical patterns after endonuclease digestion. The reason for this finding is unclear.

Both methods resulted in relatively high levels of agreement between observers, i.e., greater than that expected by chance alone. The kappa statistic demonstrated the greatest level of agreement between the observers for the two REAG methods. We would suggest the routine use of a statistical method, such as the kappa statistic, to compare different methods quantitatively in molecular typing studies.

REAG appeared to be a reliable method for delineating strains of *Candida* spp. with high discriminatory abilities, regardless of whether *BssHII* or *SfiI* was used as the restriction enzyme. Since the cost per unit is considerably lower for *SfiI*, this enzyme should be considered in typing situations in which a single restriction enzyme is needed.

In summary, we found that in the majority of patients who develop disseminated *Candida* spp. infections, the initial colonizing or infection site and subsequent bloodstream isolates are identical. Furthermore, nearly every patient was infected with a distinct or unique *Candida* strain.

Investigation of *Candida* albicans transmission in a surgical intensive care unit cluster by using genomic DNA typing methods

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Abstract

An apparent outbreak of serious *Candida albicans* infections (n=6) occurred in a surgical intensive care unit over a 4-week period. Four patients developed *C. albicans* bloodstream infections. An additional patient developed catheter-related *C. albicans* infection; the sixth patient developed an infection of cerebrospinal fluid. *C. albicans* was isolated from the hands of five health care workers (17%) and the throat of one health care worker (3%) during the outbreak investigation. Karyotyping and restriction endonuclease analysis of genomic DNA with *BssHII* of 23 *C. albicans* isolates from patients and the 6 health care worker isolates revealed 9 and 12 different patterns, respectively. Three of six patients appeared to be infected with the same *C. albicans* strain (two bloodstream infections and one cerebrospinal fluid infection). The hands of a health care worker were colonized with a strain that appeared identical to an isolate from a patient prior to infection of the patient. However, restriction endonuclease analysis with *SfiI* found differences among the isolates determined to be identical by the other two methods. Karyotyping alone does not appear to be sufficient to differentiate between outbreak and control isolates. Restriction endonuclease analysis typing may be a more sensitive method than karyotyping alone in the investigation of a cluster of *C. albicans* infections. Furthermore, the use of more than one restriction enzyme may be necessary for optimal strain discrimination in restriction endonuclease analysis of genomic DNA.

Introduction

In recent years, the *Candida* species have emerged as important nosocomial pathogens. Over the past decade, the incidence of *Candida* bloodstream infections increased by 219 to 487% in U S. teaching hospitals and by 75 to 370% in non-teaching hospitals [8 183]. Candidemia occurs most frequently in immunocompromised patients with an underlying malignancy or

hematologic disorder [115 117] and in severely ill burn patients as well as surgical intensive care unit (SICU) and neonatal intensive care unit (ICU) patients [27 29 33 155 197 216] Therefore incidence rates of candidemia are highest in tertiary care referral hospitals The incidence rate at the University of Iowa Hospitals and Clinics is 8 5 per 10 000 admissions with 57% crude and 38% attributable mortalities [237]

Until recently the development of invasive *Candida* infections was thought to be more or less exclusively due to autoinfection by endogenous *Candida* strains which initially colonize the patient Using molecular typing methods investigators have confirmed this hypothesis in neutropenic and nonneutropenic patients [158 214 222] However exogenous sources responsible for outbreaks have been described increasingly including cross-infection among ICU patients attributed to hand carriage by health care workers (HCWs) [27 71] contaminated medications pressure transducers parenteral nutrition and reused disposable devices [123 192 201 233 235 239] In half of such outbreaks reviewed by Sherertz et al [192] the source could not be identified possibly because of the lack of reliable and readily available typing methods for *Candida* species

We report an outbreak of systemic *Candida albicans* infections in a SICU in which apparently identical isolates were recovered from three patients sites of infection and from the hands of an HCW prior to development of infection in one of the patients The epidemiology of the carriage and transmission of *C albicans* among patients in the SICU was further clarified by the application of molecular typing methods

Materials and Methods

Hospital and patients The University of Iowa Hospitals and Clinics is a 900-bed teaching hospital and tertiary care referral center with approximately 200 beds designated for intensive care The SICU (Figure 1) at the time of the cluster of infections was divided into four bays with a total of 24 beds The nurse to patient ratio ranged from 1 2 to 1 1 depending on the patients needs and availability of nursing staff A mean of 164 patients per month (standard deviation ± 12) were admitted to the SICU during the 9 months prior to the outbreak During the 4-week period of the outbreak 163 patients had been admitted to the SICU The unit is prospectively surveyed by trained infection control nurses each weekday Additionally the surveillance system has been prospectively validated recently [21] No significant changes in personnel equipment or infection control surveillance or definitions occurred during the outbreak period or in the previous 9 months

Outbreak description During the period of the cluster the observed rate of *C albicans* bloodstream infections exceeded the upper limit of the predicted 95% confidence interval and an outbreak investigation was initiated Microbiological data infection control surveillance data and clinical information for all patients were reviewed in search of a common source or vehicle In order to determine current rates of carriage among SICU

workers, the hands and throats of 30 HCWs were sampled prospectively at random intervals over the next 2 weeks by using the broth-bag technique [157] and sterile premoistened rayon-tipped swabs (Culturette II Marion Scientific Kansas City, Mo), respectively Pressure transducers of patients receiving intra-arterial monitoring and the ward stock insulin in use were also cultured Similarly the throats and stools of patients in the SICU over the 5 weeks following the identification of the outbreak were also cultured at random intervals All *Candida* isolates were banked and identified to the species level by using the API 20C system (Analytab Products, Plainview, N Y) Cases were identified by reviewing microbiology reports and patient data from charts and the hospitals mainframe computer and data gathered by infection control nurses The importance of hand washing and compliance with the guidelines for prevention of nosocomial infections were reemphasized at the time of the identification of the cluster

Molecular typing Molecular typing of all isolates was accomplished by electrophoretic karyotype (EK) analysis and by restriction endonuclease analysis of genomic DNA (REAG) using the restriction enzymes *BssHII* (REAG-B) and *SfiI* (REAG-S) followed by pulsed-field gel electrophoresis Electrophoretic karyotyping and REAG were performed as described previously [39 41 222]

Analysis EK and REAG profiles were analyzed by visual inspection of photographs of ethidium bromide-stained gels Photographs were analyzed to detect similarities and differences in banding patterns by three observers who were blinded to the origin of the isolate and the results of the other observers Isolates were considered identical when all of the bands matched similar when > 95% but < 100% of the bands matched, and different when < 95% of the bands matched Interobserver reliability was calculated by using the kappa coefficient [247]

Results

Patients Between 14 October and 22 November 1990, a total of six serious *C albicans* infections occurred in an open SICU ward with adjacent beds in which patients were close together and often cared for by the same HCW (Figure 1) Four of the six patients had candidemia one had a catheter-related infection and one had a central nervous system infection The incidence density ratio for candidemia during the outbreak period was significantly elevated 4 infections per 577 patient days versus 4 per 6,430 patient days in the entire year (incidence density ratio 11 1 (95% confidence interval 2 79 to 44 56) Similarly the incidence density rate difference was 0 0063 (95% confidence interval 0 0005 to 0 01) the underlying diseases and risk factors for infection of the six patients involved in the cluster are shown in Table 1 Four patients had undergone abdominal surgery and five had diabetes mellitus as their major underlying disease Risk factors for infection included intravascular

catheters for six patients, five or more antibiotics for five patients, and total parenteral nutrition for four patients (Table 1).

Table 1. Underlying diseases and risk factors of SICU cluster patients

Risk factor	Presence of factor for patient: S	W	K	C	A	D
Infection[a]						
CRI	+					
CSF		+				
BSI			+	+	+	+
IV catheter						
Central venous	+	+	+	+	+	+
Arterial line	+	+	+	+	+	+
Peripheral line	+	+	+	+	+	+
No. of antibiotics						
≤ 4	+					
≥ 5		+	+	+	+	+
TPN[b]	+			+	+	+
Abdominal surgery	+			+	+	+
Diabetes mellitus	+	+	+		+	+

[a] CRI: catheter-related infection; CSF: cerebrospinal fluid; BSI: bloodstream infection
[b] TPN: total parenteral nutrition

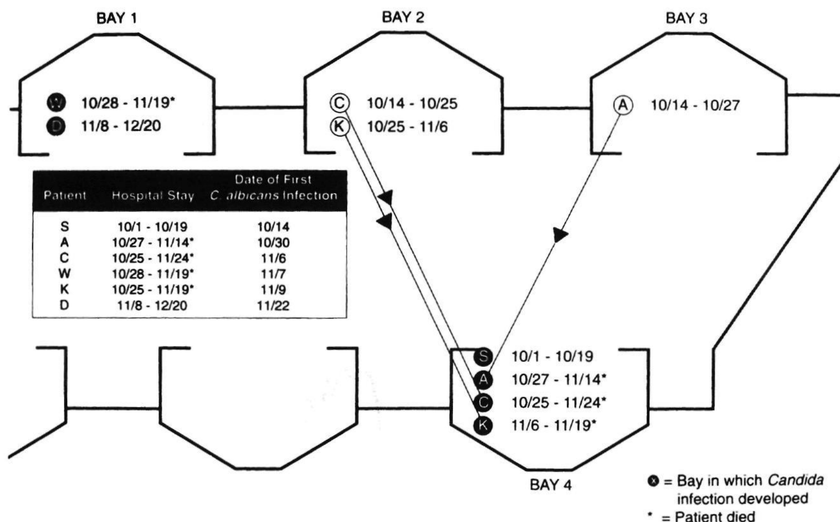

FIG. 1. Schematic diagram of bays 1 to 4 of the SICU, showing the initial localization and transfer of the cluster patients.

