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Update from a twelve-year nationwide fungaemia surveillance

increasing intrinsic and acquired resistance causes concern

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1 Update from a twelve-year nationwide fungaemia surveillance: increasing intrinsic and acquired

2 resistance causes concern

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40 ABSTRACT

41 New data from the Danish National Fungaemia Surveillance 2012-15 is reported and epidemiological trends are investigated in a 12-year perspective (2004-15). During 2012-15, 1900 42 43 out of 1939 (98%) fungal bloodstream isolates were included. The average incidence was 8.4/100,000 inhabitants and this appears to be stabilizing after the increase to 10.1/100,000 in 44 45 2011. The incidence was higher in males than females (10.0 vs 6.8) and in patients above 50 years, 46 mainly driven by an increasing incidence among 80-89 years old males (65.3/100,000 in 2014-15). The proportion of Candida albicans decreased 2004-15 (64.4% to 42.4%) in parallel with a 47 48 doubling of Candida glabrata (16.5% to 34.6%, p<0.0001). C. glabrata was more common among females (34.0% vs. 30.4% in males). Following an increase in 2004-11 the annual drug use 49 50 stabilised during the last 2-3 years but remained higher than in other Nordic countries. This was particularly true for the fluconazole and itraconazole use in the primary healthcare sector which 51 exceeded the combined national use of these compounds in each of the other Nordic countries. 52 Fluconazole susceptibility decreased (68.5%, 65.2% and 60.6% in 2004-7, 2008-11 and 2012-15, 53 respectively, p<0.0001) and echinocandin resistance in Candida emerged (0%, 0.6% and 1.7%, 54 respectively, p<0.001). Amphotericin B susceptibility remained high (98.7%). Among 16 (2.7%) 55 echinocandin-resistant C. glabrata isolates (2012-15), 13 harboured FKS mutations and five (31%) 56 57 were multidrug resistant.

The epidemiological changes and the increased incidence of intrinsic and acquired resistance emphasise the importance of continued surveillance and of strengthened focus on antifungal stewardship.

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61 INTRODUCTION

Candidaemia remains a threat to susceptible patients and continues to carry a high crude 30-days 62 mortality of 30-40% (1-3). The mortality is linked to infecting species, co-morbidity, underlying 63 64 disease, age, and time to initiation of appropriate treatment (3, 4). However, as diagnosis of invasive candidiasis is often delayed, diligent attention to intravascular devices are important and 65 empiric treatment recommendations must be founded on reliable contemporary epidemiology (5). 66 67 Institutional studies are affected by referral practices and departments served. In general, population-based studies are less prone to bias, especially when the study base becomes large and 68 representative for entire countries. Nationwide surveillance data is available from Australia, 69 Scotland, Finland, Iceland, Norway, Sweden, and Denmark (6-18). In Denmark, an increasing 70 71 candidaemia incidence was demonstrated during 8 years reaching 10.1/100,000 inhabitants in 72 2011 (18). Although increasing incidence rates have also been found elsewhere, the Danish 73 incidence is notably high compared to other reported contemporary nationwide incidence rates of 74 2.4-5.7/100.000 inhabitants (12–16).

Besides increasing incidence rates another common observation is the changing species distribution to non-*albicans* species and particularly *C. glabrata* in the northern hemisphere, Australia and Taiwan and *C. parapsilosis* in southern Europe and South America (3, 12, 13, 19, 20)) at the expense of *C. albicans*. The increase in *C. glabrata* has been coupled with an increased use of azoles for which *C. glabrata* is intrinsically less susceptible (18, 21, 22).

From 2001 the echinocandins were introduced in Europe and the USA and in 2009-12 incorporated into candidaemia management guidelines as the first-line treatment (23–27). Following the increased use, reports of acquired resistance has emerged among several different

species but especially *C. glabrata* (28–31), thereby warranting continued surveillance and monitoring. The aim of this population based nationwide study was to update and assess incidence and susceptibility patterns of fungaemia in Denmark during a 12-year period.

86 MATERIAL AND METHODS

87 Population and surveillance 2012-15

Incidences per 100,000 inhabitants were calculated using the population by January 1st each year 88 (www.statistikbanken.dk). The Danish population increased 1.4% from 5,580,516 to 5,659,715 89 inhabitants from 2012-15. The mean was used for incidence rates calculated for periods of >1 90 year. National annual number of admissions and bed days were available from The Danish Health 91 Data Authority at www.eSundhed.dk. The local study representative reported population, 92 93 numbers of admissions and bed days for hospitals within geographical capture area serviced by 94 each centre of clinical microbiology. All Danish residents have access to universal tax-supported 95 free-of-charge healthcare. All centres of clinical microbiology have specific geographic capture areas as specified previously (18), however, due to public health reforms the number was reduced 96 97 from 13 to 11 (as centres 3 and 4 were administratively merged in 2012 (geographically separated blood culturing sites remained) and centres 12 and 13 fully merged in 2013). Original numbering 98 99 of centres was retained to allow direct comparison with previous reports (17, 18).

Isolates were referred to the National Reference Mycology Laboratory for species verification and susceptibility testing (see below). Completeness was ensured through comparison with local electronic laboratory records. A total of 39 out of 1939 (2.0%) isolates were not referred and therefore excluded from the susceptibility paragraphs (14 *C. albicans,* 14 *C. glabrata,* 2 *C. tropicalis,* 2 *C. parapsilosis,* 1 each of *C. krusei, C. lusitaniae* and *C pellliculosa,* and 2 unidentified *Candida* species).

Two blood culture (BC) systems were used: BACT/ALERT (bioMérieux, Marcy l'Etoile, France) and BACTEC (Becton Dickinson, Franklin Lakes, NJ, USA), accounting for the detection of 77.9% and

22.1% of cases, respectively. For fungaemia patients with successive fungal bloodstream infections
over time, subsequent episodes were included if they occurred at least 21 days apart or were
caused by a different species consistent with previous reports (17, 18, 32).

111 Species identification

Identification was performed as previously described including matrix- assisted laser
 desorption/ionization time-of-flight mass spectrometry (Bruker, Bremen, Germany) (18) with the
 addition of DNA sequencing as described below.

