Candida and Candidaemia

Susceptibility and Epidemiology

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THE THESIS IS BASED ON THE FOLLOWING PUBLICATIONS:

I. Maiken Arendrup, Thomas Horn, Niels Frimodt-Møller. In vivo pathogenicity of eight medically relevant *Candida* species in an animal model. Infection. 2002, 30:286-291. (Ref. (1))
II. Maiken Arendrup, Bettina Lundgren, Irene Møller Jensen, Bo Sønder Hansen, Niels Frimodt-Møller. Comparison of two commercial diffusion tests - Etest and disc diffusion test, 24-h and NCCLS 48-h broth microdilution method for fluconazole and amphotericin B susceptibility of *Candida* isolates. J Antimicrob Chemother 2001, 47:521-6. (Ref. (2)).
III. Arendrup MC, Kahlmeter G, Rodriguez-Tudela JL, Donnelly JP. Breakpoints for susceptibility testing should not divide wild-type distributions of important target species. Antimicrob Agents Chemother. 2009, 53:1628-9. (Ref. (3))
IV. Maiken Cavling Arendrup, Guillermo Garcia-Effron, Walter Buzina, Klaus Leth Mortensen, Nanna Reiter, Christian Lundin, Henrik Elvang Jensen, Cornelia Lass-Flörl, David S. Perlin, Brita Bruun. Breakthrough *Aspergillus fumigatus* and *Candida albicans* double infection during caspofungin treatment: laboratory characteristics and implication for susceptibility testing. Antimicrob Agents Chemother. 2009, 53:1185-93. (Ref. (4))
V. Maiken Cavling Arendrup, Guillermo Garcia-Effron, Cornelia Lass-Flörl, Alicia Gomez Lopez, Juan-Luis Rodriguez-Tudela, Manuel Cuenca-Estrella, David s. Perlin. Susceptibility testing of candida species to echinocandins: comparison of EUCAST EDEF 7.1, CLSI M27-A3, Etest, disk diffusion and agar-dilution using RPMI and Isosensitest medium. Antimicrob. Agents Chemother. 2010, 54: 426-439. (Ref. (5))
VI. Maiken Cavling Arendrup, Juan-Luis Rodriguez-Tudela, Steven Park, Guillermo Garcia-Effron, Guillaume Delmas, Manuel Cuenca-Estrella, Alicia Gomez Lopez, David S. Perlin. Echinocandin susceptibility testing of *Candida* spp. using the EUCAST EDEF 7.1 and CLSI M27-A3 standard procedures: analysis of the influence of bovine serum albumin supplementation, storage time and drug lots. Antimicrobial Agents Chemother. 2011; 55: 1580–1587. (Ref. (6))
VII. Maiken Cavling Arendrup, Kurt Fuursted, Bente Gahrn-Hansen, Irene Møller Jensen, Jenny Dahl Knudsen, Bettina Lundgren, Henrik C. Schønheyder, Michael Tvede. Semi-national surveillance of fungemia in Denmark: notably high incidence rates of fungemia and of isolates with reduced azole susceptibility. J Clin Microbiol. 2005; 43: 4434-4440. (Ref. (7))
VIII. Maiken Cavling Arendrup, Brita Bruun,Jens Jørgen Christensen, Kurt Fuursted, Helle Krogh Johansen, Poul Kjældgaard, Jenny Dahl Knudsen, Lise Kristensen, Jens Møller, Lene Nielsen, Flemming Schønning Rosenvinge, Bent Røder, Henrik Carl Schønheyder, Marianne K Thomsen, Kjeld Truberg. National surveillance of fungemia in Denmark 2004-2009. J Clin Microbiol. 2011;49(1):325-34. (Ref. (8))
IX. Maiken Cavling Arendrup, Sofia Sulim, Anette Holm, Lene Nielsen, Susanne Dam Nielsen, Jenny Dahl Knudsen, Niels Erik Drenck, Jens Jørgen Christensen, Helle Krogh Johansen. Diagnostic issues, clinical characteristics and outcome for patients with fungaemia. J Clin Microbiol. 2011; 49: 3300-3308. (Ref. (9))

INTRODUCTION

Around the turn of the millennium reports from other countries described candidaemia as an emerging infection, particularly in the immunocompromised population. In the same time period new treatment options became available including a new drug class (the echinocandins) and new compounds with broader spectrum among the existing azole class of drugs (voriconazole and posaconazole). However, the data on the epidemiology, susceptibility and outcome of candidaemia in Denmark (DK) was scarce and limited to single centre experiences (10-14). Apparently, candidaemia was not a frequent infection in DK, and thus had attracted little attention. It was by many regarded as a rather benign condition, which in many cases could be managed simply by removing the underlying contaminated device.
The first international standard on susceptibility testing of yeast was published in 1997 by the National Committee on Clinical Laboratory Standards (NCCLS) (15), today renamed the Clinical Laboratory Standards Institute (CLSI) in the USA. Non-species specific breakpoints were provided for fluconazole, itraconazole, flucytosine and tentative ones also for amphotericin. Over the next years non-species specific breakpoints were also published for *Candida* and voriconazole and the echinocandins (16-19). At that time the clinical implication of differences in virulence among the *Candida* species were not well recognised, hence any need for caution before extrapolating susceptibility across species was not

acknowledged. Furthermore, for several of the individual drug-species combinations the breakpoints bisected wild type populations leading to a random classification of isolates with identical susceptibility (3). Finally, for some drug-species combinations the breakpoints were far higher than the upper limit of the wild type MIC range meaning isolates with acquired resistance mechanisms and elevated MIC were still classified as susceptible despite no clinical evidence suggested they were a good target for the drug in question.

At most routine laboratories for clinical microbiology in DK, *Candida* was not identified to the species level beyond *C. albicans*. Needless to say, in most laboratories susceptibility testing was not performed.

Consequently, I found it meaningful to investigate the performance and possible need for optimisation of various antifungal susceptibility test principles and to study the epidemiology of candidaemia in DK. Three publications in this thesis undertook studies and comparisons of commercial diffusion tests and reference microdilution tests and evaluated endpoint interpretation (II, IV and V). During these studies we gained valuable experience with both the various test principles including potential pitfalls, need for optimisation and limitations. One publication focussed on one of the basic tenets in the breakpoint development process, which is the importance of avoiding setting breakpoints that bisect the wild type populations (III). Finally, three publications (IV-VI) targeted the issue of potential misclassification of echinocandin resistant isolates and together with other publications from our group and others allowed the establishment of appropriate and meaningful European Committee for Antimicrobial Susceptibility Testing (EUCAST) breakpoints that correctly identify resistant isolates (20-23). Acknowledging this work the CLSI subsequently initiated a revision process of their breakpoints, which has led to a harmonisation of breakpoints and more appropriate interpretation of susceptibility results in a global perspective (24-26).

When first initiating our studies on epidemiology of candidaemia in DK, I anticipated the situation would resemble the one in the other Scandinavian countries as was the case for many bacterial infections, but our studies revealed I was clearly wrong on this point. Not only did we find a notably higher incidence rate of candidaemia in DK, we also understood that although *C. albicans* remains the predominant species, a variety of other *Candida* species are involved in human infections and that the intrinsic susceptibility pattern for these different species is as diverse as it is for bacteria. Finally, we showed that a rising number of cases involved species that are not susceptible to fluconazole which was the recommended first line treatment at that time and thereby provided local data that prompted an important change in the Danish treatment guidelines for candidaemia.

We believe our studies have contributed to the understanding of the epidemiology of fungaemia and to improved management on this infection.

The fungal universe

Fungi may divide and reproduce in two ways, asexual and sexual, and thus appear in two forms anamorph and teleomorph which are named individually. In example, the yeast *Candida krusei* refers to the asexual form whereas *Pichia kudriavtsevii* (prev. *Issatchenkia orientalis*) refers to the same fungus in the sexual state. From a taxonomic point of view, the teleomorphic name is the official or "correct" name and the fungi are divided into four groups according to their sexual state: *Zygomycota* (forming zygospores), *Ascomycota* (ascospores) and *Basidiomycota* (basidiospores) and a group of fungi for which the sexual state is not described/known: *Deuteromycota* or fungi imperfecta. However, in clinical practice the asexual name is normally used as this is the form encountered in the clinical microbiology laboratory during culture on routine media. A pragmatic and more clinically meaningful classification is used which reflects micro- and macromorphology of the different fungi as well as the pathogenic characteristics as displayed below.

Table 1. Pragmatic classification of fungi into yeasts, moulds, dimorphic fungi and dermatophytes. In the upper row the most common genera representing each of the four classes are included and those most prevalent in DK highlighted in black. In the lower rows the normal habitats are displayed as well as the most typical types of infection.

	Yeasts	Moulds	Dimorphic fungi	Dermatophytes
Genera	*Candida* *Saccharomyces* *Malassezia* *Trichosporon* *Cryptococcus*	*Aspergillus* *Fusarium* *Mucor, Rhizopus..*	*Histoplasma* *Coccidioides* *P. marneffei* *Sporotrix …*	*Trichophyton* *Microsporum* *Epidermophyton*
Normal habitat	Mucosa Skin (*Malassezia, Trichosporon*) Pigeon droppings (*Cryptococcus*)	Ubiquitous Inhalation/ inoculation	Endemic outside DK Inhalation/ Inoculation	Ringworm of humans and animals
Disease entities	Mucositis Skin infections Haematogenous dissemination Meningitis (*Cryptococcus*)	Lung-Infections Sinusitis Haematogenous dissemination (*Fusarium)*	Lung-Infections Haematogenous dissemination Skin infections	Nail, inquina, body scalp

Fungaemia, or fungal blood stream infection, is the most common form of invasive fungal infection (27,28). The vast majority involves *Candida* (candidaemia), whereas other yeasts such as *Saccharomyces cerevisiae* (strictly speaking also a *Candida* species), *Cryptococcus*, *Rhodutorula* and *Trichosporon* at decreasing incidence rates may also be involved (7,8,29,30). Moulds rarely cause fungaemia with the exception of *Fusarium*. Finally, dimorphic fungi may become invasive with a fungaemic state. However, dimorphic fungi not endemic in our part of the world.

For the purpose of this thesis, the studies are divided into three main sections: 1) Virulence of the most common human pathogenic *Candida* species (Paper I), 2) Susceptibility testing, detection of acquired resistance and implications for breakpoint setting (Papers II-VI) and 3) Epidemiology of fungaemia with focus on *Candida* (Papers VII & VIII).

1. VIRULENCE OF THE MOST COMMON HUMAN PATHOGENIC *CANDIDA* SPECIES (PAPER I)

Knowledge of the virulence and growth kinetics of the different *Candida* species in animal models is important for several reasons. First, animal models are frequently used in the evaluation of antimicrobial agents including drug-drug comparisons and optimal dosing. Second, to the extent the virulence in the animal

model mirrors that in humans, virulence in the animal model may help understanding epidemiology, course of infection and likelihood of success or failure of antifungal treatment. For example, a low virulent organism may be successfully treated with standard dosing of a given compound despite higher MICs, and adversely even a high potency compound may fail if the infection is advanced and involves a sufficiently pathogenic organism. The role virulence plays for the final outcome can be depicted in the "outcome triangle" (Fig. 1). Outcome is dependent on the fungus (virulence and susceptibility), the host status (severity of the underlying condition) and the therapy (timing in relation to infection stage, antifungal drug choice and dosing).

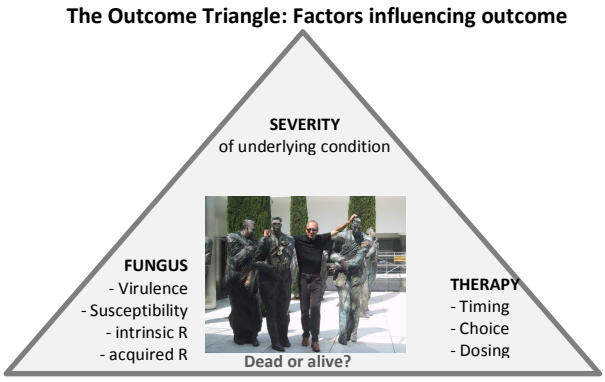


Figure 1: The outcome triangle. Outcome is dependent on the fungus (virulence and susceptibility), the host status (severity of the underlying disease) and the therapy (timing in relation to infection stage, antifungal drug choice and dosing).

In our study of the pathogenicity of *Candida* species (Paper I, (1)) we compared eight species simultaneously in a haematogenous mouse model. Endpoints were mortality and kidney burden day 2 and 7 in mice challenged intravenously (iv inoculation) with two different inoculum sizes. Two isolates of each species were selected and an additional type strain of *C. krusei* was added as a control as the two clinical strains of this species appeared surprisingly low-virulent in the initial experiments.

Overall, the isolates could be divided into three groups of decreasing pathogenic potential: I) *C. albicans* and *C. tropicalis*; II) *C. glabrata, C. lusitaniae* and *C. kefyr* and III) *C. parapsilosis, C. krusei* and *guilliermondii* as illustrated in the table 2 below. Subsequently, *C. albicans, C. tropicalis, C. glabrata* and *C. krusei* were further investigated for their relative pathoanatomical and histopathological virulence as representatives for each of the three virulence groups. The findings were consistent with those of the classification according to mortality and fungal burden. As an example, only *C. albicans* and *C. tropicalis* infected mice lost weight over the study period, had high inflammation scores in the kidneys and metastatic involvement of the eyes (Table 2). And adversely, *C. krusei* infected mice gained weight, had no metastatic eye infections and showed no or discrete inflammation of the kidney tissue.

Although virulence of different species had been subjected to prior investigations, as many as eight species had not been compared simultaneously before. Still the study is associated with several limitations. Although the iv challenge per se mimics candidaemia arising from contaminated or colonised iv catheters, the high inoculum size is artificial. Most candidaemia cases probably arise from a smaller amount of yeast cells entering the blood stream following a barrier leakage during gastrointestinal (GI) surgery or impaired integrity of the gastrointestinal mucosa during chemotherapy. Indeed, the relative virulence of *C. albicans* and *C. tropicalis* depends on the model used. Thus, whereas *C. albicans* is the more pathogenic species of the two in the iv model,*C. tropicalis* is so in the GI challenge model using animals pretreated with antibiotics and chemotherapy (1,31,32). In agreement with this *C. albicans* is still the dominating organism in invasive infections overall, but *C. tropicalis* has for a long time been recognised as a significant pathogen in the haematological setting (33-35) and ranked second, accounting for 25% of the infections in a report summarising surveys from 1952-1992 and including 1,591 cases of systemic candida infection in patients with cancer in the mainly pre-fluconazole era (34). However, outside the oncology/haematology setting *C. tropicalis* is less frequent on the Northern hemisphere. In a nationwide candidaemia survey in Norway 1991-96 *C. tropicalis* ranked only fourth

Table 2: Summary of the pathogenicity endpoints for the three virulence groups. Further details including P-values, are available in paper I (1).

Group, *Candida* species	Mortality	No. pos. kidneys (%)		Log CFU count (median)		Mouse weight change (g, mean)	Kidney weight % of mouse weight	Inflammation score	Eye infection (no. of mice)
		Day 2	Day 7	Day 2	Day 7				
I									
*albicans**	yes	100	100	5.64	6.24	-2.3	1.00	+++	1/3
tropicalis	yes	100	100	6.45	5.98	-2.1	0.92	++	2/3
II									
glabrata	no	100	100	4.42	6.04	0.2	0.79	+	0/3
lusitaniae	no	100	100	5.25	7.04	ND	ND	ND	ND
kefyr	no	100	100	5.2	6.41	ND	ND	ND	ND
III									
parapsilosis	no	100	69	4.5	3.72	ND	ND	ND	ND
krusei	no	100	38	3.65	n.y.d.	2.8	0.69	0-(+)	0/3
guilliermondii	no	50	6	4.00	n.y.d.	ND	ND	ND	ND
Uninfected control	no	0	0	ND	n.y.d	-0.1	0.73	0	0/3

* For *C. albicans* animals received an inoculum of 10^5 CFU (colony forming units) whereas animals challenged with the other species received 10^7 CFU. n.y.d: no yeast detected (detection level 10 CFU/g). ND: not done.

accounting for 6.4% of the cases and following *C. albicans* (66%), *C. glabrata* (12.5%) and *C. parapsilosis* (7.6%) each from our virulence groups I, II and III, respectively (36).
Possible explanations for this apparent contradiction could be at least two-fold. Even though *C. tropicalis* is virulent once it has entered the blood stream it is less prone to do so in absence of mucosal impairment (31). And additionally, this species is a less frequent coloniser of the mucosal surfaces, which is a necessary prerequisite for invasive infection (37).
C. parapsilosis and *C. krusei* were very low-virulent. This is in agreement with the low prevalence and mortality for *C. parapsilosis* candidaemia cases, and the fact that *C. krusei* is highly uncommon outside clinical settings involving azole exposure and poor immune status (9). Moreover, *C. parapsilosis* has been found low-virulent in the oral and vaginitis models suggesting this is a consistent finding independently on the animal model used (38). However, *C. parapsilosis* has a niche due to two features; its ability to form biofilm on plastic and other artificial surfaces and its ability to be an asymptomatic coloniser of human skin. Nosocomial infections have been reported due to contaminated iv catheters and parenteral nutrition and due to the transfer via the hands of the nursing staff to premature neonates (39-42). *C. parapsilosis* is more common in the southern hemisphere and in Asia (43). Whether this is due to poorer hygiene standards regarding hand hygiene and catheter management, or if other factors such as differences in the normal microbial flora among different populations, is not fully understood (42,44,45).
An important implication of the difference in virulence besides being related to the epidemiology of human infections is the importance of being cautious translating outcome from one species to another for a given antifungal compound unless differences in virulence are considered. This was recently done by the CLSI during the echinocandin breakpoint setting process (46). The echinocandins display very high activity (low MICs) against four of the five most common *Candida* species (*C. albicans, C. tropicalis, C. glabrata* and *C. krusei*), whereas *C. parapsilosis* is intrinsically less susceptible due to a naturally occurring alteration in the gene encoding the target enzyme glucan synthase (47). However, in the three clinical studies evaluating echinocandins for candidaemia, the outcome for *C. parapsilosis* was either equal (48,49) or only numerically and not statistically lower than that for the other species (48,50). Therefore, a non-species specific breakpoint was selected at S: MIC ≤ 2 mg/L in order to include the higher MIC distribution of *C. parapsilosis*. Hereby, it was indirectly assumed that an infection due to a *C. albicans* isolate with an MIC of 2 mg/L was equally susceptible to echinocandin treatment as was a *C. parapsilosis* with the same MIC, although an MIC of 2 mg/L was clearly elevated for a *C. albicans* isolate. As illustrated later, this was incorrect (Paper IV, (4)) which is less surprising considering the difference in virulence among these two species.