Microbiological surveillance. Following identification of the cluster, the hands and throats of 30 different HCWs were cultured. Seventeen percent of the hand cultures and 33% of the throat cultures of HCWs yielded *C. albicans*. At the same time the throats of 33% of the patients and the stools of 15% of the patients were colonized with *C. albicans*. In the subsequent 3 weeks, rates of *C. albicans* colonization among patients were 30 and 12% for the throat and stool, respectively. This difference was not statistically significant ($P>0.20$).

Molecular typing. In the analysis of the DNA profiles of the isolates a high degree of interobserver reliability was observed: the generalized kappa statistics were 0.84 or greater for EK and REAG-B and REAG-S profiles ($P<0.001$ for each). The results of the overall interpretation (consensus call of the three observers) are given in Table 2.

Table 2. Summary results of karyotyping and REAG

Patient or HCW	Isolate no.	Origin	Karyotype	REAG-B type[a]	REAG-S type[a]
S	1	Catheter	A	1a	I
	2	Catheter	A	1a	I
A	3	Blood	A	1b	II
	4	Blood	A	1a	II
	5	Urine	A	1c	II
	6	Urine	A	1c	II
	7	Urine	A	1c	II
	8	Blood	A	1c	II
W	9	CSF[b]	B	2	IIIa
	10	Trachea	B	2	IIIa
	11	CSF	B	2	IIIa
D	12	Blood	A	3	IIIa
	13	Eye	C	4	IV
HCW 1	14	Hand	A	1c	V
	15	Throat	A	1c	VI
C	16	Catheter	D	5	IIIb
	17	Blood	D	5	IIIb
	19	Catheter	D	5	IIIb
	20	Trachea	D	5	IIIb
K	21	Trachea	A	3	IV
	22	Blood	E	5	V
	23	Trachea	A	3	IV
Patient 1	24	Sputum	E	6	VI
Patient 2	25	Trachea	F	7	VII
HCW 2	26	Hand	C	8	IV
HCW 3	27	Hand	G	9	VIII
HCW 4	28	Hand	H	10	IX
HCW 5	29	Hand	I	1c	X

[a] Lowercase letters indicate subtypes with ≥95% but ≤100% matching bands
[b] CSF: cerebrospinal fluid

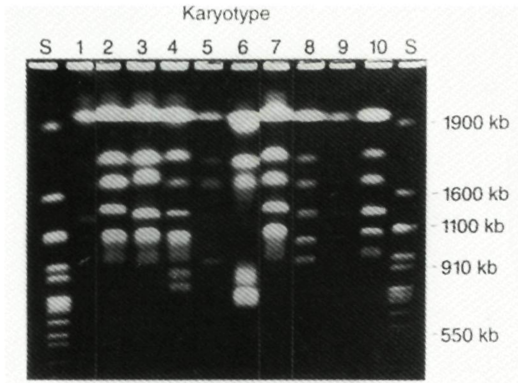

FIG. 2. Representative EK profiles of *C. albicans*. Lanes: S, *Saccharomyces cerevisiae* chromosome DNA size standards (sizes are indicated on the right); 1, *C. albicans* control strain; 2 and 3, EK E; 4, EK H; 5, EK F; 6, EK G; 7, EK D; 8 to 10, EK A.

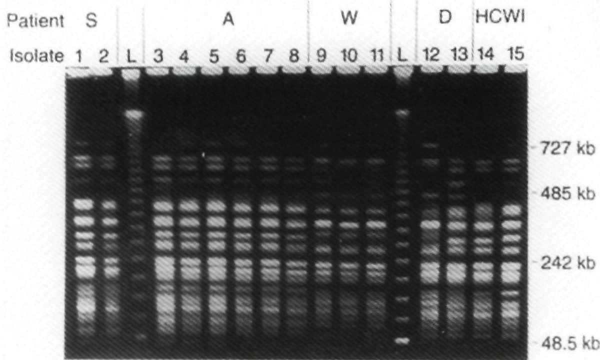

FIG. 3. Types of *C. albicans* obtained by REAG-B followed by pulsed-field gel electrophoresis. Results are shown for blood culture and other-site isolates from patients S, A, W, and D and HCW 1. Lane numbers correspond to the isolate numbers in Table 2. Banding patterns of isolates from patients S and A and HCW 1 represent identical or closely related subtypes (≥95% of bands are shared). Lanes L, molecular size standards (lambda phage DNA concatemers; sizes are indicated on the right).

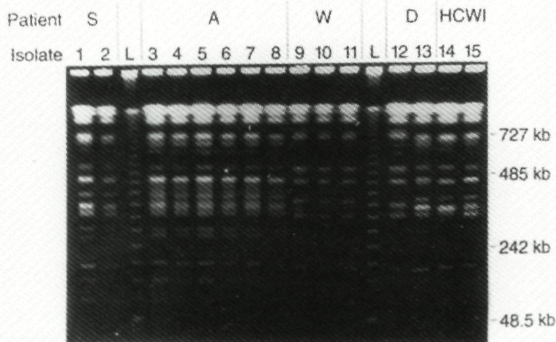

FIG. 4. Types of *C. albicans* obtained by REAG-S followed by pulsed-field gel electrophoresis. Isolates are the same as for Fig. 3. Differences in banding patterns are seen among isolates from patients S and A and HCW 1, as well as patients W and D. Lanes L, molecular size standards (lambda phage DNA concatemers; sizes are indicated on the right).

A total of 20 isolates from six outbreak patients (patients S, A, W, D, C, and K), the hand isolates from the five HCWs with positive cultures, and isolates from two additional SICU patients (patients 1 and 2) colonized with C albicans following the outbreak were typed by all three methods EK analysis and REAG-B and REAG-S identified 9, 10 and 10 different patterns, respectively (Table 2 and Figure 2 to 4) The combination of all three methods (composite DNA type) identified 16 different profiles

Identical EK patterns (EK A) were obtained for all isolates from patients S and A and from the hands and throat of HCW 1 Additionally, EK A was found in the blood and trachea of patients D and K, respectively (Table 2) In contrast, distinctly different EKs were observed for isolates from patients W and C, SICU patients 1 and 2, and HCWs 2 to 5 (Table 2) For six of the seven individuals with two or more isolates of C *albicans*, the same EK was observed on more than one occasion and/or for specimens from more than one anatomic site

The relationship among isolates determined by REAG-B generally supported that determined by EK analysis (Table 2) All isolates from patients S and A and HCW 1 had the same basic REAG-B profile (type 1), allowing for minor banding differences (shared > 95% of bands, Figure 3) REAG-B also found the blood and tracheal isolates from patients D and K to be identical (type 3) but different from the isolates of patients S, A and HCW 1 despite a common EK (Figure 3) As with EK analysis, the isolates from patients W and C, SICU patients 1 and 2 and HCWs 2 to 5 were all different as determined by REAG-B Likewise, REAG-B demonstrated that multiple isolates from the same patient generally had the same DNA banding pattern (Figure 3)

In contrast to the findings with EK analysis and REAG-B, REAG-S identified differences among the outbreak isolates (Table 2 and Figure 4) Specifically isolates from patients S and A and HCW 1 each had a different REAG-S profile despite having the same EK and REAG-B types (Table 2 and Figure 3 and 4) Several patients had the same REAG-S profile REAG-S type III was shared by patients W, D, and C, type IV was shared by patients D and K and HCW 2, type V was shared by HCW 1 and patient K and type VI was shared by HCW1 and patient 1 (Table 2) However these isolates were identified as different strains on the basis of REAG-B or EK analysis (Table 2) As with the other typing methods, multiple isolates from the same patient generally had the same REAG S profile

When the results of all three typing methods were considered (composite DNA type), a total of 16 DNA types were identified among 27 isolates from 13 individuals (Table 2) None of the composite DNA types were shared by two or more individuals Patients S, A, W and C each maintained their own unique DNA type of C *albicans* over time and from multiple anatomic sites whereas patients D and K and HCW 1 each had two different DNA types colonizing or infecting different anatomic sites

Discussion

One of the important considerations regarding the prevention and control of nosocomial candidiasis is whether the infection is endogenous or exogenous to the patient [140]. It is reasonable to assume that endogenous forms of infection may require strategies for prevention (e.g., antifungal prophylaxis) which are different from those for exogenous infections due to transmission of any organism from patient to patient (hand washing and other infection control measures). Our understanding of this area is still evolving; however, the use of molecular typing methods to fingerprint *Candida* species has been very useful in epidemiologic studies designed to address these issues [39 158 192 201 222].

The evidence for endogenous candidal infection in hospitalized patients includes the findings that unique strains of *Candida* are usually seen for each patient, that the same *Candida* DNA type is usually isolated from multiple anatomic sites over time, that colonizing and infecting strains are usually the same DNA type, and that the colonizing isolate usually precedes the infecting isolate [158 222].

