

Comparative Analysis of Epidemiological and Clinical Characteristics Between Invasive *Candida* Infection versus Colonization in Critically Ill Patients in a Tertiary Hospital in Anhui, China

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Objective: Invasive infections due to *Candida* spp. have unique epidemiology, strain distribution, antimicrobial susceptibility, and clinical features. This study aimed to compare and evaluate these characteristic variables between invasive *Candida* infection and colonization of critically ill patients in local China to potentially improve differential diagnosis and therapy.

Methods: A total of 193 critically ill patients were recruited and followed up for the study, and 133 *Candida* isolates were obtained from invasive *Candida*-infected or -colonized subjects. The strains were identified to species level through matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry, assisted by DNA sequencing. *Candida* susceptibility to common antifungals, including azoles, was determined by microbroth ATB Fungus 3 methodology. Azole resistance–related gene sequencing and homologous 3D-structure modeling were employed. Patient demographics and clinical risk factors were documented and comparatively analyzed from the hospital information-management system.

Results: Non-*C. albicans Candida* (56%) principally caused invasive *Candida* infections, while *C. albicans* (55.17%) contributed more to *Candida* colonization in critically ill patients. Additional risk factors exerted significant impact on both *Candida* cohorts, primarily including invasive interventions, cancers, and concurrent infections in common. Most colonized *Candida* spp. harbored relatively higher sensitivity to azoles. *ERG11* gene mutations of T348A and A1309G, A395T and C461T, and a novel G1193T to our knowledge were identified in azole-resistant *C. albicans*, *C. tropicalis*, and *C. parapsilosis* respectively, and their corresponding homologous 3D-structure modeling was putatively achieved.

Conclusion: Distinct epidemiological and clinical characteristics existed between invasive *Candida* infection and colonization in critically ill patients. Multiple risk factors significantly involved both the *Candida* cohorts. Colonized *Candida* exhibited generally higher azole sensitivity than invasively infectious counterparts. *ERG11* point mutations had mechanistically potential ties with local *Candida* resistance to azoles.

Keywords: invasive *Candida* infections, colonization, clinical features, *ERG11* mutations, critically ill patients, comparative analysis

Introduction

Invasive *Candida* infections are one of the most prevalent and important health care–associated fungal infections in hospitalized patients, with approximately 40% of attributable mortality among critically ill subjects.¹ Although significant improvements have been achieved in disease management and access to treatment for *Candida* infections within the last few decades, their prevalence keeps increasing globally.² In the clinic, timely pathogen identification, differential diagnosis, rational antifungal treatment, and stringent source control are still key determinants for survival to such patients.²

As per the criteria of the European Organization for Research and Treatment of Cancer and the Mycoses Study Group (EORTC-MSG), the diagnosis of invasive *Candida* infections is fundamentally based on three elements: host factors,

clinical features, and mycological evidence.³ Host factors primarily include neutropenia, hematological malignancy, stem-cell/solid-organ transplantation, and overuse of hormones/immunosuppressants within the last 2 – 3 months.³ For clinical features, the disease spectrum of invasive *Candida* infections ranges from fungemia to deep-seated candidiasis and septic shock with multiorgan failure. Its clinical symptoms vary substantially, depending on infection sites and disease burden. Knowledge of various radiological/endoscopic patterns for each form of invasive *Candida* infection facilitates early recognition and accurate diagnosis. Unfortunately, few types of these infections possess typical clinical features at the bedside.⁴ Mycological evidence regularly involves serum biomarkers, cultures, and biopsies. To date, detection of circulating antigens in patients with invasive *Candida* infections is largely restricted to serum mannans, which is a main polysaccharide component of *Candida* cell walls, using a microplate enzyme immunoassay.^{5,6} Other investigators have reported mannans assays available for diagnosis of invasive *Candida* infections with sensitivity and specificity of 41% – 87.9% and 78% – 100%, respectively.^{6,7} Mycological cultures or tissue biopsies via sterile procedures from normally sterile sites are preferred approaches to achieve a definitive diagnosis for such infections. However, their overall diagnostic sensitivity and specificity are usually unsatisfying.⁸ It turns out that host factors and clinical features in subjects with invasive *Candida* infections become increasingly complicated in current clinical settings, and relevant hematological/pathological tests are often time-consuming and lack diagnostic sensitivity/specificity. In general, clinical manifestations of invasive *Candida* infections are aspecific, and it is less likely to be able to definitively distinguish from *Candida* colonization or sepsis of bacterial origin usually occurring in hospitals. In addition, though the likelihood of invasive *Candida* infections is largely associated with prior colonization of *Candida* spp. in inpatients,⁹ specific or detailed differences of distinct clinical variables between the two types of *Candida* spp. among critically ill patients remain less extensively investigated.

In the clinic, *Candida* infections are usually treated with antifungal agents, including azoles, polyenes, fluoropyrimidine analogues, and echinocandins.¹⁰ Notably, azoles can target the ergosterol-biosynthesis process by suppressing the fungal cytochrome P450-dependent enzyme lanosterol 14- α -demethylase (*ERG11*), altering cell-membrane functions and leading to cell death.¹⁰ Reliable therapeutic effectiveness and safety have guaranteed azoles for prophylactic and empirical treatment in critically ill patients.¹¹ However, azole resistance against *Candida* has markedly increased worldwide, and its mechanism is closely associated with mutations of target genes including *ERG11*, that alter antifungal drug affinity and/or target abundance.^{12,13} As such, we sought to evaluate and/or compare the regional epidemiology, antifungal susceptibility, risk factors, and clinical outcomes of invasive *Candida* infection and colonization in inpatients in a large comprehensive tertiary hospital in central China, in order to put forward possible strategies for the improvement of differential diagnosis and therapy locally. The antifungal azole resistance-related gene *ERG11* in locally ever-increasing azole-resistant *Candida* spp. was also assessed by gene sequencing to investigate a possible underlying mechanism of the drug resistance.^{12,14}

