**In vivo imaging of disseminated murine Candida albicans infection reveals unexpected host sites of fungal persistence during antifungal therapy**

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**Objectives:** *Candida albicans* is an important fungal pathogen that can cause life-threatening disseminated infections. To determine the efficacy of therapy in murine models, a determination of renal fungal burden as cfu is commonly used. However, this approach provides only a snapshot of the current situation in an individual animal and cryptic sites of infection may easily be missed. Thus, we aimed to develop real-time non-invasive imaging to monitor infection in *vivo*.

**Methods:** Bioluminescent *C. albicans* reporter strains were developed based on a bioinformatical approach for codon optimization. The reporter strains were analysed *in vitro* and *in vivo* in the murine model of systemic candidiasis.

**Results:** Reporter strains allowed the *in vivo* monitoring of infection and a determination of fungal burden, with a high correlation between bioluminescence and cfu count. We confirmed the kidney as the main target organ but additionally observed the translocation of *C. albicans* to the urinary bladder. The treatment of infected mice with caspofungin and fluconazole significantly improved the clinical outcome and clearance of *C. albicans* from the kidneys; however, unexpectedly, viable fungal cells persisted in the gall bladder. Fungi were secreted with bile and detected in the faeces, implicating the gall bladder as a reservoir for colonization by *C. albicans* after antifungal therapy. Bile extracts significantly decreased the susceptibility of *C. albicans* to various antifungals *in vitro*, thereby probably contributing to its persistence.

**Conclusions:** Using *in vivo* imaging, we identified cryptic sites of infection and persistence of *C. albicans* in the gall bladder during otherwise effective antifungal treatment. Bile appears to directly interfere with antifungal activity.

**Keywords:** candidiasis, systemic infection, gall bladder, bioluminescence

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**Introduction**

*Candida albicans* is one of the most important human pathogenic fungi, with the capability to cause life-threatening systemic infections.1 To mimic this kind of infection an intravenous mouse model is frequently used.2 In this model the kidney is the primary target organ, and clearance of *C. albicans* from the kidney in combination with a clinical improvement in mice is commonly used as an indicator for mycological clearance of the infection.1,3,4

In some human patients therapy-refractory candidaemia associated with increased mortality occurs in the absence of detectable foci of infection.5,6 In contrast, the persistence of infection despite appropriate treatment has not been observed in mice. However, the analysis of infection in mice has relied on traditional post mortem methodologies, such as the determination of fungal burden in the organs. In this way, only a snapshot of the current situation in an individual animal can be analysed and cryptic sites of infection in subsets of animals may easily be missed.

Modern non-invasive imaging techniques, including bioluminescent imaging, allow the real-time monitoring of infection and provide new and potentially unexpected temporal and spatial information on disease development in individual animals.7–9
For *C. albicans*, reporter strains have been successfully used to visualize vulvovaginal and oropharyngeal candidiasis but have failed to visualize systemic candidiasis.10–13 Here we present the construction of a bioluminescent *C. albicans* reporter strain based on a *C. albicans*-optimized firefly luciferase that allowed the monitoring of systemic infection in mice. This technology facilitated the unexpected observation that *C. albicans* can persist in the gall bladder during otherwise effective antifungal therapy. Thus we identified a hidden focus that might serve as a source of *de novo* colonization or recurrence of infection by *C. albicans*.

