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Candida auris Antifungal Resistance, Virulence and Susceptibility to a Novel Nitric Oxide-Releasing Microparticle and Its Correlations to Clade Identification

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Abstract: *Candida auris* is a globally emerging pathogenic fungus described in Japan in 2009. This fungus has been identified mainly in nosocomial environments, associated with a high frequency of multidrug-resistant strains, and mortality rates reach 60%. *C. auris* is divided into 6 biogeographic clades, and there is a correlation between the clades and resistance against antifungals. In the current report, 8 strains of *C. auris* isolated in the Montefiore Medical Center, Bronx were analyzed to assess their clade (via ClaID) and common molecular determinants of antifungal resistance. We assessed antifungal resistance as well as the efficacy of a novel nitric oxide-donating microparticle as an alternative approach against *C. auris* in vitro through microplate susceptibility tests. Virulence was also determined in a *Galleria mellonella* model. Our results indicate that 7 out of 8 strains, belonging to clade 1, were resistant to fluconazole, while clade 2 was susceptible. Additionally, the clade 2 strain was more susceptible to treatment with the microparticle, while also being more virulent in an invertebrate model of infection. Our findings were then correlated to visualize parallels between clade identification and resistance/virulence patterns.

Keywords: *Candida auris*; resistance; antifungals; clades; microparticles

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1. Introduction

Candida auris is a multidrug-resistant pathogenic fungus first described in 2009 [1]. *C. auris* has emerged globally with four major geographic-specific clades—clade 1 (South Asian), clade 2 (East Asian), clade 3 (South African), and clade 4 (South American) [2], and recently, the fifth (Iran) [3] and sixth (Singapore) [4] clades were described. Notably, these clades also present different resistance profiles [5–10]. Misidentification of this pathogen due to its phenotypic similarities to *C. haemulonii* has led to its relatively late description and unclear distribution data [11–13]. For 2022, the CDC reports 2377 *C. auris* cases in the United States, but these numbers are potentially underreported, since the reporting of *C. auris* is not compulsory [14]. Although New York state reported only 326 cases in 2022, the

state is a hot spot for *C. auris*, as it has reported 1325 out of 5654 (~23%) total cases in the U.S. since 2013, when the CDC started tracking the disease [14].

C. auris has a wide range of disease manifestations, often identified as a severe bloodstream infection [13,15,16], but also causing disease in other sites such as the urinary tract [17–19] and the ear canal [1,20]. *C. auris* disease occurs primarily in immunocompromised individuals, with mortality rates up to 60% [16,21,22]. The pathogen is transmitted through direct contact with an infected person or environmental sources, being able to survive on surfaces and contaminating them for prolonged periods of time [23,24]. In the U.S., recent outbreaks appear to be transmitted nosocomially, beginning in healthcare facilities [21,25,26]. Unfortunately, the high resistance profile of *C. auris* against conventional antifungal therapies [1,27] and many common hospital sanitizing agents [28] makes it difficult to control the pathogen's spread, resulting in subsequent infections and disease development.

Conventional treatment approaches for fungal infections are usually limited to polyenes, azoles, and echinocandins, but the ever-increasing reports of antifungal resistance and pharmacological limitations of these drugs prompt research for alternative therapeutic options [29–31]. Recently, novel formulations of nitric oxide (NO)-releasing microparticles have been investigated with successful antifungal activity in vitro not only against *C. auris* [11] and *C. albicans* [32,33] but also against species of *Trichophyton* [34,35], demonstrating the potential of this kind of formulation for antifungal treatment.

With this in mind, we performed the clade identification of eight clinical strains collected at the Montefiore Medical Center (MMC; Bronx, NY, USA) and analyzed the genome sequence of *ERG3*, *ERG11*, and *TAC1b*, genes that are commonly linked to triazole resistance. We also characterized their antifungal susceptibility profiles and assessed the antifungal activity of a novel NO-releasing microparticle (SNO-MP) against the *C. auris* strains. Additionally, we assessed virulence profiles in an in vivo infection model using *Galleria mellonella*. These findings will help enhance our understanding of this emerging pathogen as we explore the development of more effective treatments and infection control measures, which will ultimately reduce the impact of *C. auris*.

