In vitro interaction of fluconazole and trimethoprim–sulfamethoxazole against *Candida auris* using ETEST and checkerboard methods

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ABSTRACT

Candida auris was discovered in 2009 and has rapidly emerged as a serious public health threat with cases reported in over 20 countries worldwide. As of May 8, 2020, the Centers for Disease Control and Prevention reported a total of 1122 US cases. *C. auris* is often multidrug resistant, leaving few options for treatment. Sulfonamides are known to inhibit a bacterial enzyme involved in folate synthesis and may also inhibit yeast organisms by a similar mechanism. The combination of trimethoprim and sulfamethoxazole is more commonly used than either drug alone. The objective of this study was to evaluate the combination of fluconazole and trimethoprim–sulfamethoxazole against *C. auris*. Minimum inhibitory concentrations (MICs) of fluconazole and trimethoprim–sulfamethoxazole were determined by ETEST and broth microdilution for 11 *C. auris* strains. Fluconazole MICs (µg/mL) were 4–>256 by ETEST and 2–>256 by broth microdilution (73% resistant); trimethoprim–sulfamethoxazole MICs were >32 by ETEST and 32–>128 by broth microdilution (no interpretive guidelines for *C. auris*). Using our MIC: MIC ETEST method and a checkerboard method, we investigated the interaction of fluconazole and trimethoprim–sulfamethoxazole against all isolates. These interactions were analyzed by calculating the summation fractional inhibitory concentration with synergyof ≤0.5, additivity of >0.5–1.0, indifference of >1–4, and antagonism of >4. The combination of fluconazole and trimethoprim–sulfamethoxazole revealed synergy with three (27%) and additivity with one (9%) isolate. Indifference was found for the remaining seven (64%) isolates. With the checkerboard method, synergy was seen in 1/11 (9%) isolates with fluconazole (½ MIC) plus trimethoprim–sulfamethoxazole (1/64 MIC); additivity, in 7/11 (64%) isolates with fluconazole (1/8 MIC–1×MIC) plus trimethoprim–sulfamethoxazole (1/128 MIC–½ MIC); and indifference in 3/11 (27%) isolates. Regardless, in vitro interactions may or may not correlate with clinical outcomes. Synergy testing with additional drug combinations and isolates should be performed.

INTRODUCTION

In 2009, discovery of *Candida auris* was documented after it was collected from the external ear canal of a Japanese patient. However, according to retrospective analysis dating back to 1996, this pathogen was actually present among *Candida* species collected in South Korea.[1–3] In 2016, the Centers for Disease Control and Prevention (CDC) found strains of *C. auris* with genetically distinct features and organized these into four geographical clades: South Asia (India/Pakistan) (clade I), East Asia (Japan) (clade II), South Africa (clade III), and South America (Venezuela) (clade IV).[4] Two years later, Chow *et al*[5] identified a possible fifth *C. auris* clade located in Iran.

C. auris is often misidentified as *Candida haemulonii*, but this does depend on the identification method and software that is used. Technologies such as API 20C, API ID 32C, BD Phoenix, MicroScan, and RapID Yeast Plus have been known to misidentify *C. auris*. Successful identification of *C. auris* has been obtained using specialized systems such as Bruker Biotyper matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer, GenMark ePlex BCID-FP Panel, VITEK 2 YST and bioMérieux VITEK MS MALDI-TOF according to the CDC.[6]

C. auris is a nosocomial pathogen that causes outbreaks among those who are severely ill or immunocompromised. Antifungal resistance has been reported, with roughly 90% of isolates showing resistance to fluconazole. Resistance to echinocandins is the lowest at <5%, making this antifungal class the go-to treatment for *C. auris* infections. However, the CDC has reported the existence of strains resistant to all three antifungal classes. The current guidelines for treatment of invasive *C. auris* infection is based on those for other *Candida* species but also rely on health professionals' opinions and experience.[7 8]

Antifungal combination studies have been conducted against *C. auris*, although these are few and lack congruence. Using the checkerboard method, a 2017 study evaluated micafungin and caspofungin in combination with both voriconazole and fluconazole against 10 *C. auris* isolates, including 10 that were fluconazole-resistant and 3 that were micafungin-resistant. The combination of micafungin plus voriconazole revealed a synergistic

interaction for 100% of isolates studied. The combination of micafungin plus fluconazole and the combination of caspofungin plus voriconazole or fluconazole revealed indifference for all isolates.[9] Another checkerboard study combined flucytosine with amphotericin B, voriconazole, or micafungin. Indifference was observed for the majority of isolates. For one strain, the combination of flucytosine with either amphotericin B or micafungin showed synergy.[10] More recently, O'Brien *et al*[11] evaluated eight antifungal combinations against 15 *C. auris* isolates using a checkerboard method. Fourteen isolates were fluconazole-resistant. Flucytosine plus amphotericin B inhibited 100% of the nine amphotericin B-resistant isolates. Six echinocandin-resistant *C. auris* isolates were all inhibited when anidulafungin, caspofungin, and micafungin were each combined with flucytosine. Thirteen voriconazole-resistant isolates were also inhibited with the addition of flucytosine. No other combinations were successful. The 15 samples tested were among those collected from a New York *C. auris* outbreak.[11 12]

