

1 **Rare modification in the ergosterol biosynthesis pathway leads to amphotericin B**
2 **resistance in *Candida auris* clinical isolates**

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27 **Running Head:** *Candida auris* amphotericin B resistance

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33 **ABSTRACT**

34 We determined amphotericin B (AmB) susceptibility and sequenced key genes of the ergosterol
35 biosynthesis pathway implicated in AmB resistance (*ERG2*, *ERG3*, *ERG6*, *ERG11*) of 321
36 clinical isolates of *Candida auris*. In antifungal susceptibility testing, 19 (5.9%) isolates were
37 categorized as AmB-resistant (MIC \geq 2 mg/l). Only one AmB-resistant isolate presented a unique
38 non-wild-type *ERG6* genotype that was confirmed to confer amphotericin B resistance (MIC >32
39 mg/l) when introduced into a susceptible strain (MIC = 0.5 mg/l).

40 Amphotericin B (AmB), a polyene antifungal drug, has a broad-spectrum activity against
41 pathogenic fungi, including *Candida* spp. (1). Its mode of action is rather unusual, as it does not
42 inhibit an enzyme. Instead, AmB binds to ergosterol (ERG) (2), an abundant sterol that regulates
43 permeability and fluidity of the fungal cell membrane (3). In most *Candida* species, AmB
44 resistance is rare in comparison to resistance to other antifungal drug classes (azoles and
45 echinocandins) (4). Most often, AmB resistance stems from alterations in the sterol composition
46 of the fungal cell membrane due to mutations in genes of the ERG biosynthesis pathway. Whole
47 genome sequence (WGS) analysis of AmB-resistant clinical isolates revealed a transposon
48 insertion in *ERG2* (C-8 sterol isomerase) of *C. albicans*, while a missense mutation in *ERG3* (C-5
49 sterol desaturase) and deletion of 170 nucleotides in *ERG11* (lanosterol 14 α -demethylase) were
50 detected in *C. tropicalis* (4). Moreover, a single AmB-resistant *C. albicans* isolate was reported
51 to harbor a substitution in *ERG11* and a sequence repetition (10 duplicated amino acids) leading
52 to loss of function of *ERG5* (C-22 sterol desaturase) (5). In *C. glabrata* clinical isolates, targeted
53 gene sequencing revealed the presence of AmB resistance-conferring mutations in *ERG2* and
54 *ERG6* (C-24 sterol methyltransferase) (6-8). Additionally, *in vitro* evolution experiments
55 confirmed the role of *ERG6* mutations in AmB resistance in *C. albicans* (4).

56 Clinical isolates of *C. auris*, a recently emerged nosocomial pathogen, were reported to have a
57 higher prevalence of AmB resistance (based on tentative breakpoints), e.g., 30% in the U.S. (9).
58 In contrast to relatively well-studied mechanisms of azole and echinocandin resistance (10-16),
59 information about *C. auris* response to AmB is scarce. So far, suggestions for an underlying
60 mechanism of AmB resistance in *C. auris* have come from WGS analysis. The researchers did
61 not find any loss-of-function mutations in genes previously implicated in AmB resistance in *C.*
62 *albicans* but listed various genes with non-synonymous mutations which may potentially play a

63 role (17, 18). However, no follow-up studies (e.g., targeted gene sequencing, genetic engineering)
64 were reported to confirm these observations.

65 The current antifungal armamentarium is extremely limited, with only three classes of systemic
66 drugs widely available to treat *Candida* spp. infections. Antifungal treatment of *C. auris*
67 infections is further complicated by several factors, including the scale of azole resistance (e.g.
68 90% of *C. auris* isolates in the U.S. are fluconazole-resistant) (9), emergence of multidrug
69 resistance involving two or more drug classes (19, 20), and scarce availability of first-line therapy
70 drugs, echinocandins, in resource-limited countries (21). Thus, a better understanding of the scale
71 and molecular mechanism of AmB resistance in *C. auris* is urgently needed.