**Methods**

**Generation and characterization of bioluminescent *C. albicans* reporter strains**

To generate a codon-optimized luciferase the protein sequence of the *Photorhabdus pyralis* luciferase was used as a template to create a synthetic gene (GenBank accession no. KF848404) adapted for high expression and translation in *C. albicans* in the yeast alternative nuclear code (serine encoded by CUG where applicable). Optimization was performed using the in-house program dCAIoptimizer. This program contains a reference data set of 50 highly expressed genes deduced from microarray data. In addition to a codon adaptation index (CAI) this program calculates a dCAI (dCAI) that analyses the preferred sequential arrangement of codons. The gene *CalucOPT* was synthesized with flanking 5’ HindIII and 3’ Nhel restriction sites and delivered in the pUC57 cloning vector (GenScript Cooperation, Piscataway, NJ, USA). For luciferase expression under control of the actin promoter, the gene encoding yEGFP was excised by HindIII/Nhel from plasmid pACT1-GFP (containing a sequence of the RP10 locus for targeted integration)14 and was replaced by *CalucOPT*. For construction of the reporter construct pPDC11:LucOPT that uses the promoter of the pyruvate decarboxylase gene *PDC11*, a 980 bp promoter fragment with flanking 5’ XhoI and 3’ HindIII restriction sites was amplified from the genomic DNA of *C. albicans* SC5314 with oligonucleotides pPDC11Xho_f (5’-CCT GAG TAA TGT TTT TGT CAC GAA AT-3’) and pPDC11Hind_r (5’-AAG CTT TGT AAA TTA GAT AAT TGG TGA GTT TTA-3’). The PCR fragment was used to replace the ACT1 promoter in pACT1:lucOPT. To construct a plasmid with *CalucOPT* under control of the glyceraldehyde-3-phosphate dehydrogenase (*TDH3*) promoter, an internal HindIII restriction site was amplified from the genomic DNA of *C. albicans* SC5314 with oligonucleotides pTDH3Xho_f (5’-CTC GAG TAA TGT TTT TGT CAC GAA AT-3’) and pTDH3Hind_r (5’-GTT AGA ATG AAG ACT TCA TAC G-3’; with the mutated nucleotide in bold) and an overlapping 625 bp fragment that was amplified with oligonucleotides pTDH3HindMut_f (5’-CGT AGA AGC TCA TTC TTT AAA C-3’; with the mutated nucleotide in bold) and pTDH3HindMut_r (5’-AAG GCT AGA AGC TCA TTG AAT C-3’) with the mutated nucleotide in bold) and pTDH3Hind_r (5’-AAG CTT TGT AAA TTA GAT AAT TGG TGA GTT TTA-3’). The PCR fusion product pTDH3H was used to replace pACT1 in pACT1:lucOPT, resulting in pTDH3:lucOPT. All plasmids were used to transform *C. albicans* CA14 and transformants were selected based on uracil prototrophy15 and luminescence detection. CA14 transformed with the *ACT1* promoter, the gene encoding yEGFP was excised by HindIII/HindIII restriction sites was amplified from the genomic DNA of *C. albicans* SC5314 with oligonucleotides pPDC11Xho_f (5’-CCT GAG TAA TGT TTT TGT CAC GAA AT-3’) and pPDC11Hind_r (5’-AAG CTT TGT AAA TTA GAT AAT TGG TGA GTT TTA-3’). The PCR fragment was used to replace the ACT1 promoter in pACT1:lucOPT. To construct a plasmid with *CalucOPT* under control of the glyceraldehyde-3-phosphate dehydrogenase (*TDH3*) promoter, an internal HindIII restriction site was amplified from the genomic DNA of *C. albicans* SC5314 with oligonucleotides pTDH3Xho_f (5’-CTC GAG TAA TGT TTT TGT CAC GAA AT-3’) and pTDH3Hind_r (5’-GTT AGA ATG AAG ACT TCA TAC G-3’; with the mutated nucleotide in bold) and an overlapping 625 bp fragment that was amplified with oligonucleotides pTDH3HindMut_f (5’-CGT AGA AGC TCA TTC TTT AAA C-3’; with the mutated nucleotide in bold) and pTDH3HindMut_r (5’-AAG GCT AGA AGC TCA TTG AAT C-3’) with the mutated nucleotide in bold) and pTDH3Hind_r (5’-AAG CTT TGT AAA TTA GAT AAT TGG TGA GTT TTA-3’). The PCR fusion product pTDH3H was used to replace pACT1 in pACT1:lucOPT, resulting in pTDH3:lucOPT. All plasmids were used to transform *C. albicans* CA14 and transformants were selected based on uracil prototrophy15 and luminescence detection. CA14 transformed with the *URA3*-containing plasmid pCP1015 was used as a control (designated the ‘control strain’).