2.A. SUSCEPTIBILITY AND RESISTANCE TO ANTIFUNGAL COMPOUNDS AND METHODS FOR DETERMINATION HEREOF.

Antifungal compounds and target site

The antifungal compounds used for fungal blood stream infections include amphotericin (deoxycholate and various lipid formulations), the echinocandins (anidulafungin, caspofungin and micafungin) and azoles (fluconazole). In addition voriconazole has *Candida* activity but is reserved for resistant cases or situations where coverage of *Aspergillus* is also warranted. Finally, flucytosine is used for *Cryptococcus* infections involving the CNS, but never as monotherapy. The antifungal target of each compound and drug class is illustrated below (Fig 2). Amphotericin is fungicidal and targets ergosterol, an essential sterol in the fungal cell membrane. Upon binding, pores are formed through the membrane leading to loss of intracellular substances and eventually cell death. The echinocandins inhibit the enzyme glucan synthase that is important for the cell wall formation. This target is unique as the human eukaryotic cell has no cell wall, and thereby this drug class is less prone to cause cross reaction and interference with the human cell. The echinocandins have due to their target site been compared to the ß-lactam antibiotics and are fungicidal against *Candida*. The azoles inhibit the enzyme P450 demethylase necessary for the ergosterol synthesis. This leads to reduced ergosterol formation and growth arrest. The azoles are fungistatic against *Candida*. Flucytosine is almost exclusively used in combination with amphotericin for *Cryptococcus* infections and other rare yeast infections involving the CNS or other foci where drug penetration is a limiting factor. Due to a rapid development of resistance when used as monotherapy, the compound is rarely used. It was originally developed as an anticancer agent, and does possess bone marrow depressing side effects particularly at higher concentrations. It acts by inhibiting fungal DNA and RNA synthesis and is fungistatic in lower but fungicidal in higher dosages. Finally, terbinafine is occasionally used for rare and very severe infections due to resistant moulds like *Fusarium* in combination with other agents. It inhibits an earlier step in the ergosterol synthesis pathway.

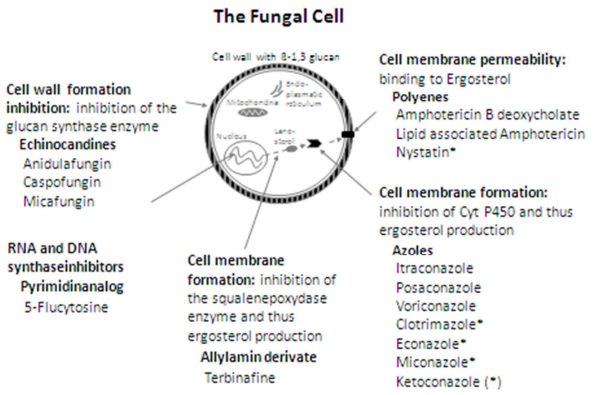


Fig 2. Target site for the antifungal drug classes. Compounds used for topical treatment only are indicated with an "*" in order to separate these from those only used for systemic treatment. Finally, compounds for systemic as well as use are denoted with an "(*)".

Intrinsic susceptibility pattern

The antifungal spectrum varies by fungal species similarly to what is well known for antibiotics and bacteria. Whereas *C. albicans, C. dubliniensis* and *C. tropicalis* are normally susceptible to all antifungals used for fungaemia, *C. glabrata* is less susceptible and *C. krusei* intrinsically resistant to fluconazole. Additionally, *C. parapsilosis* is less susceptible to the echinocandins. The intrinsic susceptibility patterns are summarised in table 3 below (51).

Table 3. Susceptibility pattern of wild type fungal blood pathogens. Susceptibility is indicated using the classical abbreviations: S denotes susceptible, I intermediate and R stands for resistant.

	Amphotericin	Echinocandins	Fluconazole	Itraconazole	Voriconazole	Posaconazole	5-flucytosine	Terbinafine
Candida								
C. albicans	S	S	S	S	S	S	S	-
C. glabrata	S	S	I-R*	S-I-R*	S-I-R*	S-I-R*	S	-
C. krusei	S	S	R	I-R	S-I-R*	S-I-R*	R	-
C. parapsilosis	S	S-I	S	S	S	S	S	-
C. tropicalis	S	S	S	S	S	S	S	-
S. cerevisiae	S	S	I-R*	S-I-R*	S-I-R*	S-I-R*	S	-
Cryptococcus	S	R	S**	S	S	S	S	-
Trichosporon	S-I-R	R	I-R	I-R	S	S	R	-
Fusarium	S	R	R	R	S-I-R	S-I-R	R	S-I-R

* The wild-type populations of *C. glabrata* and *C. krusei* (i.e. isolates without acquired resistance mechanisms) are less susceptible to all azoles than *C. albicans.* Due to methodological variation azole MIC values span the S, I and R categories for these species leading to random classification. *C. glabrata* and *C. krusei* are not regarded as optimal targets for azoles by EUCAST. Isolates with acquired resistance mechanisms to fluconazole also show elevated MICs to the other azoles.
** Hetero-resistance has been reported for the *Cryptococcus* species *C. neoformans* and fluconazole.

A number of rare species also have intrinsically reduced susceptibility to one or several drug classes. This is summarised in table 4. Among these *C. lusitaniae, C. fermentati, C. guilliermondii* and *C. palmioleophila* may be difficult to identify correctly and separate from each other in the routine laboratory, which however is important as their intrinsic susceptibility pattern differs (52).

Table 4. Rare *Candida* species with intrinsic resistance to one or several drug classes.

	Amphotericin	Echinocandins	Azoles
C. lusitaniae	X		
C. fermentati		X	
C. guilliermondii		X	X
C. metapsilosis		X	
C. orthopsilosis		X	
C. cifferrii			X
C. inconspicua			X
C. humicula			X
C. lambica			X
C. lipolytica			X
C. norvegensis			X
C. palmioleophila			X
C. rugosa			X
C. valida			X

X denotes intrinsic resistance towards the specified antifungal compound in the indicated *Candida* species.

Acquired resistance mechanisms

With the increasing use of the antifungal compounds emergence of resistance has been increasingly reported. The majority of these cases are due to selection of species with intrinsic resistance, e.g. the increasing proportion of fungaemia cases being due to *C. glabrata*, but for a minority of cases acquired resistance in species that are normally susceptible has been detected (53-57). In this context it is important to notice that only the azole compounds can be given orally, and thus are used extensively in the primary health care sector, whereas the other compounds are exclusively used in the hospital setting. Therefore, the major shift in epidemiology has been a shift from *C. albicans* to the azole resistant species *C. glabrata* whereas *C. parapsilosis* is still an infrequent pathogen and acquired echinocandin resistance has only been reported as breakthrough or failure cases in hospitalised patients (4,54,55,58).

Acquired resistance in *Candida* has been described and underlying mechanisms characterised for azoles and echinocandins. For the azoles, three mechanisms are found including 1) target gene mutation leading to affinity loss for the azole, 2) target gene up-regulation leading to lower drug efficacy simply due to competition between the drug and the target and finally 3) efflux pump induction conferring reduced intracellular drug concentration. In the individual isolate these mechanisms often act in concert leading to stepwise increases in MICs and broadening of the azole resistance spectrum (59-62). For the echinocandins so far only target gene mutations have been described as the underlying mechanisms in resistant isolates (5,6,47,63-72). The glucan synthase enzyme is an enzyme complex with at least two subunits: a catalytic subunit encoded by three related genes (*FKS1, FKS2* and *FKS3*) and a regulatory subunit, Rho1p. Mutations associated with resistance have been described in *FKS1* and *FKS2*, and naturally occurring alterations have been demonstrated in those species with intrinsic reduced susceptibility (Table 5) (5,6,47,63-72).

Table 5. Amino acid sequence of the "hot spot regions" of FKS1 and FKS2 proteins in *Candida* species. Positions associated with acquired resistance (indicated in red for high level resistance and yellow for low level resistance) are indicated as well as positions associated with intrinsic resistance (blue) or where silent alterations have been found in isolates with wild type susceptibility (green) (5,6,47,63-72).

	FKS1		*FKS2*	
	Hot spot 1 (F641-P649)*	Hot spot 2 (D1357-L1364)*	Hot spot 1 (F659-P667)*	Hot spot 2 (D1374-L1381)*
C. albicans	FL TLSLRDP	DWIRRTYL		
C. dubliniensis	FL TLSIRDP	DWIRRTYL		
C. glabrata	FL ILSLRDP	DWIRRTYL	FLILSLRDP	DWVRRYTL
C. krusei	FL TLSIRDP	DWIRRTYL		
C. tropicalis	FL TLSIRDP	DWIRRTYL		
C. guilliermondii	FMTLSIRDP	DWIRRTYL		
C. lypolytica	FL TLSIRDP	DWIRRCVL		
C. parapsilosis	FL TLSIRDA	DWIRRTYL		
C. metapsilosis	FL TLSIRDA	DWIRRTYL		
C. orthopsilosis	FL TLSIRDA	DWVRRTYL		

* Numbers indicate the amino acid number in *C. albicans* and differ for several of the other species. The amino acid abbreviations are as follows: Alanine (A), Arginine (R), Aspartic acid (D), Cysteine (C), Isoleucine (I), Leucine (L), Methionine (M), Phenylalanine (F), Proline (P), Serine (S), Threonine (T), Tryptophan (W), Tyrosine (Y) and Valine (V).

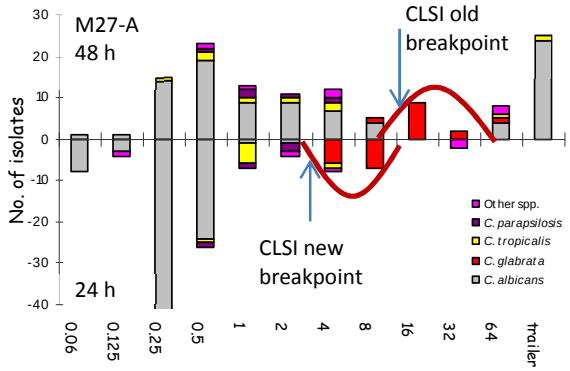
Compared to resistance incidence rates in bacteria, acquired resistance in *Candida* is a rather rare event. *Candida* is not a contagious disease and thus resistant isolates are rarely transferred from patient to patient. Moreover, resistance mechanisms cannot be transferred via plasmids among yeast cells. Hence, resistance has to arise in the each isolate during antifungal exposure, which probably is the reason for the limited level of acquired resistance in a global perspective.

Reference Methods for Antifungal Susceptibility Testing

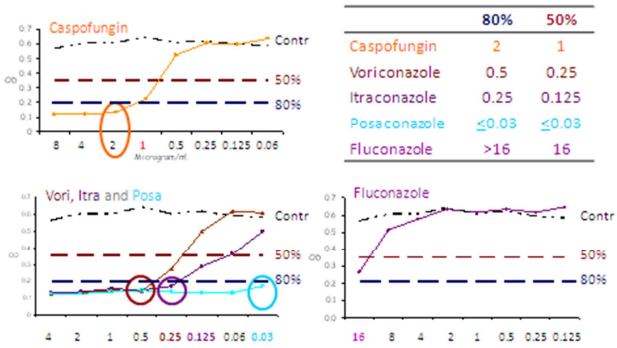
A range of factors influence the endpoint achieved when performing susceptibility testing. By modifying the test medium

(type, brand, batch), inoculum size, inoculum growth phase, incubation temperature, incubation time, definition of endpoint (50%, 80%, 100% inhibition) the MIC may vary. In example, we evaluated the influence of the duration of incubation for fluconazole and amphotericin MICs for various *Candida* species by comparing MICs obtained after 24 and 48 hours of incubation (Paper II (2)). Not surprisingly, the MIC was lower for the 24 than the 48 h reading with MIC_{50} of 0.25 mg/L (0.06-32 mg/L) vs. 2 mg/L (0.06 – ≥ 64 mg/L) for fluconazole and 0.25 mg/L (<0.03-1 mg/L) vs. 1 mg/L (0.25-2 mg/L) for amphotericin, respectively (2) (Fig 3). Subsequently, we demonstrated that using a less stringent endpoint of 50% growth inhibition rather than 80% inhibition results in a one-step difference in MIC (unpublished observation, Fig 3).

a)



b)



	80%	50%
Caspofungin	2	1
Voriconazole	0.5	0.25
Itraconazole	0.25	0.125
Posaconazole	≤0.03	≤0.03
Fluconazole	>16	16

Fig 3. Influence of changes in selected susceptibility parameters on the MIC. A one to two-fold dilution step reduction of fluconazole MICs is observed when the incubation time is shortened from 48h (above the X-axis) to 24h (below the X-axis) (2) (fig a). Similarly, a one dilution step reduction is obtained by changing the growth inhibition endpoint from 80% growth inhibition required to the less stringent 50% inhibition endpoint (fig b).

Notably, these two factors have been changed for the CLSI reference methodology during the evolution from the initial CLSI method document (M27-A) to the most recent CLSI document (M27-A3), as the incubation time has been shortened (48 h to 24 h) and a less stringent (in terms of the amount of growth allowed) endpoint criterion adopted (80% to 50% growth inhibition). The change in incubation time was motivated by the fact that, from a clinical point of view, it was desirable to shorten the incubation time and thus be able to provide the susceptibility result a day earlier. The change in inhibition endpoint was motivated by the finding that the 50% inhibition corresponds to the steepest part of the growth curve, thereby leading to less variation in the endpoint reading. Furthermore, it was avoided that a significant proportion of the isolates, the so-called trailers which were found to be susceptible in animal models, were classified as resistant since a growth inhibition of 50% but not 80% was obtained over a wide concentration range spanning the susceptibility breakpoint. Thus, the changes were reasonable and well-motivated and on the same time lead to a partial harmonisation of the CLSI and EUCAST methods. Notably, however, these changes were accompanied by a revision (lowering) of the recommended MIC ranges for the quality control strains, but unfortunately not at the same time by a change in clinical breakpoints; an issue we raised some years ago (73). A summary of the reference methods, associated MIC ranges for fluconazole and breakpoints are displayed in table 6.

Commercial Methods for Antifungal Susceptibility Testing of Candida

As the reference methods are technically demanding and time consuming they are less well suited for daily use in routine clinical microbiology laboratories. Several commercial tests have been introduced over the years including agar diffusion tests (Etest, disc and tablet diffusion), the Vitek broth based semi-automatic system and the Sensititre and Micronaut microdilution panels (to my knowledge the latter are not yet used in DK). Examples of the agar diffusion tests are shown in Fig 4. Inhibition zones for the fungicidal compounds amphotericin and echinocandins are typically clear and the margins rather sharp for *Candida*. For the fungistatic azoles on the other hand the zone margins are fuzzier and micro-colonies in the zone are often found which makes endpoint reading difficult and associated with a high degree of inter- and intra-laboratory variation (2). This implies that susceptibility testing in the busy clinical microbiology routine laboratory may be associated with a risk of misclassifications of resistant isolates. This concern was substantiated as 11% of the Nordic laboratories failed to recognise the fluconazole resistant phenotype of a *C. albicans* and 34% for a *Cryptococcus* isolate, respectively, as part of a Nordic quality assessment exercise, despite it is our experience that such external quality assessment samples receive greater attention in the laboratory than routine specimens (76).

Table 6: Method characteristics and breakpoints for the reference methods for susceptibility testing of yeasts. In the upper part of the table the key parameters recognised to influence the MIC determination for the three versions of the CLSI (formerly NCCLS) methodologies (M27-A, M27-A2 and M27-A3) (15,16,18) and for EUCAST E.DEF7.1 (74) are summarised. In the lower part the associated breakpoints are listed (15,17,19) (24-26) (20-23,75).