The repurposing of antibacterial agents has also been investigated as an alternative treatment against antifungal-resistant *C. auris* infections. The combination of colistin with caspofungin or micafungin was evaluated against 15 *C. auris* strains using a checkerboard method, where synergy was observed for 100% of isolates with the combination of colistin and caspofungin. However, with the micafungin combination, indifference was observed for all isolates.[13] Sulfonamides have shown promising activity against *Candida albicans* in combination with fluconazole using a checkerboard method.[14] In 1982, it was suggested that sulfonamides, similar to bacterial inhibition, may use the folate pathway against *Candida* species.[15]

Our objective was to determine if the combination of fluconazole and trimethoprim–sulfamethoxazole would demonstrate in vitro synergy using an ETEST minimum inhibitory concentration (MIC): MIC interaction method and a checkerboard method against 11 unique, clinical *C. auris* isolates.

MATERIALS AND METHODS

Microorganisms, media, and antimicrobial agents

Eleven *C. auris* isolates were obtained from the CDC: (5) clade I, (1) clade II, (2) clade III, (2) clade IV, and (1) clade information not available. Since *C. auris* has not been isolated at our institution, we were limited to the *C. auris* strains banked at the CDC. Sabouraud dextrose agar plates (Becton-Dickinson Microbiology Systems, Sparks, Maryland, USA) were used for initial subculture. ETEST® strips for fluconazole and trimethoprim–sulfamethoxazole (bioMérieux, Marcy L'Etoile, France) were used. Roswell Park Memorial Institute 1640 agar plates with morpholinepropanesulfonic acid and 2% glucose (Remel, Lenexa, Kansas, USA) were used for the determination of ETEST MICs and synergy testing. Standard laboratory powders of fluconazole, trimethoprim, and sulfamethoxazole and RPMI 1640 broth with glutamine, without bicarbonate, buffered with 0.165 M morpholinepropanesulfonic acid (Millipore Sigma, St. Louis, Missouri, USA) were used for determination of MICs by broth microdilution and checkerboard assay. Quality control testing was performed with *Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6258, *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853.[16 17]

Antimicrobial susceptibility testing

MICs were determined in triplicate by ETEST following the manufacturer's guidelines. First, an inoculum was prepared from a 24-hour culture on Sabouraud dextrose agar. A suspension was prepared in sterile saline and adjusted to a turbidity equivalent to a 0.5 McFarland standard. RPMI plates were inoculated twice with the suspension prior to placement of ETEST strips. Plates were incubated for 24 hours at 35°C in a loosely folded plastic bag in order to maintain moisture. The fluconazole MIC was read at the first point of significant growth inhibition or 80% inhibition, while the trimethoprim–sulfamethoxazole MIC was read at complete inhibition, which included microcolonies and hazes of growth.

MICs were also determined by broth microdilution, according to Clinical and Laboratory Standards Institute (CLSI) guidelines.[18]

Synergy testing

An ETEST MIC: MIC synergy method[19] was performed in triplicate (figure 1). Inoculum preparation and media were the same as described previously for ETEST MICs. Fluconazole and trimethoprim–sulfamethoxazole ETEST strips were placed on different sections of the RPMI plate. The agar was marked adjacent to the previously determined MIC value on each strip (1×MIC for both fluconazole and trimethoprim–sulfamethoxazole). The highest concentration was marked on the agar when the MIC exceeded this concentration (256 µg/mL for fluconazole and 32 µg/mL for trimethoprim–sulfamethoxazole). The strips were removed after incubation at room temperature for 1 hour. A new fluconazole strip was placed on the area of the previously removed trimethoprim–sulfamethoxazole strip so that the fluconazole MIC corresponded with the mark of the MIC for trimethoprim–sulfamethoxazole. The trimethoprim–sulfamethoxazole ETEST strip was applied to the area of the previous fluconazole strip so that the respective MIC values were aligned. RPMI agar plates were incubated at 35°C in a loosely folded plastic bag in order to maintain moisture. The resulting combination MICs were read at 24 hours as described previously.