2. Materials and Methods

2.1. Candida Auris Strains

A total of eight *C. auris* strains from patients with fungemia were collected over a 5-year period at the MMC and analyzed in this study (MMC1-MMC8). The samples were gathered with consent from all eight patients under the guidelines approved by the institutional review board (IRB Number: 2016–7455 approved on 2 July 2017) of the Albert Einstein College of Medicine and MMC. *C. auris* CDC type strains AR0381 and AR0387 were also used as controls. All strains were maintained at −80 °C. Prior to use, they were first plated onto Sabouraud (SAB)-agar media (Becton Dickinson and Co, Franklin Lakes, NJ, USA), and then colonies were picked and grown in liquid SAB for 24 h at 30 °C in an orbital shaker at 150 rpm.

2.2. DNA Extraction and Clade Classification

Genomic DNA was extracted using a phenol-chloroform isoamyl alcohol (PCI—25:24:1 *v*/*v*) method with minor modifications [36]. The MMC1-MMC8 and CDC type isolates AR0381 and AR0387 [37] were grown in yeast-peptone-dextrose (YPD—Becton Dickinson and Co, Franklin Lakes, NJ, USA) media. For each isolate, yeast cell pellets were suspended in lysis buffer, and cells were lysed by vigorous shaking with zirconia/silica beads. First ammonium-acetate, and then PCI were added to each tube. After centrifugation, the upper aqueous phase from each sample was collected and added to isopropanol at a 1:1 ratio. Samples were incubated at −20 °C for 45 min. The tubes were centrifuged, and the

supernatants were removed. The pellets were washed with 70% EtOH and dried at room temperature. RNase-supplemented nuclease-free water was used to suspend samples, and DNA integrity was verified by gel electrophoresis.

A recently described PCR-based clade-identification system (ClaID) was used to classify the clinical isolates [38]. For each isolate, the extracted genomic DNA was mixed with DreamTaq buffer (20 mM $MgCl_2$), DreamTaq DNA Polymerase (Thermo Scientific, Waltham, MA, USA), dNTPs mix (Roche, Basel, Switzerland), and the clade-specific forward and reverse primer pairs (Integrated DNA Technologies, Coralville, IA, USA), and nuclease-free water. PCR reactions were set up as established by Narayanan et al. [38], and the resulting amplicons were analyzed by electrophoresis on a 2% agarose gel. Verified isolates were also used as a control; therefore, PCR bands from the unclassified isolates were compared to the results of the known isolates from the CDC.

2.3. Sequence Analysis

Isolated genomic DNA was further used for targeted genome sequencing. Phusion Green master mix (Thermo Scientific, Waltham, MA, USA) was used to amplify *ERG3*, *ERG11* and *TAC1b* with primer pairs listed in Supplementary Table S1. Automated reaction cleanup with (Beckman Genomics, Brea, CA, USA) and PCR purification with Ampure (Beckman Genomics, Brea, CA, USA) were performed before Sanger sequencing each amplicons using sequencing primers. Sequence fragments were aligned and compared to type isolates from their corresponding clades (clade I: B8441; clade II: B11220).

2.4. Antifungal Microplate Susceptibility Test

The susceptibility test was performed according to the CLSI M27 4th Ed protocol [39]. Briefly, stock solutions of amphotericin B (AmB), caspofungin (Cas), and fluconazole (FCZ) were serially diluted in round-bottom 96-well plates (in RPMI 1640 buffered with MOPS—RPMI/MOPS) to curves starting at 32 µg/mL, 16 µg/mL, and 256 µg/mL, respectively. DMSO at 1% was used as a solvent control. *C. auris* suspensions in RPMI/MOPS (Corning, NY, USA) were adjusted to 2.5×10^3 cells/mL and added to the plate containing the antifungals. Following an incubation at 37 °C for 24 h, the plates were visually analyzed to determine the minimal inhibitory concentration (MIC). Resistance was established according to the tentative antifungal cutoff values provided by CDC [37]. Three experiments were performed with three replicates per condition.