	CLSI				EUCAST
	M27-A	M27-A2	M27-A3	M27-S4 (In preparation)	
Method characteristics					
Glucose Inoculum size	0.2% 0.5-2.5 x 10^3				2% 0.5-2.5 x 10^5
Plates & Reading	Round bottom & Visual				Flat & Spectrophotometer
Incubation time	48 h	24/48 h	24 h	24 h	24 h
Endpoint	80% inhib.	50% inhib.	50% inhib.	50% inhib.	50% inhib
Fluconazole QC MIC ranges					
C. parapsilosis ATCC 22019	2-8 mg/L	0.5-4 / 1-4 mg/L	0.5-4 mg/L	0.5-4 mg/L	0.5-2 mg/L
C. *krusei* ATCC 6258	16-64 mg/L	8-64 / 16-128 mg/L	8-64 mg/L	8-64 mg/L	16-64 mg/L
Breakpoints (mg/L)					
Amphotericin	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1
Anidulafungin	-	-	≤ 2	≤ 0.25; >0.5 (Ca-Ck-Ct) ≤ 0.125; >0.25 (Cg) ≤ 2; >4 (Cp)	≤ 0.03; >0.03 (Ca) ≤ 0.06; >0.06 (Cg-Ck-Ct)
Caspofungin	-	-	≤ 2		-
Micafungin	-	-	≤ 2	≤ 0.25; >0.5 (Ca-Ck-Ct) ≤ 0.06; >0.125 (Cg) ≤ 2; >4 (Cp)	-
Fluconazole	≤ 8; >32	≤ 8; >32	≤ 8; >32	≤ 2; >4 (Ca-Cp-Ct) Cg SDD: ≤ 32*; >32	≤ 2; >4 (Ca-Cp-Ct)
Itraconazole	≤ 0.125; >0.5	≤ 0.125; >0.5	≤ 0.125; >0.5	≤ 0.125; >0.5	-
Posaconazole	-	-	-	-	≤ 0.06; >0.06 (Ca-Cp-Ct)
Voriconazole	-	≤ 1; >2	≤ 1; >2	≤ 0.125; >0.125 (Ca-Cp-Ct) ≤ 0.5; >1 (Ck)	≤ 0.125; >0.125 (Ca-Cp-Ct)

"-" denotes that no breakpoints have yet been established for the given compound and method. "Ca": C. albicans, "Cg": C. glabrata, "Ck": C. krusei, "Cp": C. parapsilosis and "Ct": C. tropicalis. Breakpoints followed by a species indication are species specific in contrast to those without which are non-species specific. *For the M27-S4 set of breakpoints no S category exists for *C. glabrata* as isolates with an MIC ≤ 32 are classified as susceptible and isolates with MIC>32 are classified as resistant

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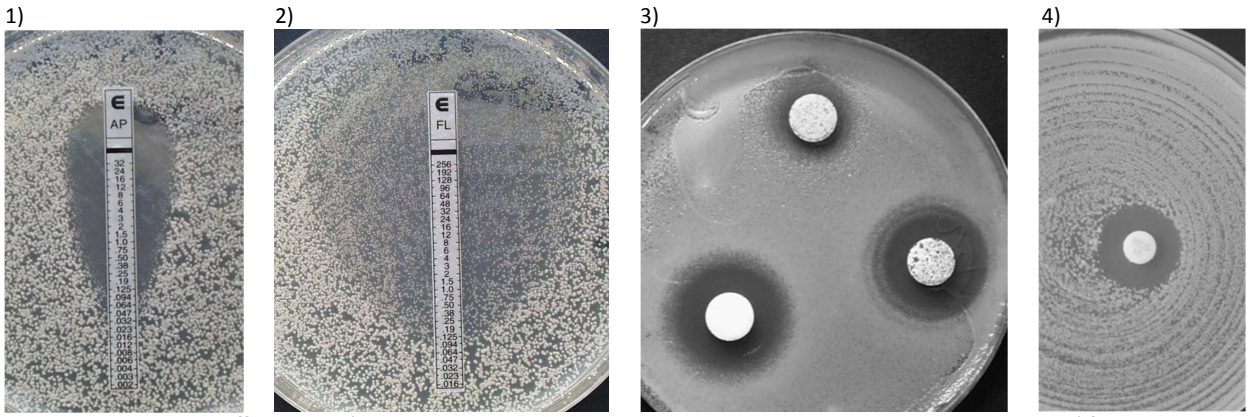


Fig 4. Etest, tablet and disc diffusion with 1) amphotericin Etest creating a clear zone with a sharp margin typically easy to read, 2) fluconazole Etest with micro-colonies in the zone and an endpoint defined as the transition from macro- to micro-colonies, 3) amphotericin, fluconazole and itraconazole tablets illustrating the fuzzy zones typical for particularly azole diffusion endpoints (lower left tablet) and 4) echinocandin disc with a clear endpoint typical for the fungicidal compounds (amphotericin and echinocandins).

Obviously, standardisation of inoculum, differences in growth rate and batch to batch variation in test media is a lot more critical for diffusion tests than for broth microdilution due to the fact that the endpoint reading is not relative to the growth control and not objective as when determined by a spectrophotometer for the EUCAST method. Another crucial matter when using commercial methods and adopting either CLSI or EUCAST breakpoints is that misclassifications are inevitable unless the MIC results for the commercial test mirror those obtained by the reference method. We recently demonstrated that the use of caspofungin Etest and the revised CLSI breakpoints resulted in misclassifications of susceptible wild type *C. glabrata* and *C. krusei* isolates in 1/3 and 2/3 of the cases, respectively. This was due to the fact that the MIC ranges obtained by Etest for these species were higher than those obtained for the CLSI methodology (77). A final disadvantage for the diffusion tests is that when mixed cultures are not recognised and therefore susceptibility tested, which is not a rare situation for double infections with *C. albicans* and *C. glabrata*, the susceptibility of the more susceptible isolate is typically reported particularly for the azoles as micro-colonies (typical for *C. glabrata*) in the zone should be ignored. In contrast, the susceptibility of the more resistant one is reported when microbroth dilution is performed as this one will grow in wells with higher drug concentrations. Pros and cons for the different commercial and reference tests are summarised below (Table 7).

Table 7. Overview of various characteristics regarding performance and friendliness for the different antifungal susceptibility test available.

	EUCAST	Etest	Disc/tablet diffusion	VITEK
General issues				
Lab-friendliness	Low	High	High	High
Sensitivity to inoculum variation	Low	Medium	High	Not tested
Reproducibility	High	Medium	Low	Not tested, probably high.
Endpoint reading				
Amphotericin	Easy, narrow conc. range limiting discriminatory potential	Easy, but recommended breakpoint bisects *C. krusei*	Small zones	Easy. Concerns for amphotericin and EUCAST possibly apply here as well.
Echinocandins	Easy	Easy, but recommended CLSI breakpoint bisects *C. glabrata* and *C. krusei*	Easy, but lab to lab variation prohibited establishment of meaningful CLSI disk breakpoints	Easy, but the test MIC range for caspofungin is too high (e.g. S and I category for *C. glabrata* cannot be differentiated. App. 20% VMEs in a recent study for caspofungin (Astvad ICAAC poster 2012).
Azoles	Easy	Difficult, potentially leading to misclassifications of resistant isolates.	Difficult, potentially leading to misclassifications of resistant isolates.	Easy

Table 7. Continued.

	EUCAST	Etest	Disc/tablet diffusion	VITEK
Specific "cons"	Not all compounds are commercially available. Most laboratories are not familiar with plate production and test principle.	MIC ranges do not mirror those of the reference methods for all species and compounds which leads to misclassifications when reference breakpoints are adopted. Pattern for the most susceptible organism reported if mixed cultures are tested.	Pattern for the most susceptible organism reported if mixed cultures are tested. Significant inter-laboratory variation potentially leading to misclassifications.	Test MIC range also for voriconazole is too high to allow detection of any minor MIC drift in normally susceptible species.
Specific "pros"	Pattern for the most resistant organism reported if mixed cultures are tested	Apparently the best test for the detection of amphotericin resistance. Most laboratories are familiar with the principle	Most laboratories are familiar with the principle	Semi-automated and electronic output improves objectivity in endpoint reading. Many laboratories are familiar with the principle

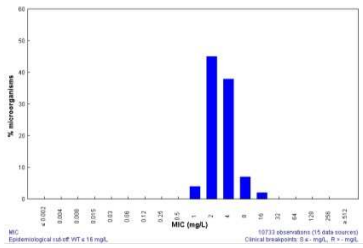
2.B. DETECTION OF ACQUIRED RESISTANCE AND IMPLICATIONS FOR BREAKPOINT SETTING (PAPERS II-VI)

Important basic tenets in susceptibility testing and development of breakpoints are that breakpoints 1) should not bisect wild type distributions of target microorganisms and 2) should not be established higher than the epidemiological cut off value (ECOFF), unless clinical data suggest infections involving such isolates can be treated efficaciously with standard dosing of the compound. The background for these fundamental rules is discussed in the following section.

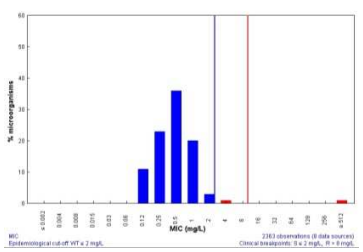
Breakpoints should not bisect wild type distributions

The scientific basis for this concept is the following. First, a well standardised antibacterial test can at best provide MICs at ± 1 dilution 95% of the times and ± 2 dilutions 99% of the times. This is evident by reviewing the wild type MIC distributions available at www.eucast.org and from where the following two examples are obtained (Fig 5a and b).

a)



b)



c)

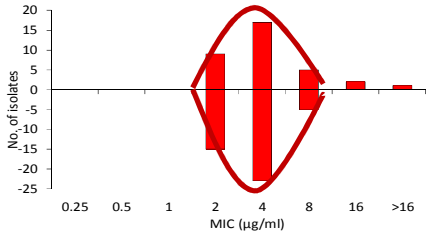
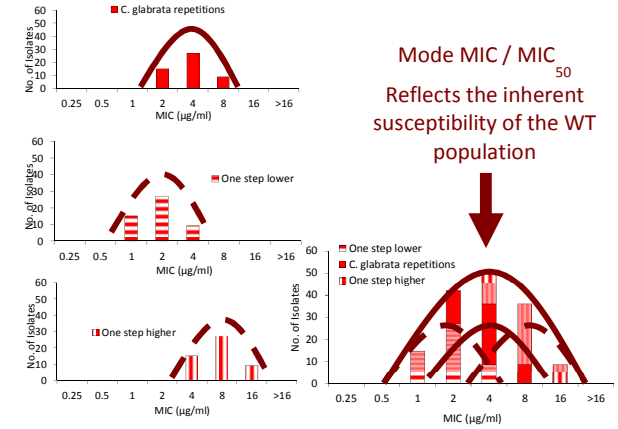


Fig 5. Examples of wild type MIC distributions for two bacteria (a) benzylpenicillin and *E. faecalis* (10,773 isolates, 15 datasets, www.eucast.org) and b) imipenem and *C. freundii* (2363 isolates, 8 datasets, www.eucast.org) and c) the comparison of fluconazole MICs obtained for 34 different *C. glabrata* isolates received for routine testing over a 3-month period (above the x-axis) and repeated testing (51 times) of a single *C. glabrata* isolate (below the X-axis) (3).

This is also the case for susceptibility testing of *Candida*, as we illustrated by repeated testing of a single *C. glabrata* isolate initially tested with a "low" MIC of 2 mg/L in parallel with isolates received in the daily routine over a 3-month period and using a single batch of susceptibility testing plates (Fig 5c) (3). Not surprisingly, we showed that the MICs of the repeated testing mirrored the MICs of the routine *C. glabrata* isolates with the exception of 3 outliers that separated from the Gaussian distribution with elevated MICs at 16 mg/L and 32 mg/L, respectively. Outlier isolates separating a single dilution step from the wild type distribution may either represent technical variation or isolates with acquired resistance mechanisms, whereas isolates separating more than a single step from the wild type distribution should be regarded as non-wild type isolates. The wild type distribution described in this study spans 3 dilution steps which is the expected variation when the analysis is performed in a single laboratory and using a single batch of test plates. As this is also the allowed MIC range for quality control strains (19,74) the entire MIC distribution of any species may generally move one step up and down as illustrated below (Fig 6a). The consequence is that when incorporating inter-laboratory variation due to different batches of test plates and different technicians, this range broadens, typically to five dilution steps as for the distributions shown

in fig 6a and as found for *C. glabrata* and fluconazole when compiling data from several laboratories (Fig 6b).

a)



b)

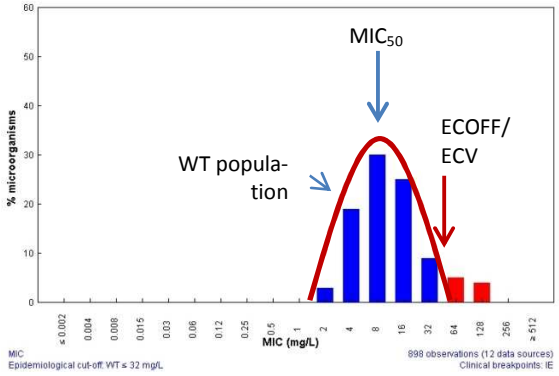
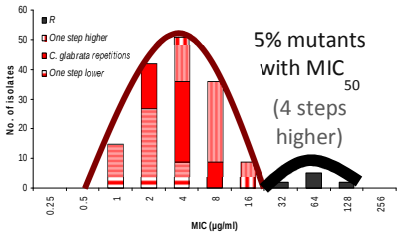


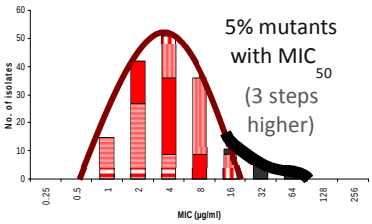
Fig 6. MIC distributions may move one step up or down and without the MICs of QC strains being off scale as the MIC range for QC strains typically spans 3 dilution steps. Hence the basic biological variation of ±1 dilution step broadens to ±2 dilution steps when data from different runs are included (Fig 6 a). A real life example of a MIC distribution for fluconazole and *C. glabrata* is shown in fig 6 b (www.eucast.org). The mode of the distribution reflects the susceptibility of the entire wild type population as variation within this can be explained solely by test variation. The upper limit of the wild type population is the ECOFF.

The implications of these facts are several. First, methodological variation alone is sufficient to explain the variation within the bell shaped population and hence the mode of the distribution reflects the susceptibility of the entire wild type population. Thus, any breakpoint bisecting this population will lead to a random classification of isolates with identical susceptibility. Second, isolates with MIC values separating from the wild type population (above the ECOFF) are non-wild type isolates and provided they belong to the same species, they possess acquired resistance mechanisms. Such isolates may or may not be clinically resistant depending on whether the drug exposure in the patient is sufficient to overcome the decreased susceptibility. Thirdly, if isolates harbour acquired resistance mechanisms leading to an MIC increase of only a few dilution steps, such isolates cannot be reliably detected using ordinary MIC testing as the MIC will overlap with the wild type population (Fig 7a-c).

a)



b)



c)

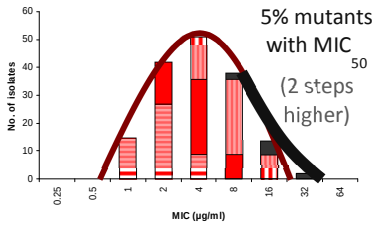


Fig 7. Fictive MIC distributions of wild type isolates from "three sources" (red) and non-wild type isolates (black) separating either 4 dilution steps (a), 3 dilution steps (b) or only 2 dilution steps (c).

If such isolates are associated with a poorer clinical response and thus important to differentiate from the wild type isolates, additional measures have to be adopted. All these scenarios are encountered for fungal infections. An example is *Aspergillus fumigatus* and itraconazole versus posaconazole. Whereas the itraconazole resistant isolates are clearly separated from the wild type population and thus easy to detect, isolates with cross resistance to posaconazole only have posaconazole MICs that are elevated a few steps from the wild type population (Fig 8) (78). This impacted the subsequent EUCAST breakpoint selection as the bioavailability of the current posaconazole formulation is not sufficient to cover isolates with increased MICs (79).

a) b)

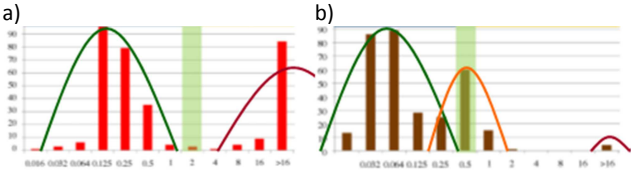


Fig 8. Itraconazole (left) and posaconazole (right) MIC distributions for *A. fumigatus* wild type isolates (red) and isolates with intermediate (orange) or high level resistance (red) (78). As seen these populations are clearly separated for itraconazole whereas the isolates with intermediate susceptibility to posaconazole overlap with the wild type isolates.

Breakpoints should not be established higher than the epidemiological cut off value (ECOFF) unless supported by clinical data

As mentioned several times in the preceding sections the clinical outcome is dependent on the severity of the underlying condition, the susceptibility and virulence of the infectious organism and the dosing and timing of the antimicrobial treatment. As illustrated in paper I (1) the pathogenicity and virulence differs considerably among the different species. The initial CLSI echinocandin breakpoints were established taking microbiological data (MIC distributions), pharmacokinetic/pharmacodynamics data (PK/PD) and outcomes from clinical trials into account (46,48-50). Based on 1) the finding that MICs of <2 mg/L for all three echinocandins encompass 98.8 to 100% of all clinical isolates of *Candida* spp. without bisecting any species group and represent a concentration that is easily maintained throughout the dosing period; 2) that data from phase III clinical trials demonstrate that there were no difference in outcome between the different species and 3) that the standard dosing regimens for each of these agents may be used to treat infections due to *Candida* spp. for which MICs are as high as 2 mg/L, a susceptibility breakpoint of ≤2 mg/L for all species and all three echinocandin compounds (19,80). An MIC predictive of resistance to these agents could not be defined based on the data from clinical trials due to the paucity of isolates for which MICs exceeded 2 mg/L (46,48-50).

The pitfalls in this procedure are the following. First, pooling MIC-outcome relationships across different species imply the presumption that these species are equally virulent. As we know this is not the case as for instance *C. parapsilosis* is clearly less virulent than *C. albicans*. In the case of the echinocandins this is of interest as *C. parapsilosis* is the species with the highest MICs for the echinocandins due to an intrinsic alteration in the target enzyme as described above. Hence, translating the outcome for the low virulent *C. parapsilosis* to the high virulent *C. albicans* with elevated MICs may be dangerous. The MICs for wild type *C. albicans* are considerably lower than for *C. parapsilosis* (e.g. caspofungin MIC_{50} of 0.06 mg/L versus 1 mg/L). Therefore, by selecting a susceptibility breakpoint of ≤2 mg/L the caspofungin MIC for the average *C. albicans* isolates may increase 5 dilution steps without exceeding the breakpoint. And although the low virulent organism with this MIC may be treated successfully, this may not necessarily be true for a more virulent isolate with the same MIC. Indeed, when more isolates from failure cases became available and the underlying resistance mechanisms were confirmed, it became evident that such isolates were misclassified according to the CLSI breakpoints.

Echinocandin susceptibility testing detection of acquired resistance

Conventional methods

We initially became aware of the risk of misclassification of echinocandin resistant *Candida* isolates by the reference methods due to a clinical case where the routine clinical microbiology laboratory had identified a potentially caspofungin resistant isolate using Etest (endpoint ≥32 mg/L) in a patient failing therapy though we by CLSI and EUCAST reference microdilution found caspofungin MICs of 1-2 mg/L and thus below the CLSI M27S-3 susceptibility breakpoint of ≤2 mg/L (4). The isolate was then subjected to a variety of susceptibility testing approaches and compared to a clinical control *C. albicans* isolate. For all tests, including repeated caspofungin CLSI and EUCAST microdilution with two different lots of pure substance, anidulafungin CLSI and EUCAST microdilution, anidulafungin and caspofungin Etest, anidulafungin and caspofungin disk testing using three different disk content amounts and two different agars and anidulafungin and caspofungin agardilution the index isolate was more resistant than the control isolate (4). This was supported in the haematogenous candidiasis mouse model as anidulafungin and caspofungin failed to reduce the kidney fungal burden in mice challenged with the caspofungin isolate whereas both compounds suppressed the fungal burden below the detection level in mice challenged with the control isolate. Finally, the *FKS1* gene hot spot region was sequenced and revealed a S645P alteration that has later been recognised as one of the most frequent and dominating resistance mutations for the echinocandins. The interpretation of these findings was obviously that the caspofungin CLSI M27S-3 breakpoint was insensitive for the detection of clinically relevant resistance, and this was in fact even more true for the anidulafungin breakpoint of ≤ 2 mg/L, as the anidulafungin MIC values obtained for this resistant isolate were even lower (CLSI: 0.25-0.5 mg/L and EUCAST: 0.06-0.25 mg/L depending on if frozen or fresh plates were used). The second notable observation was that the caspofungin MICs varied by the caspofungin pure substance lot number and most remarkably for the susceptible isolate. This suggested that for some batches the MIC values were higher than for others and that for the high MIC lots the separation between susceptible and resistant strains was less pronounced (table 2 in (4)). This observation might help understand the issue of low reproducibility of caspofungin MIC testing across studies over time and between laboratories, which is debated in this paper (Table 5 in (4)) and also addressed in our later study by comparing the MICs for quality control strains for different lots of caspofungin pure substances (6). This is still an unresolved issue as illustrated in the figure below compiling MIC values from different reference centres around the world (fig 9).

