SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

In vivo virulence of *Candida auris* isolates and efficacy of amphotericin B treatment in a neutropenic mouse model

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The PhD Defense takes place in the Lecture Hall of the Department of Internal Medicine, Building "A", Faculty of Medicine, University of Debrecen at 1:00 p.m., on the 17th of December, 2025.

Introduction

Candida auris is a multidrug-resistant, opportunistic pathogen first identified in Japan in 2009 (Satoh et al. 2009). By the mid-2010s, outbreaks of the fungus were recorded on several continents, and its global spread led to the isolation of different strains on all continents except Antarctica by the end of the decade (Sekyere et al. 2018). *C. auris* isolates were divided into six phylogenetically distinct clades (South Asian, East Asian, South African, South American, Iranian and the newly discovered Singaporean). Compared to each other, these clades show significant differences in virulence, phenotypic characteristics and antifungal agent susceptibility (Lockhart et al. 2016; Szekely et al. 2019; Borman et al. 2016; Suphavilai et al. 2024).

C. auris poses a therapeutic challenge not only because of its multidrug resistance, but also because it frequently causes infections among high-risk patients, especially those with underlying and comorbid conditions (diabetes mellitus, cardiovascular disease, immunosuppression, hematological malignancies and patients with coronavirus 2019) (Armstrong et al. 2016; Rudramurthy et al. 2017; Chowdhary et al. 2020). Data from the Centers for Disease Control and Prevention showed that 90% of *C. auris* isolates tested in the US were fluconazole resistant, 30% were amphotericin B resistant and <0.5% were echinocandin resistant (https://www.cdc.gov/fungal/candida-auris/cauris-antifungal.html).

In our previous studies, we compared the *in vitro* susceptibility of *C. auris* isolates belonging to four major clades (South Asian, East Asian, South African and South American) to antifungal agents (Papp et al. 2021; Kovács et al. 2021). Anidulafungin, caspofungin, micafungin and rezafungin were fungistatic against the four clades in time-kill studies in RPMI-1640 or 50% serum (Kovács et al. 2021). Amphotericin B (AMB) at therapeutic concentrations (1 mg/L) was shown to be fungicidal against 33%, 50%, 50% and 16.7% of isolates from the South Asian, East Asian, South African and South American clades, respectively (Papp et al. 2021). In contrast, Dudiuk et al. reported concentration-dependent but isolate-independent killing activity using >2 mg/L AMB against nine Colombian isolates they tested in their time-kill study (Dudiuk et al. 2019).

The aim of this study is to compare the *in vivo* virulence of *C. auris* isolates from four major clades (South Asian, East Asian, South African and South American) and to determine the *in vivo* efficacy of AMB in a neutropenic mouse model by letality experiments, fungal burden examinations and histopathological studies.

Objectives

In our work, we determined the *in vivo* virulence of the available *C. auris* clinical isolates in a neutropenic mouse model and compared the data with the results obtained with *C. albicans* strains used as controls. As a continuation of this series of studies, we examined the *in vivo* efficacy of amphotericin B against *C. auris* isolates that we considered representative.

4.1. We tested the *in vivo* virulence of *C. auris* isolates from four geographical clades (South Asian n = 5, East Asian n = 4, South African n = 5 and South American n = 5) against *C. albicans* controls in a neutropenic mouse model.

4.1.1. Survival experiments were performed to determine the *in vivo* virulence of the tested *C. auris* isolates based on the lethality rate, and the results were evaluated in comparison with the results of *C. albicans* control isolates.

4.1.2. Tissue fungal burden of groups infected with *C. auris* isolates and *C. albicans* isolates used as controls was determined and compared by organ culture in heart, kidney, liver, and spleen tissues.

4.1.3. Histopathological characteristics of the fungal invasion were characterized in heart, kidney, liver and spleen of mice infected with *C. auris* and *C. albicans* isolates.

4.2. We examined the *in vivo* efficacy of amphotericin B in neutropenic mice against *C. auris* isolates (South Asian n = 2; East Asian n = 2; South African n = 2; South American n = 4; two of which were environmental).

4.2.1. Survival experiments were performed to determine the *in vivo* efficacy of amphotericin B based on a comparative analysis of survival rates between treated and untreated groups.

4.2.2. Organ culture was used to determine the *in vivo* efficacy of amphotericin B therapy in terms of tissue fungal burden in the treated and untreated groups in heart, kidney and brain.

4.2.3. We used histopathological studies to characterize the *in vivo* efficacy of amphotericin B therapy on the histopathological characteristics of tissue colonization in heart, kidney and brain.