2.5. SNO-MP Synthesis and Nitrosation

Nitric oxide-releasing macroparticles, SNO-MPs, were synthesized as previously described [40,41]. The first step is synthesis of thiolated microparticles (Thiol-MP): briefly, this process consists of (i) acid-catalyzed hydrolysis of two silicate precursors, tetraethylorthosilicate (TEOS) and mercaptopropyltrimethoxysilicate (MPTS); (ii) co-condensation of hydrolyzed TEOS and MPTS to form a highly porous thiolated sol–gel monolith; (iii) a series of proprietary steps to remove any unreacted monomer and nano-sized particulate matter; and (iv) a drying step to evaporate residual water and ethanol. The second step is the nitrosation of Thiol-MP to form S-nitrosothiol-MP (SNO-MP): Thiol-MP is suspended in methanol, and HCl is added and mixed, followed by the addition of sodium nitrite. Residual HCl is then neutralized with NaOH. SNO-MPs were spun down and suspended in cold RPMI/MOPS.

2.6. SNO-MP Microplate Susceptibility Test

Working solutions of activated SNO-MP were serially diluted in round-bottom 96-well plates (using RPMI/MOPS) starting at 40 mg/mL, and *C. auris* suspensions were prepared as described above. The plates were incubated at 37 °C for 48 h. To address fungal viability,

a solution of XTT and menadione was added to the plates and incubated at 37 °C for 2 h. Color development was monitored by absorbance reading at 490 nm and MICs were labeled as the lowest concentration that dampened metabolic activity by at least 80% in comparison to the control group. The experiments were performed with two replicates per concentration in each experiment, for a total of three independent experiments.

2.7. Hemolysis by SNO-MP

Hemolytic activity was assessed using 5% sheep blood agar medium (Colorado Serum Company, Denver, CO, USA), and enzymatic activity was evaluated on 90 mm Petri dishes using the Pz value (Pz = A/B), where A is the diameter of the colony and B is the sum of the colony's and the halo's diameters. The test was performed using mSNO nanoparticles at concentrations of 40, 20, and 10 mg/mL. Candida albicans (ATCC SC5314) suspension of 1×10^6 yeasts/mL served as the positive control. Inoculations of 5 µL were performed in triplicate and incubated at 37 °C for 48 h. Hemolytic activity was categorized as follows: Pz = 1 (negative), 0.70–0.99 (low), 0.40–0.69 (moderate), and <0.39 (high).

2.8. Galleria Mellonella Infection

A *G. mellonella* infection model was used to investigate the virulence of the selected *C. auris* strains in vivo. Ten larvae (0.3 g each) were used per experimental condition. Larvae were inoculated with 10 µL of fungal suspensions totaling 5×10^5 yeasts/larva into the hemocoel through the last proleg using a 31 G insulin syringe. Prior to inoculation, yeasts were washed 3x with PBS, counted, and immediately used to infect the larvae. PBS buffer was used as a negative control of infection. All larvae were placed in sterile Petri dishes and kept in the dark at 37 °C. Larval mortality was monitored daily for 10 days. Death was assessed by the lack of movement in response to stimulation. Data were analyzed with GraphPad Prism 8 software (GraphPad, San Diego, CA, USA), and statistical analysis was performed using Mantel–Cox log-rank tests for Kaplan–Meier survival curve. A total of three independent experiments were performed.

3. Results

3.1. Clade Classification

The clades of the clinical isolates were classified based on a previously described ClaID system [38]. MMC1 and MMC3–8 all belonged to clade 1, along with the CDC's already characterized AR0387. Only MMC2 diverged from the isolate set; similarly to the control isolate AR0381, it belonged to clade 2 (East Asia) (Figure 1).