Methods

Study Design and Data Collection

We recruited and followed up a total of 193 critically ill subjects admitted to the First Affiliated Hospital of Anhui Medical University from January 2018 to June 2019. This affiliated hospital is a 2,800-bed comprehensive tertiary teaching hospital serving a broad population in central China. Three nonoverlapping patient groups were classified: invasive *Candida* infection, *Candida* colonization, and controls (no *Candida* infection or colonization). Positively cultured *Candida* strains were used for subject recruitment, *Candida* infection or colonization identified was determined for grouping *Candida* infection and *Candida* colonization, and control-group subjects were tested and found to be without *Candida* spp. during the entire hospitalization. More specific group definitions follow. Unduplicated *Candida* isolates were collected from sterile materials or unsterile body sites for each individual patient. The isolated *Candida* spp. were primarily derived from host blood, abdominal fluid, bile, dialysis fluid, pleural fluid, bronchoalveolar lavage fluid, shunt fluid, purulence, swab, secretion, urine, and sputum materials and harvested/aspirated by sterile procedures, including paracentesis of bladders and abscesses, or from deep lower respiratory tracts via ventilating devices and bronchofiberscopes for direct microscopic examinations and mycological cultures. Detailed data on mycological cultures

were documented: culture date, type of specimen source, and *Candida* genus/species identity. All subject information was obtained from the hospital's medical record-network system. The case information collected principally comprised basic demographics, length of stay, invasive interventions/procedures, immune state, underlying diseases, concurrent infections, and clinical outcomes.

Definitions of Invasive *Candida* Infections and *Candida* Colonization

Invasive *Candida* Infections

According to the criteria from the EORTC-MSG,³ candidemia is defined as the isolation of *Candida* spp. from the bloodstream in at least one blood culture. Urinary tract *Candida* infections were defined as the presence of at least 10^3 colony-forming units (CFU)/mL of *Candida* spp. in catheterized patients or $\geq 10^4$ CFU/mL in noncatheterized patients without any coisolation of other bacterial pathogens.¹⁵ The invasive *Candida* infections other than of blood or urine origin were defined as the isolation of *Candida* spp. from a host who met at least two of the systemic inflammatory response syndrome (SIRS) criteria (briefly, body temperature $>38^\circ\text{C}$ or $<36^\circ\text{C}$, heart rate >90 beats/min, respiratory rate >20 breaths/min, white blood cells $>12,000/\text{mm}^3$, or acute organ failure).¹⁶ Since not all of these criteria are definitely indicative of an underlying infectious process, we then linked each separate case to the initiation of appropriate antifungal treatment for further assessment. These criteria/definitions along with the effective initiation of antifungal therapy were combined for our final decision consequently to distinguish true invasive *Candida* infections from simply *Candida* colonization.

Candida Colonization

Candida colonization was defined as the presence of a positive *Candida* culture from an unsterile site without clinical signs of infection (eg, at least two of the aforementioned SIRS criteria) or with symptoms of infection clearly attributable to other microorganisms.¹⁶ *Candida* pneumonia must be confirmed histologically. Otherwise, only a *Candida*-positive sputum culture is considered colonization.¹⁷

Candida Spp. Identification and Antifungal-Sensitivity Testing

With automatic microbial identification equipment (BacT/ALERT 3D, BioMérieux) and/or a Sabouraud dextrose/chromogenic agar-culture system, the *Candida* isolates were strictly identified to species level by matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS; Vitek MS system; BioMérieux) within our clinical laboratory. Microscopically or macroscopically morphological analyses were employed to assist *Candida* strain identification. DNA sequencing was supplemented when unacceptable MS identification consequences occurred. MS identification was considered acceptable if its confidence value reached 99.9%. DNA sequencing for *Candida* spp. identification was carried out using universal fungal primer pairs of internal transcribed spacer 1 (ITS1) and ITS4, as previously described (Table 1).¹⁸

Antifungal in vitro sensitivity of frequently used antifungal drugs in the clinic, including amphotericin B, 5-fluorocytosine, fluconazole, itraconazole, and voriconazole, were determined in all identified *Candida* spp. with a widely used commercial antifungal-susceptibility testing method (ATB Fungus 3 panel, BioMérieux) following the manufacturer's instructions. Two quality controls consisted of standard strains of *C. parapsilosis* (ATCC 22019) and *C. krusei* (ATCC 6258). Finally, the minimum inhibitory concentration (MIC) of these antifungals was measured using an ATB-expression bacteriology device (BioMérieux), and was interpreted using the Clinical and Laboratory Standards Institute M27-A3 microbroth-dilution method,¹⁹ as well as breakpoints recommended by the antifungal-susceptibility testing kit.

Azole-Resistance Gene Sequencing and Homologous 3D-Structure Modeling

ERG11, an azole resistance-related gene, was analyzed by PCR and sequencing assays. All the primers used in the present study are listed in Table 1 and had been commercially synthesized (Shanghai Sangon Biotech).^{20–23} Yeast-DNA materials were carefully extracted as PCR templates with UNIQ-10 Column Yeast Plasmid Preps Kit (Shanghai Sangon Biotech), and PCR amplifications were conducted with an Applied Biosystems 7500 DNA analyzer (Thermo Fisher Scientific). The PCR parameters included 5 minutes at 94°C for initial denaturation, 32 cycles (30 s at 94°C for denaturation, 90 s at 50°C for annealing, and 90 s at 72°C for elongation), and 10 minutes at 72°C for last elongation.

