To Óli

Candidemia and Invasive Candidiasis: Pathogenesis, Molecular Epidemiology, and Predictors of Outcome

A population-based study

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ÁGRIP

Tíðni alvarlegra sveppasýkinga hefur aukist umtalsvert í hinum vestræna heimi á undanförnum áratugum, samhliða framförum í gjörgæslumeðferð og meðhöndlun bakteríusýkinga og illkynja sjúkdóma. Dánartíðni sjúklinga með ífarandi sveppasýkingar er mjög há. Markmið rannsóknarinnar var að kanna faraldsfræði blóðsýkinga af völdum *Candida* gersveppa (candidemia) hér á landi á 27 ára tímabili, 1980-2006, þar með talið tegundaskiptingu sveppastofna, næmi þeirra fyrir sveppalyfjum og sveppalyfjanotkun. Jafnframt var aflað upplýsinga um einkenni, áhættuþætti og forspárþætti um horfur sjúklinga sem greindust á árunum 1980-1999 (172 tilfelli). Þá var leitast við að meta umfang faraldra sveppasýkinga í blóði á landsvísu með því að beita sameindaerfðafræðilegum aðferðum. Að lokum var meinvirkni *Candida albicans* og *Candida dubliniensis* rannsökuð í músum.

Árlegt nýgengi blóðsýkinga af völdum sveppa jókst úr 1,4 sýkingum á 100.000 íbúa á árunum 1980-1984 í 5,3 sýkingar á 100.000 íbúa 2000-2006 ($p<0.001$). Mest varð aukningin hjá smábörnum (<1 árs) og meðal eldri einstaklinga (>60 ára). *C. albicans* var sá gersveppur sem oftast ræktaðist úr blóði sjúklinga (61,6%). Innflutningur á sveppalyfinu flúkonazól jókst um ~550% á landsvísu á árunum 1991 til 2006, en ekki varð vart marktækrar breytingar á næmi sveppastofna fyrir lyfinu á sama tímabili. Stærsti hluti sveppasýkinga í blóði greindist á gjörgæsludeildum (35,8%) og skurðdeildum (30,1%). Í fjölþátta aðhvarfsgreiningu hafði brottnám á miðbláæðarlegg innan tveggja daga frá greiningu marktækt forspárgildi um betri horfur (OR 0,22), en sýklalost við greiningu tengdist hærri dánartíðni (OR 8,01). Með því að stofngreina alla tiltæka sveppastofna sem ræktuðust úr blóði á tímabilinu 1991-2006 kom í ljós að 18,7-39,9% allra blóðsýkinga voru tengdar í tíma og rúmi, sem getur bent til áður ógreindra faraldra á sjúkrahúsum. Þegar meinvirkni *C. albicans* og *C. dubliniensis* var borin saman í músamódeli af blóðsýkingu var 7-daga dánartíðni hjá dýrum sem sýkt voru með *C. dubliniensis* og *C. albicans* svipuð. Meinvirkni var breytilegri milli stofna hvorrar tegundar en milli tegunda. Þráðamyndun var marktækt meiri í nýrum tilraunadýranna eftir sýkingu með *C. albicans* í samanburði við *C. dubliniensis* ($p<0,001$). Í fjölþáttagreiningu var fylgni milli bæði aukinnar þráðamyndunar (OR 2,27) og gersveppamyndunar (OR 2,06) og hærri dánartíðni, en aukin íferð einkjarna blóðfrumna hafði fylgni við betri lifun (OR 0,02).

Gersveppir valda í sívaxandi mæli blóðsýkingum inni á sjúkrahúsum hér á landi og er nýgengi þeirra svipað og í öðrum Evrópulöndum en lægra en í Bandaríkjunum. Niðurstöður okkar renna jafnframt stoðum undir mikilvægi skjótrar meðferðar, og að djúpir æðaleggir séu fjarlægðir sé þess kostur. Allt að þriðjungur ífarandi sýkinga kann að eiga rætur að rekja til sýkingaþyrpinga eða faraldra, og koma þær oftast upp á gjörgæsludeildum. Talsverð skörun er á meinvirkni *C. albicans* og *C. dubliniensis*. Niðurstöðurnar benda til að uppruni hins meinvaldandi stofns sé mikilvægur þegar rannsóknir á meinvirkni eru annars vegar. Aukin þekking á meingerð, meinvirkniþáttum og smitleiðum er mikilvæg til að bæta meðferð sjúklinga og fyrirbyggja þessar alvarlegu sýkingar.

Lykilorð: *Candida*, ífarandi sveppasýkingar, blóðsýkingar, faraldsfræði, meinvirkni.

ABSTRACT

The incidence of serious fungal infections has increased substantially in the past decades, incident to increased prevalence of susceptible hosts. Candidemia, in particular, is associated with high morbidity and mortality. This thesis outlines results from a nationwide study, conducted in Iceland from 1980 to 2006, of the incidence of candidemia. In addition, antifungal susceptibility of the pathogens was studied, as well as national consumption of antifungal agents. The clinical characteristics of candidemic patients (n=165; 172 episodes) from 1980 through 1999 are described, as well as predisposing conditions and their association with outcome. Furthermore, we studied the genetic relatedness of all available *Candida* bloodstream isolates (BSIs) in the country during a 15-year period. Finally, the virulence of *Candida albicans* and *Candida dubliniensis* were compared in a murine model of bloodstream infections.

The annual incidence of candidemia in Iceland increased from 1.4 cases/100,000 inhabitants/year during 1980-1984 to 5.3 cases/100,000 inhabitants/year during 2000-2006 ($p<0.001$), with the greatest increase in incidence occurring among infants <1 year of age and the elderly (age, >60 years). *C. albicans* was the predominant species responsible (61.6%). The national import of fluconazole increased approximately 5.5-fold from 1991 through 2006, but increased resistance to this agent was not observed. Most cases occurred in intensive care units (35.8%) and surgical wards (30.1%). In multivariate analysis, prompt removal of central venous catheters (odds ratio [OR], for death, 0.22) and septic shock (OR for death, 8.01) were the strongest independent predictors of outcome. PCR fingerprinting of *Candida* BSIs (n=219) from 94.4% of cases during 1991-2006 revealed temporo-spatial associations between 18.7-39.9% of all infections, suggestive of nosocomial clustering. When the virulence of *C. albicans* and *C. dubliniensis* was compared in a murine model, similar 7-day mortality was observed, with greater strain variation noted within species than between the two species. *C. dubliniensis* produced significantly lower levels of hyphae in kidneys than *C. albicans* ($p<0.001$). Increasing tissue burden of both hyphal forms (OR, 2.27) and yeasts (OR, 2.06) were independently associated with death, whereas greater infiltration of mononuclear cells was protective (OR, 0.02).

These results confirm that invasive candidiasis has emerged as a serious threat to hospitalized patients in recent decades. They also highlight the importance of aggressive treatment. In an unselected hospital population, as many as one-third of all cases of candidemia may be attributable to nosocomial clusters, and the risk is highest in wards providing intensive care. Our results suggest a great overlap between the virulence properties of *C. dubliniensis* and *C. albicans*. In virulence studies, the source of fungal isolates may be a neglected confounding factor. Further studies of the virulence mechanisms of different *Candida* species and modes of transmission of infecting strains in the hospital environment are warranted in order to improve treatment and develop effective prevention strategies.

Keywords: *Candida*, invasive fungal infections, bloodstream infections, epidemiology, virulence.

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DECLARATION

I performed species identification and antifungal susceptibility testing of all attainable *Candida* bloodstream isolates (BSIs) isolated at the Department of Microbiology, Landspitali-University Hospital, Reykjavik, Iceland during 1991-1999, in collaboration with Helga Erlendsdóttir, and obtained national import figures of antifungal agents. I reviewed all patient charts and collected the data. I studied the methodology regarding PCR fingerprinting of yeasts at the Department of Biology, McMaster University, Ontario, Canada, and later genotyped all obtainable BSIs by PCR fingerprinting in Iceland and analyzed the data. Three animal experiments had already been performed when work on this thesis began, but thereafter I took part in the execution of all animal studies, both regarding inoculation and viability counting. I performed all histopathological analyses myself, under the supervision of Dr. Bjarni A. Agnarsson. The multivariable logistic regression analyses of clinical prognostic factors for candidemia were performed in collaboration with Örn Ólafsson, and multiple regression analyses of histopathological variables in animal studies were performed in collaboration with Dr. Ólafur Skúli Indridason. All other statistical analyses were performed by myself. I processed the data presented in this thesis and took part in writing the papers, on which the thesis is based.

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LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. Lena Rós Ásmundsdóttir, Helga Erlendsdóttir, Magnús Gottfredsson.
Increasing incidence of candidemia: Results from a 20-year nationwide study in Iceland.
J Clin Microbiol 2002; 40(9): 3489-92.

II. Lena Rós Ásmundsdóttir, Helga Erlendsdóttir, Magnús Gottfredsson.
Improving survival of patients with candidaemia: Analysis of prognostic factors from a long-term, nationwide study in Iceland.
Scand J Infect Dis 2005; 37(2): 111-20.

III. Lena Rós Ásmundsdóttir, Helga Erlendsdóttir, Gunnsteinn Haraldsson, Hong Guo, Jianping Xu, Magnús Gottfredsson.
Molecular epidemiology of candidemia: Evidence of clusters of smoldering nosocomial infections.
Clin Infect Dis 2008; 47(2): e17-24.

IV. Lena Rós Ásmundsdóttir, Helga Erlendsdóttir, Bjarni A. Agnarsson, Magnús Gottfredsson.
The importance of strain variation in virulence of *Candida dubliniensis* and *Candida albicans*: Results of a blinded histopathological study of invasive candidiasis.
Clin Microbiol Infect 2008. Accepted for publication.

LIST OF ABBREVIATIONS

ALS	agglutinin-like sequence
AP-PCR	arbitrarily primed polymerase chain reaction
BSI	bloodstream isolate
CFU	colony forming units
CI	confidence interval
CLSI	Clinical and Laboratory Standards Institute
CVC	central venous catheter
DDD	defined daily doses
GT	genotype
HE	hematoxylin-eosin
IC	Invasive candidiasis. Defined as candidemia, disseminated candidiasis, deep organ ivolvement, endocarditis, and meningitis, excluding more superficial or less severe diseases such as oropharyngeal and esophageal candidiasis
ICU	intensive care unit
IFN	interferon
ITS	internal transcribed spacer
LD_{50}	Dose of organisms (inoculum) inducing death in 50% of experimental animals
NICU	neonatal intensive care unit
MIC	minimal inhibitory concentration
MLST	multilocus sequence typing
OR	odds ratio
PCR	polymerase chain reaction
RR	relative risk
SAP	secreted aspartyl proteinases
TLR	Toll-like receptors
TNF	tumor necrosis factor
US	United States

INTRODUCTION

Historical perspective

The first written descriptions of oral apthous lesions, that were probably thrush, date to the time of Hippocrates and Galen (1). In 1839, Langenbeck discovered fungi in the gastrointestinal tract of a patient (2), and 2 years later Berg demonstrated the fungal etiology of thrush in children (3). In 1843, Robin microscopically observed budding cells and filaments in epithelial scrapings, and named the fungus *Oidium albicans* (4). Since then, there have been more than 100 synonyms for *Candida albicans*, a denomination first used by Berkhout in 1923, which is currently the accepted name of this species (5). The first well-documented case of invasive candidiasis (IC) was described by Zenker in 1861 (6) and when the widespread use of antibiotics began in the 1940s, the incidence of practically all forms of *Candida* infections rose abruptly. Until the past 2 decades, *Candida* was often regarded as simply a contaminant or "normal flora" in laboratory results, instead of the highly prevalent and potentially aggressive pathogen we recognize today (7).

Incident to increased prevalence of susceptible hosts in recent years, fungi have emerged as major causes of human disease among hospitalized and critically ill patients (8-13). The reasons for this increase are multiple, including more extensive surgical procedures, increasing use of large vascular catheters (primarily central venous catheters, CVC) and progress in the treatment of serious bacterial infections and malignant diseases (14-17). At the same time, there has been a dramatic increase in multiple cause mortality due to invasive mycoses in the United States (US), from being the tenth most common infectious cause of death in 1980, to the seventh in 1997 (18). Candidemia, in particular, is associated with an attributable mortality as high as 49% (19), which, despite advances in the field of antifungal therapy, has remained relatively unchanged for the past decade (20, 21). The annual expenditures for IC have been estimated to approach ~$1 billion in the US alone (22), mainly due to the excessive length of hospital stay associated with these infections (23-25).

Epidemiology and incidence

The epidemiology of candidemia has been extensively studied (reviewed in 11 and 26). *Candida* species are currently the fourth most common nosocomial bloodstream

pathogens in the US, accounting for 8 to 10% of all hospital-acquired bloodstream infections, and are exceeded in frequency only by coagulase-negative staphylococci, *Staphylococcus aureus* and enterococci (27).

In the past 25 years, a number of surveys have reported a substantial increase in bloodstream infections caused by *Candida* spp. (12, 28-30) and a recent comprehensive epidemiological study of sepsis in the US reported that the annual number of episodes of fungemia increased by 207% during 1979-2000 (31). In contrast, a recent report from the National Nosocomial Infections Surveillance system (32), showed a significant decrease in bloodstream infections due to *Candida* species in intensive care units (ICUs) during 1989-1999, mostly because of a decrease in the incidence of hematogenous *C. albians* infections. However, reports from Europe, Canada and Australia all conclude that the incidence of candidemia has increased in recent years (33-35), and in the Netherlands, the incidence doubled between 1987 and 1995 (36). In contrast, a survey of candidemia in Swiss tertiary care hospitals during 1991-2000 revealed a stable incidence of the infection (37).

Population-based studies

Many surveys providing important information on incidence trends of candidemia have focused on selected patient populations or hospitals (9, 38-43). Population-based studies, on the other hand, provide information on disease incidence in both the population as a whole and in specific risk groups and have the capability to provide absolute numbers for age-specific incidence rates.

In recent years, several population-based surveys in the US, Canada, Europe and Australia have studied secular trends in the incidence of candidemia (44-52). According to these reports, incidence rates differ between the US and Europe. The annual incidence of *Candida* bloodstream infections in the US during 1992-2000 ranged from 6.0 to 8.7 cases per 100,000 population (44-46), and the highest incidence was observed in Baltimore (24.0 per 100,000 population per year) (45). At the same time, the incidence rates reported from population-based European studies were generally lower, ranging from 1.9 and 2.4 cases per 100,000 population per year in nationwide studies in Finland and Norway (49, 51), to 4.3 cases per 100,000 inhabitants per year in Barcelona, Spain (48). In contrast, a recent Australian report based on data from a countrywide, population-based, active laboratory surveillance, revealed a low annual incidence of candidemia during 2001-2004 (1.8 per 100,000)

(50), and the concurrent annual incidence rate in Canada was also low (2.9 cases per 100,000 population, 1999-2004) (47)

Most population-based studies show that the highest age-specific incidence rates of candidemia are among the very young (<1 year of age), ranging from 9.4 to 75 cases/100,000/year, and among the elderly (≥65 years), ranging from 5.2-26 cases/100,000/year (44, 45, 47-49, 51). Again, lower incidence rates have been reported from Europe compared to the US. These studies also underline the important fact that candidemia is no longer associated exclusively with the ICU, as <40% of patients in the majority of these studies were in an ICU at the time of diagnosis (45, 46, 48, 50) and 11%-28% of infections were acquired outside hospitals (45, 48, 50).

Candida - the pathogen

Candida organisms are yeasts, that is, fungi that exist predominantly in a unicellular form. They are 4-6 μm in diameter, ovoid in shape (blastospores, blastoconidia) and reproduce by budding (53). Although *Candida* are ubiquitous organisms, most are not human pathogens but exist in the environment as saprotrophs (54, 55). The genus *Candida* encompasses more than 160 species, and at least 17 of those are known to have caused bloodstream infection in humans (56, 57). Approximately 90%-95% of all cases of IC are caused by 4 species, listed here by decreasing prevalence: *C. albicans, C. glabrata*, *C. parapsilosis* and *C. tropicalis* (45, 58-60). The remaining 5 to 10% are caused by 12 to 14 species, including *C. dubliniensis*, *C. krusei*, *C. lusitaniae*, *C. guilliermondii*, and *C. rugosa* (61). The number of pathogenic *Candida* species and other fungal opportunistic pathogens isolated from clinical specimens is constantly growing (13, 56, 60, 62), in part as a result of an increased number of immunosuppressed patients, improved diagnosis and close scrutiny of patient cultures in laboratories for the optimization of antifungal treatment (57, 62).

Virtually all of the clinically common *Candida* species are diploid and capable of transition between a unicellular yeast form and a filamentous growth form (fungal polymorphism) (63) (Figure 1a-c). An exception is the haploid *C. glabrata* which grows in a unicellular form at all times. *C. parapsilosis* and *C. tropicalis* grow mainly as yeast cells or as pseudohyphae, formed when buds remain attached to the parent cell and elongate, resulting in constrictions at the cell-cell junctions of the filaments. In addition, *C. albicans* and *C. dubliniensis* are capable of forming true

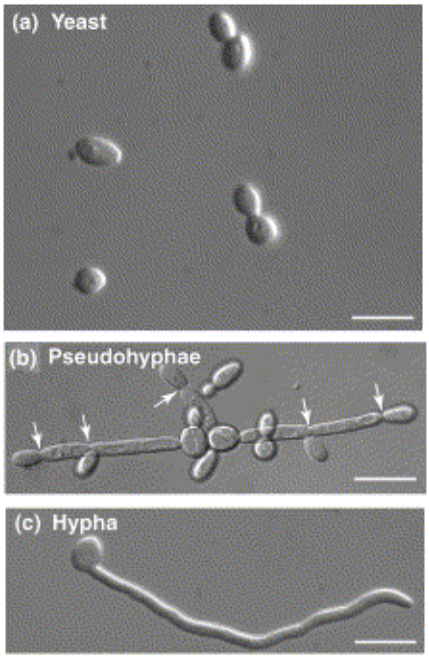
hyphae, which are outgrowths of the yeast cell that elongate by a process of apical synthesis that does not involve budding (54).

Figure 1.

Yeast, pseudohyphal and hyphal morphologies of *Candida albicans*.

a) Yeast cells. b) Pseudohyphae superficially resemble hyphae, but have constrictions at the positions of septa (arrows) and show regular branching. c) Hyphae display parallel-sided walls with no constrictions or branches. Scale bars represent 10 μm.

Reproduced from: Sudbery P, Gow N, Berman J. The distinct morphogenic states of *Candida albicans*. Trends Microbiol 2004;12(7):317-24, with permission.



Species identification and genotyping

Routine *Candida* species identification is primarily based on physiological testing. A rapid, presumptive identification of *C. albicans* can be made by observing the formation of germ tubes (the initiation of true hyphal growth) by incubation in serum (53). Isolates can be further analyzed by species specific differences in carbohydrate assimilation as well as by distinctive color when isolated on a chromogenic culture medium (64).

PCR fingerprinting

The growing field of medical mycology has benefitted from the revolutionary advances in molecular biology in recent years. New methods for species identification and genotyping of clinical isolates of human pathogenic yeasts have been developed, facilitating epidemiological studies (reviewed in 65 and 66). Among these current techniques, polymerase chain reaction (PCR) fingerprinting and random amplification of polymorphic DNA are widely used (67-72). With use of random oligonucleotide primers of approximately 10-15 bases, simple repetitive DNA sequences throughout the genome are targeted and amplified, producing individual band patterns (73). Microsatellite and minisatellite polymorphisms in

Candida species have been shown to be easily detected by PCR and to be reproducible, although intensity of bands may vary (65, 68, 69). These techniques have high discriminatory power for related and unrelated isolates of clinically relevant *Candida* species (69), especially when a number of single primers are used (74). Results of PCR fingerprinting show strong concordance with results of other established methods, such as multilocus enzyme electrophoresis, Ca3 Southern hybridization probe techniques and multilocus sequence typing (MLST) (74, 75). In addition, PCR fingerprinting requires little starting material and is rapid and simple to perform. An example of PCR fingerprints of different species of Icelandic clinical *Candida* bloodstream isolates (BSIs) is given in Figure 2.