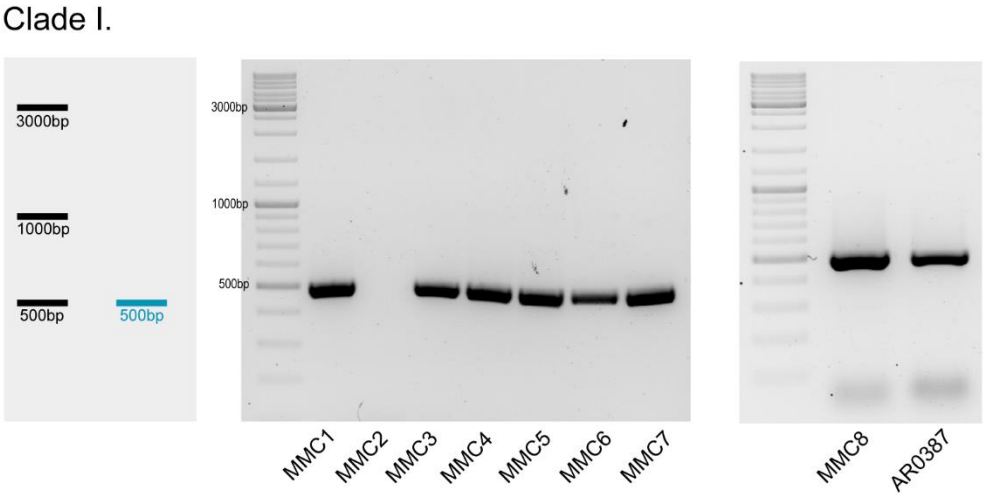

Figure 1. *Cont.*

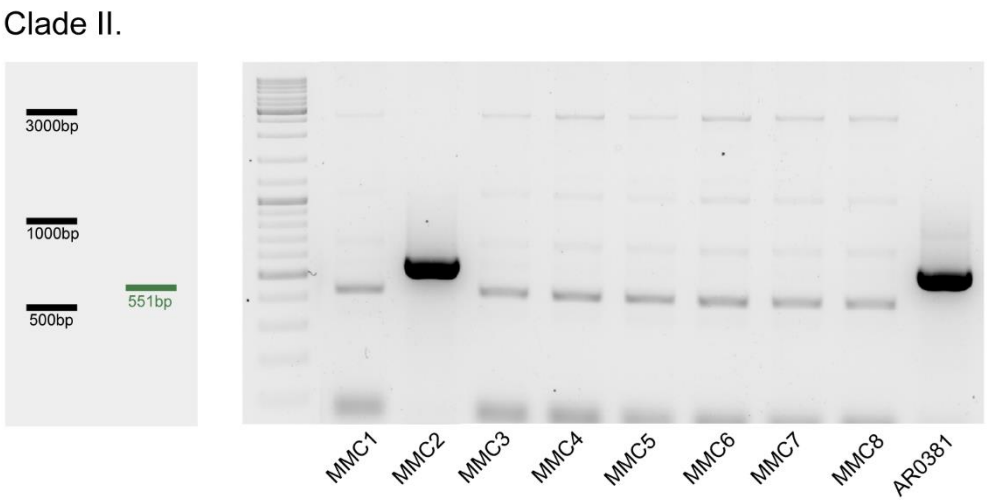

Figure 1. Clade identification. Schematic images of the expected clade I and clade II-specific fragment sizes, compared to the agarose gels, containing the clade-specific PCR fragments of the unclassified strains (MMC1–8) and the already classified clinical isolates (AR0387, AR0381).

3.2. Antifungal Susceptibility

The antifungal microplate susceptibility test showed that the tested *C. auris* strains were all resistant to fluconazole with MICs > 256 μg/mL except for MMC2, which had an MIC of 4 μg/mL (Table 1). MMC1–8 were susceptible to caspofungin (0.25 μg/mL). The MIC for amphotericin B was 1 μg/mL for all isolates except MMC2, which had an MIC of 0.25 μg/mL.