72 Here, we analyzed distribution of AmB minimal inhibitory concentration (MIC) values for 321
73 clinical isolates of *C. auris* representing five clades (I – South Asian, n=48; II – East Asian, n=6;
74 III – South African, n=30; IV – South American, n=236; V – Iranian, n=1). Antifungal
75 susceptibility testing (AFST) with AmB was performed with Etest gradient diffusion strips
76 (bioMérieux, Marcy-l'Étoile, France) according to the manufacturer's instructions. As
77 recommended by the Centers for Disease Control and Prevention (CDC), MICs of 1.5 mg/l were
78 rounded up to 2 mg/l. A tentative AmB MIC breakpoint of ≥ 2 mg/l, determined by the CDC on
79 the basis of pharmacokinetic/pharmacodynamic study results (murine model of *C. auris*
80 infection), was used to categorize isolates as resistant to AmB (9). A summary of AFST results
81 and MIC values for individual isolates are presented in Table 1 and Supplementary Table 1,
82 respectively. Three hundred and two isolates (94.1%) exhibited AmB MIC values < 2 mg/l and
83 were categorized as AmB-susceptible. A total of 19 isolates (5.9%), including 3 isolates of clade
84 I, 1 isolate of clade III, and 15 isolates of clade IV, exhibited AmB MIC values ≥ 2 mg/l and
85 therefore were categorized as AmB-resistant (Table 1). This rate is similar to the one reported
86 recently from South Africa (6%) (16), but considerably lower than majority of the studies

87 conducted so far, where anywhere from 10% to 35% of isolates (30% of U.S. isolates according
88 to the CDC) were reported as AmB-resistant (9, 22-24).

89 To decipher the molecular resistance mechanism in isolates exhibiting elevated MIC values (\geq
90 mg/l), we amplified and sequenced the following genes of ergosterol biosynthesis pathway (in the
91 entire collection of clinical isolates): *ERG2* (C-8 sterol isomerase; B9J08_004943), *ERG3* (C-5
92 sterol desaturase; B9J08_003737), *ERG6* (sterol 24-C-methyltransferase; B9J08_005340), and
93 *ERG11* (lanosterol 14- α -demethylase; B9J08_001448), which were previously implicated in
94 AmB resistance in other *Candida* spp. Gene sequences of *C. auris* strain B8441 extracted from
95 FungiDB (fungidb.org) served as a reference for primer design (Supplementary Material 2) and
96 sequence analysis. Primers were synthesized by Integrated DNA Technologies (Coralville, IA,
97 United States), and Sanger sequencing was performed by Genewiz (South Plainfield, NJ, United
98 States). A summary of sequencing results and genotypes of individual isolates are presented in
99 Table 1 and Supplementary Table 1, respectively. In 18 of 19 AmB-resistant isolates no
100 mutations in *ERG2*, *ERG3*, *ERG6*, or *ERG11* were found that could explain elevated AmB values
101 (Table 1). We noticed that amplification of *ERG6* from one South African isolate (SA18, clade
102 III; AmB MIC = 6 mg/l) yielded a much shorter PCR product (Figure 1). Sequence analysis
103 revealed that this isolate presented a unique non-wild-type *ERG6* genotype where 492 base pairs
104 (from 52 to 543) were deleted (SA18's *ERG6* is 636 bp long in comparison to the wild-type's
105 (WT's) 1128 bp), which corresponds to the deletion of 164 amino acids (from 18 to 181) and a
106 shorter Erg6 (SA18's Erg6 is 211 amino acids long in comparison to the 375 AA of WT) (Figure
107 2). Representative sequences were deposited at GeneBank (NCBI) with the accession numbers
108 OK564516 - OK564551 (*ERG2*), OK564552 - OK564587 (*ERG3*), OK564588 - OK564623
109 (*ERG6*), and OK564624 - OK564654 (*ERG11*). Accession numbers for individual isolates are
110 presented in Supplementary Table 1.

111 To confirm that the SA18's *ERG6* variant confers AmB resistance, a WT *ERG6* gene in AmB-
112 susceptible (MIC = 0.5 mg/l) isolate VPCI 717/P/14 (clade I) was replaced with an *ERG6* of
113 SA18 fused with nourseothricin (NAT) resistance gene by using CRISPR/Cas9 system as
114 described before (25), except that the cells were made electrocompetent with the Frozen-EZ yeast
115 transformation kit (Zymo Research, Irvine, CA, USA). The transformants were selected on YPD
116 plates containing 300 mg/l NAT. Correctness of transformation was validated by PCR and
117 sequencing. All PCR conditions, reagents and primers used are listed in Supplementary Material
118 2. After that, the AmB MIC of the correct transformant (MKKG066) was determined by Etest.
119 MIC >32 mg/l (no zone of inhibition around the Etest strip) was read, indicating that
120 amphotericin B resistance was induced when a wild-type *ERG6* was replaced with *ERG6* of
121 SA18 in a susceptible strain.

