Epidemiology and antifungal susceptibilities of *Candida* spp. to six antifungal agents: results from a surveillance study on fungaemia in Germany from July 2004 to August 2005

Margarete Borg-von Zepelin*, Luisa Kunz, Reinhard Rüchel, Utz Reichard, Michael Weig and Uwe Groß

Institute of Medical Microbiology, National Reference Centre for Systemic Mycoses, University Clinics, Göttingen, Germany

Received 26 September 2006; returned 15 January 2007; revised 30 March 2007; accepted 17 April 2007

**Objectives**: Data on fungal infections occurring in Germany are rare to date. The aim of the present study was to survey the epidemiological situation in Germany, to provide data on the susceptibility of the fungal isolates to antifungals.

**Methods**: Five hundred and sixty-one *Candida* isolates were collected from primarily sterile sites of patients from July 2004 to August 2005 with the aid of a nationwide established laboratory network, MykolabNet-D. The MICs of amphotericin B, flucytosine, fluconazole, itraconazole, voriconazole and caspofungin were determined using the microdilution reference procedure M27-A2 of the CLSI.

**Results**: *Candida albicans* was the most frequently isolated species (58.5%), followed by *Candida glabrata* (19.1%), *Candida parapsilosis* (8.0%) and *Candida tropicalis* (7.5%). In contrast, the isolation rate of *Candida krusei* (1.4%) was low. *Candida kefyr* appeared as a new pathogen in this profile. Amphotericin B revealed excellent activity, with only three resistant isolates (0.5%). A total of 25 isolates (4.5%) showed resistance against flucytosine. All 25 isolates were identified as *C. tropicalis* indicating a peculiarity within German isolates. The resistance rate of all tested isolates to fluconazole and to itraconazole was 3.7% and 17.6%, respectively. According to the provisional breakpoints, two isolates (0.4%) were tested as resistant to voriconazole. Caspofungin was active against the majority of isolates where an intrinsic resistance is unknown.

**Conclusions**: This latest German survey of isolates from patients with fungaemia demonstrates a favourable situation with respect to antifungal susceptibilities for the antifungal substances tested.

Keywords: azoles, azole resistance, flucytosine, caspofungin, susceptibility testing

**Introduction**

Fungal infections have substantially increased in number and severity over the past two decades especially in immunocompromised patients and those hospitalized with serious underlying diseases. Species of the genus *Candida* are most frequently isolated as the cause of invasive fungal infections and they now rank as the fourth most common cause of nosocomial bloodstream infections in the USA, accounting for 8% to 15% of all hospital-acquired sepsis cases. Longitudinal surveillance programmes in the USA and Europe have detected an increase in the prevalence of fungal infections caused by *Candida* species other than *Candida albicans* such as *Candida glabrata*, *Candida krusei* and *Candida parapsilosis*.

Moreover, it appears that marked differences exist in species distributions and antifungal drug susceptibilities between different countries, underscoring the need for continued surveillance in each country to monitor trends in pathogen distribution and drug susceptibilities. Epidemiological data on fungal infections coming from Germany are rare to date. Therefore, the National Reference Centre for Systemic Mycoses (NRZSM) has initiated a first survey on fungal infections. With the aid of the newly established laboratory network, MykolabNet-D, this survey aimed to gather epidemiological information on the spectrum of fungi.
isolated from primarily sterile clinical specimens, as well as to gather information pertaining to the situation of susceptibility to six antifungals, often used for therapy of systemic fungal infections.

Materials and methods

Study design

In order to conduct a surveillance study on fungaemia, the NRZSM has established a laboratory network, MykolaNet-D, with the assistance of 38 laboratories that are distributed all over Germany and are representative for the situation of the country. The participating laboratories were required to collect and identify the fungal strains isolated from primarily sterile sites in patients (e.g. blood culture isolates, cerebrospinal fluids, surgically obtained specimens) and to complete a questionnaire on risk factors or predisposing diseases, clinical symptoms present at the time and diagnosis of candidaemia, indwelling catheters, and current antibiotic or antifungal therapies. Using this strategy, a total of 561 Candida isolates was obtained from July 2004 to August 2005.

