In vitro synergistic combinations of pentamidine, polymyxin B, tigecycline and tobramycin with antifungal agents against Fusarium spp.

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The genus Fusarium is characterized by hyaline filamentous fungi that cause infections predominantly in immunocompromised patients. The remarkable primary resistance to antifungal agents of this genus requires a search for new therapeutic possibilities. This study assessed the in vitro susceptibility of 25 clinical isolates of Fusarium against antifungal agents (amphotericin B, caspofungin, itraconazole and voriconazole) and antimicrobials (pentamidine, polymyxin B, tigecycline and tobramycin) according to the broth microdilution method (M38-A2). The interactions between antifungal and antimicrobial agents were evaluated by the microdilution checkerboard method. Pentamidine and polymyxin B showed MIC values ≥4 µg ml⁻¹ against Fusarium spp. The highest rates of synergism were observed when amphotericin B or voriconazole was combined with tobramycin (80 % and 76 %, respectively), polymyxin B (76 % and 64 %) and pentamidine (72 % and 68 %). The most significant combinations deserve in vivo evaluations in order to verify their potential in the treatment of fusariosis.

INTRODUCTION

Fusarium is an emerging opportunistic fungal pathogen, with a significant increase in cases among immunocompromised patients (Muhammed et al., 2011). Invasive fusariosis is the second most common form of mould mycosis after invasive aspergillosis and primarily affects patients with haematological malignancies, transplant recipients and those with prolonged neutropenia (Muhammed et al., 2011; Nucci & Anaissie, 2007). Furthermore, this infection has high mortality rates (>80 %) (Torres & Kontoyiannis, 2003). The recommended treatment is amphotericin B (AMB) or voriconazole (VRC). Posaconazole is indicated for refractory cases (Tortorano et al., 2014). Nonetheless, the usage of monotherapy for treatment of systemic fusariosis is unsatisfactory owing to high rates of resistance against antifungal agents (Nucci & Anaissie, 2007). In this regard, combination therapies have been designed in an attempt to overcome antifungal resistance (Johnson et al., 2004). Combinations of antifungal and antibacterial agents have been explored for more prevalent fungi, such as Candida spp., Cryptococcus spp. and Aspergillus spp.; however, such studies are still limited with respect to therapies against Fusarium spp. (Nucci & Anaissie, 2007).

Therefore, the aim of this study was to evaluate in vitro combinations of antimicrobial agents [pentamidine (PNT), polymyxin B (PMB), tigecycline (TGC) or tobramycin (TOB)] with antifungal agents [AMB, caspofungin (CAS), itraconazole (ITC) or VRC] against Fusarium spp. The antimicrobial agents were selected according to previous reports of synergism when such drugs were combined with antifungal agents against other fungal genera and the oomycete Pythium insidiosum (Afeltra et al., 2002; Chodosh et al., 2002).
Different letters indicate significant difference between species (P<0.05).

**RESULTS AND DISCUSSION**

In vitro susceptibility of the 25 *Fusarium* isolates to the antifungal and antimicrobial agents is shown in Table 1. AMB showed greater activity against *Fusarium* spp. compared to the other antifungals. The document M38-A2 (CLSI, 2008) reports the MIC or MEC breakpoints for in vitro susceptibility testing of mould species against AMB, CAS, ITC and VRC. Based on these findings, isolates are classified as susceptible (MIC or MEC, ≤1 µg ml⁻¹), intermediate (MIC or MEC, 2 µg ml⁻¹) or resistant (MIC or MEC, ≥4 µg ml⁻¹).

The susceptibility to AMB showed MIC ≤1 µg ml⁻¹ for 28 % of isolates, MIC of 2 µg ml⁻¹ for 28 % and MIC ≥4 µg ml⁻¹ for 44 % of the isolates. With VRC, it was observed that 4 % of the isolates showed MIC ≤1 µg ml⁻¹, 8 %

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**METHODS**

*Fusarium isolates.* Twenty-five clinical isolates of *Fusarium* spp. were tested, including (number of isolates) *Fusarium chlamydosporum* (3), *Fusarium oxysporum* (5), *Fusarium proliferatum* (3), *Fusarium solani* (10), *Fusarium verticillioides* (2) and two reference strains, *F. solani* ATCC 36031 and *Fusarium sporotrichioides* CBS 41286. The strains were identified by amplifying a DNA fragment encompassing the internal transcribed spacer (ITS) region with the primers ITS1 (5'-GTAGTCATTATCCTGTGCT-3') and ITS4 (5'-CTTCGTAATTCCCTTTAAG-3') (White et al., 1990). The amplified fragments were analysed on 0.8 % agarose gel, followed by purification and sequencing. These sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

