Comparison of the Broth Microdilution Method of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) for Testing Itraconazole, Posaconazole, and Voriconazole Against Aspergillus

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ABSTRACT

We compared EUCAST and CLSI antifungal susceptibility testing methods for itraconazole, posaconazole and voriconazole against 245 *Aspergillus*. The essential agreement (EA) between methods was excellent: 100% (itraconazole), 98.4% (posaconazole), and 99.6% (voriconazole) assessing EA at +/- 2 dilutions, and 99.6% (itraconazole), 87.7% (posaconazole) and 96.3% (voriconazole) at +/- 1 dilution.
The triazole antifungals include the mould-active agents itraconazole, posaconazole and voriconazole (2). Each of these agents have good in vitro and in vivo activity against most species of *Aspergillus* (1,9,12,16,17,22,29). Although resistance (R) to triazoles is uncommon, increased R has been noted in several regions of the world since 1999 (11,12,23,25,27,28). These observations suggest that triazole resistance among *Aspergillus* spp. may be more common than acknowledged and that clinical microbiology laboratories should determine the in vitro susceptibility of clinically relevant isolates of *Aspergillus* spp. (9,11,12,17,22,28).

There are two independent standards for broth microdilution (BMD) antifungal susceptibility testing of the triazoles against *Aspergillus*: the Clinical and Laboratory Standards Institute (CLSI) method (5) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) method (7,13,15,24). The two methods are similar in that both use BMD, RPMI 1640 broth, incubation at 35-37°C for 48-hours and a complete (100%) inhibition visual MIC endpoint. They differ in inoculum density (0.4-5 x 10^4 CFU/ml [CLSI] versus 2-5 x 10^5 CFU/ml [EUCAST] ), glucose content of the medium (0.2% [CLSI] and 2.0% [EUCAST] ), and round [CLSI] versus flat-bottom [EUCAST] microdilution wells (15). Whereas numerous studies have shown that the two methods produce similar triazole (fluconazole, posaconazole, and voriconazole) MIC results when testing against *Candida* (3,6,8,20,21), very few such comparisons exist for these methods as applied to *Aspergillus* spp. (4,10). Gomez-Lopez et al. (10) demonstrated that itraconazole MICs obtained by the CLSI method were comparable to those obtained by the EUCAST method when applied to Spanish isolates of *Aspergillus* spp. More recently, Chryssanthou and Cuenca-Estrella (4) determined the susceptibilities of 40 clinical isolates of *Aspergillus* spp. for posaconazole and voriconazole by both CLSI and EUCAST BMD methods. They found that overall the level of essential agreement (EA;
agreement within +/-2 log₂ dilutions) was 92.5% and the intra-class correlation coefficient was >0.9. In an effort to further pursue the harmonization of the CLSI and EUCAST BMD methods for testing the triazoles and Aspergillus spp., we have utilized our 2009 global antifungal surveillance database (18,19) to determine the EA between CLSI and EUCAST MICs for 245 clinical isolates of Aspergillus species tested against itraconazole, posaconazole, and voriconazole. This study represents the most extensive comparison of these two BMD methods for the testing of Aspergillus spp. to date. Given the important role that both methods currently play in antifungal resistance surveillance, it is important to demonstrate the comparability of the results (11,14-17,22,28).

A total of 245 clinical isolates of Aspergillus spp. obtained from 20 medical centers worldwide during 2009 were tested against itraconazole, posaconazole, and voriconazole. The collection included 160 isolates of A. fumigatus, 32 of A. flavus, 40 of A. niger and 13 of miscellaneous species including 8 of A. terreus, 3 of A. versicolor, and 1 each of A. nidulans and A. glaucus. The isolates were obtained from a variety of sources, including sputum, bronchoscopy, and tissue biopsy specimens and represented individual infectious episodes. The isolates were collected at individual study sites and sent to the University of Iowa (Iowa City) for identification and susceptibility testing as described previously (17,18). All isolates were identified by standard microscopic morphology (26) and were stored as spore suspensions in sterile distilled water at room temperature. Before testing, each isolate was subcultured at least twice on potato dextrose agar (Remel, Lenexa, KS) to ensure viability and purity. As a screen for cryptic species within the A. fumigatus complex (e.g. A. lentulus), all A. fumigatus isolates
were tested for growth at 50°C. All isolates screened grew at 50°C, confirming that they were likely to be *A. fumigatus*.

