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Molecular characterization and antifungal susceptibility testing of *Candida nivariensis* from blood samples – an Iranian multicentre study and a review of the literature

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Abstract

Purpose. Identification of the emerging yeast species *Candida nivariensis* among presumptively identified Iranian *Candida glabrata* isolates.

Methodology. Clinical *C. glabrata* species complex isolates from blood ($n=100$; 46.9%), vaginal swabs ($n=20$; 9.4%), bronchoalveolar lavage ($n=10$; 4.7%) and sputum ($n=12$; 5.6%) from 68 patients from Iran were investigated. Isolates were characterized by CHROMagar, multiplex PCRs, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), amplified fragment length polymorphism (AFLP) fingerprinting, internal transcribed spacer (ITS)/large subunit (LSU) rDNA and *FKS1*/*FKS2* sequencing, and the European Committee on Antimicrobial Susceptibility Testing broth microdilution method. A comprehensive literature review was conducted and all the relevant clinical and microbiological data were collected.

Results. Four *C. nivariensis* isolates were recovered from blood samples of three subjects and were all consistently identified by nine-plex PCR, Bruker MALDI-TOF MS, and LSU and ITS rDNA sequencing. AFLP genotyping clustered the isolates into two groups. Sequencing of the *FKS1* and *FKS2* hotspots showed no accountable amino acid substitutions. All isolates were susceptible to amphotericin B, fluconazole, itraconazole, posaconazole, voriconazole, anidulafungin and micafungin.

Conclusion. In total, 4 out of 213 clinical *C. glabrata* species complex candidemia isolates were *C. nivariensis*. Improvement of the BioMerieux Vitek MS database is required to accurately identify *C. nivariensis* and it is advised to alternatively use CHROMagar and/or PCR-based techniques. As other species within the *Nakaseomyces* clade may cause infection and showed high MIC values for antifungals, inclusion of their spectra into the MALDI-TOF MS database seems relevant. Due to developing resistance to fluconazole and insufficient efficacy of caspofungin, the combination of catheter removal plus treatment with caspofungin, or voriconazole, or micafungin might be effective for patients.

INTRODUCTION

Candida glabrata is the second most common cause of candidemia in the USA [1] and its prevalence is increasing worldwide [2, 3]. Acquisition of resistance to azoles, echinocandins, amphotericin B and multidrug resistant (MDR) traits are alarming features of *C. glabrata* [3–5]. For a decade *C. glabrata* has been recognized as a cryptic species complex containing the emerging opportunistic yeast species *C. nivariensis* [6] and *Candida bracarensis* [7]. These species belong to the *Nakaseomyces* clade of the Saccharomycotina [8]. Comparative genomic studies showed that *C. bracarensis* and *C. nivariensis* are more related to the non-pathogenic yeast species *Nakaseomyces delphensis* than to *C. glabrata* [8].

Although some studies showed that time-consuming, but relative simple phenotypic identification techniques,

such as CHROMagar, can easily distinguish these species from *C. glabrata*, molecular assays increased the accuracy and shortened the identification turn-around time [9]. Moreover, not only is little known about their virulence, antifungal susceptibility and prevalence, but also rarity of spectra of these species has hampered the accuracy of Vitek MS to correctly identify clinical isolates of *C. bracarensis* and *C. nivariensis* [10]. Accordingly, identification and sharing data on nationwide and worldwide scales could enrich our knowledge about various aspects of these species and will contribute to diagnostic and even therapeutic improvement.

Here, we retrospectively studied a large collection of clinical *C. glabrata* isolates recovered from Iranian patients. Subsequently, amplified fragment length polymorphism (AFLP) fingerprinting [11], antifungal susceptibility patterning, using

European Committee on Antimicrobial Susceptibility Testing (EUCAST) broth microdilution, and sequencing of the *FKS1* and *FKS2* hotspot 1 (HS1) regions were performed. Lastly, a comprehensive literature review was performed to collect relevant clinical and microbiological data of *C. nivariensis* cases.

METHODS

Retrieving data of published cases

All published studies containing *C. nivariensis* cases from 2005 (year of description of the species *C. nivariensis*) to 1 October 2018 were retrieved. The keyword '*Candida nivariensis*' without any limitations in languages and dates was searched in PubMed and Google search engines.