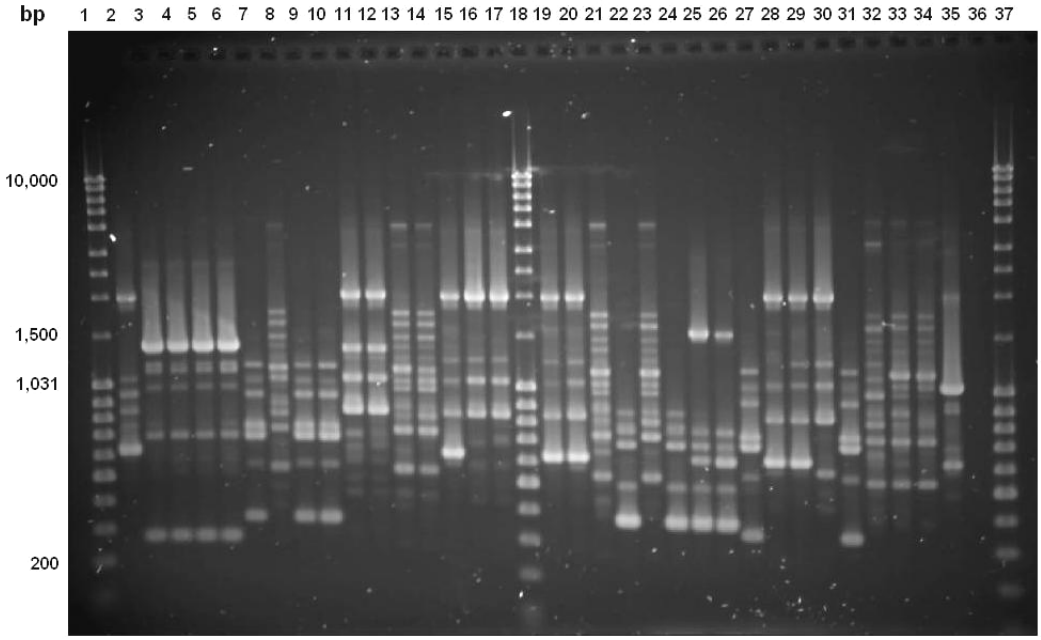


Figure 2. An example of PCR fingerprints of *Candida* isolates. Genomic DNA from 33 clinical *Candida* BSIs was amplified with the M13 core (minisatellite) sequence as a single primer. Lane 1, 18 and 37, size marker (bp: base pairs); lanes 2-17 and 19-34, *Candida* BSIs cultured from Icelandic patients; lane 35, *C. albicans* ATCC 90028; lane 36, negative control.

Multilocus sequence typing

Recently, MLST, based on DNA sequence analysis of nucleotide polymorphisms within housekeeping gene fragments, has emerged as an alternative typing tool (76, 77). A collaborative consensus MLST scheme was first developed for *C. albicans* (78), and additional MLST schemes have now been published for 4 of the common non-*C. albicans* species causing bloodstream infections in humans: *C. glabrata* (79), *C. tropicalis* (80), *C. krusei* (81) and *C. dubliniensis* (82). Although it is still costly

and not a feasible method for screening of large collections of isolates of different *Candida* species, MLST offers several advantages over PCR based methods. It is not dependent on the researcher's interpretation, the data are portable, easily stored and can therefore be easily compared (83). Although PCR-based fingerprinting methods and MLST have not been implemented for routine use for fungal diagnosis in most clinical microbiology laboratories, they have proved extremely useful for hospital epidemiology - in particular for investigating infection clusters of IC in hospitals (84, 85).

Candida dubliniensis

Thanks to major advances in species identification of isolates from clinical specimens, previously unrecognized fungal species have been discovered. In 1995, Sullivan and colleagues identified *C. dubliniensis*, which had previously been recognized as "atypical *C. albicans*" (86). Originally described in cases of oral candidosis in HIV-infected individuals, *C. dubliniensis* was initially thought to be less virulent than *C. albicans*, and hardly capable of causing invasive infections. Although it still comprises a low proportion of systemic fungal infections in large epidemiological studies (<1%) (59, 60, 87), others report a much higher recovery rate. In 2 reports from the US and Scotland, 7% and 3% of candidemias, respectively, were caused by *C. dubliniensis*, and were more commonly recovered from blood than *C. krusei* and even *C. tropicalis* in both studies (52, 88). In addition, an increasing number of reports from different parts of the world describe fatal and/or complicated cases of disseminated candidiasis caused by *C. dubliniensis* (89-96). Because not all clinical laboratories distinguish *C. dubliniensis* from the morphologically similar *C. albicans*, the prevalence of *C. dubliniensis* has probably been underreported in the past.

C. dubliniensis is phenotypically similar to *C. albicans* in many respects, being able to produce true hyphae and chlamydospores. Its phylogenic and genotypic characteristics are, however, distinct from *C. albicans* (82, 97). It can be distinguished from *C. albicans* following primary isolation from clinical specimens by dark green colonies on CHROMagar, its inability to grow at 42°C, and distinctive carbohydrate assimilation profiles (97). In addition, PCR fingerprinting with the M13 phage core sequence has been proposed as a reliable and highly reproducible method to differentiate between strains of *C. albicans* and *C. dubliniensis* (98). Nucleotide

sequence analysis of the internal transcribed spacer (ITS) region of the rRNA gene cluster, has also revealed that *C. dubliniensis* is comprised of 4 distinct genotypes, but their clinical significance has yet to be established (99).

Global trends in species distribution

Large global surveillance studies of candidemia and IC have facilitated the monitoring of longitudinal changes in species distribution and antifungal resistance. Until the late 1990s, *C. albicans* was the most common fungal pathogen cultured from blood (12, 100) and data from the ARTEMIS global surveillance program during 1997-2005, based on a total of ~200,000 yeast isolates, showed that *C. albicans* remained the most common species causing IC worldwide (60). However, recent series report that *C. albicans* currently only causes ~50% of cases of hematogenous candidiasis. Substantial geographical variances in species distribution and susceptibility patterns exist and the proportion of candidemia caused by *C. albicans* ranges from a low of 37% in Latin-America (101, 102) to a high of 70% in Norway and Finland (49, 51). *C. glabrata* is typically the second most common recovered isolate in the US and many European countries, ranging from 14%-18% of isolates (87, 102, 103), while *C. parapsilosis* and *C. tropicalis* are the most common non-*albicans Candida* species recovered in Spain, Latin-America and Asia (26, 102-105). The frequency of infections caused by non-*albicans Candida* species is increasing in many areas of the world (59, 106-112), which has implications for the regional choice of antifungal therapy, based on varying susceptibilities of different *Candida* spp. to conventional antifungal drugs.

Susceptibility of *Candida* to antifungals

Antifungal susceptibility testing

Techniques for antifungal susceptibility testing have only recently been standardized. The Clinical and Laboratory Standards Institute (CLSI) has published a reference broth microdilution method for susceptibility testing of yeasts (113), serving as standard for comparison of clinical data, and recently the European Committee on Antibiotic Susceptibility Testing proposed modification to this method (114). Agar-based methods, such as the Etest, have shown an excellent agreement with the CLSI reference method and are widely used in clinical microbiology laboratories (115).

The correlation between *in vitro* susceptibility and therapeutic outcome has been summarized as the "90-60 rule", which states that infections due to susceptible isolates respond to appropriate therapy ~90% of the time, whereas infections due to resistant isolates respond ~60% of the time (116). However, antifungal therapy failure, reflected by persistence of clinical manifestations of infection, may frequently be attributed to the severity of the host's underlying disease and/or immune status, drug pharmacokinetics and pharmacodynamics, rather than to direct resistance of the fungal strain (117).

Trends in antifungal susceptibility in relation to species of *Candida*

Candida species-specific susceptibility to the 3 main antifungal options will now be addressed briefly, but it is reviewed in detail in references 118 and 119. Interpretive susceptibility criteria have been established for fluconazole (120) and voriconazole (121), but proposed breakpoints for the echinocandins have only recently been proposed (122).

Polyenes

Amphotericin B deoxycholate is a polyene produced by *Streptomyces nodosus*, and exerts its fungicidal effect by insertion into the fungal cytoplasmic membrane and disruption of its function (123). It demonstrates rapidly cidal activity against most species of *Candida in vitro* (124). Since its discovery in the 1950s, it has served as a standard drug for the treatment of IC, but toxic effects often limit its use (125-127). Lipid-based formulations of amphotericin B have much less toxicity and favourable side effect profiles, and have proved to be an effective treatment for adult and paediatric patients with candidemia and disseminated candidiasis (128-130). *C. albicans* remains almost universally highly susceptible to amphotericin B (minimum inhibitory concentration [MIC], ≤1 μg/ml) (44, 45, 104), but MICs among *C. glabrata* and *C. krusei* isolates have been increasing (131, 132), reflected by higher dose recommendations of amphotericin B for infections with these 2 species (133). Agar-based methods, such as Etest, have shown the greatest sensitivity for detection of resistance among *Candida* spp. (134, 135).

Azoles

The azole antifungals include two broad classes: imidazoles (incl. ketoconazole, clotrimazole and miconazole) and triazoles (incl. itraconazole, fluconazole, voriconazole and posaconazole) (127). They share the same mechanism of action with both classes selectively inhibiting the fungal cytochrome P450 that brings about sterol C-14α-demethylation, resulting in decreased ergosterol synthesis and disruption of membrane synthesis in the fungal cell (123). Triazoles, however, have less effect on human sterol synthesis and are metabolized more slowly than imidazoles (123, 127).

The triazole fluconazole has been widely and increasingly used for the treatment of candidiasis since its approval in 1990 (33, 136, 137). Fluconazole has an excellent safety profile and 2 large, randomized multicenter trials showed equivalent efficacy of fluconazole to amphotericin B deoxycholate in non-neutropenic hosts (138, 139). The rate of fluconazole-resistance among the most common *Candida* bloodstream pathogens has remained infrequent worldwide; resistance (MIC $\geq$ 64 μg/ml) is reported to be < 2% among *C. albicans*, < 4% for *C. parapsilosis*, and < 5% for *C. tropicalis* (46, 51, 60, 120, 140). In contrast, the overall frequency of reduced susceptibility or resistance (MICs $\geq$ 16 μg/ml) to fluconazole among *C. glabrata* strains is ~20-30% (60, 120, 141, 142). Most *C. glabrata* isolates demonstrate "susceptibilty-dose-dependent" resistance (MIC, 16-32 μg/ml) and may respond to high dose fluconazole therapy *in vivo* (133). *C. krusei* is intrinsically resistant to fluconazole and almost all isolates have MICs $\geq$ 16 μg/ml *in vitro* (58, 60, 104).

Other available azoles include itraconazole, and new and improved formulations of this agent for oral and parentaral use have increased therapeutic options (143). The new extended-spectrum azoles, voriconazole and posaconazole, generally exhibit greater potency than fluconazole against most species of *Candida* (58, 104, 144, 145) but more importantly, are active against most clinically relevant *Aspergillus* spp. (146, 147).

Echinocandins

Echinocandins are a new class of antifungal agents that inhibit the synthesis of 1,3-β-D-glucan in the fungal cell wall, resulting in osmotic instability and cell rupture in many fungal species (118). They possess fungicidal activity against most *Candida*

species, including polyene- and azole-resistant species, and have low rates of adverse events (103, 148-151). Randomized, double-blind, multicenter trials have shown that echinocandins are as effective as amphotericin B and fluconazole for the treatment of patients with invasive *Candida* infections (152-154), and they are now an accepted first-line treatment option for IC (133).

Pathophysiology and source of invasive candidiasis

Candida species are a component of normal human flora in the majority of healthy individuals (155, 156). They are most commonly found in the gastrointestinal tract, on the mucous membranes of the mouth and vagina, and on skin (156). Surveillance cultures in ICUs have revealed that up to one-third of ICU personnel carry *Candida* species on their hands (157, 158). When the balance of the normal bacterial flora is disrupted, i.e., because of antibiotic therapy (159), or if immune defenses are compromised, *Candida* species often become pathogenic and capable of causing invasive disease (160, 161). Invasion can also occur directly by translocation, as a result of disruption of the barrier function of the skin (because of burn or the placement of intravascular catheters) or of the gastrointestinal mucosa (because of chemotherapy-induced mucositis, surgery, or perforation) (162). The pathophysiology of candidemia is summarized in Figure 3.

Although endogenous flora is often the source for nosocomial fungal infections, alternatively, exogenous strains can be transmitted to the patients from contaminated infusates, biomedical devices and the hands of health care workers (163-166). Studies using DNA fingerprinting of fungal isolates have revealed evidence of nosocomial transmission of *Candida* strains between hospitalized patients as well as between health care workers and patients (167, 168). Most molecular epidemiological studies of candidemia have been performed to investigate or confirm suspected outbreaks in single departments or hospitals (84, 85, 163, 165, 169, 170), but the overall prevalence of nosocomial clustering and/or outbreaks in patients with candidemia has not been previously elucidated.

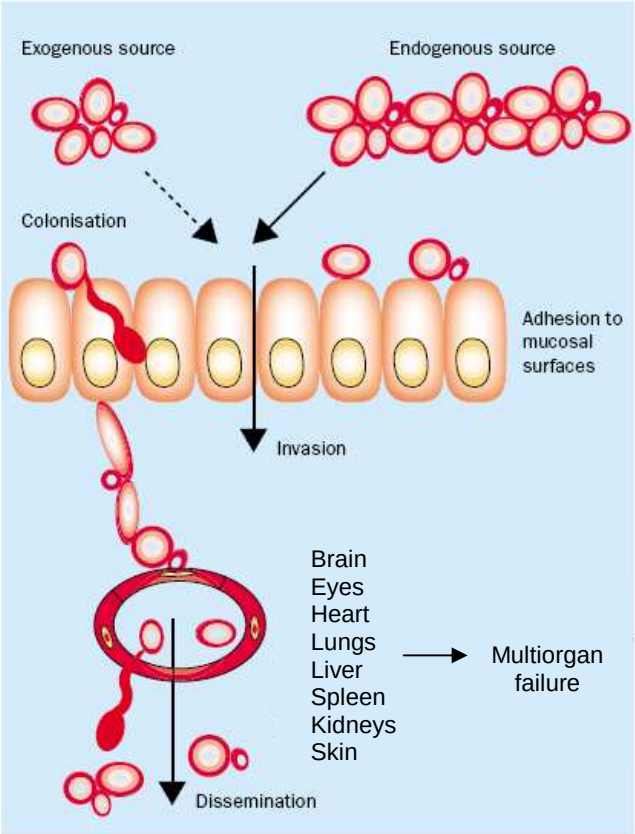
Virulence of *Candida* species

As discussed above, *Candida* has the ability to inhabit and infect several anatomically distinct sites, which requires adaptation to a variety of different environmental stresses. Virulence attributes that facilitate host tissue colonization

Figure 3.

Pathophysiology of invasive candidiasis.

Adapted from: Eggimann P, Garbino J, Pittet D. Epidemiology of *Candida* species infections in critically ill non-immunosuppressed patients. Lancet Infect Dis 2003;3(11):685-702.



and invasion have mainly been studied among *C. albicans* and include hyphal formation (morphogenesis), expression of surface recognition molecules (adhesins), phenotypic switching, and extracellular hydrolytic enzyme production (secreted aspartyl proteinases [SAPs] and phospholipases) (reviewed in references 63, 156, 171, and 172). The signal transduction pathways governing virulence may vary depending on the type of infection (i.e., mucosal or systemic) (173, 174), the site and stage of infection (175), and the nature of the host response (176).

Morphogenesis

The ability to switch between unicellular yeast cells and a filmentous growth form in the host, either pseudohyphal or hyphal, is one of the most important virulence factors of *Candida* (reviewed in ref. 176-178). However, only *C. albicans* and *C. dubliniensis* form both types of filaments. Among *C. albicans*, the yeast-hyphal transition is regulated, in part, by transcription factors that are controlled by signaling pathways responding to a variety of extracellular conditions, i.e., ambient pH, nutrient deprivation and serum (179-181). The hyphal tip functions as an effective drill-bit with which *Candida* can burrow into tissues (178), and is also the site of

apical secretion of hydrolytic enzymes (182) and antigen expression (183). *C. albicans* mutants incapable of hyphal formation are generally less virulent in experimental models of disseminated candidiasis (184-186). In contrast, strains that are unable to grow in the yeast form have also shown attenuated virulence in animal models (187). The yeast form may be better adapted for free dissemination within the circulatory system or between individual hosts, and is the mode of proliferation in target tissues (178). In *C. albicans*, the yeast form also produces its own repertoire of SAPs, all implicated in virulence (182). These observations, therefore, suggest that both growth forms may play important roles in the development and progression of invasive candidiasis.

Recent studies have shown that different growth forms induce different types of immune responses. *C. albicans* blastospores induce a protective type 1 immune response in the host´s kidneys, whereas filaments induce a non-protective type 2 immune response (188). A proposed explanation is the differential recognition of hyphae and blastospores by Toll-like receptors (TLR), which are important pattern recognition receptors for microbial ligands and modulators of host defense (189). *C. albicans* blastospores bind to TLR2 and TLR4 on mononuclear leukocytes, stimulating the secretion of tumor necrosis factor (TNF)-α as well as interferon (IFN)-γ, a key promoter of protective immunity to the pathogen (190). The hyphal form, however, binds only to TLR2, inducing large amounts of interleukin-10 secretion but much less TNF-α secretion and negligible IFN-γ secretion (190). The loss of IFN-γ production during the yeast-to-hyphal transformation may therefore be an important virulence mechanism, used to escape from the host defense.

Adherence to host constituents (adhesins)

Adhesins are biomolecules which promote the adherence of *Candida* to host cells or host-cell ligands. The *C. albicans* ALS (agglutinin-like sequence) gene family includes at least 8 different ALS genes, which encode large cell-surface glycoproteins that function as important adhesins (191). Most adhesin-encoding genes, including ALS1, ALS3, and hyphal wall protein 1 (HWP1), are hyphae-specific and co-regulated with cell morphogenesis (171, 185). Following adherence, endocytosis of *C. albicans* is mediated, in part, by hyphae-specific binding to N-cadherin on the surface of vascular endothelial cells (192).

Phenotypic switching

Phenotypic switching refers to the ability of *C. albicans* and related species, to switch reversibly and at high frequency between a number of different phenotypes (193). This is reflected by changes in colony morphology *in vitro* (194). Switching is a mode of adaption in response to environmental challenges, affecting a variety of virulence traits of *C. albicans* and several other *Candida* spp., including yeast-to-hyphae transition, antigen expression, adhesion, sensitivity to neutrophils, proteinase secretion and drug susceptibility (156, 193, 195). Phenotypic switching may therefore be a valuable trait for *C. albicans*, both as a commensal and pathogen.

Secretion of extracellular hydrolytic enzymes

The SAPs are the most extensively investiged hydrolytic enzymes secreted by *C. albicans*, and seem to play an important role in the virulence of the species (reviewed in ref. 172). Ten genes encoding SAP proteins have been cloned from *C. albicans* (196) but other pathogenic *Candida* spp. also possess SAP genes, including *C. dubliniensis* (197), *C. tropicalis* (198), and *C. parapsilosis* (199). SAPs fulfill a number of specialized functions during the infective process, which include digestion of molecules for nutrient acquisition, distortion of host cell membranes to facilitate adhesion and tissue invasion, and disruption of cell immune responses (182). SAP production is also associated with a number of other putative virulence attributes, including filamentation, biofilm formation, and phenotypic switching (172, 196). *C. albicans* also secretes phospholipase B enzymes and lipases, some of which are required for virulence in animal models of candidiasis (200).

Animal models of infection

The murine model of *Candida* sepsis is the standard animal model used to determine the host response to disseminated candidiasis, the efficacy of antifungal drugs, as well as the role of specific *Candida* virulence genes (i.e., by using mutant strains) (63, 201). Most investigators have used outbred mice (25-30g); male CF1 mice (184, 202), male BALB/c mice (188), and female NMRI mice (203). Other animal models of disseminated candidiasis include the rabbit (204), the rat (205, 206), and the guinea-pig (203). In the murine model of hematogenous candidiasis, a single dose of organism is inoculated intravenously, usually via the tail vein, and the survival of animals and tissue counts of the organisms in internal organs (generally the kidneys

and liver) are the conventional end points (201). In this model, the kidneys become the most heavily infected organs (207), whereas in the rat and the guinea-pig models, the infection is also severely spread into other organs (203, 206). The intravenous LD_{50} usually ranges from 10^4 to 10^6 yeasts, but depends on the virulence of the *Candida* strain and on the susceptibility of the mouse strain (201). It has been demonstrated that the mice die of progressive septic shock, manifested by hypotension, tachycardia, and acidosis (208), mirroring the clinical course of severe hematogenously disseminated candidiasis in humans.

Differences in virulence of *C. albicans* and *C. dubliniensis*

In the past decades, animal models of disseminated candidiasis were most extensively applied in order to study the pathogenicity of *C. albicans* and *C. tropicalis* (209-212). However, in recent years, the murine model of hematogenous candidiasis, has been employed for effective comparisons of the virulence of the most common *Candida* spp. causing bloodstream infections in humans (213). Two previous studies have used this model for direct comparisons of the virulence of *C. albicans* and *C. dubliniensis* (197, 214). Survival of mice infected by *C. albicans* was significantly impaired in comparison with animals infected with *C. dubliniensis* in one study (214), but marginal in the other study (197). *C. albicans* has also been shown to be more effective at dissemination from the gastrointestinal tract in immunocompromised animals (215). However, these studies have two major limitations in common: a low number of strains used and/or an apparent lack of criteria for selection of fungal strains for the animal experiments.