**In vivo imaging and quantification of bioluminescence**

In vivo imaging was performed on a Xenogen IVIS® Spectrum under general anaesthesia with 3% isoflurane using medium binnning, f/stop 1, subject height 1.5 cm and 5 min exposure. As the luciferase substrate, 100 µL of d-luciferin (33.3 mg/mL; Synchem OHG, Germany) was injected intraperitoneally. Paired pellets were imaged as described above after dressing with 10 µL of d-luciferin (33.3 mg/mL). IVIS Living Image® software 4.0 (PerkinElmer, Germany) was used to acquire and evaluate data, automatically generate colour scales, produce a three-dimensional reconstruction and quantify the bioluminescence in defined regions of interest (ROIs; Figure S1). ROI templates of fixed size for each anatomical location were used to ensure that areas of identical size were measured in different animals. Both the average luminescence (p/s/cm²/sr) and the total flux (p/s) were analysed, yielding nearly identical results. Statistical analysis was performed by one-way ANOVA, followed by Dunnett’s multiple comparison test or by a two-sided unpaired t-test (GraphPad Prism).

**Quantification of fungal burden and analysis of cytokine responses**

Aseptically removed kidneys were homogenized in sterile PBS and serial dilutions were plated on YPD containing 50 mg/L chloramphenicol. The statistical analysis of colony counts was performed using an unpaired two-sided t-test or one-way ANOVA (GraphPad Prism). The correlation between fungal burden and luminescence signal was evaluated by calculating the

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Spearman coefficient for correlation and non-linear (log–log) regression analysis (GraphPad Prism).

Myeloperoxidase (MPO) and cytokines were determined as previously described using murine ELISA kits [MPO: Mouse MPO ELISA kit (HyClin Biotech, The Netherlands); IL-6, IL-17A, TNF-α and MCP-1 ELISA (eBioscience, UK)], according to the manufacturers’ recommendations.

**Calcofluor white staining of fungal elements from infected tissue homogenates**

Organ homogenates were pelleted, resuspended in 20% KOH saturated with calcofluor white and incubated for 15 min at 60 °C. Following centrifugation (10 min, 8000 g), the pellet was resuspended in 0.5 mg/mL calcofluor white in Tris/HCl and incubated for 10 min at 30 °C at 1000 rpm. The mixture was centrifuged again, resuspended in PBS and evaluated using a fluorescence microscope (Zeiss AXIO Imager.M1, Carl Zeiss, Germany) equipped with a DAPI filter set.

**In vitro drug susceptibility testing by broth dilution and MIC test-strip analysis**

Drug susceptibility was tested by broth dilution following the EUCAST EDef 7.2 protocol with minor modifications. Briefly, broth dilution was performed using serial 2-fold dilutions of drugs in MOPS-buffered RPMI 1640 medium with 2% glucose in sealed plates at 37 °C and MICs were recorded using a microplate reader. In the case of trailing growth with fluconazole, MIC values were determined from the drug dilution at which the final absorbance was ≤50% of the positive control. To test the effect of bile, a sterile solution of 6.25% bile and 2% glucose in MOPS-buffered RPMI 1640 (pH 7.0) was used.

MIC test-strip analyses were performed on MOPS-buffered RPMI 1640 plates with 2% glucose with and without 6.25% bile according to the manufacturers’ instructions (Etest strips, bioMérieux, France; MIC test strips, Bestbion, Germany). For trailing growth on azoles, microcolonies were ignored and the MIC was read at 80% inhibition as recommended by the manufacturer.