Materials and Methods

Origin of *Candida auris* isolates

In our experiments with *C. auris*, the isolates tested were obtained from the UK National Mycology Reference Laboratory, and Prof. Andrew Borman and colleagues used 28S rRNA and/or ITS1 region sequencing to classify isolates at the clade level (Borman et al. 2017). *C. albicans* clinical isolates 3666 and 2606 from bloodstream infections were used as comparative controls.

Immunosuppression of the mice

In the experiments, BALB/c neutropenic female mice (21-23g) were used, 7-11 per group (Charles River Laboratories), cared for according to the guidelines described in the "Use and Care of Laboratory Animals". The *in vivo* experiments are licensed under the numbers 12/2008 and 12/2014 DE MÁB.

The experimental animals received 150 mg/kg cyclophosphamide (Endoxan, University Pharmacy, Debrecen, Hungary) intraperitoneally on the fourth day before infection and 100 mg/kg on the day before infection (Andes et al. 2010; Kovács et al. 2014). To maintain a sustained neutropenic state, 100 mg/kg cyclophosphamide was administered on the second and fifth day after infection, respectively, and every third day thereafter for the duration of the experiment.

Method of infection

24 hours before infection, the isolates were disrupted on Sabouraud agar medium and incubated at 35 °C until the onset of infection. Using a sterile cotton swab, the developed fungal phages were collected from the surface of the medium and suspended in physiological saline. The collected fungal cells were centrifuged at 3000 g for 10 min. After the supernatant was removed, 25 mL of physiological saline was pipetted to the settled cells and centrifuged for another 10 min, and this washing process was repeated twice more.

Eight mL of physiological saline was added to the settled cells after removal of the supernatant. Cell counting was performed in a Bürker chamber from a 1:100 dilution of the suspension. The cell count of the inoculum prepared was checked by quantitative inoculation. Cell aggregation was observed in *C. auris* isolates from the South African and East Asian clades (Borman et al. 2017), so to eliminate this phenomenon, these inocula were prepared in PBS instead of physiological saline.

The experimental mice were infected via the lateral tail vein and inoculated with 0.2 mL of fungal cell suspension per individual. For *C. auris* isolates, a fungal cell volume of 10^7 CFU/mL was obtained in both lethality and organ persistence experiments. Individuals infected with C. albicans isolates received 10^5 CFU/mL fungal cell volume in the lethality experiment and $5x10^4$ CFU/mL in the organ persistence experiment. The doses used in these experiments were determined during our preliminary experiments.

Amphotericin B treatment

In survival and tissue fungal burden experiments to investigate the *in vivo* efficacy of amphotericin B, individuals in the treated group received 1 mg/kg amphotericin B in 0.5 ml intraperitoneally 24 hours post-infection and repeated over the following four days. Individuals in the control groups received 0.5 mL of sterile physiological saline intraperitoneally (Bayegan et al. 2011).

Survival experiment

During the survival experiments, each group was observed for 21 days. The neutropenic status of the individuals was maintained throughout the study. Individuals in each group were checked twice daily and if they showed severe signs of systemic infection, i.e. they were no longer able to perform basic life support functions (locomotion, feeding), they were killed by cervical dislocation to prevent further senescence.

Organ culture

For tissue fungal burden studies, the test groups were killed by cervical dislocation on the sixth day post-infection and subsequently dissected. In a comparative study of virulence of *C. auris* and *C. albicans* isolates, in order to obtain information on the early stage of infection, groups were included in the experiment and dissected on the second day post infection. Kidney, spleen, liver and heart were removed at both the second day and the sixth day necropsy. In an experiment to test the efficacy of AMB in vivo, the kidney, heart and brain were removed. The weight of each organ was weighed and homogenized in a sterile rubbing cup, and a 1:10 dilution series was prepared after the addition of 1 mL of sterile physiological saline to the homogenate. From the dilutions, 100 µL was inoculated onto Sabouraud agar medium. The media were incubated at 35°C for 48 h and the colonies grown were counted. The number of cultured fungi was expressed as CFU/g, with a lower limit of detection of 50 cells/g (Bayegan et al. 2011).

Histopathological examination

In a comparative study of the virulence of *C. auris* and *C. albicans*, histopathological examinations were performed in kidney, spleen, liver and heart, and to investigate the *in vivo* efficacy of amphotericin B in kidney, heart and brain tissues. The removed organs were fixed in 10% formalin for 24 hours. Organ preparations were processed at the Department of Pathology, Kenézy Gyula Campus, University of Debrecen. Tissue sections with a thickness of 4 µm were prepared from the organ preparations using the paraffin block technique and Hematoxylin-Eosin (H&E) staining was applied for general examination of tissue morphology. Periodic acid-Schiff (PAS) reaction was used to identify fungal cells (Pupim et al. 2017). Mallory's phosphotungsticacid-hematoxylin (PTAH) staining was applied to cardiac tissue samples to detect contraction band necrosis.