Table 1 PCR primers used in the study

Primers	Primer sequences (5'–3')	Target species/ gene	Amplicon size (bp)	Reference #
ITS1	TCCGTAGGTGAACCTGCGG	Universal fungi/ITS	—	[15]
ITS4	TCCTCCGCTTATTGATATGC			
CAL-F	CAAGAAGATCATAACTCAAT	<i>C. albicans</i> /ERG11	~1,600	[17]
CAL-R	CAGAACACTGAATCGAAAGA			
CGL-F	ATGTCCACTGAAAACACTTCTTTGG	<i>C. glabrata</i> /ERG11	1,599	[18]
CGL-R	GTACTTTTGTCTGGATGTCTCTTTTC			
CTR-F	GTTTTCTACTGGATCCCATG	<i>C. tropicalis</i> /ERG11	~1,200	[19]
CTR-R	TACATCTGTGTCTACCACC			
CPA-F	GCGATAAGAATGCGGCCGCATGGCATTAGTTGATTAGC	<i>C. parapsilosis</i> / ERG11	1,569	[20]
CPA-R	ATCGTACAGGCATGCTCAGATTACACATGTATCT			

Note: ITS is a target commonly used for fungal (including *Candida*) species identification by DNA sequencing.

Abbreviation: ITS, internal transcribed spacer.

After quantitatively confirmation by the Thermo Fisher Scientific NanoDrop spectrophotometer and DNA agarose-gel electrophoresis tests, the amplified PCR products were subject to stringent sequencing analysis in both directions at Sangon Biotech using corresponding PCR amplification primers. The full length of the nucleotide sequence of *ERG11* in each typical *Candida* spp. in GenBank was used as a corresponding reference sequence (GenBank accession numbers: *C. albicans* AY856352, *C. glabrata* L40389, *C. tropicalis* XM_002550939.1, and *C. parapsilosis* GQ302972). Our obtained sequencing data then went through the GenBank BLAST database (<http://blast.ncbi.nlm.nih.gov>) for comparative analysis. Homologous modeling for 3D structures of putative mutant ERG11 protein of *Candida* spp. was conducted through Swiss-Model with the assistance of 5JLC as template (<http://www.pdb.org>),^{24,25} and the presumed protein products were visualized by PyMOL 2.2.0 (Schrödinger).

Statistical Analysis

SPSS 22.0 and GraphPad Prism 5.0 (GraphPad Software) were used for data processing and statistical analysis. All numeric data are presented as counts and percentages, and average age and hospital-stay data are expressed as means \pm SD. Comparisons among categorical data were analyzed by Pearson's χ^2 test or Fisher's exact test. $P < 0.05$ was considered statistically significant.

Results

Strain-Distribution Patterns of Invasive *Candida* Infection and Colonization

There were a total of 193 hospitalized patients successfully recruited for this study. Of these, 75 were diagnosed with invasive *Candida* infections, 58 with *Candida* colonization, and 60 were *Candida* culture-negative during hospitalization. In the present study, a total of 133 separate *Candida* isolates were detected and finally identified to the species level through microscopically or macroscopically morphological analysis and MALDI-TOF MS (Figure 1), with auxiliary gene sequencing when needed.²⁶ As listed in Table 2, *C. albicans* was the predominant species isolated from both groups of invasive *Candida* infection and colonization, accounting for 44% (n=33) and 55.17% (n=32), respectively. Among the non-*C. albicans* *Candida* (NCAC) spp., *C. glabrata* was the most dominant in both of the groups (57.14% [n=24] vs 65.38% [n=17]), followed by *C. tropicalis* (26.19% [n=11] vs 30.77% [n=8]) and *C. parapsilosis* (14.29% [n=6] vs 3.85% [n=1]). *C. krusei* only emerged in the group of *Candida* infections, accounting for the remaining 2.38% (n=1) of patients with *Candida* infections. No significant differences were observed between the distributions of *C. albicans* and NCAC ($P=0.201$).

Clinical Profiles of Patients with Invasive *Candida* Infection and Colonization

Comparison data between baseline characteristics of patients with invasive *Candida* infections and the control cohort without *Candida* are presented in Table 3. The clinical variables that were evenly distributed between the two groups

