Antagonistic *in vitro* interaction between olorofim and voriconazole against *Aspergillus fumigatus*

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Abstract

Background

The emergence of azole-resistance among *Aspergillus fumigatus* isolates has driven the development of novel antifungal compounds, which may potentially be used in combination with existing antifungal drugs.

Objectives

In this study, we evaluated the *in vitro* interaction between olorofim, a novel orotomide antifungal, and voriconazole, a triazole agent, against *A. fumigatus* using two complementary approaches.

Methods

A liquid microdilution checkerboard assay was performed in duplicate on 32 clinical isolates, including 27 voriconazole-susceptible and 5 voriconazole-resistant isolates, to determine the minimum inhibitory concentrations of both drugs alone and in combination. Interactions were quantified using the fractional inhibitory concentration index and through response surface modelling based on the Bliss independence model. Furthermore, an agar-based method was conducted on a subset of 11 isolates. Media were supplemented with either voriconazole or olorofim, on which disks containing the reciprocal drug were applied. Additionally, interaction was assessed by using Etest® strips of voriconazole on olorofim-containing agar.

Results

Antagonism was observed for 56% of isolates based by checkerboard technique and for 75% of the isolates based on response surface analysis. Antagonistic interaction was observed for all the voriconazole-resistant isolates. Across all tested isolates and agar configurations, antagonistic interactions were consistently observed, including reduced inhibition zones

around olorofim disks in the presence of voriconazole and paradoxical fungal growth surrounding voriconazole disks or strips on olorofim-containing plates.

Conclusions

These findings demonstrate the *in vitro* antagonism between olorofim and voriconazole and underscore the need for further investigation before considering their combined clinical use.

Introduction

Invasive aspergillosis caused by *Aspergillus fumigatus* is a life-threatening fungal infection that primarily affects immunocompromised individuals, such as those undergoing chemotherapy, solid organ transplantation, or hematopoietic stem cell transplantation, but also immunocompetent patients with severe viral infections. [1-3] Voriconazole or isavuconazole can be used as first-line treatment for invasive aspergillosis due to its potent antifungal activity and good clinical efficacy. [3, 4] However, the increasing prevalence of azole-resistant *A. fumigatus* strains has become a significant challenge, leading to higher mortality rates and limited treatment options. [5-7] The development of resistance, mainly associated with mutations in the cyp51A gene and alterations in its promoter, [8, 9] compromises the efficacy of azole monotherapy and necessitates alternative therapeutic strategies. [7]

Combination therapy is an attractive strategy that can enhance antifungal efficacy, expand the spectrum of activity, and potentially reduce resistance development. [10] Combination therapy using voriconazole and an echinocandin is currently recommended in case of moderate azole resistance. [11] Nevertheless, drug interactions in combination regimens are unpredictable and can lead to synergistic, indifferent (no interaction), or even antagonistic effects.

Another approach to overcome azole resistance is the development of novel antifungal agents with unique mechanisms of action. Unlike azoles, which target the ergosterol biosynthesis, these new drugs act on distinct fungal pathways, reducing the likelihood of cross-resistance. [12] One of these promising antifungals is olorofim, first member of the orotomide class, which inhibits the dihydroorotate dehydrogenase, an essential enzyme in the fungal pyrimidine biosynthesis pathway. [12-14] Olorofim has demonstrated potent *in vitro* activity against *A. fumigatus*, including azole-resistant isolates [15-18] as well as against other filamentous fungi, [19-21] and is currently undergoing clinical evaluation for the treatment of invasive fungal

infections. [13] Its novel mechanism of action and its broad-spectrum efficacy make it a strong candidate for addressing the rising challenge of azole resistance.

In the context of treating refractory infections, olorofim could potentially be used in combination with other antifungals, particularly azoles like voriconazole. However, recent studies suggest that a possible antagonism could exist between olorofim and azoles, raising concerns about the clinical implications of such combinations and highlighting the need for further investigations. [22] Concerning the potential clinical use of this combination for severe aspergillosis cases, particular attention should be given to the impact of antagonistic interactions, which could compromise treatment outcomes.

The purpose of the present study was to evaluate the *in vitro* interaction between voriconazole and olorofim against clinical isolates of *A. fumigatus*, including both azole-susceptible and azole-resistant isolates.

Materials and Methods

Isolates

A collection of 32 clinical isolates of *A. fumigatus*, including 27 voriconazole-susceptible, and 5 voriconazole-resistant isolates with known CYP51A sequence (TR34/L98H, WT, F46Y/M172V/N248T/D255E/E427K), was used. Voriconazole-resistant isolates were previously identified to the species level by sequencing a part of the beta-tubulin gene as previously described. [23] All other isolates were previously identified by Matrix Assisted Laser Desorption Ionization - Time Of Flight (MALDI-TOF) mass spectrometry. Isolates were subcultured from frozen stocks on Sabouraud dextrose agar supplemented with chloramphenicol and gentamicin to ensure purity. The two reference strains *A. fumigatus* ATCC 204305 and *Aspergillus flavus* ATCC 204304 were used as quality controls.

Drugs and medium

Double strength Roswell Park Memorial Institute (RPMI) 1640 medium with l-glutamine but without sodium bicarbonate (Sigma-Aldrich, Saint Quentin Fallavier, France), supplemented with 4% of glucose, was buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (VWR, Fontenay-sous-Bois, France), filter-sterilized, and stored at 4°C until used. The drugs included in this study were voriconazole (Sigma-Aldrich), and olorofim (CymitQuimica, Barcelona, Spain). Stock solutions were prepared in DMSO at 3200 mg/L for voriconazole and 1600 mg/L for olorofim. Stock solutions were kept at -80°C until used.

Microplate preparation for checkerboard experiments

The *in vitro* interactions between voriconazole and olorofim were first assessed by a microdilution broth testing format using 96-well flat-bottom microtiter plates (VWR). A checkerboard procedure based on the guidelines of the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antimicrobial Susceptibility Testing (EUCAST-

AFST) reference technique, was used. [24] Working solutions at four times the desired final concentrations were prepared by following the drug dilution scheme recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). A total of 50 µL of each drug concentration was dispensed into the wells of the 96-well plate. Voriconazole was distributed across the horizontal axis from column 1 to 11, while olorofim was distributed along the vertical axis from row A to H. Row H contained voriconazole alone to assess its individual activity, and column 11 contained only olorofim. Column 12 served as a growth control, containing only the culture medium with the solvent and the inoculum, but without any antifungal agents. Final concentrations ranged from 0.03 to 16 mg/L for voriconazole, and from 0.002 to 0125 mg/L for olorofim. Microplates were kept at −20°C before being used.