Urine culture

During our lethality experiments to test the efficacy of amphotericin B *in vivo*, we felt the need to obtain information on the status of the treated and untreated groups before the animals died. This intention was made possible by our observation that during intraperitoneal inoculation of animals, a slight transabdominal pressure over the bladder results in forced urination of the animals. We used this phenomenon to collect samples for urine culture. After disinfection of the periurethral region, we collected urine samples of an average volume of 50-80 µl into sterile Eppendorf tubes and immediately prepared a serial dilution on a 1:10 basis. From each dilution 100 µL were inoculated onto Sabouraud agar medium and incubated at 35°C for 48 hours. The colonies grown were counted and CFU/mL were determined from the colony counts.

Statistical analysis

In the survival experiments, statistical analysis was performed using the Kaplan-Meier test. In the tissue fungal burden examinations, the Kruskal-Wallis test (GraphPad Prism 4.03, Windows) was used for statistical analysis.

Results

Virulence of *Candida auris* clades and *Candida albicans* based on lethality

In the survival experiments, the groups infected with *C. auris* isolates did not show any particular deterioration in condition until their sudden death, and no lack of life-sustaining abilities (locomotion, feeding), except for the groups infected with strains belonging to the South American clade. There were also no symptoms suggestive of central nervous system involvement. There were significant differences in lethality between clades (South American: $P=0.0005$, South Asian: $P=0.0010$, South African: $P<0.0001$, East Asian: $P=0.0255$).

During the 21-day observation of the groups, members of the South American clade were the most virulent, with an average mortality rate of 96% (for some isolates: 90-100%). Groups infected with members of the South Asian clade also had a high mortality rate, averaging 80% (for some isolates: 50-100%). The mice infected with these isolates were characterised by very early mortality, with high numbers of deaths observed as early as the third to fourth day after infection, as shown by the steep drop in survival curves. Much more modest mortality rates were observed in groups infected with isolates belonging to the South African and East Asian clades, with an average mortality of 45% for both clades. For the South Korean isolate from blood, mortality was 50-70%. Individuals started to die relatively late, on days 7 to 8, with only one or two deaths per day in most cases, as indicated by the sloping, steep slope of the survival curve.

The mortality of the two *C. albicans* isolate-infected groups used as controls was 90-100% ($P>0.05$). The infected individuals showed signs of systemic infection, with difficulty in moving and feeding until death. On the fifth day after infection, 50-60% of the groups and 80-90% on the seventh day exited, reflecting the fact that the fungal cell volume used for infection was one hundredth of that used for groups infected with *C. auris* isolates, and is representative of the differences in virulence between the two *Candida* species.

Virulence of *Candida auris* clades and *Candida albicans* based on tissue burden

In tissue fungal burden examinations of groups infected with *C. auris* isolates, we found that the heart and kidney were more affected than the other two organs, regardless of clade. The amount of fungal cells isolated from the organs also suggested that there was a strong correlation between virulence and tissue persistence, as more virulent isolates in the lethality studies showed higher fungal burden in the organs. A difference of at least an order of magnitude was observed between the cardiac and renal tissue persistence results of isolates with high lethality (90-100%) and moderate lethality (30-60%). The difference between the heart and kidney tissue fungal load of the more virulent isolates from the second and sixth day of autopsy was also found to be more significant than for the more moderate isolates.

The results from the second day dissection showed similar levels of spleen fungal burden for all isolates ($P>0.05$), with significantly higher germ counts only in the sixth day dissection of the South American 16565 isolate group compared to East Asian isolate 15 and South Asian isolate 164 ($P<0.001$).

Of the organs tested, tissue fungal burden of the liver was the lowest. The highest germ count ($3.6x10^6$ CFU/g) was observed in samples from individuals of the group

infected with South Asian isolate 196, and the lowest in samples from isolate 164 belonging to the same clade ($6.5x10^3$ CFU/g).

Comparing the results of organ cultures of individuals in the group infected with *C. albicans* isolate (3666) on days 2 and 6, significant increases were observed in heart (P=0.016), kidney (P=0.008) and spleen (P=0.008), whereas the tissue persistence of liver was less than 104 CFU/g on both days, which is lower than the values observed for highly lethal *C. auris* isolates. Fungal cell counts of heart and kidney organ cultures from the sixth day autopsy were similar to those of the high lethality *C. auris* isolates.

