Susceptibility profile to antifungal drugs of *Pichia anomala* isolated from patients presenting nosocomial fungemia

**Running title:** Susceptibility of *Pichia anomala* to antifungal drugs

**Authors and affiliations:**

1- Vânia Lúcia Ribeiro da Matta*
Adolfo Lutz Institute. Secretary of Health, São Paulo State, Brazil

2- Márcia de Souza Carvalho Melhem
Adolfo Lutz Institute. Secretary of Health, São Paulo State, Brazil

3- Arnaldo Lopes Colombo
Division of Infectious Diseases- Federal University of São Paulo. São Paulo, Brazil

4- Maria Luiza Moretti
Infectious Diseases Division; Faculty of Medical Sciences; Universidade Estadual de Campinas, Brazil

5- Laura Rodero


6- Gisele Madeira Duboc de Almeida. Laboratório of Clinical Microbiology; Hospital das Clínicas; University of São Paulo, Brazil
7- Marilena dos Anjos Martins

Adolfo Lutz Institute. Secretary of Health, São Paulo State, Brazil

8-Silvia Figueiredo Costa- Hospital Infection Control Department and LIM 54 of Hospital das Clínicas, University of São Paulo, Brazil

9- Maria Beatriz G. Souza Dias- Hospital Sírio Libanês, São Paulo, Brazil

10- Márcio Nucci- Department of Internal Medicine, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil.

11- Anna S. Levin – Department of Infectious Diseases; Hospital Infection Control Department and LIM 54 of Hospital das Clínicas, University of São Paulo, Brazil

Institution where the work was performed: Adolfo Lutz Institute. Secretary of Health, São Paulo State, Brazil

Corresponding author:

Vânia Lúcia Ribeiro da Matta

Instituto Adolfo Lutz

Av: Dr. Arnaldo 351- 11º andar

São Paulo-São Paulo-Brasil

Zip code: 01246-901

Telephone number: 55 11 3068 2900

Fax number:55 11 3085 3505

e-mail: mattav@usp.br
ABSTRACT

*In vitro* susceptibility of 58 isolates of *Pichia anomala* to 5 antifungal drugs using two broth microdilution methods (CLSI and EUCAST) was analysed. Low susceptibility to itraconazole was observed. Fluconazole, voriconazole, amphotericin B and caspofungin showed good activity although relatively high drug concentrations were necessary to inhibit the isolates.
Several reports have pointed *Pichia anomala* (anamorph *Candida pelliculosa*) as a cause of a large spectrum of invasive infections (13;20;22;34), being fungemia the most common presentation (2;6;17;25;41). Usually, the patients have been treated with amphotericin B (with or without 5-flucytosine) or fluconazole with good clinical outcomes (3;6;12;16;25;38). Nonetheless treatment failures may occur (1;6;43) as well as cases of breakthrough fungemias in immunocompromised patients receiving prophylaxis with fluconazole (14).

Although considered an emergent hematogenous yeast pathogen, data on susceptibility to antifungal drugs of *P.anomala* are scarce (4;5;19;28;32). The aim of this study was to determine the *in vitro* antifungal susceptibility profile to 5 drugs of a large collection of *P.anomala* isolated from bloodcultures of patients with nosocomial fungemia.

For this purpose, fifty-two non-related bloodstream isolates of *P.anomala* (36 from Brazil; 13 from Argentina; and 3 from Spain) and 6 (United States) from unknown clinical specimens were tested by two broth microdilution methods according to *Clinical and Laboratory Standards Institute* (CLSI, formerly NCCLS) (35) and the *European Committee on Antibiotic Susceptibility Testing* (EUCAST) (37) guidelines.

We evaluated the activity of amphotericin B (Sigma, USA), itraconazole (Janssen Pharmaceutica), voriconazole (Pfizer Inc, USA) and caspofungin (Merck & Co., USA) at concentrations ranging from 0.015 to 8 µg/mL, and fluconazole (Pfizer Inc, USA) from 0.12 to 64 µg/mL.

The inhibition criterion (IC) adopted to determine the minimal inhibitory concentration (MIC) for amphotericin B was the lowest drug concentration which produced
complete or nearly complete (≥95%) inhibition compared with the drug-free control well (24). For azoles (35) and caspofungin (23) the lowest concentration which produced ≥50% inhibition was used.

All MICs were determined spectrophotometrically (530 nm) after incubation for 24 h (EUCAST) or 48 h (CLSI).

To categorize the isolates as susceptible we employed the CLSI (35) interpretive criteria for fluconazole (≤8 µg/mL) and itraconazole (≤0.12 µg/mL) and for voriconazole, a recently established breakpoint (BP) of ≤1 µg/mL (29). Based on pharmacokinetic data a BP of ≤1 µg/mL was assumed for caspofungin (40) and amphotericin B (21;28). Only for comparison, the same CLSI interpretive criteria were adopted for EUCAST results as BPs have not been defined yet.