Inoculum and incubation

Mature cultures of *A. fumigatus* were obtained by growing the isolates on Sabouraud dextrose agar for 2-5 days at 37°C. A standardized inoculum of conidia was prepared as follow: the conidia were carefully rubbed with a sterile cotton swab and transferred to a sterile tube containing 5 ml sterile water supplemented with Tween 20. The suspension was vortexed for 15 seconds and adjusted to a concentration of 2-5x10^6 CFU/mL by counting the conidia in a haemocytometer chamber. The conidial suspension was diluted 1/10 in sterile water. Each well of the plates was inoculated with 100 µL of the conidial suspension resulting in a final inoculation size of 1-2.5 x 10^5 CFU/mL. Plates were incubated at 37°C for 48 hours under standard conditions. The experiments were performed in duplicate. A blank plate was prepared by filling each well with 100µl of sterile water in place of the inoculum and was incubated in the same conditions.

Reading

Spectrophotometric readings were obtained using an automated Dynex MRX spectrophotometer (Dynex Technology, Chantilly, VA, USA) at 550 nm. Optical density values from the uninoculated plate were subtracted as blanks, and the percentage of growth in each well was determined by comparing it to the growth observed in drug-free control wells. A growth inhibition endpoint of 90% was used to assess the effects of the drugs both alone and in combination.

Analysis of the results

The analysis of the checkerboard assay results was performed using two complementary approaches. [25, 26] The first approach was based on the Loewe additivity model, in which the fractional inhibitory concentration index (FICI) was calculated to assess the interaction between voriconazole and olorofim. The FICI was determined as the sum of the fractional inhibitory concentrations (FICs) of each drug. The FIC was calculated as the minimum inhibitory concentration (MIC) of the drug in combination divided by its MIC alone. For calculations, high off-scale MICs were converted to the next highest concentration. The interaction was interpreted based on standard FICI thresholds, with values ≤0.5 indicating synergy, >0.5 to ≤4.0 no interaction, and >4.0 antagonism. [27] When no antagonism was observed (i.e., all FICIs were ≤4.0), the lowest FICI value was reported as the representative value for the drug interaction. However, if any well exhibited a FICI >4.0, indicating antagonism, the highest FICI value was reported. This approach ensured that potential antagonistic interactions were not overlooked and that the most synergistic effect could be captured when no antagonism was present.

The second approach was based on response surface analysis, which does not rely on a predefined inhibitory endpoint. This method assessed the overall interaction between the two drugs across the full range of tested concentrations. Experimental data, expressed as the

percentage of growth for each well, were used to fit the dose-response curve of each drug alone to a Hill equation. Based on these individual dose-response curves, a theoretical response surface was generated using the Bliss independence model, representing no interaction. The experimental response surface was then compared to this model to calculate the synergy distribution across the concentration range. For visualization, the synergy/antagonism levels were mapped onto the experimental combination dose-response surface, providing a comprehensive view of interaction patterns. To summarize the synergy/antagonism distribution, the SUM-SYN-ANT metric was calculated. This metric represents the sum of synergy and antagonism observed within the tested concentration space. To define thresholds for interpretation, a control experiment was performed using a combination of voriconazole combined with itself, which yielded a SUM-SYN-ANT value of 13.9%. Synergy was concluded when the SUM-SYN-ANT exceeded 13.9%, while antagonism was defined by a value below -13.9%. A no-interaction outcome was assigned to values between -13.9% and 13.9%. Data analysis and visualization were performed using the Combenefit software. [28]

Agar diffusion tests

A subset of 11 clinical isolates of *A. fumigatus,* including 9 voriconazole-susceptible and 2 voriconazole-resistant isolates, were randomly selected among the tested isolates. The reference strain *A. fumigatus* ATCC 204305 was included as quality control.

Double strength RPMI 1640 medium was prepared as described above and warmed at 56°C. Double strength agar solution was sterilized in an autoclave (121°C, for 30 minutes) and then let cool down to 56°C. The RPMI agar solution was obtained by mixing agar and RPMI in equal volumes. Antifungal stock solutions were then added to obtain agar plates at varying concentrations of voriconazole or olorofim. Agar plates were kept at 4°C before being used.

The *in vitro* interactions between voriconazole and olorofim were assessed using 3 different agar diffusion methods. In the first assay, disks impregnated with 4 µg of olorofim (10 µl of a solution at 400 mg/L) were placed on agar medium supplemented with varying concentrations of voriconazole ranging from 0.06 to 1 mg/L for voriconazole-susceptible isolates and from 0.5 to 8 mg/L for voriconazole-resistant isolates. In the second assay, disks impregnated with 1 µg of voriconazole (10 µl of a solution at 100 mg/L) were placed on agar medium supplemented different concentrations of olorofim ranging from 0.004 to 0.06 mg/L. In the third assay voriconazole gradient concentration strips (Etest, Biomérieux) were placed on agar medium supplemented with different concentrations of olorofim ranging from 0.004 to 0.06 mg/L. Each isolate was tested in parallel on plain agar (without antifungal). The agar plates were manually inoculated with a spore suspension adjusted to a concentration of 2-5x10^6 CFU/mL with a sterile cotton swab in three directions. Antifungal disks or strips were placed on the agar plates after inoculation and plates were incubated at 37°C for 48 hours under standard conditions. At 48 hours pictures of the agar plates were taken. Pattern of growth was recorded and inhibition zone diameters around the antifungal disks were measured using a caliper by taking two perpendicular measurements and calculating the mean value.

A paired Student's t-test was used to compare the inhibition zone diameters observed with and without the antifungal agent on agar media. A $p < 0.05$ was considered significant.

Results

Checkerboard assay

Results of the interactions between voriconazole and olorofim evaluated by checkerboard and interpretation of the results by FICI and by response surface analysis against 32 clinical isolates of *A. fumigatus* are summarized in Table 1.