Virulence of *Candida auris* clades based on histological examination of the heart, kidneys, liver, and spleen

Two individuals per clade from the groups inoculated for tissue persistence experiments were used for histopathology, and one individual from each clade was dissected 24 hours post-infection to investigate early cardiac involvement. Seven freshly dead individuals from the lethality experiments were also dissected and examined. Individuals from the South Asian 196, East Asian 12373, South African 204 and South American I-24 isolate-infected groups were used for these studies.

Large fungal cell aggregates were observed in the organs of both the specimens dissected on day 6 and those that died in lethality experiments, with the exception of the spleen. Blastoconidia and budding cells were found in the organs examined, but no hyphae or pseudohyphae were identified in any of the isolates.

Early involvement of the heart was observed as early as 24 hours after infection, with large numbers of budding fungal cells and blastoconidia in the myocardial arterioles. Irrespective of the isolate used for infection, large numbers of fungal cell aggregates with coagulation necrosis of myocardial cells were observed in myocardial fibres from individuals dissected on day 6 and killed in lethality experiments. Mallory PTAH staining also showed that myocardial fibres had lost their ciliary sheath, indicating the phenomenon of contraction band necrosis or myofibrillar degeneration.

Examination of the kidneys revealed multifocal infiltration of the renal parenchyma. Destruction of the renal tubules and large necrotising regions were observed, but the glomeruli were not affected by fungal cell colonisation. In the liver tissue, large fungal cell aggregates were found in the dilated sinusoids, in the parenchyma the fungal cells spread radially with central necrosis of the lobules. A low degree of tissue involvement of the spleen was observed, with low cellularity of fungal cell clusters, blastoconidia and budding fungal cells.

Virulence of *Candida albicans* based on histological examination of the heart, kidneys, liver and spleen

Blastoconidia and budding fungal cells were observed in tissues of individuals infected with *C. albicans* isolate 3666. Pseudohyphae and hyphae were also found in the heart, kidneys and spleen, but were not detected in liver tissue samples. Histopathological examination of the heart revealed large necrotising regions in both endocardium and myocardium, but no signs of contraction-bar necrosis in individuals infected with *C. auris* isolates.

***In vivo* efficacy of amphotericin B based on lethality**

In the group infected with two isolates from the South Asian clade, AMB therapy did not affect the survival of the mice. By day 13 post-infection, mortality was 100% in the

treated groups (isolate 196: P=0.2004; isolate 27: P=0.1459), with the steep slope of the survival curve being representative of the ineffectiveness of AMB therapy.

In contrast, in the groups infected with East Asian isolates, the survival of individuals significantly improved in response to AMB therapy (isolate 12373: P=0.0009; isolate 12373: P=0.0005), but it is important to note that signs of ataxia and imbalance were observed in several individuals in the treated groups. Three out of eight survivors in the 12372 isolate-infected group and two out of five survivors in the 12373 isolate-infected group showed such signs. Such signs were also observed in the individuals of the 12372 group that died on day 20 and in the 12373 group that died on days 17 and 21, and were present 2-3 days before death. In the group infected with two isolates from the South African clade, AMB therapy improved survival only in the group infected with strain 2 (P=0.0189), but the individual that died on day 17 of the group also showed signs of ataxia 2-3 days before death.

In the examination of the group infected with the two blood isolates of the South American clade, AMB therapy significantly reduced mortality in the I-156 isolate infected group (P=0.0017). Two cases of ataxia were observed in this group, these individuals died on days 10 and 12 of the experiment.

Survival of the groups infected with South American environmental isolates was not affected by AMB therapy, with mortality of individuals in the groups starting on days 3-4 post-infection and all groups exiting by day 7 of the experiment. The steep slope of the survival curve illustrates the ineffectiveness of AMB therapy against these isolates.

In vivo efficacy of amphotericin B based on tissue fungal burden

In tissue fungal burden examinations, AMB therapy was ineffective in the groups infected with South Asian isolates, as the amount of fungal cells isolated from the organs of the treated group did not show a decrease in any organ (P>0.05). Values of ~10^8 CFU/g were determined for kidney and heart and ~10^6 CFU/g for brain. However, a significant reduction in cell number was observed in the groups infected with East Asian isolates, with AMB therapy reducing the number of fungal cells grown from organ cultures in brain (P<0.05), kidney (P<0.01) and heart (P<0.01). The mean amount of fungi cultured from brain and heart organelles in both treated groups was ~10^5 CFU/g. One individual from the 12373 isolate-infected group showed sterility in kidney and heart organ tissues. Similar results were observed in individuals from the groups infected with isolates of the South African clade, but in the group infected with isolate 204, AMB treatment in the brain did not result in a significant reduction in fungal abundance. Sterility of the kidney and heart was observed in one individual of the isolate 2 group. The results of tissue fungal burden examinations in the groups infected with isolates of the South American clade showed close agreement with those observed in the lethality experiment. In individuals of the I-156 isolate-infected group, AMB therapy resulted in a decrease of about two orders of magnitude in the number of fungal cells grown from kidney and heart (P<0.01) and less than one order of magnitude in the number of fungal cells grown from brain (P<0.05) organ cultures. For the other three isolates, there was no significant difference between the results of the treated and control groups, with the amount of fungi cultured from organs being almost similar.