Table 1. Antifungal MIC for 8 *Candida auris* strains were determined (μg/mL). Values that are bolded indicate resistance to the specific antifungal, according to CDC's tentative MIC breakpoints for *C. auris*.

Minimal Inhibitory Concentration (μg/mL)			
Strain	**Amphotericin B**	**Caspofungin**	**Fluconazole**
MMC1	1	0.25	**>256**
MMC2	0.25	0.25	4
MMC3	1	0.25	**>256**
MMC4	1	0.25	**>256**
MMC5	1	0.25	**>256**
MMC6	1	0.25	**>256**
MMC7	1	0.25	**>256**
MMC8	1	0.25	**>256**

3.3. Detection of SNPs

Compared to the CDC standard strain, all fluconazole-resistant isolates carried non-synonymous mutations in both *ERG11* (K143R) and *TAC1b* (A640V) genes. Three additional SNPs were also identified in the *TAC1b* gene of the MMC2 isolate, but the effects of these mutations remain unknown. Although *ERG3* was also analyzed, no amino acid changes were identified in this gene in either isolate (Figure 2).

Gene ID	Gene name	Amino acid Substitution	Clade I							Clade II
			MMC1	MMC3	MMC4	MMC5	MMC6	MMC7	MMC8	MMC2
B9J08_001448	*ERG11*	K143R	■	■	■	■	■	■	■	
B9J08_004820	*TAC1b*	A640V	■	■	■	■	■	■	■	
B9J08_004820	*TAC1b*	V4A								■
B9J08_004820	*TAC1b*	F862L								■
B9J08_004820	*TAC1b*	D865A								■

Figure 2. Identified nonsynonymous amino acid substitutions. According to the targeted sequencing of three antifungal resistance-associated genes (*ERG3, ERG11, TAC1b).* Compared to the CDC isolates on the corresponding clades (B8441; B11220) non-synonymous amino acid substitution conferring SNPs were found in the *ERG11* and *TAC1b* genes, while no mutation was found in *ERG3.* Colored boxes indicate the presence of the listed substitutions.

3.4. SNO-MP Microplate Susceptibility Test and Hemolytic Activity

The microplate susceptibility test with the SNO-MP showed a similar pattern to the one with the antifungals, as the NO-releasing microparticles had antifungal activity against all 8 *C. auris* strains. The MIC for all strains was 20 mg/mL, except for MMC2, with an MIC of 5 mg/mL (Table 2). No hemolytic activity was detected, as seen by the absence of halo formation in blood-agar plates inoculated with the particles in different concentrations even higher than the MICs (Figure 3). In contrast, the positive control, *C. albicans* (ATCC SC5314), showed a halo formation (Pz = 0.397), confirming enzymatic activity.