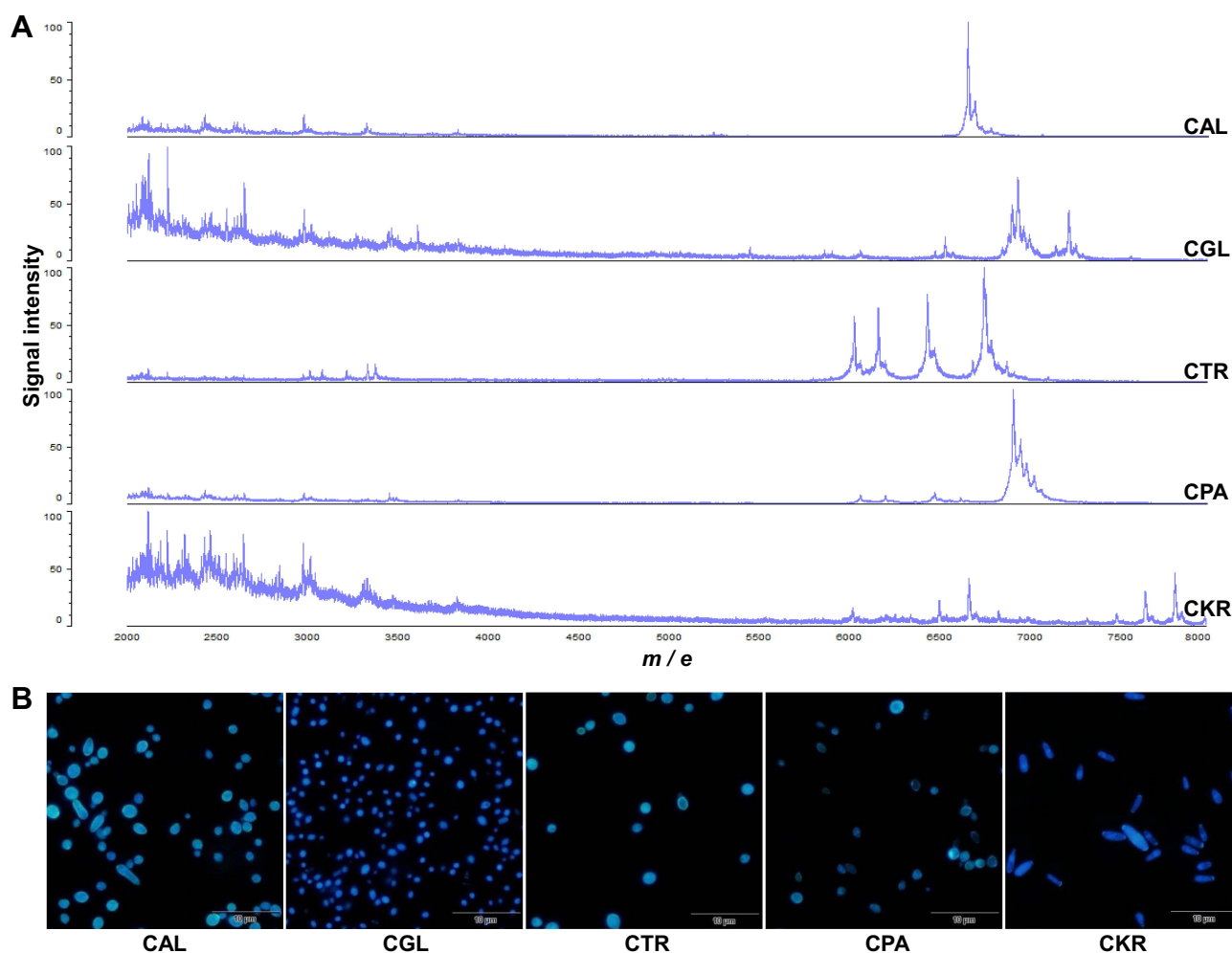


Figure 1 Data of the identification of *Candida* strains obtained from the present research. **(A)** MALDI-TOF MS analysis of *Candida* spp. typically involved in the present study. **(B)** Microscopically analyzed fungal fluorescent staining results of representative *Candida* spp. cultured on Sabouraud dextrose/chromogenic agar plates.

Abbreviations: CAL, *C. albicans*; CGL, *C. glabrata*; CTR, *C. tropicalis*; CPA, *C. parapsilosis*; CKR, *C. krusei*.

were sex, tracheal intubation, ventilatory support, arteriovenous cannulation, peritoneal dialysis/hemodialysis, immunosuppressive therapy (including immunosuppressant tacrolimus/FK506 and/or cyclosporine A treatment), diabetes mellitus, and liver cirrhosis. Comparatively, there was a significant difference in the age distribution between patients with invasive *Candida* infections and control subjects without *Candida* ($P=0.003$), especially in the age-groups 15 – 49 years ($P=0.005$) and >65 years ($P=0.003$). Of the subjects 15 – 49 years of age, the infection frequency was relatively low, whereas in those >65 years old more patients presented invasive *Candida* infections. Interestingly, that infection rate was

Table 2 Strain-distribution patterns of invasive *Candida* infection vs colonization

	Patients with invasive <i>Candida</i> infections, n=75 (%)	Patients with <i>Candida</i> colonization, n=58 (%)	P
<i>C. albicans</i>	33 (44)	32 (55.17)	0.201
Non-<i>C. albicans Candida</i>	42 (56)	26 (44.83)	
<i>C. glabrata</i>	24 (57.14)	17 (65.38)	0.739
<i>C. tropicalis</i>	11 (26.19)	8 (30.77)	0.886
<i>C. parapsilosis</i>	6 (14.29)	1 (3.85)	0.108
<i>C. krusei</i>	1 (2.38)	0	0.377

Table 3 Comparison of clinical variables of invasive *Candida* infections vs controls

	Patients with invasive <i>Candida</i> infections, n=75 (%)	Patients without <i>Candida</i> , n=60 (%)	P
Sex			
Male	33 (44)	27 (45)	0.908
Female	42 (56)	33 (55)	
Age			
Mean \pm SD (years)	57.83 \pm 19.26	50.60 \pm 15.56	0.006
0 – 14	3 (4)	0	0.117
15 – 49	21 (28)	31 (51.67)	0.005
50 – 65	21 (28)	19 (31.67)	0.643
>65	30 (40)	10 (16.67)	0.003
Hospital stay^a			
Mean \pm SD (days)	44.44 \pm 45.46	16.95 \pm 13.17	<0.001
0 – 7	4 (5.33)	12 (20)	0.009
7 – 14	22 (29.33)	23 (38.33)	0.270
>14	49 (65.33)	25 (41.67)	0.006
Clinical risk factors			
Invasive interventions			
Surgical procedures	37 (49.33)	12 (20)	<0.0001
Urethral catheters	34 (45.33)	17 (28.33)	0.043
Ventilatory support	24 (32)	13 (21.67)	0.181
Tracheal intubation	23 (30.67)	14 (23.33)	0.343
Arteriovenous cannulation	21 (28)	17 (28.33)	0.966
Tracheotomy	12 (16)	0	0.001
PD or hemodialysis	3 (4)	5 (8.33)	0.289
Immune state			
Immunosuppressive therapy	3 (4)	3 (5)	0.289
Comorbidities			
Cancers	18 (24)	5 (8.33)	0.016
Diabetes mellitus	7 (9.33)	7 (11.67)	0.659
Liver cirrhosis	1 (1.33)	0	0.369
Concurrent <i>Candida</i> infections	24 (32)	0	<0.0001
Coinfections of bacteria	49 (65.33)	28 (46.67)	0.029
Clinical outcomes			
Improvement	44 (58.67)	54 (90)	<0.0001
Deterioration	8 (10.67)	6 (10)	0.900
Moribund	2 (2.67)	0	0.203

Notes: ^aTotal length of stay for patients with invasive *Candida* infection or *Candida* colonization.