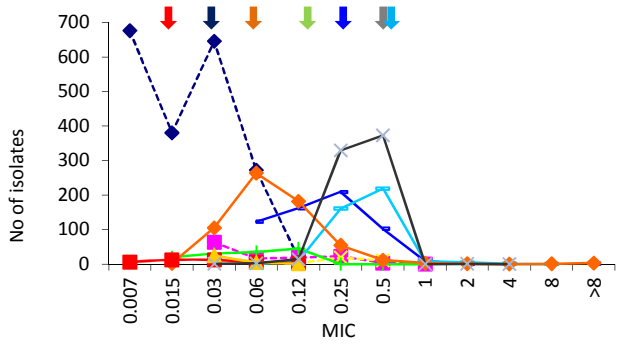


Fig 9. Diagram showing the individual caspofungin MIC distributions for *C. albicans* obtained at nine reference laboratories in Europe and the US. Three dataset were generated using the CLSI method (dotted lines) and with peaks at ≤ 0.07 and 0.03 mg/L (blue) or ≤ 0.03 mg/L (magenta and yellow). Seven dataset were generated using the EUCAST methodology (solid lines) and with peaks of the distributions at various concentrations between 0.06 and 0.5 mg/L. Obviously, it is not possible to combine such divergent datasets and select a meaningful ECOFF. (Arrows indicate the individual peaks of the distributions, marked by the colour corresponding to the dataset in question).

Consequently, EUCAST has abstained from selecting a breakpoint for caspofungin, but recommend the use of anidulafungin testing

as a marker of the echinocandin class of drugs as any potential caspofungin breakpoint leads to the risk of either being insensitive with respect to the identification of resistant isolates as illustrated in this paper or of misclassifying wild type isolates as resistant, and thereby rejecting a potentially useful drug (22,77). The findings in this study prompted further investigation into how echinocandin resistant *Candida* isolates were best identified. Obviously, the essential ingredients of such a study were the access to isolates with resistance mutations representing all the relevant species: *C. albicans, C. glabrata, C. krusei* and *C. tropicalis*. Hence, we collected such isolates via our network in Newark, Madrid and Innsbruck and achieved a unique collection including 94 clinical *Candida* isolates of which 29 harboured *fks* resistance mutations (Table 8).

Table 8. Amino acid (AA) substitutions within the Fks proteins from the *Candida* isolates included in the study. AA alterations in hot spot regions are indicated in black whereas AA alterations outside the hot spot regions are indicated in grey and in "".

C. albicans (10)	C. dubliniensis (1)	C. glabrata (11)	C. krusei (3)	C. tropicalis (4)
Fks1p	**Fks1p**	**Fks1p**	**Fks1p**	**Fks1p**
F641S	S645P	F625S	R1361G	F76S
F641S		S629P	F655F/C	S80P (n=3)
S645Y		D632G	L658W & L701M	"V213I & V265I"
S645F		**Fks2p**	"D700M"	
S645P (n=3)		F659V	"L701M"	
S645F & R1361R/H		F659S (n=2)		
D648Y		S663P		
P649H		S663F		
		D666G		
		D666E		

The amino acid abbreviations are as follows: Alanine (A), Arginine (R), Aspartic acid (D), Cysteine (C), Glycine (G), Histidine (H), Isoleucine (I), Leucine (L), Methionine (M), Phenylalanine (F), Proline (P), Serine (S), Threonine (T), Tryptophan (W), Tyrosine (Y) and Valine (V).

The isolates were susceptibility tested in a blinded fashion (for species identification as well as *FKS* genotype) as follows: 1) EUCAST microdilution with standard medium and IsoSensitest medium performed in Madrid, CLSI microdilution performed in Newark, Agar dilution using RMPI-2G agar and IsoSensitest agar in Copenhagen (Cph) and disk and Etest diffusion testing using RMPI-2G agar and IsoSensitest agar in Innsbruck. We hence took advantage of the preferred test modality for each of the centres performing the microdilution and agardiffusion tests, whereas agardilution was performed in Cph as we were the only lab that had any experience with this test principle for *Candida* and echinocandins (4). One may argue that the optimal scenario would have been to perform all tests in all laboratories or at least in two laboratories to avoid lab specific variation or errors. However, as the important question was to address the performance with respect to separation between wild type and mutant isolates rather than compare the exact MIC value across methods, we regard the chosen approach acceptable and, importantly also manageable (in the current format 3384 susceptibility results were compared). We chose to evaluate the data using the "upper limit" of the MIC distribution for the wild type isolates (WT-UL defined as the MIC_{50} + two 2-fold dilutions) of each species as a "species specific" susceptibility breakpoint and thus evaluated the number of resistant isolates misclassified as susceptible and also evaluated the degree of overlap between the endpoints for the wild type versus the mutant isolates. The goal was to identify which methods that best separated the wild type and mutant populations and also to achieve a first impression on any future appropriate breakpoints for the reference methodologies.
Overall, the proportion of MICs for resistant isolates that overlapped with MICs of wild type isolates were higher for susceptibility test results performed using the IsoSensititre medium for all tests except disk diffusion (Table 3 in (5)). Additionally, the lowest proportion of resistant isolates overlapping with the wild type distribution was observed for EUCAST (2%), agardilution (2%), Etest (5%) and CLSI (6%) and for both reference methods lower with standard 24 h than extended 48 h incubation. This was reassuring as it would be a practical issue if different media should be adopted for this particular antifungal class and as a longer incubation time is unattractive from the clinical point of view. The test results for each method are shown in the individual tables in the publication (5).
When the proportion of very major errors (VMEs, resistant isolates misclassified as susceptible by applying the WT-UL as susceptibility breakpoint) for each of the echinocandins notable differences were observed. For CLSI and EUCAST reference methodologies as well as for Etest the lowest number of VMEs (3-4%) was obtained using anidulafungin. The number of MEs was also acceptable (1% for EUCAST, 3% for CLSI and 9% for Etest). Therefore, EUCAST has recommended EUCAST anidulafungin testing as a marker of the class until breakpoints for caspofungin and micafungin have been selected (22). In DK we recommend the use of anidulafungin Etest with EUCAST breakpoints at centres not running the reference test, although realising the performance is less optimal than for the EUCAST reference method. Thus the performance if applied on this dataset was characterised by 7% (2/29) VMEs and 12% (8/68) MEs.
Etest is indeed attractive for routine laboratories as they are already familiar with the test concept for antibacterial testing, and as the reference methodologies are laborious. As discussed above the reading of Etest and other diffusion tests is, however, not always easy or straight forward and by no means objective. This is also reflected in this study in the much broader MIC and zone diameter distributions for each species and compound even though these were performed in a skilled mycology laboratory using Etest for all their antifungal susceptibility tests. Hence it is understandable that a significant variation particularly across different laboratories is observed.
The number of VMEs was remarkably high for micafungin across the methodologies which were consistently linked to a high number of misclassifications among *C. glabrata.* As this was a consistent finding it poses the question if this is in fact misclassifications of clinically resistant isolates or rather differential activity of the three echinocandins against this species. We therefore undertook an animal experiment comparing the activity of the three echinocandins against different *C. glabrata* mutants and a wild type isolate. Our findings indeed suggested that some *fks* mutations in *C. glabrata* appear to affect the susceptibility to micafungin to a lesser extent and thus these isolates may not be clinically micafungin resistant (65).
Another interesting observation was that a notably high number of VMEs were observed for caspofungin and EUCAST testing compared to CLSI testing and agardilution. EUCAST caspofungin MICs were clearly higher for both wild type and mutant isolates

as compared to those for the other echinocandins. For example the caspofungin EUCAST MICs for wild type isolates were 6-7 steps higher than the anidulafungin EUCAST MICs, whereas the caspofungin CLSI MICs were only a single step higher than the anidulafungin CLSI MICs. Moreover, the caspofungin EUCAST MICs were in the 0.5-1 mg/L range for wild type isolates except *C. parapsilosis* whereas the caspofungin CLSI were in the 0.064-0.125 mg/L range. This suggested that the caspofungin powder used for this experiment was not of optimal inhibitory potency. In our prior study we found that such inhibitory potency loss apparently affected the wild type isolates more than the resistant ones, which is also suggested by the finding that the greatest variation across caspofungin MICs published in the literature is for the most susceptible species rather than for *C. parapsilosis* (table 2 and 5, respectively in (4)). Thus, such variation in inhibitory potency apparently not only affects the value of a given breakpoint but also the ability of such a breakpoint to discriminate between susceptible and resistant isolates. The underlying reason and mechanism for this variability in inhibitory potency is not understood. The powder is delivered with an indication of the chemical potency determined by HPLC, but apparently this translates into variable inhibitory potencies despite the same source of microtitre plates and semisynthetic medium has been used for example in our laboratory. The manufacturer (MSD) currently attempts to address this important issue.

As predicted from our prior study, agardilution performed very nicely and for caspofungin was associated with no VMEs and only 1% MEs. In fact the discriminatory potential may even have been underestimated in this study as the highest concentration tested was 4 mg/L and the MICs for the vast majority of the mutant isolates were indeed ≥ 4 mg/L. Further analysis would be needed to see if the discriminatory power is even higher and if this approach could be attractive as a commercially available test.

Disk diffusion is used for antibacterial susceptibility testing worldwide and also attractive from a financial point of use as several compounds can be tested simultaneously using a single agar plate. As for agardilution caspofungin testing performed best, and again for micafungin most overlaps between wild type and mutant populations was found for *C. glabrata*. Inter-laboratory variation, however, needs to be evaluated and addressed before it becomes clear if a disc test can be developed that performs acceptably across different laboratories (Steven Brown, unpublished observation combining our data from (81) with those from his own laboratory presented at the CLSI meeting in Atlanta 2011).

Modified CLSI and EUCAST echinocandin testing

Although the studies and comparisons of the conventional susceptibility tests showed that EUCAST, Etest and CLSI all performed acceptably when applied on the well described strain collection described above, provided the MICs were interpreted using the WT-UL defined using the same set of MICs, it was still obvious that a greater separation between wild type and mutant isolates would facilitate a higher likelihood of correct classification of clinical isolates also if the tests were to be performed in different laboratories and using different batches of test plates. A preliminary study suggested the addition of serum or BSA to the growth medium of the CLSI microdilution test might lead to an improved separation (82). We therefore decided to evaluate this approach for CLSI and EUCAST using the full wild type and mutant strain collection (Paper VI, (6)). In agreement with the observations above, we demonstrated a clear increase in MICs when the test was performed in BSA supplemented medium for CLSI as well as EUCAST testing. This is not unexpected as these drugs are highly protein bound. The MIC increase was highest for the most protein bound compounds anidulafungin (>99%) and micafungin (>99%) compared to caspofungin (96.5%) (Table 9).

Table 9. Number of 2-fold dilution step increases for EUCAST MIC values by compound, *Candida* species and *FKS* genotype (wild type or mutant).

	EUCAST MIC increase (no. of 2-fold dilutions)					
	Anidulafungin		Caspofungin		Micafungin	
	WT	*fks* mutant	WT	*fks* mutant	WT	*fks* mutant
C. albicans	≥1	6	**0**	**≥6**	≥5	≥8
C. dubliniensis	≥4	≥7	**2**	**≥4**	≥7	≥6
C. glabrata	≥5	≥7	**2**	**≥5**	≥5	≥9
C. krusei	6	6	**2**	**≥4**	≥9	≥7
C. parapsilosis	≥6	-	4	-	≥7	-
C. tropicalis	≥4	7	**1**	**≥6**	≥7	≥8

Furthermore, for the EUCAST method the MIC increase was always greater for the *fks* mutant isolates compared to the wild type isolates and consequently the number of VMEs using the WT-UL as susceptibility breakpoint was either lower or unchanged for all species-compound combinations leading to a reduction of total proportion of VMEs across the drugs and species from 24% to 4-7% (depending on if *C. krusei* isolates with BSA MICs of ≥ 32 mg/L were interpreted as VMEs or not (WT-UL=64 mg/L and thus higher than the upper concentration tested) (6). More surprising was, however, the finding that the performance of the CLSI method was not improved by the supplementation of the growth medium with BSA. Overall, the number of misclassifications was 6% by CLSI but 9% by BSA modified CLSI.

Lot to lot variation of caspofungin

An issue related specifically to the susceptibility testing of caspofungin was an unacceptable variation as described earlier. Various factors might contribute to this including 1) lot to lot variation of the inhibitory potency of the powder provided, 2) poor stability of the powder in stock solution, recommended solvent was water or saline whereas glucose was stated to facilitate degradation 3) poor stability of the micro titre susceptibility plates when stored, thus a maximum of two months has been recommended in the first EUCAST standard based on unpublished data from the Spanish reference laboratory (Manuel Cuenca-Estrella personal communication) (E.DEF 7.1 (74)).

Lot to lot variation was examined by retrieving data for repeated testing of quality control strains from our Spanish colleagues' laboratory where routine use of as many as eight reference strains were used for quality control purposes. Interestingly, and although adjustment for the reported potency in the product sheet was done routinely, clear differences were observed comparing the four lots tested (Fig 10). These differences were more pronounced for the two most susceptible reference strains *C. albicans* ATCC 64548 and 64550 than for the two most widely used ones (*C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258) suggesting potency variation may have a greater effect on the

more susceptible isolates (in agreement with our previous observations discussed above) and suggesting that quite significant variation may go unnoticed though careful use of these two quality control strains is adopted. Furthermore, the lot number NEK0040, which is one of those used in our studies on echinocandin susceptibility testing, consistently generated higher MICs than the others, thus explaining our data.

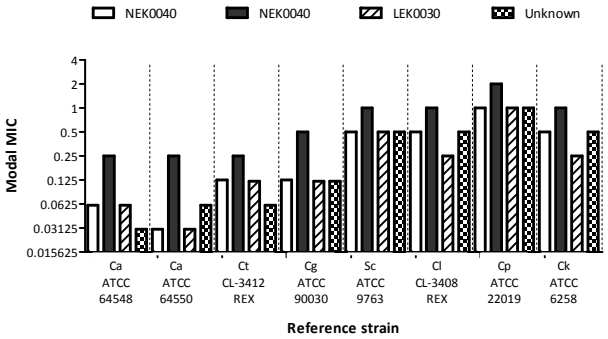


Fig 10. Modal caspofungin MICs (MIC_{50}) for eight reference strains tested according to the EUCAST methodology and using the following four different lots of caspofungin: NEK0040 (white bars; 7 repetitions), TEK0010 (black bars; 6 repetitions), LEK0030 (hatched bars; 18 repetitions), and unknown (checkerboard bars; 21 repetitions). Ca, *C. albicans*; Ct, *C. tropicalis*; Cg, *C. glabrata*; Sc, *S. cerevisiae*; Cl, *C. lusitaniae*; Cp, *C. parapsilosis*; Ck, *C. krusei*.

Stability of caspofungin in stock solution. The recommended solvent for caspofungin was water or saline whereas it for anidulafungin is DMSO. DMSO is labelled potentially carcinogenic and hence is not preferred unless it is necessary. However, from a chemical point of view all echinocandins are hydrophobic and thus hypothetically DMSO should be a more appropriate solvent. In paper IV and V ((4,5) water was used as solvent as recommended in EUCAST EDEF 7.1, however in the "BSA" study, we used DMSO for caspofungin for the EUCAST testing (6), and clearly the MICs were lower in the later study, suggesting the inhibitory potency of the powder is better preserved. This led to a following study confirming this (83) and a subsequent change in the EUCAST susceptibility guideline (84).
Finally, the stability of the micro titre plates at -80 ˚C was examined when using DMSO as solvent for all three echinocandins. As shown in table 2 in paper VI, absolutely no loss of potency was observed neither after 2 nor after 6 months of storage. Hence this extended shelf life was incorporated in the revised EUCAST E.DEF7.2 guideline (84).
So in conclusion, our studies on echinocandin susceptibility testing underlined the importance of selecting breakpoint at the species level, as combining species of different virulence and selecting genus specific breakpoint's only may lead to misclassifications. We demonstrated that part of the misclassification of resistant isolates could be avoided by adopting simple measures like using DMSO as solvent for all three echinocandins and by establishing species specific breakpoints no higher than the ECOFF. Our results have been part of the work that has led to a revision of the EUCAST susceptibility method, to the establishment of breakpoints for anidulafungin and the recommendation of using this agent as a marker for the echinocandin class of drugs until individual breakpoints for caspofungin and micafungin can be established. Our research has also helped the CLSI realise that revision and harmonisation of the CLSI breakpoints (24-26) and methodology, which will be published in the upcoming M27S-4 document, were needed.

