In Vitro Synergism Observed with Azithromycin, Clarithromycin, Minocycline, or Tigecycline in Association with Antifungal Agents against *Pythium insidiosum*

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We describe here the *in vitro* activities of azithromycin, clarithromycin, minocycline, or tigecycline alone and in combination with amphotericin B, itraconazole, terbinafine, voriconazole, anidulafungin, caspofungin, or micafungin against 30 isolates of the oomycete *Pythium insidiosum*. The assays were based on the CLSI M38-A2 technique and the checkerboard microdilution method. The main synergisms observed were through the combination of minocycline with amphotericin B (73.33%), itraconazole (70%), and micafungin (70%) and of clarithromycin with micafungin (73.33%).

Pythiosis is a life-threatening disease caused by the oomycete *Pythium insidiosum*, which can cause infections in humans and in animals, such as horses, bovines, cats, dogs, and sheep. Clinically, the disease may manifest in cutaneous, gastrointestinal, vascular, and systemic forms and has been described in tropical and subtropical areas (1). The hyphae of *P. insidiosum* are morphologically similar to those of certain mucoraceous molds, but *P. insidiosum* is not a true fungus because it does not synthesize ergosterol, which is the target of most antifungal drugs. Despite this challenge, two cases of pythiosis in humans, one case of ocular pythiosis and one case of pleuropericarditis (2, 3), have been successfully treated using combination antifungal therapy. However, combination antifungal therapy has been ineffective in cases of vascular and disseminated human pythiosis (4).

Previous studies have shown that the growth of *P. insidiosum* is inhibited *in vitro* by the glycylcycline, macrolide, and tetracycline classes of antibacterial drugs (5, 6). However, studies evaluating the antimicrobial combination of antibacterial and antifungal agents against *P. insidiosum* have not been performed. In this context, this study evaluated the *in vitro* combination of the antibacterial drugs azithromycin, clarithromycin, minocycline, or tigecycline with the antifungal drugs amphotericin B, itraconazole, voriconazole, terbinafine, anidulafungin, caspofungin, or micafungin against *P. insidiosum*.

Twenty-eight *P. insidiosum* isolates obtained from Brazilian cases of equine pythiosis and the reference strains ATCC 58.637 and CBS 101555 were evaluated in this study. The identities of the clinical isolates were confirmed using PCR-based assays (7). The antibacterial drugs azithromycin (Pharma Nostra, Rio de Janeiro, Brazil), clarithromycin (Genix, Anápolis, Brazil), minocycline (Pharma Nostra), and tigecycline (Pfizer, New York, NY) and the antifungal drugs amphotericin B (Sigma-Aldrich, St. Louis, MO), itraconazole (Frangon do Brasil Farmacêutica Ltda., São Paulo, Brazil), voriconazole (Pfizer), terbinafine (Pharma Nostra), anidulafungin (Pfizer), caspofungin (Merck, Darmstadt, Germany), and micafungin (Astellas, Chuo, Japan) were obtained commercially and diluted in dimethyl sulfoxide or distilled water, as recommended, to generate stock solutions. The concentrations of the antimicrobial agents tested were 0.03 to 16 μg/ml and 1 to 512 μg/ml for the antibacterial and antifungal drugs, respectively. The MICs and minimal effective concentrations (MECs) were determined following the Clinical and Laboratory Standards Institute (CLSI) M38-A2 guidelines (8), as adapted by Pereira et al. (9). The MICs were determined by visual observation and represent the inhibition of 100% of mycelium growth after 24 h of incubation at 37°C. The MEC endpoints for anidulafungin, caspofungin, and micafungin were defined as the lowest drug concentrations at which short, stubby, highly branched hyphae were observed after 24 h.

