In-vitro antifungal resistance of *Candida auris* isolates from bloodstream infections, South Africa

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Abstract

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Introduction

Candida auris is a multidrug-resistant fungal pathogen endemic in South African hospitals.

Materials and methods

We tested bloodstream *C. auris* isolates that were submitted to a reference laboratory for national laboratory-based surveillance for candidaemia, 2016-2017. We confirmed species identification by phenotypic/molecular methods. We tested susceptibility to amphotericin B, anidulafungin, caspofungin, micafungin, itraconazole, posaconazole, voriconazole, fluconazole and flucytosine using broth microdilution (BMD) and Etest. We interpreted minimum inhibitory concentrations (MICs) using tentative breakpoints. We sequenced the genomes of a subset of isolates and compared to the *C. auris* B8441 reference strain.

Results

Of 400 *C. auris* isolates, 361 (90%) were resistant to at least one antifungal agent, 339 (85%) to fluconazole alone (MIC of ≥32 mg/L), 19 (5%) to fluconazole and amphotericin B (MIC ≥2 mg/L) and one (0.3%) to amphotericin B alone. Two (0.5%) isolates from a single patient were pan-resistant (fluconazole, amphotericin B, echinocandins). Of 93 isolates selected for whole genome sequencing, 78 clustered in clade III including the pan-resistant isolates, 13 in clade I and two in clade IV. Eighty-four of these (91%) were resistant to at least one antifungal agent; both resistant and susceptible isolates had mutations. The common substitutions identified across the different clades were VF125AL, Y132F,

K177R, N335S, E343D in *ERG11*; N647T in *MRR1;* A651P, A657V, S195G in *TAC1b;* S639P in *FKS1;* and S58T in *ERG3* genes.

Conclusions

Most South African *C. auris* isolates were resistant to azoles, though resistance to polyenes and echinocandins was less common. We observed mutations in resistance genes even in phenotypically-susceptible isolates.

Introduction

Candida auris is an important multi-drug resistant nosocomial pathogen in healthcare settings (1). Bloodstream infection is the most frequently-reported form of invasive disease with a reported crude mortality of 30% to 60% (2, 3). The clinical impact of antifungal resistance of *C. auris* is poorly defined with no published clinical breakpoints and only tentative epidemiological cut-off values (ECVs) (4). Using breakpoints determined for closely-related *Candida* species and based on expert opinion, the U.S. Centers for Disease Control and Prevention (CDC) published a guide to assist laboratories in the interpretation of *C. auris* minimum inhibitory concentrations (MICs) (5).

The recommended first-line antifungal agent for *C. auris* bloodstream infections is an echinocandin (6). However, isolates have been reported with reduced susceptibility to these agents (7, 8, 9). Acquired resistance to fluconazole and variable susceptibility to amphotericin B and other triazoles also limit treatment options for *C. auris* infection (1, 7, 9). Furthermore, a small minority of *C. auris* isolates are resistant to all classes of systemic antifungal agents in current use for candidaemia (9, 10).

C. auris is separated into four distinct clades named for the geographic area where these were first isolated: South Asian (clade I), East Asian (clade II), African (clade III) and South American (clade IV) (11) and a potential clade V represented by a single isolate (11). Differences in genetic background, biochemical characteristics and antifungal susceptibility patterns makes each clade unique (12). Antifungal resistance seems to be clade specific; clade I, III and IV isolates may be resistant to multiple antifungal agents, while clade II isolates are generally more susceptible (13, 14). In addition, S639F/S639P mutations in the *FKS* hotspot 1 region have been detected in echinocandin-resistant isolates (14, 15). Furthermore, distinct mutations in the *ERG11* gene (F126L, Y132F, K143F) and *TACB1* gene (A640V) have been detected in azole-resistant isolates (9, 16).

87

88 Since *C. auris* was first detected in South Africa in 2009, healthcare-associated transmission events and
89 large outbreaks have led to this pathogen accounting for more than 1 in 10 cases of candidaemia (3, 17,
90 18). Some isolates have been reported to be resistant to more than one antifungal agent (19). In order to
91 determine the resistance profile of *C. auris* bloodstream isolates from South Africa and thus guide
92 empiric treatment, we performed antifungal susceptibility testing on *C. auris* isolates obtained from
93 private- and public-sector hospitals in South Africa through a national laboratory surveillance
94 programme in 2016-2017.

Materials and methods

Isolate information and case definition

We conducted national laboratory-based surveillance for candidaemia from 1 January 2016 through to 31 December 2017. Clinical microbiology laboratories affiliated to the National Health Laboratory Service (NHLS) or a private-sector pathology practice were requested to send *Candida* species isolated from blood culture specimens to the Mycology Reference Laboratory at the National Institute for Communicable Diseases (NICD). Isolates were accompanied by a laboratory report that included species identification and patient demographic details. Individual patients with more than one serial isolate were also included. At sentinel surveillance sites, we collected additional clinical information by chart review and/or interview (3).