C. albicans and *C. dubliniensis* seem to possess different virulence traits, as shown by *in vitro* and *in vivo* virulence studies. The ability to adhere to epithelial cells and to produce phospholipases, proteinases and filaments may be attenuated among *C. dubliniensis* strains (195, 215, 216). In contrast, phenotypic switching of colony morphology and adaptive resistance to fluconazole and other azole agents seems to be a more common trait in *C. dubliniensis* (195, 217). Previous work suggests that virulence properties of strains may be different, in part based on the source (systemic vs. mucosal infections) (218-220). In addition, a significant association has been found between genetic clades of *C. albicans* and anatomical source of isolates, reflecting possible differences in putative virulence properties (221). In order to adjust for this potential confounder, it is reasonable to use isolates

from analogous anatomical sources as well as comparable patients for comparisons of the virulence of the two species.

Risk factors for candidemia

Risk factors for candidemia have been identified and extensively described, especially among critically ill non-immunosuppressed patients (7, 9, 15, 17, 39, 161, 222-224). Established risk factors significantly associated with candidemia are shown in Table 1.

Table 1. Risk factors for candidemia among hospitalized patients[a,b].

Adult patients	Pediatric patients
Length of hospital and ICU stay	In addition to the adult risk factors:
Diabetes	Prematurity*
Malignancy	5 min Apgar score <5*
Renal failure/hemodialysis*	Congenital malformations
Acute pancreatitis	
Severity of disease	
Prior surgery*	
Transplantation	
Previous bacteremia	
Antimicrobial treatment (multiple or prolonged)*	
Previous colonization with *Candida* spp.*	
Parenteral nutrition*	
Immunosuppressive therapy	
Corticosteroid therapy	
Chemotherapy*	
Neutropenia (<500/mm^3)*	
Central intravascular catheters*	
Mechanical ventilation	

NOTE. APGAR, American Pediatric Gross Assessment Record.

[a] Data is compiled from references 7, 9, 15, 17, 26, 39, 161, 222, 224, and 225.

[b] *, independent risk factor.

The hitherto largest prospective study of risk factors associated with candidemia was conducted among 4,276 surgical ICU patients in the US and identified 3 major independent risk factors: prior surgery (relative risk [RR], 7.3), acute renal failure (RR, 4.2), and receipt of parenteral nutrition (RR, 3.6). Receipt of an antifungal agent was associated with decreased risk (RR, 0.3), but interestingly a higher severity of illness score was not independently predictive of poor outcome (15). In addition, ICU admission, multiple or prolonged antimicrobial therapy, neutropenia,

colonization with *Candida* spp., and indwelling vascular catheters (in particular CVCs), have been shown elsewhere to independently predispose patients to candidemia (14, 16, 20, 161, 222, 226). Among children and neonates, the most important independent risk factors are prematurity, hyperalimentation, low Apgar score, presence of CVCs, and prolonged stay in the neonatal ICU (NICU) (161, 227, 228).

Importantly the majority of candidemic patients have ≥2 significant risk factors (228), whereby the risk of infection increases exponentially (229). In addition, most of the risk factors shown in Table 1 are non- specific for candidiasis and commonly encountered in the hospital and ICU setting, potentially delaying diagnosis (224). This has prompted the development of clinical predictive rules for the early prediction of IC and for identification of patients who might benefit from early (prophylactic, empirical and pre-emptive) antifungal therapy (222, 230-232). Ostrosky-Zeichner et al. (231) recently developed a rule based on risk factors determined from a retrospective analysis of patients at 12 ICUs, which captured 34% of patients with IC. Similarly, Leon et al. (222) recently proposed a "*Candida* score" to target the best candidates for early antifungal therapy in the ICU. The score was based on 4 independent risk factors for candidemia, identified among patients in 73 ICUs: severe sepsis (2 points), surgery (1 point), total parenteral nutrition (1 point), and multifocal *Candida* spp. colonization (1 point). A score >2.5 (sensitivity 81%, specificity 74%) indicates that early therapy is warranted (222). Attempts of risk stratification are important in order to impact the incidence and mortality associated with candidemia and IC, but further validation of these models is needed.

Clinical manifestations

Clinical symptoms of candidemia and IC vary from minimal fever to full-blown sepsis syndrome and multiorgan failure, resembling those caused by bacterial pathogens (224). Acute disseminated candidiasis occurs when several organs are infected as a result of hematogenous spread, most commonly the kidneys, brain, myocardium, and eyes (Figure 1). Pathological changes in internal organs mainly comprise diffuse microabscesses with a combined acute suppurative and granulomatous reaction (53).

Clinical manifestations of candidemia and IC include characteristic skin lesions and, more rarely, eye lesions and deep abscesses. *Candida* endophthalmitis has been

reported in as high as 28% of candidemic patients (233) and presents as pain and gradual decrease in visual acuity, which can culminate in permanent blindness. Lesions are white, cotton ball-like exudates on the retina (chorioretinitis), and rapidly progress to involve the vitreous (234). Current consensus guidelines recommend at least one dilated retinal examination in all candidemic patients (133). Skin lesions associated with candidemia are characterized by non-tender firm pustules on an erythematous base and can be generalized or localized to the extremities. These can vary from tiny pustules to large, erythematous nodular lesions (53). Diagnosis can be made by demonstration of *Candida* spp. in histopathological analysis of punch biopsies from the lesions, even when blood cultures are negative.

Hepatosplenic candidiasis (chronic disseminated candidiasis) is an increasingly recognized form of disseminated *Candida* infection, that most commonly affects patients with acute leukemia after prolonged chemotherapy-related neutropenia (235). The diagnosis of hepatosplenic candidiasis is often difficult to make because of a non-specific clinical presentation and negative blood cultures (236). However, characteristic microabscesses in the liver, spleen and occasionally the kidneys can be identified by imaging techniques, in particular computed tomographic scanning or magnetic resonance imaging (237).

Diagnosis

As addressed, the diagnosis of IC remains a challenge. Culture from sterile body sites and proper histopathology remain the “golden standard” for diagnosis, but the yield of *Candida* spp. in blood cultures is suboptimal, even with current culture techniques. As a result, diagnosis is often based on clinical and/or radiological surrogate markers of disease.

Blood cultures

A positive blood culture is essentially the standard diagnostic procedure for candidemia. Studies from the early 1990s, based on autopsy records, have shown that blood cultures may only be approximately 50% sensitive for detection of disseminated candidiasis (238, 239), but this has not been recently re-evaluated.

Since the advent of modern blood culture techniques, many studies have compared the sensitivity of different blood culture systems for the detection of yeasts (240-242). Episodes of candidemia are most commonly detected with standard

aerobic and anaerobic blood culture media, and since *Candida* spp. are generally regarded as obligate aerobes, the detection rate in aerobic bottles is higher (243). A recent study compared the ability of the 2 most commonly used automated blood culture systems, BACTEC 9240 (BD Diagnostic Systems, Sparks, Md.) and BacT/ALERT 3D (bioMérieux, Inc., Durham, N.C.), to detect growth of different *Candida* species (244). The sensitivity for detection of simulated candidemia in at least 1 bottle of aerobic or anaerobic media was 92% and 100% for BACTEC and BacT/ALERT, respectively, when a standard 5-day incubation was used (244). Following blood culture, *Candida* grows rapidly on several types of solid culture media and does not need specific fungal medium for cultivation.

Non-culture based methods

In parallel with recent advances in molecular biology, there has been increased emphasis on non-culture based methods in order to potentially improve diagnostic accuracy and hasten diagnosis of candidemia and IC (245). Although various laboratory tests have been developed, that detect *Candida*-specific antibodies, antigens, or metabolites, they suffer from lack of specificity and/or sensitivity and are unable to discriminate between different *Candida* species (246-249). Recent reports suggest that the combined serological detection of mannanemia and anti-mannan antibodies may be useful for the diagnosis of systemic candidiasis (250, 251), and a new promising assay is based on the detection of β-D-glucan, which is present in the cell wall of many fungi (252-254). PCR based assays for candidemia, with use of both universal and species-specific primers, have been reported to have sensitivity close to that of blood cultures (255). Despite their great promise, non-culture based detection methods are still mainly used in reference laboratories and research and their future role in the diagnostic work-up of patients with suspected IC is still uncertain.

Burden of invasive candidiasis

Incident to the increasing incidence of serious fungal infections, there was a dramatic increase in multiple cause mortality due to invasive mycoses in the US, from 1,557 deaths in 1980 to 6,534 deaths in 1997 (18). The attributable mortality of candidemia estimated in retrospective matched-cohort studies ranges from 10%-49%, with single institutions (19-21) generally reporting higher proportions (35%-49%) than studies

based on population surveillance (10-24%) (24, 25). Furthermore, some studies indicate that candidemia carries no less risk of death during hospitalization today than it did 20 years ago (19, 21). The average length of stay in the hospital is increased approximately twofold in patients with nosocomial candidemia (21, 23, 24) resulting in significant financial burden (256). Recent cost-of-illness analyses in the US suggest that the mean cost directly attributable to an episode of IC ranges from $28,000 to $119,000 for pediatric patients (24, 257) and from $34,000 to $46,000 for adult patients (24). The variables most strongly associated with excess costs are the increased length of stay (23) and adequate antifungal treatment (25).

Predictors of mortality

Given the significant mortality associated with candidemia, many studies have assessed risk factors that could independently predict a fatal outcome. Older age, hospitalization in an ICU, neutropenia and severity of underlying illness are well known independent predictors of mortality (50, 258-261). However, prognostic factors seem to differ between adults and children. A large, prospective, multicenter study in the US showed that higher severity of illness, neoplasia, receipt of glucocorticosteroids, and candidemia due to *Candida* spp. other than *C. parapsilosis* were among the most important predictors of death in adult candidemic patients, whereas only neutropenia and endotracheal intubation were significantly associated with mortality among children (228). Mortality may also be related to the infecting pathogen, and several surveys have reported higher mortality rates associated with *C. glabrata* bloodstream infection (40, 50, 262). Among treatment related factors, the absence of antifungal treatment (48, 50, 259, 261, 263, 264) and catheter removal (48, 50, 263, 265-267) have been shown to be independently associated with poor outcome.

Prognostic factors for mortality in patients with candidemia have only recently been analyzed in population-based studies (48, 50), and these reports are based on clinical data collected during a 2 or 4 year period. Since many studies on epidemiology, management and outcomes are limited to selected hospitals (36, 37, 263, 268) or specific groups of patients (40, 264, 269), with relatively short observation periods, it is also imperative to assess continuously, whether new therapeutic options such as novel triazole antifungal agents, lipid preparations of

amphotericin B and β-glucan synthase inhibitors, have made a positive impact in the long term on outcomes among candidemic patients.

Treatment

Removal of vascular catheters

Candida spp. adhere to materials used in vascular catheters (270), and the rationale for their removal in the setting of candidemia is to eliminate a possible nidus of infection. Removal and reinsertion of vascular catheters are, however, potentially hazardous procedures among critically ill patients and the topic of catheter removal has been hotly debated (271).

Vascular catheters are an important independent risk factor for development of candidemia (161, 272) and some studies show a clear benefit in terms of a reduction in duration of candidemia and a lower attributable mortality rate when vascular catheters (in particular CVCs) are promptly removed (267, 268, 273). Other studies do not support this observation, except in cases of proven catheter-related infection (271, 274). Supporting the latter, studies have revealed strong genetic relatedness between *Candida* isolates recovered from the alimentary tract and the bloodstream in candidemic patients (275). However, it has been suggested that even when the gastrointestinal colonization is the source of infection, indwelling vascular catheters may become secondarily infected and serve as a source of sustained fungemia (276). Although prospective randomized controlled trials on this topic are lacking, current consensus guidelines emphasize that percutaneous catheters should be withdrawn from all patients with candidemia and disseminated candidiasis, if feasible (133, 277).

Antifungal treatment

Consensus guidelines also advise that all patients with positive blood culture with *Candida* species should receive antifungal therapy (133). It is recommended that treatment be continued for 2 weeks after the last positive blood culture result and resolution of the signs and symptoms of infection (133). Based on results from large randomized studies (138, 139, 152-154), amphotericin B, fluconazole, or echinocandins, are all considered suitable first choice agents in adults with bloodstream infections due to *C. albicans, C. tropicalis*, and *C. parapsilosis* (127, 133). Combination therapy potentially holds promise for immunosuppressed patients

and/or patients with severe manifestations of candidiasis (278), but awaits demonstration in clinical trials. Fluconazole prophylaxis (400 mg daily) during the neutropenic period is standard practice for patients undergoing allogenic blood and marrow transplantation and for patients with acute leukemia and prolonged neutropenia, based on decreases in superficial and invasive fungal infections and associated morbidity and mortality demonstrated in randomized-controlled trials (279-284). Recent meta-analyses of randomized-clinical trials of antifungal prophylaxis among high-risk ICU and/or surgical patients have also shown that azole prophylaxis can reduce the risk of IC by 50-80%, but the effect on mortality proved to be less robust (285-287). However, further trials are needed to identify the subgroups of patients that might benefit most from prophylaxis and to assess the effects on antifungal susceptibility patterns. Another potential strategy to reduce the morbidity and mortality of IC is to use empirical (rather than prophylactic) antifungal therapy, but in a recent, large, randomized-controlled trial among high-risk adult ICU patients, empirical fluconazole (800 mg daily) was no more beneficial than placebo (288).

Recent reports describe a surprisingly high proportion of patients with candidemia who receive inadequate antifungal therapy. Puzniak et al. (20) reported that 10% of patients that were hospitalized for at least 1 week, received no therapy, and Morgan et al. (25) reported that 30%-39% of those surviving more than 3 days from positive blood culture received <7 days of therapy. A minimal delay (>12 h from having the first positive blood sample for culture drawn) in initiating antifungal treatment in patients with candidemia has been identified as an independent predictor of mortality (289). Similarly, a recent retrospective multicenter study of candidemic patients who received fluconazole reported that increased time from blood culture until initiation of fluconazole therapy was independently associated with poor outcome (290). This underlines the current need for more rapid diagnostic techniques for the identification of *Candida* species in blood samples, as well as for the implementation of clinical prediction rules and/or bedside scores which may help clinicians select patients who will benefit from early antifungal administration.

AIMS OF THE STUDY

Our aims were:

- To define the incidence of fungal bloodstream infections on a nationwide basis in Iceland during a 27-year period, 1980-2006 (paper I and unpublished).

- To identify the spectrum of pathogens causing candidemia in Iceland, to study their antifungal susceptibility and the national consumption of antifungal agents (paper I and unpublished).

- To analyse clinical characteristics and predisposing conditions of patients with candidemia in a nationwide setting, and their association with mortality (paper II).

- To study the genetic relatedness of all available BSIs of *Candida* species in Iceland during a 15-year period using PCR fingerprinting, and thereby quantitate the contribution of nosocomial outbreaks in the overall context of candidemia (paper III).

- To compare the virulence of *C. dubliniensis*, using a range of BSIs, with *C. albicans* in a murine model of bloodstream infection in immunocompetent animals (paper IV).

- To evaluate in a blinded, systematic manner the histopathological characteristics associated with outcome in *C. dubliniensis* and *C. albicans* bloodstream infections (paper IV).

MATERIALS AND METHODS

The studies were approved by the relevant authorities in Iceland, including the National Bioethics Committee of Iceland and the Data Protection Authority of Iceland. The animal experiments were authorized by the Experimental Animal Committee of Iceland and complied with the Animal Welfare Act 15/94.

Setting and methods for blood culture

Iceland had a population of 226,948 at the beginning of 1980 and 307,672 at the end of 2006. Currently, 2 university hospitals and 14 community hospitals exist in the country. During most of the study period, 1980-2006, there were 3 adult ICUs in the country, and 1 NICU. Three clinical microbiology laboratories processed blood cultures from all the hospitals, one of them serving as a reference laboratory for the entire country. Episodes of candidemia among hospitalized patients were recorded in each laboratory. During the study period, 3 different automated blood culture systems were used. The BACTEC (Becton Dickinson Diagnostic Systems, Sparks, Maryland) radiometric blood culture system was in use until 1996, followed by the continuous monitor system ESP DIFCO (Difco Laboratories, Detroit, Michigan) during 1997-2001. From 2002, all laboratories have used the BacT/ALERT blood culture system (Organon Teknika Corp., Durham, North-Carolina).

A national population registry is available from the Bureau of Statistics in Iceland (http://www.statice.is), which contains information on every inhabitant on an annual basis, including gender, date of birth and date of death.

Candida species identification (I,II,III,IV)

All obtainable BSIs were collected from hospital laboratories across Iceland, where they had been stored in glycerol at -70°C from the time of diagnosis. In total, 217 yeast isolates cultured from blood during the period 1 January 1991 through 31 December 2006 were viable and thus available for further study. Two isolates cultured in 1990 were also attained. In total, we obtained 219 fungal BSIs identified from 198 patients with candidemia, which represent isolates from 203 (94.4%) of 215 episodes diagnosed in the country during 1991-2006. Species identification was based on germ tube production, morphology following culture on chromogenic agar

(CHROMagar, Hardy Diagnostics, Santa Maria, CA) and by analyzing sugar assimilation profiles with use of the API 20C and API id32C systems (bioMérieux, France). In addition, *C. dubliniensis* was distinguished from *C. albians* by PCR fingerprinting (described in detail on p. 26-27), and ITS sequence analysis, as described by Lan et al. (291).

Antifungal susceptibility testing (I, IV)

All isolates were tested for susceptibility to 3 antifungal agents: amphotericin B, fluconazole and itraconazole. Antifungal MICs were determined by using Etest (AB Biodisk, Solna, Sweden), an agar-based predefined concentration gradient method that has shown an excellent essential agreement with the CLSI reference broth microdilution method (115). Application of Etest and MIC readings were performed according to instructions from the manufacturer (http://www.abbiodisk.com), as follows: Organisms were inoculated onto RPMI 1640 agar plates, and when dry, the Etest strips were applied to the inoculated surface. The plates were incubated at 35°C and read at 48 h. The data reported represent concentrations of each antifungal agent necessary to inhibit 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates tested. Susceptibility results were categorized according to interpretive breakpoints recommended by the CLSI (113).

Import of antifungal agents (I and unpublished)

National import figures of antifungal agents from 1980 through 2006 were obtained from the Icelandic Association of Importers of Pharmaceuticals and the Icelandic Medicines Control Agency. The figures were expressed as number of imported packs of antifungals per year. By calculating defined daily doses (DDD) per packing and consulting the national register we were able to calculate the import as DDD per 1,000 inhabitants per year and DDD per 100,000 inhabitants per day. We applied the WHO/ATC definition of DDD (http://www.whocc.no), including our daily dose definition for lipid based amphotericin B (175 mg). Documented antifungal agents (year of first registered import) were the following: Nystatin (before 1980), griseofulvin (before 1980), amphotericin B deoxycholate (1982), 5-flucytosine (1982), ketoconazole (1985), fluconazole (1990), terbinafine (1993), itraconazole (1995), lipid-based formulations of amphotericin B (1997), voriconazole (2002), and caspofungin (2003).

Case definitions

An episode of candidemia was defined as at least 1 blood culture positive for *Candida* species with temperature of ≥38.5°C or other signs of bloodstream infection. Episodes were considered to be separate if they occurred at least 1 month apart (50, 51) or were caused by different *Candida* species. The time of onset of candidemia was defined as the date when the blood sample was obtained for culture. Cases occurring either prior to or within 2 days of hospital admission were considered community acquired (48, 50). Source of candidemia was considered catheter-related if culture of the catheter tip grew the same species isolated from blood (292). The infection was considered disseminated if evidence was found for hematogenous spread to non-contiguous sites (9). A CVC was considered promptly removed if this was done within 2 days after the first positive blood culture result was obtained (274, 293). Septic shock was defined as sepsis syndrome in association with hypotension (systolic blood pressure <90 mm Hg or reduction of 40 mm Hg from baseline) despite adequate fluid resuscitation (294), within 12 h of a positive blood culture. With use of hospital records and the national population registry of Iceland (http://www.statice.is), we calculated the case fatality proportion among patients with candidemia within 7 and 30 days, repectively, after the blood sample was obtained for culture.

Collection of clinical data (I,II,III,IV)

All patients in Iceland with *Candida* species isolated from blood from 1 January 1980 through 31 December 2006 were identified retrospectively by a nationwide search in microbiology databases. The data collected included demographic characteristics; number of blood cultures positive for *Candida* spp.; dates when blood samples were obtained for culture; original species identification; patient location (hospital and hospital ward) at time of positive blood culture; survival 7 and 30 days after the initial positive blood culture; and date of death.