**Drugs.** The drugs AMB (Sigma-Aldrich), ITC (Francon of Brazil, Pharmaceutical), VRC (Pfizer) and TGC (Pfizer) were diluted in DMSO to create stock solutions. CAS (Merck), PNT (Sigma-Aldrich), PMB (Sigma-Aldrich) and TOB (Sigma-Aldrich) were diluted in sterile distilled water to prepare stock solutions. The tested concentration (µg ml⁻¹) ranged from 0.06 to 8 for AMB; 0.25 to 32 for CAS and PNT; 0.125 to 16 for ITC, VRC, PMB and TGC; and 0.5 to 64 for TOB.

**In vitro susceptibility and drug interaction tests.** The MICs and minimal effective concentrations (MECs) were determined using the broth microdilution technique in RPMI 1640 medium according to the protocol outlined in document M38-A2 (CLSI, 2008). For all the drugs except for CAS, visual reading of the MICs was performed when there was 100 % growth inhibition compared to positive control following 48 h incubation at 35 °C. For CAS, the readings were determined in MECs, i.e. the minimal drug concentrations producing small, stubby, highly branched hyphae (CLSI, 2008). *Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6258 and *Aspergillus flavus* ATCC 204304 were used as quality control strains. The effects of interactions between antifungal and antimicrobial drugs were evaluated using the microdilution checkerboard method by determination of the fractional inhibitory concentration index (FICI) (Moody, 2007). The concentrations of antifungal and antimicrobial agents used in combination tests were obtained from the single-agent susceptibility testing performed previously. The inoculum was prepared according to M38-A2 (CLSI, 2008). To interpret the synergism between tested drugs, we used the lowest FICI from all non-turbid wells along the turbidity/non-turbidity interface after 48 h incubation at 35 °C (Moody, 2007). Interpretations for FICI values were defined as follows: ≤0.5 synergism, >0.5 and ≤4 indifference and >4 antagonism (Moody, 2007).

**Statistical analysis.** Data from MICs and FICIs for different species were analysed by the Kruskal–Wallis non-parametric test. The software used was SigmaPlot version 12.5. Significant differences were considered at P<0.05.

**Table 1. In vitro susceptibility of *Fusarium* spp. to antifungal and antimicrobial agents**

<table>
<thead>
<tr>
<th><em>Fusarium</em> spp. (n)</th>
<th>Antimicrobial agents</th>
<th>Antifungal agents</th>
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<tbody>
<tr>
<td></td>
<td>MIC range (µg ml⁻¹)</td>
<td>(GM) MIC &lt;sup&gt;1&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>PNT</td>
<td>PMB</td>
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<tr>
<td><em>F. chlamydosporum</em> (3)</td>
<td>4</td>
<td>4&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td><em>F. oxysporum</em> (5)</td>
<td>8 (1.8)</td>
<td>8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>F. proliferatum</em> (3)</td>
<td>8 (1.8)</td>
<td>8&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td><em>F. solani</em> (11)</td>
<td>16–32 (24.9)</td>
<td>32&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>F. sporotrichioides</em> (1)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16</td>
<td>16</td>
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<tr>
<td><em>F. verticillioides</em> (2)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16 (16)</td>
<td>16</td>
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<tr>
<td>Total (25)</td>
<td>4–32 (12.5)</td>
<td>32</td>
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</tbody>
</table>

PNT, pentamidine; PMB, polymyxin B; TGC, tigecycline; TOB, tobramycin; AMB, amphotericin B; ITC, itraconazole; VRC, voriconazole; CAS, caspofungin; MEC, minimum effective concentration; GM, geometric mean.

Different letters indicate significant difference between species (P<0.05).

<sup>a</sup>These species were not used for statistical analysis.

http://jmm.microbiologyresearch.org
showed MIC of 2 µg ml\(^{-1}\) and 88 % exhibited MIC $\geq 4$ µg ml\(^{-1}\). ITC and CAS exhibited no activity to 100 % of the isolates (Table 1). These findings confirmed the previously described resistance profile of Fusarium towards antifungal agents (Muhammed et al., 2011; Nucci & Anaissie, 2007).

