

Molecular Identification and Antifungal Susceptibility Profiles of Non-*albicans* *Candida* Species Clinical Isolates

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Abstract

Background: There is an increasing incidence of life-threatening systemic mycoses, specifically fulminant infections by the *Candida* species in hospitalised patients and in those who are immunocompromised. Management of the limited number of antifungal drugs currently available requires the identification of infections containing drug-resistant isolates.

Objectives: The aim of this study was to identify the non-*albicans* *Candida* species as azole-resistant fungi, isolated from sputum and bronchoalveolar lavage specimens of hospitalised cases.

Methods: The subjects included hospital-acquired infection (HAI) cases, with a primary diagnosis using a direct microscopic examination, performed for the detection of probable fungi. The molecular tests of PCR-restriction fragment length polymorphism (RFLP) and real-time PCR were performed to confirm the identity and molecular typing of the *Candida* isolates. Antifungal susceptibility testing (AFST), by the Clinical and Laboratory Standards Institute (CLSI) broth microdilution (BMD) minimum inhibitory concentration (MIC) (M27-A2) method, was performed on the hospital-isolated *Candida* species.

Results: During 24 months, from August 2014 to September 2016, a total of 198 samples were obtained from cases with proven HAI. The results of experimental studies on the specimens showed 93 (47%) positive cases for a fungal or bacterial infection, of which 54 (58%) had a fungal infection. It was hypothesised that all of the isolated organisms were causative agents of the HAI.

Conclusions: The results showed that the medium CHROMagar™ *Candida* is an accessible and easy-to-use method for the identification of infection, but not as accurate and reliable as PCR-RFLP and real-time PCR methods. Results also showed decreasing susceptibility to azoles (itraconazole in this study) of the *Candida* species.

BACKGROUND

The *Candida* species is the cause of several infections, including bloodstream infections and disseminated candidiasis. Despite recent advancements in the diagnosis and treatment of candidiasis, *Candida* is the fourth leading cause of bloodstream infection pathogens in the USA and seventh in Europe.¹ Life-threatening systemic fungal infections have increased amongst immunocompromised patients, particularly fulminant infections caused by *Candida* spp. Patients immunocompromised as a result of HIV infection, allogenic haematologic stem cell transplantation, chemotherapy for acute leukaemia, or infant prematurity, are at risk of *Candida* infections.

Although *Candida albicans* is known as the most common cause of invasive candidiasis, there has been an increase in non-*albicans* infections, such as *C. krusei*, *C. glabrata*, *C. lusitaniae*, *C. tropicalis*, and *C. parapsilosis*. Some non-*albicans* *Candida* species, including *C. auris*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, and *C. lusitaniae*, are classified as notable pathogenic fungi that cause invasive candidiasis, mainly because of their innate resistance to antifungal drugs.²⁻⁶ Treating invasive fungal infections has been difficult because of the limited availability of antifungals, as well as the relative toxicity, drug interaction, and drug-resistance challenges.⁷ Systemic and invasive candidiasis can be treated with the limited number of available antifungal medications, such as polyenes, allylamines, azoles, and the newly produced echinocandin class of molecules.¹ Adverse side effects, toxicity, and drug resistance in *Candida* species have been observed worldwide. Studies on the prevalence of *Candida* infections and antifungal susceptibility testing (AFST) can help to make decisions on clinical strategies. Studying the incidence of *Candida* infections, as well as AFST, is helpful in terms of planning clinical measures. Rapid identification of *Candida* spp. is also beneficial as early management of antifungal treatment can take place.¹ Additionally, management of the limited number of antifungals currently available requires the identification of infections containing drug-resistant isolates, and the discovery of factors that promote the evolution of drug resistance.⁴

Objectives

The aim of this study was to identify the non-*albicans* *Candida* species from azole-resistant fungi, isolated from the sputum and bronchoalveolar lavage specimens of hospitalised cases. The performance of three rapid methods of identification at the species level was evaluated, and two susceptibility tests for the *C. albicans* clinical isolates were used.