In addition, medical charts of all patients with candidemia in Iceland from 1 January 1980 through 31 December 1999 were reviewed and data were recorded on a standard computerized case report form. The data included reasons for hospital admission; duration of hospital and ICU stay; risk factors associated with candidiasis (i.e., use of antimicrobial and systemic antifungal agents, surgical operations, intravascular devices, hyperalimentation, immunosuppressive and corticosteroid

therapy); major co-morbidities; other concomitant drug therapy; clinical signs of sepsis ≤12 h of positive blood culture; results from routine blood tests at the time (≤24 h) of positive blood culture; complications of candidemia; results from imaging studies following diagnosis (≤2 weeks); and clinical outcome (≤30 days). Information regarding antifungal therapy received and the number of central intravascular catheters and catheter-days was also obtained. Central catheters included CVCs, arterial catheters, pulmonary artery catheters and umbilical catheters. Microbiology data included dates, when results of blood cultures turned positive, and results of fungal-cultures from other sites.

PCR fingerprinting (III)

In order to be able to quantitate the proportion of nosocomial clustering of *Candida* spp. BSIs, we performed PCR fingerprinting on 219 isolates, which represented 94.4% of all episodes diagnosed in the country during 1991-2006. This method was chosen, since PCR fingerprinting has high discriminatory power for related and unrelated isolates of clinically relevant *Candida* species (65, 73). Genomic DNA was extracted from each isolate as described previously by Xu et al. (68) and stored at -20°C . After measuring genomic DNA amount in each sample with a fluorometer (DyNA Quant™ 200 Fluorometer, Amersham Biosciences), all samples were diluted to 10 ng/µL. DNA was amplified by arbitrarily primed PCR (AP-PCR) using 4 single primers: i) the M13 phage core sequence (15 bases); ii) the simple repeat sequences $(GACA)_4$ (16 bases); iii) the oligonuclotide PA03 (10 bases); and iv) the intergenic spacer repeat of transfer RNA, T3B (19 bases) – that have all been previously used for genotyping *Candida* isolates (67, 68).

Amplifications were performed in volumes of 25 µL in a 200 µL Ready-To-Go-PCR tube (Amersham Biosciences) that contained ~20 ng of genomic DNA and primer at a final concentration of 0.8 µM. All PCRs were performed in a Touchgene Gradient thermal cycler (Techne Inc., Princeton, NJ) as described by Xu et al. (68), with minor alterations. Amplification products were separated by electrophoresis on 1% agarose gels with 1 x Tris-borate-EDTA buffer, pH 8, for 150 min at 6 V/cm. Amplicons were stained with ethidium bromide and were photographed digitally under ultraviolet light (ChemiImager; Alpha Innotech Co, San Leandro, CA).

Reproducibility assessment

Genomic DNA from a *C. albicans* ATCC 90028 strain was included in each AP-PCR run. PCR fingerprinting patterns of the ATCC 90028 strain were compared to assess reproducibility, and fingerprinting results from each run were included only if the ATCC 90028 fingerprint was consistent with those of previous runs.

Fingerprinting analysis and cluster definitions

The electrophoretic bands were sized and scored manually and with BioNumerics, version 4.61 (Applied Maths), using a position tolerance setting of 2%. Fingerprinting patterns were clustered, and dendrograms were generated using the Dice coefficient and the unweighted-pair group method using average linkages. Epidemiologically related isolates were clustered using 2 potential cutoff values: 100% and ≥90% relatedness. To evaluate the proportion of infections caused by clustered isolates, a nosocomial cluster was defined as isolation of closely related isolates (≥90%) from ≥2 patients in the same ward or at the same hospital within a period of 90 days. Patients with polymicrobial candidemia were excluded from these calculations.

Virulence studies in a murine model of bloodstream infection (IV)

Candida isolates

Candida isolates from different anatomical sites may differ in their virulence potential (see Introduction chapter). In order to adjust for this potential confounder, we used isolates from analogous anatomical sources as well as comparable patients in the animal experiments. The isolates were all initially cultured from blood: 9 *C. dubliniensis* and 3 *C. albicans* (paper IV, Table 1). Their original identification was based on growth phenotypes on CHROMagar (Hardy Diagnostics, Santa Maria, CA, USA) and API 20C and API id32C substrate utilization profiles (bioMérieux, Marcy l'Etoile, France). Definitive species identification was confirmed by sequence analysis of the ITS regions 1 and 2 using the fungal universal primers ITS1 and ITS4, as previously described (291).

Antifungal susceptibility testing

The MICs of amphotericin B, fluconazole and itraconazole were determined for all isolates by using Etest, as described on p. 24.

DNA fingerprinting and genotyping

Genomic DNA was extracted as described by Xu et al. (72). The 9 *C. dubliniensis* isolates were genotyped by PCR amplification with use of the genotype-specific primer pairs G1F/G1R, G2F/G2R, G3F/G3R, and G4F/G4R, as described by Gee et al. (99). These primers allow *C. dubliniensis* isolates to be grouped into 1 of 4 genotypes based on genotype-specific differences in the nucleotide sequence of the ITS1 and ITS2 regions of the rRNA gene cluster (99). Intraspecies genotypic diversity was further determined by PCR fingerprinting with 4 single primers (M13, $(GACA)_4$, PA03, and T3B), as previously described (p. 26-27).

Animal experiments

Adult (6-10 weeks of age) female non-immunosuppressed NMRI mice (mean weight 29 g) were obtained from M&B AS (Ry, Denmark). The infective inoculum was generated by suspending 2 to 3 colonies in sterile saline for a final concentration of 5-8 x 10^6 organisms/ml. The volume was adjusted appropriately by measurements of optical density (OD) and by using counting chamber. Final concentration was verified by viability counting on solid media. The mice were challenged with a volume of 200 µl of the yeast suspension by injection into the lateral tail vein, using a 27G needle and tuberculin syringes (inoculum, 1-2 x 10^6 organisms). In each experiment, a group of 10 mice were challenged with each isolate and 4 different isolates were tested each time. A total of 157 mice were infected with *C. dubliniensis* (9 strains) and 99 mice were infected with *C. albicans* (3 strains). The control strain ATCC 90028 was used in all except 2 of experiments as an internal control. In general, if the strains were consistently not associated with mortality in 10 animals they were not retested. Experiments were repeated for those strains associated with death and the results pooled. The animals were kept in cages with free access to commercial pelleted food and water under standardized conditions at the Institute of Experimental Pathology at Keldur (Reykjavik, Iceland) with regulated daylight and temperature. Mice were monitored 3 times daily for 7 days after the challenge and mortality was noted. If they appeared to be in pain or pre-terminal they were euthanized. At the end of the experiment (day 7) all live animals were sacrificed.

Viability counting

Both kidneys and the liver were removed. The liver and one kidney were homogenized (Omni tissue homogenizer; Omni, Gainesville, Va.) in 1.8 mL cold saline. Yeast densities were determined by plating serial 10-fold dilutions on Sabouraud agar with chloramphenicol (Oxoid, Basingstoke, Hampshire, UK). The colonies were counted (colony forming units [CFU])/mL) after 48 h of incubation.

Histopathological analysis

One kidney was fixed in 10% buffered formaldehyde and prepared for histopathological examination; embedded in paraffin, cut into 5-µm thick sections and stained with hematoxylin-eosin (HE) and methenamine-silver stain. Kidneys from 235 mice were available for histopathological analysis. The sections were marked with encrypted numbers and examined in a blinded manner without knowledge of the infective strain. The following characteristics were systematically scored for each specimen according to a predetermined scoring system as shown in Figure 4a-h: tissue burden of organisms (0-4); presence, localization and proportion of yeast forms vs. hyphae/pseudohyphae; inflammatory response (degree (0-4), localization and type); infiltration of neutrophils (0-4) and mononuclear cells (0-4); presence of microabscesses (+/-) and granulomas (+/-). The specimens were decrypted after all sections had been evaluated and the results recorded in a database (SPSS version 11.0; SPSS Inc., Chicago, IL, USA).

Statistical analysis

For the statistical analyses of the frequency of dichotomous variables, the Fisher´s exact test was used for comparison of two independent proportions, and the chi-square test for independence and linear trend between groups for comparison of three or four independent proportions (I, II, III, IV). Logistic regression was used to compare continuous variables (II). Statistical analyses were performed by SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). All tests were two-sided and level of significance was set at $p<0.05$.

<u>Incidence of candidemia (I,III)</u>

Information about national demographics was obtained at the National Population Registry of Iceland. These data were used to calculate the incidence (cases/100,000

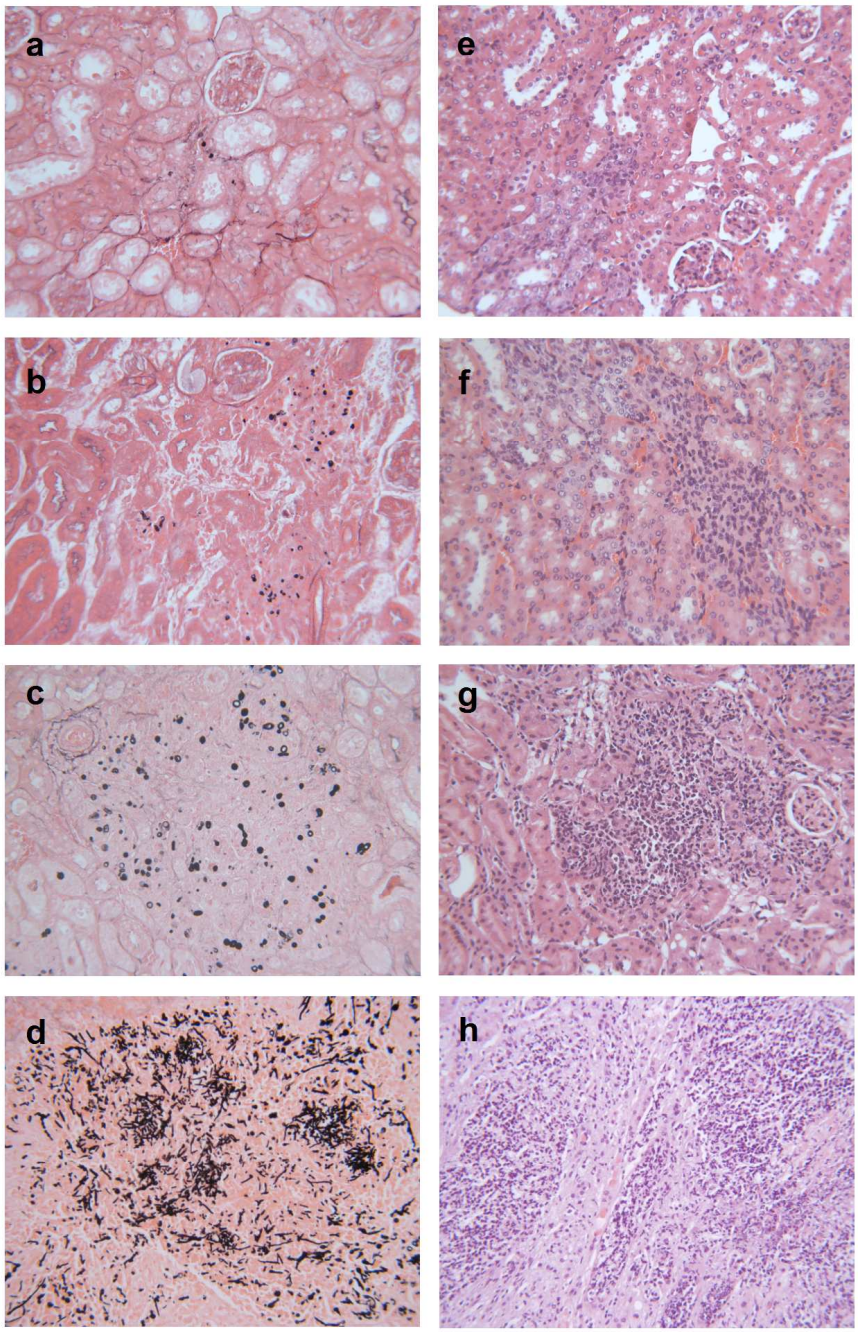


Figure 4. Quantification of tissue burden of organisms and inflammatory responses in kidneys of animals infected with isolates of *C. albicans* and *C. dubliniensis*. A scoring system of 0-4 for each parameter was developed. For quantification of fungal load, kidney slices were stained by methenamine-silver stain and scored as 0 (if no yeasts were visible) or as 1, 2, 3 or 4 (figures a, b, c and d respectively). For estimation of inflammatory responses, slices were stained with hematoxylin-eosin stain (H&E) and the amount of inflammatory cells was scored in the same way as fungal load (figures e, f, g and h respectively). Magnification 310x.

population/year) and age-specific incidence of candidemia in Iceland as well as national import of antifungals. Information regarding admissions at the 2 university hospitals was obtained from annual hospital reports and the incidence of candidemia per 1,000 and 100,000 admissions, respectively, was calculated from these numbers.

Patient survival (II,III)

Survival of adult patients was calculated from the date, when the first positive blood sample for *Candida* spp. was obtained for culture, until death or 31 December 2006. In paper III, patients with polymicrobial candidemia were excluded from analysis when we compared cluster-associated and sporadic infections with regard to case fatality.

In paper II, survival following candidemia was compared according to removal of CVCs, and by four 5-year intervals, with use of Kaplan-Meier analysis and the log-rank test. Odds ratios (OR) for mortality with 95% confidence intervals (95% CI) were calculated for the clinical variables. Univariate analyses were performed to identify risk factors associated with case fatality within 30 days after the blood sample was obtained for culture. Candidate variables, with a univariate $p<0.05$, as well as age and gender, were evaluated in a stepwise logistic regression model with death at day 30 as the dependent variable.

Virulence studies (IV)

Survival of animals was compared using Kaplan-Meier analysis and the log-rank test. We used the nonparametric Mann-Whitney test to compare yeast counts in internal organs (CFU/ml, [$\log_{10}$]) between groups. Variables significantly associated with mortality by univariate analysis were further assessed with stepwise multivariable logistic regression analysis with death at day 7 as the dependent variable. The following variables were tested: amount of hyphae (fungal load multiplied by the proportional amount of hyphae/pseudohyphae, 0-21); amount of yeast forms (fungal load multiplied by the proportional amount of yeast forms, 0-21); amount of mononuclear cells (0-4); amount of neutrophils (0-4) and fungal species (*C. albicans* vs. *C. dubliniensis*).

RESULTS

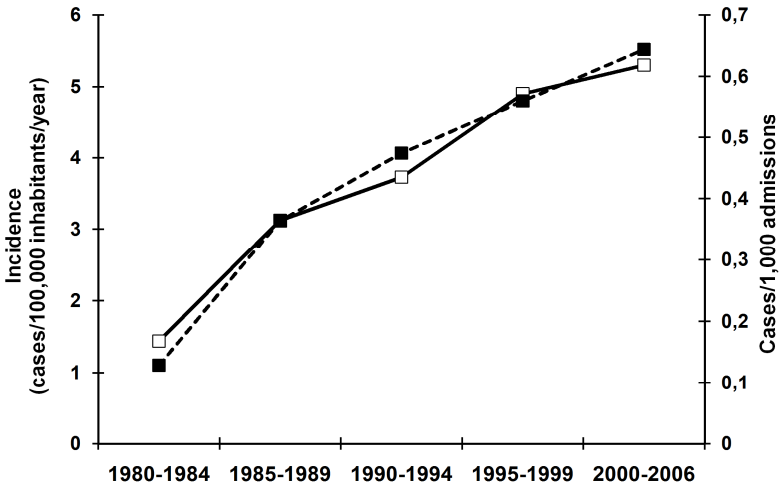
Incidence and patient demographics (I and unpublished)

During the 27-year period from 1980 through 2006, 281 episodes of candidemia in 270 patients were identified in Iceland, ranging from 2-3 cases/year during 1980-1983 to 19 cases/year in 2006. The average population-based incidence of candidemia was 3.8 cases per 100,000 population per year. The incidence increased from 1.4 during 1980-1984 to 5.3 during 2000-2006 ($p < 0.001$; Figure 5). A vast majority of cases (91%) occurred at the 2 large university hospitals, where the rate increased from 0.13 cases per 1,000 admissions during 1980-1984 to 0.64 cases per 1,000 admissions during 2000-2006 ($p < 0.001$; Figure 5). The use of blood cultures at the 2 university hospitals increased from 15,964 vials per year during 1990-1998 to 19,038 vials per year during 1999-2006. At the same time, the proportion of blood cultures positive for yeasts doubled, from 0.2% during 1990-1998 to 0.4% during 1999-2006 ($p < 0.001$).

Figure 5.

Increasing incidence of candidemia in Iceland, 1980-2006.

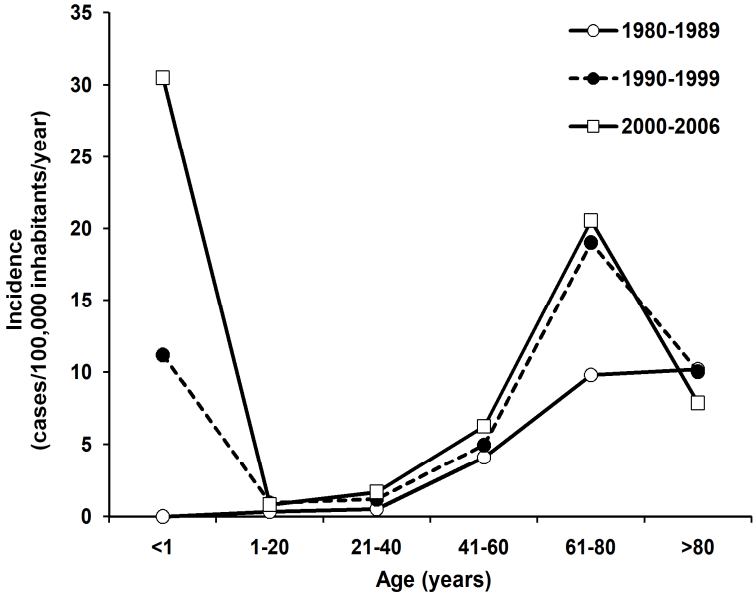
The open squares with solid lines represent the population-based incidence, whereas the filled squares with dashed lines depict the incidence as a proportion of 1,000 admissions to university hospitals.



The average population-based incidence was highest among children <1 years of age (12.0/100,000) and adults 61-80 years of age (16.5/100,000; Figure 6). No cases of candidemia were diagnosed among infants (<1 year) during 1980-1989, but the annual age-specific incidence increased from 11.2 cases per 100,000 children <1 year of age during 1990-1999 to 30.5 cases during 2000-2006 ($p = 0.003$). All these infants were <3 months of age and located in the NICU at the time of diagnosis. For

Figure 6.

Age-specific incidence of fungal bloodstream infections in Iceland from 1980-2006, expressed as cases per 100,000 population per year.



other age groups, the age-specific incidence was similarly distributed throughout the study period.

Infants <1 year of age accounted for 14 (5.0%) of 281 cases, children 1-16 years of age for 10 (3.6%) cases, adults 17-64 years of age for 126 (44.8%) cases, and elderly patients ≥65 years of age for 131 (46.6%) cases. Median age was 65 years among adults and 2 months among children. 55% of children and 58% of adults were males. There was a male dominance among infants aged <1 year (71%) and the elderly ≥65 years (60%), but the gender distribution was relatively equal among other age groups.

Most cases occurred in ICUs (100 [35.8%] of 279 cases), surgical wards (84 [30.1%] cases), and medical wards (64 [22.9%] cases). Emergency (2.5%) and gynecology services (0.4%) reported small percentages of episodes. Pediatric cases occurred in the NICU (14 cases, 5.0%), pediatric surgical or medical wards (8 cases, 2.9%) and in the ICU (2 cases).

Candida species (I and unpublished)

In total, 219 BSIs were available for definitive species identification and antifungal susceptibility testing. They were identified from 198 patients with candidemia, and represent isolates from 203 (94.4%) of a total of 215 episodes diagnosed in the country during 1991-2006. For other isolates, we report the antifungal susceptibility pattern and the species identified at the time of positive blood culture.

An overview of the different yeast species identified from blood during 1980-2006 and the frequency by which they occurred, is given in Table 2. Overall, the species distribution was stable. *C. albicans* was the most frequently isolated species (183 [61.6%] of 297 isolates), ranging between 59% and 65% of infections, and *C. glabrata* was the second most common pathogen, ranging from 13% to 15%. *C. tropicalis* emerged as the third most common pathogen (5% during 1985-1989 and 14% during 2000-2006, p = 0.027), whereas the incidence of *C. parapsilosis* decreased (12% in 1985-1989 and 5% in 2000-2006, p = 0.63). These 4 species accounted for 91.6% of the *Candida* BSIs. Species identification of all available isolates during 1991-2006, with use of phenotypic methods and PCR fingerprinting, revealed *C. dubliniensis* in 4% of episodes in 1995-1999 and 6% in 2000-2006. During the 27-year study period, the proportion of non-*C. albicans* species increased slightly, from 35% in 1980-1984 to 42% in 2000-2006 (p = 0.66).

Table 2. Species distribution of 297 fungal isolates causing bloodstream infections in Iceland, 1980-2006[a].