The interactions between the antibacterial and antifungal agents against the 30 strains were evaluated using the microdilution checkerboard method. The interpretation of the synergy testing results was determined as the lowest fractional inhibitory concentration index (FICI) of all of the nonturbid wells along the turbidity/nonturbidity interface (10) after 24 h of incubation at 37°C. FICI values were interpreted as follows: an FICI of ≤0.5, synergism; an FICI of >0.5 to ≤4, indifference; and an FICI of >4, antagonism. The tests were performed in duplicate on different days. Off-scale MICs were converted to the next higher dilution for calculation purposes.

The *in vitro* susceptibilities of the 30 *P. insidiosum* isolates are listed in Table 1. The tested antibacterial drugs were considered the most effective drugs because they required the lowest concentrations for *in vitro* inhibition of *P. insidiosum*, with MIC (geometric mean [GM]) values in μg/ml ranging from 0.25 to 4 (0.91 and 0.79, respectively) for minocycline and tigecycline, 0.125 to 8 (1.91) for azithromycin, and 0.25 to 8 (1.38) for clarithromycin. The MIC (GM) values in μg/ml for the antifungal drugs ranged from 8 to 128 (34.3) for amphotericin B, 32 to 256 (94.79) for

TABLE 1 *In vitro* activities of selected antibacterial and antifungal drugs against 30 *Pythium insidiosum* isolates

Agent	Activity (μg/ml)[a]			
	MIC range (GM)	MIC$_{50}$	MIC$_{90}$	MEC range (GM)
Antibacterials				
Azithromycin	0.125–8 (1.91)	2	8	
Clarithromycin	0.25–8 (1.38)	2	4	
Minocycline	0.25–4 (0.91)	1	4	
Tigecycline	0.25–4 (0.79)	0.5	4	
Antifungals				
Amphotericin B	8–128 (34.3)	32	128	
Anidulafungin	512 to >512 (1,000.61)	>512	>512	256 to >512 (851.18)
Caspofungin	32–256 (94.79)	128	128	8–32 (20.63)
Itraconazole	256 to >512 (707.53)	>512	>512	
Micafungin	256 to >512 (776.04)	>512	>512	32–128 (64)
Terbinafine	4–128 (25.6)	32	64	
Voriconazole	256 to >512 (871.08)	>512	>512	

[a] GM, geometric mean; MEC, minimal effective concentration.

caspofungin, 4 to 128 (25.6) for terbinafine, 256 to >512 (707.53) for itraconazole, 512 to >512 (1,000.61) for anidulafungin, 256 to >512 for micafungin (776.04), and 256 to >512 (871.08) for voriconazole. The MEC (GM) values in μg/ml for the echinocandins were 256 to >512 (851.18) for anidulafungin, 8 to 32 (20.63) for caspofungin, and 32 to 128 (64) for micafungin.

The highest synergistic interactions based on the MIC values were observed for the combinations of clarithromycin and micafungin (73.33%), minocycline and amphotericin B (73.33%), minocycline and micafungin (70%), minocycline and itraconazole (70%), minocycline and terbinafine (66.67%), tigecycline and micafungin (66.67%), clarithromycin and amphotericin B (63.33%), clarithromycin and terbinafine (63.33%), clarithromycin and caspofungin (60%), minocycline and voriconazole (60%), and tigecycline and terbinafine (60%). The other combinations produced synergistic interactions ranging from 43.33% to 53.34%. The highest synergistic interactions based on the MEC values were observed for tigecycline and micafungin (73.33%), clarithromycin and micafungin (70%), azithromycin and micafungin (66.67%), and minocycline and micafungin (63.33%). Synergistic interactions ranging from 43.33% to 53.34% were observed for the other combinations.

At least one synergistic interaction was observed in combinations of minocycline or clarithromycin with the antifungal agents. Indifference in all the combinations of tigecycline or azithromycin with the antifungal drugs was observed in 20% and 13.3% of the isolates, respectively. Antagonistic interactions (MIC and MEC) were observed when azithromycin was combined with anidulafungin, amphotericin B, caspofungin, or itraconazole (3.33% each), clarithromycin-anidulafungin (3.33%), clarithromycin-caspofungin (3.33%), tigecycline-anidulafungin (3.33%), clarithromycin-itraconazole (6.67%), and minocycline-caspofungin (6.67%) (Table 2).

