Candida tropicalis distribution and drug resistance correlated to ERG11 and UPC2 expression

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Research

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Abstract

Objective: This study is to analyze the distribution characteristics and drug resistance of *Candida tropicalis*, and the relationship between ERG11 and UPC2 expression and the resistance to azole.

Methods: The *Candida tropicalis* were cultured and identified by the Sabouraud Agar Medium, CHROM Agar Candida and France **Bio-Mérieux** ATB test. The total RNA of the collected strains was extracted, and the ERG11 and UPC2 mRNA expression levels were analyzed by the quantitative real-time PCR.

Results: Totally 2872 clinical isolates of *Candida*, including 319 strains of *Candida tropicalis* were analyzed herein, which were mainly distributed in the Departments of Respiratory Medicine and ICU. The specimen type was mainly based on the airway secretion, and the detection trend in four years was mainly related to the type of the departments and specimens. The resistance rates of *Candida tropicalis* to fluconazole, itraconazole and voriconazole have been increasing year by year. The mRNA expression levels of ERG11 and UPC2 in the fluconazole resistant group were significantly higher than the sensitive group. In addition, there was a significant linear positive correlation between these two genes in the fluconazole resistant group.

Conclusions: Over-expression of ERG11 and UPC2 genes in *Candida tropicalis* could increase the resistance to antifungal drugs. The routine detection of ERG11 and UPC2 for high-risk patients in the key departments would provide theoretical basis for the rational application of antifungal drugs.

Introduction

Candida tropicalis is widely distributed in nature, which is the main colonized bacteria in the human skin, oral cavity, and digestive tract. Moreover, *Candida tropicalis* is also an important conditional pathogenic *Candida* causing nosocomial infection, the detection rate of which is second only to *Candida albicans* [1]. Patients infected with *Candida tropicalis* might suffer from the lung infection, bloodstream infection, urogenital infections, and even the systemic infection [2]. Importantly, the increasing resistance of *Candida tropicalis* has reported over the past recent years. The global SENTRY antifungal surveillance report in 2013 has shown that, the resistance rate of *Candida tropicalis* to fluconazole is 11.6% (in totally 31 countries) [3]. Moreover, the data from the China Invasive Fungal Resistance Monitoring Network (CHIF-NET) have shown that the resistance rate of *Candida tropicalis* to fluconazole has been increased from 11.2% in 2009 to 42.7% in 2014 [4]. Therefore, the infectious factors for *Candida tropicalis*, the genotyping of drug-resistant strains, and the mechanism of drug resistance have caused widespread concern in recent year[5].

The resistance of *Candida tropicalis* to the azole antifungal drugs is mainly due to the mutations and/or the over-expression of ergosterol synthase (i.e., the 14α-demethylase, 14-DM) encoding gene ERG11, the over-expression of the MDR1 gene from the major facilitator super(MFS)-family[6, 7], and the over-expression of the ATP-binding cassette (ABC) transport box multidrug exporting transporter over-encoding CDR gene [8]. Among them, the mutation and over-expression of the 14-DM-encoding gene

ERG11 have been extensively studied for the drug resistance of *Candida tropicalis*. The UPC2 gene encodes the zinc family transcription factor Upc2p, which exerts the regulating effects on the transcriptional level in *Candida albicans* [9]. However, it is still unclear whether the UPC2 gene could regulate the expression of ERG11 in *Candida tropicalis*.

In this study, the clinical characteristics and drug resistance of *Candida tropicalis* infection were explored. Moreover, the relationship between the ERG11 and UPC2 expression and the resistance to fluconazole was investigated, trying to provide the basis for the disease diagnosis and treatment in clinic.

Materials And Methods

Strain sources

The samples obtained from each clinical department of the First Affiliated Hospital of Chengdu Medical College from January 2016 to December 2019 were sent to the laboratory, and the fungi were isolated and identified based on the conventional methods. For the repeated strains obtained from the same patient at the same site, only one strain was counted.

Fungal culture and preliminary identification

The strains were inoculated onto and cultured with the Sao Paulo medium (Antu Biotechnology Co., Ltd., Zhengzhou, Henan, China). The suspicious fungi isolated were smeared and subjected to the Gram staining. When the spores or mycelium were observed, the strain was transfected into the Chromagar color development medium (Antu Biotechnology Co., Ltd.), and cultured at 35°C for 48 h. The fungi species were initially determined according to the different colors of the colonies.