115 Susceptibility testing

Susceptibility testing was done contemporaneously for the referred isolates and included 116 117 amphotericin B, voriconazole and isavuconazole (98.0% of the isolates), anidulafungin and micafungin (97.9% of the isolates), and fluconazole (97.6% of isolates) according to the EUCAST 118 definitive document E.Def 7.3 (33). Exceptions were amphotericin B (prior to January 2015) for 119 which E-test (bioMérieux, Herlev, Denmark) and RPMI-1640 2% glucose agar buffered with MOPS 120 121 (SSI Diagnostika, Hillerød, Denmark) were used. Manufacturers and stock solutions (5000 mg/L in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Brøndby, Denmark)) were the following: fluconazole 122 and amphotericin B (Sigma-Aldrich), anidulafungin and voriconazole (Pfizer A/S, Ballerup, 123 Denmark), micafungin (Astellas Pharma Inc., Tokyo, Japan) and isavuconazole (Basilea, 124 125 Pharmaceutica Ltd., Basel, Switzerland). C. parapsilosis ATCC22019 and/or C. krusei ATCC6258 were included as guality controls in each run (34). Susceptibility classification was performed 126 127 according to established or proposed EUCAST breakpoints and ECOFFs (Supplementary table 1) 128 (35–38). Finally, amphotericin B: $\leq 1 \text{ mg/L}$: S and fluconazole: $\leq 2 \text{ mg/L}$: S was used for remaining

species to illustrate the overall susceptibility of those species or groups of fungi but should not be
 interpreted as an exact figure of clinical susceptibility or resistance.

131 Molecular identification and FKS gene sequence analysis (for selected isolates)

Sequencing of internal transcribed spacer regions ITS1 and ITS2 (ITS) (18) and translation elongation factor (TEF, for *Fusarium*) was performed as previously described (39). Echinocandin target hot-spots in *FKS1*, and for *C. glabrata* also *FKS2*, DNA sequence analysis was performed for resistant isolates and sequences compared to relevant reference sequences including Genbank accession no. JX899422 for *C. kefyr* (40).

137 **Consumption of antifungal compounds**

138 Information concerning overall use of antifungal agents in Denmark 2000-15 was retrieved from the Danish Medicines Agency (at www.medstat.dk). Posaconazole tablet and iv formulation were 139 140 marketed in Denmark in 2014. The licensed maintenance dose of these formulations is 300 mg/day compared to 800 mg/day for the oral suspension. To reflect the actual number of 141 142 individual dosages given in 2014-15, a corrected use was calculated (0.3 g enterotablet or iv infusion and 0.8 g oral solution translated to 1 DDD). The antifungal use (DDD/1000 143 144 inhabitants/year) from Norway, Sweden and Finland was retrieved from www.legemiddelforbruk.no, www.socialstyrelsen.se, and www.fimea.fi. Posaconazole formulation 145 146 information was not available and unadjusted posaconazole DDDs were used for comparison 147 between the Nordic countries and Denmark.

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148 Statistics

Chi-square test or Fisher's exact test was used for comparison of proportions and the Chi-square 149 150 test for trend used for evaluation of changes in species distribution over the 12-year surveillance period. Calculations were performed using GraphPad Prism Version 6.04 (GraphPad Software Inc., 151 152 La Jolla, CA, USA). For twelve episodes (10 in 2004 and one each in 2005 and 2007) the gender was 153 unknown. When possible, episodes were allotted evenly to genders in specific 10-year age groups, 154 but in four instances single cases within age groups were excluded from analysis of gender- and 155 age-specific incidence rates, conducted with linear Poisson regression/incidence ratio rate calculation (package: epitools). P-values <0.05 (two-tailed) were considered statistically significant. 156 Binomial univariate logistic regression was used to investigate associations between species 157 158 distribution and year, age, gender and BC system using R (R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/). For this analysis and to assure independence of 159 observations only incident cases were included. Covariates with a p<0.1 were investigated further 160 161 in a binomial multivariate analysis. Year, age, gender, and BC system were all retained in the multivariate analysis and independent significant findings displayed. The p-value also calculated 162 163 main tertiary referral hospital serviced when excluding the by centre 1-RH.

10

164 **RESULTS**

165 Current epidemiology (2012-2015) in a 12-year perspective

A total of 1939 isolates from 1883 unique episodes and 1813 patients were included in the period 2012-15. The mean and median age of patients were 65 years (range: 0-98 years) and 69 years (interquartile range 58-77 years), respectively. Overall, 59.7% of patients were males and the proportion increased (p=0.002) (Table 1). In a 12-year perspective, both the age of candidaemia patients and the male proportion increased (Table 2).

The average episode rate was 8.38/100,000 inhabitants (range 7.6-9.1) in 2012-15 and overall stable over twelve year although a significant increase in incidence was evident from 2004-11 (p=0.001). The population grew with 4.9% from 2004-15, the number of discharges increased by 20.2%, but the number of bed days decreased by 18.5% (2005-15). Consequently, the incidence rate/1,000 admissions declined whereas the incidence rate/10,000 bed days increased (Table 2). The incidence varied across centres in 2012-15, from 3.1-13.1/100,000 inhabitants, 0.2-0.7/1000 discharges and 0.6-1.8/10,000 somatic bed days (Supplementary table 2).

The highest incidence was seen at the extremes of age, i.e. 9.5/100,000 in the <1-year old and 178 179 17.2, 31.4, 39.9 and 21.2 per 100,000 inhabitants in the 60-69, 70-79, 80-89, and 90+ years old age groups, respectively, in 2012-15. Moreover, the incidence was significantly higher in males 180 compared to females (10.0 vs 6.8, IRR 1.5; 95% Cl (1.3-1.6)) (Figure 1). In the 12-y perspective, 181 182 decreasing age-specific incidence rates were observed in all age groups >50 years of age except 183 >80 years (Figure 1). Whereas the overall female incidence rate numerically decreased over the three 4-year periods (p=0.05), the male incidence remained stable and with a significant increase 184 between 80-89 years (Figure 1). 185

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186 C. albicans accounted for the majority of isolates (47.9%) in 2012-15, followed by C. glabrata (31.8%). C. tropicalis, C. krusei, C. dubliniensis and C. parapsilosis each accounted for <4.3%. Sixty-187 nine (3.6%) of the isolates belonged to other Candida species, thirty-two (1.7%) to other fungal 188 189 genera whereof eleven were mould and another eleven Saccharomyces cerevisiae isolates (Table 1). Poly-fungal episodes (n=53) involved 5.6% of isolates. Over the twelve years, the proportion of 190 C. albicans decreased (p<0.0001) whereas the proportion of C. glabrata increased (p<0.0001) 191 192 (Figure 3). This development was still observed despite conservatively assigning non-C. albicans as 193 C. glabrata among isolates from 2004-09. The proportion of C. dubliniensis increased significantly from 2.3% to 3.6% (p=0.01) whereas no change was detected for other species (Figure 3). The 194 195 increase in C. glabrata was significant for age groups 1-9, 30-39, 50-59, and 70-79 again despite 196 assigning early isolates identified as only non-albicans to be C. glabrata (p<0.03 for all groups; 197 Figure 2).