3. EPIDEMIOLOGY OF CANDIDAEMIA (PAPER VII - IX)

Background

Epidemiology of candidaemia has been the subject of numerous studies and incidence rates as different as 1.2 to 25 cases per 100,000 inhabitants or 0.19 – 2.5 per 1000 admissions have been reported, illustrating the complexity of this topic (85-89). These differences are in part related to the nature of the different surveys. Studies carried out as single- or multi-centre studies or including only a selected group of patients will naturally reflect a priori risk for candidaemia specific for the surveyed population which may be specific for the local area. Consequently, such studies are informative but not necessarily easily comparable or translated into other settings (43). On the contrary, population based surveys being either national or covering all inhabitants in a defined geographic area allow comparison of incidence rates of candidaemia between regions and countries and reveal true differences between different parts of the world.
Among the Nordic countries Norway (NO), Finland (FI) and Sweden report incidences of candidaemia around 3/100,000 inhabitants (90-94). As mentioned earlier, little was known regarding Danish epidemiology of fungaemia at the time the semi-national survey was initiated in 2003 (7). Unpublished data extracted from the data management system at the department of clinical microbiology at Herlev University Hospital showed an incidence rate of 5/100,000 inhabitants in the late nineties in the Copenhagen county (unpublished data). According to the national surveillance scheme in NO the incidence rate at university hospitals was approximately twice as high as at district hospitals (36). Thus, a figure of 5/100,000 at Herlev university hospital appeared in nice agreement with the reported Nordic national figures. Furthermore, *Candida* accounted for 1.5% of the 2,739 blood stream infections in the County of North Jutland in 1996-1998 (with a population of 493,000) equivalent to an incidence rate of 2.8/100,000 inhabitants, again in agreement with the previously published Nordic data (13).

Design

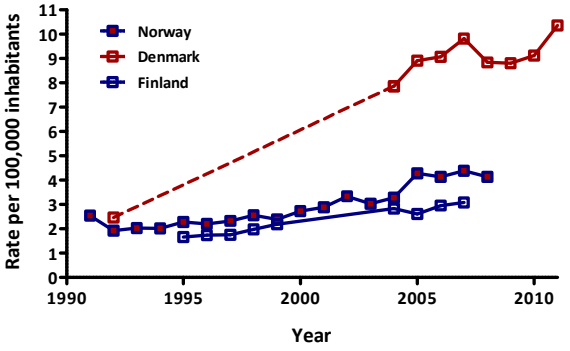
Target population: Our first study was organised as a one-year semi-national programme running May 2003 – April 2004 and including all unique fungal blood stream isolates in the uptake area from the departments of clinical microbiology at the following 6 university hospitals: Rigshospitalet, Hvidovre Hospital, Herlev Hospital, Odense Hospital, Skejby Hospital and Aalborg Hospital (2.87 mill inhabitants or 53% of the population) (Paper VII, (7)). The surveillance programme was in 2004 extended to include also Hillerød Hospital and the uptake area of Statens Serum Institut (SSI) (Roskilde and Bornholm) including a total population of 3.5 mill inhabitants or 64.5% of the population (29) and finally retrospective data from the rest of the country was retrieved leading to a national survey covering six years 2004-9 and the entire population of appr. 5.5 mill inhabitants (paper VIII, (8)). Since 2010 the surveillance programme has included prospective collection of isolates from the entire country allowing confirmation of species identification and susceptibility testing for all isolates. Data from the two most recent years were presented

at the ICAAC conference 2012 in San Fransisco and will be included as well (30).
The study on diagnostic issues, clinical characteristics and outcome (Paper IX, (9)) was undertaken to investigate underlying factors associated with fungaemia in DK. Clinical and diagnostic parameters as well as information on antifungal treatment and outcome were collected from all patients with fungaemia in 2006 from the following centres willing to do so: Rigshospitalet, Hvidovre Hospital, Herlev Hospital, Odense Hospital, Skejby Hospital and SSI (covering Roskilde and Bornholm). Data were collected using a pro forma document in order to standardise the data obtained (for details please consult (Paper IX, (9)).
Information on population sizes in DK, NO and FI has been retrieved from the publically available websites: http://www.statistikbanken.dk/statbank5a/default.asp?w=1280, http://statbank.ssb.no/statistikkbanken/Default_FR.asp?PXSid=0&nvl=true&PLanguage=1&tilside=selecttable/hovedtabellHjem.asp&KortnavnWeb=folkendrkv) and http://pxweb2.stat.fi/Dialog/Saveshow.asp

Definition of episodes and methods for species identification: Materials and methods are described in detail in each paper. Throughout these studies, unique episodes have been defined excluding re-isolation of identical species within three weeks (unless the susceptibility increased by more than 2-fold). Species identification developed gradually over time. In the first study (Paper VII, (7)) it was based on growth characteristics and colony appearance on CHROMagar, micromorphology on Rice and Tween or Corn Meal Agar, growth at 35 and 42 ˚C and carbon assimilation profiling using ATB ID32C. Later rapid identification tests were introduced including latex agglutination for the separation of *C. albicans* and *C. dubliniensis* and sequencing of the ITS region (ITS for Internal Transcribed Spacer which is a non-functional piece of ribosomal DNA) was gradually adopted for isolates with unclear identification results by the classical identification approaches. This may have resulted in improved separation of *C. dubliniensis* from *C. albicans* and improved identification for *C. guilliermondii, C. famata, C. lusitaniae, C. fermentati* and *C. palmioleophila* isolates as discussed in a separate publication (52). However, these species are uncommon and thus the changes do not affect the overall conclusions of the studies.
Susceptibility testing: The methods used for susceptibility testing changed somewhat over the years as did the interpretative breakpoints used. In paper VII (7) the American NCCLS M27-A2 document was followed and testing performed for amphotericin, fluconazole, itraconazole and caspofungin (15). In the later period (paper VIII, (8)) the EUCAST E.DEF 7.1 was used for amphotericin, anidulafungin, caspofungin, fluconazole, itraconazole, voriconazole and posaconazole (74) with the exceptions of MIC determinations for amphotericin B and caspofungin, which were performed using Etest in 2006-9 and in 2008-9, respectively (AB bioMerieux, Herlev, DK).

Findings
Fungaemia in Denmark in a Nordic perspective. Our first seminational study revealed an unexpectedly high fungaemia incidence rate of 11/100,000 inhabitants (7). However, the participating hospitals included all solid organ and haematological transplantations performed in DK as well as the major tertiary hospitals and thus this represented a skewed part of the population. Assuming the incidence rate in the remaining part of DK would be around half that in the surveyed region (as suggested by the Norwegian survey) an estimate of the national incidence rate would be 8.25/100,000 and if assuming there were no cases in the rest of the population the national incidence rate would have been 5.5/100,000. In any of these scenarios the figures were higher than expected and thus prompted the subsequent studies. In the following section the main findings are reviewed in a Nordic perspective, as each individual study can be reviewed in detail in the original publications (7-9,95).
In 2004-11, 3,982 unique isolates, from 3,867 episodes of fungaemia in 3,689 patients have been registered. The annual incidence rate has increased from 7.7-10.1 episodes/100,000 inhabitants over these 8 years and was remarkably high in a Nordic perspective as illustrated in the figure below (8,30,90-93). Notably, the incidence rate of blood stream fungal isolates was comparable and around 2/100,000 in the early 1990'ies. Since then it has increased in all three countries, but remarkably more so in DK (Fig 11a).

a)



b)

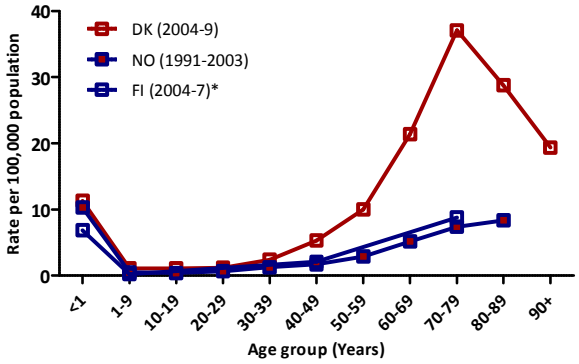


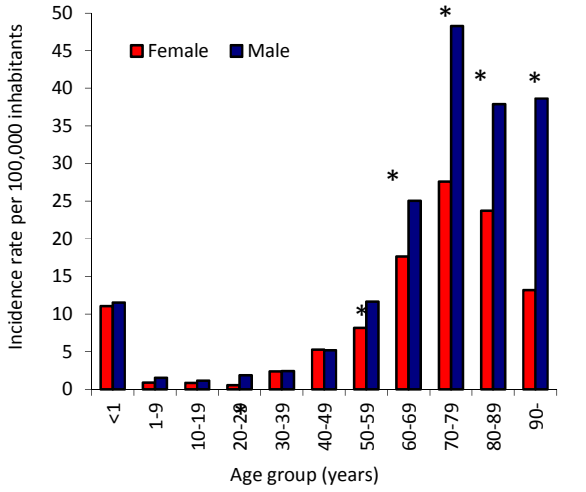
Fig 11. Incidence rate of unique fungal blood stream isolates per 100,000 inhabitants (1992-2011, fig a) and by age group compared with the similar figures from NO and FI (fig b). a) The number of isolates in DK 1992 was estimated using the number of cases (57) registered at the following four centres: Herlev Hospital, Odense Hospital, Skejby Hospital and Aalborg Hospital and the proportion of cases by which these centres contributed in the time period 2004-2011 (mean 0.45, range 0.42-0.50) leading to an estimate of 127 cases (range 113-136) in a population of 5,162,126 in 1992. b) the age specific incidence rates for FI were only available for the following age groups: <1 y-age (6.87), the 1-15 y-age (0.25), the 16-65 y-age (2.11) and the >65 y-age group (8.82) (8,90,93). These are indicated at the figure in the middle of the age group.

For all three countries the highest incidences have been observed at the extremes of age (Fig 11b). Thus, the age specific incidence

rate in the <1 year old children was 11.3, 10.3 and 6.9 isolates/100,000 in DK, NO and FI, respectively (for these age specific incidences the isolates number is used for practical reasons, however the number of episodes involving more than one isolate is less than 3% and hence insignificant in this respect). A second peak was observed in the elderly population, which in NO was 8.4/100,000 inhabitants in the 80+ age group, in FI 8.8 for the >65 year age group, but in DK at a level more than 4 times higher than in the other Nordic countries (37.1/100,000) in the 70-79 year age group. Thus, taken together, the higher incidence in DK compared to NO and FI appears to be particularly driven by a remarkably higher incidence in the elderly population.

Another characteristic finding was that the incidence was gender specific with more cases in males. In the Danish studies the proportion of cases appearing in men was 56% in 2003, 56.5% in the 2004-9 period and 59.5% in the last two years 2010-11 (and not statistically different comparing the different study periods). Again the incidence rate was highest at the extremes of age but a significantly higher incidence rate was found for men older than 50 years in 2004-9 and 2010-11, and in men in the 20-29 or 30-39 age groups in the two periods, respectively (Fig 12).

a)



b)

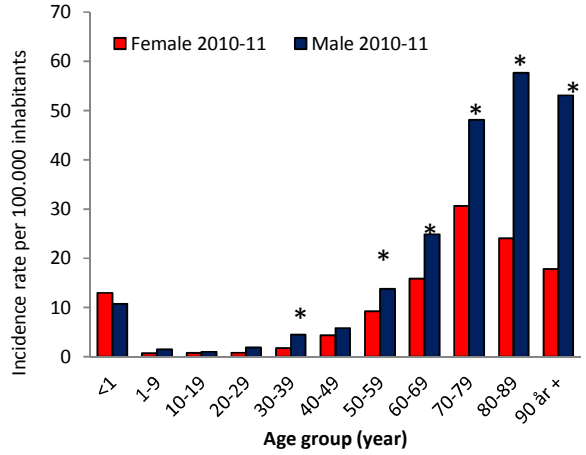


Fig 12. Incidence rate per 100,000 divided by gender for the 2004-9 period (a) (8) and the recent two years 2010-11 (b) (30). * denotes statistically significant differences in the gender specific incidence.

In NO and FI also more cases were observed among males than females. In both countries the male dominance was most prominent in the <1 years old (NO: 61% male, FI: Incidence rate ratio (male/female) 2.1) and in the >60 years old (NO: 59-63% males; FI: Incidence rate ratio 1,86). Whereas the male dominance among the elderly was thus a consistent finding in all three countries, Denmark had an equal number of cases in the <1 year old children.

Finally, the age and gender specific incidences in DK in 2004-9 and 2010-11 were compared in order to dissect the increase in incidence (Fig 13). As shown in this figure the number of isolates and the incidence rates remained unchanged for the <30 old age groups. In the 30-69 year old population, more cases were found in males despite an only marginally elevated incidence rate. In the 60-79 year old population more cases were seen for both genders despite an unchanged incidence rate and finally in the 80+ population the age specific incidence increased among men. Hence, the increasing incidence of fungaemia in DK comparing 2010-11 with the preceding six years was mainly due to the changing demography of the DK population with more elderly people whereas only among men older than 80 years of age an increase in age specific incidence rate was observed.

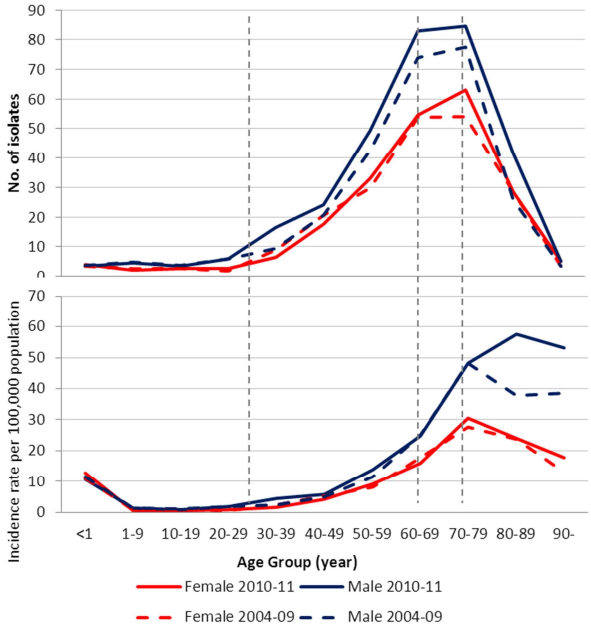


Fig 13. Age and gender specific incidence rates in DK for 2010-11 (solid lines) and 2004-9 (dotted lines). The four different patterns are indicated/separated by vertical dotted lines.

Species distribution in a national perspective

The overall species distribution has changed over the past years. Whereas *C. albicans* was the dominating pathogen with 73% even at the main tertiary hospital in Copenhagen in the mid and late 80'ties (11), it has decreased proportionally and today account for only half the isolates (Fig 14). In the same period *C. glabrata* has emerged as an important pathogen accounting for almost every third blood stream fungal infection today (Fig 14).

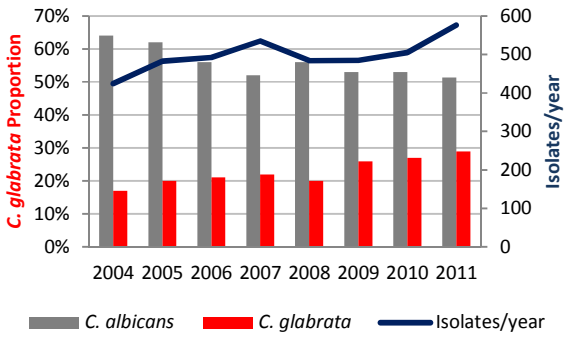


Fig 14. Changing epidemiology of fungaemia 2004-11 in DK. The total number of isolates is indicated as a blue line, whereas the proportion of isolates per year being *C. albicans* and *C. glabrata* is indicated as grey and red bars, respectively.

For comparison, *C. albicans* and *C. glabrata* accounted for 67.2% and 15.1% of the isolates in NO in 2004-8 and for 67% and 19% in FI in 2004-7, respectively (91,93). Fluconazole has been increasingly used in DK over the last decades and the majority (2/3) is used in the primary health care sector. In fact, the fluconazole use in the DK primary sector alone is twice as high as the entire use in NO (Fig 15). In addition app. 300,000 DDD of itraconazole has been used per year in DK over the last decade of which again the majority (93%) is used in the primary heath case sector providing a significant selection pressure towards azole resistant species (*C. glabrata* and *C. krusei*). In this light, it is less surprising that the proportion of *C. glabrata* is remarkably higher in DK and emerging, and that also *C. krusei*, although still a rare pathogen, is increasing (2.5% in 2004-5 but 5% in 2006-11).

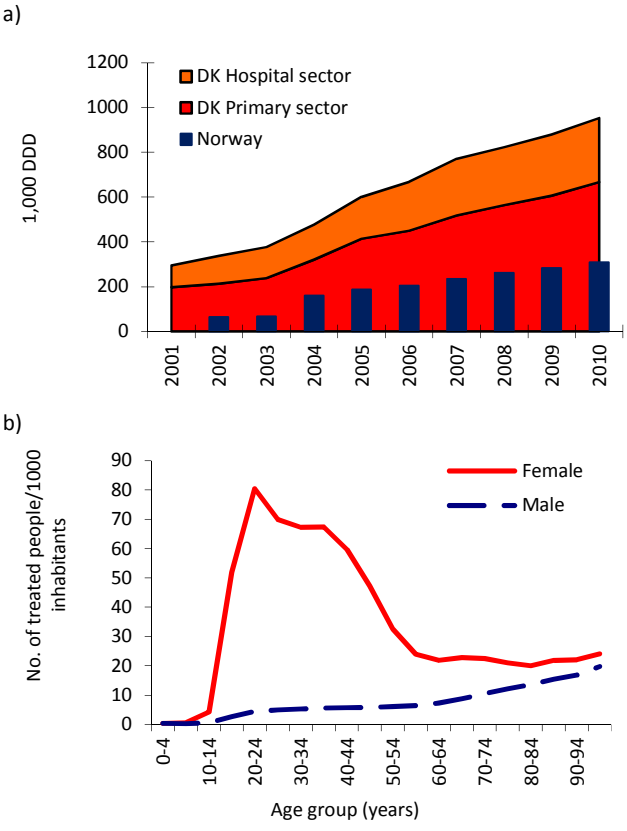


Fig 15. The use of fluconazole in the Danish hospitals (orange) and in the primary healthcare sector (red) in comparison with the total use of fluconazole in NO (blue) (panel a). In panel b the fluconazole use in the primary healthcare sector in DK is shown by age group and gender.

Fluconazole is used significantly more for women that for men due to prescriptions for vaginitis (Fig 15). In agreement with this, we also showed that *C. glabrata* was significantly more common in women than in men (23.1% vs. 19.7%, P=0.03) (8). This was also found in 2010-11 although not reaching statistical significance (*C. glabrata* F/M: 29.2% vs. 27.2%). One reason for the notable increase in fluconazole use may be that many women have already used over the counter topical azoles for vaginal symptoms before seeking the family doctor leading to an increased prescription of fluconazole for vaginal symptoms in the primary healthcare sector.
On the other hand *C. tropicalis* was more common in males (5.6% vs. 3.7%, P=0.02 and again in 2010-11: 4.2% vs. 3.9%, P>0.05). This may at least in part be related to the fact that the incidence rate of haematological and gastrointestinal cancers are 30% and 19% higher in men as *C. tropicalis* has been associated with these underlying diseases. The proportion of the remaining species not significantly different among the gender and also did not change proportionally over the years (Table 10).