METHODS

Subjects

Respiratory tract specimens were taken from patients with hospital-acquired infection (HAI) and infection symptoms occurring in the first 48 hours after arriving at the UMS University educational hospitals, in Urmia, Iran. Additionally, other clinical samples including pressure ulcers, skin abscess, vaginal discharge, and nail scrape were obtained from patients during hospitalisation.

Culture and Identifications

The collected specimens were transported to the Medical Mycology Center, School of Medicine, UMS University. The primary diagnosis included direct microscopic examination (potassium hydroxide preparation [KOH]: 10%; Giemsa: 20% stained slides), which was performed for the detection of the possible fungi causing the HAI. This was followed by growing the cultures on sabouraud dextrose agar ([SDA]: 4%), Czapek yeast extract agar (CYA), corn meal agar, and CHROMagar™ *Candida*, for the identification of yeast isolates.⁸

Molecular Identification of *Candida* Species

First, DNA extraction was performed: molecular tests (PCR-restriction fragment length polymorphism [RFLP] and real-time PCR) were performed to confirm the identity and molecular typing of *Candida* isolates. DNA extraction of *Candida* cells was performed using phenol-chloroform and the glass beads manual method (making a lysis solution with 10 mM ethylenediaminetetraacetic acid, 1% sodium dodecyl sulfate, 100 mM sodium chloride, 100 mM Tris-HCl, and 1% TritonX-100 [Sigma-Aldrich, St Louis, Missouri, USA]).⁹

There are two important non-coding regions, internal transcribed spacers (ITS) 1 and 2, containing a variable subregion inside and a conservative subregion outside. The ITS fragments are located between the 18s and 28s rRNA genes of the *Candida* species. The variability of the ITS regions have proved useful in phylogenetic studies of the *Candida* species. The target gene rRNA was amplified using ITS primers (ITS1: 5'-TCC GTA GGT GAA CCT GCG G-3'; ITS2: 5'-TCC TCC GCT TAT TGA TAT GC-3'). The PCR profile included: 5 µl of the DNA template; PCR buffer (20 mM Tris-HCl; pH 8.0); 50 mM potassium chloride; 0.1 mM each of the forward and reverse primers; and 1.5 U of Taq DNA polymerase in a total reaction volume of 50 µl. The reactions were performed in a Thermocycler model XL (Bioer Technology, Hangzhou, China). The PCR-RFLP, using the restriction enzyme MspI, was performed to create a differential pattern to identify the *Candida* species (Table 1; Figure 1).¹⁰ A real-time PCR for the confirmation of each RFLP-identified *Candida* isolate was carried out at the Molecular Research Center, EUMS, Esfahan, Iran.

Susceptibility Test

AFST, by the Clinical and Laboratory Standards Institute (CLSI) broth microdilution (BMD) minimum inhibitory concentration (MIC) (M27-A2) method, was performed on hospital-isolated *Candida* species. The medium for testing antifungal agent constituted synthetic complete medium and RPMI-1640 (Sigma-Aldrich), supplemented with glutamine but without bicarbonate, as recommended. Supplementing medium to a final concentration of 20 g/L (2%) glucose has been shown to result in better growth of yeast isolates, without markedly altering the MIC of antifungal agents.¹⁰ Preparation of the stock drug solution included the antifungal drug itraconazole (Taj Pharmaceuticals Ltd., Birmingham, UK), prepared by weighing 16 mg on an analytical balance calibrated to 2 decimal places, as it was recommended that >16 mg (32 mg) of powder was used. Antifungal drug stock solution was prepared to a concentration 100 times the highest concentration to be tested (320 mg/mL).

Table 1: Clinical specimens collected from patients with *Candida* colonisation.

Clinical specimen	Number	Percentage (%)
Bronchoalveolar lavage	12	60
Vaginal discharge	5	25
Nail scrape	2	10
Sputum	1	5
Total	20	100