The species distribution in 2012-15 varied by age, gender, and by centre (Supplementary table 2 198 and 3). Correlation between species and age, gender, BC system, and calendar year was 199 200 investigated for all incident cases (only first episode included in the full 12-year period) in a univariate and multivariate logistic regression analysis (Table 3). A decrease in C. albicans and an 201 202 increase in C. glabrata and C. dubliniensis episodes over time was found. C. glabrata was positively 203 associated with female sex whereas the opposite was the case for C. tropicalis. C. albicans, C. parapsilosis, C. dubliniensis, and "other fungi" were associated with younger patients whereas the 204 205 odds of being infected with C. glabrata increased with age. Candidaemia involving C. glabrata was 206 positively associated with BACT/ALERT whereas the opposite was true for C. parapsilosis, C. krusei, 207 and "other fungi" (multivariate analysis). For "other fungi" this association disappeared when 208 excluding centre 1-RH.

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209 Susceptibility

For the six most common *Candida* species susceptibility patterns were largely as predicted by the species identification (Table 4). However, acquired resistance in *Candida* was occasionally detected and is detailed below per drug class.

Azoles: Overall, significantly fewer 1147/1892 (60.6%) of the isolates were fluconazole susceptible 213 in 2012-15 compared to 1137/1745 (65.2%) in 2008-11 and 972/1420 (68.5%) in 2004-7, 214 215 respectively (trend test p<0.0001). Among C. albicans, C. dubliniensis, C. parapsilosis and C. tropicalis, 2.1% (24/1128) were non-wild-type (wt) for fluconazole and 1.4% (16/1128) resistant 216 (0.4% C. albicans, 4.2% C. dubliniensis, 6.5% C. parapsilosis, and 6.2% C. tropicalis) (Table 4). Two 217 218 of four fluconazole-resistant C. parapsilosis isolates were voriconazole resistant. Six of seven fluconazole non-susceptible C. tropicalis isolates had a trailing phenotype making MIC 219 determination difficult due to a 50% growth inhibition over a broad range of MIC values (which 220 221 was also the case for voriconazole and isavuconazole). Altogether fourteen isolates (1.2%) of the 222 four species were voriconazole resistant/non-wt and ten (0.9%) were isavuconazole non-wt, none of which were fluconazole susceptible. 223

For *C. glabrata* a bimodal fluconazole MIC distribution was observed, with the peaks at MIC values of 4 mg/L and 64 mg/L; 9.1% were resistant (MIC>32 mg/L). This proportion declined from 2012-13 to 2014-15 (11.4 % vs 6.6%, p=0.04). Finally, 8.1% of *C. glabrata* were also non-wt for voriconazole and 1.4% of *C. krusei* were non-wt for voriconazole.

228

Echinocandins: Acquired echinocandin resistance increased compared to the previous years:
29/1754 (1.7%) compared to 10/1581 (0.6%) and 0/1294 (0%), in 2008-11 and 2004-7, respectively
(p<0.001).

232 The twenty-nine Candida isolates displaying acquired anidulafungin resistance in 2012-15 consisted of 4.2% of C. dubliniensis, 2.7% C. glabrata, 6.8% C. krusei, 2.5% C. tropicalis, and 23% 233 234 (3/13 isolates) C. kefyr isolates (Table 4). FKS sequencing detected hot spot alterations in 13/16 C. glabrata, 1/5 C. krusei (with the remaining four having L701M outside the hotspot which has not 235 been found uniformly associated with echinocandin resistance in our laboratory), 0/3 C. 236 237 dubliniensis, 2/2 C. tropicalis, and 1/3 C. kefyr. Five of the 16 (31%) C. glabrata isolates were 238 fluconazole cross-resistant and thus multidrug resistant. For less common species see 239 supplementary data 4.

240

Amphotericin B: Acquired amphotericin B resistance was found in 1.3% of fungaemia isolates and
1.0% (18/1851) of *Candida* isolates in 2012-15 (Table 4) including 1.5% *C. glabrata* isolates, 8.2% *C. krusei* isolates, two *C. nivariensis,* and one *C. norvegensis* isolates. In all instances, the MIC was 2
mg/L and thus one dilution step above the breakpoint.

245

246 Antifungal consumption

The antifungal consumption in Denmark has increased over the first 10 years of observation but stabilised or decreased during the last 2-3 years except for posaconazole (17, 18) (Figure 4). Most fluconazole, ketoconazole, itraconazole, and terbinafine was used in the primary health care sector 2004-15 (69.9%, 87.9%, 94.7% and 99.8%, respectively).

Denmark had a higher consumption of systemic antifungal drugs per 1,000 inhabitants compared to the other Nordic countries (2015: total 790 DDDs (DK) vs 512, 321, and 762 DDDs (Norway, Sweden and Finland)) and especially of the azoles (+44-174%; 237 DDDs (DK) vs 87, 111, and 164 DDDs (Norway, Sweden, and Finland, respectively)) despite a continued annual increase in fluconazole consumption until 2012 (Finland) or 2015 (Norway and Sweden). Caspofungin was the main echinocandin used. Anidulafungin was introduced in 2009 and accounted for 6-13% of the echinocandin use until 2013 and 25% in 2015.