A standard checkerboard broth microdilution method, containing concentrations of 0.5–512 µg/mL fluconazole and 2–128 µg/mL trimethoprim–sulfamethoxazole, was used to evaluate the interaction of the antimicrobials.[20]

The fractional inhibitory concentration (FIC) was calculated for each antimicrobial to assess the combination effect: FIC of fluconazole=MIC of fluconazole in combination/ MIC of fluconazole alone, and FIC of trimethoprim–sulfamethoxazole=MIC of trimethoprim–sulfamethoxazole in combination/ MIC of trimethoprim–sulfamethoxazole alone. The ΣFIC was calculated according to the following formula: ΣFIC=fluconazole FIC+trimethoprim–sulfamethoxazole FIC.

To account for off-scale MICs (eg, >256 µg/mL for fluconazole), these MICs were converted to the nearest twofold dilution (eg, 512 µg/mL). Final ΣFIC values were rounded

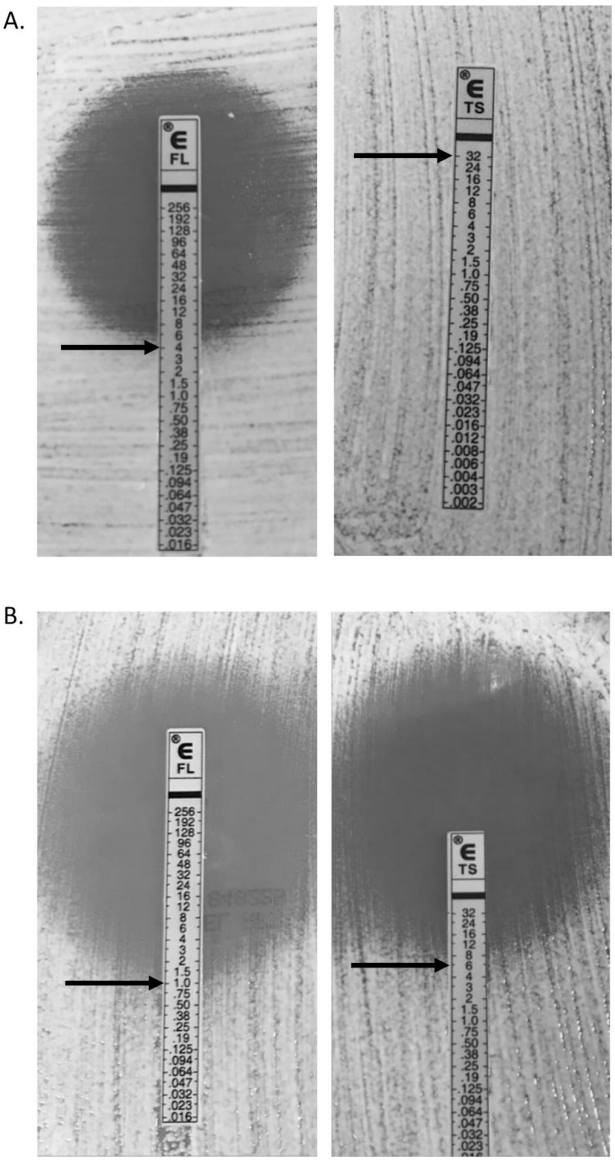

Figure 1 (A) ETEST MICs. ETEST MICs for *Candida. auris* isolate 381 were as follows: FL MIC, 4 µg/mL (left); and TS MIC, >32 µg/mL (right). (B) ETEST synergy test. Combination MICs for *C. auris* isolate 381 were as follows: fluconazole MIC after combination with TS, 1 µg/mL (left); trimethoprim–sulfamethoxazole MIC after combination with fluconazole, 6 µg/mL (right). ΣFIC=0.4 (synergy). Photos published with permission from bioMérieux, Marcy L'Etoile, France. FL, fluconazole; MIC, minimum inhibitory concentration; TS, trimethoprim–sulfamethoxazole.

up to the nearest tenth (eg, 0.37 was rounded to 0.4). The mean ΣFIC was used to interpret results of the ETEST MIC: MIC synergy method. 'Synergy' was defined as having a ΣFIC of ≤0.5; 'additivity', >0.5–1; 'indifference', >1–4; and 'antagonism', >4.[21]

RESULTS

Antimicrobial susceptibility testing

ETEST MICs (µg/mL) for fluconazole were 4–>256 (73% resistant) and trimethoprim–sulfamethoxazole, >32. Four of the isolates had on-scale fluconazole MICs (4–48 µg/mL), while the MICs for the remaining seven isolates were off-scale (>256 µg/mL) on the ETEST strip. Broth microdilution MICs (µg/mL) were 2–>256 (73% resistant) for fluconazole and 32–>128 for trimethoprim–sulfamethoxazole. The CDC tentative fluconazole breakpoint for *C. auris* is ≥32.[7] There are no CLSI interpretive guidelines for trimethoprim–sulfamethoxazole and *C. auris* (table 1).