Identification of *C. auris*

Candida isolates were submitted to NICD on Dorset transport medium (Diagnostic Media Products (DMP), NHLS, Sandringham, South Africa) and to obtain a presumptive species identification, were inoculated onto chromogenic agar (MAST ID CHROMagar *Candida*, Mast Diagnostics, Amiens, France) upon receipt. We used matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Corporation, Billerica, MA, United States) to confirm species identification. We extracted DNA from the isolates using the Zymo Fungal/Bacterial Miniprep kit (Zymo Research, Inqaba Biotec, South Africa) if repeated analysis on the MALDI-TOF MS instrument resulted in no peaks, yielded a score of <2.00, or if there was no clear identification. DNA amplification and sequencing of the internal transcribed spacer (ITS) region of the ribosomal gene was then performed using the ITS1 and ITS4 primers (20). We used the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) database to identify the species based on pairwise sequence alignment (http://blast.ncbi.nlm.nih.gov/Blast.cgi). We included only confirmed *C. auris* isolates.

Antifungal susceptibility testing

The MICs of nine antifungal agents (amphotericin B, fluconazole, voriconazole, itraconazole, posaconazole, caspofungin, anidulafungin, micafungin and flucytosine) were determined using dried broth microdilution (BMD) panels containing Alamar blue (Thermo Fisher Scientific, Cleveland, OH, USA) and following Clinical and Laboratory Standards Institute M27-Ed4 recommendations (21). All plates were incubated at 35°C and wells were visually evaluated for growth following 24 hours of incubation. MICs for echinocandins and azoles were defined as the lowest antifungal concentration that caused 50% growth inhibition compared to the positive control, while the MIC for amphotericin B was defined as the lowest concentration at which there was 100% inhibition of growth. We used CDC tentative breakpoints, which were developed using *C. auris* MIC distribution data, known molecular mechanisms of resistance and pharmacokinetic/pharmacokinetic data from a neutropenic mouse model of infection, to interpret MICs. Isolates with an amphotericin B MIC of ≥2 mg/L, with a fluconazole MIC of ≥32 mg/L or with an anidulafungin/ micafungin MIC of ≥4 mg/L were considered resistant to that agent. Micafungin and/or anidulafungin resistance was considered as a surrogate marker of resistance for the entire echinocandin class. Caspofungin MICs were not categorized due to the previously-reported inter-laboratory MIC variability noted in *Candida* species as a result of batch-to-batch variation of the powder's potency (22, 23). Multidrug-resistance was defined as resistance to more than one antifungal class. There are no breakpoints to interpret itraconazole, posaconazole, voriconazole and flucytosine MICs. *C. parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were run on all days of testing and MICs were found to be within the required quality control range. We also determined amphotericin B MICs by Etest (bioMérieux, Marcy l'Etoile, France) on RPMI 1640 plates containing 2% glucose (DMP, South Africa), according to the manufacturer's instructions. We used the Etest method since it generates a much wider and diverse range of amphotericin B MIC values than those yielded by broth microdilution testing; this

may assist to distinguish resistant and susceptible isolates (24). Isolates with resistance to echinocandins by BMD testing were re-tested by Etest for confirmation only. We calculated the range, MIC_{50} and MIC_{90} for each distribution.

Whole genome sequencing of *C. auris* isolates

We selected all echinocandin- and amphotericin B-resistant isolates and a random sample of fluconazole-resistant isolates. In total, 92 *C. auris* isolates were selected for whole genome sequencing; 62 were resistant to fluconazole, 19 were resistant to both amphotericin B and fluconazole, two were pan-resistant (amphotericin B, fluconazole and echinocandins), one was resistant to amphotericin B alone and eight were fully-susceptible. DNA extraction from these yeast isolates was performed as described above. Paired-end libraries were prepared using the Nextera DNA Flex library preparation kit, followed by 2 × 300-bp sequencing on an Illumina MiSeq instrument (Illumina, San Diego, CA, USA). The sequenced paired-end reads were quality controlled and filtered (Q>20 and length > 50 bp) using fastqc (v0.11.8) and trim Galore (v0.6.4_dev; https://github.com/FelixKrueger/TrimGalore), respectively. The clean reads from each *C. auris* isolate were analyzed for the detection of mutations using a custom targeted gene approach workflow on Qiagen CLC Genomics Server version 20 (Qiagen, The Netherlands). We determined the presence of mutations within the *ERG11* gene, *ERG9* and transcriptional regulators of efflux pumps (*MRR1* and *TAC1B* genes) responsible for azole resistance, the *FKS1* gene hot spot 1 region associated with echinocandin resistance and *ERG3*, *ERG6*, and *ERG10* genes associated with amphotericin B resistance in *C. auris*. Reference sequences and annotations for these genes were obtained from the susceptible clade I reference strain, *C. auris* B8441 (Genbank accession: PEKT00000000; https://www.ncbi.nlm.nih.gov/assembly/GCA_002759435.2/) and the clade III *C. auris* B11221 isolate (Genbank accession: PGLS00000000; https://www.ncbi.nlm.nih.gov/assembly/GCA_002775015.1/, Muñoz et al., 2018). Results tables for