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259 DISCUSSION

260 The Danish fungaemia incidence rate declined slightly in 2012-15 after an increase in the preceding 8 years. Whether this is just annual variations or an actual declining incidence is not yet 261 known. In the other Nordic countries and Scotland incidence rates have been increasing during the 262 1990s/the early 2000s, but for most parts appear now to have stabilized around a lower level 263 compared to Denmark (7-11, 13-16, 41) (Supplementary Figure 1). Outliers are Australia with a 264 265 low incidence but modest increase, and metropolitan Spain with a notable increase to 8.1/100,000 266 partly driven by a doubling of the incidence among children <1 year old (3, 12). A study of US 267 community hospital discharge records of invasive candidiasis of >1 month olds also demonstrated 268 a minor decrease from 2005-12 for both genders (42). This finding was corroborated by a 269 population based study from two metropolitan areas in the USA where their unprecedented high incidence rates declined from 2008-13 (Atlanta 14.1 to 9.5 and Baltimore 30.9 to 14.4). This 270 change was found in almost all age groups but limited to patients with central venous catheters 271 (85%) and was hypothesized to be related to the introduction of an infection control bundle 272 273 focusing on i.v. catheter management (43).

A recent study has examined the observed differences in incidence rates from 2010-11 between the Nordic countries. Denmark had a higher prevalence of malignant haematological disease compared to the other Nordic countries, but no demographic differences could justify the higher rate. Despite a similar overall antibacterial consumption (DDDs/1000 inhabitants/day), the use of penicillin, piperacillin-tazobactam, metronidazole, carbapenem, and colistin was significantly higher in Denmark (16). Metronidazole and broad-spectrum beta-lactams are associated with profound impact on the GI flora thereby potentially selecting for yeast (44, 45). Moreover, a

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higher use of broad-spectrum antibiotics and the increasing utilisation of BC reported in Denmark (particularly in the \geq 65 years old population) may be markers of Danish patients being more severely ill or the introduction of sepsis packages (including timely diagnostics) (46).

284 Despite the high overall incidence, a more diverse picture was observed among children and the elderly. The incidence rate has remained constant in the <1-year old (10.8/100.000 population) in 285 286 the 12-year perspective and was comparable to rates reported from Norway, Finland, and England 287 and Wales (6.6-11/100.000) (8, 15, 47) but low in a global perspective (20 to >90/100,000) (3, 6, 288 14, 48, 49). Such differences suggest that socioeconomic factors and infection control practises 289 may play an important role as also suggested in the study from Atlanta and Baltimore (43). In 290 contrast, the incidence rate in the elderly population was higher than in any population based 291 study in Caucasians ((3, 6, 8, 14, 15, 48, 49) and only exceeded by the rate from the mixed population in Atlanta and Baltimore (43). The high Danish rates in the elderly were mainly driven 292 by a significantly higher and increasing rate in males aged 80-89 years. The reason for this is 293 unclear. Colon cancer and haematological malignancies are recognised risk factor for candidaemia. 294 295 Both malignancies are more common in males increasing with advancing age and the total prevalence of gastro-intestinal tract (incl. pancreas and liver) cancers as well as large groups of 296 297 haematological disease (leukaemia and non-Hodgkin lymphoma) have increased more in males 298 during the last decade 2004-14 (http://www.ancr.nu).

The previously observed species-shift towards non-albicans species and particularly *C. glabrata* continued 2012-15. This trend has been observed in several population-based candidaemia surveys (3, 12, 13, 20, 50). The increase in the proportion of *C. glabrata* has happened concomitantly with an increase in azole use in Denmark and with the population getting older.

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303 Sweden, Norway, Finland, and Iceland have not witnessed the same increase in the proportion of 304 C. glabrata during the last decade of surveillance although sharing demographic characteristics. Despite an increase in systemic azole use in all four countries, the overall use was substantially 305 lower in the other Nordic countries (Figure 4, (14)). In 2015, the overall systemic azole 306 307 consumption in Norway was comparable to the fluconazole use alone in Denmark in 2004. In contrast, the use of topical azoles for vaginitis was twice as high in Norway compared to Denmark 308 309 (an average of 375 vs 213 DDD/1000 inhab./year). We speculate whether this increasing selection 310 pressure mediated by systemic azoles over the twelve-year period has facilitated the increasing proportion of *C. glabrata* in the Danish setting. 311

312 The age-dependent species distribution of C. parapsilosis and C. glabrata and the influence of blood culture system on the detection of *C. glabrata* were confirmed in a multivariate analysis (15, 313 314 17, 18, 51). Although the gradual change from BACTEC to BACT/ALERT may have contributed to the increase in C. glabrata no centres have changed to BACT/ALERT since January 2011. Less 315 importance has been placed on the impact of gender in relation to C. glabrata. C. glabrata was 316 317 correlated to the female sex and was especially common above the age of 40 years, an observation also made in our previous study but to our knowledge not reported elsewhere (18). 318 319 One reason for this could be the gender-inequality in the antifungal consumption in the primary 320 health care sector. Prior fluconazole use has been shown to be associated with emergence of C. glabrata (18, 22, 52, 53). Fluconazole is the main azole used in Denmark, and the majority is 321 322 administered in the primary sector. In this setting (2012-15), 2/3 of the sale of fluconazole was 323 prescribed for the age groups 20-65 years and with a 4.8 female/male ratio of DDDs/1000 324 inhabitants/day. Genotyping studies have confirmed that the infecting organism derives from the 325 colonizing flora (54). We therefore hypothesize that the considerable use of fluconazole in adult

326 females in the primary health care sector may play a role in the over-representation of C. glabrata in adult females. Consequently, the recommended use of topical azoles rather than systemic 327 treatment whenever possible has been reinforced in the 2012 national guidelines (55). We did not 328 329 see an increase in C. krusei. Although inherently resistant to fluconazole and potentially selected 330 for by azole treatment, this species is also less pathogenic (56). No nationwide study has, to our knowledge, reported an increase in C. krusei proportions reflecting that infections occur primarily 331 332 in a well-defined subset of patients most of whom are already recipients of prophylaxis.