Fungal identification and drug susceptibility test

The identification and susceptibility testing of the isolated fungi were performed with the ATB Expression type Bio-Mérieux microorganism identification and drug sensitivity analyzer (Bio-Mérieux Co., Ltd., Lyon, France), the yeast identification kit (colorimetric method) (Bio-Mérieux Co., Ltd.) and the yeast-like fungal susceptibility kit (micro-dilution method) (Bio-Mérieux Co., Ltd.), according to the American clinical laboratory CLSI M60 standard recommended by the Committee for Standardization (CLSI) [10]**. For the fluconazole, the sensitivity referred to the minimum inhibitory concentration (MIC) ≤ 2 μg/ml, and the drug resistance** ≥ 8 μg/ml. **The quality control strain for the identification and drug-sensitivity test was the *Candida parapsilosis* (ATCC22019).**

Quantitative real-time PCR

Totally 50 strains of *Candida tropicalis* (20 fluconazole-susceptible and 30 fluconazole-resistant strains) were collected. The total RNA was extracted with the RNA extraction kit (Sangon Biotechnology Co., Ltd., Shanghai, China). The cDNA template was obtained from the total RNA with the HiScript II Q RT SuperMix reverse transcription kit (Vazyme Biotechnology Co., Ltd., Nanjing, Jiangsu, China). Quantitative real-time PCR was performed with the ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotechnology Co., Ltd.,) on the CFX96 real-time quantitative PCR instrument (BIO-RAD, California, USA) . Primers were synthesized by the Sangon Biotech, with the following sequences: ERG11, forward 5′-TGCCTGGTTCTTGTTGCATTT-3′ and reverse 5′-AATCGTTCAAGTCACCACCCT-3′; UPC2, forward 5′-GAGTGGAACAACAACACAACAA-3′ and reverse 5′-TAAATCCCCTAAACCTGAAAGA-3′; and ACT1, forward 5′-TTTACGCTGGTTTCTCCTTGCC-3′ and reverse 5′-GCAGCTTCCAAACCTAAATCGG-3′. The reaction conditions were as follows: 95°C for 30 s; 95°C for 10 s, and 60°C for 30 s, for totally 40 times. The real-time quantitative PCR quality control strain was *Candida tropicalis* (ATCC750), and the ACT1 was used as internal reference[11].

Statistical analysis

The clinical distribution and drug sensitivity results of fungi were analyzed with the WHONET 5.6 software. The clinical distribution characteristics were analyzed by the χ^2 test using the SPSS 24.0 software. The distribution characteristics between four years were analyzed with the χ^2 test, and the Cochran Q test was performed for the distribution characteristics of each year. The paired sample *t*-test was performed for the analysis of the ERG11 or UPC2 mRNA expression levels between the drug sensitive and resistant groups, and the Spearman correlation analysis was also performed. $P < 0.05$ was considered as statistically significant.

Results

Isolation of Candida

The strain distribution of various types of *Candida* detected in the hospital from 2016 to 2019 was analyzed. As shown in Fig. 1, totally 2872 strains of *Candida* were detected. After the Cochran Q test, the composition ratio of various *Candida* species in each year showed statistical significance ($Q = 1101.094–1904.945$; all $P < 0.001$). For each year, *Candida albicans* was the major strain, accounting for more than 60% (up to 71.96% in 2017), followed by *Candida glabrata*, accounting for about 15%, and *Candida tropicalis* ranked the third, with no significant change in the proportion between 2016, 2018 and 2019 (all around 13%, which fell to 7.04% in 2017). In addition, the comparison of the composition ratio of *Candida* over these four years also had statistical differences ($\chi^2 = 33.344$; $P = 0.004$). However, the composition ratio of each kind of *Candida* was compared between these four years. Our results showed statistical significant only for *Candida albicans* ($\chi^2 = 12.620$; $P = 0.006$) and *Candida tropicalis* ($\chi^2 = 20.410$; $P <$

0.001), and the proportion of *Candida albicans* exhibited a gradual decrease (χ^2 = 4.558; P = 0.033) over these years. However, there were no differences in the detection over these years for *Candida glabrata*, *Candida tropicalis*, and *Candida krusei* (Additional file 1:Fig. S1).

Sources of Candida tropicalis samples

The *Candida tropicalis* samples detected over 2016–2019 were classified according to the sample sources, and the composition ratios were also analyzed. Our results showed that significant differences were observed in the proportion of samples from which *Candida tropicalis* was detected in each year (Q = 123.949–194.898; P < 0.001) (Fig. 2). Moreover, for each year, the major sample source was the airway secretion (more than 65%), followed by the mid-range urine (12%-25%). Although there was no statistical difference in the composition ratio of the source for *Candida tropicalis* samples for these four years (χ^2 = 14.858; P = 0.399), the proportion of urine samples detected with *Candida tropicalis* was statistically different between these years (χ^2 = 9.387; P = 0.025). Furthermore, our results from the trend test showed that, except for the proportion in the stool samples (χ^2 = 3.550; P = 0.060), the proportions in the airway secretions and urine samples were increased over the years, which were decreased in the remaining samples year by year (all P < 0.050) (Additional file 2:Fig. S2).