**Results**

**In vitro characterization of bioluminescent reporter strains**

Here, we generated a synthetic luciferase gene that was completely codon-adapted for high expression and translation in C. albicans. To ensure that reporter strains for subsequent in vivo experiments produced sufficient bioluminescence and that luciferase expression did not affect the general phenotypes in terms of growth and morphology, transformants were analysed in a series of *in vitro* experiments. Transformants containing the codon-optimized luciferase under the control of the ACT1, PDC11 or TDH3 promoter generated strong luminescence on agar plates containing -luciferin that were used to screen for positive transformants (data not shown). Transformants were selected from each construct and studied in more detail. Growth rates in liquid media with different nutrient sources (glucose, glycerol, peptone or aspartate) and colony formation on solid media (containing glucose, ethanol, casamino acids or glucose+10% serum) were indistinguishable from those of the control strain (CAI4+pCIp10), as shown in Figure S2 (available as Supplementary data at JAC Online). The formation of hyphae under different inducing conditions (growth on serum plates, embedding in YPS at 25°C and growth on plastic surfaces) was likewise comparable to the control strain (Figure S2 and data not shown). Selected transformants were additionally tested for the effect of different nutrient sources on promoter activity and the correlation of light emission with cell density (Figure S2). All selected transformants showed a very good correlation between cell number and bioluminescence regardless of the growth medium applied. However, among the three promoters used to drive luciferase expression, differences in light emission in terms...
Figure 2. Bioluminescence signals from brain and urinary bladder. (a) Example of bioluminescence signals at 72 h pi from localized brain infection with C. albicans pPDC11:lfuCPY. Top row: bioluminescence images in vivo (left) and ex vivo (right). Bottom row: brain histology (formalin-fixed, paraffin-embedded; periodic acid–Schiff stain). The region indicated by the black arrow in the left picture is magnified in the right picture. (b) Examples of bioluminescence signals from the urinary bladder of mice infected with pACT:lfuCPY. 1 and 2, image and fungal burden at 48 h pi; 3 and 4, image and fungal burden at 72 h pi. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
of the nutrient source were observed. While the ACT1 promoter produced the most stable signal on all nutrients, the PDC11 promoter was most strongly induced on YPD medium and had reduced, but similar, intensities on glucose and peptone minimal media. Likewise, the TDH3 promoter showed the strongest light emission on glucose minimal medium, whereas reduced, but similar, intensities were observed on YPD and peptone media. However, the minimal light emission was similar for all three constructs. Thus, the three promoters supported luciferase expression and bioluminescence without any obvious effects on growth capacity and filamentation, and therefore appeared suitable for in vivo imaging experiments.

**In vivo imaging allows quantitative monitoring of systemic candidiasis**

To assess virulence and in vivo bioluminescence, mice were challenged intravenously with $2.5 \times 10^5$ C. albicans cfu/g body weight.

**In vivo imaging of the moribund animals showed clear luminescence in the renal area of all the infected animals (Figure 1a).** Both the control and all the reporter strains induced lethal infection; however, while mice infected with wild-type, pACT1: lucOPT and pPDC11: lucOPT displayed comparable median survival times (5 days for wild-type and pACT1: lucOPT, and 4.5 days for pPDC11: lucOPT), the onset of mortality was reproducibly delayed in mice infected with pTDH3: lucOPT (Figure 1b; median survival time 6.5 days; $P = 0.0007$ compared with wild-type). Therefore, further characterization was carried out for C. albicans pACT1: lucOPT and pPDC11: lucOPT only. As the host immune response contributes significantly to pathogenesis in murine systemic candidiasis,22,23 renal cytokine and MPO contents were determined on Day 1 and Day 3 pi. Cytokine response and MPO content were comparable in mice infected with control and reporter strains (data not shown), confirming that the overall host response is not affected by luciferase expression.