Clinical aspects, including year of publication and location, age, sex, sample type, risk factors and underlying conditions, antifungals used and duration of therapy, outcome and microbiological data, including number of isolates, phenotype on CHROMagar, antifungal susceptibility testing (AFST) protocol, and the MIC values for the antifungals fluconazole (FLC), voriconazole (VRC), itraconazole (ITC), posaconazole (PSC), caspofungin (CSP), micafungin (MCF), anidulafungin (ANF), amphotericin B (AMB), and 5-fluorocytosine (5FC) were recorded. In order to assure the accuracy of collected clinical and microbiological data, they were checked and recorded by two individuals to reach consensus.

Patient information

Case 1 (isolate 2N)

A 67-year-old man, who underwent mitral and aortic valves' repair, two months after cardiac surgery and with clinical neurological deficits compatible with cerebrovascular accident (CVA) was admitted in Ghaem hospital, Mashad, Iran, on 5 April 2015. Brain MRI (T1 with contrast view) revealed two ring enhancement masses on right frontal and temporal lobes that suggested brain abscesses. After this primary

diagnosis, some masses were visible around the mitral valve on his Trans esophageal echocardiography (TEE). With being suspicious of mitral valve endocarditis, a blood sample was taken and incubated in Bactec device (Bactec 9420, Becton Dickinson, Franklin Lakes, NJ, USA). Broad spectrum antibiotics, including cefepime 2 g/IV/TDS, gentamicin 80 mg/IV/TDS, rifampin 300 mg/PO/BD and vancomycin 1 g/IV/BD were prescribed after blood culture sampling. After 36 h, the blood culture was positive for *Enterobacter* spp. Vancomycin was discontinued and other antibiotics continued and his fever completely resolved after 7 days. On 18 April fever started again, hence, the blood sample was taken and after 48 h it was positive for *Candida* spp. Caspofungin (70 mg/IV/stat and 50 mg/IV/daily) was added to the previous antibiotic regimen and this treatment was continued for 10 days. His follow-up blood culture was negative after 3 days. Despite being partially recovered, due to his family member's request, he was discharged while he was under treatment with fluconazole (400 mg daily/PO) 22 days post admission.

Case 2 (isolate 5N)

The same patient (case 1) after 30 days with fever complaint was referred to Ghaem hospital and admitted to the infection ward on 26 May 2015. He described his previous frequency of dysuria 5 days before admission and he was evaluated for urosepsis. Urine analysis revealed the presence of many white blood cells (leukocyturia) and yeast cells ($>10^5$ c.f.u. ml⁻¹) and accordingly AMB deoxycholate (75 mg/IV/ daily) was prescribed. A blood sample was taken and after 6 days incubation yielded yeast cells. Although, not fully recovered and his general health status was not satisfactory for the medical team, the patient left the hospital and no information was available on his follow-up.

Case 3 (isolate 35N)

A 14-year-old girl with severe burnings was hospitalized in the burn intensive care unit (ICU), Ghaem Hospital,

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Keywords: *Candida glabrata* complex; multicenter study; *Candida nivariensis*; multi-plex PCR; MALDI-TOF MS; ribosomal DNA sequencing.

Abbreviations: AFLP, amplified fragment length polymorphism; AMB, amphotericin B; AND, anidulafungin; BAL, Bronchoalveolar lavage; EUCAST, European Committee of Antifungal Susceptibility Testing; 5-FC, 5-fluorocytosine; FLC, fluconazole; ITC, itraconazole; ITS, internal transcribed spacer; LSU, large subunit; MALDI-TOF MS, matrix-assisted laser desorption ionization time of flight mass spectrometry; MCF, micafungin; PSC, posaconazole; VRC, voriconazole.

Four isolates of *C. nivariensis* were deposited in the culture collection of Westerdijk Fungal Biodiversity Institute (accession numbers CBS 15642–15645). Sequences of the large subunit and internal transcribed sequences of rDNA and hotspot 1 of *FKS1* and *FKS2* were deposited in GenBank under the accession numbers, MK503934–MK503937, MK503955–MK503958, and MK509355–MK509362.

†These authors contributed equally to this work

Two supplementary tables are available with the online version of this article.