333 Fluconazole non-susceptibility was detected in more than one third of isolates, mainly driven by an increase in C. glabrata. Of note, 9.1% of C. glabrata isolates were fluconazole and voriconazole 334 335 cross-resistant and unlikely to respond to even high dosages of azoles. Non-susceptibility to fluconazole in C. tropicalis (8.6%) was primarily caused by heavy trailing growth and affected all 336 337 azoles equally, impeding precise and reproducible MIC determination. Less than 50% trailing is commonly observed in C. tropicalis and was not associated with differential clinical efficacy of 338 fluconazole in 21 C. tropicalis cases included in multivariate analysis. The overall mortality for 339 340 these patients was less than 10% suggesting the majority were not severely ill (57, 58). Therefore, 341 it still remains to be elucidated if isolates displaying heavy trailing are indeed good targets for 342 fluconazole treatment particularly in the setting of severe disease.

Acquired echinocandin resistance remained low but increased. All isolates with an MIC elevated 343 ≥ 2 dilution step above the breakpoint had an FKS hot spot mutation whereas this was only the 344 345 case in 4/16 isolates with an MIC one dilution step above the breakpoint. The majority of isolates 346 with hot spot mutations were C. glabrata and 31% were fluconazole cross-resistant as reported 347 elsewhere (31, 59, 60). Studies from the USA has demonstrated increasing echinocandin resistance 348 rates in C. glabrata (60, 61). In this context, it is worrying that we now see emerging resistance in

2.7% of our *C. glabrata,* particularly when no isolates were found in 2004-7 and only 1.4% during 2008-12. Furthermore, we are now seeing 2.5% confirmed resistance in *C. tropicalis* and *FKS* mutations in *C. krusei* and *C. kefyr*. Echinocandin resistance has been associated with prior therapy and in *C. glabrata* particularly with the presence of mutations in the DNA mismatch repair gene MSH2 (62). The emergence of echinocandin resistance in Denmark follows a significant increase in echinocandin consumption from 2004-15. These observations suggest that longer term echinocandin should be minimized if possible including when used empirically (63, 64).

In contrast to the increasing resistance observed for azoles and echinocandins, 98% of all isolates were amphotericin B susceptible which is in agreement with previous reports showing broad activity and no indication of acquired resistance (18).

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In conclusion, Denmark remains a high incidence country for fungaemia where less than two thirds of isolates are now fully fluconazole susceptible and where acquired echinocandin resistance is on the increase. Continued epidemiological surveillance is important and efforts should be directed towards improved diagnostics and lowering the antifungal selection pressure including regulation of the fluconazole use in the primary health care sector.

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Preliminary data has been presented as a short oral poster at the 27th ECCMID in Vienna 2017.367

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372

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- 381 F2G, Gilead, MSD, Novartis, Pfizer and T2Biosystems. She is the current chairman for the EUCAST-
- 382 AFST has before this served on advisory boards for MSD (until 2014), and Pfizer (until 2012).

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621 FIGURE AND TABLE LEGENDS

Figure 1: Age- and gender-specific incidence rates per 100,000 population by three 4-year intervals(2004-2015).

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The overall male incidence rate was stable whereas the female declined numerically. Males had a slight decrease for the age 50-59 (p 0.04) and a significant increase for the age group 80-89 (p <0.0001). Females had significant decreases in age groups 40-49 and 60-69 (and only an increase in the low incidence age group 20-29 years).

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Figure 2: Species distribution of blood stream infections on age groups 2012-2015.

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632 **Figure 3:** Species distribution (isolates in %) over three 4-year periods during 2004-2015.

633

The presented percentages are based on isolate numbers during the 4-year periods. Significant p values from a Chi-square test for trend is presented. (for *C. dublinensis* a p value of 0.01 was found due to an increase in isolates 2014-2015).

637

Figure 4: Annual consumption of systemic antifungal compounds in DDDs/1000 inhabitants in
2004-2015. Hospital use is shown in orange and use in the primary healthcare sector in red. The

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total annual consumption in Norway, Sweden and Finland is inserted for comparison (dark grey,
light grey and white bars, respectively)

642

¹The DDD for caspofungin is 50 mg whereas the DDDs for micafungin and anidulafungin are 0.1g. 643 ²² For posaconazole, an enterotablet and an iv formulation was introduced on the Danish market 644 in 2014. The licensed daily dose of these new formulations is 300 mg/day (after a loading dose) 645 646 whereas the treatment dose of the suspension is 800 mg/day. The official defined DDDs was for all 647 three formulations 0.8 g (recently in 2017 changed to 0.3 g). We have translated DDDs to reflect the actual use (enterotablets and iv formulation: 1 DDD of 0.3 g (red/white stripes) and oral 648 649 suspension: 1 DDD 0.8 g (solid red)). Total use in the Nordic countries and DK (uncorrected; grey 650 line) is for a DDD of 0.8 g.

651

Table 1: Characteristics of the national fungaemia surveillance scheme on incidence rates, age,
 gender, and species distribution 2012-2015

654

a) Candida spp. includes: C. lusitaniae (19); C. kefyr (13); C. fermentati (8); C. pelliculosa (6); C.

656 guilliermondii (5); C. inconspicua (4); C. orthopsilosis (3); C. magnoliae and C. nivariensis (2 each);

657 *C. fabianii, C. metapsilosis, C. norvegensis, C. palmioleophila* and *C.utilis* (1 each) and finally two
658 *Candida* isolates not referred for species identification.

b) Other fungi include: *S. cerevisiae* (11); *Fusarium dimerum* and *Cryptococcus neoformans* (4
each); *Fusarium solani* (3); *Fusarium oxysporum, Rhodutorula mucilaginosa* and *Saprochaeta*

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clavata (2 each); *A. fumigatus, Penicillium marneffei, Trichosporon asahii* and *Williopsis saturnus* (1
each).

663

Table 2: Number of isolates, demographics of patients, and blood culture system use during three
4-year periods 2012-2015

666

For information of isolates included please see table 1 (2012-2015) and refer to references (17, 18)

ND: not determined. For the statistical analyses Chi-square trend test was used apart from age and

669 episodes rates where a linear logistic regression analysis was employed.

a) Number of bed days 2004 were not available and the figure from 2005 was used for 2004.

671

Table 3: Binomial logistic regression analysis of variables associated with changing species
distribution in Denmark 2004-15. Only incident cases were included, and only significant findings
displayed. For the multivariate analysis year, age, gender, and BC system were all kept in the
model.