Suitability for monitoring the progression of infection was investigated by repeated in vivo imaging of the mice at 8 h, 24 h, 48 h and...
Figure 4. Persistence of *C. albicans* in the gall bladder and shedding via the faeces. (a) Ventral images of selected mice from the treatment group. Mouse 8 and Mouse 3 were sacrificed on Days 5 and 10 pi, respectively, to analyse the renal fungal burden and to determine the source of the bioluminescent signal. (b) Three-dimensional reconstruction of *in vivo* bioluminescent images of Mouse 3 (left) and Mouse 8 (right) on Day 10 and
72 h pi and by quantification of the signal intensity from the raw data from defined ROIs (Figure S1). Furthermore, five mice per strain and timepoint were sacrificed after imaging to determine the kidney fungal burden and the correlation of fungal burden with bioluminescence quantification. Signals were detectable as early as 8 h pi (Figure 1c and Figure S3 (available as Supplementary data at JAC Online)). A fungal burden of ≥2500 cfu per kidney reproducibly led to clearly visible renal bioluminescence in dorsal images of mice infected with pACT1:LucOPT whereas at least 3500 cfu of pPDC11:LucOPT were required to obtain weak signals (Figure S3 and data not shown), suggesting that pACT1:LucOPT is better suited to the imaging of early infection. Signal intensity from the kidney increased from 8 h to 24 h coinciding with a significant increase in fungal burden (Figure S3). The cfu count in the kidneys and bioluminescence quantification from dorsal imaging correlated well for both pACT1:LucOPT (Spearman correlation 0.92, P < 0.0001) and pPDC11:LucOPT (Spearman correlation 0.91, P < 0.0001; Figure S3), suggesting that these reporter strains allow an accurate quantification of renal fungal burden in vivo. In additional experiments, bioluminescence of the left and right kidneys was analysed separately. The correlation between cfu count and bioluminescence in individual kidneys (Spearman correlation 0.87, P < 0.0001) was similar to the results obtained with the pooled kidneys.

In the pulmonary and gastrointestinal areas, visual signals were obtained in ventral images at 8 h pi, corresponding to a significant increase in quantification above background (Figure S3). Consistent with the transient localization of C. albicans to the lungs after intravenous infection,11 these signals decreased to background level at later timepoints (Figure S3).

Extra-renal foci of infection in individual mice

Not unexpectedly, individual mice showed distinct bioluminescence signals from the brain. The signal origin was confirmed by post mortem imaging of the brain and subsequently by histology (Figure 2a). More surprisingly, individual animals displayed signals from the urinary bladder in ventral imaging at 48 and 72 h pi (Figure 2b). The presence of C. albicans in the urinary bladder was confirmed by plating urine aseptically collected post mortem. Cultures consistently yielded bioluminescent C. albicans colonies, suggesting that either fungal cells were dislodged from the renal pelvis and collected in the urinary bladder or that the urinary bladder itself was infected.

Successful monitoring of therapeutic efficacy

To determine whether bioluminescent reporter strains allow the monitoring of therapeutic efficacy in vivo, we performed a therapy trial based on initial caspofungin therapy followed by de-escalation to fluconazole. Sixteen mice were infected intravenously with C. albicans pACT1:LucOPT at an infectious dose of 4 × 10⁶ cfu/g and randomly assigned to the treatment group and the placebo group. Treatment was initiated at 8 h pi. All mice displayed a clear bioluminescence signal at 8–24 h pi, confirming successful infection (Figure 3a). As expected, mice in the placebo group quickly developed clinical symptoms of severe disease and had to be euthanized at 56 h to 72 h pi, with a mean fungal burden of 4.3 × 10⁶ cfu in both kidneys (standard deviation ± 1.8 × 10⁵). In contrast, caspofungin-treated mice showed moderate signs of illness at 24 h pi but recovered within the following days. These clinical differences were reflected in bioluminescence imaging; signal intensity increased significantly in the placebo group from 8 to 24 h pi, while it steadily declined in treated mice (Figure 3b). Consistent with complete clinical recovery, the bioluminescence of the treated mice was similar to that of non-infected controls on Day 4 pi (Figure 3c). Fungal clearance was confirmed in the kidneys of treated clinically healthy mice sacrificed on Day 5 pi (n = 1; 80 cfu) and Day 10 pi (n = 2; 10 and 0 cfu, respectively) as well as at the end of the experiment (n = 5, 0 cfu).