Synergy testing

Results determined by the ETEST MIC: MIC synergy method are shown in table 1. Synergy was revealed with 3/11 (27%) isolates with the combination of fluconazole and trimethoprim–sulfamethoxazole and additivity with 1 (9%) isolate. Seven isolates (64%) were termed indifferent. The checkerboard method showed synergy in 1/11 (9%) isolates with fluconazole (½ MIC) plus trimethoprim–sulfamethoxazole (1/64 MIC); additivity, in 7/11 (64%) isolates with fluconazole (1/8 MIC–1×MIC) plus trimethoprim–sulfamethoxazole (1/128 MIC–½ MIC); and indifference in 3/11 (27%) isolates (data not shown).

Discussion

The objective was to determine if the combination of fluconazole and trimethoprim–sulfamethoxazole would demonstrate in vitro synergy using an ETEST MIC: MIC interaction method and a checkerboard method against 11 *C. auris* isolates. With the ETEST method, synergy was found with three isolates (27%) using the combination of fluconazole and trimethoprim–sulfamethoxazole. Additivity was observed with one isolate (9%) and indifference, with the remaining seven (64%) isolates. First, the synergy and additivity demonstrated appear to correspond with fluconazole MIC concentrations that are in range of the ETEST strip. In contrast, all isolates that showed indifference had fluconazole MICs that exceeded the highest concentration on the ETEST strip (table 1). The addition of trimethoprim–sulfamethoxazole showed a 1-2 twofold reduction in fluconazole MICs for those isolates that showed synergy or additivity. These findings suggest that, to interpret the interaction as additive or synergistic, MIC results may be limited to concentrations found on the ETEST strip.

Trimethoprim–sulfamethoxazole was tested at a 1:19 ratio. As a result, the MICs were >32 µg/mL for trimethoprim and >608 µg/mL for sulfamethoxazole for all 11 isolates (table 1). Using checkerboard assay, Eldesouky *et al* tested the interaction of fluconazole and sulfamethoxazole against one fluconazole-susceptible *C. auris* strain. It was found that sulfamethoxazole (512 µg/mL) potentiated fluconazole susceptibility, resulting in a three twofold reductions in the fluconazole MIC. However, the combination of fluconazole and sulfamethoxazole was not tested against fluconazole-resistant *C. auris*.[22]

The differences in MIC reduction may be strain dependent or due to differences in testing procedures such as our use of ETEST, which is a solid-agar medium, and checkerboard, which is a broth-based method. In addition, different concentrations of antimicrobials (often subinhibitory concentrations) are tested in combination with the checkerboard method versus a fixed concentration equal to the MIC used in the ETEST synergy method.

Potential sources of error for the ETEST method could include the alignment of strips during synergy testing and

Table 1 MICs (µg/mL) for FL and TS determined by ETEST. Synergy testing by ETEST MIC: MIC method for *Candida auris* isolates.

Candida auris isolates (n=11)	C. auris clade	FL MIC and combination MIC (µg/mL)		TS MIC and combination MIC (µg/mL)		ETEST synergy results	
		FL*	FL+TS*	TS*	TS+FL*	ETEST FICs*	Mean ΣFIC
381	II	4	1	>32	8	0.5, 0.3, 0.3	0.4, SYN
382	I	8	2	>32	12	0.4, 0.5, 0.5	0.5, SYN
387	I	12	4	>32	12	0.7, 0.5, 0.4	0.5, SYN
12	N/A	48	16	>32	24	0.6, 0.8, 0.9	0.8, ADD
384	III	>256	>256	>32	>32	2, 2, 2	2, IND
385	IV	>256	>256	>32	>32	2, 2, 2	2, IND
386	IV	>256	>256	>32	>32	2, 2, 2	2, IND
383	III	>256	>256	>32	>32	2, 2, 2	2, IND
388	I	>256	>256	>32	>32	2, 2, 2	2, IND
389	I	>256	>256	>32	>32	2, 2, 2	2, IND
390	I	>256	>256	>32	>32	2, 2, 2	2, IND

*Performed in triplicate, mean value reported.
ADD, additivity; FIC, fractional inhibitory concentration; ΣFIC, summation fractional inhibitory concentration; FL, fluconazole; IND, indifference; MIC, minimum inhibitory concentration; N/A, data not available; SYN, synergy; TS, trimethoprim–sulfamethoxazole.

subjectivity when reading MIC results. For this reason, the ETEST method was performed in triplicate, with the mean value used for interpretation, to reduce potential errors. Since these results were based on a limited number of isolates, further interaction testing against *C. auris* with trimethoprim–sulfamethoxazole should be performed.

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