synonymous or non-synonymous mutations were populated to MS Excel Workbooks using a custom R script and compared. We compared our findings to what had been previously reported by Lockhart et al. (2017), Chow et al. (2020) and Rybak et al. (2020) (9, 14, 16).

Ethics

NICD obtained annual approval for GERMS-SA laboratory-based surveillance from the research ethics committees of several South African universities. Patients, from whom surveillance data were collected prospectively through interview, provided written informed consent.

Results

Cases, species identification and selection of isolates

Between 2016 and 2017, 6669 cases of candidaemia were reported. Of these 6669, additional species identification was performed at NICD on viable isolates from 3020 cases, and only at the diagnostic laboratories for 2856 cases. Thus, the corresponding isolates from 5876 cases had a species-level identification either at NICD and/or at a diagnostic laboratory and of these, 794 (14%) cases had a *C. auris* bloodstream infection. We have previously described these cases in detail (3). Of these 794 cases, 450 had isolates that were identified as *C. auris* only at a diagnostic laboratory. Only 400 isolates from 344 cases were submitted for further testing at NICD and confirmed to be *C. auris*. The clinical details of these cases are in Table 1. The 344 cases included 45 patients with two or more serial isolates. Three hundred and ninety-four isolates (99%) were confirmed as *C. auris* by MALDI-TOF MS; the other six isolates (three with no MALDI-TOF MS identification, two with a MALDI-TOF score value of <2.00 and one with a low discrimination identification) were later confirmed as *C. auris* by *ITS* sequencing.

Antifungal susceptibility distributions

Table 2 summarizes the BMD and Etest MIC distribution, MIC_{50} and MIC_{90} of nine antifungal agents for the 400 *C. auris* isolates. The fluconazole BMD MIC_{50} and MIC_{90} values for all 400 isolates were 128 µg/ml and 256 µg/ml respectively. The amphotericin B BMD MICs ranged from 2 µg/ml to 4 µg/ml for 27% (107/400) of the *C. auris* isolates, while 73% (293/400) had MICs that ranged from 0.25 µg/ml to 1 µg/ml. Only 6% (22/400) were confirmed to be amphotericin B-resistant by Etest. The Etest MICs for these 22 isolates ranged from 2 µg/ml to 8 µg/ml. The BMD MICs for posaconazole, itraconazole and voriconazole ranged from 0.015 µg/ml to 1 µg/ml, 0.03 µg/ml to 2 µg/ml and 0.03 µg/ml to 8 µg/ml respectively. The BMD MICs for micafungin and anidulafungin ranged from 0.015 µg/ml to 8 µg/ml and 0.015 µg/ml to 2 µg/ml respectively. Two isolates from a single patient had high micafungin MICs of 4

µg/ml and 8 µg/ml but low anidulafungin MICs of 1 µg/ml and 2 µg/ml, respectively. The micafungin Etest MIC for these two isolates was 16 µg/ml. Flucytosine MICs were relatively low (range, 0.015 µg/ml to 2 µg/ml) for all 400 *C. auris* isolates.

Multi-drug resistance

Of the 400 *C. auris* isolates, 361 (90%) were resistant to at least one antifungal agent and of these, 339 (94%) were resistant to at least fluconazole. Of these 339 fluconazole-resistant isolates, 19 (6%) were also resistant to amphotericin B and thus multi-drug resistant. The flucytosine MICs for these 19 isolates ranged from 0.06 µg/ml to 1 µg/ml. Two of three *C. auris* isolates from a single patient were micafungin-resistant (MICs of 4 µg/ml and 8 µg/ml), fluconazole-resistant (MICs of 32 µg/ml and 64 µg/ml) and amphotericin B-resistant (MICs of 4 µg/ml and 2 µg/ml). This patient was a 69-year-old man who was admitted to a cardiothoracic intensive care unit at a private hospital in Pretoria. Blood cultures collected on three consecutive days in August 2016 yielded *C. auris*. The first bloodstream isolate was susceptible to all antifungal agents except amphotericin B (MIC of 2 µg/ml). The two subsequent isolates were confirmed to be resistant by Etest to micafungin (MIC of ≥16 µg/ml), anidulafungin (MIC of ≥16 µg/ml) and amphotericin B (MIC of 2 µg/ml). The flucytosine MICs for these three isolates remained low (0.25 µg/ml).