Despite the importance of endogenous infection, there are now several reports documenting transmission of *Candida* species from patient to patient, particularly in the ICU setting [27 46 71 197 216]. The open nature of many ICUs and the level of activity make it difficult to implement or enforce infection control practices, thus facilitating nosocomial transmission of pathogenic organisms such as *Candida* species. Evidence for carriage of the infecting strain of *Candida* on the hands of HCWs is mounting, suggesting that simple hand washing may be an effective infection control measure [39 40]. Unfortunately, compliance with hand-washing policies among HCW personnel is less than optimal and underscores the problem of controlling nosocomial transmission of *Candida* and other pathogens [38].

In the present study, we investigated a cluster of *Candida* infections in a single ICU in which surveillance cultures indicated hand carriage by several HCWs of the same species of *Candida* (*C. albicans*) carried by the infected patients. The clustering of these infections in time and space coupled with hand carriage of the infecting species suggested the possibility of nosocomial transmission. The epidemiology was clarified by the use of DNA-based typing methods, including EK analysis and REAG-B and REAG-S.

Application of EK analysis alone suggested infection of four patients and colonization of one HCW with a single strain of *C. albicans* Two additional patients shared a second EK of *C. albicans*, and the remaining patients and HCWs were each colonized with their own unique EK. This relationship among isolates and patients was corroborated by REAG-B. Importantly, apparently the same strain eventually causing infection in a previously uninfected patient was identified on the hands of an HCW prior to development of infection in that patient. Isolates from two patients and one HCW had the same EK and REAG-B type, suggesting limited nosocomial transmission of a single strain of *C. albicans* and linking the HCW to the two infected patients

One of the major tenets of epidemiologic typing is that although one or more typing methods may be used to demonstrate convincingly that two nosocomial pathogens are different from one another, it is very difficult to prove that they are the same strain. To this end, epidemiologic typing systems are used in combination to enhance discrimination among bacterial isolates. However, this combined approach has been used infrequently for *Candida* species. Previously, Hunter and Fraser [70] have demonstrated enhanced discriminatory power for typing isolates of *C. albicans* with the combination of resistotyping and morphotyping, suggesting that two or more methods employed in parallel or in a hierarchical fashion may be necessary for epidemiologic studies of *C. albicans*. More recently, Mercure et al. [111] demonstrated enhanced discrimination among *C. albicans* strains, using two DNA-based typing methods, restriction fragment length polymorphism analysis and Southern hybridization with DNA probe 27A. In the present study, further analysis of the *C. albicans* isolates with a second restriction enzyme, *Sfi*I, identified differences among the isolates with identical EK and REAG-B profiles, indicating that they may not be clonally related. The combination of EK analysis and REAG with two enzymes revealed unique DNA profiles for isolates from each patient and HCW. No two isolates from patients or HCWs shared all of the DNA markers.

Despite the great heterogeneity among DNA types generated by the composite (EK, REAG-B, and REAG-S) typing approach, the ability of this approach to identify related strains within an individual patient remained intact. Although we observed three individuals infected or colonized by two strains of *C. albicans*, the overall pattern was similar to that described previously [158,222] in that patients with multiple isolates were generally colonized and infected by a single strain of *C. albicans* that persisted over time and multiple anatomic sites. Overall, five of the six patients in this study were found to be infected or colonized by their own unique strain on two or more occasions.

In summary, distinct DNA types of clinical isolates of *C. albicans* were identified by using a composite approach of electrophoretic karyotyping and REAG with two different restriction enzymes. The combination improved strain discrimination compared with that of any one of the typing methods used alone. Epidemiologic investigation demonstrated that an individual patient usually harbored a single composite DNA type of *C. albicans* that was not shared with those of other patients or HCWs. These findings speak against nosocomial transmission of a single strain as a cause of the entire cluster of *C. albicans* infections. The fact that isolates from patients S and A and HCW 1 had the same strain of *C. albicans* as determined by two of the three molecular typing approaches (EK analysis and REAG-B) suggests that some transmission may have occurred on a small scale. Differences observed with the third typing approach (REAG-S) may reflect the propensity for *C. albicans* to undergo genomic rearrangements and suggests that although these isolates may have had a common origin, direct patient-to-patient or HCW-to-patient transmission is unlikely. Molecular typing methods may be used effectively to clarify the epidemiology of nosocomial infections; however, the

various typing methods must be used with the understanding that more than one approach may be necessary to achieve optimal strain discrimination.

IV. Clinical epidemiology of *Candida* bloodstream infections

Chapter 4.1

Occurence of yeast bloodstream infections between 1987 and 1995 in five Dutch university hospitals

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Abstract

The aim of this study was to identify retrospectively trends in fungal bloodstream infections in The Netherlands in the period from 1987 to 1995. Results of over 395,000 blood cultures from five Dutch university hospitals were evaluated. Overall, there were more than 12 million patient days of care during the nine-year study period. The rate of candidemia doubled in the study period, reaching an incidence of 0.71 episodes per 10,000 patient days in 1995. The general increase in candidemia was paralleled by an increase in non-*Candida albicans* bloodstream infections, mainly due to *Candida glabrata*. However, more than 60% of the infections were caused by *Candida albicans*. Fluconazole-resistant species, such as *Candida krusei* did not emerge during the study period.

The increasing rate of candidemia found in Dutch university hospitals is similar to the trend observed in the USA, but the rate is lower and the increase is less pronounced.

Introduction

In recent years, *Candida* species, most notably *Candida albicans*, have emerged as important nosocomial pathogens Over the past decade, the incidence of *Candida* bloodstream infections increased by two- to fivefold in teaching hospitals and by one- to fourfold in non-teaching hospitals in the USA [8 13 183]. Candidemia occurs most frequently in high-risk patients, such as immunocompromised patients with an underlying malignancy or hematological disorder [115 117], severely ill burn-patients, and surgical and neonatal intensive care patients [27 29 117 155 197 216] Therefore, the incidence of candidemia is highest in tertiary care referral hospitals, with an incidence of 5-10 per 10,000 admissions [8] Nosocomial candidemia now accounts for 10%-15% of all hospital-acquired bloodstream infections in hospitals in the USA, *Candida* thus being among the four predominant microorganisms causing nosocomial bloodstream infections [8 144] Clearly, candidemia is posing an increasing-

ly serious problem in infectious diseases, and has been shown to be associated with 57% crude and 38% attributable mortality rates [237].

As neither the prevalence nor incidence of nosocomial fungal bloodstream infections in The Netherlands is known, the aim of this study was to identify trends in yeast bloodstream infections retrospectively in this country, by evaluating computerized laboratory and census data from five of the eight Dutch university hospitals.

Patients and Methods

Five university hospitals in The Netherlands participated in the study, including two hospitals in Amsterdam and one each in Rotterdam, Utrecht, and Nijmegen. The overall bed capacity of these five hospitals, which serve as tertiary-care referral centers for roughly two-thirds of the population of The Netherlands, is approximately 5,500.

Episodes of fungemia were identified retrospectively from microbiology data systems using computer-generated lists of patients whose blood cultures yielded yeasts during the period from 1 January 1987 to 31 December 1995. All patients admitted to the study hospitals were eligible for enrollment An episode of fungal bloodstream infection was defined as at least one positive blood culture yielding yeast during a single hospitalization period. The number of blood culture sets examined per year and their outcomes (negative, positive bacterial, positive fungal) were reported. All microbiological data were taken at face value, disregarding the different blood culture- and identification-systems used in the participating hospitals. Between 1991 and 1992 automated blood culture systems were implemented in all participating hospitals. None of the hospitals used the lysis centrifugation system. Census data to determine patient days were retrieved from the hospital's information systems.

Results

During the nine-year study period from 1987 to 1995, the five university hospitals delivered a total of 12,353,861 patient days of care, averaging 1,372,600 ± 32,300 (mean ± SD) patient days per year. The number of patient days decreased over the years from 1,426,319 in 1987 to 1,322,338 in 1995. In contrast, the number of admissions (118,966 in 1987; 128,864 in 1995), and the number of blood cultures taken per year (34,450 in 1987; 49,203 in 1995), increased by 8.3% and 42.8%, respectively.

The proportion of positive blood cultures (with no regard for clinical significance) was 19% ± 2.9% (mean ± SD), remaining stable over the years. The proportion of positive blood cultures yielding yeasts ranged from 3.2% in 1988 to 5.6% in 1993.

Overall, 671 episodes of yeast bloodstream infections occurred during the study period (Table 1); 635 episodes (94.6% ± 2.9%) were due to *Candida* species, and 46 episodes (6.8%) were due to *Cryptococcus* species.

Table 1: Overall episodes of yeast bloodstream infections in the study period.

	1987	1988	1989	1990	1991	1992	1993	1994	1995	Mean/SD
Yeast (n=671)										
Overall no	57	48	70	64	73	70	111	78	100	74.6 / 19.9
No. per 10,000 PD	.40	.34	.51	.46	.54	.50	.82	.58	.76	.55 / .16
Candida (n=625)										
Overall no	53	45	63	59	67	67	101	75	95	69.4 / 18.4
No. per 10,000 PD	.37	.32	.46	.43	.50	.48	.74	.56	.72	.31 / .15
Cryptococcus (n=46)										
Overall no	4	3	7	5	6	3	10	3	5	5.1 / 2.3
No. per 10,000 PD	.03	.02	.05	.03	.04	.02	.07	.02	.04	.035 / .017

The rates of yeast bloodstream infections in The Netherlands during the study period are given as episodes per 10,000 patient days (Table 1). The lowest rate was seen in 1988 (0.34 episodes per 10,000 patient days); the highest rate in 1993 (0.82 episodes per 10,000 patient days). Figure 1 depicts the individual rates for *C. albicans* and non-*C. albicans*. During the study period, the rate of bacteremia increased from 15.2 episodes per 10,000 patient days to 23.5 episodes per 10,000 patient days in 1987 and 1995, respectively. Therefore, all rates showed the same, increasing trend. Importantly, there was no significant difference between the rate of candidemia due to *C. albicans* and non-*C. albicans* species. During the study period, fluconazole was licensed for sale in The Netherlands. The annual use of fluconazole, estimated from the nation-wide sale figures (data supplied by Pfizer, The Netherlands), increased by factor four from 15.5 kg in 1990 to 63.0 kg in 1995 (Figure 1).