The 32 *A. fumigatus* isolates exhibited MICs for voriconazole ranging from 0.25 to 4 mg/L with a geometric mean of 0.52 mg/L and olorofim exhibited MICs ranging from 0.008 to 0.03 mg/L with a geometric mean of 0.017 mg/L. Similar results were observed for the two replicates with MICs within 2 $\log_2$ dilutions in 100 % of the cases. The interaction was antagonistic (FICI > 4) for 56% of the isolates with FICIs ranging from 4.25 to 16.5. For all other isolates, no interaction was observed. This antagonism concerned 100% (5/5) of voriconazole-resistant isolates and 48% (13/27) of the voriconazole-susceptible isolates.

Analysis of the checkerboard data by the response surface approach led to similar results compared to the FICI results (Table 1). The SUM-SYN-ANT metric, calculated by the Bliss model, was indicative of an antagonistic interaction between the two drugs for 75% of the isolates and ranged from -15.3 to -76.7, with a mean of -25.1. There was no interaction between the drugs for the other isolates. Synergy was never observed. Similar to the FIC study, antagonism was observed for 66% of voriconazole-susceptible isolates and 100% of voriconazole-resistant isolates.

An example of the response surface analysis for an azole-susceptible and -resistant isolate is presented in Figure 1 and 2, respectively.

Agar diffusions assays

The interaction of voriconazole with olorofim was also evaluated using three different agar diffusion methods on a subset of 12 *A. fumigatus* comprising of 10 azole-susceptible and 2 azole-resistant isolates.

In the first assay, growth inhibition around olorofim-impregnated disks was evaluated on agar containing different concentrations of voriconazole. Compared to plain agar, the addition of voriconazole led to a significant reduction in olorofim inhibition diameters ($p < 0.0001$), indicating an antagonistic interaction. Without voriconazole, the mean inhibition diameter was 29.6 mm for azole-susceptible isolates and 29.5 mm for azole-resistant ones. In presence of 0.06 mg/L of voriconazole, the diameter decreased to 20.0 mm for susceptible isolates, and to 22.3 mm for resistant isolates in presence of 0.5 mg/L of voriconazole (Table 2, Figure 3). As expected, no fungal growth was observed at voriconazole concentrations ≥ 0.25 mg/L for susceptible isolates and ≥ 2 mg/L for resistant ones. This antagonism seemed to be concentration-dependent ($p < 0.005$), with higher voriconazole levels leading to smaller inhibition zones. At 0.125 and 1 mg/L respectively, the mean inhibition diameter was 17.2 mm for voriconazole-susceptible isolates and 17.0 mm for voriconazole-resistant isolates. (Table 2).

In the second assay, voriconazole-impregnated paper disks were placed on agar plates containing olorofim at concentrations above the MIC of the isolate. For azole-susceptible isolates, the addition of 0.004 mg/L of olorofim to the agar also led to a significant reduction in the mean inhibition diameter of voriconazole, from 28.2 mm to 26.3 mm ($p < 0.05$). This antagonistic effect appeared to be concentration-dependent ($p < 0.01$), as higher olorofim levels further decreased inhibition zones, with a mean diameter of 23.5 mm observed at 0.008 mg/L. Additionally, a ring-like growth pattern was seen around the voriconazole disk, indicating an antagonistic interaction between the two drugs (Figure 4A).

For azole-resistant isolates, no inhibition zone was observed around the voriconazole disk on plain agar. However, when olorofim was added at supra-MIC concentrations, a zone of paradoxical growth appeared around the voriconazole disk, again indicating an antagonistic interaction (Figure 4B).

In the third assay, voriconazole MICs determined by gradient strips were measured on agar containing increasing concentrations of olorofim. For azole susceptible isolates MICs for voriconazole ranged from 0.125 to 0.19 mg/L with a geometric mean of 0.151 mg/L. For azole resistant isolates, MICs ranged from 2 to 4 mg/L with a geometric mean of 2.82 mg/L. There was no difference in MIC when olorofim was added to the agar plates. However, as in the previous experiment, at supra-MIC concentration of olorofim a zone of paradoxical growth around the voriconazole strips was observed, indicative of an antagonistic interaction between the two drugs (Table 3, Figure 5).

Discussion

In the present study, the *in vitro* interaction between olorofim and voriconazole against *A. fumigatus* isolates was assessed using checkerboard and agar diffusion methods. Moreover, the results of the checkerboard experiments were analysed by two different methods: calculation of the FICI based on the Loewe additivity model, and by response surface analysis based on the Bliss independence model. Both techniques consistently demonstrated antagonism between the two drugs. Antagonism was observed in both azole-susceptible and azole-resistant *A. fumigatus* isolates.

Van Rhijn and colleagues previously investigated the interaction between olorofim and triazole antifungals (voriconazole and itraconazole) against an azole-susceptible *A. fumigatus* isolate and its laboratory-derived TR34/L98H mutant. [22] Despite the distinct mechanisms of action of olorofim and azoles, they observed a strong antagonism, with azoles reducing the activity of olorofim. In checkerboard assays, high FIC indices of 4 to 6 indicated significant antagonism, accompanied by a fourfold increase in olorofim MIC. Agar diffusion tests also showed that voriconazole promoted fungal growth inside the olorofim inhibition zone. In the present study, we confirmed and expanded upon these observations by demonstrating antagonism between the two drugs against a large collection of clinical *A. fumigatus* isolates, including both voriconazole-susceptible and -resistant isolates. The antagonism observed between olorofim and voriconazole may be explained by the impact of azoles on fungal metabolism, particularly the upregulation of pyrimidine biosynthesis pathways. Azoles inhibit ergosterol synthesis, leading to a cellular stress response in *A. fumigatus* that enhances the activity of pathways involved in nucleotide production, including pyrimidine biosynthesis. Since olorofim targets dihydroorotate dehydrogenase, a key enzyme in this pathway, the increased flux through pyrimidine biosynthesis could diminish the drug's efficacy. This

metabolic compensation likely contributes to the reduced susceptibility to olorofim observed when combined with azoles. [22] It has been previously shown that the combination of flucytosine (a fluorinated cytosine analogue) and voriconazole exhibits antagonistic activity against *A. fumigatus* and *Aspergillus terreus*, [29] suggesting a possible interplay between the antifungal effects of azoles and pyrimidine-related activity.