Mashhad, Iran, on 26 October 2015. The whole upper body part, neck and face were involved with second to third degree burns. In the beginning of her admission, she was alert and in order to relieve the pain, fentanyl (50 µg/IV/infusion/hour) was prescribed for a few days and daily washing and dressing of wounds were performed. The first episode of fever was noticed 7 days post admission. Physical examinations showed that there was a significant infection of the burn wounds. Hence, wound drainage and blood were subjected to culture, and imipenem (500 mg/IV/QID) and vancomycin (1 g/IV/BID) treatments were started. The blood and wound drainage culture yielded both *Acinetobacter baumannii*, vancomycin was replaced by colistin (9 mu/IV/loading and then 4.5 mu/IV/BID) and she was treated with both colistin and imipenem. After 2 days, fever signs were eliminated and first debridement surgery was performed. On the fourteenth admission day, the affected site was repaired with allograft skin surgery. Then 3 days after the surgery (seventeenth day of admission), the patient became febrile and discharges were observed around the skin graft. A blood sample yielded an imipenem and amikacin resistant strain of *Pseudomonas aeruginosa* and *Candida* spp., while the wound culture remained negative. Imipenem treatment was stopped and meropenem (2 g/TDS/IV) and fluconazole (200 mg/daily/PO) were added to colistin. On the twenty-first day of admission, blood cultures remained positive with *Pseudomonas aeruginosa* and *Candida* spp. On 26 November 2015, in spite of the extensive debridement of the graft and the replacement of fluconazole with amphotericin B deoxycholate (50 mg/daily/IV) the patient succumbed.

Case 4 (isolate 81)

A 62-year-old female with left limb sarcoma was admitted with necrotizing fasciitis and severe sepsis in the emergency ward in Imam Khomeini complex hospital, Tehran, Iran, on 23 July 2018. Treatment with meropenem (1 gr/TDS/IV) and vancomycin (1 g/IV/BID) was started and amputation of the left limb was immediately performed. During 24–26 July, despite antibiotic therapy and amputation, the patient was suffering from fever and sepsis. Hence, being suspicious for an infection caused by other micro-organisms, blood samples were taken and incubated in Bactec 9420 device (Becton Dickinson). On 26 July, following the isolation of *Pseudomonas aeruginosa* from a wound culture of the amputated leg discharges, colistin (9 mu/IV/Loading and then 4.5 mu/IV/BID) and gentamycin (240 mg/IV/TDS) were administered and treatment with vancomycin was stopped. On 27 July, blood cultures yielded yeast cells and subsequent PCR reported them as *C. glabrata* and antifungal therapy with 300 mg of liposomal amphotericin B was started the following day. On 29 July, liposomal AMB was replaced with 50 mg of caspofungin. On 8 August 2018, the patient died due to septic shock and cardiac arrest.

Isolates, identification, and part sequencing of *FKS1* and *FKS2* genes

Preserved clinical isolates of *C. glabrata* (2015–2018) from four metropolitan cities of Iran, including, Tehran, Shiraz,

Isfahan and Mashhad, were retrospectively collected. Isolates were obtained from blood ($n=71$; 33.3%), urine ($n=100$; 46.9%), vaginal swabs ($n=20$; 9.4%), bronchoalveolar lavage (BAL) fluid ($n=10$; 4.7%) and sputum ($n=12$; 5.6%) samples. Isolates were streaked on Sabouraud dextrose agar at 37 °C for 24–48 h. Colonies that showed white and pink colonies on CHROMagar were selected and subjected to a comprehensive multiplex 21-plex PCR [12]. Further analysis by a nine-plex PCR [9], Bruker MALDI-TOF MS (Microflex LT, Bruker Daltonics, Bremen, Germany), large subunit (LSU) and internal transcribed spacer (ITS) rDNA sequencing, antifungal susceptibility testing (EUCAST v9.0) and AFLP was performed. Identification by MALDI-TOF MS was performed using a full extraction method [13]. In order to evaluate the accuracy of MALDI-TOF MS and the nine-plex PCR, sequencing of LSU of ribosomal DNA (rDNA) using primers of LROR (5'-ACCCGCTGAAGTAAAGC-3') and LR5 (5'-TCCTGAGGGAAACTTCG-3'), and ITS rDNA using primers of ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC GTA GGT GAA CCT GCG G-3') was performed [14].

C. glabrata increasingly shows resistance to echinocandins that is mainly associated with acquisition of mutations in the hotspot 1 (HS1) of *FKS1* and *FKS2* genes [15]. *C. nivariensis* is phylogenetically closely related to *C. glabrata*, therefore, we sequenced HS1 of these two genes for the isolates of *C. bracarensis* and *C. nivariensis*. Primers targeting HS1 of *FKS1* (389 bp) and *FKS2* (718 bp) of *C. glabrata* were used for this purpose (unpublished data).

