In vitro Evaluation of Antifungal Drug Combinations against Multidrug-resistant *Candida auris* isolates from New York Outbreak

Brittany O'Brien[1], Sudha Chaturvedi[1,2], Vishnu Chaturvedi[1,2]*

Mycology Laboratory[1], Wadsworth Center New York State Department of Health, and Department of Biomedical Sciences[2], University at Albany School of Public Health, Albany, NY, USA

Running title: *C. auris* antifungal combination

*Corresponding author: Mycology Laboratory, Wadsworth Center, New York State Department of Health, 120 New Scotland Ave., Albany, NY 12208, USA

Tel: +1-518-474-4177; FAX: +1-518-486-7971; E-mail: Vishnu.Chaturvedi@health.ny.gov

orcid.org/0000-0002-3922-9676

Abstract

Since 2016, New York hospitals and healthcare facilities have faced an unprecedented outbreak of pathogenic yeast *Candida auris*. We tested over one thousand *C. auris* isolates from affected facilities and found high-resistance to fluconazole (FLC, MIC_{50} >256 mg/L), and variable resistance to other antifungal drugs. Therefore, we evaluated if two-drug combinations are effective *in vitro* against multidrug-resistant *C. auris*. Broth micro-dilution (BMD) plates were custom-designed, and quality controlled by TREK Diagnostic System. We used MIC_{100} endpoints for the drug combination readings as reported earlier for the intra- and inter-laboratory agreements against *Candida* species and *Aspergillus fumigatus*. The study results were derived from 12,960 MIC_{100} readings, for fifteen *C. auris* isolates tested against 864 possible two-drug antifungal combinations for nine antifungal drugs. Flucytosine (5FC) at 1.0 mg/L potentiated the most successful combinations with other drugs. Micafungin (MFG), Anidulafungin (AFG), Caspofungin (CAS) at individual concentrations of 0.25 mg/L in combination with 5FC (1.0 mg/L) yielded MIC_{100} for 14, 13, and 12 of 15 *C. auris* test isolates. AMB / 5FC (0.25/1.0 mg/L) yielded MIC_{100} for 13 isolates. None of the combinations were effective for *C. auris* 18-1, which tested resistant against FLC and 5FC, except POS/5FC (0.12/1.0 mg/L). The simplified two-drug combination susceptibility test format would permit laboratories to provide clinicians and public health experts with additional data to deal with multidrug-resistant *C. auris*.

39 **Introduction**

40 *Candida auris* is an emerging multidrug-resistant yeast, causing invasive
41 healthcare-associated infection with high mortality worldwide (1). It was described in
42 2009 as a new yeast species from the ear discharge of a Japanese patient (2). In the
43 ensuing years, many reports of multidrug-resistant *C. auris* infections came from South
44 Asia, South Africa, South America and travel-related cases from other parts of the
45 world (3-6). The largest, localized, uninterrupted *C. auris* outbreak in the US healthcare
46 facilities continues to afflict the New York metro areas (2013-2019); *C. auris* caused 23
47 deaths among the first 51 clinical case-patients in New York (7). In response, the New
48 York State Department of Health (NYSDOH) laboratory scientists and epidemiologists
49 developed enhanced protocols for the unprecedented surveillance and testing for *C.*
50 *auris* (8). To date, over 15,000 clinical samples from 150 facilities were processed in the
51 NYSDOH laboratory. The CLSI broth microdilution method (BMD) was used to test
52 nearly one thousand *C. auris* isolates, which revealed high-resistance to fluconazole
53 (MIC_{50} >256 mg/L), and variable resistance to other broad-spectrum antifungal drugs
54 (Chaturvedi et al. unpublished data). Therefore, we investigated the efficacy of two-
55 drug combinations against a representative set of fifteen drug-resistant *C. auris*.