122 *ERG6* encodes C-24 methyltransferase, which converts zymosterol to fecosterol in the ERG
123 biosynthesis pathway. In *C. albicans*, a fragment between amino acids 127 and 135 of Erg6 is a
124 highly conserved S-adenosylmethionine binding site (26). This fragment (amino acids 128-136 in
125 Erg6 of *C. auris*) is not present in SA18's Erg6 due to the deletion of amino acids 18-181 (Figure
126 2). It was previously shown that lack of Erg6 activity leads to the accumulation of zymosterol,
127 which can support fungal cell growth, but the absence of ergosterol in the cell membrane confers
128 AmB resistance (6).

129 In conclusion, we found that mutations in key genes of ergosterol biosynthesis, which can be
130 linked directly to AmB resistance, are extremely rare. Only 1 of 313 clinical isolates screened
131 (0.3%) had an *ERG6* variant which induced AmB resistance in a WT strain. It is possible that
132 mechanisms other than *ERG6* mutations may also contribute to reduced AmB susceptibility in *C.*
133 *auris*, although this remains to be determined.

134

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232

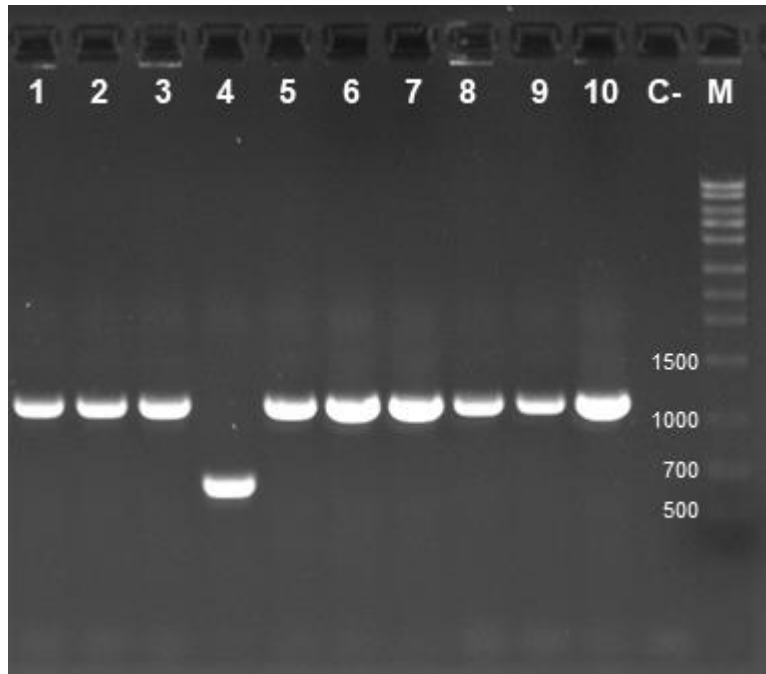
233 **Table 1.** Results of AFST and gene sequencing performed for 321 *C. auris* isolates belonging to
 234 five geographic clades: I – South Asian (n=48); II – East Asian (n=6); III – South African (n=30);
 235 IV – South American (n=236); V – Iranian (n=1).

236 AmB – amphotericin B; MIC – minimal inhibitory concentration

237 * The actual range is 1.5-2 mg/l, but all AmB MIC values of 1.5 mg/l obtained by Etest were
 238 rounded up to 2, as recommended by the CDC.

239 ** K177R/N335S/E343D – present in all isolates of clade IV

Clade	Number of isolates	AmB MIC range [mg/l]	Erg2	Erg3	Erg6	Erg11
I	45	0.19 - 1	WT	WT	WT	WT; Y132F; K143R
	3 (6.3%)	2*	WT	WT	WT	Y132F
II	6	0.094 - 0.5	D39E	WT	WT	WT
	0	≥2	-	-	-	-
III	29	0.19 - 0.38	D39E	WT	WT	V125A/F126L
	1 (3.3%)	6	D39E	WT	deletion of AA 18-181	V125A/F126L
IV	221	0.25 - 1	D39E	S58T	WT	WT**; E102K; Y132F; K143R; G459S; I466M; Y501H
	15 (6.4%)	2*	D39E	S58T	WT	WT**
V	1	0.19	WT	I31V/S37N/L63M	A287T	K177R/N335S/E343D/T366I
	0	≥2	-	-	-	-



240

241 **Figure 1.** Results of PCR amplification of *ERG6* gene from *C. auris* clinical isolates SA15-SA24
242 (lanes 1-10).

243 C-, negative control; M: molecular size marker

