Candida albicans Airway Colonization Facilitates Subsequent Acinetobacter baumannii Pneumonia in a Rat Model

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The objective of the study was to determine the effects of Candida albicans respiratory tract colonization on Acinetobacter baumannii pneumonia in a rat model. Rats were colonized with C. albicans by instillation of 3 × 10⁶ CFU into their airways, while sterile saline was instilled in the control group. The colonized rats were further divided into two groups: treated with amphotericin B or not. The rats were subsequently infected with A. baumannii (10⁶ CFU by tracheobronchial instillation). A. baumannii lung CFU counts, cytokine lung levels, and rates of A. baumannii pneumonia were compared between groups. In vitro expression of A. baumannii virulence genes was measured by reverse transcription (RT)-PCR after 24-hour incubation with C. albicans or with Mueller–Hinton (MH) broth alone. Rats with Candida colonization developed A. baumannii pneumonia more frequently and had higher A. baumannii CFU burdens and heavier lungs than controls. After A. baumannii infection, lung interleukin 17 (IL-17) concentrations were lower and gamma interferon (IFN-γ) concentrations were higher in Candida-colonized rats than in controls. Candida-colonized rats treated with amphotericin B had a decreased rate of A. baumannii pneumonia and lower IFN-γ levels but higher IL-17 levels than untreated rats. Expression of basC, burB, baauA, ptk, plc2, and pld2 was induced while expression of ompA and abal was suppressed in A. baumannii cultured in the presence of C. albicans. C. albicans colonization facilitated the development of A. baumannii pneumonia in a rat model. Among Candida-colonized rats, antifungal treatment lowered the incidence of A. baumannii pneumonia. These findings could be due to modification of the host immune response and/or expression of A. baumannii virulence genes by Candida spp.

Candida albicans is a commensal of the human skin and mucosa but also the most common fungal human pathogen (1–3). Recent data are suggestive of clinically significant interactions between C. albicans and bacteria, potentially affecting their virulence (2, 4, 5) and propensity to develop antibacterial resistance (6–9).

Specifically, clinical and laboratory investigations have focused on the interplay between C. albicans and Pseudomonas aeruginosa (10). In animal models and observational studies, C. albicans airway colonization has been associated with increased incidence of P. aeruginosa pneumonia (11, 12). Prior antifungal treatment was found to reduce the risk of P. aeruginosa pulmonary infection (11, 13). However, in burn patients, P. aeruginosa inhibited the growth of Candida spp. on the wound surface (4).

Acinetobacter baumannii is an emerging nosocomial pathogen associated with significant morbidity and multidrug resistance (14–16). A. baumannii can firmly adhere to the hyphae of C. albicans and inhibit their growth via production of the OmpA protein (17). Nevertheless, A. baumannii exhibited enhanced growth in the presence of another yeast, Saccharomyces cerevisiae (18).

These observations indicate complex, possibly both synergistic and antagonistic yeast-bacterial interactions that have not been adequately investigated. Therefore, we aimed to study the relationship between C. albicans respiratory tract colonization and bacterial pneumonia caused by A. baumannii in a rat model, as well as the effect of culture in the presence of C. albicans on the expression of A. baumannii virulence genes.

MATERIALS AND METHODS

Animals. We used 2.5- to 3-month-old pathogen-free male Wistar rats, weighing 250 to 275 g (11), purchased from the Laboratory Animal Center (Southern Medical University, Guangzhou, China). All experiments were approved by the Ethics of Animal Experiments Committee of Nan Fang Hospital, an affiliate of the Southern Medical University, Guangzhou, China. The rats were housed under standard conditions (12 h light/12 h dark; 22 to 24°C) in the Animal Care Facility Service (Southern Medical University, Guangzhou, China). The study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

C. albicans respiratory tract colonization. Candida colonization was achieved by transthoracic instillation of 3 × 10⁶ CFU C. albicans SC5314 on the first day of experiments, as previously described (11). To establish the model, 4 animals were sacrificed at each time point, 4, 24, and 72 h and 7 and 14 days after instillation, and their lungs were removed. The lungs from one animal were homogenized and used for Candida CFU counts, whereas those from the other three were used for histopathologic analyses. We compared lung cytokine levels between Candida-colonized and control groups.
trol rats without *A. baumannii* infection 48 h after *Candida* or saline instillation, respectively.