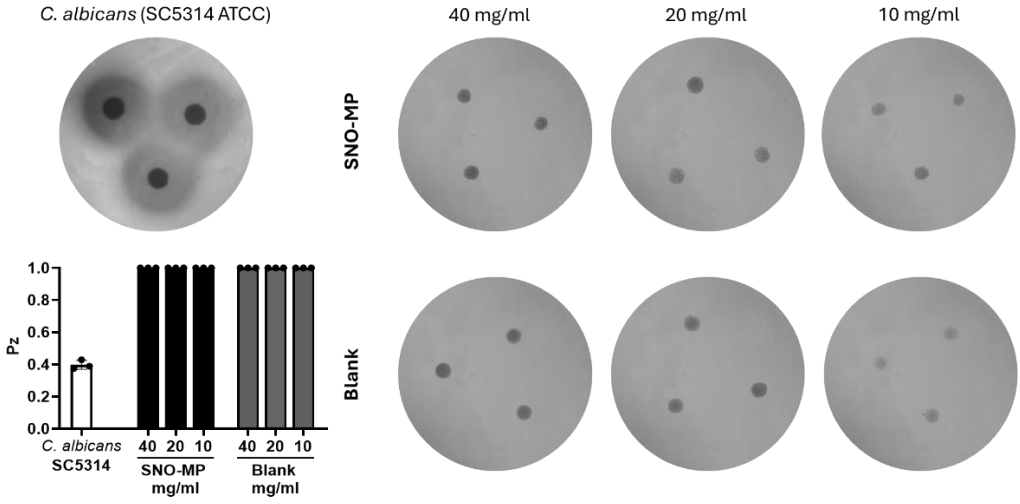

Figure 3. Hemolytic activity by SNO-MP. Hemolytic activity was evaluated using. C. albicans SC5314 was used as a positive control. Different concentrations of SNO-MP and blank particles were inoculated on plates containing sheep blood agar and incubated for 48 h at 37 °C until development of halo indicating hemolytic activity. Pz values were calculated as Pz = A/B, where A is the colony diameter and B is the total diameter of the colony plus the enzymatic activity halo (Pz = 1 indicates no hemolytic activity).

Table 2. SNO-MP MIC for 8 *Candida auris* strains was determined in mg/mL.

Minimal Inhibitory Concentration (mg/mL)			
Strain	**SNO-MP**	**Strain**	**SNO-MP**
MMC1	20	MMC5	20
MMC2	5	MMC6	20
MMC3	20	MMC7	20
MMC4	20	MMC8	20

3.5. Virulence in Galleria mellonella

G. mellonella injected with a lethal inoculum of *C. auris* were observed up to 10 days. Survival curves demonstrate that the clade 2 strain MMC2 was significantly more virulent than the clade 1 strains MMC1, MMC4, and MMC5. Other clade 1 strains were not significantly different than MMC2. Control larvae injected with PBS displayed a 100% survival rate for the experimental timeframe (Figure 4).

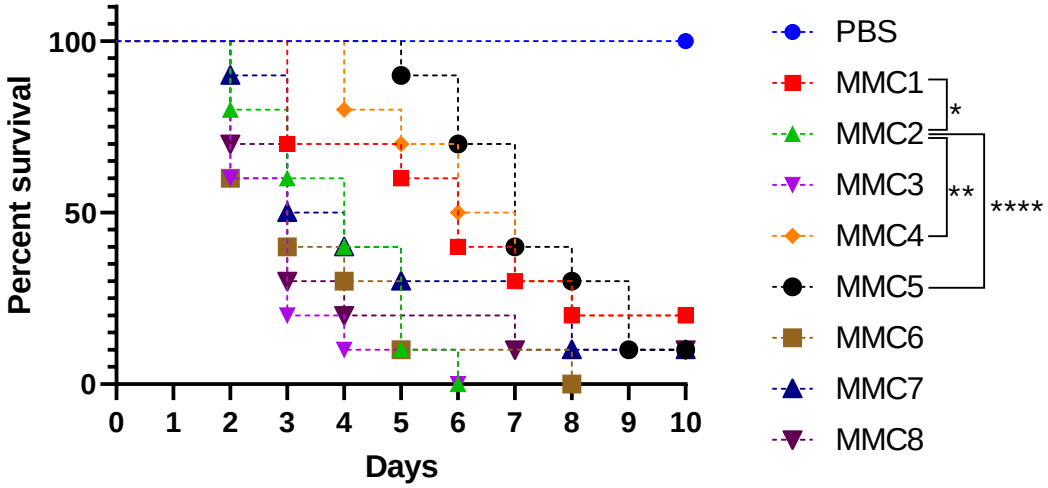

Figure 4. *Galleria mellonella* survival rates following infection with *Candida auris*. *G. mellonella* were infected with a lethal inoculum of *C. auris* yeasts, and survival was monitored for 10 days. Infection with the clade 2 strain, MMC2, resulted in a significantly higher mortality rate compared to the clade 1 isolates MMC1, 4, and 5. Infection with other clade 1 isolates had no significant survival rates compared to MMC2. Statistical analysis was performed with log-rank Mantel–Cox, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