In vivo efficacy of amphotericin B based on histological examination

Histopathological examination of dissected specimens on the second day post-infection showed early involvement of the kidneys and heart in both treated and control groups, but no fungal cells were detected in brain tissue. In the infected organs, mostly small fungal

cell aggregates were observed in the renal parenchyma and myocardial arterioles. Multifocal infiltration of hearts and kidneys was observed in autopsies of individuals that died between days 4 and 6 of lethality experiments, irrespective of clade. Examination of the myocardium revealed the presence of contractile-bar necrosis by Mallory PTAH staining. Brain tissue examination identified involvement of both cerebrum and cerebellum. Histopathological examination of individuals that died on days 17, 20 and 21 of the survival experiment in East Asian isolates (12372, 12373) confirmed cerebral and cerebellar involvement, but no fungal cells were identified in the kidneys or heart.

***In vivo* efficacy of amphotericin B based on urine culture**

The 196 (South Asian clade), the 12372 (East Asian clade), the 204 (South African clade), I-156 (bloodstream isolate from the South American clade) and 13112 (environmental isolate from the South American clade), the urine of control groups infected with isolates I-156 (bloodstream isolate from the South American clade) contained 3.2-5.4x10^3, 2-6x10^3, 1.6-4x10^3, 1.08-6.1x10^3 and 8.8x10^3-7.4x10^4 CFU/ml *C. auris*. The two-day AMB treatment (day 3) resulted in an average reduction of at least 1 log CFU compared to the control group results ($p < 0.001$ in all cases).

Discussion

Comparative study of virulence of *Candida auris* and *Candida albicans* in a neutropenic mouse model

The most common clinical presentation of *C. auris* infection is candidemia in high-risk patients in intensive care units. The main risk factors are diabetes mellitus, cardiovascular and gastrointestinal disease, hematological malignancies and corticosteroid treatment (Lockhart et al. 2016; Sekyere et al. 2018; Armstrong et al. 2016; Rudramurthy et al. 2017). Interestingly, neutropenia is not considered a major risk factor for invasive *C. auris* infection. This clinical experience is perhaps related to the in vitro finding that C. auris is more efficient than *C. albicans* in evading neutrophil engulfment and neutrophil-derived extracellular traps (Johnson et al. 2018). Despite apparently appropriate targeted antifungal therapy, candidemia often persists; septic metastatic complications (e.g. spondylodiscitis, endo- and pericarditis) are also common. The kidneys are also frequently involved in pathogenesis, both in cases with and without candidemia (Chowdhary et al. 2018; Morales-López et al. 2017; Adams et al. 2018; Vallabhaneni et al. 2017). In *C. auris* candidemia, the hospital mortality rate can reach 68-80% (Lockhart et al. 2016; Vallabhaneni et al. 2017). Mortality from *C. auris* is difficult to quantify accurately due to the severe underlying disease in patients; limited estimates range from 23% to 67% (Kim et al. 2024).

Data on clade-specific mortality are incomplete. In the first reported cases of *C. auris* candidemia, two of three patients infected with the East Asian clade died in Korea and persistent candidemia was observed in their cases despite FLU and AMB treatment (Lee et al. 2011). Lockhart et al. reported mortality rates of 47-72%, 60% and 33% for South Asian, South American and South African clades, respectively (Lockhart et al. 2016).

Data on clade-specific mortality are incomplete. The results of our survival experiments in the first *C. auris* candidemia in a neutropenic mouse model were consistent with results from other research groups showing that *C. auris* is less virulent than *C. albicans* (Wang et al. 2018; Ben-Ami et al. 2017; Torres et al. 2020). In our study, the virulence ranking of the four *C. auris* clades was as follows: South American clade > South Asian clade > South African clade = East Asian clade. Our lethality results obtained with the South Asian clade (50-100% lethality) correlate well with those previously reported in neutropenic BALB/c or CD-1 mice (40-100% lethality) at the same or higher (10^7-10^8 CFU/mouse) infective doses (Torres et al. 2020; Singh et al. 2019). The lack of similar studies with the South African clade precludes comparison with our results. However, we confirmed a previous observation in the *G. mellonella* model that aggregating South African *C. auris* isolates are less virulent than non-aggregating South African isolates (Borman et al. 2016). In addition, for virtually all clades, we found significant differences in virulence between isolates from the same clade. These results are in agreement with previous *in vitro* results showing that *C. auris* isolates can produce a number of virulence factors in a strain-dependent manner, but to a lesser extent than *C. albicans* (Sekyere et al. 2018; Larkin et al. 2017). It is worth noting that *C. auris* isolates belonging to the South American clade from bloodstream infection and hospital settings were not detected in the 21st century. This highlights the ability of *C. auris* to maintain its virulence in the hospital environment for long periods of time, and these isolates represent a potential source of colonization and infection for high-risk patients (Lockhart et al. 2016; Sekyere et al. 2018; Armstrong et al. 2016; Ruiz-Gaitán et al. 2018; Al Maani et al. 2019).