56

57

58 **Materials and Methods**

59 Isolates–*C. auris* isolates were initially processed on Sabouraud dulcitol agar at 40℃,
60 and single colonies used to confirm identify with MALDI-TOF-MS and ITS -D1/D2
61 sequencing as described earlier. The isolates were stored at -80℃ in 20% sterile glycerol.
62 Drug combinations–Fluconazole (FLC) was not tested in combinations because of wide-
63 spread resistance in our isolate collection. Nine drugs were selected for the combination
64 testing: amphotericin B (AMB), anidulafungin (AFG), caspofungin (CAS), micafungin
65 (MFG), flucytosine (5FC), isavuconazole (ISA), posaconazole (POS), voriconazole (VRC)
66 and itraconazole (ITC). An advisory from the Centers for Diseases Control and
67 Prevention was used for C. auris antifungal susceptibility testing and interpretation
68 (https://www.cdc.gov/fungal/candida-auris/c-auris-antifungal.html).
69 Combination testing–Broadly, we followed a method described earlier from a multi-
70 laboratory evaluation of antifungal combination testing (9). Two fixed concentrations of
71 each drug were tested, a value conforming to Cmax in the product insert, and a
72 concentration considered resistant for a majority of pathogenic yeasts. The drug
73 combinations were dispensed in a total volume of 100 µl. The 96-well microtiter plates
74 were custom-manufactured and tested for sterility by TREK Diagnostic System, Part of
75 Thermo Fisher Scientific, Oakwood Village, OH, USA. They were transferred under dry
76 ice to NYSDOH Mycology Laboratory and stored at -80℃until used. The schematic of
77 plate design is provided in supplementary file 1. We also prepared two-fold dilutions of

78 each drug combination by adding 100 µl sterile RPMI 1640 to the initial well and
79 transferring 100 µl of 200 µl mix into micro-well of a new 96-well microtiter plate. This
80 dilution scheme provided 1/2, 1/4, 1/8, 1/16, and 1/32 concentrations of each drug in the
81 initial well. The inoculation, incubation, and MIC_{100} end-point reading, and *Candida*
82 *krusei* ATCC was the quality control strain, were as published earlier from a multi-
83 laboratory evaluation of antifungal combination testing (9). Briefly, *C. auris* cultures
84 were streaked on Sabouraud dextrose agar for 24 hours at 35℃, cells suspended in
85 sterile water to absorbance (A_{530}) 0.08-0.1 absorbance as measured with a Mettler Toledo
86 UV5 bio spectrophotometer; 20 µl of the cell suspension was added to the 11 ml of
87 RPMI 1640 broth tube. One-hundred-microliter of the suspension in RPMI 1640 was
88 added to each well of the 96-well microtiter plate with the drug combinations. The
89 plates were incubated at 35℃ for 48-hrs, and MIC_{100} reading was obtained with an
90 illuminated convex mirror plate reader. Twenty microliters of the drug-fungal
91 suspension from each well, after MIC_{100} determination, was transferred to the well of a
92 fresh 96-well microtiter plate that contained 180 µl of sterile RPMI-1640 to set-up the
93 sterility testing. We incubated the sterility plates at 35℃ for 48-hrs, and determined the
94 minimum fungicidal concentration (MFC) from each well with no visible growth. In
95 preliminary experiments, a good correlation was observed between MFC values
96 obtained from microbroth plates and Sabrouad dextrose agar (details not shown).
97