**Assessing the effect of *C. albicans* respiratory tract colonization on *A. baumannii* pneumonia.** Rats were divided into *C. albicans* respiratory tract colonization group (n = 16) and saline control (n = 18) groups. A suspension of 10^7 CFU of *A. baumannii* ATCC19606 was transglottally instilled in each animal on the second day of the experiment. At this inoculum, *A. baumannii* pneumonia develops in <50% of immunocompetent rats. The animals were sacrificed on the third day. Lung weights, *A. baumannii* CFU counts, and lung cytokine concentrations were compared between the two groups. Pneumonia was defined as macroscopic and/or microscopic lung inflammation with a bacterial burden of >10^4 CFU per lung (11).

**Antifungal treatment.** The *Candida*-colonized rats were divided into those treated with 1 mg/kg of body weight/day of intraperitoneal amphotericin B (treatment group; n = 16) or normal saline intraperitoneal injections (control group; n = 16), as previously described (11), for 3 days after airway colonization with *Candida*. On the 4th day, the rats were infected with *A. baumannii* as described above, and on the 5th day, they were sacrificed for lung tissue bacterial CFU and cytokine measurements. In a separate experiment, we compared lung cytokine levels between *Candida*-colonized rats treated for 3 days with amphotericin B or not and without Acinetobacter infection.

**Cytokine measurements.** Levels of interleukin 2 (IL-2), IL-5, IL-6, IL-10, IL-17, and gamma interferon (IFN-γ) were determined using the Milliplex Map Rat Cytokine/Chemokine Magnetic Bead panel (Millipore Corporation, USA) on a Luminex (Austin, TX) 100 IS system according to the manufacturer’s instructions to evaluate the host local immune response.

**In vitro experiments, RNA isolation, and real-time PCR analysis.** For in vitro experiments, 1 ml of 5 × 10^6 CFU/ml *A. baumannii* in Mueller-Hinton (MH) broth suspension was cultured in 1 ml of *C. albicans* MH broth suspension (3 × 10^7 cells/ml) (*Candida*) or 1 ml of MH broth (control). After a 24-hour incubation at 37°C, 1 ml was moved to a 1.5-ml tube and centrifuged for 10 min at 5,000 × g to obtain bacterial pellets; 100 μl of TE buffer (10 mM Tris · Cl, 1 mM EDTA, pH 8.0) with 1 mg/ml lysozyme was added to the precipitate, which was resuspended and incubated for 5 min at room temperature.

Total *A. baumannii* mRNA was extracted using an RNeasy Mini RNA isolation kit (Qiagen, Shanghai, China). RNA integrity, concentration, and purity were assessed using a Nanodrop spectrophotometer. Samples with a 260/280-nm ratio between 1.9 and 2.1 were used for further analyses. Any potential carryover genomic DNA (gDNA) contamination was removed using gDNA Eraser (TaKaRa, Dalian, China); 1 μg of mRNA was used to synthesize cDNA with the PrimeScript RT reagent kit with gDNA Eraser (Perfect Real Time; TaKaRa, Dalian, China). Quantitative analyses of virulence genes of *A. baumannii* (*ompA*, *pgc*, *lpca*, *basC*, *basD*, *barb*, *baua*, *ptk*, *plc2*, *pld2*, and *abaI*) were performed on a LightCycler 480 real-time PCR system (Roche, Switzerland) using the SYBR Premix *Ex Taq* II (Tli RNaseH Plus) kit (TaKaRa, Dalian, China).

Using 16S rRNA as the reference gene (19), reverse transcription (RT)-PCR data from at least 3 independent experiments were analyzed by the 2^-ΔΔCt^ method. Changes in the expression of *A. baumannii* virulence genes in the presence of *C. albicans* were expressed as a ratio relative to the control group.

**Statistical analysis.** Data were analyzed using SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA). All data are presented as means and standard deviations (SD) unless otherwise specified. Normality of distribution was tested with the Kolmogorov-Smirnoff test. We determined statistical significance (*P* < 0.05) by t-test, analysis of variance (ANOVA), or Mann-Whitney U test, where appropriate.

**RESULTS**

**C. albicans** respiratory tract colonization model. On the basis of the model provided by Roux et al. (11), the respiratory tracts of Wistar rats were colonized with *C. albicans*. The 14-day survival of the colonized rats was 100%, and there were no differences in activity, fecal shape, and average daily weight gain between animals colonized with *C. albicans* and controls (airway instillation of normal saline) (Fig. 1). We found no histopathologic evidence of pneumonia in *Candida*-colonized rats.