Department distribution of Candida tropicalis

The department sources of *Candida tropicalis* in 2016–2019 were analyzed. Our results showed that, from 2016 to 2019, significant differences were observed in the distribution of *Candida tropicalis* in these departments (Q = 40.746–92.691; all P < 0.001). The departments with relatively high detection rates included the Departments of Respiratory Medicine, ICU, and Geriatrics (Fig. 3). Moreover, among the proportions of *Candida tropicalis* detected in different departments, the detection rates of *Candida tropicalis* in the Department of Respiratory Medicine were more than 20% in 2016, 2017 and 2019, which fell to 14.82% in 2018. Furthermore, the amount of *Candida tropicalis* detected in the ICU had been increasing year by year, from 8.16% in 2016 to 37.03% in 2018, which was decreased to 12.35% in 2019. The proportions of *Candida tropicalis* detected in the Department of Geriatrics were higher in 2016 (18.37%) and 2019 (23.46%), while lower in 2017 and 2018 (about 9%). The composition ratio of *Candida tropicalis* detected in different departments between four years suggested statistically significant differences (χ^2 = 58.045; P = 0.002). However, for the comparison of the *Candida tropicalis* amount detected in each department between four years, statistically significant differences were observed in the amount of *Candida tropicalis* in the Department of Geriatrics (χ^2 = 8.623; P = 0.035) and ICU (χ^2 = 27.148; P < 0.001) (Additional file 3:Fig. S3).

Resistance of Candida tropicalis to antifungal drugs

The resistance of *Candida tropicalis* to azoles such as fluconazole, itraconazole and voriconazole, from 2016 to 2019, was analyzed. Our results showed that *Candida tropicalis* had high resistance rates to fluconazole, itraconazole, and voriconazole, with even the cross-resistance phenomenon. In 2018, the resistance rate of *Candida tropicalis* to fluconazole was up to 39.2%. There was no statistically

significant differences in the resistance rates of *Candida tropicalis* to azole antifungal drugs over these four years (χ^2 = 1.156; P = 0.979).

Relative mRNA expression levels of ERG11 and UPC2 in Candida tropicalis

In order to further study the resistance-related genes of *Candida tropicalis*, the mRNA expression levels of ERG11 and UPC2 were detected with the quantitative real-time PCR. Our results showed that the data of the relative mRNA expression levels of ERG11 and UPC2 genes in the *Candida tropicalis* from the fluconazole-resistant groups (27/30 strains fully resistant to fluconazole, itraconazole, and voriconazole) and fluconazole-sensitive groups were in the normal distribution. Based on the paired sample *t*-test, the relative mRNA expression levels of ERG11 in the drug-resistant group was 1.579 ± 0.896, while the relative mRNA expression of ERG11 in the sensitive group was 0.483 ± 0.259, with statistical significance (t = 4.511; P < 0.001) (Fig. 4A). On the other hand, the relative mRNA expression levels of UPC2 in the resistant group was 1.400 ± 0.919, while the relative mRNA expression levels of UPC2 in the sensitive group was 0.448 ± 0.272, with statistically significant difference (t = 3.970; P < 0.001) (Fig. 4B). These results suggest that the resistance of *Candida tropicalis* to fluconazole is related to the expression levels of ERG11 and UPC2.

Correlation analysis of UPC2 and ERG11 mRNA expression in Candida tropicalis

The mRNA expression levels of resistance-related genes (i.e., the ERG11 and UPC2) in *Candida tropicalis* were detected, and the correlation between the gene expression levels was further analyzed. Our results from the Spearman correlation analysis showed that there was no liner correlation between the expression levels of UPC2 and ERG11 in the sensitive group (r = − 0.074; P = 0.757) (Fig. 5A). However, the UPC2 and ERG11 expression levels were positively correlated in the drug resistance group (r = 0.571; P = 0.001) (Fig. 5B). These results suggest that the over-expression of fluconazole-resistant ERG11 in *Candida tropicalis* may be related to the regulation of UPC2.

Discussion

Candida tropicalis is a kind of common conditional pathogenic fungus, with increasing detection rates over the past years. In this study, totally 2872 strains of *Candida* were detected from the samples collected from 2016 to 2019, in which *Candida albicans* accounted for about 70%, while *Candida tropicalis* ranked the third, accounting for 12%. Moreover, the overall composition rates of *Candida* showed statistically significant differences between the detection years. The analysis of the detection rate and drug sensitivity of *Candida tropicalis* in our hospital showed that the resistance rate of *Candida tropicalis* to azoles had been sharply increased. In this study, based on the increasing isolation rate and drug resistance rate of *Candida tropicalis*, the clinical distribution characteristics and drug resistance of *Candida tropicalis* were analyzed, as well as the relationship between the ERG11/UPC2 gene expression and the resistance to azoles.

Our results showed that there was no statistical difference in the overall composition rate of *Candida tropicalis* samples tested over these four years. Moreover, the main detection source was the airway secretion, which might be related to the fungus being one of the upper respiratory tract-colonized fungus, and *Candida tropicalis* could form biofilms on the invasive catheters (such as the tracheal intubation) to resist disinfection [12]. *Candida tropicalis* with reproductive growth would often be detected in the airway secretions. Therefore, it is necessary to rule out the normal colonization bacteria or device contamination during diagnosis. Moreover, comprehensive prognosis should involve the patients' clinical manifestations and other test results. In addition, *Candida tropicalis* was detected in the mid-range urine samples, with a detection rate of 13%-25%, which was increasing over the years. This phenomenon might be related to the increasing number of the patients treated for the invasive urinary tract operation and the subsequently increasing mid-range urine samples. *Candida tropicalis* has strong adhesion, penetration and destructive abilities to the mucous membranes, with strong resistance to antifungal drugs, which are stronger than *Candida albicans* [13]. When the patients receive the invasive urinary tract operation, the urinary tract would communicate with the outside, and the body could not effectively kill the fungi. These conditions would provide convenient environment for the reproduction and growth of *Candida tropicalis*, eventually leading to *Candida* infection.