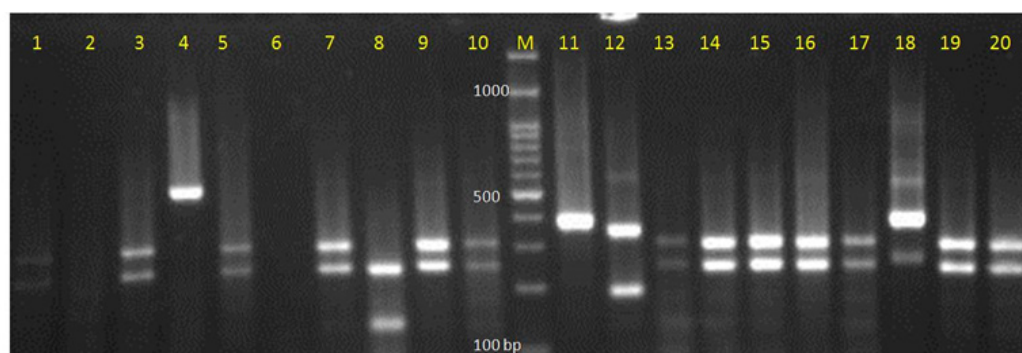


Figure 1: Electrophoretic picture of digestion with restriction enzyme MspI in the PCR-restriction fragment length polymorphism method.

Lanes: 1, 3, 5, 7, 9, 10, 13, 14, 15, 16, 19, 20 show *C. albicans*; lane 11: *C. parapsilosis*; lane 12: *C. glabrata*; lane 8: *C. tropicalis*; lane 4: undigested PCR product; and lane M: 100 base pair DNA marker.

Dimethyl sulfoxide (DMSO) was suitable for dissolving itraconazole, as well as other antifungal drugs (ketoconazole and flucytosine). The drug's stock solutions were used, and the DMSO was prepared and sterilised beforehand.

For the yeast cell suspension, a 24-hour culture was prepared on 4% SDA and adjusted by a spectrophotometry system (wavelength: 530 nm; optical density: 75–77), ensuring that the yeast cell suspensions had equal concentration to the 0.5 McFarland standard.

Treatment

MIC testing of itraconazole was performed as per the procedure in the CLSI guidelines M27-A2,^{9–20} using RPMI-1640 broth, an inoculum of $0.5\text{--}2.5 \times 10^3$ colony-forming units (cfu)/mL, incubation at 35°C, and MIC endpoint criteria of prominent inhibition of growth.¹⁰ A serial dilution of the drug solution was made adding 100 µl of free bicarbonate RPMI-1640 into the first well (each lane of a 96-well plate), followed by a reduction in concentration (16.0000 to 0.0313 µg/mL). For the treatment, 100 µl of 1,000 times diluted yeast cells suspension was added to 10 wells, plus a negative control (without fungal cells) and a positive control (fungal cells only). After incubating for 24–48 hours at 35°C, turbidities of fungal growth were detected on a light screen. The MIC for the azole family were described as the lowest concentration of the drug that could reduce 50% of fungal growth. MIC of itraconazole for *Candida* cells were obtained by observing the first well without any growth.

RESULTS

Throughout the 24 months between August 2014 and September 2016, 198 clinical specimens were tested for fungal isolation. Bronchoalveolar lavage and sputum were the most frequent specimens with *Candida* isolation (as pathogens or colonising) (Table 1). *Candida* species, dominantly *C. albicans*, were obtained from 20/198 clinical specimens used in this study. Clinical cases were in the age range of 1–78 years. The identification results showed 93 (47.0%) patients with fungal or bacterial colonisation, and 25 (26.9%) had a fungal infection. It is thought that all of the isolated organisms were causing agents of infection in the tested cases. Among all isolated *Candida*

species, *C. albicans*, *C. dubliniensis*, *C. glabrata*, and *C. tropicalis* were confirmed. These findings are based on the PCR-RFLP results confirmed by real-time PCR (Table 2). With the exception of *C. dubliniensis*, the results of molecular identifications were approximately similar in the two molecular methods. *C. dubliniensis* isolates in this study were identified by CHROMagar *Candida* and confirmed by real-time PCR. Additionally, a drug-resistant *Candida* isolate was identified as *C. parapsilosis* by PCR-RFLP and real-time PCR. Susceptibility testing (MIC) of *Candida* isolates results showed that 48% were resistant and 52% were sensitive to itraconazole. MIC results showed that tested *C. parapsilosis* were resistant against itraconazole (MIC: 0.125–0.250 µg/mL). Moreover, of the 23 tested samples of *C. albicans*/*C. dubliniensis*, 11 (47.8%) were resistant (MIC range: 0.125–0.250 µg/mL). Only one case of *C. glabrata* was sensitive to itraconazole with an MIC range of ≤ 0.125 µg/mL.