4. Discussion

With its rapid emergence, *C. auris* has attracted intense attention from the medical community for its multidrug-resistant profile and nosocomial pattern of transmission. In fact, the threat of *C. auris* infections was recently recognized by the World Health Organization 2022 Fungal Priority Pathogen List, with *C. auris* being added to the critical group alongside *Cryptococcus neoformans*, *Candida albicans*, and *Aspergillus fumigatus* [42].

In this study, we characterized clades and antifungal resistance profiles for eight different strains isolated in one healthcare facility in the Bronx. In line with previous studies, clade classification further verifies that clade 1 is the most common type in New York state, as seven out of the eight MMC isolates (87.5%) showed this genotype [8,43]. The only diverging strain, MMC2, belonged to the rarely isolated clade 2 (Figure 1). Generally, isolates from clade 2 are associated with sporadic cases, while clade 1 has been linked to excessive outbreaks of systemic candidiasis [8,44]. This difference in clinical manifestations may explain the differences in isolation frequencies between the clades.

Our results also show a resistance pattern that correlates to the literature reports of the initial four clades of *C. auris* that showed 93% resistance to fluconazole, 35% resistance to amphotericin B, and 7% resistance to echinocandins (including caspofungin) [2]. Seven out of eight strains (87.5%) tested in this study showed resistance to fluconazole (MMC1 and MMC3–8), and no strains resistant to AmB and caspofungin were found (Table 1). The seven FCZ-resistant isolates belong to clade 1, a clade that often exhibits a high frequency of antifungal resistance [8,43]. The only identified strain from clade 2, MMC2, was susceptible to FCZ, Cas, and AmB (Table 1). It is also important to highlight that MMC2, the only strain from clade 2, was not only susceptible to fluconazole but also the most susceptible to AmB, in accordance with previous data from our group [45] and the literature [7,8].

Genomic analysis from Chow et al. indicated that in clade 1 isolates, the resistant phenotype is predominantly associated with single nucleotide polymorphisms (Y132F, K143R) in the coding region (*ERG11*) of the 14-α-demethylase enzyme. Since 96% of the 118 studied clade 1 isolates contained either of these mutations, we can hypothesize that they might also be present in the MMC strains [8]. With that in mind, we verified the genomic background of the resistant phenotypes in our isolates and found out that resistance profiles corresponded with sequencing results, as all resistant strains harbored both one of the most common resistance-inducing amino acid substitution (K143R) in Erg11 and the likewise commonly present mutation A640V in Tac1b [46]. Additional non-synonymous mutations were also identified in the MMC2 strain (V4A; F862L; D865A), but they seem to have no definitive effect on antifungal resistance (Figure 2).

There are several pharmacological limitations associated with conventional antifungal therapy, such as low bioavailability, lack of selectivity, and high toxicity [30,31]. This, in addition to *C. auris's* high resistance to antifungal drugs, makes alternative treatments in high demand. As one of the few novel platforms for antifungal delivery currently in development, micro- and nano-drug delivery systems (MiNaDDS) offer an interesting alternative to counter both pharmacological limitations and antifungal resistance [47].

Our results demonstrate that the microparticle has antifungal activity similar to what is described for other NO-releasing particles that were recently tested with MMC1 and MMC2 [11]. Other than that, the susceptibility pattern was also similar to what was observed for the conventional antifungal agents, with MMC2 being more susceptible than strains belonging to clade 1 (Table 2). Moreover, the microparticle did not promote hemolysis (Figure 3), suggesting that mSNO nanoparticles may exhibit antifungal properties without promoting hemolytic-associated toxicity, making them promising candidates for further investigation in therapeutic applications. The microparticle treatment approach for *C. auris* could be applied to systemic approaches for candidemia as well as topically for either ear infections or as part of a colonization eradication method [48–50]. The latter is especially notable as there currently is no markedly effective de-colonization method, which is essential for mitigating risks for outbreaks [51].