Yeast species	No. (%) of isolates					Total no. (%) of isolates
	1980-1984 *n* = 17	1985-1989 *n* = 41	1990-1994 *n* = 49	1995-1999 *n* = 72	2000-2006 *n* = 118	
C. albicans	11 (65)	26 (63)	32 (65)	45 (63)	69 (59)	183 (62)
C. glabrata		6 (15)	7 (14)	11 (15)	15 (13)	39 (13)
C. parapsilosis		5 (12)	4 (8)	9 (12)	6 (5)	24 (8)
C. tropicalis		2 (5)	4 (8)	4 (6)	16 (14)	26 (9)
C. dubliniensis				3 (4)	7 (6)	10 (3)
Other *Candida* spp.[b]	1 (6)		2 (4)		5 (4)	8 (3)
Other fungi[c]	5 (29)	2 (5)				7 (2)

[a] Episodes were caused by a single fungal species in 267 cases; 2 different *Candida* sp. were isolated simultaneously in 12 cases and 3 different *Candida* sp. in 2 cases.
[b] Other *Candida* spp. are, as follows: *Candida famata* (2 isolates), *Candida guilliermondii* (1), *Candida lusitaniae* (3 isolates), and *Candida krusei* (2 isolates).
[c] Other fungi are *Pichia anomala* (1 isolate) and yeast without definitive species identification (6 isolates, no longer available, presumed to be *Candida* isolates).

C. albicans was the most common cause of candidemia in all age groups (range, 57% to 86%; Table 3). *C. glabrata* showed a gradual increase with age, and caused 31 (19%) of 162 episodes in patients >60 years of age.

Table 3. Distribution of fungal isolates according to age group.

Yeast species	No. (%) of isolates					
	<1 y[a] *n* = 14	1-20 y *n* = 16	21-40 y *n* = 24	41-60 y *n* = 81	61-80 y *n* = 146	≥80 y *n* = 16
C. albicans	12 (86)	11 (69)	17 (71)	48 (59)	83 (57)	12 (75)
C. glabrata	1 (7)		1 (4)	6 (7)	28 (19)	3 (19)
C. parapsilosis	1 (7)	3 (19)		8 (10)	11 (8)	1 (6)
C. tropicalis			2 (8)	9 (11)	15 (10)	
C. dubliniensis			3 (13)	2 (3)	5 (3)	
Other fungi		2 (12)	1 (4)	8 (10)	4 (3)	

[a] All infants <3 months of age, in the NICU.

The majority of candidemic episodes among patients in ICUs and surgical wards were caused by *C. albicans* (68.6% and 64.8%, respectively). In contrast, 36 (52%) of 69 isolates cultured from patients in medical wards were identified as non-*C. albicans*, most commonly *C. glabrata* (16%) and *C. tropicalis* (12%).

Antifungal susceptibility testing (I and unpublished)

Susceptibility testing for amphotericin B, fluconazole, and itraconazole was performed for all available BSIs (219 isolates). A summary of the *in vitro* susceptibility test results is given in Table 4. All isolates tested were susceptible to amphotericin B (MIC, ≤ 1 μg/ml). In total, 214 isolates (97.7%) were susceptible to fluconazole (MIC, ≤8 μg/ml), including all *C. albicans*, *C. tropicalis*, *C. parapsilosis* and *C. dubliniensis* isolates. Four *C. glabrata* isolates (13%) and 1 *C. krusei* isolate had decreased susceptibility to fluconazole (MICs, ≥ 16 μg/ml), but no isolates were fully resistant (MIC, ≥ 64 μg/ml). The proportion of isolates highly susceptible to fluconazole (MIC, ≤ 1 μg/ml) remained relatively stable at ~80% during 1991-2006, without significant change in the distribution of MICs of fluconazole between the first (1991-1999) and second half (2000-2006) of the study period (p = 0.90), with a median MIC of 0.25 μg/ml during both time periods. Most isolates (86.3%) were susceptible to itraconazole (MIC, ≤ 0.125 μg/ml). Itraconazole resistance (MIC, ≥ 1 μg/ml) was observed for 5% of all *Candida* isolates tested and was highest among *C. glabrata* (30%).

Table 4. *In vitro* susceptibilities of *Candida* bloodstream isolates.

Species (no. of isolates tested)	Antifungal agent	MIC (μg/ml)		
		MIC range	MIC_{50} [a]	MIC_{90} [b]
C. albicans (135)	Amphotericin B	0.002-0.38	0,094	0,125
	Fluconazole	0.047-8.0	0,19	0,5
	Itraconazole	0.004-0.75	0,023	0,094
C. glabrata (30)	Amphotericin B	0.064-0.75	0,25	0,38
	Fluconazole	0.25-48.0	4,0	12,0
	Itraconazole	0.064-32.0	0,38	3,0
C. tropicalis (20)	Amphotericin B	0.002-0.5	0,125	0,38
	Fluconazole	0.125-4.0	0,38	1,0
	Itraconazole	0.003-0.064	0,016	0,047
C. parapsilosis (19)	Amphotericin B	0.047-0.25	0,125	0,25
	Fluconazole	0.125-8.0	0,75	2,0
	Itraconazole	0.008-0.19	0,064	0,125
C. dubliniensis (10)	Amphotericin B	0.012-0.125	0,023	0,094
	Fluconazole	0.047-8.0	0,19	2,0
	Itraconazole	0.004-0.5	0,064	0,38

[a] MIC at which 50% of the isolates tested are inhibited.
[b] MIC at which 90% of the isolates tested are inhibited.

Import of antifungal agents (I and unpublished)

The national import of selected antifungal agents from 1990 through 2006 is shown in Figure 7a-c (shown for all registered antifungals during 1990-1999 in paper I, Table 3). Amphotericin B and fluconazole (Figure 7a and 7b) were probably mainly used for the treatment of IC, whereas the primary indications for itraconazole, terbinafine and ketoconazole in recent years have been onychomycosis and fungal skin infections (Figure 7c). Fluconazole was approved for oral and parenteral use in 1990. During the period from 1991 to 2006, the use of oral formulations increased from 2.6 to 15.9 DDD per 100,000 inhabitants per day (9.4 to 58.1 DDD/1,000 inhabitants/year) (512%) and the import of fluconazole for parenteral use increased from 0.14 to 1.65 DDD per 100,000 inhabitants per day (0.5 to 6.0 DDD/1,000 inhabitants/year) (1080%). Voriconazole and caspofungin were first imported in 2002 and 2003, respectively. Voriconazole use increased from 0.03 to 0.13 DDD/100,000 inhabitants/day during 2002-2006 and caspofungin use increased from 0.02 to 0.05 DDD/100,000 inhabitants/day during 2003-2006.

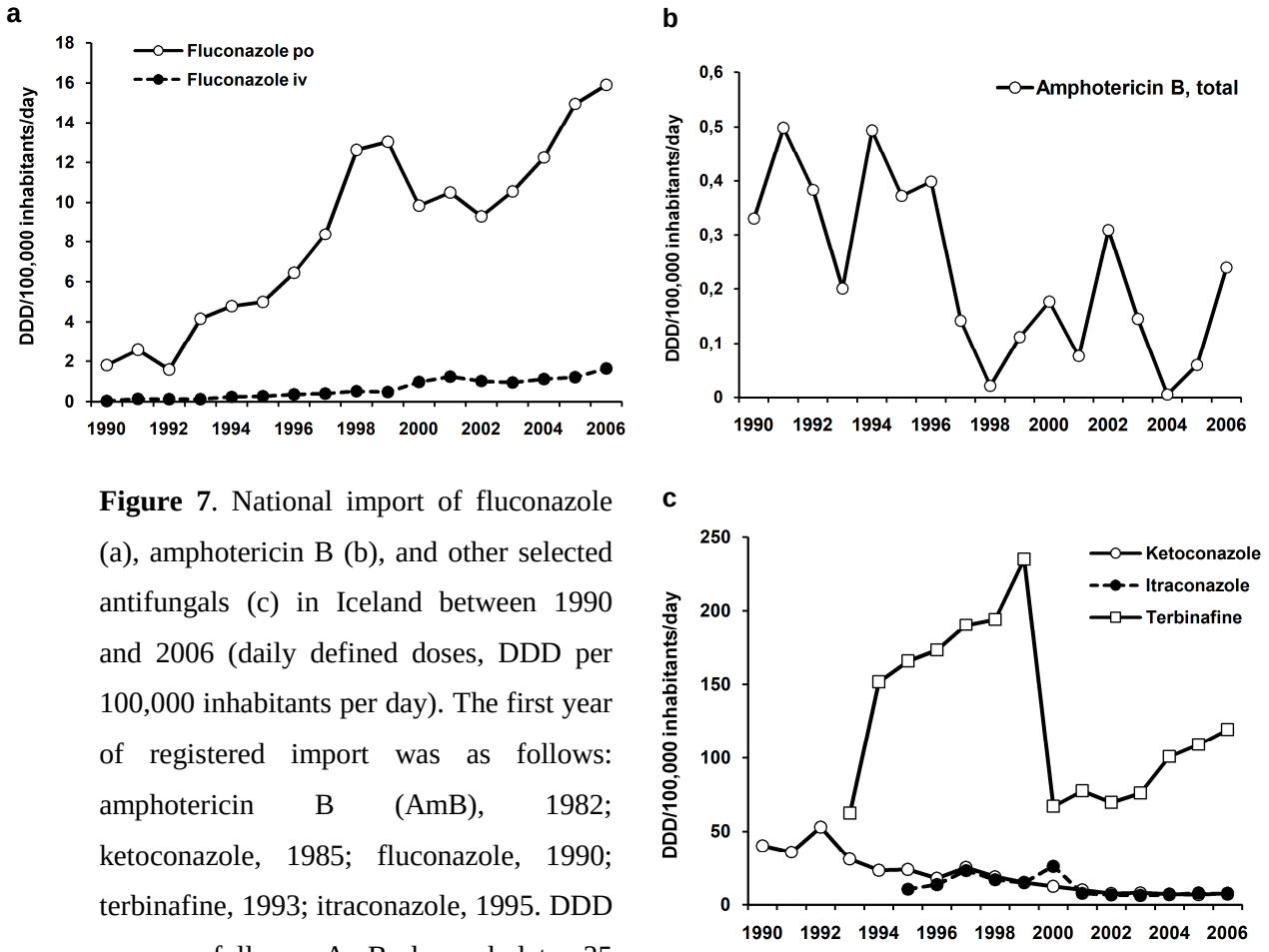


Figure 7. National import of fluconazole (a), amphotericin B (b), and other selected antifungals (c) in Iceland between 1990 and 2006 (daily defined doses, DDD per 100,000 inhabitants per day). The first year of registered import was as follows: amphotericin B (AmB), 1982; ketoconazole, 1985; fluconazole, 1990; terbinafine, 1993; itraconazole, 1995. DDD were as follows: AmB deoxycholate, 35 mg; lipid-based AmB, 175 mg; fluconazole, itraconazole, and ketoconazole, 200 mg each; terbinafine, 250 mg.

Risk factors for candidemia and association with outcome (II)

Patient demographics

Medical charts of all patients with candidemia in Iceland from 1 January 1980 to 31 December 1999 were reviewed. During the 20-year study period, 172 episodes of candidemia, in 165 patients, were diagnosed. Data on hospital characteristics and outcome were available for all episodes and clinical data were available for 162 episodes (94.2%) in 155 patients.

A detailed description of the study cohort is given in Table 5. In total, 4% of patients had recurrent episodes, occurring at least 1 month apart; 5 had 2 separate episodes and one patient had 3 episodes. A single *Candida* sp. was cultured from blood in 166 cases (96.5%), 2 different *Candida* sp. were isolated simultaneously in 5 cases, and 3 different *Candida* sp. were isolated in 1 case.

Table 5. Summary of 172 candidemic episodes in Iceland, 1980-1999.

Variable	No. (%), by age group		
	≤16 years	>16 years	Total
Patients (*n* = 165)			
Male	7 (64)	84 (55)	91 (55)
Female	4 (36)	70 (45)	74 (45)
Median age in years (range)	1 (0-2)	66 (17-96)	64 (0-96)
Median days in hospital before candidemia (range)	33 (12-384)	22 (0-166)	23 (0-384)
Median days of hospital stay (range)	121 (50-771)	53 (3-269)	56 (3-771)
Single episode	10 (91)	149 (97)	159 (96)
Recurrent episodes [a]	1 (9)	5 (3)	6 (4)
Episodes (*n* = 172)			
Hospital location[b]			
ICU	2 (16)	58 (37)	60 (35)
Adult surgery	…..	62 (39)	62 (36)
Adult medicine	…..	39 (24)	39 (23)
NICU	5 (42)	…..	5 (3)
Other pediatric wards	5 (42)	…..	5 (3)
Source of infection[c]			
Catheter-associated	2 (17)	78 (54)	80 (51)
No demonstrated source	8 (67)	48 (33)	56 (36)
Disseminated infection	2 (17)	16 (11)	18 (12)
Other sources[d]	0	3 (2)	3 (2)
Fungal species (*n* = 179)			
Candida albicans	9 (75)	105 (63)	114 (64)
Candida glabrata	1 (8)	23 (14)	24 (13)
Candida parapsilosis	2 (17)	16 (10)	18 (10)
Candida tropicalis	0	10 (6)	10 (6)
Candida dubliniensis	0	3 (2)	3 (2)
Other fungal species	0	10 (6)	10 (6)

NOTE. There are minor differences in species distribution and total number of *Candida* BSIs compared with Table 1 in paper II, since PCR fingerprinting analyses revealed a previously unidentified polyfungemia in 2 cases.

[a] Five patients had two separate episodes of candidemia and one patient had three episodes.
[b] Information was missing for 1 episode.
[c] Information was available for 157 cases.
[d] Urosepsis in 2 cases and factitious sepsis in 1 case.

Most cases occurred in adult surgery wards (62 [36%] of 171 cases), ICUs (60 [35%] cases) and adult medicine wards (39 [23%] cases). Seven cases (4%) were community acquired. The first cases of candidemia among children (≤ 16 years) were diagnosed in 1993.

Overall, 51% of candidemic episodes were catheter-related, as demonstrated by a positive culture of fungi from catheter tips (Table 5). The proportion of catheter-related infections was significantly higher among adults (54% [78 of 145]) compared to children (17% [2 of 12]; p = 0.016).

An overview of concomitant conditions and risk factors is shown in Table 6.

Table 6. Selected concomitant conditions, risk factors and symptoms for 172 episodes of candidemia, Iceland, 1980-1999.

	n/N (%) of episodes		
Variable	Children (≤16y)	Adults (>16y)	p value
Underlying condition			
Solid organ malignancy	0	53/150 (35)	0.009
Gastrointestinal diseases	3/12 (25)	26/150 (17)	0.45
Renal failure	2/12 (17)	21/150 (14)	1.0
Hematological malignancy	0	20/150 (13)	0.36
Prematurity	5/12 (42)	…..	…
Diabetes mellitus	0	11/150 (7)	1.0
Risk factor			
Preceding infections[a]	8/12 (67)	131/149 (88)	0.06
Other bloodstream infections	5/12 (42)	54/149 (36)	0.76
Line infections	1/12 (8)	25/149 (17)	0.69
Abdominal surgery[b]	0	80/149 (54)	0.001
Antimicrobial agents[a]	11/12 (92)	131/149 (88)	1.0
Corticosteroids[c]	7/11 (64)	33/147 (22)	0.006
Hyperalimentation	9/12 (75)	118/150 (79)	1.0
Neutropenia[d]	0	17/150 (11)	0.62
Vascular catheters, any	12/12 (100)	135/148 (91)	0.60
Central venous catheter[e]	12/12 (100)	130/148 (88)	0.36
Arterial catheter	6/12 (50)	46/148 (31)	0.21
Other catheters	2/12 (17)	13/148 (9)	0.31
Symptoms[f]			
Fever ≥38,5°C	6/12 (50)	144/148 (97)	<0.001
Septic shock	2/12 (17)	23/148 (16)	1.0
Dyspnoea	5/12 (42)	3/148 (2)	<0.001

NOTE. n, number of patients with a given characteristic; N, total number of patients evaluated.

[a] Within 2 weeks before positive blood culture sample was obtained.
[b] Within 1 month of candidemia.
[c] Within 24 hrs of blood culture.
[d] Absolute neutrophil count of ≤500 cells/mm^3.
[e] All umbilical catheters in pediatric patients are classified as CVCs.
[f] Within 12 hrs from positive blood culture for fungi.

None of the patients had HIV infection. Common treatment-related risk factors included indwelling vascular catheters (92%), antimicrobial agents (88%), hyperalimentation (78%) and prior surgery (68%). There were no significant differences between adults and children with respect to intravascular catheters in place, preceding infections, and previous receipt of antibiotics or hyperalimentation. The clinical presentation of candidemia differed between age groups as shown in Table 6.

Therapy

Data on antifungal therapy were available for 148 episodes among adults (Table 7). In total, systemic antifungal therapy was administered in 70% of cases; the proportion increased from 61% in 1980-1989 to 74% in 1990-1999 (p = 0.013). In the subset of adults who received treatment, amphotericin B (monotherapy or

Table 7. Treatment approaches in adult patients with candidemia[a].

Variable	No (%) within each period			
	1980-1984	1985-1989	1990-1994	1995-1999
Number of patients	17	38	45	60
Number of patients with CVCs	16 (94)	30 (91)	37 (90)	47 (83)
CVC management				
Promptly removed	6 (38)	25 (83)	22 (60)	39 (83)
Retained	10 (63)	5 (17)	15 (41)	8 (17)
Antifungal therapy	5 (29)	25 (78)	30 (71)	43 (75)
Amphotericin B alone	-	12 (38)	12 (29)	6 (11)
Fluconazole alone	-	-	7 (17)	25 (44)
Amphotericin B and fluconazole[b]	-	-	6 (14)	11 (19)
Amphotericin B and 5-flucytosine[b]	2 (12)	9 (28)	3 (7)	1 (2)
5-flucytosine monotherapy	2 (12)	2 (6)	1 (2)	-
Other[c]	1 (6)	2 (6)	1 (2)	-
None	12 (71)	7 (22)	12 (29)	14 (25)
Combined prompt CVC-removal and antifungal therapy:	3 (19)	21 (70)	18 (49)	32 (68)

NOTE. Discrepancies between Table 7 and Table 3 in paper III result from re-analysis of the data with use of more stringent classification of antifungal therapy. N/A, not applicable.

[a] Information was available for 148 episodes.
[b] In combination or in any sequence.
[c] Treatment with amphotericin B, fluconazole and 5-flucytosine in combination or any sequence or ketoconazole monotherapy.

combination therapy) was administered in 80% of cases during 1980-1989, compared with 55% during 1990-1999 (p = 0.03). During 1990-1999, 68% of treated adults received fluconazole alone or in combination.

All children and 88% of adults had a CVC in place when blood sample was obtained for culture and the CVC was promptly removed (i.e., <2 days from diagnosis of candidemia) in 71% and 57% of infections in adults and children respectively. A significant shift towards more aggressive treatment among adult patients was observed during the study period (p = 0.02, Table 7).

Patient outcomes

Survival analyses were performed for adult patients only (1980-1999, 160 cases; 2000-2006, 96 cases). A statistically significant improvement in patient survival among adult patients with candidemia was noted during 1980-1999 (p = 0.02, log-rank test; paper II, Figure 2). The survival rate during 1980-1984 was 41.2% with a steady improvement noted during the ensuing 15 years, with 73.3% survival during 1995-1999. However, during 2000-2006, the 30-day survival rate among adults decreased to 60.4% (Figure 8).

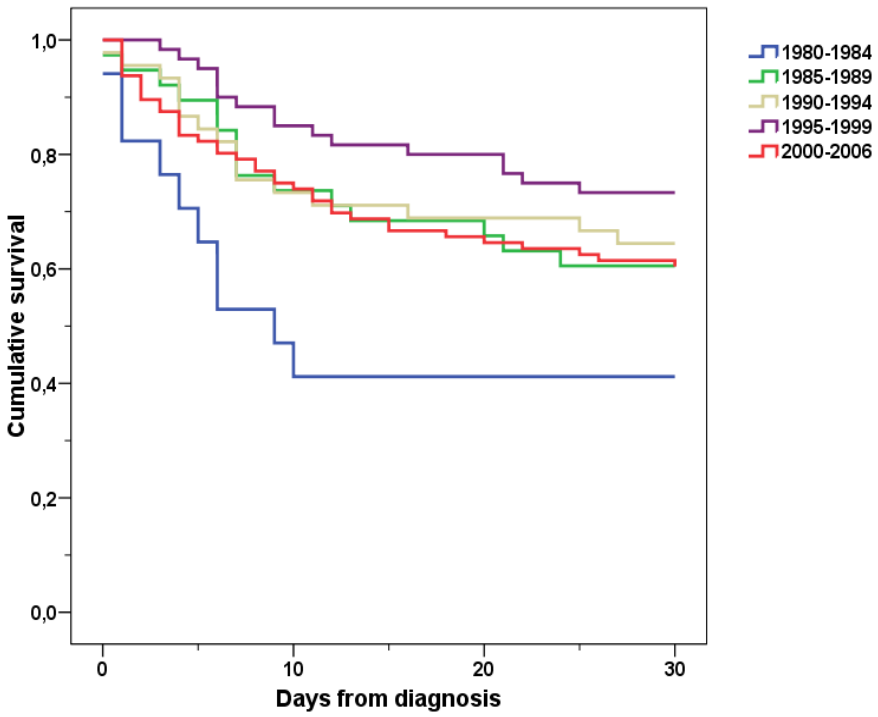


Figure 8. Kaplan-Meier survival analysis for adult patients with candidemia in Iceland, 1980-2006. As shown, the 30-day case fatality for candidemic patients decreased steadily during 1980-1999, but increased in 2000-2006 (p = 0.046, log-rank test).