Table 10. Species distribution in DK in the recent 8 year period. The total number of isolates is 3,983.

	2004	2005	2006	2007	2008	2009	2010	2011
No. of isolates	424	482	492	535	484	485	505	576
Species distribution								
C. albicans	64%	62%	56%	52%	56%	53%	53%	**51%**
C. dubliniensis	1%	2%	3%	3%	3%	3%	2%	2%
C. glabrata	17%	20%	21%	22%	20%	26%	27%	**29%**
C. krusei	3%	2%	6%	4%	6%	4%	5%	**5%**
C. parapsilosis	4%	2%	5%	4%	4%	4%	5%	3%
C. tropicalis	4%	5%	4%	5%	6%	4%	4%	4%
C. species*	3%	2%	2%	4%	2%	3%	2%	4%
Non-albicans**	3%	3%	2%	2%	1%	2%	-	-
Other fungi	1%	2%	1%	3%	2%	0.4%	2%	2%

**C.* species denotes species other than the ones specifically included in the table and include *C. ciferrii* 1, *C. colliculosa* 1, *C. fermentati 3, C. guilliermondii* 20, *C. holmii* 1, *C. inconspicua* 2, *C. inconspicua/norvegensis* 1, *C. intermedia* 2, *C. kefyr* 17, *C. lambica* 1, *C. lipolytica* 2, *C. lusitaniae* 33, *C. magnolia* 1, *C. nivariensis* 1, *C. norvegensis* 5, *C. orthopsilosis* 2, *C. palmioleophila* 11, *C. pelliculosa* 7, *C. pulcherima* 1, *C. rugosa* 1, *C. utilis* 1 and *C. valida* 1. **Non-albicans indicates isolates other than albicans that were not referred for species identification at SSI and therefore may be any other species than *C. albicans*.

<u>The species distribution varied by age group.</u> *C. albicans* and *C. parapsilosis* were the two dominant species in the neonatal and paediatric setting, whereas particularly *C. glabrata* became increasingly more important in the elderly population in agreement with observations in NO (90) and globally (43) (Fig 16). As mentioned previously, *C. parapsilosis* has been associated with skin and intravenous catheters. Both these factors may contribute to a higher frequency in the neonates and paediatric setting. On the other hand the risk of *C. glabrata* infection may increase as a consequence of an increasing azole selection pressure over time via azole exposure in medical treatment (as discussed above) but potentially also in food items and wine due to the increased use

of azole fungicides for crop protection. Although purely speculative it is interesting in this perspective that the annual intake of alcohol is twice as high in DK as compared to NO and that wine often contains various azole fungicides (Paul Verweij, personal communication).

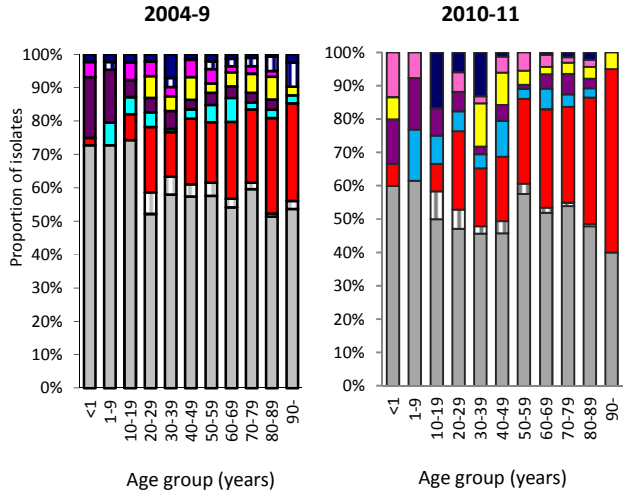


Fig 16. The age specific national species distribution (proportion of isolates for each species) in DK is shown for the two periods 2004-9 (left panel) (8) and 2010-11 (right panel) (30). Grey: *C. albicans*, white and grey: *C. dubliniensis*, red: *C. glabrata*, light blue: *C. krusei*, purple: *C. parapsilosis*, yellow: *C. tropicalis*, pink: *Candida* spp., dark blue: other fungi, and dark blue and white: non-*albicans Candida* (not identified to the species level).

Centre differences in incidence and species distribution

The incidence and species distribution varied by medical centre and was highest at Rigshospitalet, Funen, N-Jutland and Aarhus (9.5-16.6/100,000 inhabitants and 0.44-0.98/somatic discharges) in agreement with these centres being referral centres for complicated diseases for the country (Table 11). The lowest incidence was found at Herning and S-Jutland (3.9-4.6/100,000 inhabitants and 0.24-0.27/1000 discharges). The species distribution also varied among the centres and for the two time periods. For all centres the proportion of *C. glabrata* increased comparing 2004-9 with 2010-11. And whereas no centres had a mean *C. albicans* proportion in 2004-9 below 50%, this was the case for six centres in 2010-11 (RH, Herlev, Frederiksborg, SW-Zealand, Vejle and Herning). Also, *C. glabrata* and *C. krusei*, which are both intrinsically resistant to fluconazole, together accounted for more than 41-43% at four centres in 2010-11 (Herlev, Frederiksborg, SW-Zealand and Herning) whereas these species accounted for only 23% in Funen. Finally, three of the six centres which did not initially participate in the surveillance programme failed to species identify a significant proportion of the blood stream isolates beyond *C. albicans* even though as described above the susceptibility pattern is strongly linked to the species identification.

Table 11. Fungaemia incidence and species distribution by centre in DK in 2004-9 and 2010-11.

	Time period	1-RH*	2-Cph City Hospitals	3-Cph County Herlev*	4-Frederiksborg	5-7-SW-ZealandSlagelse	8-Funen*	9-S-Jutland	10-Esbjerg	11-Vejle	12-Herning	13-Viborg	14-N-Jutland	15-Aarhus
Incidence														
/100,000														
inhab.	2004-9	na	7.3	7.8	7.2	7.8/4.2	11.5	4.1	5.4	7.4	6.1	4.5	8.9	10.0
	2010-11	na	7.9	3.9	6.5	6.3	16.6	3.9	8.2	6.7	4.6	7.2	10.3	9.5
/1,000														
discharg.	2004-9	1.22	0.39	0.35	0.36	0.37/0.21	0.53	0.25	0.30	0.37	0.37	0.20	0.42	0.49
	2010-11	0.98	0.39	0.44	0.25	0.33	0.77	0.24	0.48	0.33	0.27	0.32	0.51	0.44
Species distribution														
C. albicans	2004-9	51%	51%	61%	54%	55%/54%	60%	52%	68%	58%	52%	73%	62%	61%
	2010-11	48%	50%	45%	49%	49%	58%	56%	59%	45%	46%	55%	54%	58%
C. dubliniensis	2004-9	4%	3%	2%	4%	3%/1%	3%	0%	0%	0%	1%	0%	2%	4%
	2010-11	2%	2%	3%	0%	0%	4%	0%	0%	3%	0%	0%	2%	1%
C. glabrata	2004-9	20%	30%	19%	25%	18%/15%	17%	22%	16%	23%	37%	13%	21%	20%
	2010-11	24%	34%	31%	38%	38%	21%	33%	26%	29%	42%	24%	25%	26%
C. krusei	2004-9	9%	3%	5%	4%	4%/1%	4%	0%	3%	1%	1%	3%	3%	4%
	2010-11	8%	5%	10%	5%	5%	2%	0%	3%	0%	0%	0%	7%	4%
C. parapsilosis	2004-9	7%	3%	4%	3%	4%/1%	5%	2%	4%	3%	1%	2%	2%	3%
	2010-11	7%	0%	3%	3%	3%	5%	11%	8%	5%	0%	6%	3%	3%
C. tropicalis	2004-9	4%	6%	5%	5%	11%/5%	4%	0%	9%	3%	5%	3%	3%	4%
	2010-11	2%	2%	5%	6%	6%	3%	0%	0%	8%	8%	6%	3%	6%
Candida spp	2004-9	3%	2%	5%	3%	3%/0%	2%	5%	0%	0%	2%	3%	5%	3%
	2010-11	4%	5%	1%	0%	0%	5%	0%	5%	11%	4%	0%	2%	3%
non-albicans	2004-9	0%	0%	0%	1%	1%/23%	0%	20%	1%	10%	1%	2%	0%	1%
Other Fungi	2004-9	3%	2%	0%	1%	2%/0%	4%	0%	0%	1%	0%	0%	2%	1%
	2010-11	4%	2%	1%	0%	0%	1%	0%	0%	0%	0%	9%	4%	0%

Susceptibility of the Danish fungaemia isolates

The susceptibility pattern was determined for the bloodstream isolates referred to the SSI as part of the national surveillance programme. This was done for 272 (88%) of the isolates in the 1 year semi-national survey running 1st of April 2003 – 31st of March 2004, for 72% of the isolates in the 6 year national survey (as data from 6 centres were only collected retrospectively) and for 98% of the isolates in the prospective national survey covering 2010-11. In the following paragraph the trends in susceptibility over the last 8 years will be reviewed separately for each of the three drug classes, amphotericin, azoles and echinocandins.

Amphotericin. The susceptibility to amphotericin has remained high throughout the study period in agreement with the broad spectrum activity of this agent and similar findings worldwide (96,97). Thus, the vast majority of the isolates were categorised as susceptible (MIC ≤1 mg/L: to the left of the dotted line at Fig 17). This was the case even though the MIC was determined using Etest since 2006 as Etest has been shown to be more sensitive in detecting resistant isolates than microdilution tests (55,98,99).

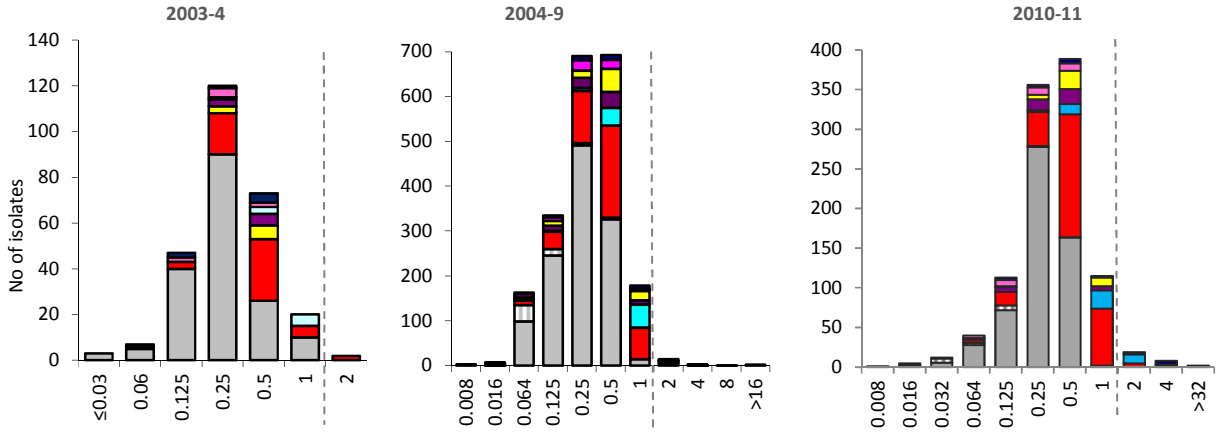


Fig 17. The amphotericin MICs of the individual fungaemia isolates in each of the three time periods 2003-4, 2004-9 and 2010-11 are displayed. The individual *Candida* species are denoted by the following colours: *C. albicans*: grey, *C. glabrata*: red, *C. krusei*: turquoise, *C. parapsilosis*: purple, *C. tropicalis*: yellow, other *Candida* spp.: pink and other fungi dark blue. The dotted line indicates the susceptibility breakpoint (S: ≤1 mg/L), hence all values to the left of the dotted line are categorised as S.

The amphotericin MICs for *C. albicans* and *C. dubliniensis* were slightly lower than those for the other species and particularly the MICs for *C. glabrata* and *C. krusei* were very close to the clinical breakpoint of 1 mg/L. This suggests that these species are slightly less susceptible than *C. albicans* as has been reported elsewhere and is supported by time kill studies (97,100). It also implies, however, that even small changes in the performance of the susceptibility testing itself may lead to differences in the proportion of isolates classified as non-susceptible, as a single-step increase in MIC is enough to bring a number of these isolates above the breakpoint. Indeed, an unexpected high proportion of 26.9% of the *C. krusei* isolates were classified as resistant in 2010-11 in contrast to 4.7% in 2004-9 (30). A closer look at the Etest MIC distribution for the 2010-11 *C. krusei* dataset is shown below (Fig 18).

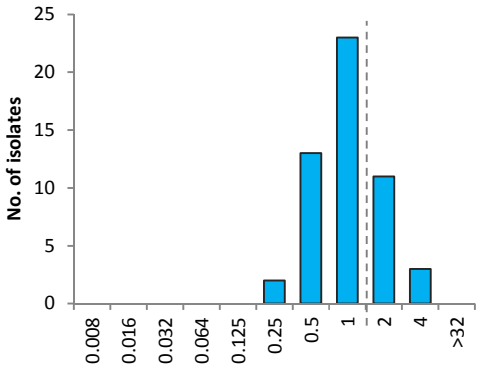


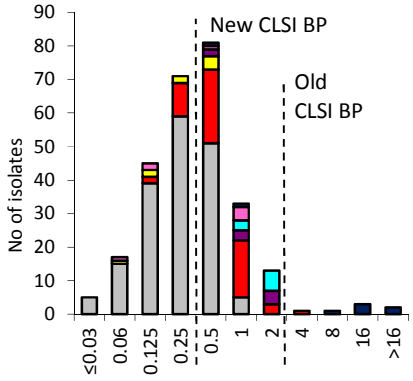
Fig 18. The amphotericin MICs of the *C. krusei* isolates obtained and tested in 2010-11 by Etest. The MIC distribution is Gaussian (suggesting no difference in true susceptibility). However, the dotted line indicating the susceptibility breakpoint S: ≤1 mg/L bisects the distribution leading to a probably artificial classification into S and R isolates.

The MICs form a perfect Gaussian distribution spanning 5 dilution steps and with the mode at 1 mg/L and no outliers separating from the distribution. This suggests that no separate resistant population exists for this species, but rather that it is in general two dilution steps less susceptible than *C. albicans* when tested by Etest (3). The proportion of *C. glabrata* with MICs higher than 1 mg/L remained stable, with 1.6% (1/63) in 2003-4, 1.6% (7/449) in 2004-9 and 2.0% (6/298) in 2010-11, and no isolates truly separated from the main population which displayed a mode of 0.5 mg/L. Thus, again these isolates with MIC >1 mg/L probably represent the right-side tail of the *C. glabrata* wild type distribution which overall is one dilution step less amphotericin susceptible than *C. albicans*. Finally, comparing the overall proportion of fungaemia cases for which amphotericin was not an appropriate choice (regarding MIC >1 mg/L as not appropriate and thus probably overestimating the true resistant population somewhat) a slight increase was noted: 0.7% in 2003-4, 1.0% in 2004-9 and 2.7% in 2010-11. However, as explained in this section this is most likely a technical issue associated with the change to Etest rather than a true emergence of amphotericin resistance, and thus

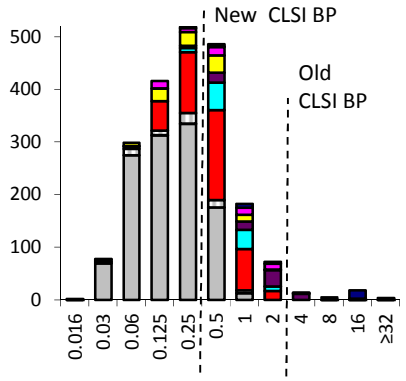
amphotericin still is the agent with the broadest spectrum in the setting of fungaemia.

Echinocandins. The susceptibility to the echinocandins was investigated using caspofungin and CLSI microdilution in the first study (7), followed by anidulafungin EUCAST microdilution in the second study (except for caspofungin in 2008-9 which was analysed using Etest) (8) and finally anidulafungin and EUCAST microdilution only as a marker for the class in 2010-11 (30). The change from caspofungin to anidulafungin was motivated by the findings of an unacceptably high batch to batch variation for caspofungin (addressed above (4,6)).
Using the initial ("old") CLSI breakpoints for the susceptibility interpretation of the caspofungin MIC for 2003-4 and 2004-9, very few isolates among the common *Candida* species were classified as non-susceptible (Fig 19). This was the case in 2003-4 for one *C. glabrata* isolate with an MIC of 4 mg/L (1.6% of the *C. glabrata* isolates) (this isolate is further investigated and reported in (55); and in 2004-9 for two *C. dubliniensis* isolates (3.1%, one with a Fks1p S645P alteration) and one *C. albicans* isolate (0.1%), all three of which were clearly resistant with caspofungin MICs ≥16 mg/L. Of note the CLSI caspofungin breakpoints have since been revised (S: ≤0.25 mg/L for *C. albicans, C. krusei* and *C. tropicalis* and S≤0.125 mg/L for *C. glabrata*). Application of these ("new") breakpoints would have bisected the distributions and obviously lead to a high proportion of misclassification of susceptible isolates as resistant as illustrated below (Table 19 left panel).