98 **Results**

99 Among the test group, *C. auris* 16-1 was susceptible to all antifungals tested
100 while the remaining fourteen isolates showed resistance to FLC (Table 1). The
101 distribution of resistance in remaining *C. auris* was triazole resistance (2 isolates),
102 echinocandin resistance (6 isolates), and AMB resistance (4 isolates) (Table 1). Only *C.*
103 *auris* 18-1 and 18-13 showed resistance to 5FC. The combination test results were
104 derived from 12,960 MIC_{100} readings, for fifteen *C. auris* isolates tested against 864 two-
105 drug antifungal combinations for nine antifungal drugs (Supplementary Table 1).
106 Flucytosine (5FC) at 1.0 mg/L potentiated the most successful combinations with other
107 drugs (Table 2). MFG, AFG, and CAS at individual concentrations of 0.25 mg/L
108 combined well with 5FC (1.0 mg/L) to yield MIC_{100} for 14, 13, and 12 out of 15 *C. auris*
109 isolates tested. AMB / 5FC (0.25/1.0 mg/L) yielded MIC_{100} for 13 isolates. Triazoles POS,
110 ISA and VRC also combined well with 5FC (0.25/2.0 mg/L) to yield MIC_{100} for 12, 13,
111 and 13 isolates, respectively. The summary results for the remaining combinations are
112 presented in supplementary table 2. Remarkably, MIC_{100} readings were obtained for all
113 selected combinations. *C. auris* 18-1, resistant to FLC and 5FC initially, also tested
114 resistant to all antifungal combinations except POS/5FC (0.12/1.0 mg/L) (Table 2).
115
116
117

118 **Discussion**

119 The clinical and public health microbiology community is at high alert due to the
120 alarming rise in the incidence and spread of drug-resistant *C. auris* globally (10). An
121 aggressive therapeutic intervention is indicated for invasive *C. auris* infections
122 associated with high mortality(7, 11). The results of the current study fill a gap in the
123 published literature, as there is no report on the efficacy of antifungal combinations
124 against *C. auris*. Our findings suggested that *C. auris* with various resistance patterns are
125 susceptible to low dose combinations of existing drugs. Several of the effective
126 combinations of antifungals exerted fungicidal activities, which was an additional
127 indicator of the drug combination efficacy. The earlier multi-laboratory evaluation
128 showed that MIC_{100} endpoints provide a reliable method for antifungal combination
129 testing vis-a-vis ∑FIC (the summation of fractional inhibitory concentration) index (9).
130 We further optimized the method by using fixed drug concentrations instead of the
131 checkerboard dilutions, as described by other authors in earlier publications (12, 13). As
132 almost all NY isolates belonged to the South Asian clade, it remains to be determined by
133 other investigators if the resistant *C. auris* strains from South Africa and South America
134 clades respond similarly to the antifungal combinations. Further clinical relevance of
135 the findings reported in the study could be evaluated by
136 pharmacokinetic/pharmacodynamic studies and testing in the animal models (14, 15).

137 In conclusion, a limited set of two drug-combinations of antifungals were efficacious *in*
138 *vitro* against fifteen multidrug-resistant *C. auris* strains.
139 The simplified two-drug combination susceptibility test format would permit
140 laboratories to provide clinicians and public health experts with additional data to deal
141 with multidrug-resistant *C. auris*.
142

143 **Acknowledgments**
144 We are grateful to the members of the New York State Healthcare Epidemiology
145 and Infection Control Program, *Candida auris* Investigation Workgroup, and the staff
146 members from various hospitals and long-term-care facilities for assistance with
147 surveillance samples.
148

149 **Funding**
150 This publication was supported in part by Cooperative Agreement number
151 NU50CK000516, funded by the Centers for Disease Control and Prevention. Its contents
152 are solely the responsibility of the authors and do not necessarily represent the official
153 views of the Centers for Disease Control and Prevention or the Department of Health
154 and Human Services.
155

156 **Transparency declaration**

B. O'Brien performed the experiments and tabulated and analyzed the data, S. Chaturvedi supervised single drug testing and interpretation, helped interpreting combination testing and critiqued draft manuscript, and V. Chaturvedi conceived and designed the study, interpreted data, and wrote the manuscript.

