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Checkerboard Analysis To Evaluate Synergistic Combinations of Existing Antifungal Drugs and Propylene Glycol Monocaprylate in Isolates from Recalcitrant Tinea Corporis and Cruris Patients Harboring Squalene Epoxidase Gene Mutation

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ABSTRACT Recalcitrant dermatophytic infections of the glabrous skin (tinea corporis/cruris/faciei) pose a huge challenge to health care systems. Combinations of oral and topical drugs may potentially improve cure rates, but the same has never been objectively assessed for this condition in laboratory or clinical studies. The present study was undertaken with the aim of identifying synergistic combinations of oral and topical antifungals by testing clinical isolates obtained from patients with recalcitrant tinea corporis/cruris. Forty-two patients with tinea corporis/cruris who had failed oral antifungals or had relapsed within 4 weeks of apparent clinical cure were recruited. Twenty-one isolates were identified by sequencing (all belonging to the *Trichophyton mentagrophytes/T. interdigitale* species complex) and subjected to antifungal susceptibility testing (AFST) and squalene epoxidase (SQLE) gene mutation analysis. Finally, five isolates, four with underlying SQLE gene mutations and one wild-type strain, were chosen for checkerboard studies using various combinations of antifungal agents. Most isolates ($n = 16$) showed high MICs of terbinafine (TRB) (0.5 to >16 μg/ml), with SQLE gene mutations being present in all isolates with MICs of $\geq$0.5 μg/ml. Synergistic interactions were noted with combinations of itraconazole with luliconazole, TRB, and ketoconazole and propylene glycol monocaprylate (PGMC) with luliconazole and with the triple combination of PGMC with luliconazole and ketoconazole. *In vitro* synergistic interactions provide a sound scientific basis for the possible clinical use of antifungal combinations. Hence, these synergistic combinations may be tested for clinical utility in the wake of rising resistance among dermatophytic infections of the glabrous skin.

KEYWORDS synergy, dermatophytes, tinea corporis/cruris, terbinafine, itraconazole, PGMC, resistance, SQLE mutations, fractional inhibitory concentration, checkerboard analysis, ketoconazole, fluconazole, luliconazole

Recalcitrant dermatophytic infection is an emerging health care issue in many parts of Asia and Europe and has various causes, including *in vitro* resistance, lack of compliance, steroid abuse, variations in the quality of antifungals (especially itraconazole), and, possibly, immunological dysfunction (1–12). However, the rationale and utility of combining oral and topical antifungal drugs in tinea corporis/cruris are as yet unclear and unsupported by robust laboratory or clinical data. However, clinicians still often combine different antifungal drugs, sometimes even oral drugs at unapproved doses, to ensure a clinical cure (13, 14). The latter is a worrisome trend and is fraught with potential side effects and medicolegal consequences (13). Any use of combination

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TABLE 1 MICs for various tested antifungals[a]

Drug	MIC (μg/ml)			
	T. mentagrophytes/*T. interdigitale* (*n* = 21)			Reference strain (*T. mentagrophytes* MTCC 7687)
	GM MIC	MIC_{50}	MIC_{90}	
KTC	0.457	0.25	2	1
FLU	25.398	16	128	16
CLOT	0.591	0.5	2	<0.03
LLCZ	0.026	0.03	0.03	<0.03
BFZ	0.792	0.03	0.5	0.25
EBZ	0.118	0.06	0.5	0.13
ITC	0.183	0.06	0.5	0.13
TRB	1.36	8	16	<0.03
AMO	0.03	0.03	0.03	<0.03
CPX	1.259	1	2	2
AMB	6.361	8	16	8
FTCZ	0.197	0.13	0.5	<0.4

[a]KTC, ketoconazole; FLU, fluconazole; CLOT, clotrimazole; LLCZ, luliconazole; BFZ, bifonazole; EBZ, eberconazole; ITC, itraconazole; FTCZ, fenticonazole; TRB, terbinafine; AMO, amorolfine; AMB, amphotericin B; CPX, ciclopirox; GM, geometric mean.

antifungals should be based on *in vitro* checkerboard studies, which predate clinical trials and use in patients (13, 14).

The aim of this study was to select clinically recalcitrant patients with tinea corporis and cruris, study the antifungal susceptibility to common antifungal drugs in them, perform squalene epoxidase (SQLE) mutation studies, and run a checkerboard analysis of various antifungals on the isolates obtained to arrive at a scientifically sound combination of regimen of systemic and topical antifungals that can be translated into clinical practice.