In our study we found an excellent agreement (≤ 2-fold dilutions) between MIC results generated by the EUCAST and CLSI methods for all antifungal drugs (table 1). The lowest agreement rate was observed for itraconazole assays as already reported in a previous study testing other Candida species (10). When evaluating the difference of MICs obtained by the two methods, EUCAST produced statistically significant lower MICs for voriconazole, amphotericin B and caspofungin (table 1). However, despite these differences, all isolates were categorized as susceptible by both methods, according to the assumed BPs. Although both methods were suitable to test P. anomala, the 24 hours of incubation of EUCAST method provided a clear spectrophotometric endpoint reading, which represents an advantage by reducing the incubation time.

No remarkable differences were observed considering the geographical distribution of the isolates (data not shown), so Table 2 presents in a continuous fashion the cumulative percentage of all 58 P. anomala isolates susceptible at each dilution
throughout the serial dilutions. We could observe that MICs tended to concentrate from the middle to the high end of the range for all the drugs. Although almost all isolates were categorized as susceptible to fluconazole (table 2), similarly to other investigators (19;28;32;42), some studies showed the opposite (5;36). Indeed, the modal values, CIM$_{50}$ and CIM$_{90}$ of our collection were close to those reported for C. glabrata (8;9;11;26;27;33), a species of Candida that is considered to be less susceptible to azoles than C. albicans.

Itraconazole was the triazole with the lowest in vitro activity against P.anomala, as more than 60% of all isolates showed reduced sensitivity to the drug (table 2). Also, MIC$_{50}$ and MIC$_{90}$ for itraconazole were much higher than those frequently obtained for C. albicans (8;11;24;33) and similar to those determined for C. glabrata, C. krusei and C. guilliermondii (4;10;11;18;24;33).

Voriconazole was very active against all P.anomala isolates (table 2) but MIC values were usually higher than those reported for C. albicans, C. parapsilosis and C. tropicalis (7;18;27;28;30;32). Actually, they were similar to MIC results obtained by other investigators testing C. glabrata and C. krusei (7;18;27;28;30).

In our study, amphotericin B MICs were tightly clustered between 0.12 and 1.0 $\mu$g/mL and all isolates were considered susceptible to the drug (table 2). In fact, the resistance of Candida species to amphotericin B is a rare phenomenon that may be associated with the low sensitivity of broth microdilutions methods in discriminating resistant strains.

Caspofungin presented good in vitro activity against P.anomala as all isolates were inhibited by $\leq$ 1$\mu$g/mL (table 2). However their MIC values were higher than those usually necessary to inhibit most isolates of C. albicans, C. tropicalis and C. glabrata.
The only other study on susceptibility of *P. anomala* to caspofungin generated MIC$_{50}$ values 8 times lower than in our report. This finding may be partially explained by differences in the incubation time used in both studies as we read the CLSI-MICs at 48 hours instead of 24 hours as suggested by Pfaller et al (31). In fact, we were not able to determine confident MICs at 24 hours by using the CLSI inoculum, in contrast with the 100-fold higher EUCAST inoculum. Finally, neither trailing nor paradoxical growth were seen in our series of *P. anomala* against caspofungin, as observed for some *Candida* species (24;39).

In summary, *P. anomala* did not show intrinsic resistance to any of the studied drugs. Nonetheless, susceptibility to itraconazole was poor. In contrast, fluconazole; voriconazole; amphotericin B and caspofungin presented good activity against *P. anomala* although relatively high drug concentrations were necessary to inhibit the isolates. Altogether, the susceptibility profile of *P. anomala* was more similar to that of *C. glabrata* than to that of *C. albicans*. Trailing and paradoxical growth were not observed in the presence of any antifungal drug tested.

Finally, our study analyzed the largest series of *P. anomala* bloodstream isolates tested against 5 antifungal drugs by two different methods and could establish a reliable susceptibility profile for *P. anomala*, as an attempt to guide the therapeutic choice of antifungal drugs in cases of invasive infections.

**Acknowledgments**

Laboratórios Pfizer Ltda. Brasil; Merck Co. and Janssen Pharmaceutica for donating the antifungal powders.
Manuel Cuenca- Estrella and Richard Hollis for providing isolates from Spain and USA.

Giovane Coutinho for technical assistance in experiments with caspofungin

Geraldo R. Godoy for organizing the tables.
REFERENCES


26-Pfaller M. A., D.J. Diekema, R.N. Jones, H.S. Sader, A.C. Fluit, R.J. Hollis, S.A. Messer; and SENTRY Participant Group. 2001. International surveillance of
bloodstream infections due to *Candida* species: frequency of occurrence and *in vitro*
susceptibilities to fluconazole, ravuconazole, and voriconazole of isolates collected
from 1997 through 1999 in the SENTRY antimicrobial surveillance program. J Clin

of voriconazole, posaconazole, and four licensed systemic antifungal agents against

28-Pfaller M.A., D.J. Diekema, S.A. Messer, L. Boyken, R.J. Hollis, and R.N.
Jones. 2004. *In vitro* susceptibilities of rare *Candida* bloodstream isolates to
ravuconazole and three comparative antifungal agents. Diagn Microbiol Infect Dis.