High tissue persistence regardless of clade supported our histological results; in all cases, multifocal, large fungal cell aggregates were found in the heart, kidney and liver of individuals dissected on day 6, whereas only discrete fungal cell clusters were observed

in the spleen. Interestingly, these lesions were observed in both aggregative and non-aggregative isolates, suggesting that both phenotypes behave similarly *in vivo*. These large fungal cell aggregates in tissues may protect the fungus from an effective immune response and promote fungal persistence and proliferation (Sekyere et al. 2018; Borman et al. 2016; Alfouzan et al. 2019). Furthermore, it cannot be excluded that the homogenization of these large tissue aggregates was not perfectly successful and some cells remained aggregated despite homogenization, which could result in underestimation of CFU values. This may explain why some isolates (e.g. isolate 164 from the South Asian clade) unexpectedly showed lower CFU on day 6 than on day 2.

Large, multifocal fungal lesions in the myocardium without involvement of the endocardium suggest a haematogenous origin; groups of fungal cells adherent to the coronary arteries were observed on day 1 post-infection in all groups infected with *C. auris* isolates. Our results are in agreement with those of studies in which high fungal loads ($\geq\times10^5$ CFU/g) were observed in myocardium in BALB/c, A/J, neutrophil elastase-deficient, C57BL/6 neutropenic and non-neutropenic mouse models of *C. auris* isolates from South American and South Asian clades (Torres et al. 2020; Xin et al. 2019). In contrast, endocardial involvement was found in virulent *C. albicans*, and myocardial involvement was predominantly subendocardial, suggesting that fungal cells penetrated the myocardium from the endocardium.

Despite the high fungal burden ($\geq5.2 \times 10^5$ CFU/g) in the heart as early as day 2, no increased early mortality was observed in the less virulent isolates from the South African, East Asian and South Asian clades. Our results suggest that delay in diagnosis and treatment of *C. auris* candidemia may increase the chances of developing myocardial failure induced by infection and the resulting sudden mortality.

High fungal burden in the kidney and/or heart is often associated with high fungal burden in the brain and lungs, as reported in both immunocompetent and neutropenic mice. Singh et al. observed simultaneously high tissue burden in the kidneys (2×10^8 CFU/g) and brain (2×10^6 CFU/g). Interestingly, the *C. auris* cells found in the brain were mainly localised in the capillaries, not in the brain tissue itself. *Candida* pneumonia is an uncommon manifestation of invasive *Candida* infections in humans, but in immunocompetent ICR and BALB/c mice, higher infective doses ($\geq10^7$ CFU/g) resulted in a lung tissue burden of 10^3->10^5 CFU/g (Fakhim et al. 2018; Wang et al. 2018). Although we did not investigate the fungal burden on the brain and lungs in this mouse model experiment, the high fungal burden on the heart and kidney suggests that invasion of the CNS and lungs may be possible. However, no evidence of meningeal involvement was observed (Torres et al. 2020).

High fungal burden was observed in organs, especially in the myocardium, confirming our hypothesis that the heart is one of the main targets in cases of *C. auris* infection. However, failure of other organs, especially the kidneys, may also contribute to mortality. Isolates belonging to the same clade showed differences in virulence; significantly higher virulence of the South American clade was clearly detectable in isolates from both bloodstream and hospital environments.

In vivo efficacy of amphotericin B against *Candida auris*

Among *Candida* infections, an increasing number of diseases caused by *non-albicans* species have appeared in recent years, which may be of concern due to their higher antifungal agent resistance (Esmailzadeh et al. 2018; Zarrinfar et al. 2021; Arastehfar et al. 2019). In our neutropenic mouse model experiment, 1 mg/kg AMB daily showed clade- and isolate-dependent activity against *C. auris*. The intravenous infection spread rapidly to internal organs, including the central nervous system; AMB treatment

significantly increased the survival rate of mice only in isolates from the East Asian clade and one isolate from a South African and one from a South American clade.