The number of episodes of fungemia caused by the different *Candida* species and the proportion due to the individual non-*C. albicans* species is shown in Table 2. The increase of non-*C. albicans* species was mainly due to *Candida glabrata*. Interestingly, the number of episodes of candidemia due to *Candida krusei* was very low throughout the study period (Figure 2).

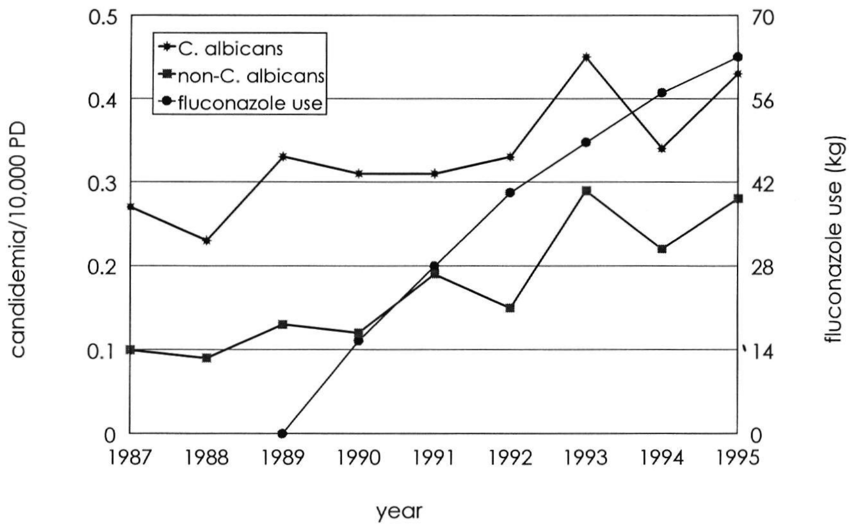

Figure 1: Episodes of *C. albicans* and non-*C. albicans* fungemia per 10,000 patient days (PD) in five Dutch university hospitals, and the yearly use of fluconazole estimated from the nationwide sale of capsules (50, 150, 200 mg) and vials(100 and 200 mg).

Table 2: Episodes of fungemia (n) caused by individual *Candida* species and proportion of candidemia due to non-*candida*-albicans species (NCAS)

	1987	**1988**	**1989**	**1990**	**1991**	**1992**	**1993**	**1994**	**1995**
C. albicans	39	32	45	43	42	46	61	46	57
Non-*C. albicans*									
C. glabrata	3	3	5	3	5	2	14	10	16
C. tropicalis	2	1	2	1	3	1	3	4	4
C. krusei	2	1	0	0	1	0	0	2	2
C. parapsilosis	4	1	1	4	2	4	6	2	2
C. guillermondii	0	1	0	0	1	1	3	1	2
Other species	0	0	1	1	5	4	3	3	3
Not determined*	3	6	9	7	9	9	11	7	9
Subtotal (NCAS)	14	13	18	16	26	21	40	29	38
%	26.4	28.9	28.6	27.1	38.2	31.3	39.6	38.7	40.0
Total	53	45	63	59	68	67	101	75	95

NCAS = non-*C. albicans* species
* mostly originating from one study center that only differentiated *C. albicans* and NCAS

In the last year of the study period, 40% of all *Candida* bloodstream infections were due to non-*C. albicans* species, with *C. glabrata* accounting for 42% or 55% of those episodes (depending on whether the figures for the non-determined species were included or not, see Table 2).

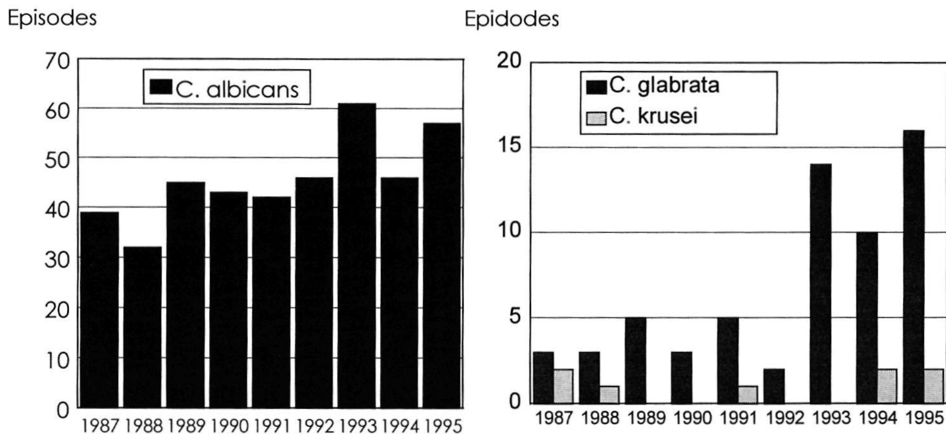

Figure 2: Occurrence of *C. albicans*, *C. glabrata*, and *C. krusei*, among blood culture isolates in five Dutch university hospitals from 1987 to 1995.

Discussion

Major changes have occurred in hospital populations, health care technology, and the use of antimicrobial drugs during the last two decades. Data from the National Nosocomial Infections Surveillance System and other hospitals in the USA show that these changes have had a profound impact on the epidemiology of fungal infections, including the increasing incidence of *Candida* bloodstream infections [8,13,189,231]. So far, only one European study has addressed the increasing problem of fungemia. Bruun et al. [24] showed an increase in the annual incidence of fungemia at a Danish university hospital from 19 episodes in 1989 to 57 episodes in 1994. Unfortunately, the authors gave no rates, thereby making it impossible to compare their findings with the literature and introducing the possibility of bias due to a likely increase in the number of blood cultures taken, as demonstrated in our study.

Pittet and Wenzel [150] reported a linear increase in the rates of nosocomial bloodstream infections at the University of Iowa Hospitals and Clinics (UIHC) between 1981 (67 episodes per 10,000 patient days) and 1992 (184 episodes per 10,000 patient days). Statistically significantly increased rates were demonstrated for the four predominant blood culture isolates, coagulase-negative staphylococci, *Staphylococcus aureus*, enterococci, and

Candida species Rates of candidemia increased 12-fold during the study period described, reaching a peak of two episodes per 10,000 patient days in 1991 [150] The rates of bacteremia and candidemia found in Dutch university hospitals show the same, increasing trend, but on a lower scale In 1991, we found 0 5 episodes of candidemia per 10,000 patient days, a rate, fourfold lower than the UIHC rate Even three years after the end of the study in the USA, the rate of candidemia in Dutch university hospitals is still 2 8-fold lower (0 72 episodes per 10,000 patient days) In general, the rates of candidemia in The Netherlands are not only lower but the increase is less dramatic (2 3-fold compared to 12-fold, at the UIHC) This difference between the USA and The Netherlands might be due to the very restricted antibiotic policy of Dutch physicians both within and outside the hospital, which is reflected in the low incidence of other multi-resistant pathogens, such as penicillin-resistant pneumococci, methicillin-resistant *Staphylococcus aureus*, and vancomycin-resistant enterococci [88 108 113 199]

Overall, 60% of all candidemic episodes in the study period were caused by *C albicans*, but the proportion of candidemic episodes due to non-*C albicans* species increased from 26% to 40% This increase was due mainly to a rising number of episodes due to *C glabrata*, a *Candida* species with reduced susceptibility to fluconazole During the study period, the rates of candidemia due to *Candida* species with reduced fluconazole susceptibility (*C glabrata and C krusei*) increased 4 7-fold (0 03 per 10,000 patient days in 1987 versus 0 14 per 10,000 patient days in 1995) compared to an 2 8-fold increase (0 06 per 10,000 patient days in 1987 versus 0 17 per 10,000 patient days in 1995) for the other non-*C albicans* species However, other factors besides the increased use of fluconazole in The Netherlands may have contributed to this species shift and should be identified in future investigations
Changes in blood culture technique, most importantly the introduction of agitation (1991/92), may have influenced our general findings, but were not the only factors influencing the general trend, since this would have resulted in a sudden increase with only minor changes in the period before and after the introduction
To what degree demographic changes in patient population may have contributed to the observed trend is not clear During the study period the number of admissions and thus the number of patients who could develop candidemia increased, but at the same time, the average time of hospitalization decreased (from 12 1 days in 1987 to 10 3 days in 1995) thereby reducing the chance of the individual patient acquiring systemic candidosis
The introduction of new diagnostic and therapeutic techniques, and the increased and prolonged use of multiple antimicrobial agents in a growing proportion of severely ill patients (reflected in the growing number of blood cultures taken) may be other factors contributing to the increase of nosocomial *Candida* bloodstream infections in Dutch university hospitals

Chapter 4.2

Candidemia in intensive care unit patients: risk factors for mortality

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Abstract

Aim of this study was to evaluate whether risk factors which predict the development of candidemia may furthermore predict death in ICU patients with candidemia. During a 8-year-period all ICU patients whose blood cultures yielded *Candida* species (n=40) were retrospectively evaluated in a case-control fashion. The average incidence of *Candida* bloodstream infections was 5.5 per 10,000 patient days, ranging from 2.4 in 1990 to 7.4 in 1994. C. *albicans* was the most common pathogen in candidemic patients, but the proportion of non-C. *albicans* strains showed an increasing trend during 1989-1993, with a major shift towards non-C. *albicans* species in 1994. The overall mortality of patients with candidemia was 58%. Mortality was highest in the group of patients with multi-organ dysfunction syndrome, especially among those in need of hemodialysis. Risk factors for the development of candidemia, such as age, malignancy, steroid use, iv catheterization, and the use of broad spectrum antibiotics were not correlated with mortality in our ICU patients.