Supplementary Data

Supplementary File 1-Design of 96-well microtiter plates for the antifungal combination testing

Supplementary Table 1–Master file for all antifungal combination test results

Supplementary Table 2–Summary of less effective antifungal combinations against *Candida auris*

170 Table 1. Broth microdilution determination of antifungal MIC_{50} (mg/L) and interpretative

171 criteria (S, I, R) for the test isolates of *Candida auris*

172

C. auris	Source	Antifungal drug									
Susceptible		FLC	VOR	ITC	ISA	POS	AFG	CAS	MFG	AMB	5FC
16-1	Ear	8	0.25	0.5	0.25	0.25	0.12	0.12	0.12	0.5	0.5
		S	S	S	S	S	S	S	S	S	S
17-12	Urine	32	0.5	0.06	0.015	0.03	8	8	8	1	0.032
		S	S	S	S	S	R	R	R	S	S
18-14	NA	>256	1	0.25	0.5	0.03	0.06	0.015	0.06	1	0.047
		R	S	S	S	S	S	S	S	S	S
18-15	Blood	>256	1	0.25	0.5	0.03	0.12	0.03	0.06	1.5	0.064
		R	S	S	S	S	S	S	S	S	S
17-1	Blood	>256	2	0.5	0.5	0.25	4	4	4	1	0.094
		R	S	S	S	S	R	R	R	S	S
17-13	Urine	>256	1	0.25	0.5	0.03	0.06	0.03	0.06	3	0.032
		R	S	S	S	S	S	S	S	R	S
17-14	Groin swab	>256	2	0.5	0.5	0.12	4	2	4	0.75	0.032
		R	S	S	S	S	R	R	R	S	S
17-15	Skin rash swab	>256	2	0.5	0.5	0.25	4	2	4	0.75	0.032
		R	S	S	S	S	R	R	R	S	S
17-16	Urine	>256	2	0.5	0.5	0.12	4	2	4	1	0.023
		R	S	S	S	S	R	R	R	S	S
17-17	Nares swab	>256	4	0.5	0.5	0.06	0.25	0.03	0.06	3	0.064
		R	R	S	S	S	S	S	S	R	S
17-18	Nares swab	>256	2	0.5	1	0.12	0.25	0.06	0.06	3	0.064
		R	S	S	S	S	S	S	S	R	S
18-1	Wound swab	128	0.5	0.12	0.12	0.03	0.25	0.03	0.12	1	>32
		R	S	S	S	S	S	S	S	S	R
18-2	Ascites fluid	>256	2	1	1	0.25	8	2	4	1.5	0.064
		R	S	R	R?	S	R	R	R	S	S
18-5	Urine	>256	2	0.25	0.5	0.06	0.12	0.03	0.12	2	0.094
		R	S	S	S	S	S	S	S	R	S
18-13	Rectal swab	256	0.5	0.12	0.12	0.03	0.12	0.03	0.12	1	>32
		R	S	S	S	S	S	S	S	S	R