Antifungal combinations are often used to treat invasive aspergillosis, particularly in severe or refractory cases. The combination of azoles with echinocandins is among the most studied and used in patients, as it involves drugs with distinct mechanisms of action and has not demonstrated antagonistic interactions. [30-32] Nevertheless, in a multicentre, randomized trial, combination therapy with voriconazole and anidulafungin did not significantly improve overall mortality compared to voriconazole monotherapy in patients with invasive aspergillosis, although a trend towards benefit was observed in certain subgroups. [33] Since olorofim is intended for the treatment of severe and refractory infections, it will likely be used in combination with other antifungals, particularly azoles. Combination therapy involving olorofim has already been reported, for example, in the treatment of refractory *Microascus* spp. infections with olorofim and terbinafine. [34] Therefore, the observed *in vitro* antagonism between olorofim and azoles raises concerns about the potential efficacy of such combinations in clinical settings. It is crucial to confirm these *in vitro* findings with *in vivo* studies and clinical data before considering combination therapy in patients.

Although our study used two complementary techniques (checkerboard and agar diffusion) to validate the results and included both azole-susceptible and azole-resistant isolates, it has some limitations. First, *in vitro* findings may not fully translate to clinical outcomes, as we did not perform *in vivo* validation using experimental animal models. Second, mechanistic

investigations were not conducted, and a deeper understanding of the causes of antagonism may be important for the potential clinical use of this combination.

Therefore, further studies are needed to determine whether antagonism occurs *in vivo* and whether it impacts clinical outcomes. Additionally, it is important to explore the *in vitro* interaction between olorofim and voriconazole against other fungal pathogens, including non-*fumigatus Aspergillus* species and *Scedosporium*/*Lomentospora* species. It should also be evaluated whether the degree of antagonism is similar with other azoles, such as itraconazole, posaconazole, and isavuconazole.

In summary, this study provides strong evidence of *in vitro* antagonism between olorofim and voriconazole against *A. fumigatus*. These findings underscore the need for caution when considering combination therapy involving olorofim and azoles. Future research should focus on mechanistic studies and validation in experimental animal models to guide optimal antifungal treatment strategies.

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Transparency declarations

During the past 5 years, Eric Dannaoui has received research grants from Biomérieux; travel grants from Gilead, Pfizer, and Mundipharma, and speaker's fee from Pfizer and Mundipharma. Patrick Schwarz has received research grants from Basilea Pharmaceutica, Gilead, and Pfizer; travel grants from Gilead and Pfizer; and speaking fees from Pfizer and Insmed.

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Table 1: Interaction of voriconazole with olorofim against 32 *Aspergillus fumigatus* clinical isolates and 1 reference strain by checkerboard and interpretation by fractional inhibitory concentration index and response surface analysis.

Isolate	Checkerboard MICs ug/mL					Response surface analysis	
	VRZ alone	OLO alone	VRZ/OLO in combination	FICI	INTPN	SUM-SYN-ANT	INTPN
AF 94	4	0.015	2/0.25	16.5	IND	-76.65	ANT
AF 2372	4	0.03	2/0.25	8.5	ANT	-59.01	ANT
AF 2676	1	0.015	0.5/0.06	4.5	ANT	-39.10	ANT
AF 4083	4	0.015	4/0.13	9	ANT	-48.40	ANT
AF R 52	4	0.015	2/0.06	4.5	ANT	-31.32	ANT
AF 4017	0.5	0.015	0.5/0.06	5	ANT	-28.57	ANT
AF 7802	0.5	0.015	0.25/0.06	4.5	ANT	-30.19	ANT
AF 7858	2	0.015	0.25/0.002	0.25	IND	-33.61	ANT
AF 7883	1	0.015	0.5/0.002	0.63	IND	-33.84	ANT
AF 7957	0.25	0.015	0.25/0.002	1.13	IND	-11.75	IND
AF 7984	0.25	0.015	0.25/0.002	1.13	IND	-3.67	IND
AF 7896	0.25	0.015	0.03/0.015	1.13	IND	-24.39	ANT
AF 8013	0.5	0.015	0.25/0.06	4.5	ANT	-41.03	ANT
AF 8032	0.25	0.015	0.25/0.06	5	ANT	-26.03	ANT
AF 8037	0.25	0.015	0.25/0.002	1.13	IND	-29.26	ANT
AF 8041	0.5	0.015	0.03/0.015	1.06	IND	-24.71	ANT
AF 8053	0.5	0.015	0.03/0.015	1.06	IND	-29.42	ANT
AF 8054	0.25	0.015	0.25/0.06	5	ANT	-27.02	ANT
AF 8058	0.25	0.008	0.25/0.03	5	ANT	-17.10	ANT
AF 8064	1	0.015	0.25/0.06	4.25	ANT	-26.25	ANT

AF 8070	0.25	0.015	0.13/0.06	4.5	ANT	-13.10	IND
AF 8072	0.25	0.015	0.25/0.06	5	ANT	-15.26	ANT
AF 8073	0.25	0.008	0.13/0.03	4.5	ANT	-16.47	ANT
AF 8076	0.25	0.015	0.25/0.002	1.13	IND	-6.54	IND
AF 8089	0.5	0.015	0.03/0.015	1.06	IND	-11.86	IND
AF 8090	0.5	0.015	0.25/0.06	4.5	ANT	-9.01	IND
AF 8093	0.25	0.015	0.25/0.002	1.13	IND	-20.05	ANT
AF 8102	0.25	0.015	0.25/0.002	1.13	IND	-18.34	ANT
AF 8110	0.25	0.015	0.13/0.06	4.5	ANT	-9.45	IND
AF 8120	0.5	0.015	0.25/0.003	0.75	IND	-17.08	ANT
AF 8126	0.5	0.015	0.25/0.06	4.5	ANT	-12.70	IND
AF 8164	0.25	0.015	0.03/0.015	1.13	IND	-11.86	IND
ATCC 204305	0.25	0.015	0.25/0.002	1.13	IND	-11.01	IND

442 ANT: antagonism; ATCC: American Type Culture Collection; FICI: fractional inhibitory concentration index; IND: no interaction; INTPN:

443 interpretation; MIC: minimum inhibitory concentration; OLO: olorofim; VRZ: voriconazole.

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Table 2: Olorofim inhibition diameter on agar plates with two sub-inhibitory concentrations of voriconazole.