There are no MIC breakpoints for in vitro susceptibility testing of fungi against antifungal agents. Lionakis et al. (2003) evaluated the activity of PNT against Fusarium spp. and reported similar MIC ranges: 8–32 µg ml\(^{-1}\) for F. solani and 4–8 µg ml\(^{-1}\) for non-\(F.\) solani. This drug reached plasma concentrations from 0.5 to 3.2 µg ml\(^{-1}\), but concentrations in peripheral tissues were higher: 56 µg g\(^{-1}\) in lungs, 123 µg g\(^{-1}\) in kidneys, 300 µg g\(^{-1}\) in the liver and 368 µg g\(^{-1}\) in the spleen (Bernard et al., 1985). We report MICs for PNT, which are lower than the tissue concentration of the drug. The MIC range observed for PMB (4–16 µg ml\(^{-1}\)) was also compatible with its serum concentrations (6.25–50 µg ml\(^{-1}\)) (Sarria et al., 2004). Other authors have reported in vitro activity of PMB to Cryptococcus neoformans (8 µg ml\(^{-1}\)), Candida spp. (32–256 µg ml\(^{-1}\)) and Rhizopus oryzae (32 µg ml\(^{-1}\)) (Zhai et al., 2010). However, this activity has not been described for Fusarium spp.

TOB and TGC did not show activity in the concentration range tested. Some authors have reported potent activity of TGC against biofilms of \(C.\) albicans (128 µg ml\(^{-1}\)) (Ku et al., 2010) and \(P.\) insidiosum (0.25–4 µg ml\(^{-1}\)) (Jesus et al., 2014). Against Fusarium spp, TOB was classified by others as being active, showing MICs of $\approx 500$ µg ml\(^{-1}\) (Chodosh et al., 2000; Day et al., 2009). In this study, we tested lower concentrations of this drug, i.e. closer to the plasma concentrations (Beringer et al., 2000; Rodvold et al., 2006), and no antifungal activity was detected.

When MICs were compared among the Fusarium spp., the statistical analysis showed significant differences ($P<0.05$) between \(F.\) chlamydosporum and \(F.\) solani to AMB and PNT. For confirmation of \(Fusarium\) spp., it has been suggested to sequence for the gene coding for the elongation factor \(\alpha\) (O’Donnell et al., 2010). According to recent studies (Al-Hatmi et al., 2016; Espinel-Ingroff et al., 2016), identification of Fusarium spp. is important because there are differences in relation to the antifungal susceptibility between different species. We used the ITS region for identification of the strains. However, the ITS region does not have the required discriminatory power to accurately identify Fusarium isolates at the species level. Therefore, this question is a limitation of this study. All combinations of antifungals with AMB or VRC resulted in synergistic interactions. The most synergistic combinations were (percentage of synergisms): AMB+TOB (80 %), VRC+TOB (76 %), AMB+PMB (76 %), VRC+PMB (64 %), AMB+PNT (72 %) and VRC+PNT (68 %). Combinations with CAS demonstrated the lowest rates of synergism. All combinations with ITC resulted in indifferent interactions for 100 % of the isolates. Antagonistic interactions were not observed for any of the combinations (Table 2). The concentrations of antifungal agents in synergistic combinations showed the following ranges: 0.25–8 µg ml\(^{-1}\) for PNT, 0.25–4 µg ml\(^{-1}\) for PMB, 2–8 µg ml\(^{-1}\) for TGC and 4–32 µg ml\(^{-1}\) for TOB. In the combinations studied, the MICs of the antifungal agents were consistent with the clinically achievable serum or tissue concentrations (Beringer et al., 2000; Bernard et al., 1985; Rodvold et al., 2006; Sarria et al., 2004).

The combinations with PNT showed strong synergism with AMB and VRC. Other studies also demonstrated synergism when PNT was combined with ketoconazole or AMB against \(Candida\) albicans (St-Germain, 1990) or \(Scedosporium\) prolificans (Afeltra et al., 2002). Several mechanisms of action have been proposed, such as inhibition of DNA, RNA, phospholipids and protein synthesis (Sands et al., 1985). Synergistic relations were also observed for AMB+TGC and VRC+TGC. TGC is a glycyclycin with a structure similar to that of tetracyclines, and acts to inhibit protein synthesis (Pankey, 2005). Synergistic interactions were reported for AMB+TGC and azoles+TGC against \(P.\) insidiosum (Jesus et al., 2014). The synergism that we observed in AMB or VRC combined with PNT or TGC can be explained by the same mechanism that underlies the synergism between AMB and azithromycin, i.e. fungal cell membrane damage by the antifungal agent, allowing the entrance of antibacterial agents and thus leading to inhibition of protein synthesis (Clancy & Nguyen, 1998).