Abbreviation: PD, peritoneal dialysis.

found to be highly associated with patient hospitalization duration ($P<0.001$). The average hospital stay was 44.44 vs 16.95 days between patients with or without *Candida* infections. Moreover, certain invasive interventions or surgical procedures, as well as concurrent infections, were generally considered as highly relevant risk factors of invasive fungal infections. In the present study, those risk factors were urethral catheters ($P=0.043$), tracheotomy ($P=0.001$), surgical procedures ($P<0.0001$), cancers ($P=0.016$), concurrent *Candida* infections (ie, different *Candida* spp. or the same species but with different drug sensitivity, $P<0.0001$), and concurrent bacterium infections (ie, infections with bacteria identified during the period of *Candida* infection or colonization, $P=0.029$). Eventually, the overall clinical outcomes of the uninfected control group were remarkably improved compared with their *Candida*-infected counterpart.

As summarized in Table 4, similar comparisons were conducted between patients with *Candida* colonization and the control cohort without *Candida*. Despite that ventilatory support, peritoneal dialysis/hemodialysis, immunosuppressive therapy, diabetes mellitus, and liver cirrhosis were not markedly different between them, it was extremely interesting to note that in patients with *Candida* colonization, the distribution of sex, age, length of stay, urethral catheters, tracheal intubation, arteriovenous cannulation, tracheotomy, surgical procedures, cancers, concurrent *Candida* infections and concurrent bacterium infections differed significantly, and most were dominant when compared with those in patients without *Candida* ($P<0.05$ for all the variables). Likewise, the control cohort without *Candida* exhibited more improved prognosis than the group with *Candida* colonization ($P=0.005$).

Next, we further compared the groups of patients with invasive *Candida* infection or colonization. As shown in Table 5, patients with *Candida* colonization were mainly male ($P=0.002$) and older ($P=0.007$). Not surprisingly, patients

Table 4 Comparison of clinical variables of *Candida* colonization vs controls

	Patients with <i>Candida</i> colonization, n=58 (%)	Patients without <i>Candida</i> , n=60 (%)	P
Sex			
Male	41 (70.69)	27 (45)	0.005
Female	17 (29.31)	33 (55)	
Age			
Mean \pm SD (years)	66.12 \pm 14.17	50.60 \pm 15.56	<0.001
0 – 14	0	0	/
15 – 49	5 (8.62)	31 (51.67)	<0.0001
50 – 65	18 (31.03)	19 (31.67)	0.941
>65	35 (60.34)	10 (16.67)	<0.0001
Hospital stay^a			
Mean \pm SD (days)	29.55 \pm 32.90	16.95 \pm 13.17	<0.001
0 – 7 days	1 (1.72)	12 (20)	0.002
7 – 14 days	14 (24.14)	23 (38.33)	0.097
>14 days	43 (74.14)	25 (41.67)	<0.0001
Clinical risk factors			
Invasive interventions			
Surgical procedures	26 (44.83)	12 (20)	0.004
Urethral catheters	32 (55.17)	17 (28.33)	0.003
Ventilatory support	22 (37.93)	13 (21.67)	0.053
Tracheal intubation	29 (50)	14 (23.33)	0.003
Arteriovenous cannulation	30 (51.72)	17 (28.33)	0.009
Tracheotomy	8 (13.79)	0	0.003
PD or hemodialysis	4 (6.90)	5 (8.33)	0.769
Immune state			
Immunosuppressive therapy	3 (5.17)	3 (5)	0.495
Comorbidities			
Cancers	27 (46.55)	5 (8.33)	<0.0001
Diabetes mellitus	9 (15.52)	7 (11.67)	0.541
Liver cirrhosis	2 (3.45)	0	0.147
Concurrent <i>Candida</i> infections	9 (15.52)	0	0.001
Coinfections of bacteria	42 (72.41)	28 (46.67)	0.004
Clinical outcomes			
Improvement	40 (68.97)	54 (90)	0.005
Deterioration	2 (3.45)	6 (10)	0.157
Moribund	1 (1.72)	0	0.307

Notes: ^aTotal length of hospital stay for patients with invasive *Candida* infection or *Candida* colonization.

Abbreviation: PD, peritoneal dialysis.

Table 5 Comparison of clinical variables of invasive *Candida* infection vs colonization

	Patients with Invasive <i>Candida</i> Infections, n=75 (%)	Patients with <i>Candida</i> Colonization, n=58 (%)	P
Sex			
Male	33 (44)	41 (70.69)	0.002
Female	42 (56)	17 (29.31)	
Age			
Mean \pm SD (years)	57.83 \pm 19.26	66.12 \pm 14.17	0.007
0 – 14	3 (4)	0	0.123
15 – 49	21 (28)	5 (8.62)	0.005
50 – 65	21 (28)	18 (31.03)	0.703
>65	30 (40)	35 (60.34)	0.020
Hospital stay^a			
Mean \pm SD (days)	44.44 \pm 45.46	29.55 \pm 32.90	0.037
0 – 7	4 (5.33)	1 (1.72)	0.278
7 – 14	22 (29.33)	14 (24.14)	0.504
>14	49 (65.33)	43 (74.14)	0.276
Clinical risk factors			
Invasive interventions			
Surgical procedures	37 (49.33)	26 (44.83)	0.606
Urethral catheters	34 (45.33)	32 (55.17)	0.260
Ventilatory support	24 (32)	22 (37.93)	0.476
Tracheal intubation	23 (30.67)	29 (50)	0.023
Arteriovenous cannulation	21 (28)	30 (51.72)	0.005
Tracheotomy	12 (16)	8 (13.79)	0.724
PD or hemodialysis	3 (4)	4 (6.90)	0.458
Immune state			
Immunosuppressive therapy	3 (4)	3 (5.17)	0.747
Comorbidities			
Cancers	18 (24)	27 (46.55)	0.006
Diabetes mellitus	7 (9.33)	9 (15.52)	0.277
Liver cirrhosis	1 (1.33)	2 (3.45)	0.415
Concurrent <i>Candida</i> infections	24 (32)	9 (15.52)	0.029
Coinfections of bacteria	49 (65.33)	42 (72.41)	0.384
Clinical outcomes			
Improvement	44 (58.67)	40 (68.97)	0.222
Deterioration	8 (10.67)	2 (3.45)	0.117
Moribund	2 (2.67)	1 (1.72)	0.717

Notes: ^aTotal length of stay for patients with invasive *Candida* infection or *Candida* colonization.