Isolate	OLO inhibition diameter (mm) on agar plates with the following VRZ concentration (mg/L)		
	0	**C1**	**C2**
AF 8064	29	17	15
AF 2372*	31	21	19
AF 7896	29	21	13
AF 4083*	28	23.5	15
AF 7883	30	21	18
AF 8073	30	20	19
AF 8058	29	18.5	19
AF 7984	32.5	21	16
AF 8041	27	19	17
AF 8072	29	18	16
AF 8076	31	25	21.5
ATCC 204305	29	19.5	15

*VRZ-resistant isolates; OLO: olorofim; VRZ: voriconazole; C1 = 0.06 and C2 = 0.125 mg/L for VRZ-susceptible isolates while C1 = 0.5 and C2 = 1 mg/L for VRZ-resistant isolates.

Table 3: Voriconazole inhibition diameter and gradient strip MIC on agar plates with two sub-inhibitory concentrations of olorofim.

Isolate	VRZ Inhibition diameters (mm) with the following OLO concentration (mg/L)			VRZ gradient strip MICs (mg/L) with the following OLO concentration (mg/L)		
	0	0.004	0.008	0	0.004	0.008
AF 8064	28	26	24	0.19	0.19	0.19
AF 2372*	≤6	≤6	ND	2	2	2
AF 7896	29	29	23	0.125	0.125	0.125
AF 4083*	≤6	≤6	ND	4	4	4
AF 7883	26.5	25	23	0.125	0.125	0.125
AF 8073	28	25	23	0.19	0.19	0.19
AF 8058	27	25	23	0.19	0.19	0.19
AF 7984	26	27	NG	0.125	0.125	0.125
AF 8041	29	26	24	0.19	0.19	0.19
AF 8072	28.5	27	26	0.125	0.125	0.125
AF 8076	32	27	22	0.125	0.125	0.125
ATCC 204305	29	30.5	28	0.125	0.125	0.125

*VRZ-resistant isolates; ATCC: American Type Culture Collection; MIC: minimum inhibitory concentration; NG: no fungal growth observed; ND: not done; OLO: olorofim; VRZ: voriconazole.

Figure legends:

Figure 1: Combination of olorofim with voriconazole against azole-susceptible *Aspergillus fumigatus* isolate AF 8053 analysed by response surface modelling based on the Bliss model. (A, B) Dose-response curve of voriconazole and olorofim alone. (C) Predicted response surface (prediction of effect if there is no interaction between the drugs). (D) Synergy mapped on the experimental response surface. Analyses were performed by combining results from two independent experiments. VRZ: voriconazole; OLO: olorofim.

Figure 2: Combination of olorofim with voriconazole against azole-resistant *Aspergillus fumigatus* isolate AF 2372 analysed by response surface modelling based on the Bliss model. (A, B) Dose-response curve of voriconazole and olorofim alone. (C) Predicted response surface (prediction of effect if there is no interaction between the drugs). (D) Synergy mapped on the experimental response surface. Analyses were performed by combining results from two independent experiments. VRZ: voriconazole; OLO: olorofim.

Figure 3: Agar-based combination assay of voriconazole and olorofim on *Aspergillus fumigatus* AF8072 (row A, azole-susceptible) and *A. fumigatus* AF2372 (row B, azole-resistant). Disks containing 4 µg of olorofim were placed on plain agar or agar supplemented with increasing concentrations of voriconazole. Growth was assessed after 48 hours. A reduction of the inhibition diameter of olorofim is observed as the concentration of voriconazole increases and approaches the MIC. Growth is completely inhibited at voriconazole concentration of 0.25 mg/L for isolate AF 8072 and 2 µg/ml for isolate AF 2372. VRZ: voriconazole.

Figure 4: Agar-based combination assay of voriconazole and olorofim on *A. fumigatus* AF8072 (row A, azole-susceptible) and *A. fumigatus* AF2372 (row B, azole-resistant). Disks containing 1 µg of voriconazole were placed on plain agar or agar supplemented with increasing concentrations of olorofim. Growth was assessed after 48 hours. For the azole-susceptible isolate AF 8072 (A), the voriconazole inhibition diameter is reduced in presence of olorofim and a paradoxical ring of fungal growth is observed at olorofim concentration of 0.004 µg/mL and 0.008 µg/mL, indicative of an antagonistic interaction between the two drugs. For the azole-resistant isolate AF 2372 (B), voriconazole is inactive with no visible inhibition zone on plain agar. In presence of olorofim at supra-MIC concentrations (0.008 µg/mL and above), growth around the voriconazole disk is enhanced indicative of antagonistic interaction between the two drugs. OLO: olorofim.

Figure 5: Agar-based combination assay of voriconazole and olorofim on *A. fumigatus* isolate AF 7896 (row A, azole-susceptible) and *A. fumigatus* isolate AF 2372 (row B, azole-resistant). Gradient concentration strips (Etest) of voriconazole were placed on plain agar or agar supplemented with increasing concentrations of olorofim. Growth was assessed after 48 hours. Paradoxical growth around the voriconazole strip is indicative of antagonistic interaction between the two drugs. OLO: olorofim.

Figure 1.