Clade-specific susceptibility and resistance mutations

Of the 92 isolates with WGS analysis, 84 (91%) were resistant to one or more antifungal agents, while the remaining eight were susceptible to all tested antifungal agents. The majority of the isolates (n=77) belonged to clade III, 13 belonged to clade I and two belonged to clade IV. Among the 77 clade III isolates, 69 (90%) in total had some evidence of resistance: 59/69 (86%) were resistant to fluconazole alone, 7/69 (10%) were resistant to both fluconazole and amphotericin B, 2/69 (3%) were resistant to

224 micafungin, fluconazole and amphotericin B and 1/69 (1%) was resistant to amphotericin B alone (Table
225 3). Ninety-nine per cent (76/77) of the clade III isolates had two substitutions (F126L and V125A; now
226 referred as VF125AL) based on *ERG11* gene mutations. These mutations were observed in 68 isolates
227 with a fluconazole MIC of ≥32 µg/ml and eight isolates with MICs of 8 µg/ml to 16 µg/ml. These 76 clade
228 III isolates also had a N647T *MRR1* substitution. In addition, only 16 of the clade III isolates had A651P
229 *TAC1b* substitutions, with a single isolate having an extra S195G *TAC1b* substitution. These isolates had a
230 BMD fluconazole MICs ranging from 16 µg/ml to 256 µg/ml (Table 1, supplementary). Distribution of
231 common specific substitutions within the sequenced resistance genes in each clade is shown in Figure 1.
232
233 Twelve of 13 (92%) clade I isolates were resistant to both fluconazole and amphotericin B and one isolate
234 to fluconazole alone. One of the two clade IV isolates was resistant to fluconazole but not to any other
235 agent. The 13 clade I isolates all had Y132F *ERG11* substitutions, while eight clade I isolates had A657V
236 *TAC1b* substitutions and only two isolates had A651P *TAC1b* substitutions. The two clade IV isolates with
237 fluconazole MICs of 16 µg/ml and 64 µg/ml had M351V and A27T *ERG9* substitutions and K177R, N335S,
238 E343D *ERG11* substitutions. The fully-susceptible clade IV isolate also had 12 uncommon substitutions
239 within the *MRR1* gene (S30T, N70S, E76_P77delnsDS, D80E, N133S, K138E, K167N, L211V, R249K, R280G,
240 R413K, K534N), while the resistant clade IV isolate had a A651P *TAC1b* substitution.
241
242 Three clade III isolates from a single patient, two with an Etest micafungin MIC of ≥16 µg/ml and one
243 with a BMD micafungin MIC of ≥2 µg/ml, had an S639P substitution due to a mutation within the *FKS1*
244 hotspot 1 region. Another three clade I echinocandin-susceptible isolates (anidulafungin BMD MIC of ≤1
245 µg/ml; micafungin BMD MIC of 0.5 µg/ml) from different patients had an uncommon D642Y substitution
246 due to a *FKS1* hotspot 1 region mutation, while a single clade III echinocandin-susceptible isolate
247 (anidulafungin and micafungin BMD MICs of 0.06 µg/ml) also had three uncommon different

248 substitutions (T125I, C1253fs (fs=frameshift), G1250S) due to mutations within the *FKS1* hotspot 1 region
249 (Table 2, supplementary).
250
251 Both clade IV isolates (amphotericin B BMD MICs of ≤2 µg/ml and Etest MICs of ≤0.75 µg/ml) had
252 uncommon S58T substitutions due to mutations within the *ERG3* gene. No mutations were observed
253 within the *ERG6* and *ERG10* genes for any of the 92 isolates. Of the 8 fully-susceptible isolates, only one
254 clade III isolate with a micafungin BMD MIC of 0.5 µg/ml, anidulafungin BMD MIC of 0.12 µg/ml,
255 fluconazole BMD MIC of 4 µg/ml, and an amphotericin B Etest MIC of 0.38 µg/ml did not have any
256 mutation within any genes and was considered a wild-type.
257
258 **Clinical treatment and outcomes**
259 Clinical data on antifungal treatment and in-hospital outcome were available for 25% (87/344) of
260 patients with *C. auris* candidaemia. Table 3 (Supplementary) shows the 75 patients with documented
261 receipt of systemic antifungal therapy, MIC data, and in-hospital outcome. The overall in-hospital case
262 fatality ratio was 47% (35/75).
263
264 Of the 35 who died, 11 were treated with amphotericin B alone and 15 with amphotericin B plus either
265 an echinocandin (n=11) or azole (n=4). The remaining nine were either treated with an echinocandin
266 (n=7), fluconazole (n=1) or an unspecified agent (n=1). Thirty-two of the 35 isolates from patients who
267 died were resistant to fluconazole alone (MIC of ≥32 µg/ml), while one isolate was resistant to both
268 fluconazole and amphotericin B (MIC of 3 µg/ml). The remaining two isolates were susceptible to all
269 tested antifungal agents. Sixteen of the patients who died were previously treated with fluconazole
270 (n=5), micafungin (n=3), anidulafungin (n=2), amphotericin B and fluconazole (n=2), posaconazole (n=1),

amphotericin B, caspofungin and micafungin (n=1), caspofungin and anidulafungin (n=1), posaconazole, amphotericin B, fluconazole, itraconazole, voriconazole, micafungin and anidulafungin (n=1).