RESULTS

Out of the 42 patients who fulfilled the inclusion criteria, 26 patients showed positive cultures for dermatophytes. Subculture was achieved from 23 of these, and subsequently, in 21 cases, DNA sequencing of the 28S rRNA gene internal transcribed spacer (ITS) region was performed, which identified all isolates as belonging to the *Trichophyton mentagrophytes*/*T. interdigitale* species complex. Of these 21 patients, 8 had previously been treated with oral terbinafine (TRB), 11 had been treated with oral itraconazole, and 3 had been treated with oral fluconazole.

Antifungal susceptibility test (AFST) results (Table 1) revealed that compared to the standard strain (*T. mentagrophytes* MTCC7687), all clinical isolates were less susceptible to fluconazole but were susceptible to ketoconazole, itraconazole, and luliconazole. Most isolates had comparable MIC values of bifonazole, eberconazole, fenticonazole, amorolfine, and ciclopirox. MICs of amphotericin B and clotrimazole were higher for the clinical isolates than for the MTCC strain, while about 76% of strains showed high MICs of terbinafine, ranging from 0.5 to >16 μg/ml.

SQLE sequencing found three types of point mutations in the isolates. Eleven isolates had a TTC-CTC point mutation at nucleotide position 1189 (1189TTC-CTC), and 2 isolates had a 1189 TTC-TTA mutation. Both of these mutations cause the same amino acid substitution, Phe393Leu, in the SQLE protein. Three strains had a 1177 TTA-TTC mutation, which corresponds to the amino acid substitution Leu393Ser. Five strains showed no mutation. While the mutations conferred various levels of susceptibility to terbinafine (Table 2), the isolates with the Leu393Ser substitution had lower MICs than the isolates with the Phe393Leu substitution.

To perform synergy analysis, five *T. mentagrophytes*/*T. interdigitale* species complex clinical isolates (DA02, DA17, DA19, DA26, and DA42) were selected based on their susceptibility pattern against terbinafine and type of point mutation in the SQLE gene

TABLE 2 Terbinafine MICs and squalene epoxidase gene mutations among the 21 isolates

T. mentagrophytes/*T. interdigitale* species complex clinical isolate no.	Nucleotide substitution within the squalene epoxidase gene	Terbinafine MIC (μg/ml)
DA02	1189 TTC-TTA (Phe397Leu)	4
DA07	No mutation	<0.03
DA09	1189 TTC-CTC (Phe397Leu)	8
DA12	No mutation	<0.13
DA13	1189 TTC-CTC (Phe397Leu)	8
DA19	1189 TTC-CTC (Phe397Leu)	>16
DA17	1177 TTA-TCA (Leu393Ser)	0.5
DA20a	1177 TTA-TCA (Leu393Ser)	0.25
DA21	1189 TTC-CTC (Phe397Leu)	8
DA18	1177 TTA-TCA (Leu393Ser)	0.5
DA23	1189 TTC-CTC (Phe397Leu)	4
DA26	1189 TTC-CTC (Phe397Leu)	>16
DA27	1189 TTC-CTC (Phe397Leu)	8
DA29	1189 TTC-CTC (Phe397Leu)	8
DA30	1189 TTC-CTC (Phe397Leu)	>16
DA32	No mutation	0.03
DA37	1189 TTC-CTC (Phe397Leu)	8
DA36	1189 TTC-CTC (Phe397Leu)	>16
DA38	No mutation	<0.03
DA40	1189 TTC-TTA (Phe397Leu)	>16
DA42	No mutation	<0.03

(Table 3). The various combinations tested and the respective outcomes are mentioned in Table 4. The combinations of itraconazole with luliconazole, itraconazole with terbinafine, and itraconazole with ketoconazole showed synergistic effects (fractional inhibitory concentration [FIC] index of $\leq$0.5). The combination of ketoconazole with luliconazole showed synergistic effects against 3 clinical isolates and an additive effect against the remaining strains, while no antagonism was observed in any combination tested.

Propylene glycol monocaprylate (PGMC) is a fatty acid derivative that is known to destabilize the fungal cell membrane and cause increased drug permeation in fungal cells (15, 16). Analysis of the combination of PGMC with luliconazole was performed against five clinical isolates (DA02, DA17, DA19, DA26, and DA42). The combination was found to be synergistic against 4 clinical isolates and additive against 1 clinical isolate (Table 4). No antagonism was observed.