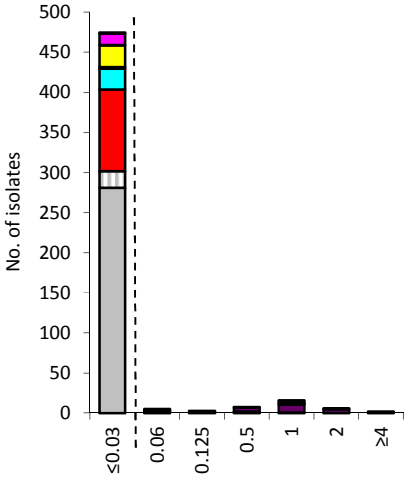
Caspofungin 2003-4



Caspofungin 2004-9



Anidulafungin 2004-9



Anidulafungin 20010-11

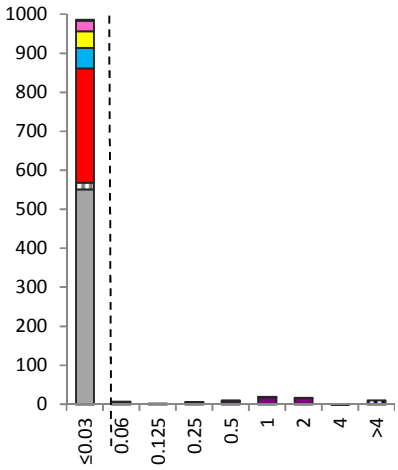
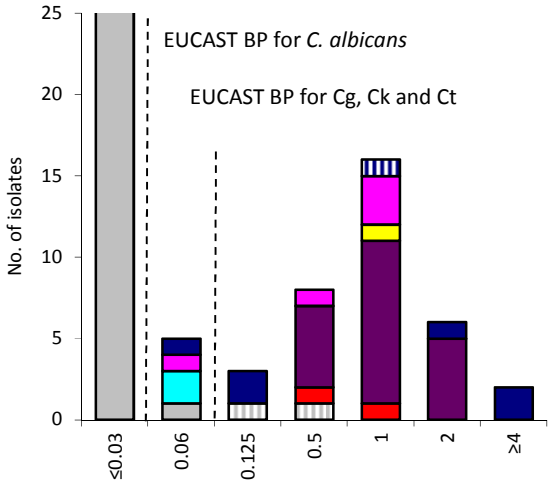


Fig 19. The caspofungin (left panel) and anidulafungin (right panel) MICs of the individual fungaemia isolates in each of the three time periods 2003-4, 2004-9 and 2010-11 are displayed. The individual *Candida* species are denoted by the following colours: *C. albicans*: grey, *C. glabrata*: red, *C. krusei*: turquoise, *C. parapsilosis*: purple, *C. tropicalis*: yellow, other *Candida* spp.: pink and other fungi dark blue. The dotted lines indicate the susceptibility breakpoints for *C. albicans* according to CLSI for caspofungin (initial breakpoint (Old CLSI BP) S: ≤2 mg/L, revised breakpoint (New CLSI

BP) S: ≤0.25 mg/L) and according to EUCAST for anidulafungin (S: ≤0.03 mg/L).

Anidulafungin testing was adopted in 2008. The vast majority of the isolates were highly susceptible with MICs below 0.03 mg/L (Fig 19). The susceptibility breakpoint for anidulafungin is S: ≤0.03 mg/L for *C. albicans* and *C. dubliniensis* but ≤0.06 mg/L for *C. glabrata, C. krusei* and *C. tropicalis*. One *C. albicans* (0.4%), two *C. dubliniensis* (8.7%), two *C. glabrata* (2%) and one *C. tropicalis* (3.6%) isolates were classified as non-susceptible in 2004-9, whereas in 2010-11 this was true for two *C. glabrata* (0.7%) and one *C. tropicalis* (2.3%, Fks1p S80S/P) (Fig 20). Thus, no indication of increasing acquired echinocandin resistance was found. Finally, a considerable proportion of the other *Candida* species and other fungal isolates were not susceptible due to intrinsic resistance (including *C. parapsilosis, C. guilliermondii, Cryptococcus, Fusarium, Geotrichum* and *Rhodothorula*).

Anidulafungin 2004-9



Anidulafungin 2010-11

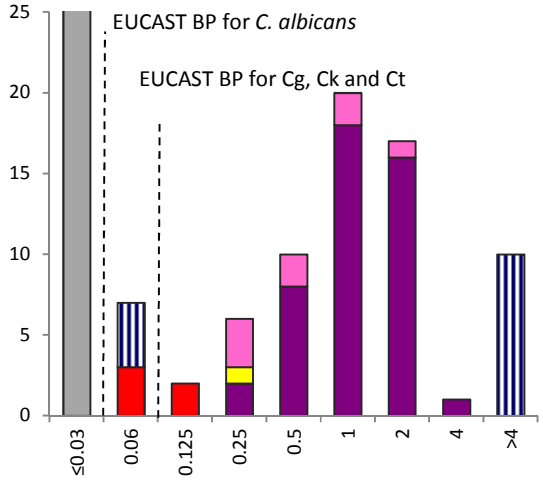
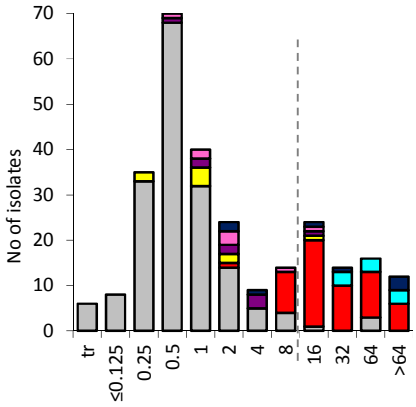


Fig 20. The anidulafungin MICs for blood isolates with higher MICs for each of the two time periods 2004-9 and 2010-11 are displayed. The individual *Candida* species are denoted by the following colours: *C. albicans*: grey, *C. glabrata*: red, *C. krusei*: turquoise, *C. parapsilosis*: purple, *C. tropicalis*: yellow, other *Candida* spp.: pink and other fungi dark blue. The dotted lines indicate the EUCAST susceptibility breakpoints for *C. albicans* and *C. dubliniensis* (S: ≤0.03 mg/L) and for *C. glabrata* (Cg), *C. krusei* (Ck) and *C. tropicalis* (Ct) (S: ≤0.06 mg/L).

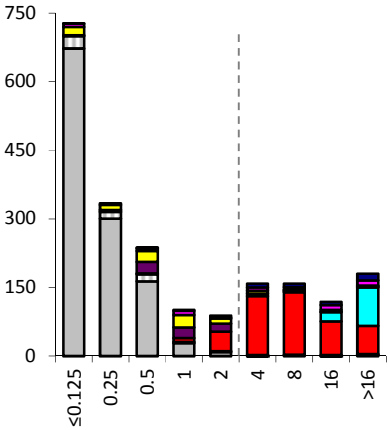
The overall proportion of fungaemia cases that was not appropriate targets for the echinocandin class of drugs either due to the species being intrinsically less susceptible or resistant or due to acquired resistance was unchanged 6.2% in 2003-4, 6.6%% in 2004-9 and to 6.2% in 2010-11, respectively.

Azoles. Among the four azoles available for systemic treatment, fluconazole is the one that has been most extensively used for fungaemia and therefore susceptibility trends for this compound is discussed in detail below.

Fluconazole 2003-4



Fluconazole 2004-9



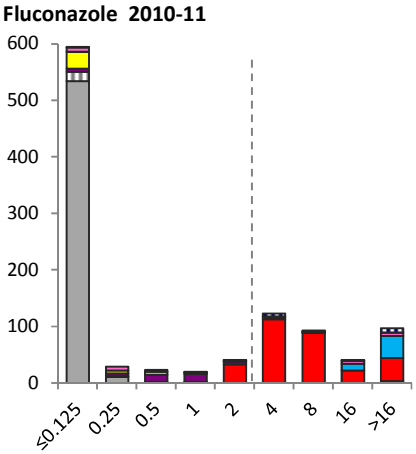


Fig 21. The fluconazole MICs of the individual fungaemia isolates in each of the three time periods 2003-4, 2004-9 and 2010-11 is displayed. The individual *Candida* species are denoted by the following colours: *C. albicans*: grey, *C. glabrata*: red, *C. krusei*: turquoise, *C. parapsilosis*: purple, *C. tropicalis*: yellow, other *Candida* spp.: pink and other fungi dark blue. The dotted line indicates the susceptibility breakpoint (S: ≤8 mg/L for the CLSI method and 48h incubation used in 2003-4 and S: ≤2 mg/L for the EUCAST method and *C. albicans, C. parapsilosis* and *C. tropicalis* used in 2004-9 and 2010-11).

For fluconazole the shift in methodology from CLSI with 48 h incubation and an 80% inhibition endpoint used in the first study (7) to EUCAST with 24 h incubation and a 50% endpoint (8,30) had a clear impact on the MIC values obtained as is evident when the panels above are compared. For example, the mode of the *C. albicans* distribution decreased from 0.5 to ≤0.125 mg/L and for *C. glabrata* from 32 to 8 mg/L. However, as the EUCAST susceptibility breakpoint (2 mg/L) was also two dilution steps lower than the original CLSI breakpoint (8 mg/L), the overall susceptibility classification of the isolates remained unchanged. Another evident observation was the intrinsically lower susceptibility of *C. glabrata* and *C. krusei* for which the MICs are clearly higher than those for the other *Candida* spp. No change in susceptibility pattern for individual species was observed throughout the three study periods. In example the proportion of *C. albicans* for which the MIC was above the breakpoint was 3.1% (6/193) in 2003-4, 0.6% (7/1183) in 2004-9 and 0.5% (3/551), respectively, in 2010-11, and similarly the fluconazole resistance incidence rates for *C. parapsilosis* and *C. tropicalis* declined from 2004-9 to 2010-11 from 6 and 6.7% to 2.2 to 2.3%, respectively. However, the overall proportion of fungaemia cases that was not appropriate targets for fluconazole either due to the species being intrinsically less susceptible or resistant or due to acquired resistance increased from 29.3% in 2003-4, 31.1% in 2004-9 and to 36.2% in 2010-11, respectively, due to an increased proportion being *C. glabrata*. Therefore, fluconazole cannot be recommended as first line treatment before species identification is available.

Overall, the level of acquired resistance among the blood isolates included in this study was low. One concern is however, that we may only detect the tip of the iceberg due to the design of the epidemiological studies where only the initial blood culture isolates from each patient is included. That this may be a real concern is suggested by the fact that we have observed cases involving acquired echinocandin resistance although these were not captured via the surveillance programme, a few of which are published (4,55,101), and as we see a significant level of acquired azole resistance among mucosal isolates (Table 12) as also observed elsewhere (56).

Table 12. Comparison of the proportion of invasive versus superficial isolates that are either fluconazole (Denmark) or voriconazole (Spain (56)) resistant.

	Proportion of isolates with fluconazole MIC >4 mg/L (Denmark)		Proportion of isolates with voriconazole MIC >0.125 mg/L (Spain)	
	1907 blood isolates	176 mucosal isolates	2806 deep site isolates	402 oropharynx isolates
C. albicans	0.6%	15%	2.4%	20.3%
C. dubliniensis	3.1%	20%	-	-
C. parapsilosis	6.0%	0%	1.1%	7.7%
C. tropicalis	6.7%	50%	10.4%	10%

Host factors and outcome (Paper IX)

As evident from the previous sections DK is unique in the Nordic perspective due to a notably high and still increasing incidence rate and due to a higher and increasing proportion of the isolates belonging to species that due to intrinsic susceptibility pattern are poor targets for fluconazole. It was therefore obvious to investigate possible reasons for this difference. Several reasons had been proposed including difference in blood culture systems and practices, differences in underlying diseases and differences in management. We therefore undertook a study in 2006 where underlying microbiological, clinical and management issues and outcomes for the Danish fungaemia patients were characterised at six centres willing to retrieve the necessary information (9).

Microbiological issues

Blood culture systems

When presenting the DK data, it is obvious too ask if differences in blood culture (BC) systems or practises might explain the different incidence rates of fungaemia in the Nordic countries. In Denmark two BC systems were used throughout the survey periods, BACTEC and BacT/ALERT. In the initial study covering 2003-4, three centres used each of the two systems, whereas towards the end of this decade more centres used BacT/ALERT (eight centres versus four centres and additionally two switching to BacT/ALERT during the study period). In Norway, however, these two systems were also used by the majority of the centres (19/21 centres) with 12 using BACTEC and 7 using BacT/ALERT, and thus differences in choice of blood culture system hardly explains the notable differences in overall incidence rates (90).

However, we extended the findings by Sandven et al (90) that *C. glabrata* was less frequently detected when the BACTEC system was used. Thus, we initially reported that the variation in distribution of *C. glabrata* between centres using BACTEC (16.7%, 97/580) and centres using BacT/ALERT (24.4%, 135/553) was statis-

tically significant (p=0.0015) in the semi-national survey (29). This difference was still significant when the surveillance programme was extended to include the entire country and even when the analysis was performed for the elderly population only, in order to avoid possible bias related to different age distribution among centres using the two BC systems as *C. glabrata* is more common in the elderly population (8). As a consequence of this finding, and the in vitro demonstration of the same phenomenon for experimentally inoculated BC bottles (102), it was recommended to include a mycosis BC bottle for patients at risk of *C. glabrata* infection (elderly and fluconazole exposed patients) at centres using the BACTEC system. Our preliminary data from one hospital suggested this might improve detection (9). However, in the present study we included data also on concomitant bacteria and we were surprised to find a significant difference in favour of the BACTEC system as concomitant bacteraemia was found significantly more often at centres using BACTEC (28%, 53/192) compared to centres using the BacT/ALERT system (9%, 11/124) (P<0.0001). This has subsequently been supported by similar findings in Sweden (103,104) and France (105) and suggests that a BC bottle with selective fungal medium should be included for all patients at risk for *Candida* infections independently on whether the BACTEC or the BacT/ALERT system is used.

The incubation time before BC positivity did not vary by BC system but by species. Thus, 50% of the cases were detected day 1 for *C. tropicalis* and *C. krusei*, day 2 for *C. albicans* whereas days of incubation were needed to detect 50% of the cases for *C. parapsilosis* and *C. glabrata.* The incubation time needed for detection of 75% of the cases were 2 days for *C. tropicalis* and *C. krusei*, 3 days for *C. albicans* and *C. parapsilosis* and 4 days for *C. glabrata*, respectively. The slow growth of *C. glabrata* and *C. parapsilosis* is well recognised, and may delay appropriate treatment.

Colonisation and species distribution

Colonisation was not systematically investigated except at one centre (Rigshospitalet). Yet it was reported in almost half of the episodes and the species identified in two thirds of these cases. It has previously been shown that the same genotype is often represented among invasive and colonising isolates in the same patient, in agreement with the assumption that invasive *Candida* infections are opportunistic and originate from the patient's normal microflora (106-108). Thus, it was somewhat surprising that 11/100 invasive isolates were different from the ones found in colonisation samples. Notably, this was never the case when a less frequent species was identified either alone or as part of polymicrobial colonisation. This suggests that careful evaluation of colonisation cultures is needed, if colonisation samples are used to guide antifungal treatment for invasive infections in order not to miss less common species that might be intrinsically resistant.

Notable differences were observed in the species distribution for the different clinical settings and depending on whether the patient had received antifungal prophylaxis. *C. albicans* accounted for all but one isolate in the neonatal-paediatric setting and for at least half of the isolates in all other clinical specialties except a) haematology, where the fluconazole resistant species *C. glabrata* and *C. krusei* accounted for 61% and *C. tropicalis* for 11%, and b) medical gastroenterology, where again *C. glabrata* was a frequent pathogen (52%) but also *C. parapsilosis* (12%). Finally, subsequent infection with *C. glabrata, C. krusei* or *S. cerevisiae* was significantly more common in patients with at least 1 week of prophylaxis than in patients with shorter or no exposure. Similarly, a recent French study has demonstrated that recent exposure to fluconazole and caspofungin influences the aetiology of subsequent candidaemia, with more isolates resistant to the compound in question (109) and the unique species distribution in the haemato-oncology setting has been reported at other centres as well (110). These differences in local epidemiology among the clinical settings and the impact of prior antifungal exposure should be considered when local prophylaxis and treatment guidelines are established.

Host factors, antifungal treatment and outcome

The majority of patients was found in the ICU (161/314, 51%) or had undergone surgery (177/314, 56% particularly abdominal 144/177, 81% of surgical patients and 46% overall), and together two thirds (208/314, 66.2%) of the patients belonged to either or both of these groups. Compared to other epidemiological surveys conducted since the year 2000 (Table 13) this is a high proportion, but otherwise the well-recognised host factors are similar across the surveys with the vast majority of the patients having a central venous catheter, undergone surgery, suffering from underlying malignant disease or being premature/neonate, respectively (9,85-87,111,112).

Table 13. Comparison of patient groups and underlying host factors for patients with candidaemia in European (111), American (86,87), Australian (85), Spanish (112) and Danish (9) surveys over the past 15 years. The most recent American survey (87) cover two regions: Atlanta and Baltimore and hence figures for both regions are mentioned with the Atlanta figures first.

Patient group and host factor	Country/region No. of cases Study period (Years)					
	Europe 2,089 1997-99	USA 1,143 1998-2000	Australia 1,093 2001-04	Denmark 314 2006	Spain 171 2004-08	USA 2,675 2008-11
Central Venous Catheter	-	78%	73%	92%	92%	86-82%
Surgery	45%	50%	37%	56%	-	38-33%
Intensive Care Unit	40%	36%	25%	51%		33-30%
Solid Organ Tumour	23%	24%	16%	24%	27%	22-19%**
Haematological Malignancy	12%	11%	16%	9%	13%	
Solid Organ Transplant	3.5%	5%*	2.2%	2.2%	5%	-
Premature/ Neonate	6%	5%	3.3%	1.0%	-	4-2%
Burn-patient	1.4%	-	-	1.9%	-	-
30-days mortality	38%	36%	25%	37%	22%	29-28%

* including bone morrow transplant patients; ** Solid organ as well as haematologic malignancies.

In the Danish study, antifungal treatment was given before the blood culture was obtained in 49 patients (16.6%). For comparison this was also the case for 18 and 10% of the patients in a recent survey from Atlanta and Baltimore. In 278 episodes antifungal treatment was given after the blood culture was drawn (88.5%) which again is in agreement with recent experiences (90 and 83% in the Atlanta and Baltimore survey (87)). Fluconazole was the preferred agent prescribed in 57% of the cases and clearly more often in the elderly population than in the children, which is somewhat paradoxical as the fluconazole resistant species *C.*

glabrata and *C. krusei* are significantly more common among the elderly population. Indeed, the initial treatment was judged inappropriate in 15% of the cases, most often due to the prescription of fluconazole for cases that were later found to involve *C. glabrata, C. krusei*, *S. cerevisiae* or *Trichosporon*, respectively. For *C. glabrata* episodes a significant difference in outcome was found for patients receiving caspofungin initially versus fluconazole. Thus, the mortality was 12 and 48% respectively, P=0.023). Additionally, outcome varied by underlying age and severity of disease (increasing significantly by age (P=0.009) and was significantly higher for patients at the ICU (46% vs. 24%, P=0.0001). Finally, outcome varied numerically by species being lowest for cases involving *C. parapsilosis* or other *Candida* species in agreement with the outcome triangle dogma.