*Candida* colonization of the respiratory tract facilitates the development of subsequent *A. baumannii* pneumonia. Twenty-four hours after lung instillation of *A. baumannii*, rats with prior *C. albicans* respiratory tract colonization had higher *A. baumannii* lung CFU counts (*P* = 0.026) and lung weights (*P* = 0.034) than controls (Fig. 2). Ten of 16 rats with *C. albicans* airway colonization developed *A. baumannii* pneumonia as opposed to 4/18 in the control group (*P* = 0.017). Two lungs infected with *A. baumannii* are shown in Fig. 3. Consolation is evident in the lung of a *Candida*-colonized rat (Fig. 3B). Microscopically, animal lungs colonized with *C. albicans* prior to *A. baumannii* infection demonstrated heavier infiltration of inflammatory cells and alveolar damage (Fig. 4D) than lungs with *A. baumannii* infection in the absence of prior *C. albicans* colonization (Fig. 4C).

**C. albicans** colonization downregulated the effect of *A. baumannii* on host IL-17 production and upregulated its effect on IFN-γ production. We measured lung IFN-γ, IL-2, and IL-17 levels in *C. albicans*-colonized rats and controls in the absence of (48 h after *Candida* or saline instillation) and 24 h after *A. baumannii* infection. Levels of IL-17 (41.4 ± 7.0 versus 17.7 ± 4.2 pg/ml; *P* = 0.01) (Fig. 5A), IL-2 (84.6 ± 8.3 versus 54.3 ± 7.1 pg/ml; *P* = 0.02) (Fig. 5B), and IFN-γ (130.2 ± 33.9 versus 54.3 ± 10.8 pg/ml; *P* = 0.005) (Fig. 5C) were higher in rats colonized with *Candida* than in controls in the absence of bacterial infection.

In the control group, IFN-γ (*P* = 0.002), IL-17 (*P* < 0.001),
and IL-2 ($P = 0.02$) concentrations were significantly increased with *A. baumannii* instillation, whereas in *Candida*-colonized rats, only IFN-γ levels were increased with bacterial infection ($P < 0.001$). *A. baumannii*-infected rats with prior *C. albicans* airway colonization had significantly lower IL-17 ($34.9 \pm 6.53$ versus $47.8 \pm 7.0$ pg/ml; $P = 0.029$) (Fig. 5A) and higher IFN-γ ($480.5 \pm 11.7$ versus $301.8 \pm 97.4$ pg/ml; $P = 0.011$) (Fig. 5C) concentrations than *A. baumannii*-infected controls. There was no difference in IL-2 levels ($79.8 \pm 19.4$ versus $67.8 \pm 2.8$ pg/ml; $P = 0.265$) (Fig. 5B).

**Effect of antifungal therapy.** In rats with *C. albicans* airway colonization, we studied the effects of systemic amphotericin B (1 mg/kg daily), starting on the day of *Candida* instillation and administered for 3 days, on the lung fungal burden, subsequent *A. baumannii* pneumonia, and host cytokine production.

The pulmonary fungal burden was significantly decreased in amphotericin B-treated rats compared to those that did not receive antifungal treatment ($P = 0.015$) (Fig. 6A). Fewer rats in the treatment group developed *A. baumannii* pneumonia (5/19 versus 10/16; $P = 0.031$). Lung weights ($P = 0.027$) (Fig. 6B) and lung tissue *Acinetobacter* CFU counts ($P = 0.038$) (Fig. 6C) were also significantly lower in *Candida*-colonized rats that were treated with amphotericin B than in those that were not.

In *Candida*-colonized rats without *A. baumannii* infection, IL-2 (Fig. 7B) and IFN-γ (Fig. 7C) lung concentrations were decreased with antifungal treatment ($P < 0.05$), whereas there was no significant difference in IL-17 levels (Fig. 7A). After *A. baumannii* instillation, IL-17 levels were higher ($P = 0.005$) (Fig. 7D) whereas the concentration of IFN-γ was lower ($P = 0.032$) (Fig. 7F) in *Candida*-colonized rats that received antifungal treatment than in those that did not. IL-2 levels were similar in the two groups ($P = 0.693$) (Fig. 7E). We did not find any significant effects of *Candida* colonization or antifungal treatment on IL-5, IL-6, or IL-10 levels (see Fig. S1 and S2 in the supplemental material).

**Effect of *C. albicans* on expression of *A. baumannii* virulence genes.** The expression of *A. baumannii* virulence genes was assessed by RT-PCR after 24 h of culture with *C. albicans* compared to MH broth alone. The primers used in this experiment are listed in Table S1 in the supplemental material. In the presence of *C. albicans*, the expression of *basC, barB, bauA, ptk, plc2*, and *pld2* was upregulated, whereas the expression of *abaI* and *ompA* was suppressed (Table 1).