Abbreviation: PD, peritoneal dialysis.

in the *Candida*-infected group had longer average hospital stay ($P=0.037$) and suffered more concurrent *Candida* infections ($P=0.029$) than patients with *Candida* colonization. Unexpectedly, a higher incidence of tracheal intubation ($P=0.023$) and arteriovenous cannulation ($P=0.005$) occurred in patients with *Candida* colonization. Interestingly, the clinical outcomes were not statistically different between the two groups ($P>0.05$). Additionally, to assess the relationship of the multiple risk factors and clinical outcomes for all three groups, an overall comparative analysis on these significant clinical variables was conducted and is presented in [Supplementary Table S1](#).

Antifungal-Sensitivity Patterns of Invasive *Candida* Infection and Colonization

The in vitro sensitivity of five frequently used antifungal drugs in clinic were examined for all the isolated *Candida* spp.; amphotericin B, 5-fluorocytosine, fluconazole, itraconazole, and voriconazole. As shown in Table 6, for subjects overall, amphotericin B and 5-fluorocytosine demonstrated excellent in vitro activity against all *Candida* spp., although 5.90% of *C. glabrata* collected from patients with *Candida* colonization were not susceptible to 5-fluorocytosine. Nonetheless, sensitivity to azoles varied among *Candida* spp. and between groups. Of note, certain *C. tropicalis* from both groups were, with relatively high frequency, resistant to fluconazole, itraconazole, and voriconazole. Comparative analysis of antifungal-susceptibility patterns between patients with invasive *Candida* infection and colonization was carried out, and the data are summarized in Table 6. In general, most *Candida* spp. from patients with *Candida* colonization harbored considerably higher sensitivity to azoles than those with invasive *Candida* infections, except *C. glabrata*, with relatively lower susceptibility to itraconazole and 5-fluorocytosine in the colonization group. In addition, our clinical data demonstrated that 50.67% of patients with invasive *Candida* infections and 18.97% of patients with *Candida* colonization had received prior treatment with azoles (Supplementary Table S2).

Lastly, in order to investigate possible azole-resistance mechanisms in our common local *Candida* spp. of *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. parapsilosis*, clinical isolates of azole-resistant *Candida* strains were harvested and the azole resistance-related gene *ERG11* was analyzed by PCR and sequencing assays. After successfully being analyzed and confirmed by molecular biological assays, the gene-fragment PCR products of interest underwent precise sequencing

Table 6 Antifungal-sensitivity patterns of patients with invasive *Candida* infection and colonization

Strain	Antifungal drug	Patients with invasive <i>Candida</i> infections				Patients with <i>Candida</i> Colonization				P ^a
		Sensitivity n (%)	MIC (μg/mL)			Sensitivity n (%)	MIC (μg/mL)			
			Range	MIC ₅₀	MIC ₉₀		Range	MIC ₅₀	MIC ₉₀	
<i>C. albicans</i>	AmB	33 (100)	0.50–1.00	0.50	0.50	32 (100)	0.50–1.00	0.50	0.50	/
	FC	33 (100)	4.00–4.00	4.00	4.00	32 (100)	4.00–4.00	4.00	4.00	/
	Flu	31 (93.90)	0.50–64.00	1.00	2.00	32 (100)	0.50–8.00	1.00	2.00	0.038
	Vrc	33 (100)	0.06–1.00	0.06	0.06	32 (100)	0.06–0.50	0.06	0.13	/
	Itr	31 (93.90)	0.12–2.00	0.13	0.13	32 (100)	0.12–0.25	0.13	0.13	0.038
<i>C. glabrata</i>	AmB	24 (100)	0.50–0.50	0.50	0.50	17 (100)	0.50–0.50	0.50	0.50	/
	FC	24 (100)	1.00–4.00	4.00	4.00	16 (94.10)	4.00–16.00	4.00	4.00	0.038
	Flu	24 (100)	1.00–4.00	1.00	4.00	17 (100)	1.00–8.00	2.00	8.00	/
	Vrc	23 (95.80)	0.06–2.00	0.06	0.25	17 (100)	0.06–0.50	0.06	0.50	0.130
	Itr	19 (79.20)	0.12–0.50	0.13	0.50	9 (52.90)	0.12–2.00	0.13	1.00	<0.001
<i>C. tropicalis</i>	AmB	11 (100)	0.50–1.00	0.50	1.00	8 (100)	0.50–1.00	0.50	1.00	/
	FC	11 (100)	4.00–4.00	4.00	4.00	8 (100)	4.00–4.00	4.00	4.00	/
	Flu	7 (63.60)	0.50–128.00	2.00	64.00	7 (87.50)	1.00–64.00	1.00	64.00	<0.001
	Vrc	9 (81.80)	0.06–8.00	0.13	4.00	7 (87.50)	0.06–4.00	0.06	4.00	0.314
	Itr	6 (54.50)	0.12–4.00	0.13	2.00	7 (87.50)	0.12–2.00	0.13	2.00	<0.001
<i>C. parapsilosis</i>	AmB	6 (100)	0.50–0.50	0.50	0.50	1 (100)	/	0.50	0.50	/
	FC	6 (100)	4.00–4.00	4.00	4.00	1 (100)	/	4.00	4.00	/
	Flu	5 (83.30)	1.00–64.00	4.00	64.00	1 (100)	/	4.00	4.00	/
	Vrc	5 (83.30)	0.06–2.00	0.06	2.00	1 (100)	/	0.13	0.13	/
	Itr	4 (66.70)	0.12–0.50	0.13	0.50	1 (100)	/	0.13	0.13	/
<i>C. krusei</i>	AmB	1 (100)	/	0.50	0.50	/	/	/	/	/
	FC	1 (100)	/	4.00	4.00	/	/	/	/	/
	Flu	/	/	/	/	/	/	/	/	/
	Vrc	1 (100)	/	0.06	0.06	/	/	/	/	/
	Itr	/	/	/	/	/	/	/	/	/

Notes: ^aComparisons of the indicated antifungal sensitivities between the two cohorts in terms of the percentage of *Candida* strains, except colonized *C. parapsilosis* and *C. krusei*.