DISCUSSION

The prevalence of systemic *Candida* infections, for which hospital care is needed, has recently elevated significantly for several reasons. Patients at risk of systemic fungal infections (including candidiasis), such as those with a serious underlying disease, immunosuppression caused by cytotoxic/anti-rejection chemotherapy, corticosteroid therapy, and long-term antibiotic therapy, are on the increase.¹¹ Furthermore, fungal infections are recognised as a serious concern amongst the elderly population, as age is a risk factor because of its effect on mortality rates.^{12,13} Presence of *Candida* colonisation on the skin surface and mucosa is a potential source of systemic infection, and the hands of medical personnel have been evidenced to play an important role in nosocomial infections with transmission from healthcare workers to patients in hospitals.¹⁴ Moreover, transmission from common sources such as contaminated intravenous fluids, hospital food, and medical equipment has been shown.^{14,15}

In the present study, most of the subjects were admitted to specialist medical wards, including nephrology and pulmonology. A significant number of the patients hospitalised were immunosuppressed after a kidney transplant, an important risk factor for *Candida* colonisation and infection.

Table 2: Molecular identifications and minimum inhibitory concentration of itraconazole on clinical *Candida* isolates.**

Number	Strain number	Age/sex	Source	CHROM agar <i>Candida</i>	PCR-RFLP	Real-time	MIC 24	MIC 48
1	KDY1072	35/F	BAL	<i>C. glabrata</i>	<i>C. albicans</i>	<i>C. albicans</i>	S	S
2	KDY1155	48/M	BAL	<i>C. albicans</i>	<i>C. glabrata</i>	<i>C. glabrata</i>	S	S
3	KDY1237	36/M	BAL	<i>C. dubliniensis</i>	<i>C. albicans</i>	<i>C. dubliniensis</i>	4	R
4	KDY1271	43/F	Nail scrape	<i>C. albicans</i>	<i>C. albicans</i>	<i>C. albicans</i>	S	S
5	KDY1244	60/F	Vaginal discharge	<i>C. albicans</i>	<i>C. albicans</i>	<i>C. albicans</i>	S	S
6	KDY1108	55/M	BAL	<i>C. albicans</i>	<i>C. albicans</i>	<i>C. albicans</i>	8	R
7	KDY1239	28/F	Vaginal discharge	<i>C. albicans</i>	<i>C. albicans</i>	<i>C. albicans</i>	R	R
8	KDY1253	26/M	BAL	<i>C. albicans</i>	<i>C. albicans</i>	<i>C. dubliniensis</i>	R	R
9	KDY1210	59/M	BAL	<i>C. albicans</i>	<i>C. albicans</i>	<i>C. albicans</i>	S	S
10	KDY1201	66/F	BAL	<i>C. albicans</i>	<i>C. tropicalis</i>	<i>C. tropicalis</i>	8	R
11	KDY1110	73/M	BAL	<i>C. albicans</i>	<i>C. albicans</i>	<i>C. albicans</i>	S	S
12	KDY1147	78/F	BAL	<i>C. albicans</i>	<i>C. albicans</i>	<i>C. albicans</i>	8	R
13	KDY1130	11/F	BAL	<i>C. albicans</i>	<i>C. albicans</i>	<i>C. albicans</i>	R	R
14	KDY935	34/M	Vaginal discharge	<i>Candida</i> spp.	*	<i>C. parapsilosis</i>	R	R
15	KDY1203	1/M	Sputum	<i>C. albicans</i>	<i>C. albicans</i>	<i>C. albicans</i>	8	R
16	KDY1159	56/M	BAL	<i>C. albicans</i>	<i>C. albicans</i>	<i>C. albicans</i>	8	R
17	KDY1208	57/F	Nail scrape	<i>C. albicans</i>	<i>C. albicans</i>	<i>C. albicans</i>	S	S
18	KDY1061	50/F	Vaginal discharge	<i>C. albicans</i>	<i>C. albicans</i>	<i>C. albicans</i>	8	R
19	KDY1162	17/F	Vaginal discharge	<i>C. albicans</i>	<i>C. albicans</i>	<i>C. albicans</i>	S	S
20	KDY1253	26/M	BAL	<i>C. dubliniensis</i>	<i>C. albicans</i>	<i>C. dubliniensis</i>	S	S
21	KDY1227	9/M	Urine	<i>C. albicans</i>	<i>C. albicans</i>	<i>C. albicans</i>	S	S
22	KDY1116	42/M	BAL	<i>C. dubliniensis</i>	<i>C. albicans</i>	<i>C. albicans</i>	S	S
23	KDY1658	-		<i>C. albicans</i>	<i>C. albicans</i>	<i>C. albicans</i>	S	S
24	KDY953	34/F	Sputum	<i>C. albicans</i>	<i>C. albicans</i>	<i>C. albicans</i>	S	S
25	KDY618	80/F	BAL	<i>Candida</i> spp.	<i>C. parapsilosis</i>	<i>C. parapsilosis</i>	R	R

