Fenticonazole activity by the EUCAST and CLSI methods against 260 Candida vulvovaginitis isolates from two European regions and annotations on the prevalent genotypes.

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Abstract

Fenticonazole activity was studied against 260 West and the South-East European vulvovaginal candidiasis isolates and displayed low MICs. The EUCAST and CLSI microdilution methods assessed fenticonazole for the first time and showed excellent agreement (97%) and significant interclass correlation coefficient (P<0.0001). Also, agreement for itraconazole was 84%, fluconazole 90%, and ketoconazole 98% (P<0.0001). Multilocus typing by PCR fingerprinting and subsequent cluster analysis delineated geographically-associated alignments for C. albicans and fluconazole resistance-related clusters for C. glabrata.
Uncomplicated vulvovaginal candidiasis (VVC) affects approximately 75% of women at reproductive age (13, 17, 22) where Candida albicans is a major cause and C. glabrata accounts for approximately 5% of cases worldwide (30). The recommended first-line therapy for uncomplicated VVC is topical azoles, (4, 7, 25, 27, 28) unless resistance of isolate is substantiated or azole hypersensitivity is diagnosed (4, 8).

Identifying antifungal resistance in vitro is clinically important but variable host responses to treatment and unpredictable fungal load in the vulvovaginal mucosa (in loco) invariably weaken in vitro with in vivo correlations. However, standardized susceptibility testing of isolates to local antifungals could provide data on the in vitro activity of newer topical antifungals.

Recording agreement of the CLSI (24) and EUCAST (10) reference methods on susceptibility of VVC isolates from Belgian and Greek patients to the topical imidazole, fenticonazole (8, 12) forms the basis of this report. Subsequently, PCR fingerprinting investigated whether distinct geographical and azole-resistant clinical isolate subpopulations can be recognized.

A total of 260 base-line C. albicans and C. glabrata isolates from pregnant, non-pregnant and diabetic women, were tested (Table 1). Isolates were identified in CHROMagar medium (CHROMagar, Paris, France) and identified with the API ID 32 C system (Bio-Rad, Marnes-la-Coquette, France). All C. albicans isolates were screened for C. dubliniensis (3, 18, 31) and C. glabrata stains were screened for C. nivariensis and C. bracarensis (1, 19) to ensure that susceptibility testing and PCR fingerprints corresponded only to C. albicans and C. glabrata isolates.

Stock fluconazole (Pfizer Inc., Sandwich, Kent, U.K) solutions and range of concentrations of itraconazole, and ketoconazole (Janssen, Beerse, Belgium), were
prepared as per each reference method. Fenticonazole compound (Recordati S.A, Milan, Italy and Galenica, Athens, Greece) was prepared as 100× stock in dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany) at final concentration range 0.0312 µg/ml to 32 µg/ml. Test medium, inoculum preparations, and reading of results were as per respective (10, 24) guidelines. C. parapsilosis ATCC 22019 and C. krusei ATCC 6258 were used as control stains for both methods (Table 2). No CLSI and EUCAST out of range MICs were observed for itraconazole, fluconazole and ketoconazole.

No differences in susceptibilities among isolates from the three patient groups were observed but, contrasting previous reports, (21) no geographical associations in susceptibility were recorded for isolates from the two European regions. Fluconazole resistance (Table 2) in C. albicans was rare (6.9%), whereas 45% of the C. glabrata isolates were resistant (6, 11). Fluconazole and ketoconazole cross-resistance was inferred for 20/249 (8.03%) C. albicans and 3/11 (27.2%) C. glabrata. Generally, lower fenticonazole MICs were recorded (Table 2) but their clinical relevance cannot be assessed without correlating in vitro responses and in loco fenticonazole pharmacokinetics and pharmacodynamics with in vivo response. Topical ketoconazole efficacy and drug levels have thus far been assessed ex vivo in human skin stripplings and have successfully supported standardized susceptibility testing and clinical investigations (2). However, bioassay systems to complement in vitro studies have not been assessed with topical VVC agents.

The CLSI and EUCAST agreement (29) within ±1 dilution was 84-98% (Table 3) and interclass correlation coefficients were statistically very significant (P<0.0001) suggesting that fenticonazole testing with both reference methodologies gives concordant MICs.
Possible susceptibility-associated relatedness of strains and the population structure of\n*C. albicans* and *C. glabrata* isolates from the two geographic regions was studied by\nPCR fingerprinting using the minisatellite specific oligonucleotide [5'-GAGGGTGGCGGTTCT-3'] M13 (23, 35) as described before (15, 34). All Greek\nVVC isolates originated exclusively from Greek Caucasians, whereas Belgian strains\nwere isolated from patients of mixed ethnic origin including African immigrants\nresiding in Belgium.