Table 8. Univariate analysis for 30-day case fatality among adult patients with candidemia. Variables significantly associated with outcome.

Variable	n/N (%) Lived (n = 103)	Died (n=57)	p value	OR (95% CI)
Clinical characteristics				
Recent or ongoing pneumonia	19/97 (20)	24/52 (46)	0.001	3.52 (1.68-7.38)
Fever ≥38.5°C	97/97 (100)	47/51 (92)	0.013	1.09 (1.00-1.18)
Hypotension/shock	7/97 (7)	16/51 (31)	<0.001	5.88 (2.23-15.51)
Change of mental status	9/97 (9)	12/51 (24)	0.035	3.01 (1.17-7.72)
Use of corticosteroids	13/97 (13)	20/50 (40)	0.001	4.31 (1.91-9.72)
Serum-sodium (Na^+) (mmol/l, mean ± SD)	136 ± 5.7	141 ± 9.7	0.004	1.09 (1.03-1.15)[a]
Duration of symptoms preceding diagnosis (days, mean ± SD)	2.7 ± 1.3	3.4 ± 2.4	0.030	1.24 (1.02-1.51)[b]
Treatment-related factors				
CVC promptly removed	70/85 (82)	22/45 (49)	<0.001	0.21 (0.09-0.46)
Any antifungal therapy	75/97 (77)	29/52 (56)	0.011	0.37 (0.18-0.76)

NOTE. n, number of patients with a given characteristic; N, total number of evaluable patients with a given characteristic. N/A, not applicable; OR, odds ratio; CI, confidence interval.

[a] Increase in OR for each 1mmol/l increase (range:110-175 mmol/l).
[b] Increase in OR for each 1 day increase in duration of symptoms.

Parameters that were significantly associated with 30-day mortality by univariate analysis are shown in Table 8, including septic shock at the time of blood culture, prior pneumonia and corticosteroid use. In addition, a change in mental state at the time of blood culture was associated with poor prognosis. When patient outcome was studied according to therapeutic interventions, antifungal therapy and prompt removal of potentially infected CVCs were both significantly associated with improved outcome. Kaplan-Meier survival analysis further revealed that the mortality of patients who had their CVCs retained was significantly higher than for patients who had their catheters removed ($p < 0.001$, log-rank test; paper II, Figure 3).

Multivariable logistic regression analysis was performed to study the association of these parameters with outcome, along with age and sex of the patient. In the final model (paper II, Table IV), 3 parameters were independently associated with outcome 30 days from the time of blood culture. Prompt removal of CVCs was significantly associated with improved outcome (OR, 0.22; 95% CI, 0.08-0.61; p=0.004), whereas septic shock (OR, 8.01; 95% CI, 2.25-28.55; p=0.001), and

increasing levels of serum-sodium (Na^+) (OR, 1.15; 95% CI, 1.05-1.25; p = 0.002) were associated with increased mortality. Eleven patients (7%) died before the diagnosis of candidemia was made. Consequently they did not have their vascular catheters removed nor did they receive antifungal treatment. We, therefore, repeated the analysis after excluding the patients who died within 72 h of the infection (paper II, Table IV, right column), but this did neither change the hierarchy nor the significance of the individual parameters.

Molecular epidemiology of candidemia (III)

Epidemiology

In order to estimate the proportion of clusters of candidemic episodes we studied the genetic relatedness of all obtainable fungal BSIs in Iceland during a 15-year period, 1991-2006. A description of the study cohort is included in paper III, Table 1. The mean incidence of candidemia in Iceland from 1991 to 2006 was 4.8 cases per 100,000 population per year; there was an increase from 3.7 cases per 100,000 population per year during 1991-1994 to 5.8 cases per 100,000 population per year during 2003-2006. The number of episodes varied greatly during the study period, ranging from 4 in 1991 to 19 in 2006.

Microbiology

We obtained 219 BSIs which were cultured from 198 patients with candidemia and represent isolates from 94.4% of all episodes diagnosed during 1991-2006. The isolates were most commonly cultured from patients in ICUs (35.2%) and adult surgery wards (28.7%) (paper III, Table 1). Thirteen isolates (6.0%) were cultured from children in the NICU. *C. albicans* represented 61.6% of the isolates (135 isolates), *C. glabrata* 13.7% (30), *C. tropicalis* 9.1% (20), *C. parapsilosis* 8.7% (19), *C. dubliniensis* 4.6% (10), and other *Candida* species 2.3% (5) (paper III, Figure 1).

PCR fingerprinting

PCR with primers M13 and $(GACA)_4$ identified the greatest number of genotypes (paper III, Table 2): M13 had the greatest discriminatory power for identifying *C. albicans* and *C. parapsilosis*, $(GACA)_4$ had the greatest power for identifying *C. glabrata* and *C. tropicalis*, and both primers identified an equal number of genotypes of *C. dubliniensis*.

Molecular epidemiology

A summary of the molecular epidemiology of bloodstream infections attributable to *C. albicans* is given in paper III, Table 3. PCR fingerprinting with the M13 primer revealed 35 different genotypes (GTs). The 2 most prevalent genotypes (GT-2 and GT-4) caused 24% of all infections and were endemic during almost the entire study period. GT-32 caused 8% of all infections and was prevalent during 1994-2001 but was not identified after that. During 2005-2006, 2 new genotypes emerged (GT-1 and GT-22) that caused almost one-half (11 of 23) of all infections during the 2-year period. A dendrogram showing 18 of the most common *C. albicans* genotypes and their corresponding PCR profiles is depicted in paper III, Figure 2.

The molecular epidemiology of bloodstream infections caused by non-*C. albicans* species is summarized in Table 9. Two endemic genotypes of *C. glabrata* (GT-39 and GT-45), and 1 of *C. parapsilosis* (GT-54) were identified. Oher non-*C. albicans* GTs were sporadically encountered.

Table 9. Molecular epidemiology of *Candida* non-*albicans* bloodstream infections in Iceland, 1991-2006.

		Genotype														
		C. glabrata				*C. tropicalis*					*C. parapsilosis*			*C. dubliniensis*		
Year	No. of isolates	39	43	45	Other[a]	46	47	49	52	Other	53	54	Other	57	60	Other
1991	1			1												
1992	1						1									
1993	2			1								1				
1994	6			2			1					3				
1995	3	1			1							1				
1996	6						1					3	1		1	
1997	8	1		1	1		1			1		2			1	
1998	4	1		2							1					
1999	5	1			2								1		1	
2000	5	1							1	1	1	1				
2001	3			1	1									1		
2002	6	1			1	1		1				2				
2003	9	1	1	2				2	1	1						1
2004	8	1	1	2				1				1		2		
2005	6	1						2				1			1	1
2006	6				1	4										1
Total	79	9	2	12	7	5	4	6	2	3	2	15	2	3	4	3

NOTE. Results for each species are based on PCR fingerprinting with the primer with the greatest discriminatory power for that particular fungal species: $(GACA)_4$ for *C. glabrata* and *C. tropicalis*, and M13 for *C. parapsilosis* and *C. dubliniensis*. Strains with a similarity coefficient of ≥ 90% were classified within the same genotype. Genotypes are arbitrarily numbered.

[a] Other genotypes comprised 1 isolate each.

Table 10. Clustered episodes of candidemia.

Criteria for clonality	No. of infections caused by clustered *Candida* spp.					No. (%) of episodes caused by clustered isolates
	Candida albicans (*n* = 128)	*Candida glabrata* (*n* = 22)	*Candida tropicalis* (*n* = 16)	*Candida parapsilosis* (*n* = 16)	*Candida dubliniensis* (*n* = 7)	
Same hospital						
100%	27	0	4	5	0	36 (18.7)
≥90%	29	0	4	5	0	38 (19.7)
Same hospital ward						
100%	12	0	2	0	0	14 (7.3)
≥90%	12	0	2	0	0	14 (7.3)

NOTE. Clusters comprised clonal isolates that were cultured from ≥2 patients at the same hospital within a period of 90 days. Results for each species are based on PCR fingerprinting with the primer with the greatest discriminatory power for that particular fungal species: M13 for *C. albicans*, *C. parapsilosis* and *C. dubliniensis*, and $(GACA)_4$ for *C. glabrata* and *C. tropicalis*. Two clusters involved 3 cases, the remainder involved 2 cases. Patients with polymicrobial candidemia (n=12) are excluded.

Nosocomial clusters.

The proportion of infections caused by clustered isolates is summarized in Table 10. When 100% similarity for the primer with the greatest discriminatory power was set as the definition of clonality, the proportion was 18.7% (36 cases). If ≥90% similarity was used as the cutoff, the proportion of candidemia cases caused by strains within the same genotype clusters was 19.7% (38 cases). For comparison, when data was analyzed by ward, with use of both 100% and ≥90% as the cutoff, the proportion was 7.3% (14 cases) for both criteria. These clusters occurred in ICUs (6 cases), the NICU (6 cases) and in a surgical ward (2 cases).

When the PCR results from all 4 primers were combined and used for analysis, the proportion of infection clusters at the same hospital was 39.9% (n = 77) if ≥ 90% similarity was required.

With use of 19.7% as a reference, the average rate of cluster-associated cases of candidemia was 11.3 per 100,000 hospital admissions per year. Clusters were small; >80% involved 2 cases, and the remainder involved 3 cases. The time and species distribution of infection clusters is shown in Figure 9. The majority of clustered isolates (22 isolates, 58%) were cultured from samples from patients in ICUs and the NICU, followed by surgery wards (26%), medicine wards (11%) and other wards (5%). The proportion of clustered isolates with similarity coefficient ≥90% was significantly higher in wards providing intensive care (i.e., ICUs and the NICU) than

in other wards (27% [22 of 82] and 15% [16 of 108], respectively; OR, 2.11; 95% CI, 1.03-4.34; p = 0.045). Clusters were particularly prevalent in the NICU, where 53.8% of isolates (7 of 13), all *C. albicans*, were part of clusters. *C. albicans* was the pathogen in 85% of all candidemias in the NICU. The proportion of clustered isolates was significantly higher among pediatric patients than among adults (45% [9 of 20] vs. 17% [29 of 170]; p = 0.007).

Figure 9.

Clusters of candidemia as a function of time, *Candida* species, and location of patients at the time of positive blood culture.

ICU, intensive care units and neonatal intensive care unit; S, surgery; M, medicine; O, other wards; BSIs, bloodstream isolates.

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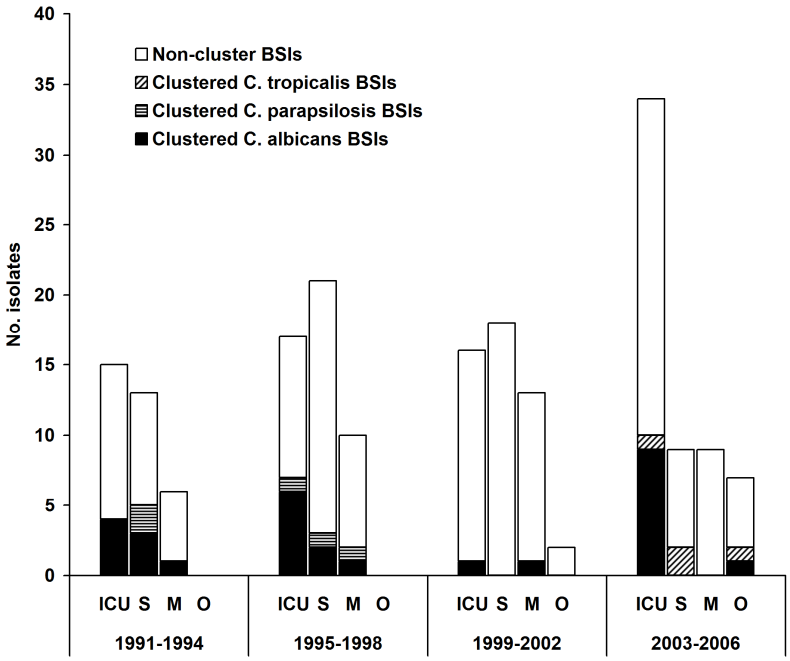

When different *Candida* species were compared, the proportion of clustered isolates was highest for *C. parapsilosis* (31%), which was significantly higher than among other non-*C. albicans* species (8%; p = 0.034). The clustered *C. parapsilosis* isolates all belonged to the prevalent GT-54. Clustered episodes of *C. tropicalis* infections were caused by GT-46 (in 2006) and GT-49 (in 2005), respectively. For *C. albicans*, 29 isolates (23%) were part of clusters, with 3 genotypes (33 [24% of isolates]) causing 8 (53%) of 15 clusters. GT-2 caused 4 clusters in the study period, and 50% of strains of that genotype were implicated in infection clusters. GT-32 caused 2 clusters during 1994-1995. GT-22 emerged in 2006 and caused 2 clusters of candidemia, 1 in the NICU and 1 in an ICU/surgery ward. All 4 strains of that genotype were part of clusters.

Case fatality

Survival data were available for 197 patients. During the 16 years of the study, 32 (16%) patients died within 7 days and 63 (32%) patients died within 30 days after blood samples were obtained for culture. The 7-day and 30-day case-fatality rates for cluster-associated cases did not differ statistically significantly from those of sporadic nosocomial infections.

C. albicans and *C. dubliniensis*: comparison of virulence and histopathological changes (IV)

Candida isolates

The virulence of *C. albicans* and *C. dubliniensis* was compared using a murine model of bloodstream infection. A description of the 3 *C. albicans* and 9 *C. dubliniensis* isolates used is given in paper IV, Table 1. The source patients had a wide variety of underlying conditions, most commonly hematological and solid organ malignancies with or without neutropenia.

All *Candida* isolates were susceptible to fluconazole (MIC, ≤ 8 μg/ml) and itraconazole (MIC, ≤ 0.125 μg/ml). One Irish *C. dubliniensis* isolate was resistant to amphotericin B (MIC, >1 μg/ml); all others were susceptible.

All isolates were identified to the species level with use of ITS sequence analysis and the *C. dubliniensis* isolates were genotyped by PCR with genotype-specific primer pairs (99) (paper IV, Figure 2). Six (67%) of the 9 *C. dubliniensis* strains, including 4 of the 5 Icelandic strains, belonged to genotype 1. Two strains belonged to genotype 2 and 1 Icelandic strain (IS-124) yielded amplimers with both genotype 1- and genotype 2-specific primer pairs, respectively. By PCR, all isolates had distinct PCR fingerprinting profiles without evidence of clonality (paper IV, Figure 2).

Survival of animals

After 7 days of observation, a total of 21.0% (33/157, 7 experiments) of mice infected with *C. dubliniensis* strains had died, compared with 23.2% (23/99) for *C. albicans* ($p = 0.65$). Marked variation in survival was observed depending on the strains used (Figure 10). This applied to both *Candida* species, with most fatalities occurring on days 4 and 5. The 2 most virulent *C. dubliniensis* strains (IS-30 and IS-

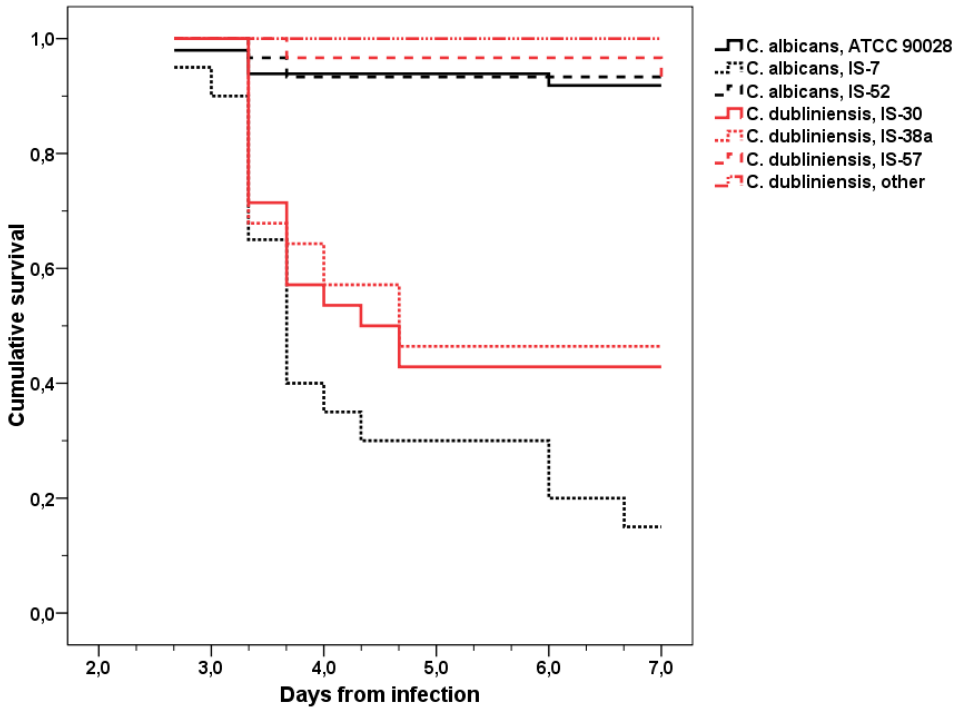

Figure 10. Survival of mice infected by *C. albicans* (3 isolates, *n* = 99) compared to *C. dubliniensis* (9 isolates, *n* = 157). Survival was monitored 3 times per day for one week. Other *C. dubliniensis* strains: IS-124; IS-176; CD-541; 1329; 1862 and CBS 8500.

38a) showed virulence comparable to the most virulent C. albicans strain (IS-7), but 6 of the 9 C. dubliniensis strains tested were not associated with any deaths.

Load of organisms in internal organs

A detailed overview of the results from colony counts in internal organs of infected animals is given in Table 11. For both species a significantly greater load of yeasts was noted in kidneys (median, 2.7 x 10^4 CFU/ml, range, 0 - 3.5 x 10^7) compared with livers (median 7.3 x 10^2, range, 0 - 2.5 x 10^6; p = 0.001). Colony counts in both organs did not differ significantly between fungal species for animals given the same infective dose (1-2 x 10^6 organisms; p > 0.4). A wide variation in tissue burden was noted, with as high as 1000-fold difference between strains belonging to the same *Candida* species.

Histopathological analysis in kidneys

The results from the blinded histopathological analysis are summarized in paper IV, Table 3. Kidneys from 235 animals were examined; 89 were seeded with *C. albicans*

Table 11. *In vivo* virulence of *C. albicans* and *C. dubliniensis* in a murine model of blood stream infection.

Species	Isolate	No. of exp.	No. of mice	Median inoculum (organisms)[a]	Mean survival time (d)	No. of survivors at day 7 (%)	Liver (CFU/ml), median (range)	Kidney (CFU/ml), median (range)
C. dubliniensis	IS-30	3	28	1.0×10^6	5.11	12 (42.9)	3.1×10^4 (0-2.5×10^6)	2.4×10^5 (0-3.5×10^7)
	IS-38a	3	28	1.1×10^6	5.24	13 (46.4)	2.0×10^4 (9.2×10^1-1.9×10^6)	1.2×10^5 (1.2×10^4-1.2×10^7)
	IS-57	3	30	1.2×10^6	6.89	28 (93.3)	9.9×10^1 (0-1.8×10^4)	2.2×10^4 (3.3×10^2-1.8×10^6)
	IS-124	2	21	1.0×10^6	7	21 (100)	9.7×10^2 (9.2×10^1-9.8×10^4)	6.3×10^4 (7.4×10^1-6.6×10^5)
	IS-176	1	10	1.0×10^6	7	10 (100)	2.9×10^2 (9×10^1-1.5×10^3)	1.7×10^4 (4×10^3-5.5×10^4)
	CD-541	1	10	1.0×10^6	7	10 (100)	5.7×10^3 (1.5×10^3-2×10^4)	1.3×10^4 (2.4×10^3-5.8×10^4)
	1329	1	10	1.1×10^6	7	10 (100)	1.5×10^3 (2.3×10^2-9.5×10^3)	2.4×10^3 (6.1×10^2-8.6×10^3)
	1862	1	10	1.0×10^6	7	10 (100)	6.3×10^1 (1.3×10^1-5.3×10^2)	4.1×10^3 (9.6×10^2-7.4×10^3)
	CBS 8500	1	10	1.1×10^6	7	10 (100)	2.8×10^2 (9.8×10^1-1.2×10^4)	1.9×10^4 (5.7×10^3-6.7×10^4)
C. albicans	ATCC 90028	5	49	1.1×10^6	6.74	45 (91.8)	2.3×10^2 (0-1.1×10^5)	1.0×10^4 (4.7×10^2-3.3×10^6)
	IS-7	2	20	1.0×10^6	4.43	3 (15.0)	2.9×10^4 (3×10^3-5.3×10^4)	3.4×10^6 (8.6×10^3-7.9×10^6)
	IS-52	3	30	1.1×10^6	6.77	28 (93.3)	5.5×10^2 (2×10^1-1.5×10^6)	2.2×10^4 (3.1×10^3-1.2×10^5)

[a] The lethal dose needed to kill 50% of the animals (LD_{50}) was determined for *C. albicans* ATCC 90028 and subsequent experiments were conducted using this strain as a reference.

strains and 146 with *C. dubliniensis* strains (89% and 93% of infected animals respectively).