Only three of the 35 had their isolates sequenced. These three isolates had fluconazole MICs of ≥32 mg/L and VF125AL mutations within the *ERG11* gene. In addition to fluconazole resistance, one isolate also had an amphotericin B MIC of >3 µg/ml; however, no mutations were detected within the *ERG3*, *ERG6* and *ERG10* genes. For the two fluconazole-resistant isolates, one patient was previously treated with a combination of antifungal agents including posaconazole, amphotericin B, fluconazole, voriconazole, micafungin and anidulafungin before being treated with amphotericin B for 18 days during the episode of candidaemia. The other patient had been previously treated with fluconazole and was then initially treated for the episode of candidemia with amphotericin B for 1 day followed by fluconazole for 24 days. The third patient with a fluconazole- and amphotericin B-resistant isolate had no prior antifungal treatment and was treated for the episode of candidaemia with amphotericin B for 10 days.

Of the 40 who recovered, nine were treated with amphotericin B alone, while the others were treated with more than one antifungal agent (Table 3, supplementary). Of the 40 *C. auris* isolates from the patients who recovered, 38 (95%) were only resistant to fluconazole (MIC of ≥32 µg/ml). Nine of these patients had been previously treated with micafungin (n=4), fluconazole (n=3), voriconazole (n=1) and amphotericin B (n=1). One patient of the nine had their isolate sequenced. This isolate had a fluconazole MIC of ≥32 µg/ml and had VF125AL mutations within the *ERG11* gene. The patient had received prior fluconazole treatment before being treated for the episode of candidaemia with anidulafungin for 11 days.

Discussion

We performed antifungal susceptibility testing on 400 South African *C. auris* bloodstream isolates from national surveillance. Ninety per cent of the 400 isolates were resistant to fluconazole but only 5% were amphotericin B-resistant and fluconazole-resistant. Two isolates from a single patient were resistant to three antifungal classes. Of 92 isolates which were sequenced, 84% belonged to clade III, 14% belonged to clade I and 2% belonged to clade IV. A much larger proportion of clade I isolates were multi-drug resistant (>90% resistant to fluconazole and amphotericin B) than clade III isolates. Mutations were observed within resistance genes in both susceptible and resistant clade III and clade IV isolates.

C. auris was the third most common *Candida* species isolated from South African patients with bloodstream infections (3). The increase in the number of *C. auris* infections reported worldwide is of major concern because this fungus is difficult to identify using standard identification methods, is often multidrug-resistant and can cause large outbreaks in acute hospital and long-term healthcare settings (9, 25). Recently, Magobo et al. reported the emergence of multidrug-resistant isolates (8%) among 85 tested South African *C. auris* isolates. However, our study sample derived from 2-year national surveillance of candidaemia provides a much more representative picture of the antifungal susceptibility profile (19). The vast majority of our study isolates were fluconazole-resistant. Fluconazole must be avoided as first-line empiric treatment for candidaemia in hospitals and units where *C. auris* is endemic. We also observed resistance to amphotericin B and echinocandins in our setting, albeit at a much lower relative frequency. We found that resistance to azoles and amphotericin B was the most common resistance combination (5%), while 2 (0.5%) isolates were resistant to azoles, amphotericin B and echinocandins. A multicenter study of 350 Indian *C. auris* isolates collected between 2009 and 2017 reported that 14% were resistant to both azoles and flucytosine, 7% to both azoles and amphotericin B and 2% to azoles and echinocandins (2). In contrast, all our isolates had low flucytosine MICs. A combination of amphotericin B and flucytosine

is a potent regimen and known to be efficacious for other serious fungal infections such as cryptococcal meningitis (26). This combination may potentially be useful in resource-limited settings for treatment of invasive *C. auris* infections and this should be explored in prospective studies.