Fungal isolates

Species identification was performed at the participating laboratories and confirmed by the NRZSM, Germany, using standard morphological and physiological methods, including fermentation and growth on different carbon sources and on nitrogen sources as well as growth at various temperatures. Additionally, a commercially available test kit was used (Api 32C aux). Candida dubliniensis, for example, was differentiated by the use of chromagar, growth at 45°C and chlamydospore formation on Niger seed agar. For the antifungal drug susceptibility testing, C. parapsilosis ATCC 22019 and C. krusei ATCC 6258 were used as controls.

Antifungal drugs

Standard powders of amphotericin B (Sigma–Aldrich, Taufkirchen, Germany), flucytosine (Sigma–Aldrich), fluconazole (Pfizer, Karlsruhe, Germany), itraconazole (Ortho Biotech, Bridgewater, NJ, USA), voriconazole (Pfizer Ltd, Sandwich, Kent, UK) and caspofungin (MSD, Rahway, NJ, USA) were used. Amphotericin B, itraconazole and voriconazole were dissolved in dimethyl sulphoxide at the proposed stock solutions and stored at −20°C. Caspofungin and flucytosine were dissolved in aqua bidest. Fluconazole was dissolved according to reports recently published that analysed influences of methodological variables on susceptibility testing of caspofungin against Candida species, though an interpretive cut-off value is not yet available for this echinocandin. Therefore, the endpoint is given as the concentration of the drug in the assay at which 50% of growth control was observed.

Analysis of results

Interpretive breakpoints proposed by the CLSI for flucytosine, fluconazole and itraconazole were used. Provisional interpretive breakpoints for voriconazole were used as proposed by Pfizer Inc. and recently confirmed by Pfaffer et al. Isolates were classified according to their MIC as susceptible or as showing decreased susceptibility. The latter category included the dose-dependent (S-DD), intermediate and resistant (R) categories according to the CLSI. In the Results section, MIC50 and MIC90 values are given. These are average concentrations where 50% or 90%, respectively, of all fungal isolates are susceptible to a certain antifungal substance. The quality control isolates C. parapsilosis ATCC 22019 and C. krusei ATCC 6258 were included in all runs, and all results were within the published limits. No differences were observed over the whole test period.

Results

In the present study, the majority of fungal strains were isolated from adult patients. Only 10 isolates originated from infants younger than 1 year and 10 further isolates came from children aged 1 to 10 years. These account for 1.7%. In contrast, 212 fungal strains (36.7%) were isolated from patients older than 70 years.

In all age groups, the fungal isolates were found more frequently in males (male:female ratio = 305:256). Of the fungal isolates, fluconazole and itraconazole were used for therapy of systemic fungal infections.

The pH of the test medium was 7. An inoculum size of $10^5$ cfu/mL was used. MIC endpoints were determined spectrophotometrically at 24 h and in the case of C. parapsilosis at 48 h. For amphotericin B, the endpoint of the MIC was defined as the lowest drug concentration that resulted in a reduction in growth by 90% or more, compared with that of a drug-free growth control well. For flucytosine and azoles, the MIC endpoint was defined as a 50% reduction in optical density. For caspofungin, the endpoint was defined according to reports recently published that analysed influences of methodological variables on susceptibility testing of caspofungin against Candida species, though an interpretive cut-off value is not yet available for this echinocandin. Therefore, the endpoint is given as the concentration of the drug in the assay at which 50% of growth control was observed.

The MIC endpoints were determined spectrophotometrically at 24 h and in the case of C. parapsilosis at 48 h. For amphotericin B, the endpoint of the MIC was defined as the lowest drug concentration that resulted in a reduction in growth by 90% or more, compared with that of a drug-free growth control well. For flucytosine and azoles, the MIC endpoint was defined as a 50% reduction in optical density. For caspofungin, the endpoint was defined according to reports recently published that analysed influences of methodological variables on susceptibility testing of caspofungin against Candida species, though an interpretive cut-off value is not yet available for this echinocandin. Therefore, the endpoint is given as the concentration of the drug in the assay at which 50% of growth control was observed.