This study demonstrated that, individually, the antifungal drugs have weak or no *in vitro* antimicrobial activities compared with the clear *in vitro* inhibition against *P. insidiosum* by the selected antibacterial agents, for which the observed MIC GMs were <2 μg/ml. Interestingly, considering a general review of the pharmacology of the antimicrobials in this study (11), we observed that the MIC/MEC GMs of the combined antimicrobials are compat-

ible with the plasma and tissue concentrations achieved by these drugs (Table 2).

The results of the effects of the single drugs found in this study are similar to those of previous studies that evaluated the susceptibility of *P. insidiosum* to antifungal (9, 12–15) and antibacterial (5, 6) drugs. The combination of antifungal agents against *P. insidiosum* showed the highest synergisms between terbinafine and amphotericin B (41.18% [14]), terbinafine and caspofungin (41.2% to 46.7% [12, 13]), terbinafine and fluconazole (41.2%), terbinafine and ketoconazole (29.4%), terbinafine and miconazole (11.8%) (13), terbinafine and itraconazole (17% to 40% [12, 15]), and terbinafine and voriconazole (17% [15]). Interestingly, these findings demonstrated that the combination of antifungal and antibacterial agents produces higher synergistic interactions than either produces alone, varying from 46.67% to 73.33% (minocycline plus antifungals) to 33.33% to 66.67% (tigecycline plus antifungals), 30% to 53.33% (azithromycin plus antifungals), and 43.33% to 73.33% (clarithromycin plus antifungals).

The favorable *in vitro* interactions of antibacterial and antifungal drugs against fungi have been observed since the 1970s, particularly with the synergism observed between amphotericin B with tetracycline or minocycline against *Candida* spp., *Cryptococcus neoformans*, and *Saccharomyces cerevisiae* (16, 17). Since then, several studies demonstrated the *in vitro* synergisms between amphotericin B or fluconazole with azithromycin, clarithromycin, doxycycline, minocycline, or tetracycline against *Aspergillus* spp., *Candida* spp., and *Fusarium* spp. (18–23), as well as other combinations of antibacterial and antifungal agents against pathogenic fungi, as reviewed by Afeltra and Verweij (24) and Liu et al. (25).

Despite these favorable *in vitro* interactions, there are few or no *in vivo* or clinical data that support the use of such associations as the therapy of choice in the treatment of most fungal infections (24, 25). The different methods of interpreting the *in vitro* interactions between drugs (26) and the contradictory results observed in *in vitro* and *in vivo* correlations (27–29) may contribute to the divergent results, indicating the need for standardization of the methods used to evaluate the drug interactions.

Given that *Pythium* species are unable to synthesize their own sterols, which are essential for their reproduction (30), they must take up sterols from their plant or animal hosts. As already de-

TABLE 2 *In vitro* combinations of MIN, TIG, AZT, or CLT with AMB, ITZ, VRZ, TRB, AND, CSF, or MCF against 30 *Pythium insidiosum* isolates[a]