Introduction

ICU patients account for only a small part of the hospital population, but contribute considerably (about one fourth) to the overall rate of endemic and epidemic nosocomial infections [107 212] Development of hospital-acquired infections is a major determinant of morbidity and mortality in these patients

Candidemia occurs most frequently in immunocompromised patients with an underlying malignancy or hematological disorder as well as surgical and neonatal intensive care unit patients [27 29 38 216] In recent years the incidence of *Candida* bloodstream infections increased dramatically with *Candida* advancing to be the third to fourth most common pathogen causing bloodstream infections [8 21 147 195] In general, critically ill patients who develop nosocomial bloodstream infection are at greater risk to die, than patients with comparable severity of underlying disease without this complication [195] Patients with fungal bloodstream

infection, such as those due to *Candida*, where shown to have the shortest survival prospects of any blood-borne infection [181]. Wey et al. [238] reported in a well matched case-control study a crude and attributable mortality of 57% and 38%, respectively.

Various risk factors for the development of systemic *Candida* infections have been reported, among which the severity of underlying illness (assessed by APACHE II), number and extend of prior antibiotic therapy, concomitant isolation of *Candida* species from other sites, the presence of central venous catheters, and prior hemodialysis were proven in multiple logistic regression models, or were unanimously identified by all investigators [23 149 238].

The purpose of this paper was to evaluate whether risk factors for the development of candidemia may be used to identify ICU patients at risk to die from *Candida* bloodstream infections. Improved stratification of the risk of candidemia would help to identify ICU patients in whom pre-emptive treatment with toxic and/or expensive (new) antifungal agents would be reasonable. Furthermore, we examined whether an increased incidence of candidemia and mortality is attributed to the emergence of non-*C. albicans* species.

Patients and Methods

The University Hospital Nijmegen is a 980-bed tertiary care teaching hospital with several intensive care facilities. The central ICU encompasses 37 beds, primarily directed at patients recovering from thoracic and abdominal surgery. Results of all blood cultures from patients admitted to the ICU between 1-1-1987 and 31-12-1994 were screened for *Candida* species.

All patients with at least one blood culture positive for a *Candida* species were objected to a retrospective chart review and analyzed in a case-control fashion. Candidemic patients who died during their ICU stay (non-survivors) were considered as cases; those surviving candidemia and being discharged from the ICU served as controls (survivors).

Census data of the department of intensive care were used to calculate incidence of candidemia, which was expressed as cases per 10,000 patient days. Furthermore, the distribution between incidence of *Candida albicans* versus non-*C. albicans* strains was determined. Medical records of all selected cases were reviewed for patient demographics, severity of underlying diseases (SAPS II score[91]), the time on ventilator, the time with indwelling intravascular catheters, colonization with *Candida* species, the length of stay in the ICU before development of candidemia and antimicrobial/-fungal therapy. Multi-organ dysfunction syndrome (MODS) was classified according to the following criteria: respiratory failure, acute respiratory distress syndrome (ARDS), hypotension, renal failure requiring acute hemodialysis, and hepatic dysfunction with coagulation system disorders or gastrointestinal dysfunction.

To analyze risk factors for mortality in cases of candidemia, difference between the groups were evaluated by crosstabs and multiple regression analysis (SPSS for MS windows, version 6.1). The dependent variable was survival, independent variables were risk-factors for candidemia.

Results

A total of 40 patients with positive blood cultures for *Candida* and available medical records were identified. Demographic features of the patients are depicted in Table 1.

Table 1: Patient demographics.

	Survivor (n=17)	Non-Survivor (n=23)
Male/female ratio	10/7	12/11
Age	54 ± 5 yr	48 ± 5 yr
ICU duration	46 ± 9 days	29 ± 8 days
Days in ICU until first isolation of *Candida* spp.	14 ± 3 days	14 ± 4 days

The male/female ratio was similar in survivors (n=17) and non-survivors (n=23). The mean age of non-survivors was slightly lower (48 years as compared to 54 years in survivors) but not significantly different. The mean length of stay before development of candidemia was 14 days in cases and controls.

The incidence of candidemia is shown in Figure 1. The average rate of candidemia was 5.5 per 10,000 patient days per year, ranging from 2.4 in 1990 to 7.4 in 1994.

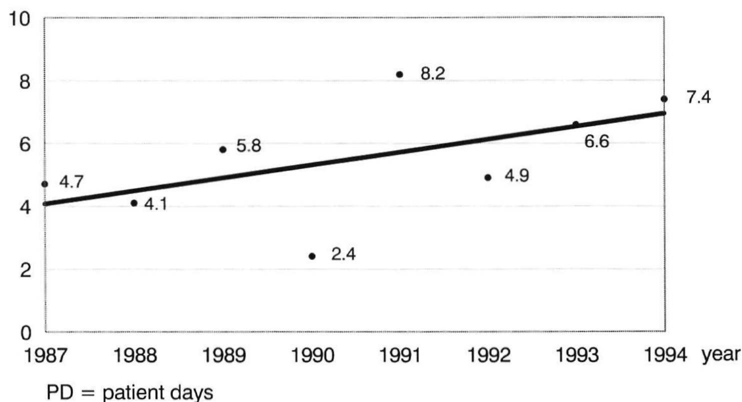

Figure 1: Incidence of *Candida* bloodstream infections; 1987-1994.

During the 8-year-period (1987-1994) *C. albicans* accounted for 75% of all blood culture isolates (Figure 2).

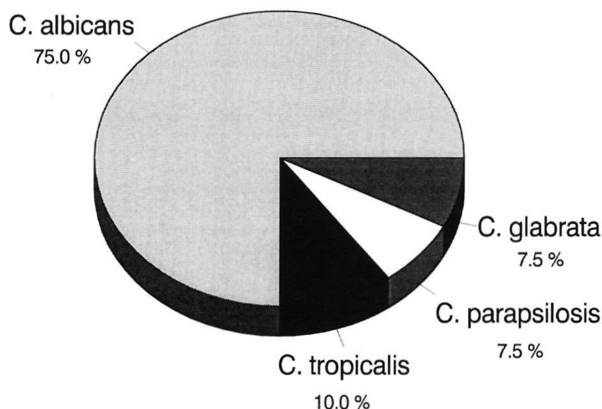

Figure 2: *Candida* species causing bloodstream infections 1987-1994.

Other *Candida* species found were *Candida tropicalis* (10%), *Candida parapsilosis* (8%) *and Candida glabrata* (8%). *C. albicans* remained the most prominent pathogen until 1994. In 1994 the proportion of non-*C. albicans* species among the *Candida* isolates from blood cultures rose to 56% (Figure 3).

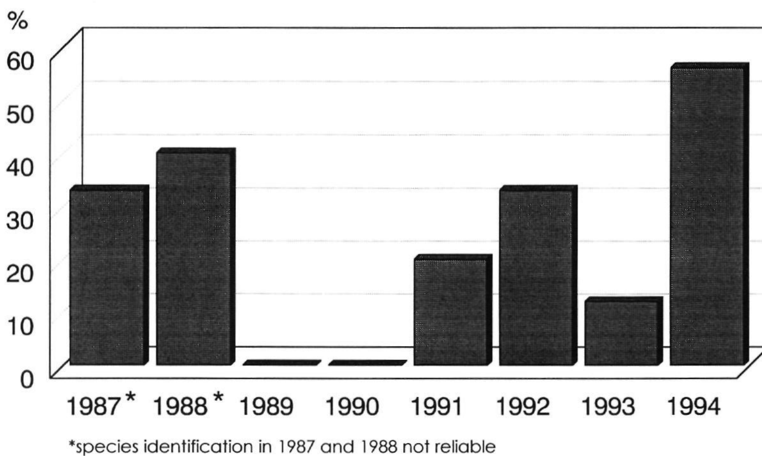

Figure 3: Proportion of non-*C. albicans* species causing bloodstream infections; 1987-1994.

Antifungal treatment (mostly fluconazole, rarely amphotericin B) was started in 31 of the 40 patients (77.5%); 82% of the survivors versus 74% of the non-survivors. Nine out of 40 patients of

whom six died, had no antifungal treatment. None of the patients received prophylactic antifungal treatment.

Table 2 depicts the underlying diseases and co-morbidity by survival group. All patients were on mechanical ventilation, had intravascular devices, and were undergoing concomitant broad spectrum antibiotic treatment throughout their ICU stay. All possible determinants for mortality were equally distributed among survivors and non-survivors, except acute hemodialysis, ARDS, and systemic inflammatory response syndrome with MODS. The mortality among patients with MODS was 91%. Within the group of non-survivors 22 patients died due to intractable septic shock, ARDS or MODS. One patient died due to an intracerebral hemorrhage. The crude mortality of all ICU patients with candidemia was 58%.