676

^a 95% confidence intervals. Due to an interaction between calendar year and BC system, the year
 factor is split on ¹BACT/ALERT (top) and ²BACTEC (bottom).^b p value when excluding the main
 tertiary hospital Rigshospitalet (Centre 1-RH).

680

36

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685	S: susceptible; R: resistant; non-wt: non-wildtype (as the MIC was above the ECOFF).
686	Empty cells indicate that there were no isolates for which the MIC had the indicated value.
687	- The indicated concentration of that particular drug was not tested
688	Bold numbers indicate isolates for which the MIC were above the ECOFF and with underlined
689	numbers indicating isolates that were resistant.
690	EUCAST breakpoints and ECOFFS were used. For Candida species and "other fungi" an
691	amphotericin B breakpoint of 1 mg/L was used. For "other fungi" a fluconazole breakpoint of 2
692	mg/L was used. These last cut-offs are only an indication of the susceptibility profile for the given
693	species/group of isolates (and a conservative estimate of the proportion of cases that are likely
694	good targets for the compound in question).
695	¹ For the echinocandins and fluconazole 29 and 23 isolates were tested, respectively. ² For the
696	echinocandins and fluconazole, 1898 and 1892 isolates were tested, respectively.

Table 4: Susceptibility pattern of Danish fungaemia isolates collected in 2012-15 to six antifungal

682 compounds. The EUCAST breakpoints and ECOFFs (Supplementary table 1) are used for the

classification as susceptible (S), resistant (R), and non-wildtype (non-wt).

684

JCM

Table 1: Characteristics of the national fungaemia surveillance scheme on incidence rates, age, gender, and species distribution 2012-2015.

	•				
	2012	2013	2014	2015	2012-2015
mber of Isolates	492	523	441	483	1939
nber of Episodes	479	508	429	467	1883
nber of Patients	461	490	419	443	1813
n patient age (95% CI)	66.1 (64.5;67.7)	64.7 (63.2;66.3)	64.8 (63.1; 66.5)	65.7 (63.1; 66.5)	65.3 (64.5; 66.2)
an patient age (interquartile range)	69 (58; 78)	68 (59; 77)	69 (58; 77)	69 (59; 77)	69 (58; 77)
er % male	55.1%	56.8%	63.5%	64.0%	59.7%
de rate					
000 inhabitants	8.58	9.07	7.62	8.25	8.38
discharges	0.35	0.37	0.31	0.34	0.34
00 bed days	1.05	1.14	0.97	1.008	1.06
es on species (%)					
cans	230 (47%)	268 (51%)	225 (51%)	205 (42%)	928 (47.9%)
oliniensis	13 (3%)	12 (2%)	20 (5%)	26 (5%)	71 (3.7%)
abrata	170 (35%)	155 (30%)	125 (28%)	167 (35%)	617 (31.8%)
ısei	19 (4%)	19 (4%)	20 (5%)	17 (4%)	75 (3.9%)
rapsilosis	16 (3%)	18 (3%)	10 (2%)	20 (4%)	64 (3.3%)
picalis	18 (4%)	23 (4%)	15 (3%)	27 (6%)	83 (4.3%)
ida species ^a	18 (4%)	19 (4%)	17 (4%)	15 (3%)	69 (3.6%)
r fungi ^b	8 (2%)	9 (2%)	9 (2%)	6 (1%)	32 (1.7%)
ida spp. includes: C. lusitaniae (19); C. kefyr (13)	; C. fermentati (8); C. pe	lliculosa (6); C. guilliierm	ondii (5); C. inconspicua	(4); C. orthopsilosis (3); C.	magnoliae and C. nivarien

a) Candida spp. includes: C. lusitaniae (19); C. kefyr (13); C. fermentati (8); C. pelliculosa (6); C. giulliliermondii (5); C. inconspicua (4); C. orthopsilosis (3); C. magnoliae and C. nivariensis (2 each); A. fabianii, C. metapsilosis, C. norvegensis, C. palmioleophila and C.utilis (1 each) and finally two Candida isolates not referred for species identification.
b) Other fung include: S. cerevisiae (11); Fusarium dimerum and Cryptococcus neoformans (4 each); Fusarium more field, Trichosporon asahii and Williopsis saturnus (1 each).

Table 2: Number of isolates, demographics of patients, and blood culture system use during three 4-year periods 2012-2015.

	2004-7	2008-11	2012-15	Time trend (p value)
Isolates	1932	2049	1939	
Episodes	1874	1994	1883	
Patients	1795	1895	1813	
Mean age (95% CI)	61.9 (60.2; 63.6)	63.0 (61.3; 64.7)	65.3 (64.5; 66.2)	0.0002
Median age (25% quartiles)	65 (54; 75)	66 (56; 74)	69 (58; 77)	ND
Male gender (%)	56.4	59.2	59.7	0.01
Episode rate				
/100,000 inhabitants	8.64	9.03	8.38	0.34
/1000 discharges	0.39	0.38	0.34	< 0.0001
/10,000 bed days	0.90 ^a	1.03	1.06	< 0.0001
Proportion of isolates from BACTEC (%)	45.0	36.2	22.0	<0.0001

For information of isolates included please see table 1 (2012-2015) and refer to references (17, 18).

ND: not determined. For the statistical analyses Chi-square trend test was used apart from age and episodes rates where a linear logistic regression analysis was employed. a) Number of bed days 2004 were not available and the figure from 2005 was used for 2004.

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 Table 3: Binomial logistic regression analysis of variables associated with changing species distribution in Denmark 2004-15. Only incident cases were included, and only significant findings displayed. For the multivariate analysis year, age, gender, and BC system were all kept in the model.