Each strain was tested on five independent occasions to assure reproducibility of\nresults. Cluster analysis was performed by Bionumerics, version 4 (Bio-Maths,\nBilthoven, Belgium- National Centre for Meningococcal Disease, Athens School of\nPublic Health, Greece) using the Dice Coefficient of similarity and cluster analysis\nwith the unweighted pair-group method with arithmetic averages (UPGMA) using\n1.00% position tolerance and no optimization, to obtain the greatest variation in\nsimilarity.

Discrete non-nosocomial and epidemiologically unrelated *C. albicans* subpopulations\nin the two European regions were identified (Fig. 1). Despite microsatellite\nfingerprinting inference to the contrary, (5, 20) our minisatellite typing did not\nassociate fluconazole resistant *C. albicans* with any particular cluster. Similarly,\nMLST did not significantly ally isolates with specific azole susceptibility profiles to\nparticular clades (26). At a global level MLST analysis of *C. albicans* with different\ngeographical and anatomical origins has shown clades with geographical enrichment\n(32, 33). Also, microsatellite analysis has even separated German from Austrian *C.\nalbicans* clades in Central Europe (14), though with no reference to the ethnic origin\nof the population studied. Our minisatellite assay assembled all strains from Greek
Caucasians in a single group, (Fig. 1) but irrespective of their geographic origin fluconazole-resistant *C. glabrata* isolates grouped in a single cluster (Fig.2).

Association of fluconazole resistant strains with specific clades has been also shown by MLST analysis (9). M13 typing is not MLST equivalent, as each method assays different elements of the genome. However, the acute discrimination of the fluconazole-resistant *C. glabrata* subpopulation among only 11 isolates, adds confidence that M13 typing may be dependably used in discriminating *C. glabrata* fluconazole-resistant strains. Notably, *C. albicans* and *C. glabrata* isolates from pregnant, non-pregnant and diabetic women did not associate with specific clusters.

This study showed excellent agreement between the EUCAST and CLSI methods (97%) in testing fenticonazole against *C. albicans* and *C. glabrata* from uncomplicated VVC and limited *C. albicans* fluconazole resistance. Comparative multilocus typing by PCR fingerprinting has clustered fluconazole-resistant *C. glabrata* isolates in a separate group irrespective of their geographic origin, whereas *C. albicans* isolates clustered in geographically distinct groups with no susceptibility associations. The influence of marker choice (16) and sample size on the *C. albicans* geographic distinction patterns cannot be excluded. However, assuming no deviations from the Hardy-Weinberg principle the observed clustering of VVC strains from Greek Caucasian patients may reflect an *ad hoc* geographically restricted event; nonetheless requiring further investigation.
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The Authors declare no conflicts of interest.
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Molecular identification of unusual pathogenic yeast isolates by large
ribosomal subunit gene sequencing: 2 years of experience at the United


Table 1. Origin of isolates from 260 patients with uncomplicated vulvovaginitis

<table>
<thead>
<tr>
<th>Patients (n)</th>
<th>Mean age</th>
<th>Belgium</th>
<th>Greece</th>
<th>Total No of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C. albicans</td>
<td>C. glabrata</td>
<td>C. albicans</td>
</tr>
<tr>
<td>Pregnant (65)</td>
<td>30</td>
<td>61</td>
<td>4</td>
<td>NI</td>
</tr>
<tr>
<td>Non-pregnant (152)</td>
<td>40</td>
<td>132</td>
<td>2</td>
<td>17</td>
</tr>
<tr>
<td>Diabetic (43)</td>
<td>42</td>
<td>NI</td>
<td>NI</td>
<td>39</td>
</tr>
<tr>
<td>Total No of patients</td>
<td>193</td>
<td>6</td>
<td>56</td>
<td>5</td>
</tr>
</tbody>
</table>

*a Patient group not included*
Table 2. Susceptibilities of 260 vulvovaginal candidiasis (VVC) isolates and quality control (QC) strains by the CLSI M27-A2 and EUCAST broth microdilution methods.