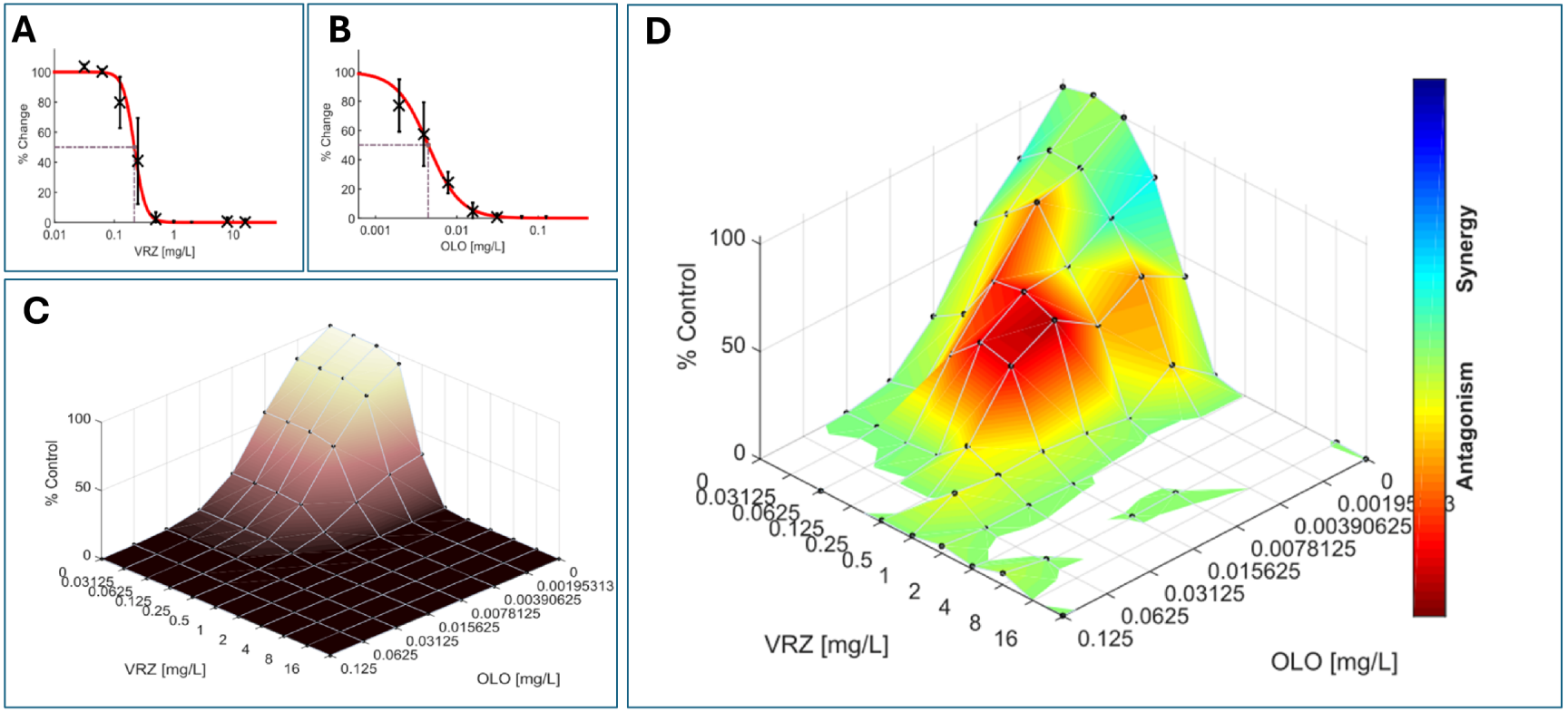

A
% Change
VRZ [mg/L]
B
% Change
OLO [mg/L]
C
% Control
VRZ [mg/L]
OLO [mg/L]
D
% Control