The IDSA Invasive Candidiasis Clinical Practice Guidelines (2016) has pointed out that the high-risk factors for *Candida* infection include staying in the ICU for more than 3 days, severe disease and broad-spectrum antibiotic use for more than 3 days[14]. The information suggests that, when staying in different clinical departments, the infection rates of *Candida* may be different. In this study, our results showed that, from 2016 to 2019, the departments with relatively high detection rates of *Candida tropicalis* in the hospital included the Department of Respiratory Medicine and ICU, mainly from the airway secretion samples. There were significant differences in the overall composition rates of *Candida tropicalis* between these clinical departments and between these detection years. The detection inconsistencies might be related to the high-risk factors of *Candida* infection and the disease type of patients in these departments. There were many patients with basic respiratory diseases in the Department of Respiratory Medicine, and therefore the detection rate of *Candida tropicalis* was high, which had been decreasing over these years. The results may be related to the correct collection of the airway secretion samples and standardized submission for test in the department. In addition, the detection rate of *Candida tropicalis* in the ICU was high, which had been increasing year by year. These results might be because of the increasing number of critical and complicated patients, and the subsequent repeated application of high-efficiency broad-spectrum antibacterial drugs [15]. Moreover, in the ICU, invasive procedures would be often performed, such as the tracheal intubation, tracheotomy and/or intubation [16], which might easily cause infection by conditional pathogenic fungi.

In this study, our results showed that there were no significant differences in the resistance rate of *Candida tropicalis* to azole antifungals between these four years, and the resistance rate had been increasing year by year (with the resistance rate for fluconazole as high as 39.2%). The increased resistance rate of *Candida tropicalis* to azoles might be related to the easy application of such drugs and the relatively mild adverse reactions[17]. In clinic, a large number of patients with severe fungal infections and long-term application of these drugs were subjected to the prophylactic treatments, which would lead

to the drug resistance [18]. Moreover, in this study, the ERG11 and UPC2 genes of 50 strains of *Candida tropicalis* were detected, and our results showed that the relative expression level of ERG11 gene in the drug-resistant group was significantly higher than the sensitive group, in line with the findings from Jiang *et al*. [19] concerning the high expression of ERG11 in fluconazole-resistant fungi. The ERG11 over-expression could increase the amount of 14-DM in cells, which ensures the ergosterol synthesis and the normal growth and reproduction of *Candida*, therefore leading to azole drug resistance [20, 21]. Moreover, Jiang *et al*. [19] have cloned the Y132F and S154F mutations of *Candida tropicalis* ERG11 into *Saccharomyces cerevisiae* and shown that the sensitivity of *Saccharomyces cerevisiae* to azole drugs, especially fluconazole, has been decreased. These results suggest that Y132F and S154F are involved in resistance of *Candida tropicalis* to fluconazole. Moreover, our results showed that, the expression level of UPC2 in the resistant group was also higher than the sensitive group, indicating that the over-expression of UPC2 may cause *Candida tropicalis* to become resistant to azoles, which was consistent with the findings from Jiang *et al*. [22]. In this study, the correlation analysis of the ERG11 and UPC2 mRNA expression levels in *Candida tropicalis* showed that there was a linear positive correlation between the UPC2 and ERG11 genes in the drug-resistant group, while no significant linear correlation was found in the sensitive group. These results indicated that the over-expression of UPC2 in the fluconazole-resistant *Candida tropicalis* was likely to induce the over-expression of ERG11. In clinically isolated *Candida albicans*, the functional mutations in UPC2 have been shown to cause the ERG11 over-expression and lead to the fluconazole resistance [23, 24]. Choi *et al*. [25] have sequenced the UPC2 gene in *Candida tropicalis*, and their results have shown that the amino acid substitutions caused by the mutant gene appeared not only in the resistance group over-expressing ERG11, but also in the sensitive group with no ERG11 over-expression. In this study, our results showed that the over-expressions of ERG11 and UPC2 in the *Candida tropicalis* were related to the fluconazole resistance, with a linear relationship. The drug resistance might be related to multiple factors, and in a few drug-resistant bacteria without ERG11 and UPC2 over-expression, the mechanisms underlying the drug resistance might be related to efflux pumps [26] and biofilm formation [27]. To fully understand the drug resistance mechanisms of *Candida tropicalis*, it is still necessary to comprehensively study the impacts of the mechanism on drug sensitivity. Based on these findings, further in-depth studies are still needed to investigate the transcriptional regulatory function of Upc2p in the drug-resistant fungi, and to explore how the UPC2 over-expression regulates ERG11, thus leading to the drug resistance to azoles.