Persistence of C. albicans in the gall bladder during antifungal treatment

Within the treatment group, individual mice (three out of eight) displayed a circumscribed bioluminescence signal below the sternum. The first signal occurred at 48 h pi (during caspofungin treatment) and persisted with intermittent intensity up to the end of the experiment (Figure 4a). Three-dimensional reconstruction of the bioluminescence imaging located the source of the signal to the liver (Figure 4b). This was unexpected as the fungal burden in the liver generally declines over time22,26 and the signal occurred in mice showing successful clearance of C. albicans from the kidneys. Post mortem imaging revealed that the signal derived from the gall bladder (Figure 4c). Microscopically, long C. albicans filaments were found in gall bladder homogenate and plating yielded pure cultures of bioluminescent C. albicans (~3 × 10⁵ cfu/organ; Figure 4c). Biliary excretion might lead to a shedding of pathogens from the gall bladder to the intestinal lumen, as shown for Listeria monocytogenes and Salmonella Typhi.25,26 Indeed, some faecal pellets from mice showing a gall bladder signal produced clear bioluminescence signals and contained viable fungi (Figure 4d), suggesting that C. albicans is excreted with bile into the intestinal tract. Similar results were obtained in a second treatment experiment; 4 out of 10 mice displayed luminescence signals from the gall bladder with varying signal intensity. Histologically, C. albicans filaments were found within the lumen of the gall bladder without obvious involvement of the gall bladder epithelium (data not shown). The onset of signal detection in these mice ranged from Day 4 to Day 13 after infection. Thus, persistence in the gall bladder was reproducibly observed in a subset of infected mice under treatment.

In vitro susceptibility of C. albicans to antifungal compounds in the presence of bile extracts

To determine whether bile components interfered with antifungal efficacy, thereby promoting the persistence of C. albicans in the
gall bladder, we analysed MICs in vitro. Due to the unavailability of mouse bile in sufficient amounts, we used porcine and bovine bile extract. Using the broth dilution method, the MIC increased from 0.25 mg/L to 2.5 mg/L for fluconazole and from 0.078 mg/L to 0.10 mg/L for caspofungin in the presence of 6.25% (w/v) bile extract. Although this concentration is only \( \approx 50\% \) of the natural bile concentration, a dilution experiment revealed that a minimum of 0.5%–1% (w/v) of bile was sufficient to confer resistance to caspofungin. Additionally, the pH of the medium did not influence the protective effect. Although standard media for drug susceptibility testing were generally buffered to pH 6.5, similar protective effects against caspofungin were observed at pH 6.0, 7.0 and 8.0 (Figure S4, available as Supplementary data at JAC Online). Similarly, on solid media using Etest strips, the MIC of caspofungin was 0.064 mg/L on standard MOPS-buffered RPMI medium, while no inhibition zones were observed in the presence of bile extracts, independent of the strip manufacturer and the addition of glucose to the bile extract (Figure 5a). Likewise, susceptibility to fluconazole was clearly decreased in the presence of porcine bile (Figure 5b).

To test whether bile also alters the susceptibility to other antifungal substances, we performed additional test-strip analyses for amphotericin B, anidulafungin, posaconazole and voriconazole. For all the tested antifungals, bile extracts increased the MIC by factors of 10 to \( >1000 \) (Figure 5b).

This effect of bile was also observed in five additional \( C. \) albicans wild-type strains susceptible to caspofungin and anidulafungin on RPMI medium. The presence of bile completely abolished the inhibitory effect of caspofungin, and the tolerance to anidulafungin increased at least by a factor of 2000 (Figure 6). Thus, the results suggest that bile decreases the susceptibility of \( C. \) albicans to various antifungals, which could support the persistence in the gall bladder during therapy.