VRZ [mg/L]
OLO [mg/L]
Synergy
Antagonism

Figure 2.

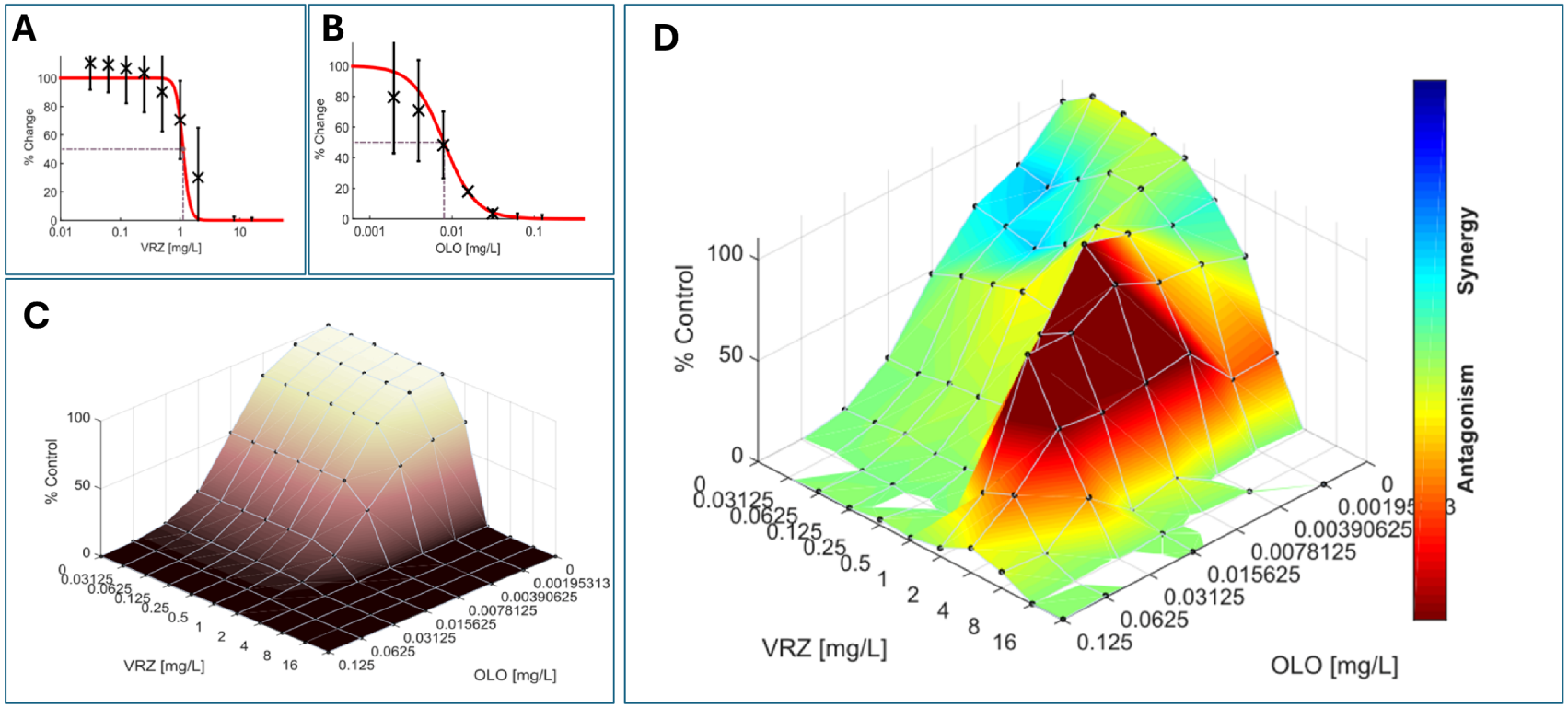
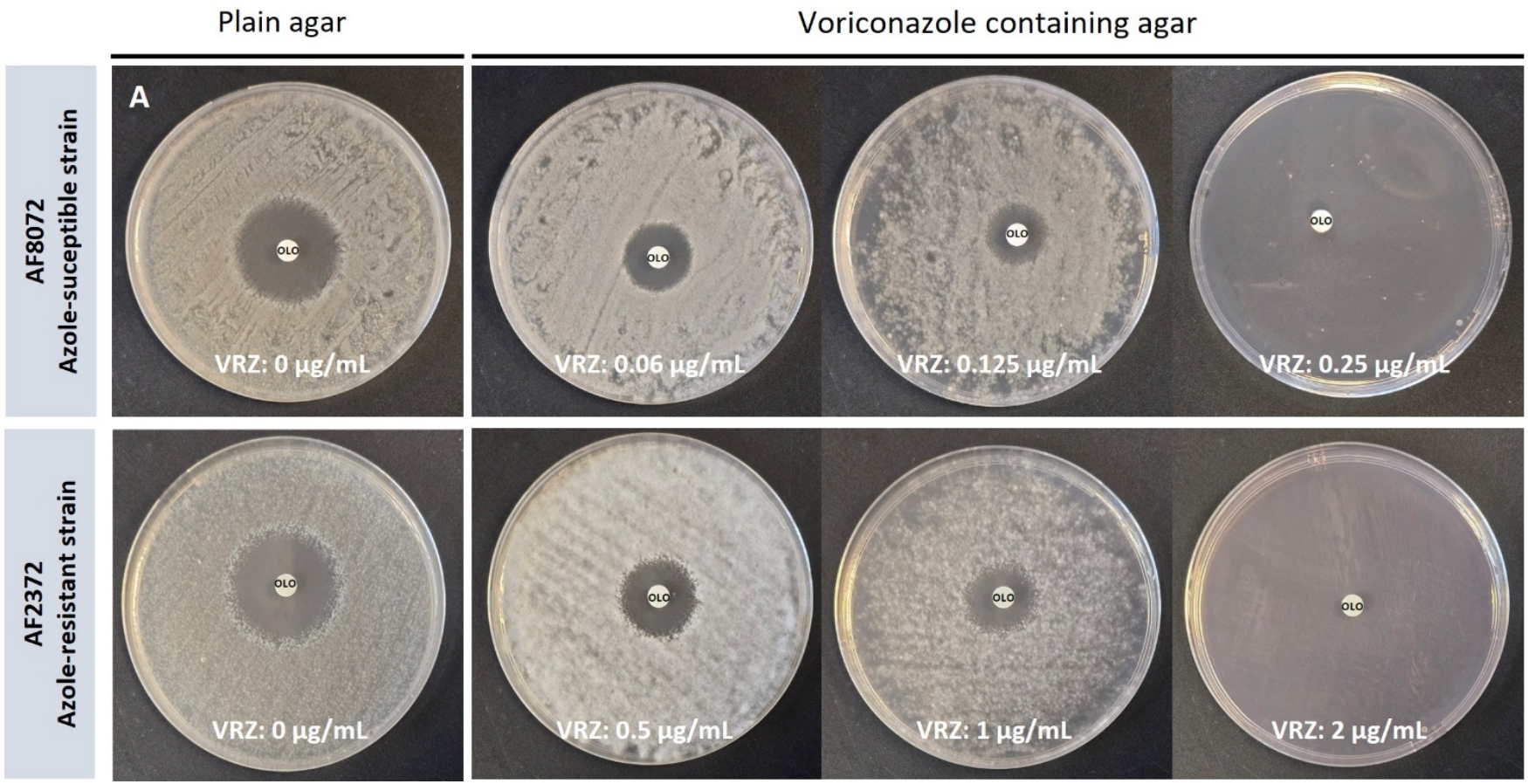