Abbreviations: AmB, amphotericin B; FC, 5-fluorocytosine; Flu, fluconazole; Vrc, voriconazole; Itr, itraconazole; MIC, minimum inhibitory concentration.

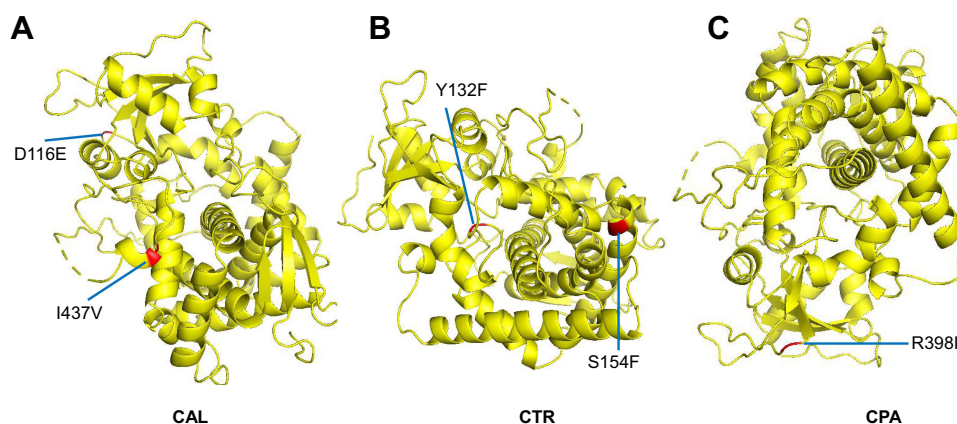


Figure 2 Data of homologous 3D-structure modeling for putatively mutant *ERG11* gene products in *Candida* spp. assisted by 5JLC as template (<http://www.pdb.org>). Presumably altered 3D steric conformations of ERG11 enzymes from azole-resistant *C. albicans* (A), *C. tropicalis* (B), and *C. parapsilosis* (C) are shown. Red spots indicate mutation sites at the amino-acid level in each *Candida* spp. resistant against azoles when compared with corresponding reference sequences in GenBank.

Abbreviations: CAL, *C. albicans*; CTR, *C. tropicalis*; CPA, *C. parapsilosis*.

analyses. The corresponding *Candida ERG11* gene-nucleotide sequences in GenBank were used as reference sequences. Upon alignment with the GenBank reference sequences, the sequencing data identified multiple major point mutations: T348A, C1203T, T1284C, and A1309G in *C. albicans*, C1148T, C1478T, and A1583G in *C. glabrata*, T225C, G264A, A395T, and C461T in *C. tropicalis*, and T591C and G1193T in *C. parapsilosis*. Their corresponding amino-acid substitutions were (except silent or synonymous mutations) D116E and I437V in *C. albicans*, Y132F and S154F in *C. tropicalis*, and R398I in *C. parapsilosis*. With the assistance of homologous 3D-structure modeling based on the 5JLC template, corresponding putatively altered 3D steric conformations of the ERG11 enzyme are presented in Figure 2. Locations of the mutated residues on ribbon cartoons of the molecule are indicated, and visualization of its substrate entry and exit channels is clearly available.

Discussion

Invasive *Candida* infections are a rapidly emerging condition largely associated with contemporary biomedical advances and nosocomial infections.¹ Strikingly, critically ill patients in hospital are at high risk of invasive *Candida* infections, especially in cases of trauma, organ transplantation, surgery, and burns, suggesting that the incidence of such infections is likely linked to concomitantly compromised barriers of skin and mucous membrane.^{27–29} In particular, invasive candidiasis is closely related to empirical treatment with abuse of antifungal drugs. Therefore, early critically differential diagnosis of invasive *Candida* infections to exclude colonization or other types of infections and standardized treatment with rational antifungals are expected to improve prognosis of patients with invasive *Candida* infections.² Comparatively evaluating the relationship between local likelihood of invasive *Candida* infection and colonization in critically ill patients would potentially contribute to prophylaxis of such infections.

Among human-borne *Candida* spp., *C. albicans* is a leading member in both healthy and disease states, which is even confirmed by animal models.^{30,31} However, the number of infections caused by NCAC spp. has elevated significantly in the last three decades.^{28,30,32} There has been a substantial increase in NCAC strains identified in human candidiasis, partly owing to improvement in clinical laboratory diagnostic approaches, such as extensive use of chromogenic media with an ability to differentiate distinct *Candida* spp. and introduction of MS and molecular biological techniques during the routine diagnosis of fungal diseases.⁸ The present findings revealed that *C. albicans* was a principal agent causing *Candida* colonization within critically ill subjects at our hospital, whereas NCAC spp. were the major pathogens in the *Candida*-infected group. The high prevalence of NCAC spp. in the infected group could be a reflection of their inherently high-level resistance to certain antifungals (eg, azoles).^{33,34} In comparison to infections of *C. albicans* alone, traditional antifungal agent therapy would promote the persistence of NCAC strains when mixed infections of *Candida* spp. are present.³³