A majority of our isolates belonged to clade III, while smaller proportions belonged to clade I (14%) and clade IV (2%). We found that a much higher proportion (92%) of clade I isolates were multi-drug resistant (both fluconazole and amphotericin B) compared to 13% of clade III isolates. Chow et al. also reported a larger proportion of clade I isolate (45%) as multidrug resistant than clade III (8%) and clade IV (10%) (14). Furthermore, two of our clade III isolates were pan-resistant. So far, pan-resistant isolates have been reported only in 3% of clade I isolates (14). Fluconazole resistance has been reported in clade I isolates (India and Pakistan), clade III isolates (South Africa) and clade IV isolates (Venezuela) (9). In contrast, a very low prevalence of fluconazole resistance has been reported in clade IV Colombian isolates and clade II Japanese isolates (2, 27, 28). Fluconazole resistance is associated with clade-specific mutations within the *ERG11, ERG9, MRR1* and *TAC1b* genes. Some of our clade I, clade III and clade IV isolates had similar mutations to those reported previously, while other mutations were uncommon (9, 14, 16). Uncommon mutations within the susceptible *C. auris* isolates may be related to natural evolutionary divergence rather than a mechanism of resistance. Ninety-nine of the clade III isolates had *ERG11, MRR1* and *TAC1b* mutations. In *C. albicans*, *MRR1* and *TAC1b* are zinc-cluster transcription factors reported to play a role in the regulation of the expression of the multidrug resistance-related gene *MDR1* and *CDR1*, respectively, while *ERG11* encodes a microsomal and membrane-bound protein that functions as a lanosterol 14, α-demethylase of the cytochrome P450 family (29). All clade I isolates had *ERG11* mutations but no *MRR1* mutations. *MRR1* mutations have only been noted in clade III isolates (16, 30). However, one of the clade IV isolates had 12 *MRR1* mutations and an fluconazole MIC of 16 µg/ml. It is difficult to establish if these mutations contributed to an elevated MIC, since the other clade IV isolate did not have these mutations but had an MIC of 64 µg/ml. Both clade IV isolates had

three mutations within the *ERG11* gene which is commonly reported in Colombian isolates but are not associated with fluconazole resistance (27). All clade I isolates had a fluconazole MIC of ≥128 µg/ml and had *ERG11* mutations. Of the clade III isolates with *ERG11* and *MRR1* mutations, seven were considered susceptible with fluconazole MICs ranging from 8 µg/ml to 16 µg/ml. A larger number of clade III genomes with MICs of ≤4 µg/ml should be sequenced to determine if these isolates have mutations. A proportion of clade I, III and IV isolates had *TAC1b* mutations. Mutations in *TAC1b* are associated with fluconazole resistance in *C. auris* isolates (16). The A657V *TAC1b* substitution has been reported in 15 clade I isolates with the Y132F *ERG11* substitution and elevated fluconazole MICs, which is similar to our findings (16). However, two of our clade I isolates with MICs of 256 µg/ml and a single clade IV isolate with MIC of 64 µg/ml had a A651P *TAC1b* substitution. This A651P *TAC1b* substitution was most common among our clade III isolates with MICs of 64 µg/ml to 256 µg/ml. Rybak et al. reported 16 clade IV isolates harboring A651T *TAC1b* substitutions (16). None of our isolates harbored the A640V *TAC1b* substitution which is mostly common in clade I isolates with fluconazole MIC of >64 µg/ml and K143R *ERG11* substitution . Our isolates also lacked the K247E, M653V, A15T or P595L/H *TAC1b* substitutions which has been found to occur naturally in *C. auris* (16).

Amphotericin B resistance is not commonly described among *Candida* species (11, 31). However, Chow et al. reported that 47% of clade I isolates and 11% of clade IV isolates were resistant to amphotericin B, while all clade III and clade II isolates were susceptible by the Etest method (14). Based on the Etest method, we confirmed resistance in 6% (22/400) of South African isolates. The Etest method yields a much wider MIC range compared to BMD, partly because the strip includes lower antifungal concentrations (32). Twelve clade I and 10 clade III isolates did not have mutations within the *ERG3*, *ERG6* and *ERG10*, despite having high amphotericin B Etest MICs. In a large clade I *C. auris* outbreak involving 72 patients in the United Kingdom, Rhodes et al. reported five amphotericin B-resistant isolates with an MIC of 2 mg/L, none of which had any *ERG3, ERG5* and

ERG6 mutations (33). Of the 22 amphotericin B-resistant isolates in our study, none had any mutations. Only the two clade IV isolates had a single mutation within the *ERG3* gene; these two isolates had an amphotericin B Etest MICs of 0.75 µg/ml and 0.5 µg/ml and would be considered susceptible. We do not know the relevance of these mutations. In fungi, *ERG3* encodes a C-5 sterol desaturase that is involved in one of the final reactions in the ergosterol biosynthesis pathway (29). A missense mutation in *ERG3* gene results in azole resistance in some clinical isolates of *Candida albicans and Candida parapsilosis* (34, 35). In this study, both the fluconazole-resistant and-susceptible clade IV isolates had a mutation within the *ERG3* gene.