All isolates were tested for in vitro susceptibility to itraconazole, posaconazole, and voriconazole using the CLSI and EUCAST BMD methods. Reference powders of each agent were obtained from their respective manufacturers. Personnel performing the in vitro susceptibility studies were blinded to the results of the CLSI method as compared to the EUCAST method.

CLSI BMD testing was performed exactly as outlined in document M38-A2 (5) by using RPMI 1640 medium with 0.2% glucose, inocula of 0.4 x 10^4 to 5 x 10^4 CFU/ml, and incubation at 35°C for 48-hours. MIC values were determined visually as the lowest concentration of drug that caused complete inhibition of growth (first clear well) relative to that of the growth control.

EUCAST BMD testing was performed exactly as detailed by EUCAST (24) by using RPMI 1640 medium with 2.0% glucose, flat-bottom microdilution trays, inocula of 2 x 10^5 to 5 x 10^5 CFU/ml, and incubation at 35°C. MIC values were determined visually, after 48-hour incubation, as the lowest concentration of drug that resulted in complete growth inhibition.

Quality control was ensured by testing the following strains recommended by CLSI (5) and EUCAST (7): *C. parapsilosis* ATCC22019, *C. krusei* ATCC 6258, and *A. flavus* ATCC 204304.

The MIC results for each triazole obtained with the EUCAST method were compared to those of the CLSI BMD method. High off-scale MIC results were converted to the next highest concentration and low off-scale MIC results were left unchanged. Discrepancies of more than +/- 1 log_2 dilutions and of more than +/- 2 log_2 dilutions among MIC results were used to calculate the EA.
The Table summarizes the in vitro susceptibilities of 245 isolates of *Aspergillus* spp. to itraconazole, posaconazole, and voriconazole as determined by both methods. The MIC results for each agent were typical of those for each species of *Aspergillus* (1, 9, 17, 19, 22, 28).

The overall EA determined as the percentage of results within ± 1 log₂ dilution ranged from 87.7% (posaconazole) to 99.6% (itraconazole) and improved to 98.4% (posaconazole) to 100.0% (itraconazole) when the more standard criteria of ± 2 log₂ dilutions was used (Table).

Of the discrepancies (> +/- 2 log₂ dilutions) noted between the EUCAST and CLSI BMD results, the MIC values generated by the CLSI method were higher than those obtained by the EUCAST in 4 of 5 (80%) instances (4 of 4 with posaconazole and 0 of 1 with voriconazole). The largest number of discrepancies observed with the EUCAST and CLSI comparison occurred with *A. fumigatus* tested against posaconazole (4 discrepant results). Notably, 3 of the later discrepant results would result in isolates of *A. fumigatus* being categorized as wild-type (WT) strains by EUCAST and as non-WT strains by CLSI according to the criteria published by Espinel-Ingroff et al (9).

Regarding the individual species, the EAs between the EUCAST and CLSI BMD MIC results were ≥90% for all organism-drug combinations with the exception of *A. niger* and posaconazole (80.0%) using the +/- 1 log₂ dilution criterion and were ≥97% for all comparisons using the +/- 2 log₂ dilution criterion (Table). Among the 8 discrepancies noted for *A. niger* and posaconazole using the +/- 1 log₂ dilution criterion, only one resulted in what would be considered a very major discrepancy (WT by EUCAST and non-WT by CLSI). The remaining 7 discrepant results would still be categorized as WT by both methods.