Survival data were consistent with the results of tissue fungal burden studies. In the otherwise less virulent East Asian and South African clades, a 5-day AMB treatment was able to reduce the fungal burden in the heart and kidney tissues, as confirmed by histological studies, leading to longer survival of the mice. AMB was ineffective in sterilizing the central nervous system, as indicated by the high fungal burden (at least 10^5 CFU/g) on day 6 and the appearance of neurological signs (torticollis, ataxia) observed in lethality experiments. Interestingly, in the most virulent South Asian and South American clades, clinical signs of CNS involvement were rarely or not observed in AMB-treated mice, as severe myocardial damage led to early death of the individuals before signs of CNS involvement could develop. The fact that mice infected with environmental isolates responded poorly to AMB treatment may be of some concern.

Our results are consistent with those of other studies. Lepak et al. found that 1.25, 5, and 20 mg/kg AMB administered 2 h post-infection was fungistatic against 8 of 9 *C. auris* isolates (from the four major clades) used in a neutropenic mouse model of renal fungal burden (Lepak et al. 2017). AMB showed the best results (more than 1 log CFU reduction) against a single Japanese isolate (East Asian clade). In 2021, Herrada et al. determined the efficacy of AMB against a single *C. auris* isolate in their lethality and renal tissue fungal burden assays. Antifungal agents (liposomal AMB, micafungin, rezafungin) compared to AMB were found to have significantly better activity in a neutropenic mouse model than conventional AMB when administered 2 hours after infection (Herrada et al. 2021; Hager et al. 2018). However, the efficacy of AMB in the treatment of fungal myocarditis and central nervous system infection caused by *C. auris* has not been investigated. The lack of previous studies investigating the efficacy of AMB against the four major *C. auris* clades precludes the possibility of comparing our results with those of other authors.

Another limitation of the comparison is that we examined tissue fungal burden in the kidney, heart, and brain. Other authors have found that intravenous *C. auris* infection in neutropenic mice resulted in high tissue fungal burdens in the lungs, cecum, uterus, and stomach (Pichowicz et al. 2022). However, previous studies have shown that the heart and kidneys are the main targets of invasive *C. auris* infections, along with involvement of the central nervous system (Forgács et al. 2020; Torres et al. 2020; Singh et al. 2019; Xin et al. 2019); involvement of these organs, with particular emphasis on the heart, is the primary cause of mortality in mice (Forgács et al. 2020). The significance of our study is that we investigated the *in vivo* efficacy of AMB against the four major clades in both lethality and tissue persistence assays, as well as the effect on urinary CFU after 2 days of AMB treatment. In addition, the histological studies performed had an important role in understanding the early stages of the pathogenesis of invasive *C. auris* infections. However, the Iranian and Singaporean clades were not investigated.

Candidemia is the most common clinical presentation of *C. auris* infections and its recognition requires immediate antifungal therapy (with echinocandins or AMB) (Sekyere et al. 2018; Lockhart et al. 2016;). Failure of treatments with echinocandins and AMB in patients with persistent or recurrent candidemia is a consequence of insufficient elimination of fungal cells (Ruiz-Gaitán et al. 2018; Alatoom et al. 2018). Metastatic dissemination further complicates the complete eradication of the infection, which can lead to endophthalmitis, spondylodiscitis, endo-/myocarditis (Ruiz-Gaitán et al. 2018). Although CNS involvement is rare, such cases have been observed in adult patients undergoing neurosurgery or treated for subarachnoid hemorrhage (Khatamzas et al. 2019;

Singhal et al. 2018). An Iranian infant developed meningitis due to a *C. auris* isolate belonging to the South Asian clade (Mirhendi et al. 2022).