Analysis of results

Interpretive breakpoints proposed by the CLSI for fluocytosine, flucytosine and itraconazole were used. Provisional interpretive breakpoints for voriconazole were used as proposed by Pfizer Inc. and recently confirmed by Pfaffer et al. Isolates were classified according to their MIC as susceptible or as showing decreased susceptibility. The latter category included the dose-dependent (S-DD), intermediate and resistant (R) categories according to the CLSI. The quality control isolates C. parapsilosis ATCC 22019 and C. krusei ATCC 6258 were included in all runs, and all results were within the published limits. No differences were observed over the whole test period.

Susceptibility testing—broth microdilution method

The MICs of amphotericin B, flucytosine, fluconazole, itraconazole, voriconazole and caspofungin were determined according to the reference procedures of the CLSI described in document M27-A2. The methods included some modifications to allow automation of the method and to permit the incubation period to be shortened from 48 to 24 h.

Briefly, testing was performed with RPMI 1640 medium supplemented with 0.2% glucose in flat-bottomed microdilution plates.
Table 1. Spectrum of fungal isolates in all primarily sterile sites (left) and in blood cultures (right)

<table>
<thead>
<tr>
<th>Species</th>
<th>Blood Cultures (n=328)</th>
<th>All Sterile Sites (n=420)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td>328 (58.5%)</td>
<td>250 (58.4%)</td>
</tr>
<tr>
<td>C. glabrata</td>
<td>107 (19.1%)</td>
<td>80 (18.7%)</td>
</tr>
<tr>
<td>C. parapsilosis</td>
<td>45 (8.0%)</td>
<td>40 (9.3%)</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>42 (7.5%)</td>
<td>27 (6.3%)</td>
</tr>
<tr>
<td>C. kefyr</td>
<td>11 (2.0%)</td>
<td>9 (2.1%)</td>
</tr>
<tr>
<td>C. krusei</td>
<td>8 (1.4%)</td>
<td>7 (1.6%)</td>
</tr>
<tr>
<td>C. guillermondii</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>C. dubliniensis</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>C. inconspicua</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>C. catemulata</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>C. colliculosa</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>C. famata</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>C. intermedia</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>C. lusitaniae</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>C. norvegensis</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>C. rugosa</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>C. utilis</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>642</td>
<td>428</td>
</tr>
</tbody>
</table>

specimens. Of the C. tropicalis strains, 64% were blood culture isolates.

When data were analysed for the range of susceptibility, the MIC50 was low for all fungal isolates and all antifungals tested (Table 2). The MIC90 revealed a broader spectrum of inhibitory concentrations of the tested antifungal substances. The MIC90 of fluconazole was found to be at 16 mg/L and thus in the range of dose-dependent susceptibility. The MIC90 of itraconazole was determined to be 1 mg/L, a concentration that is already in the resistant range. The resistant isolates were further analysed. With flucytosine, 25 fungal isolates were tested resistant corresponding to 4.5%. All of these isolates belonged to the species C. tropicalis. No differences in underlying diseases between patients harbouring flucytosine-resistant and -susceptible C. tropicalis isolates have been observed. The percentage of fluconazole-resistant isolates amounted to 3.7%. The data on itraconazole showed a percentage of 17.6% resistant fungal isolates under the conditions tested. More than 60% (62 out of 99) of this group were represented by C. glabrata. Of the fungal isolates 19.1% were additionally tested as being dose-dependently susceptible. With voriconazole, the susceptibility tests according to CLSI revealed low resistance (0.4%). As was expected, we hardly saw any resistance to amphotericin B. Only three such isolates were found, one each of Candida rugosa (MIC 2 mg/L), C. glabrata (MIC 2 mg/L) and Candida kefyr (MIC 4 mg/L), respectively. At present, no interpretable breakpoints for caspofungin exist, therefore MIC50, MIC90 and the range of measured MICs are given and no statement concerning the clinical resistance could be made (Table 2).