Species (no.)		Univariat	e		Multivariate			
	Odds ratio	95% CI ^a	р	pb	Odds ratio	95% CI ^a	р	pb
C. albicans (2967)								
Calendar year (Year)	0.94	0.93-0.96	< 0.001	< 0.001	0.95 ¹	0.94-0.97	<0.001	<0.001
					0.92 ²	0.89-0.94	<0.001	<0.001
Age (Year)	0.995	0.992-0.998	<0.001	0.001	0.995	0.992-0.998	0.002	0.002
C. glabrata (1369).								
Calendar year (Year)	1.08	1.06-1.10	< 0.001	< 0.001	1.06	1.04-1.08	< 0.001	< 0.001
					1.11	1.07-1.15	<0.001	<0.001
Blood culture system (BACTEC)	0.65	0.57-0.75	< 0.001	<0.001	0.78	0.68-0.90	< 0.001	0.004
Gender (Female)	1.15	1.01-1.30	0.032	0.023	1.17	1.03-1.33	0.014	0.009
Age (Year)	1.02	1.02-1.03	<0.001	<0.001	1.02	1.02-1.02	<0.001	<0.001
C. tropicalis (237)								
Gender (Female)	0.74	0.56-0.97	0.034	0.018	0.74	0.56-0.97	0.033	0.018
C. parapsilosis (171)								
BC system (BACTEC)	1.87	1.38-2.54	< 0.001	0.005	1.70	1.24-2.34	0.001	0.045
Age (Year)	0.98	0.98-0.99	<0.001	<0.001	0.98	0.98-0.99	<0.001	<0.001
C. krusei (219)								
BC system (BACTEC)	2.00	1.52-2.62	<0.001	<0.001	2.07	1.56-2.74	<0.001	<0.001
C. dubliniensis (149)								
Calendar year (Year)	1.06	1.01-1.12	0.014	0.010	1.07	1.02-1.13	0.007	0.001
Age (Year)	0.99	0.982-0.997	0.007	0.009	0.989	0.981-0.997	0.008	0.005
Other fungi (83)								
BC system (BACTEC)	1.87	1.21-2.89	0.005	0.046	1.71	1.09-2.69	0.019	0.10
Age (Year)	0.98	0.97-0.99	< 0.001	< 0.001	0.98	0.97-0.99	< 0.001	0.001

^a 95% confidence intervals. Due to an interaction between calendar year and BC system, the year factor is split on ¹BACT/ALERT (top) and ²BACTEC (bottom).^b p value when excluding the main tertiary hospital Rigshospitalet (Centre 1-RH).

	comp	
16	32	64
-	-	-
-	-	-
-	-	-
	<u>3</u> -	-
-	-	-
-	-	-
-	-	-
-	-	-
-	-	-
<u>2</u>	<u>1</u>	-
-	-	-
-	-	-
-	-	-
-	-	-
- 15	- 14	38
-	-	
-		-
-		-
-	-	-
-	-	-
<u>17</u>	55	-
-	-	-
-	-	-
-	-	-
-	-	-

1 2 2

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1

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2

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s

%

100

100

99.5

99.6

99.7

ND

100

95.8

94.4

95.8

97.2

ND

98.5

97.3

98.0

0.0

IE

ND

91.8

93.2

IE

0.0

IE

ND

100.0

0.0

0.0

88.7

No

914

914

909

910

911

ND

71

68

67

68

69

ND

594

587

591

0

IE

ND

67

68

IE

0

IE

ND

62

0

0

55

128

_

<u>17</u>

R

%

No

0 0.0

0 0.0

5 0.5

4 0.4

3 0.3

ND ND

0 0.0

3 4.2

4 5.6

3 4.2

2 2.8

ND ND

9 1.5

16 2.7

12 2.0

55 9.1

IE IE

ND ND

6 8.2

5 6.8

IE E

73 100

IE IE

ND

0 0.0

ND ND

ND

4 6.5

ND

ND

non-wt

No %

0 0.0

0 0.0

5 0.5

6

3

3 0.3

0 0.0

3 4.2

4 5.6

4

2 2.8

1 1.4

9 1.5

16

12 2.0

55 9.1

49 8.1

ND

6 8.2

5 6.8

1 1.4

ND ND

1

ND ND

0 0.0

ND ND

ND

7 11.3

0.7

0.3

5.6

2.7

ND

1.4

ND

Accepted Manuscript Posted Online

<u><</u>0.008

-

880

-

43

-

434

_

C. albicans (914)

Micafungin

Fluconazole

Voriconazole

Isavuconazole

C. dubliniensis (71) Amphotericin B

Anidulafungin

Micafungin

Fluconazole

Voriconazole

Isavuconazole

C. glabrata (603)

Amphotericin B

Anidulafungin

Micafungin

Fluconazole

Voriconazole

Isavuconazole

Amphotericin B

Anidulafungin

Micafungin

Fluconazole

Voriconazole

Isavuconazole

C. parapsilosis (62) Amphotericin B

Anidulafungin

Micafungin

Fluconazole

C. krusei (73)

Amphotericin B Anidulafungin 0.015

874

29

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16

43

24

-

-

1

129

132

11

0.032

4

40

5

909

911

18

25

4

68

70

5

200

25

108

426

16

21

0.064

67

1

15

<u>3</u>

1

13

258

<u>3</u>

248

78

41

15

19

0.125

155

520

1

1

20

28

1

86

<u>6</u>

2

157

20

1

4

46

16

24

6

Table 4: Susceptibility pattern of Danish fungaemia isolates collected in 2012-15 to six antifungal compounds. The EUCAST breakpoints and ECOFFs (Supplementary table 1) are used for the classification as susceptible (S), resistant (R), and non-wildtype (non-wt).

1 2 4 8

7

24 17 15 0

32

6

<u>1</u> - -

<u>1</u>

1

1

No. of isolates with the given MIC (mg/L)