Conclusions

In conclusion, *Candida tropicalis* has become the main pathogen of non-*Candida albicans* infection, and the drug resistance rate has been gradually increased. It can often cause infections in patients with low immunity, basic diseases, invasive procedures, and long-term and large-dosage application of broad-spectrum antibiotics. Our results showed that the fluconazole resistance of *Candida tropicalis* with ERG11 over-expression may be related to the regulation of the zinc family transcription factor Upc2p. Therefore, when applying and selecting the antifungal drugs, in addition to the drug sensitivity findings, clinicians should fully understand the clinical distribution, the formation of drug resistance, and the over-

expression of ERG11 and UPC2 genes. Routine detection of ERG11 and UPC2 for the high-risk patients in clinic, would contribute to the disease early diagnosis and timely treatment, to delay and prevent the development of resistance of *Candida tropicalis*.

Declarations

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Author's contributions

DW and NA conducted experiments,participated in data analysis,and drafted the initial manuscript.YY,XY and YF collected information and performed **statistical analysis.JF designed the study and critically revised the manuscript.**All authors readed and approved the final manuscript.

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Availability of data and materials

The data analysed in this study are included in the manuscript.

Ethics approval and consent to participate

This was a retrospective study in which did not contain experiments using animals and human studies.The anonymised patient data and strains were collected and used during routine clinical practice,with patient consent.The research was approved by the Ethics Committee of the First Affiliated Hospital of Chengdu Medical College, and it was performed in accordance with the approved guidelines. Written informed consent were obtained from all participants.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

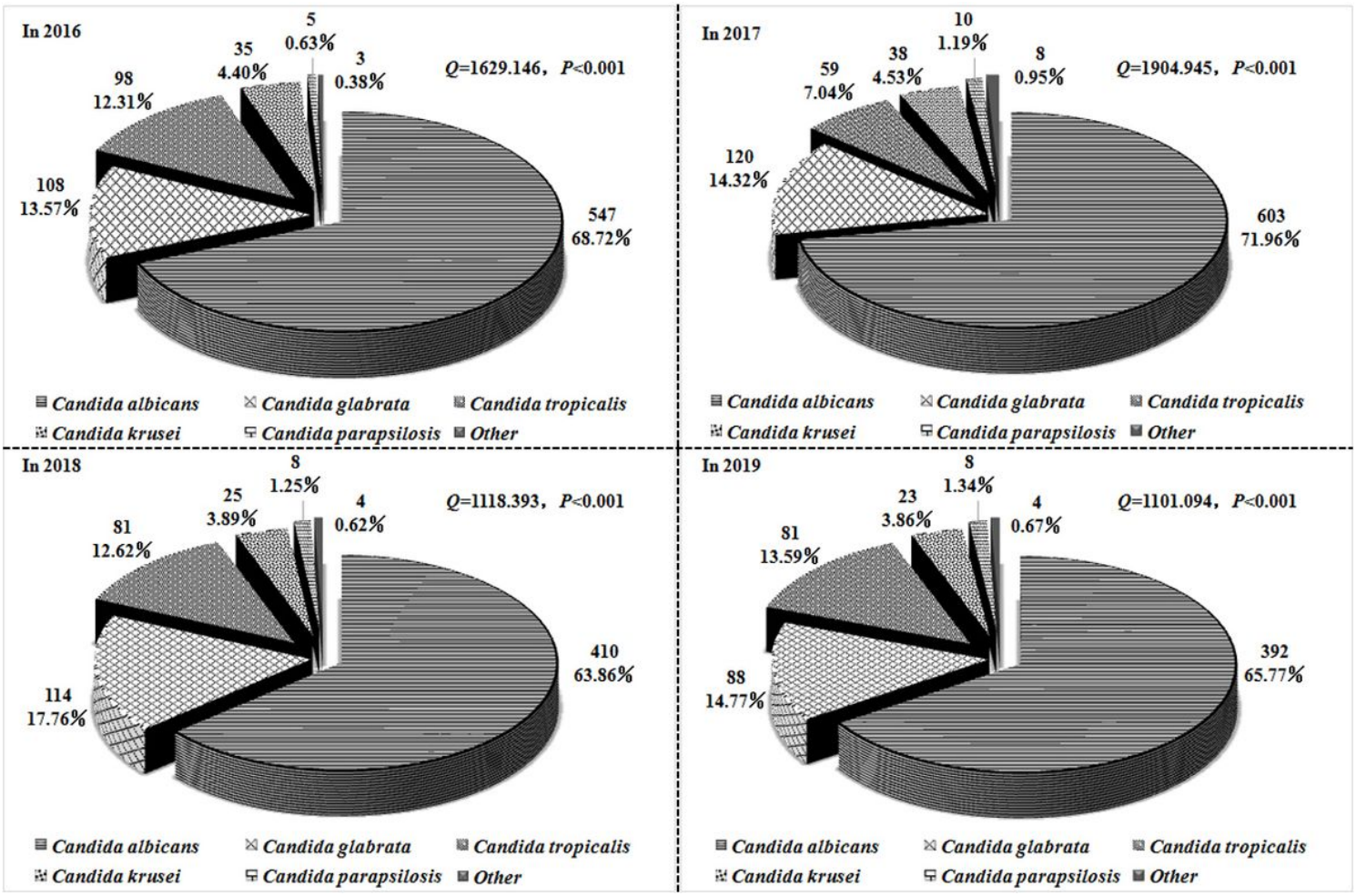

Figure 1

Composition of Candida species detected from 2016 to 2019. The composition rates (cases and percentage) of Candida detected in 2016, 2017, 2018, and 2019.