Certain potential risk factors have previously been identified for *Candida* infections, mainly composed of prior antifungal exposure, immunosuppressive therapy, malignancy, surgery, presence of central venous catheters, trauma, and bacterial sepsis.^{35–38} When compared with the control cohort without *Candida*, we found that age and length of hospital stay had close ties with both invasive *Candida* infection and colonization. Generally, patients with invasive *Candida* infection or colonization were older and had longer hospitalization than those without. Meanwhile, patients with invasive *Candida* infections presented further prolonged hospitalization than their counterparts with *Candida* colonization. To be noted, various other risk factors (such as urethral catheters, tracheotomy, surgical procedures, cancers, and concurrent infections) were also evaluated to exert significant impacts on invasive *Candida* infection and colonization in this study. Nearly half the female subjects that were followed up developed invasive *Candida* infections. The elevated frequency of vaginal colonization and the short length of female urethrae may predispose women to such fungal infections.^{39,40} However, risk factors of systemic infections of *Candida* spp. (due to indwelling catheters, renal failure, total parenteral nutrition) are difficult to prevent or modify, especially among critically ill patients that usually need urgent interventions.^{41,42}

The variability of antifungal susceptibility in *Candida* spp. is geographically dependent.^{43–46} Specifically, we comparatively analyzed the regional susceptibility of common antifungals against *Candida* spp. between the invasive *Candida*-infection and *Candida*-colonization groups in the present study. Almost all the *Candida* isolates were sensitive to amphotericin B and 5-fluorocytosine, except that some *C. glabrata* in patients with *Candida* colonization resisted against 5-fluorocytosine. The local *Candida* spp. showed a differential level of sensitivity to azoles, especially in the group of invasive *Candida* infections. *C. tropicalis* and *C. parapsilosis* exhibited lowered sensitivity to voriconazole, fluconazole, and itraconazole. *C. glabrata* was relatively less susceptible to itraconazole. Other *Candida* spp. displayed higher sensitivity to azoles (>90%). Though itraconazole lacks commercial intravenous formulations to treat invasive infections, the data we obtained on its in vitro sensitivity to *Candida* spp. would benefit the research on azole cross-resistance.⁴⁷ More interestingly, we found that *Candida* isolates from the *Candida*-colonization group generally demonstrated relatively higher azole sensitivity than those from their infected counterparts. Prior azole treatment is expected to confer selection pressure and raise resistance against azoles in patients with *Candida* infections or colonization.⁴⁸ The difference in antimicrobial susceptibility between the two cohorts is worth further examination.

The azole-resistance mechanism among *Candida* spp. involves multiple pathways, of which inhibition of azole activity by altering target sites has been frequently described.^{12,13} In this context, nonsynonymous mutations in *ERG11* are likely to change the 3D structure of lanosterol 14- α -demethylase and to weaken protein affinity to azoles, consequently reducing the antifungal inhibition of ergosterol biosynthesis.⁴⁹ In the present study, two nonsynonymous gene mutations (A395T and C461T), subsequently leading to corresponding amino-acid substitutions (Y132F and S154F) in the ERG11 protein, were identified in all *C. tropicalis* resistant against azoles. The homologous 3D-structure modeling data of ours and others all presumably presented that Y132F and S154F were located in the opening and inner structures of the channel for entrance and exit of azole drugs in the ERG11 enzyme, speculating that synergistic effects of the two mutations are likely to suppress ERG11 binding to antifungal azoles and increase azole resistance.⁵⁰ Furthermore, we observed two mutations of T348A (D116E) and A1309G (I437V) in *ERG11* of azole-resistant *C. albicans*, and to our knowledge, a novel mutation of G1193T (R398I) in *ERG11* was found in azole-resistant *C. parapsilosis*. Since previous studies have reported that T348A (D116E) and A1309G (I437V) of *ERG11*, present in both fluconazole-susceptible and -resistant *C. albicans*, were scarcely thought to enable azole resistance,⁵¹ there might exist other pathways responsible for *C. albicans* azole resistance, which together with the newly identified G1193T mutation in *ERG11* of *C. parapsilosis*, needs more mechanistic investigations in future.

Conclusion

This study comparatively evaluated *Candida* spp. distribution, clinical antifungal-sensitivity profile, and various clinical features of critically ill patients with invasive *Candida* infection or colonization. Our findings revealed that *C. albicans* contributed more to *Candida* colonization, whereas NCAC spp. largely engaged with the *Candida*-infected group. Colonized *Candida* exhibited higher azole sensitivity than the invasive type. The risk factors of age, hospital stay, typical invasive interference, certain comorbidities, and concurrent infections significantly influenced invasive *Candida*

infection and colonization. On average, patients with invasive *Candida* infections were older or had longer hospital stays than those with *Candida* colonization or without any *Candida*. Notably, *ERG11* polymorphisms suggest a potential relationship with *Candida* resistance against azoles.

Abbreviations

NCAC, non-*C. albicans Candida*; EORTC-MSG, European Organization for the Research and Treatment of Cancer–Mycoses Study Group; CFU, colony-forming units; SIRS, systemic inflammatory response syndrome; MALDI-TOF MS, matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry; ITS, internal transcribed spacer; MIC, minimum inhibitory concentration; PCR, polymerase chain reaction.

Ethics Approval

The research protocol was reviewed and approved by the Ethics Committee of Clinical Medicine Research of the First Affiliated Hospital of Anhui Medical University (PJ2022-01-21). Each subject provided written informed consent as per the Declaration of Helsinki.

Author Contributions

All authors made a significant contribution to the work reported, whether in conception, study design, execution, acquisition of data, analysis and interpretation, or all these areas, took part in drafting, revising, or critically reviewing the article, gave final approval to the version to be published, have agreed on the journal to which the article has been submitted, and agree to be accountable for all aspects of the work.

Funding

This work was supported by the National Natural Science Foundation of China (21604079 [JX] and 81601446 [BW]).

Disclosure

The authors declare no competing interests in this work.

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