*Undigested DNA in RFLP profile

**MIC range: 0.063–0.25µg/ml

BAL: bronchoalveolar lavage; F: female; M: male; MIC: minimum inhibitory concentration; R: resistant; RFLP: restriction fragment length polymorphism; S: sensitive.

Two studies from the USA have shown a medieval prevalence of *Candida* yeasts at nephrology wards.^{16,17} This study showed that there was no difference in sex preponderance with regards to either colonisation or infection by *Candida* yeasts; however, the elderly patients (>50 years old) were more at risk of infection. Similar to other studies, kidney transplant, immune suppression, and haemodialysis were shown to be the main risk factors in this study. Several other studies have indicated that immunosuppression, broad spectrum antibiotic therapy, and diabetes are important predisposing factors of candidiasis.^{15,17} Another recent study classified antibiotic treatment 3 months after kidney transplant, cytomegalovirus infection, and diabetes as independent risk factors associated with invasive fungal infections.

Amongst all collected specimens in this study, most of the *Candida* cells were isolated from bronchoalveolar lavage, which indicated *Candida* colonisation of the mucosal layer of the oropharynx and the lower respiratory tract. Post-transplant immunosuppression may have predisposed the patients to mucosal colonisation by *Candida*. Several researchers have also reported a high prevalence of *Candida* species causing pulmonary colonisation infection. This study's findings of *Candida* identification showed that *C. albicans* was the most common yeast isolate. This result is consistent with that of several other studies.¹⁷ Another study from the USA reported *C. albicans* as the most common fungal isolate from 28 kidney transplant, 17 liver transplant, six lung transplant, and four heart transplant patients.⁹ Nonetheless, non-*albicans Candida* species, such as *C. glabrata* and *C. dubliniensis*, have been identified as leading isolates in other studies. The importance of real-time PCR in this research was highlighted by its ability to differentiate *C. dubliniensis*, which could not be differentiated by PCR-RFLP and a restriction enzyme. Using real-time PCR for the

molecular identification of *Candida* yeasts has been reported by several researchers. A recent study from China reported the identification of four *Candida* species, including *C. albicans*, *C. tropicalis*, *C. glabrata*, and *C. dubliniensis*. Another study from Germany used real-time PCR assays for identifying *C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, and *C. dubliniensis*.¹⁰ There were no coincidences amongst the three methods used in the present study, which involved at least seven *Candida* isolates at the species level. For example, *C. tropicalis* identified by the CHROMagar™

differential medium was not confirmed by PCR-RFLP and real-time PCR. However, one case of *C. albicans* identified by real-time PCR was identified as *C. glabrata* by CHROMagar™

and PCR-RFLP. These results of AFST (MIC) indicated that approximately half of the clinical isolates of *C. albicans* exhibited itraconazole resistance. Two thirds of cases of *C. dubliniensis* and both isolates of *C. parapsilosis* were also resistant. A study from Turkey reported 11/23 isolates of *C. albicans* and 3/5 isolates of *C. glabrata* were resistant to itraconazole (MIC: $\geq 16 \mu\text{g/mL}$).¹²

CONCLUSION

In conclusion, the authors found that immunosuppressions and/or iatrogenic interventions predispose patients hospitalised at medical wards to yeast colonisation/infection. *C. albicans* is the predominant species amongst the isolates that colonises in the upper respiratory tract. It was also found that *C. albicans* had a decreasing susceptibility to itraconazole (an azole antifungal drug). Molecular identification of *Candida* species and their resistance patterns is essential to optimising patient management and reducing unnecessary drug use.