0.5

0.25

356 331 1

349 34 5 **2**

1

2

19 13

<u>1</u> <u>1</u>

128 218 143 <u>9</u>

<u>5 1 2 2</u>

1

1 1 22 164 278 53

13 10 18 **31 17 1**

6

4 30

11

40

7

34 20 2

2 7 37 16

1

5 31 16 3 **3 <u>1</u> <u>1</u>**

<u>3 1 2</u>

17

15 1

2

13 35 13

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						-	-	-	-	ND	
26	41	14		-	-	-	-	-	-	81	
	<u>1</u>			-	-	-	-	-	-	79	
			1	-	-	-	-	-	-	80	
22	26	15	1	2	<u>1</u>	<u>2</u>	2	-	-	74	
<u>3</u>		<u>1</u>		<u>2</u>	<u>1</u>	-	-	-	-	74	
				2	1	-	-	-	-	ND	
19	19	6	<u>3</u>	-	-	-	-	-	-	62	
2	7	7	3	-	-	-	-	-	-	ND	
3	9	3	3	-	-	-	-	-	-	ND	
11	16	3	6	8	<u>3</u>	<u>6</u>	<u>8</u>	-	-	40	
3	5	1			1	-	-	-	-	ND	
2	1	3			1	-	-	-	-	ND	
5	7	7	6	-	-	-	-	-	-	25	
2	0	0	18	-	-	-	-	-	-	ND	
2	0	0	18	-	-	-	-	-	-	ND	
				7	4	5	<u>7</u>	-	-	0	
5	2	1	2	7	2	-	-	-	-	ND	
1	3	3	1	1	8	-	-	-	-	ND	
574	666	205	<u>24</u>	-	-	-	-	-	-	1876	
11	16	46	40	-	-	-	-	-	-	ND	
18	25	39	38	-	-	-	-	-	-	ND	
407	121	68	177	298	64	48	147	-	-	1147	
66	33	23	35	26	7	-	-	-	-	ND	
16	23	30	18	18	12	-	-	-	-	ND	
was ab	ove the	ECOF	F).								
MIC ha				le.							
tested											
the EC	OFF an	d with	under	lined r	numb	ers ind	dicating	isolat	es that v	vere resistan	t.
										used For "	

<u>1</u> 1

53

61

33

24

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66

76

9

12

35

46

1

3

27

323

71

1239

1614

33

28

_

12

6

-

17

1102

219

-

S: susceptible; R: resistant; non-wt: non-wildtype (as the MIC v Empty cells indicate that there were no isolates for which the - The indicated concentration of that particular drug was not to

23

-

2

1382

Voriconazole Isavuconazole

C. tropicalis (81)

Amphotericin B

Anidulafungin

Micafungin

Fluconazole

Voriconazole

Isavuconazole

Amphotericin B

Anidulafungin

Micafungin

Fluconazole

Voriconazole

Isavuconazole

Other fungi (31)¹

Amphotericin B

Anidulafungin

Micafungin

Fluconazole

Voriconazole

Isavuconazole

Anidulafungin Micafungin

Fluconazole

Voriconazole

Isavuconazole

Overall (1900)² Amphotericin B

Candida sp. (65)

5

1

13

5

-

4

6

17

23

6

6

2

4

1

4

7

103

336

47

269

111

2

1

10

4

2

12

8

4

4

14

6

4

5

7

8

4

284

24

59

562

202

58

Bold numbers indicate isolates for which the MIC were above EUCAST breakpoints and ECOFFS were used. For Candida species and "other fungi" an amphotericin B breakpoint of 1 mg/L was used. For "other fungi" a fluconazole breakpoint of 2 mg/L was used. These last cut-offs are only an indication of the susceptibility profile for the given species/group of isolates (and a conservative estimate of the proportion of cases that are likely good targets for the compound in question).

2

ND ND

0 0.0

2

1 1.2

5

7 8.6

ND ND

3 4.6

ND

ND

17

ND

ND

2

ND

ND

16

ND

ND

24 1.3

ND

ND

177

ND

ND

3.2

2.5

6.2

6.5

2 3.2

1 1.6

0 0.0

2 2.5

1 1.2

7

7 8.6

5 6.2

ND ND

8.6

ND

96.8

ND

100

97.5

98.8

91.4

91.4

ND

95.4

ND

ND

61.5

ND

ND

80.6

ND

ND

0.0

ND

ND

98.7

ND

ND

60.6

ND

ND

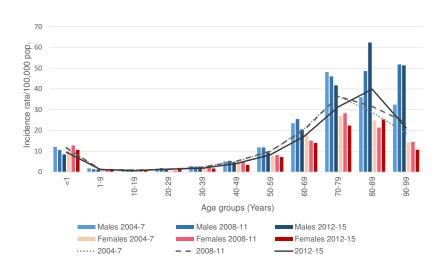
60

ND

MOU

¹ For the echinocandins and fluconazole 29 and 23 isolates were tested, respectively. ² For the echinocandins and fluconazole, 1898 and 1892 isolates were tested, respectively.

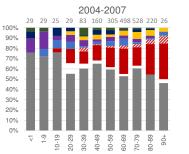
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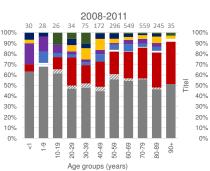


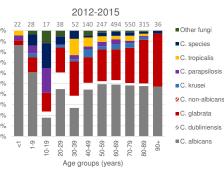
JCM

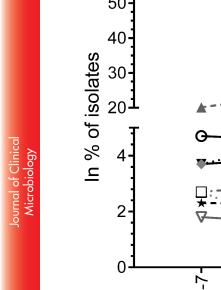


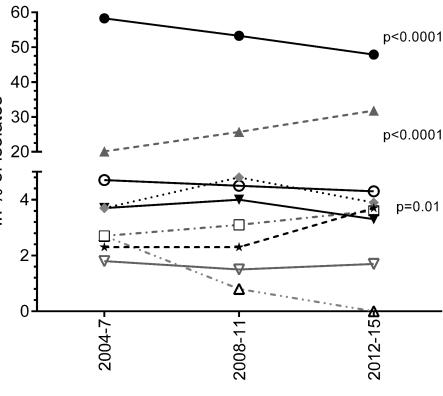


Age groups (years)





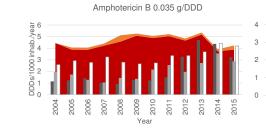




Time periods

- C. albicans
- C. dubliniensis
- C. glabrata
- C. krusei
 - C. parapsilosis
- C. tropicalis 0
- Candida spp. ----
 - non-C. albicans -Δ·
 - Other fungi -

JCM



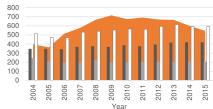
Fluconazole 0.2 g/DDD



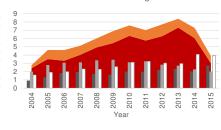
Itraconazole 0.2 g/DDD

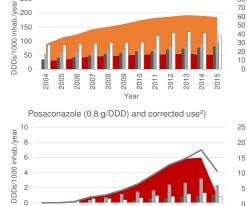
Year



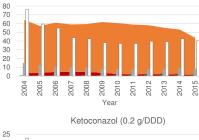


Voriconazole 0.4 g/DDD





Year



Year