We were somewhat surprised to find no correlation at all between the timing of therapy and mortality. This has been elegantly demonstrated in two highly cited studies (113,114). Basically, both these studies showed that the mortality increased from 10-15% to 35-40% when antifungal treatment was delayed from day zero (the day of blood culture) until day 3 (Fig 22). In general it makes sense to assume that it is not beneficial for the patient to delay treatment, however, our study is far from the only one that has failed to confirm such a clear-cut relationship (115-118)(Fig 22). As discussed in our paper, several factors may contribute to this unexpected finding. First, the initiation of therapy was not standardised according to underlying disease. We cannot exclude, that a significant part of the patients who received early treatment were those with the most severely underlying conditions thus prompting the early treatment. Second, our dataset is not standardised by choice of treatment or age. It is possible that patients with treatment initiated after the blood culture became positive were more likely to receive echinocandins, which have been shown overall to be more efficacious than fluconazole (119) as well as than those treated early before documentation of the fungaemia.

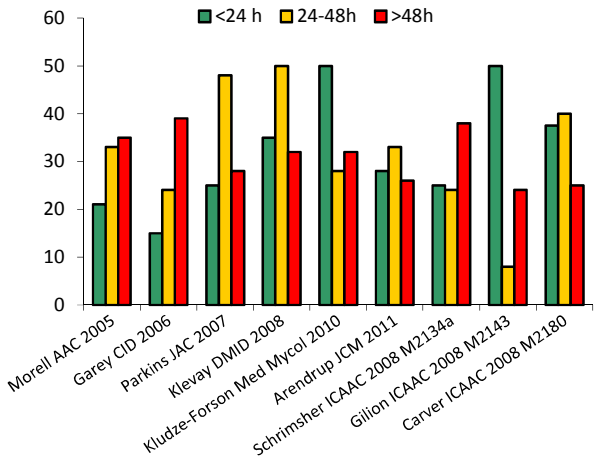


Fig 22. Histogram showing the overall mortality in patients with candidaemia/fungaemia depending on if treatment was initiated within the first 24 hours (green columns), after 24-48 hours (yellow columns) or after at least 48 hours (red columns). Three studies show a correlation between timing of antifungal treatment (Morrell, Garey and Schrimser) whereas the rest fail to do so.

In conclusion, our findings confirmed the hypothesis that outcome is driven by three factors: the severity of the underlying condition, the pathogenicity and susceptibility of the infecting organism and the choice of antifungal treatment (se Fig 1 above). Important findings were that 1) outcome is significantly better for *C. glabrata* cases if caspofungin rather than fluconazole is used as first line agent. This has subsequently been supported additionally (119); 2) adoption of the recommendation of prescribing echinocandin as the first line agent would have resulted in fewer patients receiving inappropriate treatment. This is now implemented in the Danish guideline available at Promedicin.dk, the American guidelines (120) and in the upcoming ESCMID guidelines (XX); and finally, 3) more sensitive and thus earlier diagnosis may be achieved if a mycosis BC bottle is included not only for the BACTEC system to increase detection of *C. glabrata* but also for the BacT/ALERT system to improve detection of fungaemia in patients with polymicrobial infection, a finding that has subsequently been supported by other reports (103-105).

CONCLUSIONS AND IMPLICATIONS FOR FUTURE RESEARCH

In summary, our research has provided detailed and updated knowledge of the interplay between virulence, epidemiology, antifungal treatment and susceptibility.

The fact that the incidence rate is approx. three times higher in DK than in the other Nordic countries is still surprising and unexplained. This was apparently not the case 15-20 years ago and the difference in incidence rate is limited to the elderly population, and therefore we hypothesise the underlying reason is related to host factors rather than genetic differences in susceptibility to the infection. Knowledge on the epidemiology and underlying host factors is a necessary ingredient for the identification of risk groups for whom intensified diagnostics and prophylaxis, empiric or pre-emptive therapy can assure that the individual patient receives treatment before the infection is advanced beyond therapeutic reach. In this context it is interesting that as many as two thirds of the cases occur in patients exposed to abdominal surgery or intensive care treatment, and that we have been able to reduce the number of infections in this particular setting via specific interventions (121). We also showed that the incidence rate was stable in most age groups over the later years apart from the older men. It is plausible, that advances in therapeutic options for severe diseases including the performance of complicated surgical procedures also in the oldest patient groups are factors that have contributed to this change in epidemiology. However, our study on underlying factors was not national and therefore may not be representative for the entire country. We have undertaken a study collecting data on underlying host factors, treatment and outcome for all cases of candidaemia in 2010 and 11 with the goal of providing updated and specific background information for Denmark. Such data may hopefully help better identification of high risk patients suitable for targeted and intensified diagnostics and therapeutic interventions. Ultimately, we need better diagnostics that can allow an earlier diagnosis and thereby hopefully allowing a better outcome, particularly as our mortality rate observed in the semi-national study appeared in the high end as compared to that in recent international reports. However, again this may be biased as the studied patient population probably included the most severely ill population among those with fungaemia. A better understanding of this will hopefully emerge from our ongoing study.

Another important observation was the epidemiological change in species distribution. A significant increase in number and proportion of cases involved fluconazole resistant isolates. Azoles are extensively used in the primary healthcare sector for benign

conditions like vaginitis and toenail infections. In agreement with this we showed that *C. glabrata* was significantly more common by age and over the surveillance period, and with a higher incidence among women. Obviously, changed practices particularly in the primary healthcare sector may potentially help reducing the selection pressure. Such changes should include the use of topical agents rather than oral fluconazole for vaginitis thereby minimising the systemic selection pressure on the normal mucosal *Candida* flora in the gastrointestinal tract. This however may require that azoles for vaginal application do not remain as over the counter agents as many women have used one of these without mycological or clinical confirmation of the diagnosis, leaving the treating physician with no other options than systemic fluconazole if *Candida* vaginitis is suspected despite the self-medication. Secondly, terbinafine rather than itraconazole should be prescribed for toenail infection unless specific reasons dictate otherwise thus limiting the use of itraconazole. Another hypothetic parameter driving the selection towards less azole susceptible species is the increasing use of azole fungicides for crop protection. Azole fungicide use has been linked to an increasing proportion of *A. fumigatus* isolates being azole resistant and similarly some years ago a presentation at the ICAAC conference reported that more *Candida* isolates on the surface of fruit from non-ecological farms were azole resistant than from ecological farms (122). In addition, remaining azole compounds in food product and wine may contribute to a low grade azole exposure on the mucosal surfaces favouring a change in the *Candida* micro-flora. These possible links between azole use in general practice and agriculture should be further explored and steps taken to limit their use when alternative agents are available.

Acquired resistance was a rare event and with no signs of emergence of such isolates over the eight years the surveillance of fungaemia has been on-going. This is somewhat in contrast to the experience from our daily routine, where we regularly encounter not only acquired azole resistance but also echinocandin resistance. Several factors may explain this discrepancy. In fact, the design of the surveillance programme is not sensitive with respect to detection of emerging resistance as only the initial isolate from each episode is included. This is typically obtained at a time point with the lowest antifungal exposure (particularly for the echinocandins which are mainly used for documented invasive infections). From an epidemiological point of view this is a sound strategy as otherwise individual patients from which several fungal isolates were obtained would bias and skew the epidemiological data set. However, it is likely that by doing so we only detect the tip of the resistance iceberg as subsequent isolates obtained after longer duration of antifungal treatment are not included unless the local laboratory realise an increase in MIC of more than two dilution steps. We find that this question deserves a focussed investigation and therefore plan to collect mucosal post-treatment isolates from patients with fungaemia and investigate the susceptibility pattern in such isolates compared to the initial invasive isolates.

Another reason for the low prevalence of acquired resistance is that it possibly goes underdiagnosed due to suboptimal susceptibility tests or MIC interpretation. Not only were the initial CLSI breakpoints for many drug-species combinations too high and thus insensitive with respect to detect resistant isolates, EUCAST also only recently established European breakpoints for instance for the echinocandins. This limited the ability for the routine laboratory to understand the pitfalls and limitations of susceptibility testing and particularly of their interpretation of their susceptibility endpoints. Another challenging issue is that it requires expertise to perform and interpret antifungal susceptibility testing and hence that misclassifications may occur in the busy routine laboratory. We therefore undertook several studies comparing the performance of commercial susceptibility tests and reference methodologies and demonstrated that susceptibility testing using commercial tests is challenging even when conducted outside the busy clinical routine laboratory. We next focused on the reference methods in order to provide data that facilitated the necessary development, revision and refinements of EUCAST and CLSI methods and breakpoints. We highlighted the importance of neither selecting breakpoints that bisect wild type populations nor breakpoints that were substantially higher than the ECOFF unless supported by clinical data. Also we demonstrated the variability of caspofungin pure substance from lot to lot and in solution, particularly if stock solutions were prepared in water/saline rather than DMSO. These findings have played an important role in optimising the reference susceptibility tests and facilitated the development of useful EUCAST breakpoints and the harmonisation and revision of the CLSI breakpoints. New antifungal agents are under clinical evaluation and hence will need appropriate testing and breakpoint development. The CLSI organisation is under significant influence of the pharmaceutical industry as only one third of the members are academic researchers whereas the remaining members are either representatives from the industry or regulatory bodies. This is an important reason for the prolonged process regarding revision of the antifungal breakpoints despite it for several years has been clear for the academic community that the initial breakpoints lead to an unacceptably high proportion of resistant isolates being misclassified as susceptible. In this light is it of outmost importance that the industry independent EUCAST antifungal subcommittee's work is continued despite the increasing challenges faced due to limited financial support and that the commercial susceptibility test providers acknowledge the recent advances for the reference methodologies and breakpoints and ensure harmonisation of their test results and recommendation with these. With the shortcomings for particularly the commercially available antifungal susceptibility test methods and heavy demand for standardisation in mind, it is obvious that novel, rapid and less challenging tests for detection of antifungal resistance mechanisms in the routine laboratory are urgently needed. Future research in this area should focus on the increasingly easier available molecular methods including real-time PCR and gene sequencing. Particularly for the detection of echinocandin resistance such tests should be rather straight forward to develop as resistance has not yet been detected in any examined isolates without accompanying mutations in the target gene. For the detection of azole resistance, however, it may be slightly more challenging as several different mechanisms often play in concert in the individual isolate and thus should be detected simultaneously in isolates of different species.

We hope our studies and findings have increased the awareness and knowledge on how to optimise the diagnosis and antifungal treatment choices for patients with fungaemia and other forms of fungal infections. However, for every piece we have brought to the puzzle it becomes evident that many more are still missing.

SUMMARY

In our part of the world invasive fungal infections include invasive yeast infections with *Candida* as the absolutely dominating path-

ogen and invasive mould infections with *Aspergillus* as the main organism. Yeasts are part of our normal micro-flora and invasive infections arise only when barrier leakage or impaired immune function occurs. On the contrary, moulds are ubiquitous in the nature and environment and their conidia inhaled at a daily basis. Hence invasive mould infections typically arise from the airways whereas invasive yeast infections typically enter the bloodstream causing fungaemia. *Candida* is by far the most common fungal blood stream pathogen; hence this genus has been the main focus of this thesis.

As neither the Danish epidemiology nor the susceptibility of fungal pathogens was well described when we initiated our studies we initially wanted to be able to include animal models in our work. Therefore, a comprehensive animal study was undertaken comparing the virulence in a haematogenous mouse model of eight different *Candida* species including the five most common ones in human infections (*C. albicans, C. glabrata, C. krusei, C. parapsilosis* and *C. tropicalis* and in addition three rarer species *C. guilliermondii, C. lusitaniae and C. kefyr*). We found remarkable differences in the virulence among these species and were able to group the species according to decreasing virulence in three groups I: *C. albicans* and *C. tropicalis*, II: *C. glabrata, C. lusitaniae* and *C. kefyr*, and III: *C. krusei, C. parapsilosis* and *C. guilliermondii*. Apart from being necessary for our subsequent animal experiments exploring *in vivo* antifungal susceptibility, these findings also helped us understand at least part of the reason for the differences in the epidemiology and the pit falls associated with the establishment of genus rather than species specific breakpoints. In example, it was less surprising that *C. albicans* has been the dominant pathogen and associated with a significantly higher mortality than *C. parapsilosis* and that *C. glabrata* and *C. krusei* mainly emerged in the post fluconazole era and in settings with azole selection pressure. Moreover, it was less surprising that infections due to mutant *C. albicans* isolates with echinocandin MICs of 1-2 mg/L were not good targets for the echinocandins despite the fact that the outcome for infections involving wild type *C. parapsilosis* for which similar echinocandin MICs were similar was not inferior.

This last observation highlights the importance of providing optimal, reproducible and sensitive reference susceptibility testing methods and notably accompanied by appropriate breakpoints that allow a separation and detection of susceptible and resistant isolates against which the commercial tests can be validated. Correct detection of resistant isolates is for obvious reasons crucial in order to avoid inappropriate treatment. And if the test method cannot correctly identify resistant isolates it makes little sense performing susceptibility testing at all. On the other hand misclassification of susceptible isolates as resistant is also an issue as the patient is thereby deprived an appropriate treatment option among the few available. These comments may seem very basic; nevertheless, it has taken a lot of effort and patience to optimise the susceptibility tests, understand the variability issue for caspofungin testing, to provide appropriate breakpoints that reduced misclassifications to a minimum and not the least to facilitate a harmonisation of breakpoints across the Atlantic sea. We initially realised that the CLSI method and echinocandin breakpoint misclassified resistant isolates. This was due to the endorsement of a single susceptibility breakpoint across all *Candida* species and the three echinocandins and therefore set as high as 2 mg/L in order to include and not bisect the *C. parapsilosis* population. Through our comprehensive comparisons of echinocandin susceptibility testing using EUCAST, CLSI, Etest, disk diffusion and agardilution with different media with and without the supplementation of bovine serum albumin we provided data that supported the current reference methodologies, provided that drug and species specific breakpoints were selected. Moreover, the issues of caspofungin variability and of overlap between micafungin MICs for wild type and mutant *C. glabrata* populations were handled and understood. Anidulafungin EUCAST breakpoints are now published and publically available at the www.eucast.org website and anidulafungin testing recommended as a marker for the echinocandin class. Our antifungal EUCAST breakpoint setting approach has been adopted by the CLSI leading to revision and harmonisation of breakpoints for the three echinocandins, fluconazole and voriconazole.

Our epidemiological studies developed gradually over the years following our observation of a notably high incidence rate of fungaemia compared to our Nordic neighbours. Initially, we anticipated that our high incidence was at least in part related to the fact that the capture area for our initial studies was skewed with dominance of university hospitals and inclusion of all centres performing solid organ or bone marrow transplantation. However, when the surveillance was extended to the entire country, the high incidence remained a consistent finding and we even demonstrated that the incidence rate is still increasing. Additionally we demonstrated a changing epidemiology as a high and increasing proportion of the cases involved fluconazole resistant isolates and that this proportion also was significantly higher than in the other Nordic countries. This appears to be related to a significantly higher and increasing fluconazole use in DK than in the other Nordic countries. Exploring the incidence rate for the individual hospitals and age groups we demonstrated not unexpectedly that the incidence rate was highest at the university centres, but also that whereas the age specific incidence rate was comparable in children and the younger adults with that in the other Nordic countries it was notably higher in the elderly population. This in combination with the fact that it is increasing specifically in the elderly men and that the incidence rates in the Nordic countries were comparable two decades back suggest that host specific factors including antifungal consumption rather than genetic differences in susceptibility to fungaemia account for the differences, and hence that it is possibly modifiable by implementing relevant measures. Hence, it was important to investigate the underlying clinical conditions and diagnostic factors and the outcome in Danish patients with fungaemia.

In this study we demonstrated that two thirds of the patients had received abdominal surgery or intensive care treatment prior to the development of the fungaemia, a proportion that is higher than in most other studies. We also demonstrated that unless surveillance cultures are handled with careful attention the detection of non-*C. albicans* may go un-noticed which imply a risk of inappropriate treatment in cases involving intrinsically resistant species. Finally, we demonstrated the necessity of using a fungal blood culture flask in addition to the conventional aerobic and anaerobic ones if all *C. glabrata* infections (BACTEC) and all polymicrobial infections (BacT/ALERT) are to be diagnosed. Hence close monitoring with the use of improved diagnostic options (such as frequent BC including a mycosis bottle, surveillance cultures and mannan antigen and antibody screening) of particularly ICU and abdominal surgery patients may help better identify patients with fungaemia and allow early treatment. With respect to treatment and outcome we found that the fluconazole re-

sistant species *C. glabrata, C. krusei* and *S. cerevisiae* were significantly more common in patients exposed to at least 7 days of antifungal prophylaxis (mainly fluconazole). We also demonstrated that a significant proportion of the patients initially received inappropriate antifungal treatment and that the outcome was significantly improved when patients with *C. glabrata* received caspofungin as their first line agent. This has today been incorporated in the Danish and international treatment guidelines.
The prevalence of acquired antifungal resistance remained very low throughout the study period, however, we may only have detected the tip of the resistance iceberg due to the study design, where for epidemiological purposes only the initial isolate was included with the lowest antifungal exposure, and as the susceptibility tests and breakpoints were not optimal for the detection of resistance at all centres. Most DK laboratories either do not susceptibility test or use commercial tests such as the Etest and later the VITEK system. These are FDA approved with the CLSI breakpoints which, as we have shown, have been far too high to reliably detect resistance and which despite having now been revised and harmonised are not yet in formal CLSI print and hence not incorporated in the product inserts for the commercial tests on the market. Finally, even for laboratories aware of these issues challenges are still ahead as the official breakpoints not always lead to a correct classification for MIC endpoints obtained using the commercial systems or as the commercial tests do not include a relevant concentration range for all drug bug combinations (77)(Astvad ICAAC 2012 and submitted).
I thus believe, the studies included in this thesis have contributed significantly to the understanding of the interplay between the *Candida* virulence, epidemiology and susceptibility and the importance of appropriate diagnostics and treatment choice. It is my hope that we thereby have contributed to the improved options and outcome for patients with candidaemia.

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