Genotyping of isolates of *C. nivariensis* using AFLP

In order to investigate the genetic relationship and genotypic diversity of our isolates, AFLP was followed as described before [11]. Besides our clinical isolates of *C. nivariensis* ($n=4$), CBS reference and type strains of *C. nivariensis* (CBS 9984, CBS 9985 and CBS 10161), *C. bracarensis* (CBS 10154) and a clinical isolate of *C. bracarensis* (H111) generously provided by Professor W. Liao (Shanghai, China), *C. glabrata* (CBS 138), *C. uthaithanina* (CBS 10932), *C. kungkrabaensis* (CBS 10927), *N. delphensis* (CBS 2170) and *N. bacillisporus* (CBS 7720) were included in the AFLP experiment.

Antifungal susceptibility testing

To define the MIC of our isolates, antifungal susceptibility testing was performed using broth microdilution procedure of EUCAST version 9.0 (www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Clinical_breakpoints/Antifungal_breakpoints_v_9.0_180212.pdf). Amphotericin B (AMB), 5-flucytosine (5FC; Sigma Chemical Corporation, St. Louis, MO, USA), fluconazole (FLC), voriconazole (VCZ) and anidulafungin (AND; Pfizer, New York, NY, USA), itraconazole (ITC; Santa Cruz Biotech, Dallas, TX, USA), posaconazole (PSC; MSD, Kenilworth, NJ, USA), and micafungin (MCF; Astellas Pharma, Tokyo, Japan) were included. As caspofungin is associated with interlaboratory variations and obtained MIC values of this drug cannot differentiate wild-type and mutated strains of *C. glabrata* [16], we did not

include this drug in our antifungal susceptibility assessment. It is known that MIC values obtained for anidulafungin and micafungin better predict the presence of mutations in *FKS1* and *FKS2*, and thus both echinocandins were included [16]. AMB, AND, FLC and MCF were interpreted based on clinical breakpoints, while ITC, PSC, VRC and 5FC were interpreted based on epidemiological cut-off values. For antifungal susceptibility testing, apart from our clinical isolates of *C. nivariensis* ($n=4$), four reference and type strains of *C. nivariensis* (CBS 9983, CBS 9984, CBS 9985 and CBS 10161) were included.

RESULTS

Data obtained from published cases of *C. nivariensis*

All clinically and microbiologically relevant data of published literature and our cases are presented in Tables S1 and S2 (available in the online version of this article). In total, 75 *C. nivariensis* isolates were found in 13 countries. Isolates of *C. nivariensis* were recovered from four continents and Europe with 39 isolates had the highest number of isolates, followed by Asia with 30 isolates, America with 4 isolates and Australia with only 1 isolate. Country-wise, China contained the highest number of isolates of *C. nivariensis* ($n=20$), followed by the UK ($n=16$), Poland ($n=14$), Spain ($n=8$), India ($n=6$), Argentina ($n=3$), Malaysia ($n=2$), and Australia, Brazil, France, Indonesia, Italy and Japan reported only one isolate. The majority of infections caused by *C. nivariensis* were found in adults aged >20 years (89.65%) with a median age of 62 years. Two-thirds of cases of infections ($n=29$) (with known data of age and sex) were acquired by women and the rest by men ($n=10$). *C. nivariensis* isolates were recovered from a wide ranges of clinical samples, including blood ($n=18$), vagina ($n=17$), urine ($n=6$), BAL and tracheal aspirate ($n=4$), peritoneal fluid ($n=6$), oral samples ($n=3$), abscess ($n=2$), urine and renal catheter ($n=2$), vascular tip catheter

($n=2$), sputum ($n=2$), CSF ($n=2$), pleural fluid, urostomy fluid, lung biopsy, pelvic collection, nasal secretion, exit site swab, nail with each one sample (for three samples no source was mentioned). Candidemia (24%), vaginitis (22.66%) and candiduria (8%) were among the most prevalent clinical manifestations caused by *C. nivariensis*. Broad-spectrum antibiotic therapy and catheter insertion were the most encountered risk factors for development of candidiasis due to *C. nivariensis*. Among 18 patients with candidemia due to *C. nivariensis*, treatment option and clinical outcome (death/survival) were reported for only three and four patients, respectively. These three patients were primarily treated with fluconazole and during the course of infection, fluconazole was changed to voriconazole and micafungin followed by micafungin alone ($n=1$), or catheter removal and CSP ($n=1$), or CSP treatment ($n=1$). From these three patients two were treated with VCZ plus MCF or CSP plus catheter removal survived, while the one treated with CSP died. With respect to cases with vaginitis, FLC was the most frequently used antifungal ($n=10/16$), followed by miconazole ($n=3/16$), boric acid in combination with ITC and chlorhexidine ($n=2/16$), and FLC in combination with nystatin ($n=1/16$). From these 16 vaginitis cases only 12 mentioned clinical outcome, among them four out of eight individuals administered with FLC, two out of three patients treated with miconazole, and one individual treated with the combination of FLC and nystatin showed recurrent vaginitis.