173

174 NA not available

175

176 Table 2. Most promising antifungal combinations for *Candida auris*

177

Combi-nation	End -reading	*Candida auris*														
		16-1	17-1	17-12	17-13	17-14	17-15	17-16	17-17	17-18	18-1	18-2	18-5	18-13	18-14	18-15
MFG + 5FC	MIC_{100}	0.12*/ 1.0	0.12/ 1.0	0.06/ 0.5	0.12/ 1.0	0.12/ 1.0	0.12/ 1.0	0.12/ 1.0	0.12/ 1.0	0.25/ 2.0	NA	0.12/ 1.0	0.12/ 1.0	0.12/ 1.0	0.12/ 1.0	0.12/ 1.0
AMB + 5FC	MIC_{100}	0.25/ 1.0	0.25/ 1.0	0.25/ 1.0	0.25/ 1.0	0.25/ 1.0	0.25/ 1.0	0.25/ 1.0	0.25/ 1.0	0.25/ 1.0	NA	0.25/ 1.0	0.25/ 1.0	NA	0.25/ 1.0	0.25/ 1.0
AFG + 5FC	MIC_{100}	0.12 / 1.0	0.12/ 1.0	0.12/ 1.0	0.12/ 1.0	0.12/ 1.0	0.25/ 2.0	0.12/ 1.0	0.12/ 1.0	0.25/ 2.0	NA	0.12/ 1.0	0.12/ 1.0	NA	0.12/ 1.0	0.12/ 1.0
CAS + 5FC	MIC_{100}	0.12/ 1.0	0.12/ 1.0	0.12/ 1.0	0.12/ 1.0	0.12/ 1.0	0.12/ 1.0	0.25/ 2.0	0.12/ 1.0	NA	NA	0.12/ 1.0	0.25/ 2.0	NA	0.12/ 1.0	0.12/ 1.0
POS + 5FC	MIC_{100}	0.12/ 1.0	0.12/ 1.0	0.12/ 1.0	NA	NA	0.12/ 1.0	0.12/ 1.0	0.12/ 1.0	0.12/ 1.0	0.12/ 1.0	0.12/ 1.0	0.12/ 1.0	NA	0.12/ 1.0	0.25/ 2.0
ISA + 5FC	MIC_{100}	0.12/ 1.0	0.12/ 1.0	0.12/ 1.0	0.12/ 1.0	0.12/ 1.0	0.12/ 1.0	0.12/ 1.0	0.12/ 1.0	0.12/ 1.0	NA	0.12/ 1.0	0.12/ 1.0	NA	0.12/ 1.0	0.25/ 2.0
ITC + 5FC	MIC_{100}	0.03/ 0.5	0.06/ 1.0	0.06/ 1.0	0.06/ 1.0	NA	0.06/ 1.0	0.06/ 1.0	0.06/ 1.0	0.06/ 1.0	NA	0.06/ 1.0	0.06/ 1.0	NA	0.06/ 1.0	0.12/ 2.0
VOR + 5FC	MIC_{100}	0.25/ 1.0	0.25/ 1.0	0.125/ 0.5	0.25/ 1.0	0.25/ 1.0	0.25/ 1.0	0.25/ 1.0	0.25/ 1.0	0.25/ 1.0	NA	0.25/ 1.0	0.25/ 1.0	NA	0.25/ 1.0	0.5/ 2.0