Based on the double-combination results, the triple combination of PGMC plus ketoconazole and luliconazole was tested against 5 clinical isolates. The triple-combination results indicate that the combination of PGMC plus ketoconazole and luliconazole was synergistic against 2 clinical isolates and additive against 3 clinical isolates. No antagonism was observed in any isolate.

DISCUSSION

Our checkerboard analysis of clinical isolates from patients with recalcitrant tinea corporis/cruris harboring SQLE gene mutations found the combinations of itraconazole

TABLE 3 Terbinafine susceptibility of isolates chosen for checkerboard analysis

Isolate	Point mutation in the squalene epoxidase gene	Terbinafine MIC (μg/ml)
DA02	1189 TTC-TTA (Phe397Leu)	4
DA17	1177 TTA-TCA (Leu393Ser)	0.5
DA19	1189 TTC-CTC (Phe397Leu)	>16
DA26	1189 TTC-CTC (Phe397Leu)	>16
DA42	No mutation	<0.03

TABLE 4 Isolate-wise results of the checkerboard analysis[a]

Combination	Result for strain				
	DA02	DA17	DA19	DA26	DA42
Ketoconazole + ciclopirox	Additive	ND	Indifferent	Additive	Additive
Ketoconazole + amphotericin B	Additive	ND	Indifferent	Additive	Additive
Ketoconazole + terbinafine	Synergy	ND	Additive	Synergy	Additive
Ketoconazole + fluconazole	Additive	ND	Additive	ND	ND
Ketoconazole + luliconazole	Synergy	Additive	Synergy	Synergy	Additive
Itraconazole + ciclopirox	Synergy	ND	ND	Additive	Additive
Itraconazole + amphotericin	Additive	ND	ND	Indifferent	Indifferent
Itraconazole + luliconazole	Synergy	Synergy	Synergy	Synergy	Additive
Itraconazole + terbinafine	Synergy	Synergy	Synergy	Synergy	ND
Itraconazole + ketoconazole	Synergy	Synergy	Synergy	Synergy	Additive
Itraconazole + fluconazole	Synergy	ND	ND	ND	ND
Fluconazole + amphotericin	Additive	Additive	ND	ND	Additive
Fluconazole + terbinafine	Synergy	Additive	ND	ND	Additive
Fluconazole + luliconazole	Additive	Additive	ND	ND	Additive
Fluconazole + ciclopirox	Additive	Synergy	ND	ND	Additive
Fluconazole + ketoconazole	Additive	ND	ND	ND	Additive
PGMC + luliconazole	Synergy	Synergy	Additive	Synergy	Synergy
PGMC + itraconazole	Indifferent	Synergy	Synergy	Additive	Additive
PGMC + ketoconazole	Additive	Additive	Additive	Synergy	Additive
PGMC + ciclopirox	Additive	Synergy	Additive	Additive	Additive
PGMC + terbinafine	Additive	Additive	Synergy	Additive	Additive
PGMC + fluconazole	Additive	Synergy	Additive	Additive	Additive
PGMC + amphotericin	ND	Additive	Additive	ND	ND

[a]Synergy, FIC index of ≤0.5; additive, FIC index of >0.5 to 1; indifferent, FIC index of >1 to 4; antagonistic, FIC index of >4. ND, not determined.

plus luliconazole, itraconazole plus ketoconazole, and itraconazole plus terbinafine to be synergistic. The membrane-disrupting agent PGMC also showed synergistic activity with luliconazole.

The changing dynamics of recalcitrant dermatophytic infections in certain regions of the world have important clinical and therapeutic implications (3, 17) and warrant studies on *in vitro* susceptibility, resistance mechanisms, and synergy data to achieve better clinical outcomes. In concurrence with previous studies (4–6, 18), we also observed the predominance of *T. mentagrophytes*/*T. interdigitale* species complex strains as the causative agents of tinea corporis/cruris. Although the taxonomic aspects of the prevalent species are still being worked on, a recent genome analysis of the species has confirmed it as a unique clade related to the *T. mentagrophytes*/*T. interdigitale* complex and likely belongs to an early-diverging clade of the complex (6). An important clinical and therapeutic aspect is the high frequency of terbinafine resistance reported in this new species type (4–6), which has also been seen in *Trichophyton rubrum* and *T. interdigitale* strains from other countries (7–12). Our study revealed that the majority of clinical isolates had high terbinafine MICs (MIC_{50}, 8 μg/ml; MIC_{90}, 16 μg/ml) compared to the MIC of the reference strain (<0.03 μg/ml) (Table 1). The MICs of clinical isolates were also high for fluconazole, clotrimazole, and amphotericin B. Luliconazole and ciclopirox had comparable MICs between the clinical and reference strains.