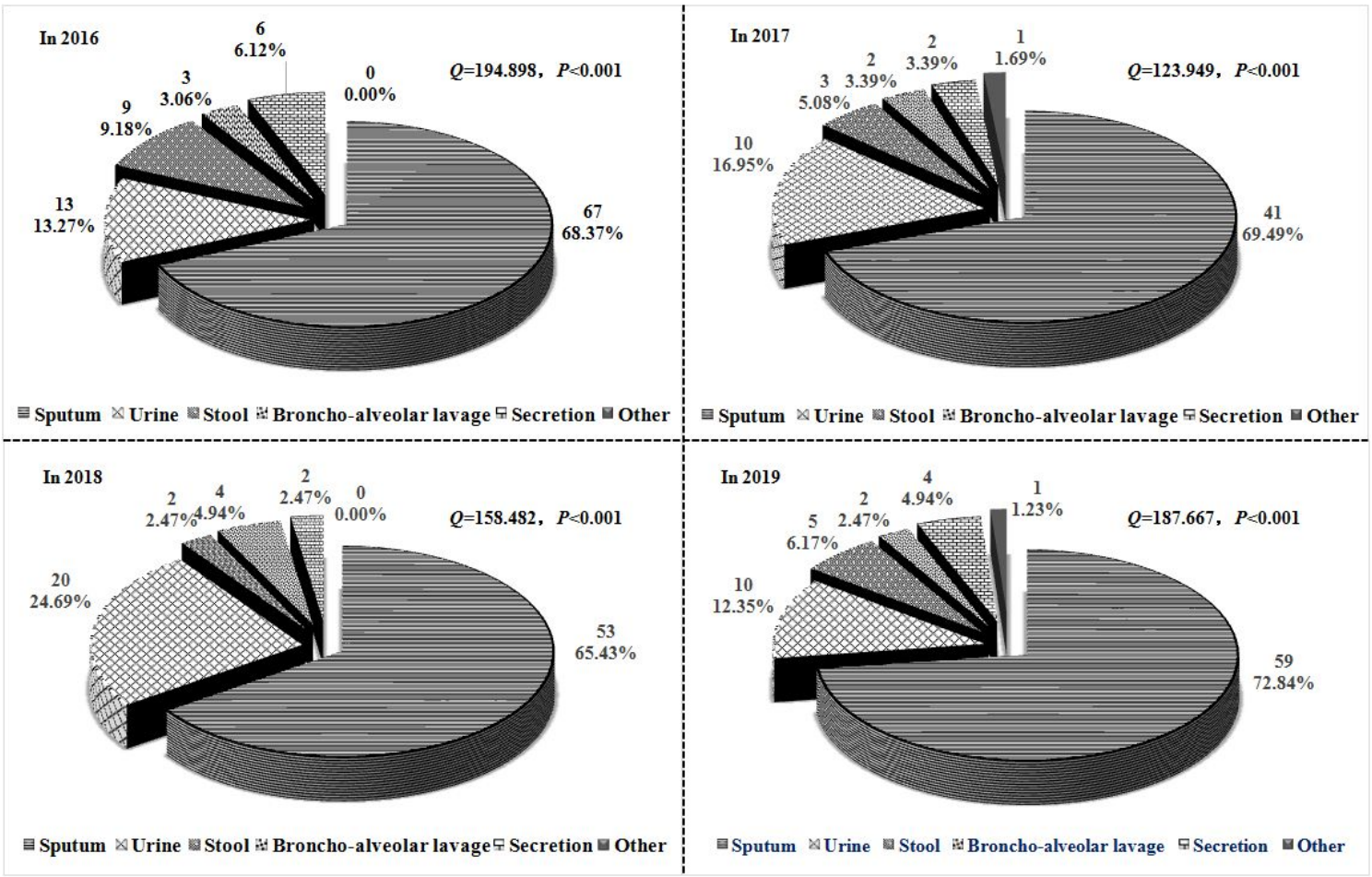

Figure 2

Sample composition of Candida tropicalis from 2016 to 2019. The sample sources (cases and percentage) of Candida tropicalis detected in 2016, 2017, 2018, and 2019.

