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In Vitro Activities of Voriconazole, Itraconazole, and Terbinafine Alone or in Combination against *Pythium insidiosum* Isolates from Brazil[▽]

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We evaluated the in vitro activities of voriconazole, itraconazole, and terbinafine against 30 clinical isolates of *Pythium insidiosum* using a checkerboard macrodilution method. The combined activity of terbinafine plus itraconazole or plus voriconazole was synergic against 17% of the strains. Antagonism was not observed.

Pythium insidiosum is classified in the kingdom *Stramenopila*, class *Oomycetes* (3). It causes pythiosis, a disease mainly diagnosed in horses, dogs, and humans (14). Human pythiosis was first documented in 1985 (3). Since then, several cases have been reported, with high rates of morbidity and mortality (12). It is found mostly in Thailand, and two factors contribute to importance of pythiosis in that country: the prevalence of β-thalassemia and the presence of large flooded areas used for agriculture (18). Combinations of antifungal agents have been poorly studied in medical mycology, and their activities against *P. insidiosum* are almost unknown (17).

The aim of the present study was to investigate the in vitro activity of terbinafine (TRB) combined with itraconazole (ITC) and of TRB combined with voriconazole (VRC) against 30 isolates of *Pythium insidiosum* from animal pythiosis by using a macrodilution methodology based on the M38-A technique (10).

This study included 28 Brazilian *P. insidiosum* strains obtained from animal pythiosis (horses, dogs, and sheep) and two standard strains (ATCC 58637 and CBS 101555). The identities of the isolates were confirmed by a PCR-based assay (13). The inocula consisted of *P. insidiosum* zoospores obtained as previously described (11). These were counted in a hemacytometer and diluted in RPMI 1640 broth containing L-glutamine and buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid, yielding a final concentration of 2×10^3 to 3×10^3 zoospores/ml. *Candida parapsilosis* (ATCC 22019) and *Aspergillus flavus* (ATCC 204304) were used as quality control organisms (10).

The antifungal agents tested were TRB (Novartis) at 1 to 64 mg/liter, ITC (Sigma Pharma) at 0.125 to 32 mg/liter, and VRC (Pfizer) at 0.125 to 32 mg/liter. The interactions of the combinations (TRB-ITC and TRB-VRC) were evaluated by using the checkerboard technique according to the broth macrodilution design (2). The range of drug concentrations for use in the checkerboard assay was the same used in individual tests. Aliquots (50 μl) containing different concentrations of each antifungal agent (seven of TRB and nine of triazole agents) were placed in tubes to provide 63 drug combinations; 0.9 ml of inoculum was added to each tube. The interactions were interpreted as synergistic (fractional inhibitory concentration index [FICI] ≤ 0.5), indifferent ($0.5 < \text{FICI} \leq 4$), or antagonistic (FICI > 4) based on the respective FICI (5), using the following formula: FICI = (MIC A in combination/MIC A) + (MIC B in combination/MIC B). Off-scale MICs were converted to the next higher dilution for calculation purposes.

MIC-1 and MIC-0 were used as the reading criteria for TRB and were determined as the lowest drug concentrations at which slight growth (25%) or no growth were evident compared to the positive control (hyphae under optimal growth conditions), respectively. Only MIC-0 was determined for VRC, ITC, and the combinations tested (TRB-VRC and TRB-ITC). The MIC readings were visual and assessed the presence (i.e., growth) or absence of hyphae after 24 h of incubation at 37°C. The tests were carried out in duplicate on the same day; whenever the values obtained were not coincident, the assay was repeated. Immediately after the MICs were determined, the minimal fungicidal concentrations (MFCs) were assayed by transferring 0.1 ml from each culture with a drug concentration equal to or greater than the established MIC-0 to tubes containing 0.9 ml of Sabouraud broth. The MFC was defined as the lowest drug concentration at which no growth could be observed after 24 h of incubation at 37°C.

The results revealed that TRB was the most effective drug, with MIC-0 and MFC values ranging from 0.5 to 8 mg/liter (Table 1). TRB's MIC-1 ranged between 1 and 4 mg/liter. The fungicidal activities for ITC and VRC were >16 mg/liter. The effects of both combinations, TRB-VRC and TRB-ITC, were synergistic on 17% and indifferent on 83% of the isolates. The interpretations of both interactions were equivalent for 26 isolates (87%): 23 were indifferent, and 3 showed synergistic effects (Table 2).

Regarding the methodology used, we emphasize that *P. insidiosum* zoospores can be counted, allowing for the obtainment of a standardized inoculum; furthermore, the growth in

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TABLE 1. In vitro susceptibilities of *P. insidiosum* (n = 30) to ITC, VRC, and TRB

Antifungal agent	MIC (mg/liter)				MFC_{50}[b] (mg/liter)
	Range (MIC-0)[a]	Mode	MIC_{50}	MIC_{90}	
Itraconazole	16–>32	>16	≥16	≥32	>16
Voriconazole	16–>32	>16	≥16	≥32	>16
Terbinafine	0.5–8	4	4	4	4

[a] That is, the range of the lowest drug concentration at which complete growth inhibition was observed.
[b] That is, the minimal concentration exerting fungicidal effects on 50% of the isolates.