Mutational analysis revealed mutations leading to the amino acid substitutions Phe397Leu in 13 isolates and Leu393Ser in the SQLE protein in 3 isolates (Table 2), which concur with previous data (4–11, 18). The mutation-harboring isolates had MICs between 0.25 μg/ml and >16 μg/ml, while the 5 tested isolates that did not show mutations had MICs of between ≤0.03 μg/ml ($n = 4$) and 0.13 μg/ml ($n = 1$). This is in contrast to the observations of Singh et al. (4), Khurana et al. (5), and Rudramurthy et al. (18), where mutations were observed only for isolates with MICs of ≥4 μg/ml, but in line with the observations of Yamada et al. (8) and Saunte et al. (7), where mutations were reported in isolates with MICs of as low as 0.1 μg/ml. Although the clinical

breakpoints for terbinafine in dermatophytic infections of the glabrous skin are not defined, a cutoff value of between 4 and 5 μg/ml has been suggested, based on limited existing data (17).

While clinical failure is associated with a high frequency of resistance to the primary oral agent (terbinafine), alternative therapeutic options need to be considered. Griseofulvin and fluconazole have been proven to be largely ineffective (3), and with itraconazole, there are quality concerns such as variation in the pellet quality, number, and polymers among different brands that can affect the serum levels and, consequentially, its clinical results (1, 2, 19). As there is no new oral antifungal drug approved for dermatophytes, there is an emergent need to use existing drugs in an effective manner. Clinicians often use antifungals in combinations, but there are no mycological or clinical data to support this approach in tinea corporis/cruris (13). Any assumption that the use of two or more effective drugs with different mechanisms of action will produce an improved outcome compared to the use of a single agent alone is simplistic and unscientific. The use of combination antifungals based on such a premise could actually reduce antifungal killing and clinical efficacy, may lead to drug interactions, and may increase the overall cost of treatment (20).

There are 3 phases for demonstrating and validating synergistic antifungal drug combinations: *in vitro* checkerboard testing, *in vivo* animal model validation, and clinical trials (13). Combinations of systemic and topical antifungals have been tested in clinical studies on dermatophytic onychomycosis (21, 22, 36), but no such study has been conducted on dermatophytoses of the glabrous skin: tinea corporis/cruris. The few *in vitro* checkerboard studies done previously with dermatophytes have been performed on limited spectra of drugs (23, 24), with laboratory rather than clinical isolates (25, 26), and with isolates that had a mixture of *Candida* spp., nondermatophyte molds, and dermatophytes (21).

One hindrance in testing combinations with terbinafine so far has been the low prevalent MICs, making it difficult to test at levels 4 to 5 dilutions below the MIC value (21). Furthermore, if the MIC of the clinical isolates is low, combination therapy is inherently not required. In our study, the high terbinafine MICs encouraged us to perform a checkerboard analysis on 5 isolates, 4 of which harbored SQLE gene mutations and 1 of which was a wild-type strain. Interestingly, the combination of terbinafine and itraconazole showed synergistic interactions in 4 of the isolates and indifference/antagonism in none, despite terbinafine resistance (Table 4). Luliconazole is a relatively new antifungal and has been consistently shown to have low MICs against dermatophytes (4–6). Synergistic interactions of oral itraconazole and topical luliconazole may be clinically relevant, although cost may be a deterrent to their widespread use. Topical ketoconazole, on the other hand, is a less expensive alternative, and synergy with itraconazole will make this a useful and cost-effective option.

Thus, our work shows that there are certain useful combinations that can be clinically tested and prescribed to enhance the effectiveness of treatment with the available basket of antifungals. Notably, we also observed that propylene glycol monocaprylate (PGMC) (15, 27) can potentiate luliconazole, and this can be used as a monotherapeutic topical agent in applicable scenarios. PGMC is already approved by the U.S. FDA as a generally recognized as safe (GRAS) excipient. This molecule has been repurposed as an antifungal agent, and its approved uses include as a viscosity-increasing agent, a nonaqueous skin-conditioning agent, and a surfactant-emulsifying agent.