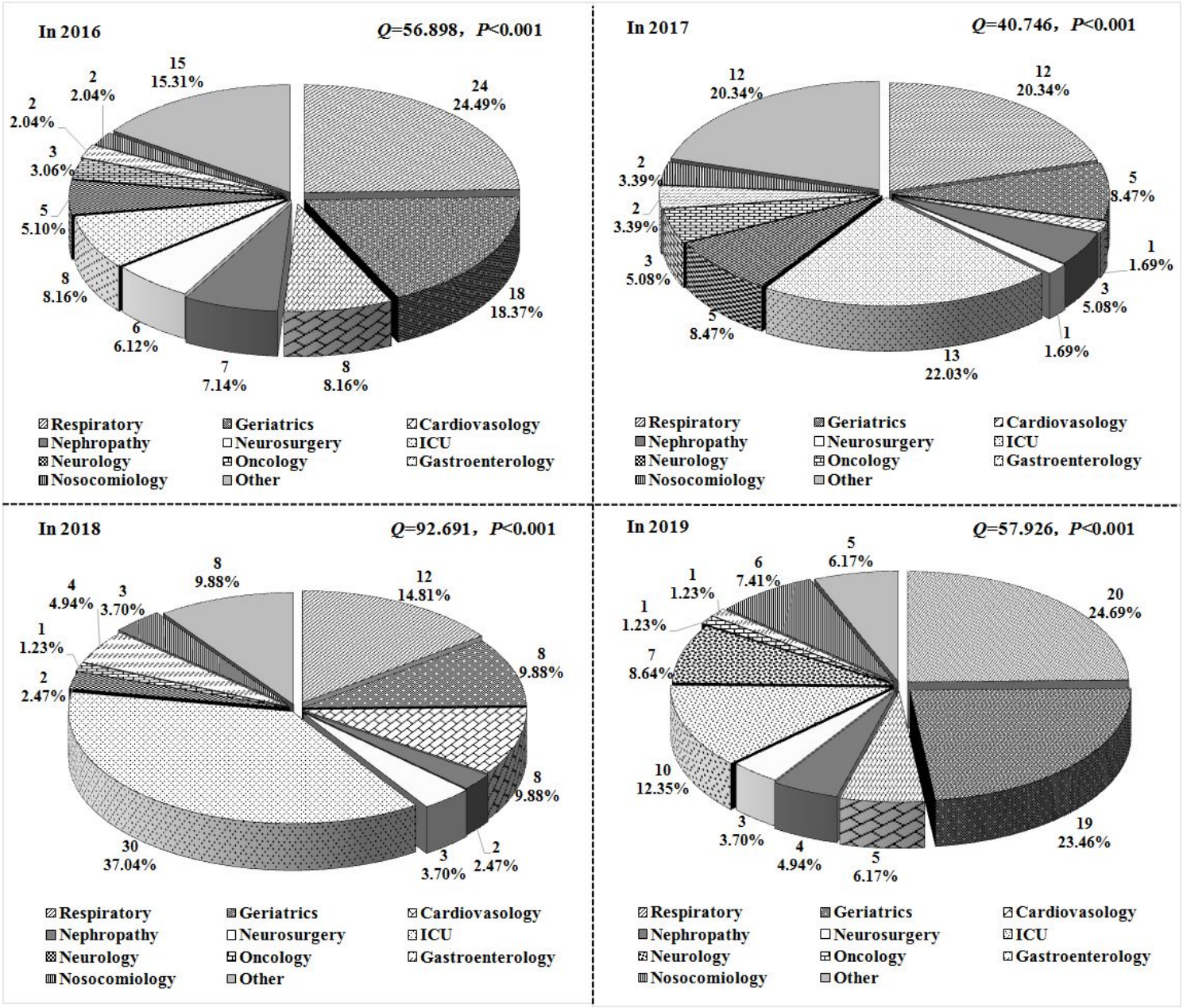

Figure 3

Distribution of Candida tropicalis in different departments from 2016 to 2019. The distribution of Candida tropicalis in different departments (cases and percentage) in 2016, 2017, 2018, and 2019.