RPMI broth was excellent. Similar results were obtained by Pereira et al. (11). The disadvantages of the methodology used include the difficulty in obtaining the required amounts of zoospores and the fact that the zoospore is not a pathological form. Nevertheless, zoospores and hyphae have the same organelles and cell membrane composition (9).

In vitro susceptibility studies on *P. insidiosum* were previously performed by Sekhon et al. (16) and Shenep et al. (17), who did not describe the procedures used for inoculum preparation, the incubation time and temperature, or the reading criteria. Shenep et al. (17) showed a pharmacological cure of pythiosis utilizing TRB plus ITC. In the present study, which describes the first results of the use of antifungal agent combinations against *P. insidiosum* in vitro, the two combinations tested displayed a synergistic effect on 17% of the 30 isolates studied and no synergism on 83% of them. We must emphasize that three isolates demonstrated synergism of both combinations, consistent with biochemical variability or inconstancy among *Pythium* strains (7). Conversely, Schurko et al. (15) showed that genotypic variability between American *P. insidiosum* strains does not exist.

Triazoles and TRB block different steps of the same fungal ergosterol biosynthesis pathway (8). It has been suggested that, when combined, one of them might increase the cell permeability to the other drug, providing support for a synergistic action. As pointed out by Dykstra et al. (4), *Pythium* is not a true fungus and does not utilize ergosterol as the main sterol in cellular membranes. Thus, it is not surprising that antifungal agents that interfere with ergosterol synthesis are ineffective against oomycetes. Moreover, the results obtained in the present study need to be correlated with in vivo assays. We believe that the MICs of the synergic combinations can be considered therapeutic because these concentrations are achievable in human and animal sera (1, 6, 17).

TABLE 2. In vitro activity of ITC plus TRB and of VRC plus TRB against *P. insidiosum* (n = 30)

Isolate[a]	ITC and TRB			TRB and VRC		
	MIC of combination (mg/liter)		FICI (interpretation)[b]	MIC of combination (mg/liter)		FICI (interpretation)
	ITC	TRB		TRB	VRC	
ATCC 58637*	0.5	2	0.5 (S)	2	0.25	0.5 (S)
CBS 101555	0.5	4	1.0 (I)	2	0.5	0.5 (S)
LAPEMI 123*	0.5	4	2.0 (I)	2	2	1.0 (I)
LAPEMI 124*	2	2	0.3 (S)	2	0.5	0.2 (S)
LAPEMI 125*	0.5	4	1.0 (I)	4	0.5	1.0 (I)
LAPEMI 126*	0.5	4	1.0 (I)	4	0.25	1.0 (I)
LAPEMI 127*	0.5	4	2.0 (I)	4	0.5	2.0 (I)
LAPEMI 128	0.5	4	1.0 (I)	2	0.5	0.5 (S)
LAPEMI 129*	1	2	1.0 (I)	4	16	2.2 (I)
LAPEMI 134	0.5	2	0.5 (S)	4	0.5	1.0 (I)
LAPEMI 135*	0.5	4	1.0 (I)	4	0.5	1.0 (I)
LAPEMI 136*	2	4	1.0 (I)	4	0.5	1.0 (I)
LAPEMI 137*	0.5	4	2.0 (I)	4	0.5	2.0 (I)
LAPEMI 138*	0.5	2	1.0 (I)	4	0.5	2.0 (I)
LAPEMI 141*	0.5	2	4.0 (I)	2	0.5	4.0 (I)
LAPEMI 142*	0.5	2	1.0 (I)	2	0.5	1.0 (I)
LAPEMI 143*	0.5	4	1.0 (I)	4	0.5	1.0 (I)
LAPEMI 144*	0.5	4	2.0 (I)	8	0.5	4.0 (I)
LAPEMI 145*	0.5	4	1.0 (I)	4	0.5	1.0 (I)
LAPEMI 147*	0.5	4	1.0 (I)	4	8	1.2 (I)
LAPEMI 148*	0.5	4	1.0 (I)	4	0.5	1.0 (I)
LAPEMI 152*	0.5	4	1.0 (I)	4	0.5	1.0 (I)
LAPEMI 156	0.5	2	0.5 (S)	4	0.25	1.0 (I)
LAPEMI 167*	0.5	4	1.0 (I)	4	0.5	1.0 (I)
LAPEMI 175*	0.5	4	2.0 (I)	4	2	2.0 (I)
LAPEMI 177*	0.5	4	2.0 (I)	4	0.25	2.0 (I)
LAPEMI 178*	0.5	4	1.0 (I)	4	0.5	1.0 (I)
LAPEMI 179*	0.5	4	2.0 (I)	4	0.5	2.0 (I)
LAPEMI 187*	0.5	1	0.1 (S)	4	0.5	0.5 (S)
LAPEMI 198*	0.5	4	2.0 (I)	4	0.5	2.0 (I)

[a] *, Isolates with similar fractional inhibitory concentration index interpretations in both assays. ATCC, American Type Culture Collection; CBS, Centraalbureau voor Schimmelcultures; LAPEMI, Laboratory of Mycological Research.
[b] Interpretations: S, synergistic; I, indifferent.

In conclusion, the findings of the present study are very encouraging because the drugs tested showed synergistic or indifferent effects but never antagonistic interactions. Combination therapy provides an alternative to monotherapy, especially for patients with invasive infections that are difficult to treat.

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