While our data have some unique aspects as we analyzed clinical isolates from patients who failed oral antifungal drugs instead of laboratory strains, we acknowledge the need for clinical validation. We also admit the potential flaws of checkerboard analysis, including an inability to provide a graded response as needed to determine dose-response curves and providing only a static rather than a dynamic view of antimicrobial interactions (13, 14). The ideal analysis is based on "time-kill" studies, which provide a more dynamic assessment of the interaction between an antimicrobial agent and a

given organism (13). We hope that these will be addressed in future studies, but our data give firm scientific data on the possible synergistic combinations of systemic and topical antifungals that can be used in recalcitrant dermatophytosis of the glabrous skin.

MATERIALS AND METHODS

The study was approved by the institutional ethics committee. It was a prospective cross-sectional analysis conducted in the Department of Dermatology at Dr Ram Manohar Lohia hospital in collaboration with Vyome Laboratories, Delhi, India. Patient recruitment was performed between July 2017 and July 2018 from the outpatient clinic of the department.

Forty-two patients with tinea corporis or cruris who had taken 4 to 6 weeks of oral antifungals (itraconazole, terbinafine, fluconazole, or ketoconazole) in approved/higher doses with no/minimal improvement in clinical morphology and itching and those who had relapsed following successful systemic treatment within 4 weeks after stopping therapy were included after obtaining informed consent. Patients who were using/had used topical steroids were excluded.

Sample collection. To avoid external contaminants, skin lesions were cleaned with 70% ethanol-soaked cotton. By using a sterile scalpel, skin scrapings were collected from the active edges of the lesion and inoculated into tubes containing 5 ml of Sabouraud dextrose (SD) broth with chloramphenicol at 0.05 mg/ml. All tubes were brought to the microbiology laboratory as early as possible and processed for *Trichophyton* isolation by inoculating 100 μl of the fungal sample on SD agar (SDA) with chloramphenicol at 0.05 mg/ml by the spread plate method. Plates were incubated at 37°C for 7 days. After incubation, dermatophyte characteristic colonies, white to off-white filamentous colonies, were subcultured into a fresh SD agar plate and subsequently processed for identification (28–30).

Dermatophyte identification. Cultured dermatophyte isolates were subjected to genomic DNA extraction using a DNA extraction kit (Qiagen) according to the manufacturer's instructions. Dermatophyte identification was carried out by amplifying the internal transcribed spacer (ITS) region of 23S rRNA using the ITS1 and ITS4 primers (31). PCR amplification was performed by an initial denaturation step at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 1 min, and extension at 72°C for 1 min, with a final extension step at 72°C for 10 min. Amplified products were separated by 1.5% agarose gel electrophoresis. The 650-bp PCR products were cleaned using a Definity rapid tip (Sigma) and submitted for DNA sequencing. The obtained DNA sequences were analyzed by a BLAST search against the NCBI database, and species-level identification was performed.

Antifungal susceptibility testing. (i) Antifungal drugs. All clinical isolates were tested for their antifungal susceptibility to ketoconazole (Himedia), fluconazole (Alfa Azar), clotrimazole (Sigma-Aldrich), luliconazole (Sigma-Aldrich), bifonazole, eberconazole, itraconazole (Intas Pharma), fenticonazole (Optimus Drug Pvt. Ltd.), terbinafine (Sigma-Aldrich), butenafine, amorolfine (Optrix Lab), and ciclopirox (Sigma-Aldrich).

(ii) Inoculum preparation. A 7-day-old *Trichophyton* sp. culture grown on SDA was used for inoculum preparation. A sterile moist cotton swab was gently rubbed over the fungal growth and inserted into a Falcon tube containing sterile water to release the fungal components. The tube was kept undisturbed for 5 min to allow hyphae to settle, and the upper portion was carefully transferred into a fresh tube for UV-visible spectroscopy measurement. We performed uniform turbidity measurement (i.e., 80% transmittance at 530 nm using a UV-visible spectrometer) for preparing the inoculum for MIC testing.

(iii) MIC determination. The antifungal susceptibility of *Trichophyton* species isolates was determined by the broth microdilution method according to CLSI guidelines (CLSI M38-A2) (32), with slight modification. We used SD broth in our MIC testing scenario because we have seen that SD broth is very conducive to the uniform growth of most clinical isolates of dermatophytes as well as reference strains. This observation is also supported by the literature (33). To avoid data reproducibility issues, we included a standard strain (*T. mentagrophytes* MTCC 7687) in every instance while testing antifungals alone or in combination against clinical isolates.