**DISCUSSION**

We studied a simple, reproducible rat model of *C. albicans* respiratory tract colonization previously described by Roux et al. (11) and extended their findings for the evaluation of *C. albicans*- *A. baumannii* (an emerging opportunistic pathogen) coinfection. After instillation, *Candida* organisms were retrieved from animal lungs with slow clearance over time, 14-day survival was 100%, and there were no differences in weight or health status between *Candida*-instilled animals and controls (Fig. 1). These findings are consistent with colonization rather than clinical infection. How-

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**FIG 2** Fungal colonization facilitates development of subsequent *A. baumannii* pneumonia. Median *A. baumannii* CFU counts per lung (log transformed) (A) and lung weights (B) (horizontal lines) are shown. Mann-Whitney test; *, $P < 0.05$.

**FIG 3** Macroscopic appearance of *A. baumannii* pneumonia. (A) Normal macroscopic appearance of the lungs from a rat that was not colonized with *Candida* and did not develop pneumonia after transglottal instillation of $10^6$ CFU of *A. baumannii*. (B) Marked consolidation of the upper and middle lobes in the right lung of a rat colonized with *C. albicans* that developed pneumonia after instillation of the same *A. baumannii* inoculum.
ever, instillation of *C. albicans*, but not of normal saline, elicited a strong host immune response (Fig. 5) similar to that observed previously (11). Therefore, even though there was no evidence of clinical disease, it seems that *C. albicans* in the respiratory tract is not just a commensal bystander.

Importantly, we found convincing evidence that *C. albicans* respiratory tract colonization in rats facilitated the subsequent development of severe *A. baumannii* infection, as evidenced by higher bacterial CFU counts, lung weights, histopathology (Fig. 2 to 4), and percentages of animals that developed pneumonia than for controls. To our knowledge, this is the first report of an association between *Candida* airway colonization and *Acinetobacter* pneumonia, which is consistent with animal studies (11) and clinical data (12, 13) for other pathogens. Such observations could be due to (i) modification of the host immune response by *C. albicans* and/or (ii) fungal-bacterial interactions resulting in enhanced bacterial virulence.

As noted above, *C. albicans* colonization increased IFN-γ secretion, in agreement with a previous report (11). In both studies, this effect was completely reversed by administration of antifungal agents (Fig. 7), which also led to significantly lower rates of bacterial pneumonia, lung weights, and *A. baumannii* CFU counts (Fig. 6). These effects were previously attributed to a strong Th1 response to the presence of *C. albicans* (11), leading to increased levels of IFN-γ. However, it should be noted that innate immune cell populations, such as NK cells, also produce IFN-γ in the context of pulmonary infection and likely contribute to cytokine production, especially at early time points before priming of Th1 cells.

IFN-γ has been shown to hamper host antibacterial defenses. Specifically, overnight incubation with IFN-γ decreased phagocytosis of *Escherichia coli* and *Staphylococcus aureus* by alveolar macrophages by >90% (11). Moreover, IFN-γ downregulated the expression of a class A scavenger receptor on the surfaces of alveolar macrophages, thus impairing phagocytosis of *Pneumococcus*, which was restored to normal by anti-IFN-γ specific antibodies or opsonization, which bypasses scavenger receptors (20). Therefore, upregulation of IFN-γ production is a potential determinant of the increased susceptibility to bacterial infections associated with *C. albicans* airway colonization.

IL-17 levels were significantly lower in *Candida*-colonized rats than in controls after *A. baumannii* instillation, in agreement with previous observations in a rat model of *P. aeruginosa* pneumonia (11). Similar to the study by Roux et al. (11), we found no difference in IL-17 lung concentrations between *Candida*-colonized rats treated with amphotericin B and untreated rats in the absence of *A. baumannii* instillation. However, in the prior study, the effect of antifungals on IL-17 production in *Candida*-colonized rats as a response to bacterial infection was not evaluated (11). Interestingly, in our study, treatment of *C. albicans* colonization with amphotericin B led to a significant increase in post-*A. baumannii* instillation IL-17 levels. These findings support the well-described role of IL-17 in host defenses against extracellular bacteria and...
fungi (21, 22). Notably, amphotericin B has antifungal as well as immunomodulatory effects, and further studies are needed to determine if our observations are the result of either or both (23).