29-Pfaller M. A., D. J. Diekema, J. H. Rex, A. Espinel-Ingroff, E. M. Johnson, D.
Andes, V. Chaturvedi, M. A Ghannoum, F. C. Odds, M. G. Rinaldi, D. J.
Sheehan, P. Troke, T. J. Waish, and D. W. Warnock. 2006. Correlation of MIC with
Outcome for *Candida* Species Tested against Voriconazole: Analysis and Proposal

30-Pfaller M.A., S.A. Messer, A. Houston, K. Mills, A. Bolmstrom, and R.N.
susceptibilities of 312 clinical isolates of *Candida* species by using three different

31-Pfaller M.A., S. A. Messer, L. Boyken, C. Rice, S. Tendolkar, R. J. Hollis, and
D.J. Diekema. 2004. Further standardization of broth microdilution methodology for
*in vitro* susceptibility testing of caspofungin against *Candida* species by use of an
international collection of more than 3,000 clinical isolates. J Clin Microbiol.


Table 1: Distribution of *P. anomala* isolates according to differences in minimal inhibitory concentration (MIC) results obtained by EUCAST method compared with CLSI method.

<table>
<thead>
<tr>
<th><em>P. anomala</em> (nº of isolates)</th>
<th>Antifungal drug</th>
<th>Nº of isolates for which EUCAST-MICs differed from CLSI-MICs</th>
<th>Agreement within nº of dilution (%)</th>
<th>Mean difference (log₂ values)</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-3</td>
<td>-2</td>
<td>-1</td>
<td>0</td>
</tr>
<tr>
<td>All organisms (58)</td>
<td>Fluconazole</td>
<td>6</td>
<td>8</td>
<td>33</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Itraconazole</td>
<td>1</td>
<td>2</td>
<td>14</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Voriconazole</td>
<td>3</td>
<td>23</td>
<td>28</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Amphotericin B</td>
<td>1</td>
<td>22</td>
<td>27</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Caspofungin</td>
<td>8</td>
<td>32</td>
<td>16</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 2: Susceptibility profile of *Pichia anomala* determined by CLSI and EUCAST broth microdilution methods against 5 antifungal drugs

<table>
<thead>
<tr>
<th>Antifungal drug</th>
<th>Microdilution Method</th>
<th>Cumulative % of susceptible isolates at MIC (µg/mL) of 0.015 0.03 0.06 0.12 0.25 0.5 1 2 4 8 16</th>
<th>Mode</th>
<th>GM-MIC a</th>
<th>%S b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluconazole</td>
<td>CLSI</td>
<td>0 0 0 0 0 0 0 0 0 15.5 56.8 93 100</td>
<td>4</td>
<td>5.08</td>
<td>96.6</td>
</tr>
<tr>
<td></td>
<td>EUCAST</td>
<td>0 0 0 0 0 0 0 0 0 22.4 55.1 96.5 100</td>
<td>8</td>
<td>4.79</td>
<td>93</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>CLSI</td>
<td>1.7 0 12 36.1 74.1 96.5 100</td>
<td>0.25</td>
<td>0.21</td>
<td>36.2</td>
</tr>
<tr>
<td></td>
<td>EUCAST</td>
<td>0 0 7 39.5 91.2 98.2 100</td>
<td>0.25</td>
<td>0.19</td>
<td>39.6</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>CLSI</td>
<td>0 3.4 18.9 39.6 96.5 100</td>
<td>0.25</td>
<td>0.16</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>EUCAST</td>
<td>0 3.4 29.3 65.5 100</td>
<td>0.12</td>
<td>0.12</td>
<td>100</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>CLSI</td>
<td>0 0 0 12 36 88 100</td>
<td>0.5</td>
<td>0.45</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>EUCAST</td>
<td>0 0 0 15.5 55.1 94.7 100</td>
<td>0.25</td>
<td>0.37</td>
<td>100</td>
</tr>
<tr>
<td>Caspofungin</td>
<td>CLSI</td>
<td>0 1.7 17.2 84.5 100</td>
<td>0.12</td>
<td>0.12</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>EUCAST</td>
<td>0 12 70.6 100</td>
<td>0.06</td>
<td>0.07</td>
<td>100</td>
</tr>
</tbody>
</table>

a Geometric mean of MICs
b Percentage of susceptibility (EUCAST and CLSI MICs were interpreted according to CLSI breakpoints for fluconazole and itraconazole. For voriconazole, amphotericin B and caspofungin the adopted breakpoint was ≤ 1µg/mL)