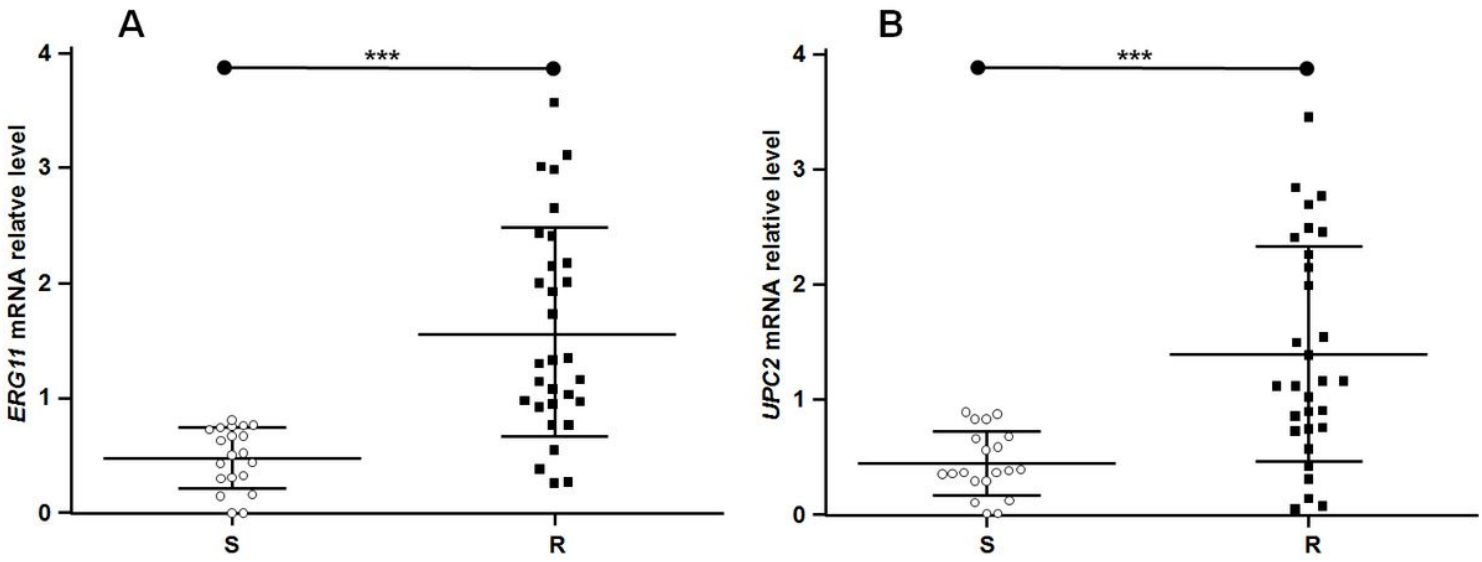

Figure 4

ERG11 and UPC2 expression levels in Candida tropicalis. (A-B) Relative mRNA expression levels of ERG11 (A) and UPC2 (B) were analyzed and compared between the sensitive group (20 strains) and the resistant group (30 strains). P < 0.001. S, the fluconazole-sensitive group of Candida tropicalis; and R, the fluconazole-resistant group of Candida tropicalis.

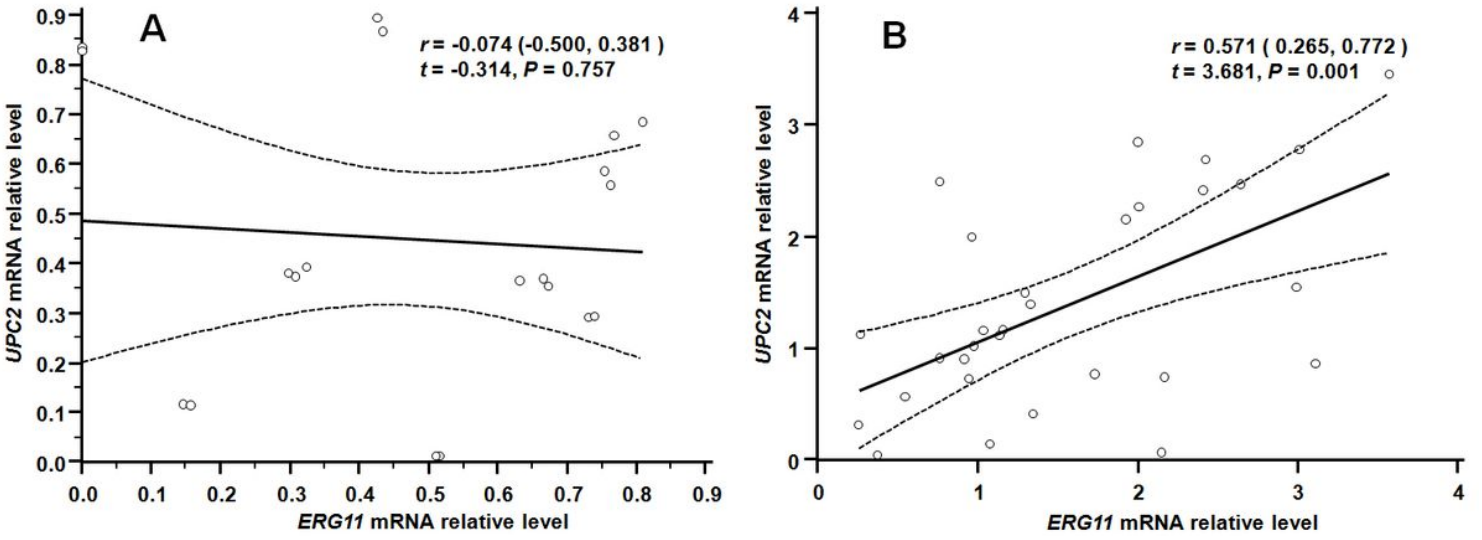

Figure 5

Correlation analysis between UPC2 and ERG11 expression levels. (A-B) The correlation between the UPC2 and ERG11 expression levels in the fluconazole-sensitive (A) and -resistant (B) groups of Candida tropicalis. P < 0.05.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigS3.jpg
- FigS2.jpg
- FigS1.jpg