Drug combination	MIC[b] Range (GM) (µg/ml) ATB	MIC[b] Range (GM) (µg/ml) ATF	MIC[b] FICI range (GM) (µg/ml)	MIC[b] Interpretation (%) S	I	An	MEC[c] Range (GM) (µg/ml) ATB	MEC[c] Range (GM) (µg/ml) ATF	MEC[c] FICI range (GM) (µg/ml)	MEC[c] Interpretation (%) S	I	An
MIN and TRB	0.06–0.5 (0.14)	0.25–32 (0.72)	0.03–2.18 (0.23)	66.67	33.33	0.00	NA[d]	NA	NA	NA	NA	NA
MIN and AMB	0.03–1 (0.11)	0.5–16 (1.05)	0.03–2.5 (0.27)	73.33	26.67	0.00	NA	NA	NA	NA	NA	NA
MIN and ITZ	0.03–1 (0.17)	0.125–2 (0.35)	0.02–2.01 (0.20)	70.00	30.00	0.00	NA	NA	NA	NA	NA	NA
MIN and VRZ	0.125–2 (0.20)	0.5–256 (3.10)	0.05–1.5 (0.26)	60.00	40.00	0.00	NA	NA	NA	NA	NA	NA
MIN and MCF	0.06–0.5 (0.19)	0.5–64 (2.96)	0.05–1.13 (0.23)	70.00	30.00	0.00	0.03–0.5 (0.16)	0.5–64 (3.32)	0.08–1.13 (0.30)	63.33	36.67	0.00
MIN and CSF	0.06–0.5 (0.21)	0.5–8 (2.14)	0.08–2.04 (0.46)	46.67	53.33	0.00	0.06–0.5 (0.19)	0.5–8 (2.24)	0.13–4.81 (0.65)	46.67	46.66	6.67
MIN and AND	0.06–1 (0.23)	0.5–64 (3.32)	0.05–1.25 (0.28)	53.33	46.67	0.00	0.06–1 (0.22)	0.5–64 (3.82)	0.04–2.29 (0.33)	43.33	56.67	0.00
TIG and TRB	0.03–0.5 (0.15)	0.5–32 (0.74)	0.05–2.02 (0.27)	60.00	40.00	0.00	NA	NA	NA	NA	NA	NA
TIG and AMB	0.03–1 (0.15)	0.5–16 (0.85)	0.04–2.16 (0.32)	56.67	43.33	0.00	NA	NA	NA	NA	NA	NA
TIG and ITZ	0.06–0.5 (0.26)	0.25 (0.25)	0.02–2.01 (0.31)	46.67	53.33	0.00	NA	NA	NA	NA	NA	NA
TIG and VRZ	0.03–1 (0.31)	2–256 (2.35)	0.04–2.01 (0.40)	40.00	60.00	0.00	NA	NA	NA	NA	NA	NA
TIG and MCF	0.03–0.25 (0.15)	2–32 (2.30)	0.02–1.01 (0.20)	66.67	33.33	0.00	0.03–0.25 (0.11)	2–32 (2.46)	0.05–1.06 (0.21)	73.33	26.67	0.00
TIG and CSF	0.03–0.5 (0.19)	0.5–64 (2.35)	0.02–2.68 (0.45)	33.33	66.67	0.00	0.03–0.25 (0.12)	0.5–16 (2.09)	0.05–3.42 (0.53)	46.67	53.33	0.00
TIG and AND	0.03–0.5 (0.29)	2 (2)	0.02–2.01 (0.39)	40.00	60.00	0.00	0.03–0.5 (0.29)	0.03–2 (1.74)	0.02–4.01 (0.41)	43.34	53.33	3.33
AZT and TRB	0.125–4 (0.59)	0.5–32 (0.72)	0.05–2.16 (0.49)	33.33	66.67	0.00	NA	NA	NA	NA	NA	NA
AZT and AMB	0.03–4 (0.37)	0.5–32 (0.95)	0.05–4.13 (0.45)	40.00	56.67	3.33	NA	NA	NA	NA	NA	NA
AZT and ITZ	0.03–4 (0.71)	0.25–32 (0.42)	0.02–8.01 (0.53)	30.00	66.67	3.33	NA	NA	NA	NA	NA	NA
AZT and VRZ	0.125–4 (0.55)	2 (2)	0.02–2.01 (0.31)	53.33	46.67	0.00	NA	NA	NA	NA	NA	NA
AZT and MCF	0.03–2 (0.28)	2–128 (3.32)	0.02–2.01 (0.24)	53.33	46.67	0.00	0.03–1 (0.20)	2–64 (2.24)	0.05–1.81 (0.25)	66.67	33.33	0.00
AZT and CSF	0.03–4 (0.56)	0.5–64 (2.14)	0.02–2.03 (0.38)	50.00	50.00	0.00	0.03–1 (0.28)	0.5–32 (1.82)	0.09–8.07 (0.57)	43.34	53.33	3.33
AZT and AND	0.06–4 (0.76)	0.03–256 (2.7)	0.04–8.01 (0.48)	36.67	60.00	3.33	0.03–2 (0.64)	0.5–2.56 (2.24)	0.04–8.01 (0.41)	43.34	53.33	3.33
CLT and TRB	0.03–2 (0.20)	0.03–16 (0.66)	0.03–3.29 (0.24)	63.33	36.67	0.00	NA	NA	NA	NA	NA	NA
CLT and AMB	0.03–2 (0.16)	0.5–64 (0.89)	0.03–2.05 (0.24)	63.33	36.67	0.00	NA	NA	NA	NA	NA	NA
CLT and ITZ	0.125–4 (0.56)	0.25 (0.25)	0.04–2.01 (0.42)	43.33	50.00	6.67	NA	NA	NA	NA	NA	NA
CLT and VRZ	0.06–1 (0.33)	2 (2)	0.04–2.01 (0.25)	56.67	43.33	0.00	NA	NA	NA	NA	NA	NA
CLT and MCF	0.03–2 (0.20)	2–64 (2.24)	0.04–1.1 (0.17)	73.33	26.67	0.00	0.06–2 (0.18)	2 (2)	0.05–3.63 (0.21)	70.00	30.00	0.00
CLT and CSF	0.03–1 (0.18)	0.5–16 (2.41)	0.03–2.08 (0.28)	60.00	40.00	0.00	0.06–0.5 (0.16)	0.5–2 (1.82)	0.06–4.16 (0.47)	53.34	43.33	3.33
CLT and AND	0.06–2 (0.35)	2 (2)	0.04–4.01 (0.27)	50.00	46.67	3.33	0.06–2 (0.36)	2 (2)	0.04–4.01 (0.29)	46.67	50.00	3.33