The molecular mechanism of echinocandin resistance is highly specific and not affected by multidrug transporters (36). In *C. auris*, echinocandin resistance is associated with mutations in the hot spot regions of the *FKS* genes (*FKS1*, *FKS2* and *FKS3*) which encode β-1,3-D-glucan synthase (37). In this study, only three clade I isolates and four clade III isolates had mutations within the *FKS1* hotspot 1 region. Three of the clade III isolates were from a single patient but only two were resistant to micafungin. However, all three isolates had S639P substitutions caused by *FKS1* hotspot1 mutations. The S639P substitution has been reported in echinocandin-resistant isolates from clade I and IV though not previously from clade III isolates (14). Clade I and III echinocandin-resistant isolates have been reported to have either the S639F or S639Y substitutions (7, 14). Three of the clade I isolates had a D642Y substitution due to a *FKS1* region hot spot 1 mutation, though their anidulafungin and micafungin MICs were ≤1 and 0.5 µg/ml respectively. The relevance of this D642Y substitution in *C. auris* needs further investigation. Furthermore, another clade III isolate had three mutations in the *FKS1* region hot spot 1 region; however, this isolate had anidulafungin and micafungin MICs of 0.06 µg/ml. It is possible that these mutations may not be related to resistance but other phenotypic characteristics of the organism.

The clonal expansion or transmission of pan-drug resistant or multidrug-resistant isolates may severely compromise the treatment of *C. auris* infection. We found that 33% of patients had been exposed to antifungal agents before they were treated for *C. auris* infection. This might have resulted in the development of resistance and poorer clinical outcomes (38). However, we did not perform a multivariable analysis to look at the association between antifungal resistance and in-hospital outcome in our study. The FIDSSA guideline recommends echinocandins as a first-line treatment option for patients with invasive *C. auris* infection and amphotericin B as an alternative agent in clinical settings where echinocandins are unavailable (6). These agents are still good empiric treatment options in South African hospitals though robust infection control and antifungal stewardship programmes are essential to limit further emergence of resistance. This large study provides a representative national antifungal profile of *C. auris* in South Africa and a baseline to monitor emerging resistance to the approved antifungal agents. We used CDC tentative breakpoints to interpret MICs which allows comparison of our results with other published studies. We performed WGS for only a sub-set of resistant isolates so the clade distribution may not be completely representative.

Conclusions

C. auris isolates from national surveillance were almost all resistant to fluconazole with a smaller proportion resistant to amphotericin B or echinocandins. We observed mutations within resistance genes even in susceptible *C. auris* isolates and further studies are required to understand the mechanism of resistance and the relevance of mutation within genes among South African isolates using a larger WGS dataset.

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443 **AUTHOR CONTRIBUTIONS**

444 Surveillance methods: EV, NPG

445 Processing of isolates: TGM, SDN, RSM

446 Whole genome sequencing: SM, IA, SK

447 Data analysis: TGM, SK, JFM, NPG

448 Manuscript writing: TGM, NPG

449 Critical review of manuscript: TGM, EV, SDN, SK, JFM, RSM, SM, IA, NPG

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612 **Table 1: Demographic and clinical characteristics of cases with *C. auris* infections, 2016-2017**
613 **(n=344)**

Demographic and clinical features (N)	n (%)
Median age (IQR), years	53 (34-64)
Sex (n=340)	
Male	215 (63)
Female	129 (37)
Province (n=344)	
Gauteng	327 (95%)
Other province	17 (5%)
HIV status and CD4 count (n=42)	
HIV-seropositive	10 (24)
Patients with known CD4 counts	4 (10)
(CD4 count range, 19-86 cells/µl)	

614 **Table 2: Antifungal susceptibility of 400 South African *C. auris* bloodstream isolates, 2016-2017**

Antifungal agent	Test method	Number of isolates with MIC (µg/ml) of:																MIC_{50}	MIC_{90}	MIC range	% resistant
		0.015	**0.03**	**0.06**	**0.125**	**0.25**	**0.5**	**1**	**2**	**3**	**4**	**8**	**12**	**16**	**32**	**64**	**128**	**256**				
Itraconazole	BMD		3	54	204	122	12	3	2										0.12	0.25	0.03-2	0
Voriconazole	BMD		3	8	18	52	121	141	55		1	1							0.5	2	0.03-8	0
Posaconazole	BMD	26	91	156	104	19	3	1											0.06	0.12	0.015-1	0
Fluconazole	BMD										3	5		31	**58**	**83**	**110**	**110**	128	256	4-256	90
Caspofungin	BMD	5	21	198	131	26	12	3				2		2					0.06	0.25	0.015-16	-
Micafungin	BMD	2	12	198	159	14	8	4	1		**1**	**1**							0.06	0.12	0.015-8	0.5
Anidulafungin	BMD	2	4	95	232	54	7	5	1										0.12	0.25	0.015-2	0
Flucytosine	BMD	2		79	256	57	4	1	1										0.12	0.25	0.015-2	0
Amphotericin B	BMD					1	23	269	**104**			**3**							1	2	0.25-4	27
Micafungin	Etest													**2**								0.5
Amphotericin B	Etest	14	4	9	28	122	145	55	**15**	**2**	**4**	**1**							0.38	1	0.015-8	6

615 BMD: broth microdilution; MIC: minimum inhibitory concentration; *tentative breakpoints: itraconazole and voriconazole ≥2 µg/ml; fluconazole ≥32 µg/ml; micafungin and
616 anidulafungin ≥4 µg/ml; amphotericin B ≥2 µg/ml. Resistant isolates are highlighted in bold.