Discussion

This report provides a new description of the causative Candida species and in vitro drug susceptibilities of such fungal strains isolated from patients with fungaemia in Germany. These data were obtained from a surveillance study performed with the aid of a network of German laboratories. Our findings indicate that the distribution of Candida species from primarily sterile deep sites, in general, and from blood cultures, in particular, is comparable to that documented in recent reports from several other European countries.5

The predominance of C. albicans was reported by nearly all European and USA surveys.1,5,11,12 However, for the ranking of the non-albicans Candida species, each European country has to be analysed separately. C. glabrata ranked second in our study as well as, for example, in Switzerland.11 In contrast, in data from Spain, high percentages of C. parapsilosis have been noted.13 This species has often been reported to be an emerging pathogen in neonates.14

Among the non-albicans Candida spp., we found 11 isolates of C. kefyr, 9 of these strains having been isolated from blood cultures. Whether this species is an emerging pathogen, as has been reported recently by Reuter et al.,15 is unclear at present. Further observations and detailed analysis of the respective patients are needed.

For 4% of all tested isolates, our findings revealed a low rate of fluconazole resistance and confirmed the low level of fluconazole resistance among C. albicans isolates from all deep sites (3%).16 A high proportion of 206 isolates (36.8%) demonstrated decreased susceptibility to itraconazole (MIC >0.25 mg/L). However, the MIC90 for all isolates tested against itraconazole was found to be 1 mg/L, an antifungal concentration that is achievable in serum and plasma of patients under therapy.17 When Pföller et al.18 reported the in vitro activities of flucytosine against more than 8000 clinical isolates of Candida spp. obtained from blood and other deep sites at more than 200 hospitals worldwide, resistance to flucytosine (MIC >16 mg/L)

Table 2. Antifungal susceptibilities of 561 clinical isolates of fungal species by the CLSI reference microdilution method

<table>
<thead>
<tr>
<th>Antifungal Drug</th>
<th>MIC50 (mg/L)</th>
<th>MIC90 (mg/L)</th>
<th>Range of MICs</th>
<th>R (%)</th>
<th>S-DD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flucytosine</td>
<td>&lt;0.125</td>
<td>2</td>
<td>&lt;0.125 to &gt;16</td>
<td>25 (4.5%)</td>
<td>10 (1.8%)</td>
</tr>
<tr>
<td>Flucconazole</td>
<td>0.5</td>
<td>16</td>
<td>&lt;0.25 to &gt;64</td>
<td>21 (3.7%)</td>
<td>33 (5.9%)</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>0.125</td>
<td>1</td>
<td>0.0313 to 16</td>
<td>99 (17.6%)</td>
<td>107 (19.1%)</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>0.0313</td>
<td>0.5</td>
<td>&lt;0.0313 to 16</td>
<td>2 (0.4%)</td>
<td>3 (0.5%)</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>0.5</td>
<td>1</td>
<td>&lt;0.0313 to 4</td>
<td>3 (0.5%)</td>
<td>ND</td>
</tr>
<tr>
<td>Caspofungin</td>
<td>0.0313</td>
<td>0.25</td>
<td>&lt;0.0313 to 16</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
Epidemiology and antifungal susceptibilities of *Candida* spp.

was observed in only 3% of *C. albicans* and in 1% of *C. glabrata*. In this study, we documented flucytosine MICs in the range 0.125–16 mg/L with an MIC of 2 mg/L. No resistant isolate of *C. albicans*, *C. glabrata* and *C. parapsilosis* was observed, which is consistent with reports from other European countries. However, we observed 25 *C. tropicalis* isolates (59.5%) that were resistant to flucytosine. Since this is not consistent with other European reports, we assume it is a situation peculiar to Germany. Such geographic peculiarities of flucytosine resistance have also been reported by Cuenca-Estrella *et al.* This group compared *Candida* spp. from Spain and Argentina and reported striking differences for *C. tropicalis* with 1.8% flucytosine resistance in Spain and 10.6% flucytosine resistance in Argentinian isolates.