**Discussion**

Non-invasive imaging techniques allow the real-time monitoring of infection, providing temporal and spatial information on disease progression in individual animals.\(^7,8,27\) However, previous attempts at using bioluminescence imaging for investigating deep-seated \( C. \) albicans infections were unsuccessful. While a surface-anchored \( \text{Gaussia luciferase} \) produced highly luminescent strains and was suitable for studying superficial infections\(^\text{11} \) and the colonization of implanted subcutaneous catheters,\(^\text{50} \) this system failed to monitor systemic infections. One explanation for this
failure might derive from the limited body distribution of the substrate coelenterazine, which may not have reached the site of infection in sufficient quantity. However, an even more likely explanation derives from the light emission of around 480 nm by Gaussia luciferase. This wavelength is highly absorbed by haemoglobin and thus strongly dampens the penetration of light to the outside.7 Firefly luciferase, as used in our study, emits light at >600 nm under in vivo conditions, which reduces its light absorption by haemoglobin. However, previous studies using firefly luciferase for the in vivo detection of disseminated candidiasis also failed.10 One reason suggested is that luciferin uptake was hampered by a morphological switch from yeast to hyphae, which might show reduced permeability for luciferin.

However, firefly luciferase has been successfully used for in vivo imaging of the filamentous fungi Aspergillus fumigatus and Aspergillus terreus,29–32 although limited oxygen availability in necrotizing tissues can reduce light emission from infected tissues, as shown for A. fumigatus infections.33

A more detailed analysis of the previous firefly luciferase constructs used for the in vivo imaging of C. albicans infections revealed two other reasons why this approach may have failed.53 First, the codon usage of the firefly luciferase was only partially adapted to C. albicans. All the CUG codons of a luciferase gene that was previously adapted to high-level expression in mammalian cells were converted into CUG codons, but all other codons were maintained. Second, the peroxisomal targeting sequence of the firefly luciferase had not been deleted. In this respect, studies on yeast have shown that a peroxisomal targeting of the luciferase strongly reduces light emission.34 To overcome these construct-derived limitations, we generated a completely synthetic firefly luciferase that was adapted to high-level gene expression in C. albicans and from which the peroxisomal targeting sequence was omitted. Furthermore, we tested different promoters to drive gene expression. In vitro experiments showed that all three promoters generated highly bioluminescent strains, with the actin promoter pACT1 showing the least influence of the nutrient source on light emission. Nevertheless, although the pyruvate decarboxylase promoter pPDC1 and the glyceraldehyde-3-phosphate promoter pTDH3 were slightly affected by the nutrient source, light emission remained at a high level under the in vitro conditions tested. Indeed, all the strains allowed a visualization and quantification of fungal burden in mice across the full time

Figure 6. Susceptibility of different C. albicans strains to echinocandins on RPMI 1640 and bile medium. (a) Anidulafungin. (b) Caspofungin.
course of the systemic infection. Thus, limitations of in vivo bioluminescence due to restricted oxygen availability and substrate distribution have not been observed in this model, and the codon adoption of the synthetic luciferase strongly increased the sensitivity of the system.

Although no phenotypic defects were observed for any of the reporter strains in vitro, pTDH3:LucOPT displayed a moderate, but reproducible and significant, reduction in virulence. As the luminescence of this strain was comparable to that of the other reporters, it appears unlikely that the virulence defect was a direct consequence of luciferase expression. Alternatively, polar effects described for the URA Blaster method could lead to this effect. In contrast, the virulence of and immune response to C. albicans pACT1:LucOPT were indistinguishable from those for the wild-type and mediated highest reproducibility and imaging sensitivity. Thus, this strain was chosen to further investigate systemic candidiasis in real time.