[a] MIN, minocycline; TIG, tigecycline; AZT, azithromycin; CLT, clarithromycin; AMB, amphotericin B; ITZ, itraconazole; VRZ, voriconazole; TRB, terbinafine; AND, anidulafungin; CSF, caspofungin; MCF, micafungin.
[b] GM, geometric mean; ATB, antibacterial; ATF, antifungal; FICI, fractional inhibitory concentration index; S, synergism; I, indifference; An, antagonism.
[c] MEC, minimal effective concentration for echinocandins.
[d] NA, not applicable.

scribed for non-*P. insidiosum* isolates, some sterol-targeting anti-fungal agents, while ineffective on mycelium grown in the absence of sterol, demonstrated antimicrobial activity in culture media containing cholesterol (31, 32). Conversely, the presence of cholesterol decreased the growth-inhibitory action of the antibacterial drugs that act by inhibiting the protein synthesis of *Pythium* isolates (33, 34). In this context, the possible changes in the permeability of the plasma membrane of *P. insidiosum* caused by antifungal drugs may facilitate the entry of antibacterial drugs in the cell, resulting in the synergistic interaction of these drugs.

These results have a direct impact on the clinical treatment of pythiosis because empirical therapy can be better adjusted in suspected cases of pythiosis before microbiological confirmation of the pathogen. The striking difference between the MICs of antibacterial and antifungal agents may suggest that the evaluated antibacterial drugs are a better treatment option than the antifungal drugs. However, further studies using models of experimental pythiosis are needed to reveal the therapeutic efficacies of the *in vitro* synergisms observed in this study, which can then be utilized to suggest the best treatment for pythiosis.

In conclusion, we found that the combination of azithromycin, clarithromycin, minocycline, or tigecycline with antifungal agents may be an effective alternative in the treatment of pythiosis, because these combinations result in synergistic interactions. However, a small percentage of antagonistic interactions was observed, mainly in the combination of azithromycin or clarithromycin with echinocandins. Future studies should consider this antagonistic, though small, potential between antibacterial drugs and the echinocandins evaluated.

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