These results confirm and extend those of Chryssanthou and Cuenca-Estrella (4) demonstrating that, as with *Candida* spp., susceptibility results obtained by the two methods are...
comparable when testing the triazoles against *Aspergillus* spp. Similar to these investigators, we found a higher level of intermethod agreement with itraconazole and voriconazole than with posaconazole. Both methods may be used with confidence for both clinical testing and in antifungal resistance surveillance. One limitation of this study is that for most of the agents and species, the range of MICs is quite narrow reflecting the fact that triazole-resistance is uncommon in clinical isolates of *Aspergillus* from most geographic regions. Thus, this study does not address how the methods would compare in their ability to detect isolates with elevated MICs. Further evaluation is warranted using a multicenter study design.
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REFERENCES


evolution of azole resistance in *Aspergillus fumigatus* associated with treatment failure.


Table. In vitro susceptibilities of *Aspergillus* spp. isolates to itraconazole, posaconazole, and voriconazole as determined by the CLSI and EUCAST broth microdilution methods

<table>
<thead>
<tr>
<th>Species (no. tested)</th>
<th>Antifungal agent</th>
<th>Test method</th>
<th>MIC (µg/ml)</th>
<th>% Essential agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Range Mode</td>
<td>+/-1 dil b</td>
<td>+/-2 dil b</td>
</tr>
<tr>
<td><em>A. fumigatus</em> (160)</td>
<td>Itraconazole</td>
<td>EUCAST 0.25-&gt;8 1</td>
<td>99.4 100.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CLSI</td>
<td>0.5-&gt;8 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Posaconazole</td>
<td>EUCAST 0.06-1 0.5</td>
<td>90.0 97.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CLSI</td>
<td>0.25-2 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Voriconazole</td>
<td>EUCAST 0.25-2 0.5</td>
<td>95.6 100.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CLSI</td>
<td>0.12-1 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. flavus</em> (32)</td>
<td>Itraconazole</td>
<td>EUCAST 0.25-2 0.5</td>
<td>100.0 100.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CLSI</td>
<td>0.5-1 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Posaconazole</td>
<td>EUCAST 0.25-1 0.25</td>
<td>90.6 100.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CLSI</td>
<td>0.25-1 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Voriconazole</td>
<td>EUCAST 0.5-2 1</td>
<td>96.9 100.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CLSI</td>
<td>0.25-1 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. niger</em> (40)</td>
<td>Itraconazole</td>
<td>EUCAST 0.25-4 2</td>
<td>100.0 100.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CLSI</td>
<td>0.5-8 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Posaconazole</td>
<td>EUCAST 0.12-1 1</td>
<td>80.0 100.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CLSI</td>
<td>0.25-2 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Voriconazole</td>
<td>EUCAST 0.25-2 1</td>
<td>100.0 100.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CLSI</td>
<td>0.25-2 0.5</td>
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</tr>
<tr>
<td>Total (245)</td>
<td>Itraconazole</td>
<td>EUCAST 0.25-&gt;8 1</td>
<td>99.6 100.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CLSI</td>
<td>0.5-&gt;8 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Posaconazole</td>
<td>EUCAST 0.06-4 0.5</td>
<td>87.7 98.4</td>
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<tr>
<td></td>
<td>CLSI</td>
<td>0.25-&gt;8 0.5</td>
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<tr>
<td>Drug</td>
<td>EUCAST (log2)</td>
<td>CLSI (log2)</td>
<td>EUCAST %</td>
<td>CLSI %</td>
</tr>
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<td>--------------</td>
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<td>-------</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>0.12-4</td>
<td>0.12-8</td>
<td>96.3</td>
<td>99.6</td>
</tr>
</tbody>
</table>

EUCAST, European Committee on Antimicrobial Susceptibility Testing; CLSI, Clinical and Laboratory Standards Institute.

\(^{a}\) +/- 1 dil, % of results within plus/minus 1 log₂ dilution of one another; +/- 2 dil, % of results within plus/minus 2 log₂ dilutions of one another.

\(^{b}\) In addition to listed species, the total number of isolates tested included *A. terreus* (8 isolates), *A. versicolor* (3 isolates), *A. nidulans* (1 isolate), and *A. glaucus* (1 isolate).