PMB also showed synergistic interactions with AMB and VRC. This antifungal agent is a cationic heptapeptide with a hydrophobic tail and has bactericidal activity (Zhai et al., 2010). Based on the activity of PMB against bacteria, Zhai et al. (2010) hypothesized that its antifungal effect is mediated by its attachment to anionic lipids of the fungal membrane, thus facilitating membrane rupture. The presence of sterols in the eukaryotic membrane inhibits the insertion of cationic peptides into anionic structures on the fungal membrane, minimizing the formation of pores (Mason et al., 2007). Because of the previous ergosterol reduction promoted by azoles, the action of PMB potentiates antifungal activity (Zhai et al., 2010). We hypothesize that PMB could also act in a similar manner when combined with AMB, as it can also interact with the ergosterol membrane.

Furthermore, TOB demonstrated potent antifungal activity when combined with AMB or VRC. A patient with \(F.\) oxysporum-induced keratitis showed clinical improvement after treatment with TOB and topical natamycin (Chodosh et al., 2000). According to Dalhoff (1987), aminoglycosides can increase permeability of the cell wall and cell membrane of fungi. This observation may explain the synergistic interactions observed in our study.

Our results suggest that combinations of AMB or VRC with the antimicrobial agents tested here warrant further studies in vivo. Our in vitro results are a preview of the effects of antifungal and antimicrobial drugs when used in combination against a pathogen. Although in vitro studies have limitations, they are necessary for the development of valid hypotheses regarding new treatments. The antimicrobials tested here are frequently used in medical practice because
<table>
<thead>
<tr>
<th>Fusarium spp. (n)</th>
<th>Antifungal agents</th>
<th>PNT</th>
<th>PMB</th>
<th>TGC</th>
<th>TOB</th>
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<tr>
<td></td>
<td>%S %I</td>
<td>%S %I</td>
<td>%S %I</td>
<td>%S %I</td>
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<tr>
<td>AMB</td>
<td>FICI range (GM)</td>
<td>FICI range (GM)</td>
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<tr>
<td>F. chlamydosporum (3)</td>
<td>AMB 0.31 (0.28)</td>
<td>0.24–0.31 (0.28)</td>
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<td></td>
<td>AMB 0.37 (0.30)</td>
<td>0.12–0.75 (0.36)</td>
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<td></td>
<td>AMB 0.75 (0.35)</td>
<td>0.37–1.25 (0.77)</td>
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<tr>
<td>F. oxysporum (5)</td>
<td>AMB 0.28–1.25 (0.48)</td>
<td>0.24–0.75 (0.36)</td>
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<tr>
<td></td>
<td>AMB 0.37–1.00 (0.64)</td>
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<tr>
<td>F. proliferatum (3)</td>
<td>AMB 66.7 33.3</td>
<td>0.28–0.53 (0.38)</td>
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<td></td>
<td>AMB 66.7 33.3</td>
<td>0.12–0.75 (0.36)</td>
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<td>F. solani (11)</td>
<td>AMB 54.5 45.5</td>
<td>0.15–1.00 (0.46)</td>
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<td></td>
<td>AMB 63.6 34.6</td>
<td>0.15–1.75 (0.38)</td>
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<tr>
<td>F. sporotrichioides (1)</td>
<td>AMB 100 0.18</td>
<td>0.12–0.75 (0.30)</td>
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<tr>
<td>F. verticillioides (2)</td>
<td>AMB 100 0.27–0.50 (0.37)</td>
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<tr>
<td>Total (25)</td>
<td>AMB 72 28</td>
<td>0.15–1.25 (0.41)</td>
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</table>

| Fusarium spp. (n) | Antifungal agents | AMB, amphotericin B; CAS, caspofungin; ITC, itraconazole; VRC, voriconazole; PNT, pentamidine; PMB, polymyxin B; TGC, tigecycline; TOB, tobramycin; %S, percentage of synergisms; %I, percentage of indifference; FICI range, fractional inhibitory concentration index range; GM, geometric mean. No significant difference between species was observed (P>0.05).
they have a good record of clinical safety. Therefore, these are promising candidates for new studies on combination treatments for fusariosis.

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REFERENCES