Table 2: Underlying diseases and co-morbidity by survival group

	Survivor (n=17)	Non-Survivor (n=23)	Odds ratio (95% CI)
Abdominal surgery	1	11	3.2 (1.0-10.5)
Acute hemodialysis	1	11	1.8 (1.2-2.7)
Antibacterial use	17	23	
• > 7 days	12	18	1.3 (0.5-3.9)
• > 2 classes	7	11	1.1 (0.7-2.0)
ARDS	0	21	10.8 (2.9-40.9)
COPD	0	1	1.0 (0.9-1.1)
CVC	15	23	0.9 (0.7-1.0)
Diabetes mellitus	0	1	1.0 (0.9-1.1)
Malignancy	5	6	1.0 (0.6-1.4)
MODS	2	21	10.1 (2.7-38.6)
Pneumonia	2	4	1.0 (0.8-1.4)
Splenectomy	3	2	0.9 (0.7-1.2)
Steroid use	4	8	1.2 (0.8-1.7)
TPN	14	20	1.4 (0.3-5.9)

ARDS = acute respiratory distress syndrome, COPD = chronic obstructive pulmonary disease, CVC = central venous catheter, MODS = multi-organ dysfunction syndrome, TPN = total parenteral nutrition, 95% CI = 95% confidence interval

In the stepwise multiple regression analysis only ARDS and MODS remained significant. The mean (SD) severity illness score determined by SAPS II at admission to the ICU were 47.4 (15.6) and 38.7 (11.0) in survivors and non-survivors, respectively. The corresponding risk of hospital

death in survivors and non-survivors was 55% and 30% respectively The overall SAPS II score of all study patients was 42 corresponding with an 38% chance of hospital death Given a crude mortality of 58% and a predicted mortality of 38% the attributable mortality in patients with candidemia was 20%

In 51% of the study patients *Candida* was the first pathogen isolated from blood cultures Thirty-three percent of the patients initially had suffered from bacteremia due to gram-negative or gram-positive pathogens prior to development of candidemia and in 7% of the patients bacteremia and candidemia occurred simultaneous In the remaining patients (9%) candidemia was followed by bacteremia

Discussion

The management of patients with severe diseases and life-threatening conditions routinely involves the use of mechanical ventilation invasive monitoring parenteral nutrition and indwelling catheters Obviously these patients are prone to nosocomial infections The rate of hospital-acquired infection among ICU patients is accordingly 5-10 times higher than among general ward patients [212]

Over the past decade the incidence of *Candida* bloodstream infections has been reported to markedly increase in hospitals in the USA [8 21 147] A similar but less striking increase has been shown for most of the Dutch university hospitals [223] Given the "natural" accumulation of high risk patients in tertiary care referral hospitals incidence rates of candidemia are highest in these hospitals and were described to be as high as 8 5 per 10 000 admissions [238]

Mortality in most studies is high ranging from 38% to 79% The overall mortality in our patients with candidemia was 58% which is in accordance with previous reports In a case-control study performed by Wey et al [238] 57 % of all patients with candidemia died whereas 38% died as a direct result of candidemia (attributable mortality) Our analysis showed a lower attributable mortality (20%) probably reflecting the severity of the already existing underlying disease at the onset of candidemia The low attributable mortality observed makes it even more difficult to explain the difference between survivors and non-survivors concerning the risk of hospital death at ICU admission (estimated by SAPS II) which was considerably higher in survivors In order to exclude concomitant bacterial sepsis which might have contributed to the non-fungal-related mortality we evaluated the sequential occurrence of pathogens isolated from blood cultures 84% of the patients had primary candidemia or candidemia following bacteremia In both cases it might be assumed that the bacteremia did not contribute to the death of the patient which more likely was the case in those patients who suffered from simultaneous bacteremia (7%) or who's candidemia was followed by bacterial sepsis (9%)

Risk factors for the development of candidemia were of no further value to predict mortality once candidemia has developed Other clinical conditions associated with impaired immunity

that may facilitate opportunistic infections, such as age, sex, gastrointestinal bleeding, pneumonia, diabetes mellitus, chronic obstructive pulmonary disease, steroid use, or prior alcohol abuse, did not significantly contribute to mortality in patients with candidemia.

In recent years epidemiological changes of *Candida* infections have been documented by several authors indicating a shift toward (less azole-susceptible) non-*C. albicans* species especially in patients with AIDS and cancer [153 242]. Whether or not these changes are due to the excessive use of fluconazole is debated in the literature, and would expand the spectrum of this discussion [86 241]. Overall, 75% of all blood culture isolates during the 8-year-study period were *C. albicans*. Until 1995, no patient with *C. krusei* candidemia was observed among ICU patients in our hospital [220], and the proportion of other non-*C. albicans* species remained low. However, we demonstrated an upward trend during 1989 to 1993, and a steep increase of non-*C. albicans* species in 1994, when for the first time less than 50% of the *Candida* blood culture isolates were *C. albicans*.

Frequently, ICU clinicians consider candidemia as an epi-phenomenon in already severely ill patients with a past history of bacterial sepsis. On the basis of our results candidemia should be considered as an independent variable for morbidity and mortality. Risk factors that might be helpful to predict the development of candidemia among non-neutropenic critically ill patients had no further value in predicting mortality among ICU patients with candidemia. The SAPS II score taken at ICU admission was insufficient to indicate the actual risk of hospital death, since the clinical outcome was primarily determined by the development of multiple organ dysfunction reflecting the severity of illness at the moment of candidemia

V. Summary

Samenvatting

Zusammenfassung

Chapter 5

Summary

The increasing rate of fungal infections during the last decade most notably due to *Candida* species - is soundly documented in the Anglo-American literature Today *Candida* species are the most important fungal pathogens causing hospital-acquired infections and are recently among the top four nosocomial pathogens causing bloodstream infections in the US Given the increasing incidence high attributable mortality and increased health care costs associated with nosocomial candidemia many hospitals feel the pressing need for more and new effective preventive diagnostic and therapeutic measures to deal with this emerging group of pathogens Implicit in these issues is the need for an increased understanding of the epidemiology of systemic *Candida* infections Advances in the antifungal treatment and prophylaxis of *Candida albicans* infections may promote the emergence of other more resistant species The incidence and clinical significance of this phenomenon especially in the growing non-immunocompromised patient population needed to be surveyed Furthermore the reservoir and mode of transmission of exogenous *Candida* infections should be assessed New molecular typing techniques are important tools in clinical and epidemiologic investigations to solve the above mentioned problems These studies are essential to the rational development of effective measures to prevent and to control nosocomial infections caused by *Candida* species

In chapter 2 1 the usefulness of karyotypes established by contour-clamped homogenous electric field (CHEF) gel electrophoresis CHEF restriction fragment analysis (RFA) and isoenzyme profiles to investigate outbreaks was analyzed in a set of epidemiologically related *Candida tropicalis* strains earlier characterized by conventional RFA Karyotyping by conventional CHEF gel electrophoresis was inadequate to delineate related and unrelated strains whereas CHEF RFA patterns allowed easy delineation However the limits to the discriminative power of this procedure were not fully explored

An evaluation of PCR-fingerprinting CHEF karyotyping and RFA in multiple reference strains from seven *Candida* species (chapter 2 2) made clear that despite recent progress to standardize and validate typing systems only insufficient discriminatory power is achieved in *Candida* species other than C *albicans* To fulfill recently published standards the available typing systems still need further improvement The ability to delineate *Candida* strains should be evaluated in a great number of unrelated strains (>20) and typing standards should include specific restriction enzymes or PCR primers validated on species level

Nevertheless at the moment genomic DNA typing methods are the ones most appropriate for molecular epidemiology studies as those described in chapter 3 1 and 3 2

For a long time it was believed that *Candida* infections solely originated from the patient's own flora Proof of such endogenous infections in hospitalized patients was given by the findings that unique strains of *Candida* were seen for each patient, that colonizing and infecting strains usually shared the same DNA type, and that the colonizing isolate usually preceded the invasive isolate (chapter 3 1) Particularly in the ICU setting patient to patient transmission of *Candida* species was assumed to be of importance, next to the endogenous infections The use of two molecular typing methods suggested that some transmission may have occurred among patients of an ICU (chapter 3 2) Differences observed with a third typing approach may reflect the propensity for *C albicans* to undergo genomic rearrangements, but suggests that although these isolates may have had a common origin, direct patient-to-patient or health care worker-to-patient transmission was unlikely

Recent data from the USA indicated major changes in the epidemiology of fungal infections, including the increasing incidence of *Candida* bloodstream infections Chapter 4 1 describes the results of a retrospective multi-center study defining secular trends in yeast bloodstream infections in The Netherlands The candidemia rate found among Dutch university hospital patients (0 70 per 10,000 patient days in 1995) show the same trend observed among patients in the USA, but on a much lower scale and with a less dramatic increase The trend of candidemias due to non-*C albicans* species parallels the one due to *C albicans*, the species that still causes 60% of all episodes of *Candida* bloodstream infections