The fungal load observed by histopathological analysis did not differ between the two species ($p = 0.078$). When all kidneys were analyzed, the yeasts were predominantly in the yeast form in 77.9% of sections whereas the hyphal form prevailed in 20.4%. *C. albicans* strains formed pseudohyphae and true hyphae to a significantly greater degree than *C. dubliniensis* strains ($p < 0.001$), in most part due to the intense formation seen in animals infected with the highly virulent IS-7 (paper IV, Table 3). Similar results were obtained when the highly virulent strains of both species were analyzed independently; the hyphal form dominated in 16 (80%) of 20 kidneys infected with IS-7, compared with 20 (36%) of 56 kidneys infected with the highly virulent *C. dubliniensis* strains, IS-30 and IS-38a ($p = 0.001$). Yeasts and inflammatory cells were equally distributed throughout different parts of the kidneys and their location was not associated with fungal species or mortality. In 4 sections (1.7%), no yeasts were visible.

The type of inflammatory infiltrate was not significantly different between fungal species. Mixed infiltrate of neutrophil and mononuclear cells, representing acute and chronic inflammation, was observed in almost half of kidney sections (115/235), with neutrophils predominating in 76 (32.3%) specimens and mononuclear cells in 44 (18.7%). Significantly greater inflammation was noted in the kidneys after infection with *C. albicans* compared to *C. dubliniensis* ($p < 0.001$), although marked variation was again observed for different strains of each species. This difference remained significant when highly virulent strains of both species were independently analyzed (IS-7 vs. IS-30 and IS-38a; $p < 0.001$). *C. albicans* was also more commonly associated with preponderant chronic granulomatous inflammation ($p = 0.003$) and greater amount of mononuclear cells ($p < 0.001$).

Correlates of outcome

The association between histopathological variables and outcome by univariate and multivariate analysis is summarized in paper IV, Table 4. Higher fatality ratios at day 7 were significantly associated with greater fungal load in kidneys ($p < 0.001$), level of neutrophil infiltration ($p < 0.001$) and increasing degree of formation of hyphae/ pseudohyphae relative to the budding yeast form ($p < 0.001$). High levels of

mononuclear cell infiltration were, however, associated with reduced mortality ($p<0.001$).

When multivariable stepwise logistic regression analysis was performed to study the association of these parameters with outcome, 3 parameters were independently associated with outcomes (paper IV, Table 4B). Higher levels of mononuclear cell infiltration observed in infected tissue were significantly associated with lower mortality (OR, 0.02; 95% CI, 0.00-0.15; $p < 0.001$), whereas increasing tissue burden of both hyphal forms (OR, 2.27; 95% CI, 1.07-4.80; $p = 0.032$) and budding yeasts (OR, 2.06; 95% CI, 1.14-3.72; $p = 0.016$) were independently associated with death.

DISCUSSION

In this population-based study we have shown that the incidence of diagnosed candidemia in Iceland has almost quadrupled in the past 27 years, with the greatest increase in incidence occurring among patients at the extremes of the age spectrum. The study highlights the significant morbidity and mortality associated with candidemia as well as the importance of aggressive treatment. Our results show, that in an unselected hospital population, as many as one-third of all cases of candidemia may be attributable to nosocomial clusters, and the risk is highest in wards providing intensive care. We have also revealed that strains of different *Candida* species exhibit wide variation in virulence and that choice of strains is of paramount importance in performing virulence comparisons.

Increasing incidence of candidemia (I and unpublished)

Invasive fungal infections, caused by yeasts of the *Candida* genus, have emerged as a serious threat to hospitalized patients worldwide in recent decades (27, 31, 262). Despite the widespread use of antifungals for prophylaxis and treatment of invasive fungal infections, candidemia remains the most frequent life-threatening fungal disease and is associated with a prolonged hospital stay and a resulting rise in cost (24, 25). Our study further reflects on that observation by revealing that the average annual incidence of candidemia in Iceland increased steadily and significantly during a 27-year study period, from 1.4 cases per 100,000 population during 1980-1984 to 5.3 cases per 100,000 population during 2000-2006.

When work on this thesis began, the epidemiology of candidemia had primarily been studied in selected hospitals (9, 36, 42, 295, 296) and/or among specific patient populations (39, 40, 43), but few studies had focused on this problem on a nationwide basis. Since then, a number of reports from population-based studies of candidemia in the US, Canada, Europe and Australia have been published, either based on a geographical region (44-48) or a whole country (49-52). However, to our knowledge, none of them have looked at secular trends in incidence rates during such a long observation period. Population-based studies have several advantages. They provide information on disease incidence and trends both in the population as a whole and specific risk groups. In addition, they include both nosocomial and

community-acquired infections and enable researchers to calculate absolute numbers for age-specific incidence rates. Population-based collection of isolates can also provide a more representative estimate of species distribution and the incidence of antifungal resistance within the population. Still it has been pointed out, that these studies have the potential to both overestimate and underestimate the incidence of candidemia (297). Positive cultures may reflect colonization or environmental contamination and, conversely, low sensitivity of culture (239) can lead to underestimation of incidence. However, according to a recent report, the sensitivity of currently used automated blood culture systems has markedly improved (244).

Population-based studies from the US all report a higher incidence of *Candida* bloodstream infections compared to Iceland. The average annual incidence of candidemia in the San Francisco and Atlanta metropolitan areas from 1992 to 1993 was 8 infections per 100,000 population (44), and in Iowa and Connecticut in 1998-2001 the mean annual incidence was 6.0 and 7.1 cases per 100,000, respectively (45, 46). The population-based incidence in Baltimore during 1998-2000 was substantially higher (24.0 cases per 100,000) (45). The annual incidence of candidemia in Iceland is, however, slightly higher than that observed in other population-based European studies, when the incidence for analogous time intervals is compared. The incidence in Iceland was substantially higher than in Finland (1.9 cases/100,000 population in 1995 to 1999) (49) and Norway (2.4 cases/100,000 population in 1991 to 2003) (51) but similar to that reported from Barcelona, Spain, during 2002-2003 (4.3 cases/100,000) (48) and Scotland in 2005-2006 (4.8 cases/100,000) (52). The only exception is a semi-national surveillance of fungemia in Denmark conducted during 2004-2006, reporting an annual average incidence of 10.4 cases of candidemia per 100,000 population (137). That is almost double the incidence rate we found in Iceland at the same time. Two other population-based studies conducted during 1999-2004, one in Canada and the other in Australia, also reported a lower annual incidence of 2.9 cases and 1.8 cases per 100,000 population, respectively (47, 50). Importantly, all these studies documented an increase in incidence over the course of the study.

For comparison with hospital-based surveillance studies, the incidence of candidemia at the 2 university hospitals in Iceland mirrored the increase in population-based incidence, reaching 0.64 cases per 1,000 admissions during 2000-2006. This was considerably less than the estimated incidence rates in the US during

1996-2006, based on data from the National Hospital Discharge Survey (range, 1.9 to 2.3 cases per 1,000 hospital discharges) (26), but similar to previously reported rates from other European tertiary care hospitals (37, 136, 137, 296, 298). Interestingly, less than 5% of candidemic episodes in Iceland during 1980-1999 were non-nosocomial in origin, which is similar to that reported from Finland (1.2%) (49), but less than reported from the US (28%) and Spain (11%) (45, 48).

Differences in incidence rates between countries may also result from demographic variations and clinical practice differences, such as CVC use and patterns of health-care delivery. Differences in antibiotic use patterns and antifungal prophylaxis might also be important, and restricted antibiotic use policy has been suggested to have contributed to the low incidence of candidemia in Norway and Finland (49, 51). The frequency of blood culture use in diagnostics could also have an effect. Although the use of blood cultures increased at the university hospitals, the proportion of cultures that turned out positive for yeasts remained stable during the 1990s, as reported in paper I. However, the proportion increased significantly thereafter. The observed increase in incidence of candidemia may be due, in part, to improved detection and identification of yeasts, but other factors could be important, such as greater use of invasive devices, broad-spectrum antibacterial agents and cytotoxic chemotherapies, more extensive surgical procedures and advanced life support (15, 299). The increasing number of susceptible hosts with immunocompromise due to mucosal or cutaneous barrier disruption, defects in mucosal and cell-mediated immunity, neutropenia, metabolic dysfunction, and extremes of age, are also important (26, 300). The increased rate of candidemia in Iceland during 2000-2006 can, in fact, be largely explained by the increase in incidence observed among infants <1 years of age.

The high incidence of *Candida* bloodstream infections among patients at the extremes of the age spectrum is consistent with previous studies (30, 44, 45, 47, 50, 51, 137). The annual incidence among infants <1 year of age during 2000-2006 is, surprisingly, two to three times higher than that reported from the other Northern European countries (30.5 vs. 9.4-16.3 cases per 100,000 population) (49, 51, 137), but lower than the rates reported from Spain in 2002 and 2003 (38.8/100,000 population) (48) and among white neonates in 2 population-based US studies (37 and 41/100,000 population) (44, 45). Our study, therefore, confirms and emphasizes the risk of candidemia in this vulnerable population. Most of these children were

premature infants, less than 3 months of age, and located in the NICU at the time of diagnosis. Low birth weight, complications associated with prematurity and fungal colonization following vaginal deliveries are all documented risk factors for candidemia in neonates as well as aggressive use of intravascular catheters in the NICU setting (161, 228, 301). We have also shown that nosocomial clustering of candidemia is particularly prevalent in this setting (paper III). Accordingly, preventive measures could, therefore, be particularly effective to reduce the rate of candidemia in NICUs.

Our results underline the fact that candidemia is no longer associated predominantly with critical care but affects patients with a wide variety of underlying diseases throughout the hospital, as only 36% of patients were located in an ICU and 5% in an NICU at the time of diagnosis. Similarly, the proportion of candidemias arising in the ICU setting has been less than 40% in most other population-based studies (37, 45, 46, 48, 50). Although the annual incidence of candidemia decreased significantly among ICU patients in the US in the 1990s (32), we did not observe a similar trend in Iceland during the 27-year study period.

Candida species distribution (I and unpublished)

Knowledge of the distribution of *Candida* species in each country is essential for establishing efficient management strategies. *C. albicans* remained the most frequently isolated species from blood throughout the study (overall, 62% of all *Candida* spp.), and the species distribution remained relatively stable during the first 2 decades. However, from 2000 to 2006, there was trend for decreased isolation of *C. albicans* and *C. parapsilosis* from blood and a significant increase in isolation of *C. tropicalis,* which emerged as the third most commonly isolated species during 2000-2006. A similar trend was noted in a global antifungal surveillance study of IC during 1997-2005 (60), and in 2 population-based European studies in the 1990s (37, 136).

A shift in the epidemiology of hematogenous candidiasis towards greater isolation of non-*C. albicans* species has been a global concern in the past 10-15 years. This was first reported from the US in neutropenic recipients of bone marrow transplants in 1989 and 1990 (302). Since then, many other series have confirmed these observations (100, 107, 157, 303) and although *C. albicans* has remained the most common species causing IC worldwide, it currently only causes approximately

50% of cases of hematogenous candidiasis (87, 102, 103). Widespread use of azole antifungals, especially fluconazole, for treatment and prophylaxis of IC, may have played a role in this observed shift, since it has largely been manifested in a rise in infections caused by species with reduced susceptibility to azole antifungal agents, such as *C. glabrata* (32, 43, 304). Fluconazole prophylaxis is used infrequently in Iceland, which may explain the stable proportion of *C. glabrata* bloodstream infections during the long study period. The increased frequency of *C. glabrata* bloodstream infections among older patients is in agreement with previous studies (50, 228, 305) and others have noted that elderly patients (age, >60 years) may also be at increased risk of dying from *C. glabrata* bloodstream infections (306). The elderly have higher rates of oropharyngeal colonization with *C. glabrata* (307), but the relationship to candidemia is not clear.

Our results confirm that bloodstream infections due to *C. parapsilosis* are more commonly encountered in pediatric patients in comparison with adults (137, 305, 308-310). Surprisingly, this species was not a major pathogen among infants, however. Given the known propensity of *C. parapsilosis* to adhere to foreign material (311, 312) and the low proportion of *C. parapsilosis* infections diagnosed during the last 7 years of the study, these results might suggest improved catheter care and infection control procedures in the country in recent years,

At the same time the proportion of infections caused by *C. tropicalis* increased significantly from 6% of infections during 1995-1999 to 14% in 2000-2006. It is an important pathogen in patients with neutropenia and/or hematologic malignancies (43, 313) and, in fact, one-third of patients with *C. tropicalis* bloodstream infections from 1980 to 1999 in Iceland suffered from these conditions. A relatively large proportion of *C. tropicalis* infections from 2000-2006 was outpatient-acquired (15%), but a more detailed analysis of clinical data from that period is pending.

Antifungal drug susceptibility and relation to antifungal use (I and unpublished)

This shift in species distribution is particularly important in relation to the predictable susceptibility patterns associated with *Candida* species. Overall, 97.7% of the Icelandic BSIs cultured from 1991 to 2006 were susceptible to fluconazole and there was no significant change in distribution of fluconazole MICs, despite an approximately 5.5-fold increase in use of that agent during the same period. Our

findings confirm the reports of infrequent fluconazole resistance among *C. albicans*, *C. parapsilosis* and *C. tropicalis* worldwide in the past decade (51, 60, 105, 120, 140, 142) but the proportion of *C. glabrata* isolates with reduced susceptibility to fluconazole (MIC, ≥16 μg/ml) was considerably lower than that recently reported from a global antifungal surveillance program (13% vs. 31%) (120).

Several other studies in the US and Europe have reported a substantial increase in fluconazole use in the past 15 years (33, 36, 37, 136, 137). Our data are directly comparable to national data on fluconazole usage reported from 2 Scandinavian studies. In 2003, the combined use of fluconazole and itraconazole was 36.5 DDD/1,000 inhabitants/year in Norway (51), 64.9 DDD/1,000 inhabitants/year in Iceland, and 146 DDD/1,000 inhabitants/year in Denmark (137). Similarly, fluconazole consumption increased by 152% in Denmark during 2001-2006 (137), which is considerably greater than a 50% increase observed in Iceland at the same time. This could explain why decreased azole susceptibility in *Candida* species that are normally susceptible to fluconazole (e.g., *C. albicans*) occurs more frequently in Denmark than in Norway and Iceland.

Amphotericin B susceptibility profiles were consistent with previous reports. The MIC_{90}s for itraconazole were <1 μg/ml for all *Candida* species, except *C. glabrata*. Resistance to itraconazole was found in 30% of *C. glabrata* isolates, which is a proportion compatible with previously published reports from large multicenter surveys in the US and Europe (58, 314).

In summary, this study has shown that, on a national level, the incidence of candidemia in Iceland has increased almost fourfold in the past 27 years, with the highest incidences of infection occurring in the youngest and older age groups. Although *C. albicans* remained the most common cause of candidemia throughout the study period, non-*C. albicans* species have been increasingly identified in recent years. Fluconazole use has increase approximately 5.5-fold during the last 17 years, but the vast majority of strains are still susceptible to this agent.

Concomitant conditions and risk factors (II)

We performed a population-based analysis of risk factors and predictors of mortality among the subset of patients diagnosed with candidemia during 1980-1999 (paper II). When work on this thesis began, risk factors for candidemia and prognostic factors had mainly been studied in selected hospitals (14, 16, 315) or among several

selected patient groups, including patients with hematologic or solid organ malignancies (261, 272, 316, 317), recipients of bone marrow transplants (304, 318, 319), preterm neonates (161), and burn victims (320, 321). Since then, several other population-based studies have been published, documenting risk factors for candidemia and IC (45, 47, 49) and their association with mortality (48, 50).

Important risk factors for candidemia and IC in adults (14, 15, 228, 260, 272) and children (30, 225, 227) have been well established. In general, the comorbid conditions and risk factors identified in this study were in concordance with previous reports (44, 45, 48, 50, 268, 298). However, infections in patients with solid tumors (45%) and post-surgical patients (68%) were particularly common (10%-28% and 48%-56%, respectively, elsewhere) (49, 50, 268, 298) and diabetes and renal failure were rare (29% and 35% in the US) (45). Among both children and adults the most prominent risk factors were central intravascular catheters and prior bacterial infections/use of antibacterial agents, both of which are well documented independent risk factors for candidemia (14, 15, 26). Our findings also highlight the difference between children and adults as risk groups. Adults were more likely to have malignancy and to have undergone abdominal surgery, whereas the children more commonly received corticosteroids, primarily used for respiratory distress or bronchopulmonary dysplasia in premature neonates (322). Similarly, a large study of candidemia in the US revealed different underlying conditions and risk factors among children and adults, with significantly higher survival rates among adult patients (76% vs. 54%) (228). Because of the small number of pediatric cases we were unable to analyze independent risk factors for mortality among children and a comparison of survival between children and adults was not performed, due to the low number of children with candidemia.

Long-term trends in patient outcomes and treatment (II)

Candidemia is associated with high crude mortality, in part reflecting the severe underlying illnesses of the infected patients. In Iceland, 37% of adult patients with candidemia during 1980-2006 died within 30 days from blood culture. This is in concordance with case-fatality rates reported from European tertiary-care centers, ranging from 26% to 44% (48, 49, 87, 268), but lower than those reported from the US, ranging from 39% to 75% (9, 27, 303, 323).

Furthermore, our study reflects on the progress in management of these life-threatening infections over a 20-year period, 1980-1999. We have shown that the prognosis of patients with candidemia improved significantly during the 1980s and 1990s. A similar observation was noted in another retrospective European study, reporting a decrease in the crude mortality rate among candidemic patients in a tertiary care hospital from 49% during 1992-1994 to 38% in 1995-1997 (259), but the number of patients with rapidly fatal underlying diseases was low. An inverse trend was observed in 2 matched cohort studies conducted at a tertiary care hospital in Iowa, US, where the attributable mortality increased from 38% (crude mortality, 57%) during 1983-1986 to 49% (crude mortality, 61%) during 1997-2001 (19, 21).

In the early 1980s therapeutic options were limited and the clinical relevance of positive blood cultures with *Candida* spp. was commonly disputed. Removal of potentially infected catheters was considered the mainstay of treatment, whereas antifungal therapy was often reserved for the few patients who remained fungemic and yet stable enough to tolerate potentially toxic drug therapy (324). A shift towards a more aggressive approach is well illustrated in the current study. Consensus recommendations by experts in infectious diseases advocate antifungal treatment for all patients who have *Candida* spp. cultured from peripheral blood, as well as removal of vascular catheters (133). According to our results, this shift towards a more uniformly aggressive approach to candidemia, in combination with other advances in the management of critically ill patients, seems to be associated with improved outcomes.

By multivariate analysis, prompt removal of CVCs was independently associated with lower death rates. The exclusion of patients who died within 3 days after the diagnosis did not significantly modify the results. The importance of catheter removal in the setting of candidemia has been extensively reviewed and debated (271, 276, 325). Available data, which correct for severity of underlying illness and other confounding variables, suggest that survival of candidemic patients is significantly improved if catheters are removed (48, 259, 265, 266). Several other studies suggest that catheter exchange may be beneficial, both in terms of reduction in duration of candidemia and lower mortality rates among pediatric and adult patients (225, 267, 298). However, a recent study based on data from a population-based surveillance did not demonstrate that early removal (<2 days of incident candidemia) of CVCs in candidemic patients was beneficial in terms of outcome

(293). Similarly, a retrospective study of CVC management in cancer patients with candidemia showed that early (<72 h) CVC-removal was only beneficial in patients with probable or confirmed catheter-related candidemia and not in patients with a definite non-catheter source or evidence of dissemination (274). Although prospective controlled trials on this topic are lacking, recent treatment guidelines recommend removal of vascular catheters, if feasible (133).