Ventral imaging revealed involvement of the urinary bladder in individual mice. Urinary bladder colonization probably developed in an antegrade fashion by a dislocation of mycelial casts from the collecting tubuli, renal pelvis or proximal ureter. Similar mechanisms have been suggested in human candidiasis associated with candidemia and discussed for candiduria in systemically infected rabbits. As the prognostic value of candiduria in critically ill surgical patients is unclear, the mouse model of disseminated candidiasis in combination with imaging might be useful to systematically investigate the relevance of candiduria in disseminated candidiasis and to clarify whether the urinary bladder is actively infected or rather serves as a reservoir for C. albicans cells dislodged from the upper urinary tract. Furthermore, bioluminescence imaging was suitable for monitoring the efficacy of antifungal therapy. Unexpectedly, it also revealed that C. albicans persisted in the gall bladder of some treated mice. The presence of C. albicans in the gall bladder on Days 1 and 2 after systemic infection with a comparably high dose of 8.5 × 10^6 C. albicans cfu/g body weight has previously been described and attributed to a slow, but steady, filtration of C. albicans through the bile system. We likewise assume that the fungus was introduced into the gall bladder via the bile ducts in the early phase of infection, before renal infection had been controlled by treatment. However, gall bladder signals were detected at a time when the kidney infection had been cleared by antifungal treatment. Furthermore, long, branching hyphae were observed in gall bladder homogenates, which are unlikely to have passed via the bile ducts. Thus, rather than merely serving as a reservoir, it appears that the gall bladder provides an environment in which C. albicans is able to grow. This is supported by the observation that C. albicans readily grows on bile extracts, forming hyphae in vitro. Consistent with the previous observation by Rozell et al., we did not observe infection of the gall bladder epithelium but hyphae within the gall bladder lumens. Whether infection of the gall bladder epithelium occurs thus remains to be determined.

Persistence in the gall bladder has previously been demonstrated for pathogenic bacteria such as L. monocytogenes and Salmonella Typhi but, to our knowledge, not for any fungi. Bacteria are excreted with the bile into the intestine and subsequently shed with the faeces. This was similarly observed for C. albicans and might allow a recolonization of the intestinal tract after the cessation of antifungal treatment. As colonization of the mucosa with C. albicans is a risk factor for the development of candidiasis in critically ill patients, this finding might be of clinical relevance. Data on the occurrence and clinical relevance of gall bladder infections by C. albicans is sparse; however, in one study acalculous cholecystitis was observed in 10% of fatal cases of disseminated candidiasis in children, and another prospective study reported the isolation of Candida spp. in 5% of bile samples. Candida in the gall bladder or biliary tract has also been reported in surgical patients. The persistence of Candida infections despite appropriate antifungal therapy and in the absence of drug resistance, as well as the recurrence of infection with an identical C. albicans strain after apparent resolution of the first episode, occurs in some patients, especially in neonates, and can be associated with increased mortality. Importantly, in many cases the site of persistence could not be identified. Our finding that persistence in mice occurs in the gall bladder provides a hint for the identification of persistent foci in humans. However, further studies are needed to determine whether persistence in the gall bladder provides a source for relapsing candidiasis, and which additional confounding factors might contribute to the recurrence of infection.

Drug failure due to pharmacokinetics appears unlikely to contribute to persistence in the gall bladder as caspofungin and fluconazole are excreted in the bile. Biofilm development might also prevent mycological clearance and thereby contribute to gall bladder persistence, as described for Salmonella Typhi. However, based on our data that the presence of bile extracts significantly decreases the susceptibility of C. albicans to various antifungal substances, we propose a direct interference of bile with antifungal agents as the mechanism of persistence. Increased resistance to azoles in the presence of bile has been previously described. The exact mechanisms of bile-mediated resistance remain unclear as C. albicans appears unable to take up exogenous sterols from bile and no up-regulation of genes known to mediate azole resistance has been observed. As tolerance was observed not only for azoles, but also for echinocandins and amphotericin B, the interference of bile with susceptibility for antifungals appears to be a general effect.

In summary, we demonstrate that monitoring the establishment and progression of systemic candidiasis, including the in vivo determination of kidney fungal burden, in mice is feasible by real-time in vivo imaging. Our results not only confirm the course of infection as previously determined by conventional methods, but also provide novel insights by detecting antegrade involvement of the urinary bladder and persistence of C. albicans in the gall bladder during caspofungin/fluconazole treatment. Persistence in the gall bladder and subsequent shedding with the faeces has not yet been described for pathogenic fungi and might be of clinical relevance for persistent candidaemia and recurrence of infection.

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Transparency declarations

None to declare.

Supplementary data

Figures S1 to S4 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References

41 Echeverria PM, Kett DH, Azoulay E. Candida prophylaxis and therapy in the ICU. Semin Respir Crit Care Med 2011; 32: 159–73.