References

1. Bhattacharjee P. Epidemiology and antifungal susceptibility of *Candida* species in a tertiary care hospital, Kolkata, India. *Curr Med Mycol*. 2016;2(2):20-7.
2. Kaur R et al. Identification and antifungal susceptibility testing of *Candida* species: a comparison of Vitek-2 system with conventional and molecular methods. *J Glob Infect Dis*. 2016;8(4):139-46.
3. Wiederhold N et al. The fungal Cyp51 specific inhibitor VT-1598 demonstrates *in vitro* and *in vivo* activity against *Candida auris*. *Antimicrobial agents and chemotherapy*. *Antimicrob Agents Chemother*. 2019;63(3).

4. Demers E et al. Evolution of drug resistance in an antifungal-naive chronic *Candida lusitanae* infection. *Proc Nat Acad Sci U S A*. 2018;115(47):12040-5.
5. Chew KL et al. Delay in effective therapy in anidulafungin-resistant *Candida tropicalis* fungaemia: potential for rapid prediction of antifungal resistance with whole-genome sequencing. *J Glob Antimicrob Resist*. 2019;16:105-7.
6. Thomaz D et al. An azole-resistant *Candida parapsilosis* outbreak: clonal persistence in the intensive care unit of a Brazilian teaching hospital. *Front Microbiol*. 2018;9:2997.
7. Nami S et al. Current antifungal drugs and immunotherapeutic approaches as promising strategies to treatment of fungal diseases. *Biomed Pharmacother*. 2019;110:857-68.
8. Naji S et al. Interspecies differences of *Candida* species causing recurrent vulvovaginal candidiasis in response to fluconazole treatment. *Tehran Univ Med J*. 2017;75(4):280-7.
9. Shoham S, Marr K. Invasive fungal infections in solid organ transplant recipients. *Future Microbiol*. 2012;7(5):639-55.
10. Zhang J et al. Development of *Candida*-specific real-time PCR assays for the detection and identification of eight medically important *Candida* species. *Microbiol Insights*. 2016;9:21-8.
11. Vincent J et al. Epidemiology, diagnosis and treatment of systemic *Candida* infection in surgical patients under intensive care. *Intensive Care Med*. 1998;24(3):206-16.
12. Kucukates E et al. Identification of *Candida* species and susceptibility testing with Sensititre YeastOne microdilution panel to 9 antifungal agents. *Saudi Med J*. 2016;37(7):750-7.
13. Guimarães T et al. Epidemiology and predictors of a poor outcome in elderly patients with candidemia. *Int J Infect Dis*. 2012;16(6):e442-7.
14. León C et al. What's new in the clinical and diagnostic management of invasive candidiasis in critically ill patients. *Intensive Care Med*. 2014;40(6):808-19.
15. Ahmed A et al. Risk prediction for invasive candidiasis. *Indian J Crit Care Med*. 2014;18(10):682-8.
16. Khan A et al. Fungal infections in renal transplant patients. *J Clin Med Res*. 2015;7(6):371-8.
17. Chakrabarti A et al. Incidence, characteristics and outcome of ICU-acquired candidemia in India. *Intensive Care Med*. 2015;41(2):285-95.
18. Sahin SZ et al. Invasive fungal infections in renal transplant recipients: epidemiology and risk factors. *Mycopathologia*. 2015;180(1-2):43-50.
19. Aboutalebian S et al. Molecular epidemiology of otomycosis in Isfahan revealed a large diversity in causative agents. *J Med Microbiol*. 2019;68(6):918-23.
20. Mirhendi H et al. Identification of pathogenic *Aspergillus* species by a PCR-restriction enzyme method. *J Med Microbiol*. 2007;56(11):1568-70.

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