Clade 2 isolates have been correlated to a less virulent profile, mainly because they are rarely isolated and usually not linked to invasive disease, as most isolates originate from ear infections, while isolates from clade 1 are usually associated with invasive disease and large outbreaks and are therefore considered more virulent [8,44]. However, this correlation was not observed in our in vivo model of infection (Figure 4), with clade 2 strain MMC2 not only being as virulent as four clade 1 strains in our wax moth model but also being significantly more virulent than 3 clade 1 strains (MMC1, 4, and 5). It is important to highlight that even though *G. mellonella* has been increasingly used as an infection model for fungal pathogenesis due to its multiple advantages [52–54], this model also has its shortcomings, like the lack of an adaptive immune system, and experiments with more

complex in vivo models and a larger variety of strains are needed in order to achieve more definitive results.

Taken together, our data corroborates the correlation between clades and the resistance profiles of the tested strains. The findings also support the need for more studies on MiNaDDS, as NO-releasing platforms have significant potential as alternative treatments for *C. auris* infections, but further development of the platform, in vivo experiments, and full characterization of the microparticle formulation are still necessary. Additionally, as far as the model limitations go, our present data suggests that clade 2 isolates can be at the very least as virulent as clade 1 isolates, going against initial correlations between these two clades and virulence profiles.

5. Conclusions

In conclusion, our study highlights the complex interplay between clade classification, antifungal resistance profiles, and virulence in *C. auris*. While clade 1 remains predominant at our institution, our findings demonstrate that clade 2 isolates, often considered less virulent, can exhibit comparable or even greater virulence in an invertebrate model of infection. Our study also emphasizes the potential of innovative treatment strategies like MiNaDDS, particularly NO-releasing platforms, to address the challenges posed by *C. auris*. Although further experiments featuring more sophisticated in vivo models and a broader pool of strains are needed, our findings highlight the importance of continued research into alternative therapeutic options.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microbiolres16010015/s1. Table S1: Primers used for the amplification of the selected resistance related genes.

Author Contributions: Conceptualization, J.D.N. and D.Z.-M.; Methodology, A.F.V., F.B., J.P.G., A.B.J., A.D., L.N., D.Z.-M. and D.C.-J.; Formal analysis, A.F.V., F.B., J.P.G., A.B.J., E.V.C.d.A., D.Z.-M. and D.C.-J.; Investigation, A.F.V., F.B., A.B.J., E.V.C.d.A., D.Z.-M. and D.C.-J.; Resources, A.D. and J.D.N.; Data curation, A.F.V., L.N., J.D.N. and D.Z.-M.; Writing—original draft, A.F.V. and F.B.; Writing—review and editing, A.D., L.N., J.D.N. and D.Z.-M.; Visualization, A.F.V., F.B., J.P.G., A.B.J. and E.V.C.d.A.; Supervision, A.F.V., F.B., L.N., J.D.N. and D.Z.-M.; Project administration, J.D.N. and D.Z.-M.; Funding acquisition, J.D.N. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Study was performed according to guidelines supported by IRB Number: 2016–7455 (approved on 2 July 2017) of the Albert Einstein College of Medicine and Montefiore Medical Center. All in vivo experiments were performed in a *Galleria mellonella* model of infection and therefore exempt from legal and ethical considerations.

Informed Consent Statement: *C. auris* strains were gathered with informed consent from all eight patients under the guidelines approved by the institutional review board (IRB Number: 2016–7455 approved on 2 July 2017) of the Albert Einstein College of Medicine and Montefiore Medical Center.

Data Availability Statement: The original sequencing data presented in the study is openly available in NCBI SRA database under the following reference number: PRJNA1189548.

Conflicts of Interest: A.D. is employed by Zylo Therapeutics. All remaining authors have no conflicts of interest to declare.

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