Initial stock solutions (~2 mg/ml) of antifungals were prepared in dimethyl sulfoxide (DMSO) or water, and further dilution was carried out in SD broth. In a 96-well plate, 100 μl of SD broth was added to all the wells, and 100 μl of SD broth containing the desired concentration of antifungals was then added to the wells of the first column. Serial dilutions were performed from wells of the 1st column up to the 10th column. Wells of the 11th and 12th columns were kept as positive and negative growth controls, respectively. The fungal inoculum was prepared in sterile water, adjusted to 80% transmittance at 530 nm containing 1×10^5 to 2×10^5 conidial spores/ml, and then further diluted 100 times with SD broth. Finally, 100 μl of the inoculum was added to all the wells except the negative control. Plates were incubated at 35°C for 7 days. We performed MIC assays at 35°C $\pm$ 2°C in line with previous literature showing that the incubation temperature (28°C or 35°C) does not influence the MIC of antifungals significantly (33, 34). The MICs of all antifungal compounds were determined by observing the lowest concentration of the drug that had no visible fungal growth compared to the growth control. As all isolates were identified as belonging to the *T. mentagrophytes*/*T. interdigitale* species complex, we used *T. mentagrophytes* MTCC 7687 as a standard strain for comparison of MIC values. The MIC values of the reference strain are mentioned in Table 1.

(iv) Synergy testing. Synergy testing was performed for commonly used antifungal drugs (oral and topical) (Table 1). Combinations of antifungals with each other and combinations of PGMC (propylene glycol monocaprylate) with antifungals were tested by the checkerboard method. In a 96-well plate, initially, all the wells were filled with 100 μl SD broth. Drug A was initially dissolved in DMSO and further diluted in SD broth, and 100 μl of an 8$\times$ concentration of drug A was added to the initial column (wells

A1 to H1) and serially diluted up to the 10th column. The 11th and 12th columns served as the drug B-alone control and the growth control, respectively. Similarly, drug B was initially dissolved in DMSO and finally diluted in broth to obtain a 4× concentration of the desired drug concentration range. It was prepared separately in the tube for each concentration intended to be used. One hundred microliters of the first concentration of drug B was added to first row A wells A1 to A11 but not A12 (as it served as a growth control). This process was repeated for rows B to G but not H with the respective drug concentrations. All wells were mixed thoroughly by pipette aspiration to obtain proper drug disruption. One hundred microliters of the contents of each well was transferred to another 96-well plate, marked as a replica plate. Finally, 100 μl of the inoculum (1×10^3 to 3×10^3 CFU/ml) was added to all the wells. Plates were incubated at 37°C for 7 days.

The triple-combination method was performed exactly like the double-combination checkerboard method as explained above except that SD broth was supplemented with the sub-MIC of drug C intended to be synergistic with the combination of drugs A and B.

The synergism of drugs A and B was determined by calculating the fractional inhibitory concentration (FIC) of drug A (FICA) and drug B (FICB). The FICA and FICB were determined by dividing the MIC value of the drug in combination with the MIC value of the drug alone. The FIC index (FICI) was calculated as FICA + FICB. If the FICI value was ≤0.5, it was considered synergy; values of greater than 0.5 but less than 1 were considered additive; values of ≥1 to 4 were interpreted as indifferent; and values of >4 were considered antagonism (35).

Squalene epoxidase sequencing. *T. interdigitale* isolate genomic DNA was amplified with in-house-designed primers specific for the squalene epoxidase (SQLE) gene (TMS F [5′-TGTCGTTCTCCTCCGGAATC-3′] and TMS R [5′-GGGAGGAGGTAGATGGGTTTG-3′]). PCR amplification was performed by an initial denaturation step at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 54°C for 1 min, and extension at 72°C for 1 min, with a final extension step at 72°C for 10 min. Amplified products were separated by 1.5% agarose gel electrophoresis. The 410-bp PCR products were cleaned using a Diffinity rapid tip (Sigma) and submitted for DNA sequencing. The obtained DNA sequences were analyzed by a BLAST search against the NCBI database and compared with the squalene epoxidase gene of the *T. interdigitale* H6 strain.

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