617 **Table 3: Antifungal resistance and susceptibility across different *C. auris* clades (n=92)**

Resistance and susceptible *C. auris* isolates	Clades n (%)		
	Clade I	Clade III	Clade IV
Resistant isolates (n=84)			
Fluconazole	**1 (8)**	**60 (77)**	**1 (50)**
Amphotericin B	0	**1 (1)**	0
Micafungin	0	0	0
Fluconazole, amphotericin B	**12 (92)**	**7 (9)**	0
Fluconazole, amphotericin B, micafungin	0	**2 (3)**	0
Susceptible (n=8)			
Fluconazole	0	0	0
Amphotericin B	0	0	0
Micafungin	0	0	0
Fluconazole, amphotericin B	0	0	0
Fluconazole, amphotericin B, micafungin	0	**7 (10)**	**1 (50)**
Total # of isolates (n=92)	**13 (14)**	**77 (84)**	**2 (2)**

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621 **Figure 1: Distribution of known drug mutations within the *ERG11* (n=5; Y132F, VF125AL, K117R, N335S, E343D), *MRR1* (n=1; N647T) and TAC1b (n=3;**
622 **S195G, A651P, A657V), *FKS1HP1* (=1; S639P), and *ERG3* (n=1; S58T) genes in 92 *C. auris* isolates. *ERG11, MRR1* and *TAC1b* mutations are associated with**
623 **fluconazole resistance, *FKS1* mutations with echinocandin resistance and *ERG3* mutations with amphotericin B resistance.**

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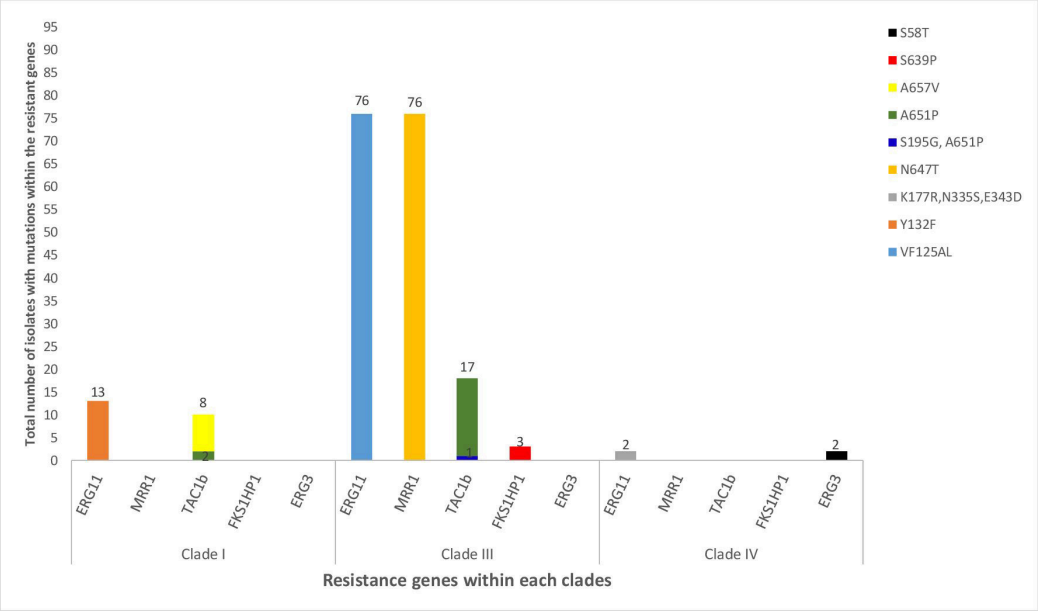

Figure 1: Distribution of known drug mutations within the *ERG11* (n=5; Y132F, VF125AL, K117R, N335S, E343D), *MRR1* (n=1; N647T) and TAC1b (n=3; S195G, A651P, A657V), *FKS1HP1* (=1; S639P), and *ERG3* (n=1; S58T) genes in 92 *C. auris* isolates. *ERG11, MRR1* and *TAC1b* mutations are associated with fluconazole resistance, *FKS1* mutations with echinocandin resistance and *ERG3* mutations with amphotericin B resistance.