A
% Change
VRZ [mg/L]
B
% Change
OLO [mg/L]
C
% Control
VRZ [mg/L]
OLO [mg/L]
D
% Control
VRZ [mg/L]
OLO [mg/L]
Synergy
Antagonism

Figure 3.


Plain agar
Voriconazole containing agar
AF8072
Azole-suceptible strain
AF2372
Azole-resistant strain
A
VRZ: 0 µg/mL
VRZ: 0.06 µg/mL
VRZ: 0.125 µg/mL
VRZ: 0.25 µg/mL
VRZ: 0 µg/mL
VRZ: 0.5 µg/mL
VRZ: 1 µg/mL
VRZ: 2 µg/mL
OLO

508 **Figure 4.**

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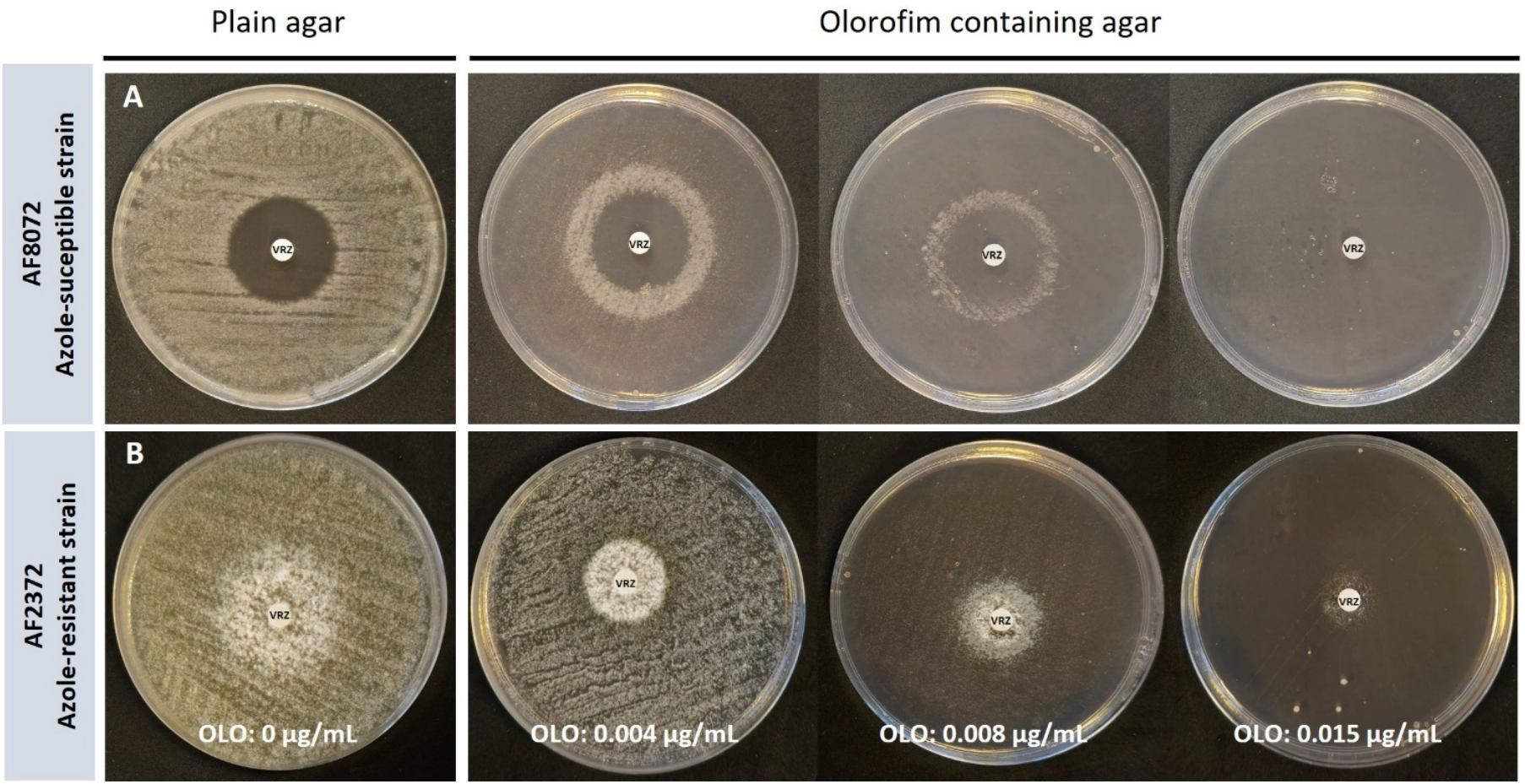

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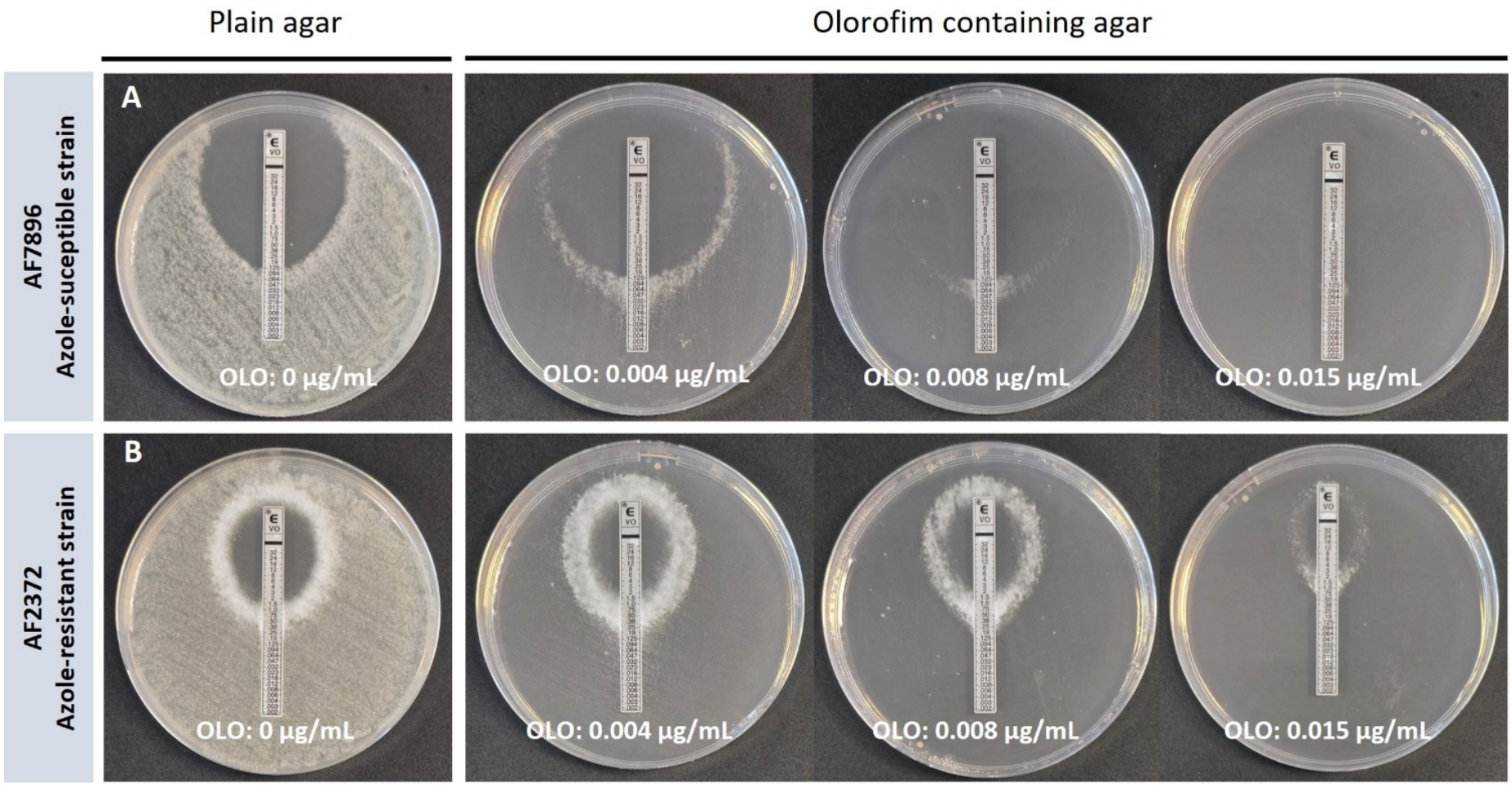
Figure 5.


Plain agar
Olorofim containing agar
AF7896
Azole-suceptible strain
A
OLO: 0 µg/mL
OLO: 0.004 µg/mL
OLO: 0.008 µg/mL
OLO: 0.015 µg/mL
AF2372
Azole-resistant strain
B
OLO: 0 µg/mL
OLO: 0.004 µg/mL
OLO: 0.008 µg/mL
OLO: 0.015 µg/mL