Meanwhile, interpretive breakpoints are also available for voriconazole. The critical analysis of the MIC values revealed that only a few *Candida* isolates were resistant. Although no established interpretive breakpoint is available for caspofungin as yet, the MIC of 0.25 mg/L for all tested isolates was low.

In conclusion, this new national survey of German clinical fungal isolates shed light on antifungal susceptibilities of *Candida* spp. isolated from primarily sterile deep sites between July 2004 and August 2005. In general, the situation of antifungal resistance is favourable for the German fungal isolates.

**Acknowledgements**

This work includes parts of the medical thesis of L. K. We thank S. Kellner, D. Hermann and M. Schaffrinski for skillful technical assistance. The help of C. Maueicke, BSc, in preparing the manuscript is gratefully acknowledged. We thank Pfizer, Ortho Biotech and MSD for supplying the antifungal substances. This study has been supported by the German Ministry of Health (BMG).

The MykolabNet-D consists of the following laboratories: Institute of Microbiology and Hygiene, Homburg; Niedersächsisches Landesgesundheitsamt, Hannover; Institute of Med. Microbiology, Mannheim; Gemeinschaftspraxis Wisplinghoff und Kollegen, Köln; Institute of Medical Microbiology, Magdeburg; Department of Medical Microbiology and Immunology of Infections, Berlin; Gemeinschaftspraxis Stein und Partner, Mönchengladbach; Friedrich-Loeffler-Institute of Medical Microbiology, Greifswald; Gemeinschaftspraxis Wagner, Stibbe und Partner, Göttingen; Institute of Laboratory Medicine, Städtische Kliniken, Frankfurt (Main)-Höchst; Institute of Clinical Chemistry and Laboratory Medicine, Suhl; Gemeinschaftspraxis Drs Schotdorf und Kollegen, Augsburg; Institute of Medical Microbiology, Münster; Landesamt für Verbraucherschutz, Magdeburg; Institute of Medical Microbiology and Hygiene, Mainz; Gemeinschaftspraxis Drs Bartl, Wimmer and Partner, Augsburg; Institute of Hygiene and Laboratory Medicine, Krefeld; Institut of Medical Microbiology/ Medizinuntersuchungsamt, Kiel; Institute of Medical Microbiology, Rostock; Institut of Clinical Chemistry and Laboratory Medicine, Dresden-Friedrichstadt; Ärztesches Labor Dr Berthold und Kollegen, Frankfurt(Oder); Thüringer Landesamt für Lebensmittel und Verbraucherschutz, Erfurt; Labor Dr Arnold, Würzburg; Institute of Clinical Microbiology, Immunology and Hygiene, Erlangen; Landesunter suchungsanstalt für Gesundheits- u. Veterinärwesen Sachsen, Chemnitz; Institut of Laboratory Medicine and Microbiology, Helios Kliniken, Schwerin; Laborzentraxus Dr Runnebaum und Partner, Eppelehim; Robert-Koch-Institute, Berlin; Institute of Microbiology and Hygiene (Charité), Berlin; Fachärzte für Laboratoriumsmedizin Dr Becker und Kollegen, München; Zentrallabor des Klinikums, Duisburg; Institute of Medical Microbiology, Köln; Institute of Medical Microbiology, Düsseldorf; Institute of Medical Microbiology, Marburg; Institut of Medical Microbiology/Medizinaluntersuchungsamt, Bochum; Institute of Medical Microbiology, Göttingen.

**References**

13. Cuenca-Estrella M, Rodriguez D, Almirante B *et al.* In vitro susceptibilities of bloodstream isolates of *Candida* species to six antifungal agents: results from a population-based active surveillance