Despite it's relatively rare occurrence, candidemia poses a serious threat to immunocompromised and ICU patients in which it was shown to have the shortest survival prospects of any blood-borne infections In chapter 4 2 we tried to evaluate whether risk-factors predicting the development of candidemia were of further value to predict patients who are most likely to die from the fungal infection With the exception of the multiple organ dysfunction syndrome reflecting the severity of illness at the moment of candidemia no further risk-factors hold through in a multi variant analysis

Regarding the increasing incidence of invasive *Candida* infections and the unpredictability of its clinical outcome, future investigations should be aiming at improving prevention and treatment Especially the group of seriously ill non-neutropenic patients deserves more attention regarding further data on the epidemiology (carrier-ship and transmission) and the development of treatment strategies that might help to prevent a further shift to *Candida* species which are less-susceptible to presently available antifungals

Samenvatting

De toenemende incidentie van gist- en schimmelinfecties tijdens de laatste decennia - in het bijzonder door *Candida* soorten - is goed gedocumenteerd in de Anglo-Amerikaanse literatuur Hendentendage behoren *Candida* soorten tot de belangrijkste verwekkers van ziekenhuis-infecties en zijn de 4^{e} in de rij van nosocomiale verwekkers die in de V S uit bloedkweken geisoleerd worden Gezien de toenemende incidentie de hoge attributieve letaliteit en de hoge kosten die geassocieerd zijn met nosocomiale candidemieen is het belangrijk voor de gezondheidszorg nieuwe effectieve maatregelen ter preventie diagnostiek en therapie van infecties door deze micro-organismen te ontwikkelen Om te komen tot een nationaal beleid ten aanzien van nosocomiale *Candida* infecties is het in kaart brengen van het probeem een eerste vereiste

Vooraleerst is het noodzakelijk de epidemiologie van systemische *Candida* infecties beter te begrijpen Daarbij moet men een onderscheid maken tussen infecties door *Candida albicans* en *Candida niet-albicans* Er zijn aanwijzingen dat er naast een toegenoemen incidentie in het algemeen ook een shift ten gunste van de niet-albicans species waar te nemen is

Het toenemende gebruik van antifungale therapie en profylaxe van *Candida albicans* infecties zou voor het optreden van andere en resistentere *Candida* species verantwoordelijk zijn Een surveillance van de incidentie en klinische significantie van dit fenomeen was daarvoor noodzakelijk vooral in de groeiende groep van niet-immuno-gecompromitteerde patienten Ook de bron en de mogelijke transmissieweg van exogene *Candida* infecties behoefde verder onderzoek

In hoofdstuk 2 1 werd het nut van contour-clamped homogenous electric field (CHEF) karyotypering CHEF restrictie fragment analyse (RFA) en isoenzyme profielen voor het onderzoek van epidemieen geanalyseerd aan de hand van een set epidemiologisch gerelateerde *Candida tropicalis* stammen die eerder door middel van conventionele RFA gekarakteriseerd zijn Karyotypering middels conventionele CHEF gel electrophoresis bleek inadequaat om gerelateerde en niet gerelateerde stammen te differentieren Wel was dit met CHEF-RFA mogelijk maar het discriminerende vermogen van deze procedure was nog niet helemaal uitgezocht

De evaluatie van PCR fingerprinting CHEF karyotypering en RFA in verschillende referentiestammen van 7 *Candida* species (hoofdstuk 2 2) maakte duidelijk dat ondanks recente verbeteringen om typeringssystemen te standaardiseren en te valideren het discriminerend vermogen van deze systemen met betrekking tot *Candida* species anders dan *Candida albicans* onvoldoende lijkt Om recent gepubliceerde standaarden te kunnen naleven moeten de huidige methoden nog verbeterd worden Het vermogen *Candida* stammen te differentieren zou in een groot aantal ongerelateerde isolaten (>20) geevalueerd moeten worden Standaard c q normen met betrekking tot de typering van

gisten zouden informatie met betrekking tot species specifieke restrictie enzymen c q PCR primers moeten bevatten

Desondanks zijn genomische DNA typeringsmethoden momenteel het meest geschikt voor moleculaire epidemiologische studies zoals die beschreven zijn in hoofdstuk 3 1 en 3 2 Oorspronkelijk was men ervan uitgegaan dat *Candida* infecties uitsluitend door stammen van de patienten zelf veroorzaakt werden (endogene infecties) Dit stoelde op de waarneming dat er voor iedere patient eigen" *Candida* stammen werden gevonden dat de koloniserende en infecterende stam meestal van een identiek DNA type was en dat het koloniserende isolaat meestal op een eerder tijdstip dan het invasieve isolaat gevonden werd (hoofdstuk 3 1) Bij intensieve-zorg patienten rees het vermoeden dat naast de endogene infectieweg ook een horizontale overdracht van *Candida* species van patient naar patient belangrijk zou kunnen zijn Het gebruik van twee moleculaire typerings-methoden suggereerde dat een dergelijke transmissie onder intensieve zorg patienten zeer wel mogelijk was (hoofdstuk 3 2) Wel zijn bij gebruik van een derde typeringsmethode verschillen tussen de stammen gevonden die laten vermoeden dat ook als het voor *C albicans* mogelijk zou zijn genomische veranderingen te ondergaan en daarmee de bovengenoemde isolaten een gemeenschappelijke oorsprong hadden directe overdracht van patient op patient of van gezondheidsmedewerker naar de patient onwaarschijnlijk was

Recente gegevens uit de V S toonden aan dat ingrijpende veranderingen in de epidemiologie van gist infecties plaatsgevonden hebben waaronder ook een toenemende incidentie van candidemieen Hoofdstuk 4 1 beschrijft de resultaten van een retrospectieve multicentrische studie naar het voorkomen van candidemieen in Nederland over de tijd De incidentie van candidemieen bij patienten van 5 academische ziekenhuizen (0 70 per 10 000 patientendagen in 1995) laat dezelfde trend zien als gesignaleerd bij patienten uit de V S maar op een lager niveau en met een minder duidelijke stijging De toename van candidemieen veroorzaakt door *C niet-albicans* species loopt parallel met die veroorzaakt door *C albicans* de soort die tot nu toe verantwoordelijk was voor 60% van alle candidemieen

Hoewel candidemien zeldzaam zijn vormen zij een ernstige bedreiging voor immuno gecompromitteerde en intensieve-zorg patienten patienten met een candidaemie hebben een lagere kans op overleving dan patienten met sepsis door een andere verwekker In hoofdstuk 4 2 worden risicofactoren onderzocht die het mogelijk maken om het ontstaan van een candidemie te voorspellen en een verdere voorspellende waarde met betrekking tot de letaliteit van deze patienten te stellen De ernst van het onderliggend lijden is de belangrijkste zelfs enige risicofactor voor het verkrijgen van een candidemie Dit werd fraai aangetoond bij patienten met een multiple dysfuntion syndrome

Gezien de toenemende incidentie van invasieve *Candida* infecties en de onvoorspelbaarheid van het klinische beloop zou het onderzoek zich zeker ook op een

verbetering van preventie en therapie moeten richten In het bijzonder bij ernstig zieke niet neutropene patienten zouden meer gegevens verzameld moeten worden over epidemiologie (dragerschap en transmissie) Van evenzoveel belang is het ontwikkelen van therapeutische strategieen die een verdere shift naar *Candida* species die minder gevoelig zijn voor de huidige antimycotica, kunnen voorkomen

Zusammenfassung

Die steigende Inzidenz von Pilzinfektionen wahrend des letzten Jahrzehntes insbesondere durch *Candida* Spezies ist in der anglo-/amerikanischen Literatur gut dokumentiert Heute sind *Candida* Spezies die wichtigsten Erreger von Krankenhaus-erworbenen Pilzinfektionen Sie gehoren in den USA seit kurzem zu den vier haufigsten Sepsiserregern Angesichts der zunehmenden Inzidenz der hohen attributiven Mortalitat und der steigenden Kosten die mit Krankenhaus-erworbenen Candidamien verbunden sind ist die Notwendigkeit entstanden nach neuen und effektiven Maßnahmen zur Pravention Diagnostik und Therapie dieser aktuellen Erreger zu suchen Voraussetzung hierfur ist eine verbesserte Einsicht in die Epidemiologie systemischer *Candida* Infektionen Eine Zunahme der antimykotischen Therapie und Prophylaxe von *Candida albicans* Infektionen kann zum Auftreten anderer und/oder resistenterer Spezies fuhren Die Inzidenz und klinische Relevanz dieses Phanomens sollten ebenso wie mogliche Reservoirs und Ubertragungswege von *Candida* Infektionen untersucht werden Molekulare Methoden zur Typisierung sind wichtige Mittel um deratige klinische und epidemiologische Studien uberhaupt zu ermoglichen Die Ergebnisse dieser Studien bilden eine rationale Basis zur Entwicklung von effektiven Praventions- und Kontrollmaßnahmen gegen *Candida* Infektionen

In Kapitel 2 1 wurde der Einsatz von contour clamped homogenous electric field (CHEF) Gelelektrophorese CHEF Restriktionsfragment Analyse (RFA) und Isoenzym Profilen zur Aufklarung von Epidemien untersucht Benutzt wurden epidemiologisch zusammenhangende *Candida tropicalis* Stamme die zu einem fruheren Zeitpunkt bereits mittels konventioneller RFA typisiert worden waren Wahrend eine Unterscheidung in relatierde und unrelatierte Stamme mittels CHEF Karyotypierung nicht moglich war gelang dies anhand der CHEF-RFA Banden Zum Zeitpunkt der Untersuchung waren die diskriminierenden Eigenschaften dieser Methoden noch nicht vollstandig exploriert