178

179 * mg/L

180 NA Not available

181 **References**

182 1. Lockhart SR, Etienne KA, Vallabhaneni S, Farooqi J, Chowdhary A, Govender NP,
183 Colombo AL, Calvo B, Cuomo CA, Desjardins CA. 2017. Simultaneous Emergence of
184 Multidrug-Resistant *Candida auris* on 3 Continents Confirmed by Whole-Genome
185 Sequencing and Epidemiological Analyses. Clinical Infectious Diseases 64:134-140.
186 2. Satoh K, Makimura K, Hasumi Y, Nishiyama Y, Uchida K, Yamaguchi H. 2009. *Candida*
187 *auris sp. nov*., a novel ascomycetous yeast isolated from the external ear canal of an
188 inpatient in a Japanese hospital. Microbiology and immunology 53:41-44.
189 3. Vallabhaneni S, Kallen A, Tsay S, Chow N, Welsh R, Kerins J, Kemble SK, Pacilli M, Black
190 SR, Landon E, Ridgway J, Palmore TN, Zelzany A, Adams EH, Quinn M, Chaturvedi S,
191 Greenko J, Fernandez R, Southwick K, Furuya EY, Calfee DP, Hamula C, Patel G, Barrett P,
192 Lafaro P, Berkow EL, Moulton-Meissner H, Noble-Wang J, Fagan RP, Jackson BR,
193 Lockhart SR, Litvintseva AP, Chiller TM. 2017. Investigation of the First Seven Reported
194 Cases of *Candida auris*, a Globally Emerging Invasive, Multidrug-Resistant Fungus-United
195 States, May 2013-August 2016. Am J Transplant 17:296-299.
196 4. Govender NP, Magobo RE, Mpembe R, Mhlanga M, Matlapeng P, Corcoran C, Govind C,
197 Lowman W, Senekal M, Thomas J. 2018. *Candida auris* in South Africa, 2012-2016.
198 Emerg Infect Dis 24:2036-2040.
199 5. Chowdhary A, Kumar VA, Sharma C, Prakash A, Agarwal K, Babu R, Dinesh K, Karim S,
200 Singh S, Hagen F. 2014. Multidrug-resistant endemic clonal strain of *Candida auris* in
201 India. European journal of clinical microbiology & infectious diseases 33:919-926.
202 6. Calvo B, Melo ASA, Perozo-Mena A, Hernandez M, Francisco EC, Hagen F, Meis JF,
203 Colombo AL. 2016. First report of *Candida auris* in America: Clinical and microbiological
204 aspects of 18 episodes of candidemia. Journal of Infection 73:369-374.
205 7. Adams E, Quinn M, Tsay S, Poirot E, Chaturvedi S, Southwick K, Greenko J, Fernandez R,
206 Kallen A, Vallabhaneni S, Haley V, Hutton B, Blog D, Lutterloh E, Zucker H, Workgroup.
207 *Ca*l. 2018. *Candida auris* in Healthcare Facilities, New York, USA, 2013-2017. Emerging
208 Infectious Diseases 24:1816-1824.
209 8. Leach L, Zhu Y, Chaturvedi S. 2018. Development and Validation of a Real-Time PCR
210 Assay for Rapid Detection of *Candida auris* from Surveillance Samples. J Clin Microbiol
211 56.
212 9. Ren P, Luo M, Lin S, Ghannoum MA, Isham N, Diekema DJ, Pfaller MA, Messer S,
213 Lockhart SR, Iqbal N, Chaturvedi V. 2015. Multilaboratory testing of antifungal drug
214 combinations against *Candida* species and *Aspergillus fumigatus*: utility of 100 percent
215 inhibition as the endpoint. Antimicrob Agents Chemother 59:1759-66.
216 10. Tsay S, Kallen A, Jackson BR, Chiller TM, Vallabhaneni S. 2018. Approach to the
217 Investigation and Management of Patients With *Candida auris*, an Emerging Multidrug-
218 Resistant Yeast. Clin Infect Dis 66:306-311.
219 11. Ruiz-Gaitan A, Moret AM, Tasias-Pitarch M, Aleixandre-Lopez AI, Martinez-Morel H,
220 Calabuig E, Salavert-Lleti M, Ramirez P, Lopez-Hontangas JL, Hagen F, Meis JF, Mollar-
221 Maseres J, Peman J. 2018. An outbreak due to *Candida auris* with prolonged
222 colonisation and candidaemia in a tertiary care European hospital. Mycoses 61:498-505.
223 12. Pfaller MA, Barry AL, Fuchs PC, Gerlach EH, Hardy DJ, McLaughlin JC. 1993. Comparison
224 of fixed concentration and fixed ratio options for dilution susceptibility testing of gram-

225 negative bacilli to ampicillin and ampicillin/sulbactam. Eur J Clin Microbiol Infect Dis
226 12:356-62.
227 13. Bradford PA, Huband MD, Stone GG. 2018. A Systematic Approach to the Selection of
228 the Appropriate Avibactam Concentration for Use with Ceftazidime in Broth
229 Microdilution Susceptibility Testing. Antimicrobial Agents and Chemotherapy
230 62:e00223-18.
231 14. Lepak AJ, Andes DR. 2015. Antifungal Pharmacokinetics and Pharmacodynamics. Cold
232 Spring Harbor Perspectives in Medicine 5.
233 15. Hager CL, Larkin EL, Long L, Zohra Abidi F, Shaw KJ, Ghannoum MA. 2018. *In Vitro* and *In*
234 *Vivo* Evaluation of the Antifungal Activity of APX001A/APX001 against *Candida auris*.
235 Antimicrobial Agents and Chemotherapy 62:e02319-17.
236
237