One limitation of the study is the lack of information on severity of illness, a well-known predictor of outcomes in candidemia (9, 228). The infections were diagnosed in different locations around the country and the quality of registration and patient charts differed greatly. As a result, a score for severity of illness could not be calculated. The second limitation is the lack of a control group which makes it impossible to estimate the attributable mortality in this cohort of patients.

In summary, this long-term nationwide study of candidemia included patients with wide variety of underlying diseases, in contrast to many other studies which have focused on selected patient groups. Our results emphasize the difference in clinical presentation between children and adults and they underline the strong association between prompt removal of CVCs and favourable outcome. In addition, we demonstrated a steady improvement in prognosis among patients with candidemia during 1980s and 1990s, an era of increasingly aggressive antifungal management. However, despite increasing efforts in management of these infections in recent years, we did not observe a continuing improvement in prognosis during 2000-2006, which suggests that prevention should currently be the highest priority. Promising prevention strategies include those that improve adherence to current recommendations for placement and care of CVCs (326, 327), control of antibiotic use, and the implementation of risk stratification rules to identify patients that may benefit from early diagnostic and therapeutic interventions (222, 230, 231).

Molecular epidemiology of candidemia: evidence of clusters of smoldering nosocomial infections (III)

In this part of the thesis we used PCR fingerprinting to study the genetic relatedness of clinical *Candida* BSIs, responsible for 94.4% of all cases of candidemia in Iceland during a 16-year period. To our knowledge, this is the first long-term, nationwide study of the molecular epidemiology of candidemia.

Our results indicate that in an unselected hospital population 18.7-39.9% of candidemic episodes are caused by nosocomial clusters of infection, defined as isolation of closely related strains (≥90% similarity of fingerprinting profiles) from ≥2 patients at the same hospital within a period of 90 days. The risk of nosocomial clustering was dependent on both the *Candida* species and location of the patients within the hospital. The average population-based rate of cluster-associated candidemia was at least 11.3 cases per 100,000 hospital admissions per year during the 16-year study period. Literature review did not provide comparable information. However, a recent study of all invasive group A streptococcal infections diagnosed in Ontario, Canada, during 1992-2000, reported that the average rate of outbreak-assocated disease was 0.5 per 100,000 hospital admissions per year (328).

Several other molecular epidemiological studies of candidemia have been performed to investigate or confirm suspected outbreaks in single departments or hospitals, but none of them were population-based. For example, Marco et al. (168) analyzed 110 *C. albicans* isolates by this method; the isolates were collected from candidemic patients in surgical and neonatal ICUs of 4 hospitals in the US. They observed a higher degree of clustering of isolates in 3 of the 4 hospitals, compared with unrelated control isolates (168). Other studies have demonstrated that single strains have been responsible for a number of temporally associated outbreaks of candidemia in the same hospital or ICU, which have smoldered over a long period of time, even years (84, 85, 163, 169, 170, 275). This observation is in strong contrast to common outbreaks of bacterial infections which traditionally have a more abrupt onset and shorter duration (328-331).

Random amplification of polymorphic DNA and PCR fingerprinting with the 4 single primers provided an effective method for assessing both interspecies and intraspecies genetic variability of the large number of isolates belonging to different yeast species. The M13 core sequence and the simple repeat sequences $(GACA)_4$ had the highest discriminatory power for the 5 most common fungal species; *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and *C. dubliniensis*. PCR fingerprinting with the M13 primer revealed 35 different genotypes of *C. albicans*, 2 of which (GT-2 and GT-4) were endemic throughout most of the study period and caused 5 of 15 clusters. Other common genotypes (GT-32 and GT-26) were infrequently identified among clustered isolates. In 2006, a new distinct genotype, GT-22, was identified

that caused 2 clusters in separate hospitals. These results may, therefore, indicate variable density in the environment, transmissibility or invasive potential.

The proportion of clustered isolates was significantly higher for *C. parapsilosis* than for other non-*C. albicans* species. An environmental source is more commonly implicated in infections caused by *C. parapsilosis*, when compared with other *Candida* species, which could partly explain this difference (164, 332). Since it is usually exogenously acquired, detection of *C. parapsilosis* bloodstream infection can be an indication of breaks in catheter care or infection control procedures (333). It is noteworthy, however, that during the study period no clusters or outbreaks of candidemia were identified by the hospital surveillance team, underlining the smoldering nature of these infections. Thus, when the characteristics of nosocomial candidemia are taken into account, prospective hospital surveillance with use of molecular typing might be particularly effective for identifying nosocomial clusters.

In the current study, the majority of BSIs were detected in culture samples from patients in ICUs (41%), including the NICU, but the proportion of clustered isolates in ICUs was higher than expected (58%). The frequency of clusters was highest in the NICU, and 54% of isolates cultured from NICU patients, all *C. albicans*, were implicated in infection clusters. Furthermore, when data was analyzed by ward, 86% of clustered isolates were cultured from patients in ICUs and the NICU. Several reports have revealed evidence of outbreaks of candidemia in ICUs and NICUs, highlighting the risk of nosocomial transmission of infecting strains in these wards (170, 275, 334). Possible explanations include widespread use of broad spectrum antibiotics, total parenteral nutrition and intravascular devices in the ICU setting. Frequent contact with patients by hospital personnel has also previously been suggested to facilitate cross-contamination in the ICU environment (165, 168, 334, 335).

No consensus threshold values have been established for cluster analysis by PCR for *Candida*. The definition of cluster in the current study takes into account DNA fingerprinting data from the isolates, as well as temporal-spatial relationships between the patients, but it is still somewhat arbitrary. In comparison, by fingerprinting *C. albicans* isolates with the complex probe Ca3, a similarity coefficient (S_{AB}) of 0.80 has been found to be a reasonable threshold for defining clusters (73). By limiting the definition of clusters to ± 90 days, we may be underestimating the extent of the problem, because, as previously discussed, it has

been shown that nosocomial outbreaks of *Candida* infections can persist over a long period of time.

To summarize, we have revealed that as many as one-third of all cases of candidemia in an unselected patient population are caused by clusters of epidemiologically and genetically related strains, suggesting that many of the strains have the ability to persist over long periods. Therefore, clusters of candidemia lack the general characteristics of nosocomial outbreaks, tend to be unnoticed and can only be identified by prospective nosocomial surveillance using molecular typing. Nosocomial candidemia may, therefore, pose new challenges for infection control in hospitals. Prospective clinical epidemiology studies are warranted to identify a source of these infections.

The importance of strain variation in the comparative virulence of *C. albicans* and *C. dubliniensis* (IV)

Screening of all available BSIs cultured in Iceland during 1991-2006, with use of phenotypic methods and PCR fingerprinting, revealed *C. dubliniensis* in 4% of episodes in 1995-1999 and 6% in 2000-2006. At the same time, *C. dubliniensis* "emerged" as an increasingly important bloodstream pathogen, both in the US and Europe, in patients with a wide variety of comorbid conditions (59, 87-91, 268). It is closely related to *C. albicans*, and shares many of its characteristic phenotypic traits, but has distinct phylogenic and genotypic characteristics. We, therefore, asked whether its virulence properties and pathogenesis of infection were comparable to that of *C. albicans*.

To our knowledge, this is the first study to compare the virulence of *C. dubliniensis* and *C. albicans* in a murine model of bloodstream infection, using a wide array of *C. dubliniensis* isolates from comparable infections. In addition, we evaluated the accompanying histopathological changes systemically and in a blinded manner. We did not observe a significant difference in the virulence of *C. albicans* and *C. dubliniensis*, when results from all isolates of each species were combined. Indeed, a greater variation in virulence was noted among different strains of each species than between the fungal species themselves. Both species comprised strains of high and low virulence, and 6 of the 9 *C. dubliniensis* strains were virtually avirulent.

A murine model of bloodstream infection is an established animal model for effective comparison of the pathogenicity of different *Candida* species (201, 213). Although studies using isolates from comparable patients and clinical sites are lacking, 2 other studies have used this model to compare the virulence properties of *C. albicans* and *C. dubliniensis*. Gilfillan et al. (197) compared the virulence of 4 mucosal isolates of *C. dubliniensis* with 1 systemic isolate of *C. albicans*. The mice infected with *C. dubliniensis* survived longer, although the difference was marginal (197). Vilela et al. (214) compared 7 isolates of *C. albicans* from other sites than blood with 7 *C. dubliniensis* isolates of unspecified origin. In general, the composite survival rate was significantly lower in *C. albicans* infected mice in comparison with *C. dubliniensis*. A major limitation of these studies was an apparent lack of criteria for selection of the fungal strains used, which could explain the discrepancy between our results and these reports. Previous work suggests that virulence properties of strains (i.e., production of SAPs and biofilm formation) may be different, in part based on the source (systemic vs. mucosal infections) (218, 219, 336). In addition, significant association has been found between genetic clades of *C. albicans* and anatomical source of isolates, reflecting possible differences in putative virulence properties (221). We, therefore, believe that careful selection of isolates is of paramount importance in performing virulence comparisons.

All *Candida* BSIs tested in the current experiments had distinctive PCR fingerprinting profiles, representing 12 different strains. Among *C. dubliniensis* isolates, genotype 1 isolates predominated (6/9, 67%), which is consistent with results from the study of Gee et al. (99), where 72% (71/98) of isolates, recovered from 15 different countries, belonged to genotype 1. We did not observe an association between genotype and virulence in the murine model.

Although the composite suvival rate of animals did not differ significantly when analyzed by species, a marked variation in virulence was noted between strains of each species. The most virulent *C. dubliniensis* strains were IS-30 and IS-38a, with 43% and 46% 7-day survival rates, respectively. However, 6 of the 9 *C. dubliniensis* strains were not associated with any deaths. Among *C. albicans* strains, IS-7 was by far the most virulent, with only 15% survival at day 7. The slope of the survival curve for mice infected with the 3 most virulent strains was comparable, with most animals succumbing to the infection on day 4-5. Since consistent results were generally obtained between experiments for those strains associated with death, we

found it reasonable to pool the results in the data analysis. Our results are not directly comparable to the observations reported by Gilfillan et al. and Vilela et al., but both papers reported a great variation in survival of animals infected with different *C. dubliniensis* isolates, ranging from virtually avirulent to highly virulent isolates (197, 214). However, this variation was not described in detail. We did not observe an association between the virulence of the strains in the murine model and the mortality of the patients from whom these strains were cultured (paper IV, Table 1), emphasizing the importance of host factors for disease progression.

In order to explain the observed variations in virulence among the different strains, we evaluated the accompanying histopathological changes in kidneys of the infected animals in a blinded, systematic manner. Higher levels of mononuclear cells in kidney sections were independently associated with lower death rates at day 7, reflecting a successful immunoresponse. A significantly greater inflammatory infiltration was noted in kidneys seeded by *C. albicans*, compared with *C. dubliniensis*. Granulomatous inflammation and greater mononuclear infiltrate were also more commonly noted with *C. albicans*. The inflammatory responses to systemic infections with *C. albicans* and *C. dubliniensis* have not been systematically recorded previously, but contrary to our results, Vilela and co-workers (214) reported a generally stronger inflammatory reaction in histopathological analysis of kidneys seeded with *C. dubliniensis* compared with *C. albicans*. However, the type or amount of inflammatory infiltrates was not specified in detail and observations were not specified in detail. Virulence differences on the strain level may in part be attributed to the amount and type of systemic and local immune responses, both innate and adaptive response, evoked by the different strains (337, 338).

One of the most extensively studied virulence traits of *C. albicans* and *C. dubliniensis* is their ability to form true hyphae and pseudohyphae in order to invade host tissues and cause deep seated infections, as reviewed in the Introduction section of this thesis. Our results show, that the *C. albicans*, especially the highly virulent strain IS-7, formed hyphae and pseudohyphae to a significantly greater degree than *C. dubliniensis*. However, when all strains were analysed, hyphal formation was not an independent predictor of mortality. Similarly, previous studies have shown, that hyphal production of *C. albicans* exceeds *C. dubliniensis* both *in vivo* (214) and under most conditions that promote this morphological transition *in vitro*, including growth in serum and shifts in pH and temperature (215). A recent survey comparing

the genome of *C. dubliniensis* and *C. albicans* with use of DNA microarrays revealed that several hyphae-specific virulence-associated genes of *C. albicans* were absent (SAP5 and SAP6) or divergent (HWP1) in *C. dubliniensis*, which could in part explain this difference (339). Differences in gene expression have also been described, relating to differential control of the NRG1 regulator gene, a well known repressor of filamentation in *C. albicans* (340-342).

Although the importance of hyphal production for *Candida* pathogenicity is undisputed, the yeast form can also disseminate and multiply in internal organs. The ability of *C. dubliniensis* to traverse the mucosal barrier of the gastrointestinal tract may be attenuated compared with *C. albicans* (215), but nevertheless, our results show that once inside the bloodstream it can cause serious deep seated infections with mortality comparable to *C. albicans*. The fact that filamentation remained significantly more common among animals seeded with *C. albicans* in comparison with *C. dubliniensis*, when highly virulent strains of both species were independently analysed, suggests that other virulence factors may be more important for the pathogenesis of invasive *C. dubliniensis* infections.

The most important limitation of our study was the relatively low number of *C. albicans* isolates tested. However, to our knowledge, no other studies have used a commensurable number of systemic *C. albicans* isolates to compare its virulence with *C. dubliniensis*. The *C. albicans* isolates tested also had an acceptable virulence range, comprising strains of high and low virulence. Furthermore, it can be argued that by using BSIs we are selecting strains that are intrinsically more virulent, surpassing the natural barrier of infection. The scoring of the accompanying histopathological changes in internal organs of animals can be viewed as a second limitation. As we are unaware of any previously published guidelines in this field, we propose a scoring method for systemic evaluation of these changes using blinding to reduce bias. Finally, we used non-immunocompromised animals, but the correlation between virulence in animal models and humans has not been formally studied. Given the lack of a better alternative, we believe, however, that it provides more realistic information than *in vitro* studies.

In summary, we compared the virulence of *C. albicans* and *C. dubliniensis* in a murine model using an unprecedingly large number of *C. dubliniensis* BSIs. We observed greater strain to strain variation within species than between the two species, with both species comprising highly virulent strains. Our results also show

that factors other than fungal morphology in tissues may account for the observed variation in the mortality of the animals, in particular the balance between the infiltration of acute and chronic inflammatory cells. The origin of the strains may be a neglected confounding factor in virulence studies. Ideally, for meaningful comparisons, many representative strains from each species should be used.

GENERAL SUMMARY AND CONCLUSIONS

Incident to increased prevalence of susceptible hosts in recent years, fungi have emerged as major causes of human disease among hospitalized and critically ill patients worldwide. The purpose of this project was to study secular trends in the epidemiology and clinical mycology of *Candida* bloodstream infections during a long observation period, in a population-based, nationwide setting, with special reference to trends in incidence rates, species distribution and antifungal susceptibility patterns.

- We have shown that the incidence of candidemia in Iceland has increased almost 4-fold in the past 27 years, with the greatest increase in incidence occurring among patients at the extremes of the age spectrum. Our results demonstrate that the incidence of candidemia in Iceland is similar og slightly higher than reported from population-based studies in other European countries but lower than in the US. Surveillance is needed to track trends of this serious infection and provide guidelines for treatment and infection control strategies.

- Although *C. albicans* remains the most common cause of candidemia in Iceland, there has been a trend towards greater isolation of non-*C. albicans* species from blood samples in recent years. Fluconazole use has increased approximately 5.5-fold in the past 2 decades, but no change in susceptibility to this agent was observed during the study period. However, prospective surveys are warranted to detect possible emergence of antifungal resistance among *Candida* BSIs, in the context of constantly increasing use of antifungals in clinical practice.

- Our results highlight the significant morbidity and mortality associated with candidemia and invasive candidiasis as well as the importance of aggressive treatment, especially the strong association between prompt removal of CVCs and favourable outcome, supporting current treatment guidelines. Our results underline the important fact that candidemia is no longer associated

predominantly with ICUs, but affects patients with a wide variety of underlying diseases throughout the hospital. Despite steady improvement in prognosis noted among patients with candidemia in Iceland during the first 2 decades of this study and intense efforts in the management of these infections in recent years, prognosis has not continued to improve. This emphasizes the need for continuous surveillance, clinical vigilance and judicious use of antifungal agents.

Previous molecular epidemiological studies of candidemia had been performed to investigate or confirm suspected outbreaks in single hospital departments or selected hospitals, but the overall prevalence of nosocomial clustering in candidemic patients remained unknown.

- We have shown that, in an unselected patient population, up to one-third of all cases of candidemia are caused by strains that are epidemiologically and genetically related. The risk of nosocomial clustering is dependent on both the species of the pathogen and the location of the patients, being highest in ICUs and the NICU. These clusters lack the general characteristics of nosocomial outbreaks, tend to be unnoticed, and can be identified only by prospective nosocomial surveillance with use of molecular typing – posing new challenges for infection control in hospitals.

The aforementioned parts of the study revealed a surprisingly high and increasing proportion of infections to be caused by *C. dubliniensis.* Its virulence was compared to *C. albicans* in an established murine model of bloodstream infection, using a unprecedingly wide array of *C. dubliniensis* isolates. The associated histopathological changes in kidney sections were evaluated and in the absence of previously published guidelines in this field, we propose a scoring method for systemic evaluation of these changes using blinding to reduce bias.

- We did not observe a significant difference in the virulence of *C. albicans* and *C. dubliniensis*, when results from all isolates of each species were combined. Importantly, a greater variation in virulence was noted among different strains of each species than between the fungal species themselves.

Both species comprised strains of high and low virulence, and 6 of the 9 *C. dubliniensis* strains were virtually avirulent. The origin of the strains may be a neglected confounding factor in virulence studies and we conclude that, for meaningful comparisons in future studies, many representative strains from each species should be used.

- In histopathological analysis both yeast and hyphal forms were independently associated with mortality, suggesting similar virulence for both. Factors other than fungal morphology in tissues may account for the observed strain variation in virulence, which could include differences in cell-mediated, humoral or innate immune responses evoked by the different yeast strains, supported by the protective effect of increased infiltration of mononuclear cells demonstrated in this study.

FUTURE DIRECTIONS

While these studies shed some light on the magnitude, consequences and molecular epidemiology of serious *Candida* infections as well as the virulence of selected causative species, much work remains to be done in order to understand more fully the transmission and virulence mechanisms of these pathogens.

The results presented in this thesis suggest that a substantial proportion of candidemic episodes in the hospital is caused by genetically and temporally related strains. These results may provide the framework for a strategy for more definitive testing of the origins of *Candida* strains responsible for nosocomial infections. Prospective surveillance studies are needed that will address the relationship between commensal organisms and subsequent infecting strains, the impact of transfer from health-care workers to patients, and the microevolution of endemic strains in hospital settings. The observation that nosocomial clusters disproportionally affect patients in ICUs and NICUs suggests that such studies would be most effectively conducted in these settings. PCR fingerprinting proved to be a highly discriminating method for detecting inter- and intraspecies genetic variability among *Candida* isolates. Although still costly and not fully developed for all the common *Candida* species, MLST may be better reproducible, with the added advantage that data are portable and can, therefore, be easily compared. Both methods could be used to genotype isolates if an outbreak is suspected.

When we compared the virulence of *C. dubliniensis* with that of *C. albicans*, a greater variation in virulence was noted among different strains of each species than between the fungal species themselves, an observation that may have implications for the choice of strains in future virulence studies of these species.

The observed variation in virulence of the strains might potentially be explained by strain-related differences in the expression of antigens in the fungal cell wall. An attractive candidate is fungal heat-shock protein 90 (hsp-90), and effective serological responses to this antigen in patients have been shown to correlate closely with a good prognosis (343). It is present in large amounts in the hyphal tips of *Candida* yeasts (183), which indicates that it may be important for the pathogenesis of invasive *Candida* infections. Another fertile area of research relates to antimicrobial peptides (i.e., cathelicidin), important effector molecules of innate

immunity and key factors in mucosal defenses against bacteria (344, 345). However, their role in fungal infections has been incompletely defined (346). Studies of strain-related differences in hsp-90 expression and susceptibility to the effects of the cathelicidin peptide LL-37 are pending at our institution. Studies of differences in cytokine profiles and T-cell responses among candidemic patients are also needed for better understanding of the balance between protective immune responses and immunopathology and their relation to outcome in the clinical setting.

The increasing incidence of candidemia and IC as well as the permanently high attributable in-hospital morbidity and mortality, despite advances in the field of antifungal therapy, suggest that prevention should currently be the highest priority. Promising prevention strategies include programs to improve use, placement and care of CVCs, prudent use of antimicrobial agents, and the implementation of risk stratification rules to identify patients that may benefit from early diagnostic and therapeutic interventions. Further investigations of the virulence mechanisms of different *Candida* species and modes of transmission in the hospital environment are essential for improving our understanding of these life-threatening – but often preventable – complications of a hospital stay.

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PAPERS I-IV