Die diskriminierenden Eigenschaften von PCR-fingerprinting CHEF Karyotypierung und RFA wurden an Referenzstammen von sieben *Candida* Spezies ausgewertet Trotz aktueller Verbesserungen in der Standardisierbarkeit und Validierung der Typisierungsmethoden zeigten sich Mangel bei den sogenannten nicht *C albicans* Spezies Um entsprechend einer vor kurzem publizierten Norm arbeiten zu konnen mussen die im Moment verfugbaren Methoden verbessert werden Die Methode sollte in der Lage sein innerhalb der verschiedenster *Candida* Spezies eine grossere Anzahl (>20) nicht-relatierter Stamme zu unterscheiden Auch sollten Normen explizit Restriktionsenzyme und PCR primers beschreiben die auf Speziesniveau validiert wurden

Trotzdem sind die Gentypisierungen zur Zeit die besten Methoden die uns fur molekular-epidemiologische Studien (wie in Kapitel 3 1 und 3 2 beschrieben) zur Verfugung stehen

Relativ lange war man der Ansicht, daß Candida Infektionen ausschließlich von der patienteneigenen Flora ausgehen Daß bei jedem hospitalisierten Patienten individuelle *Candida* Stämme gefunden wurden, daß kolonisierende und infizierende Stämme meistens den gleichen DNA-Typ aufwiesen und daß überwiegend erst eine Kolonisation und danach eine Infektion erfogte, waren weitere Hinweise für diesen endogenen Infektionsweg (Kapitel 3 1). Vor allem bei Intensivpatienten wurde jedoch vermutet, daß es auch zu Kreuz-infektionen mit *Candida* kommen kann Mittels zweier molekulärer Typisierungstechniken haben wir solche Kreuzinfektionen bei Intensivpatienten nachgewiesen (Kapitel 3.2). Die mittels einer dritten Methode gefundenen Unterschiede könnten durch kleine genetische Veränderungen der *Candida* Isolate zustande gekommem sein, lassen aber auch die Annahme zu, daß - trotz eines möglichen gemeinsamen Ursprungs - in der beschriebenen Situation keine Kreuzinfektion stattgefunden hat

Aktuelle Daten aus den USA zeigen erhebliche Veränderungen in der Epidemiologie mykotischer Erkrankungen inklusiv einer Zunahme der Inzidenz von Candidämien. Kapitel 4.1 gibt die Ergebnisse einer niederländischen multi-zenter Studie wieder. Der Trend von Candidämie unter niederländischen Patienten (Inzidenz 0,70 per 10.000 Patiententagen) war vergleichbar dem unter US Patienten, aber auf einem niedrigeren Niveau bei deutlich weniger steilem Anstieg. Die Trendkurven der durch C. *albicans* und nicht-C. *albicans* verursachten Septikämien verliefen parallel. Auch 1995 war C. *albicans* noch der häufigste Verursacher von Candidämien (60%).

Trotz des geringen Vorkommens stellen Candidämien eine ernste Bedrohung dar, besonders bei abwehrgeschwächten Patienten und bei Patienten der Intensivstation. *Candida* Septikämien weisen in diesen Patientengruppen eine höheren Letalität auf als Septikämien, die durch andere Mikroorganismen hervorgerufen werden. In Kapitel 4.2 wurde versucht zu klären, ob Risikofaktoren, die zur Entstehung einer Candidämie beitragen, auch Einfluß auf die Mortalität der Patienten haben. Mit Ausnahme des multiplen Organversagens, das den Ernst der Erkrankung zum Zeitpunkt der Candidamie wiederspiegelt, erwies sich keiner der untersuchten Risikofaktoren in der Multivarianz Analyse als signifikant.

Im Hinblick auf die zunehmenden Inzidenz von invasiven Candida Infektionen und der Unvorhersagbarkeit des klinischen Verlaufs, sollten zukünftige Studien darauf gerichtet sein, Maßnahmen der Prävention und Therapie zu verbessern. Besonders im Hinblick auf die Gruppe der schwersterkrankten, nicht-neutropenen Patienten auf den Intensivstationen ist vermehrte Aufmerksamkeit im Bezug auf die Erhebung von epidemiologischen Daten zur *Candia* Trägerschaft und Übertragung erforderlich. Notwendig ist ebenfalls die Entwicklung von Therapieformen, die einen weiteren Shift zu *Candida* Spezies mit geringer Empfindlichkeit gegenüber den zur Zeit verfügbaren Antimykotika verhindern.

VI. References

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Appendix

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Jacques Meis and Mieke Hoogkamp not only started the idea on a second (Dutch) thesis but completely switched around my private and professional live by asking me to come to Nijmegen, a move so much enjoyed by my family and me, that it could be called a transformation to be Dutch

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2

Curriculum vitae

De auteur van dit proefschrift werd geboren op 14 februari 1961 te Warstein Duitsland In 1979 behaalde hij zijn gymnasium diploma aan het Neusprachliche Gymnasium te Warstein Na de militaire dienstplicht te hebben vervult, begon hij met de studie geneeskunde, de eerste twee jaar aan de Westfalische Wilhelms Universitat te Munster, daarna aan de Technische Universitat te Munchen Hier werd het artsexamen met goed gevolg in mei 1987 afgelegd

In februari 1989 werd de graad van Doktor der Medizin (Dr med) aan de Technische Universitat Munchen verkregen

Van juni 1987 tot en met december 1993 was hij assistent geneeskundige in opleiding tot medisch microbioloog en volgens staflid aan het Institut fur Medizinische Mikrobiologie und Hygiene Universitatsklinikum Rechts der Isar te Munchen (hoofden Prof Dr I Braveny, Prof Dr R Wagner) Met een subsidie van de Walter-Marget Stichting voor Infektiologie en de B Braun Stichting was hij van juni 1992 tot en met juni 1993 als "research fellow" verbonden aan de Division of Hospital Epidemiology and Quality Assurance in The University of Iowa Hospitals and Clinics, te Iowa City USA (Prof Dr R Wenzel)

Vanaf januari 1994 is hij werkzaam als medisch microbioloog en hoofd van de sectie Hygiene en Infectiepreventie op de afdeling Medische Microbiologie van het Academisch Ziekenhuis Sint Radboud te Nijmegen (Prof Dr J A A Hoogkamp-Korstanje)

Hij is getrouwd met Angela en heeft twee kinderen Timothy en Allegra

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Stellingen

behorend bij het proefschrift

Epidemiology of systemic *Candida* infections:

Studies to pursue the molecular and clinical epidemiology.

Nijmegen, 13 juni 1997

Andreas Voss

1. Even with the use of modern DNA-based typing methods, the classical epidemiological investigation is still the corner stone, since a single chromosomal deletion or insertion event (eg. acquisition or loss of bacteriophage) may result in different banding patterns.

2. Discrimination between coincidental but independent infection and epidemics caused by a single isolate is a major concern that directly affects the preventive and hygienic measures to be taken (A. van Belkum, Clin Microbiol Rev, April 1994).

3. It is not only the biased observation of an eager scientist looking for new research opportunities that systemic fungal infections are rising and gaining clinical importance.

4. Fungi Imperfecti can't have sex - therefore they attack man (M. Rinaldi, San Franciso 1995)

5. One of the most important breakthroughs of the 19th century was that physicians learned to wash their hands (von Bergmann, 1876). One of the most important breakthroughs of the 20th century could be that physicians remembered what they learned a century ago.

6. You can get to see other people, sleep in peace, off-load decisions, feel important, and impress (or bore) your colleagues - and all in work time: " Meetings" the practical alternative to work.

7. Diet Coke® is proven to be fattening! Significantly more Diet Coke® drinkers are overweight than Classic Coke® consumers (own experience and observation).

8. Op de ICs van alle ziekenhuizen zou een teken moeten staan: " Handen wassen verboden". Misschien leert men dan uiteindelijk dat handen bijna altijd gedesinfecteerd moeten worden. (C. Vandenbroucke-Grauls, 1996)

9. Dat in veel instellingen de formatie van hygiënisten nog niet aan de GHI norm is aan gepast, berust op het feit dat ziekenhuisbestuurders twee normen kennen: "wettelijke" normen die direct kosten besparen (b.v. minder ruimte tussen IC bedden) en "nieuwe te onderbouwen" normen die (in eerste instantie) kosten veroorzaken (b.v. tenminste 1 hygiënist per 250 bedden)

10. Dank aan de Nederlandse Taalunie! De introduktie van de nieuwe spelling heeft het gat tussen mijn taalniveau en dat van de gemiddelde Nederlander kleiner gemaakt.

11. Dat medici ondanks E. coli O157, BSE, VRE en andere micro-organismen nog vlees eten moet iets met de natuurlijke ontkenning van doktoren met betrekking tot de eigen gezondheid te maken hebben ("The Swan with two Nicks" , Little Bullington, Cheshire, UK)

12. Insofern ein vereintes Europa in der Zukunft eine gemeinsame Sprache sucht stimme ich für ein Gemisch aus **Nie**derländisch, **Deut**sch und Eng**lisch** (= "**Niedeutlisch**", *sprich: nie-deut-lich*), eine Sprache die ich schon jetzt perfekt beherrsche.

13. " Ein Mann ohne Bauch ist ein Krüppel" (Chinese gids, Beijing - Verboden Stad, juni 1996)

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