In previous studies using the same rat model (11, 24), \textit{C. albicans} airway colonization primed the host for development of \textit{S. aureus} and \textit{P. aeruginosa} pneumonia; however, the \textit{T}H2 lineage did not seem to be activated, in agreement with our results (see Fig. S1 and S2 in the supplemental material). On the other hand, in BALB/c mice, \textit{C. albicans} intranasal instillation had a beneficial effect on \textit{Pseudomonas} pneumonia, with increased \textit{T}H2 cytokine levels (25). Therefore, it seems that the interplay between \textit{Candida} airway colonization, host immune response, and subsequent development of bacterial pneumonia is host dependent. In human cells, where a \textit{T}H1-\textit{T}H17 response is protective against \textit{Candida} infections (26), IL-26 was recently identified as a potent mediator of the \textit{T}H17 axis antibacterial properties (22). Since the gene for IL-26 is absent in rodents (21, 22), other mechanisms are likely implicated in our rat model observations, and it is not known whether they are conserved in humans, as well.

We demonstrated induction of the \textit{A. baumannii} phospholipase (\textit{plc2} and \textit{pld2}) and \textit{basC, basD} \textit{(P}/H110050.066), and \textit{bauA} genes in the presence of \textit{C. albicans} (Table 1); \textit{basC}, \textit{basD}, and \textit{bauA} are essential for the biosynthesis and utilization of acinetobactin, the main siderophore in \textit{A. baumannii}. Furthermore, we observed induction of \textit{barB}, also part of the siderophore-mediated iron acquisition system (27, 28). Siderophores facilitate iron acquisition from the host and consequently enhance bacterial fitness and virulence, given its limited availability as free iron in mammalian tissues (28). Therefore, the intensification of (i) iron uptake and (ii) use of carbon as a source of energy from host phosphatidylcholine breakdown by \textit{Acinetobacter} phospholipases (29) are potential mechanisms through which \textit{C. albicans} enhances the ability of \textit{A. baumannii} to cause pneumonia. On the other hand, suppression of the \textit{ompA} and \textit{abaI} genes could be indicative of an antagonistic relationship between the two organisms. The \textit{ompA} product has been found to adhere to hyphae, inhibiting \textit{C. albicans} growth (17), whereas \textit{abaI} encodes an autoinducer synthase that contributes to quorum sensing and biofilm formation (30, 31).

Our study has limitations that should be taken into consideration and addressed in future experiments. First, we used only one \textit{A. baumannii} and one \textit{C. albicans} strain. Also, we studied a model of acute infection in previously healthy animals, and it is difficult to draw firm conclusions that will be applicable to intensive care unit (ICU) patients, who are frequently immunosuppressed, are hospitalized for days or weeks, have structural lung abnormalities, and have received multiple antibiotics. Third, we studied cytokine levels in lung homogenate tissue, consisting of diverse immune and other cell populations. Fourth, we assessed only the short-term in vitro effect of \textit{A. baumannii} coinfection with \textit{C. albicans}
on the expression of *A. baumannii* virulence genes, which might not adequately simulate the complex *in vivo* bacterial-yeast interactions in the setting of chronic airway colonization and biofilm physiology. Notably, though, in a recent clinical study of 618 intubated patients, we found that *Candida* colonization was significantly associated with the subsequent development of *A. baumannii* ventilator-associated pneumonia, after robust adjustment for all other confounders and propensity score matching (32).

In conclusion, we found that respiratory tract colonization with *C. albicans* facilitated the development of *A. baumannii* pneumonia in a rat model by modulating the expression of *A. baumannii* virulence genes and/or through modification of the host immune response. In the setting of established *Candida* colonization, antifungal treatment lowered the incidence of *A. baumannii* pneumonia, potentially by suppressing IFN-γ production and enhancing IL-17 production. Prospective clinical studies on the association between *Candida* sp. airway colonization, preemptive antifungal treatment, and subsequent *A. baumannii* ventilator-associated pneumonia should be considered.

### ACKNOWLEDGMENTS

This work was supported by grants from the Guangdong Natural Science Foundation (grant 2014A030313305), the Science and Technology Planning Project of Guangzhou, Guangdong Province (grant 201510010046), the Science and Technology Planning Project of Guangdong Province (grant 2015A050502026), the Science and Technology Innovation Fund of Guangdong Provincial Department of Education (grant 2014KTSCX039), and Projects of the National Natural Science Foundation of China (grant 81570012) to Xiaojiang Tan. We have no other financial disclosures.

### TABLE 1

*In vitro* expression of virulence genes relative to housekeeping gene 16S rRNA in *A. baumannii* ATCC 19606

<table>
<thead>
<tr>
<th>Gene</th>
<th><em>C. albicans</em></th>
<th>Controls</th>
<th>P value</th>
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<td><em>lpxA</em></td>
<td>0.47 ± 0.23</td>
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<td><em>basC</em></td>
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<td><em>pgdC</em></td>
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<tr>
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<td><em>abaI</em></td>
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REFERENCES