<table>
<thead>
<tr>
<th>Candida isolates (n) and QC strains</th>
<th>Method</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt; range (µg/ml)</th>
<th>GM</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt; range (µg/ml)</th>
<th>GM</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt; range (µg/ml)</th>
<th>GM</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt; range (µg/ml)</th>
<th>GM</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans (249)</td>
<td>CLSI</td>
<td>0.03-0.5</td>
<td>0.07</td>
<td>0.03-0.5</td>
<td>0.14</td>
<td>0.12-16</td>
<td>1.86</td>
<td>0.12-4.0</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>EUCAST</td>
<td>0.03-0.5</td>
<td>0.06</td>
<td>0.03-0.25</td>
<td>0.10</td>
<td>0.12-32</td>
<td>1.84</td>
<td>0.12-4.0</td>
<td>0.54</td>
</tr>
<tr>
<td>C. glabrata (11)</td>
<td>CLSI</td>
<td>0.03-0.5</td>
<td>0.10</td>
<td>0.03-1</td>
<td>0.34</td>
<td>2.0-≥64</td>
<td>7.51</td>
<td>0.5-4.0</td>
<td>1.86</td>
</tr>
<tr>
<td></td>
<td>EUCAST</td>
<td>0.03-0.5</td>
<td>0.10</td>
<td>0.03-0.5</td>
<td>0.28</td>
<td>2.0-≥64</td>
<td>7.51</td>
<td>2.0-8.0</td>
<td>2.13</td>
</tr>
<tr>
<td>C. parapsilosis ATCC 22019</td>
<td>CLSI</td>
<td>0.06-0.25</td>
<td>0.14</td>
<td>0.03-1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.16</td>
<td>2-8</td>
<td>3.73</td>
<td>0.12-0.25</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>EUCAST</td>
<td>0.06-0.12</td>
<td>0.09</td>
<td>0.03-0.25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.13</td>
<td>0.5-4</td>
<td>2.14</td>
<td>0.12-0.5</td>
<td>0.21</td>
</tr>
<tr>
<td>C. krusei ATCC 6258</td>
<td>CLSI</td>
<td>0.12-0.5</td>
<td>0.10</td>
<td>0.06-2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.23</td>
<td>16-64</td>
<td>45.25</td>
<td>0.25-0.5</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>EUCAST</td>
<td>0.03-0.12</td>
<td>0.08</td>
<td>0.06-1&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.20</td>
<td>32-64</td>
<td>45.25</td>
<td>0.12-1</td>
<td>0.26</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results obtained for 20 independent tests.
<sup>b</sup> 1/20 tests (value: 1 µg/ml) out of the observed range: 0.03 - 0.5 µg/ml; <sup>c</sup> No test out of range; <sup>d</sup> 1/20 tests (MIC: 2 µg/ml) out of the observed range: 0.06 - 0.5 µg/ml; <sup>e</sup> 1/20 tests (MIC: 1 µg/ml) out of the observed range: 0.06-0.25 µg/ml.
Table 3. Agreement and Intraclass Correlation Coefficients (ICCs) on log2-transformed data (SPSS, version 10.0; SPSS Inc., Chicago, Ill, USA, 1999) between the CLSI and EUCAST reference methods for azole drugs against vulvovaginitis isolates.

<table>
<thead>
<tr>
<th>Antifungal drugs</th>
<th>Agreement*</th>
<th>ICC</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>itraconazole</td>
<td>84</td>
<td>0.64</td>
<td>&lt;10^-4</td>
</tr>
<tr>
<td>fenticonazole</td>
<td>97</td>
<td>0.88</td>
<td>&lt;10^-4</td>
</tr>
<tr>
<td>fluconazole</td>
<td>90</td>
<td>0.90</td>
<td>&lt;10^-4</td>
</tr>
<tr>
<td>ketoconazole</td>
<td>98</td>
<td>0.88</td>
<td>&lt;10^-4</td>
</tr>
</tbody>
</table>

*A value of 85% was selected to validate the results. Agreement and ICCs calculated from the results obtained for all *C. albicans* and *C. glabrata* isolates.
**Figure 1.** Summary of results on 249 *Candida albicans* vulvovaginal candidiasis (VVC) isolates. Dendrogram representing the three *C. albicans* clusters of VVC isolates. Groups defined by 75% similarity. Of the 194 Belgian isolates 137 (70.6%) grouped in cluster ii and 57 (29.40%) grouped in cluster iii. All 55 Greek isolates grouped in a single cluster i.
Figure 2. *Candida glabrata* clusters. Groups were defined by 86% similarity. Isolates Cg 3, 4, 5, 6, 10 and 11, with fluconazole resistance, clustered in group ii.