Our results strongly suggest that myocardial damage is primarily responsible for mortality in both AMB-treated and untreated control mice. Histological examinations of animals necropsied on day 2 post-infection confirmed our previous observations that intravenous administration of *C. auris* cells resulted in high tissue fungal burden in the heart and kidney as early as 2 days post-infection (Forgács et al. 2020). AMB treatment failed to eliminate or reduce the fungal burden in the organs tested for the more virulent South Asian and South American clades, as demonstrated by histological examination of mice killed on days 3-4 (appearance of contraction-band necrosis in the hearts and extensive fungal infiltration in the kidneys and brain). Although we did not measure AMB concentrations in serum, heart, kidney and brain, which may be considered a further limitation of the study, other authors found AMB concentrations of 0.1 mg/l, 0.7 μg/g and 0.3 μg/g in the serum, heart and kidney of mice 4 hours after 1 mg/kg AMB treatment (Shadkchan et al. 2003). In addition, AMB is highly protein-bound, further reducing the concentration of free, and therefore biologically active, AMB in serum and tissues, which explains the poor *in vivo* efficacy of AMB (Lewis et al. 2003). This is particularly evident against environmental isolates from the South American clade, which are the least susceptible isolates in lethality assays (Papp et al. 2021). In contrast, AMB treatment significantly reduced the burden on kidney and heart tissue by day 6 in mice infected with less virulent isolates from the East Asian and South African clades; the majority of mice remained alive after 21 days, even if they showed clinical signs of meningitis. It is important to note that in the histological examinations of the dead individuals in these groups, fungal cells were not found in the heart or kidney, but they were always found in the cerebrum and cerebellum. Our histological results suggest that at least 4 days are required after infection for *C. auris* to enter the central nervous system. In contrast to the study by Singh et al., we identified fungal cells in the brain tissue, not in the capillaries (Singh et al. 2019). These clade-specific data suggest that AMB concentrations during the first 6 days of the experiment were high enough to reduce or, in some cases, completely eliminate fungal cell numbers in the heart, kidneys, and urine, but not in the central nervous system (Lewis et al. 2003; Dudiuk et al. 2019; Shadkchan et al. 2003; Caballero et al. 2021). *C. auris* strains isolated from patients are genetically identical to strains from hospital surfaces and healthcare workers, suggesting that the source of *C. auris* may be the hospital environment or healthcare workers (Armstrong et al. 2016). Recent data have confirmed the importance of environmental isolates of *C. auris*, as Arora et al. isolated *C. auris* from both a sandy beach and a tidal marsh in India. These environmental strains were genetically close to clinical isolates from India (South Asian clade) and all but one isolate showed multidrug resistance (Arora et al. 2021). In addition, *C. auris* was isolated from the surface of stored apples; these strains were closely related to strains from Indian patients, hospitals, and clinical strains from other parts of the world (South Asian clade) (Yadav et al. 2022). Since hospital environmental isolates may be the primary source of infection for high-risk patients, determining the antifungal susceptibility of these isolates is of paramount importance. Notably, AMB at a daily dose of 1 mg/kg was completely effective.

Summary

Due to its global spread, hospital outbreaks linked to Candida auris, this multidrug-resistant opportunistic pathogen, were recorded on several continents by the late 2010s. The available data suggest that echinocandins are the antifungal agents of choice for invasive C. auris infection, but even with appropriate therapy, mortality is 60%. Today, C. auris is considered a priority pathogen according to the WHO list.

The first aim of my PhD project was to determine the in vivo virulence of C. auris clinical isolates from the four available geographical clades in a neutropenic mouse model and to compare the data with the results obtained with C. albicans strains used as controls. While the second objective was to investigate the in vivo antifungal activity of amphotericin B against C. auris. To test our objectives, we performed survival experiments, determined the extent of fungal burden by organ culture, and characterized the histopathological characteristics of the tissue invasion by histopathological examination.

In the virulence experiments, the highest mortality was observed in the South American clade (96%) on day 21 of the survival experiment, followed by the South Asian (80%), South African (45%) and East Asian (44%) clades. Regardless of clade, C. auris strains were found to be less virulent than C. albicans. Tissue fungal burden examination results showed a strong correlation with lethality. Histopathological examination revealed large aggregates of fungal cells in the heart, kidney and liver, with the heart and kidney being the most severely affected organs, irrespective of clade. In experiments on the in vivo antifungal activity of amphotericin B, AMB at a dose of 1 mg/kg/day increased survival and reduced tissue fungal burden only in the less virulent and more AMB-sensitive in vitro East Asian and South African clades. However, the effectiveness of AMB against other clades is questionable. Tissue examinations suggest that C. auris can invade the heart, kidneys and central nervous system despite early AMB therapy.

In summary, our results show that in cases of suspected invasive C. auris infection, daily high-dose echinocandin or AMB therapy should be started as soon as possible to protect patients from the serious consequences of fungal myocarditis or meningitis.

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List of publications related to the dissertation

1. **Forgács, L.**, Borman, A. M., Kovács, R. L., Balázsi, D., Tóth, Z., Balázs, B., Chun-Ju, C., Kardos, G., Kovács, I., Majoros, L.: In Vivo Efficacy of Amphotericin B against Four Candida auris Clades.
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List of other publications

3. Kovács, F., Jakab, Á., Balla, N., Tóth, Z., Balázsi, D., **Forgács, L.**, Harmath, A., Bozó, A., Ragyák, Á., Majoros, L., Kovács, R. L.: A comprehensive analysis of the effect of quorum-sensing molecule 3-oxo-C12-homoserine lactone on Candida auris and Candida albicans.
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