Comparison of identification systems and sequencing of HS1 *FKS1* and *FKS2*

Using 21-plex PCR, 213 clinical isolates were identified as *C. glabrata*. In contrast, the nine-plex PCR identified four isolates of *C. nivariensis* and no *C. bracarensis* in agreement with MALDI-TOF MS (green scores >2) and sequencing of LSU rDNA (Fig. 1). In total, 4 out of the 213 Iranian *C. glabrata* species complex isolates were *C. nivariensis* and the rest were *C. glabrata*. Interestingly, all isolates of *C. nivariensis*

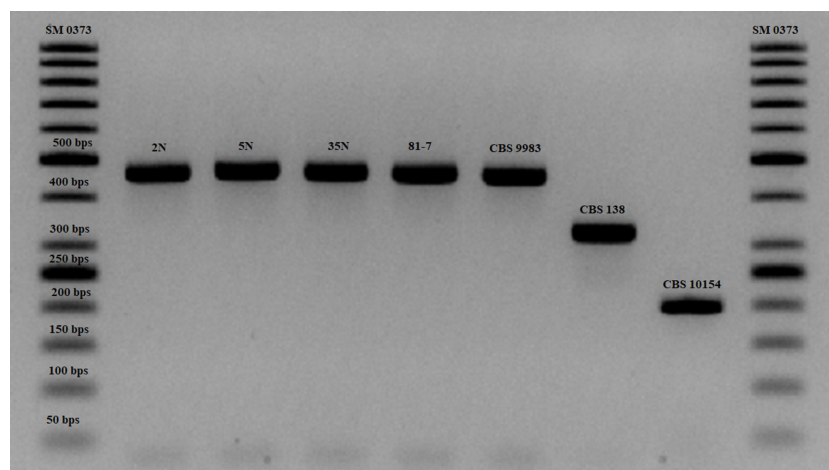


Fig. 1. Successful identification of *C. nivariensis* isolates using nine-plex PCR.

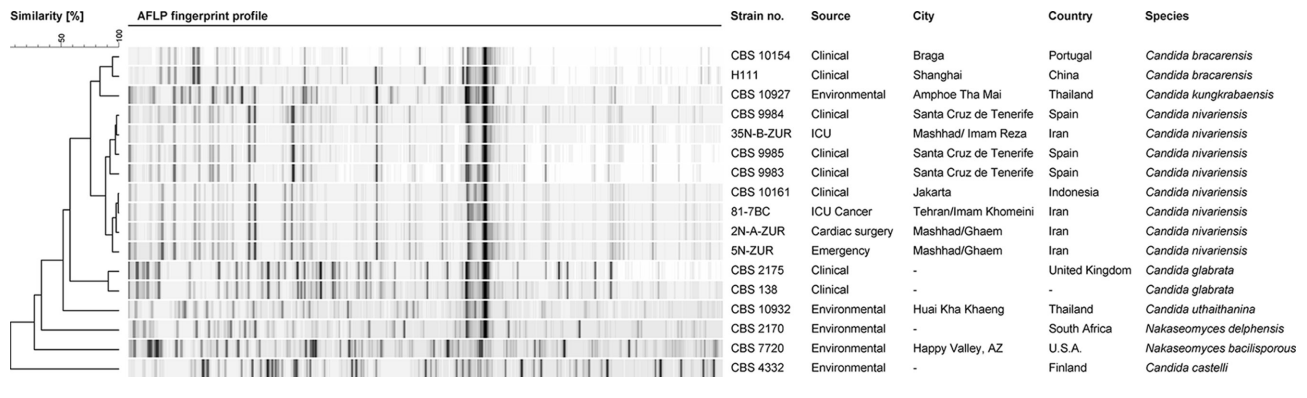


Fig. 2. AFLP fingerprinting profiles generated for *C. nivariensis* isolates.

were from blood samples and were obtained from Mashhad in 2015 ($n=3$) and Tehran in 2018 ($n=1$) that comprised 4.41% of the Iranian candidemia cases caused by *C. glabrata*.

Despite the fact that primers targeting HS1 of *FKS1* and *FKS2* were based on the corresponding sequences of *C. glabrata*, *FKS2* was successfully amplified for all four isolates of *C. nivariensis*, while *FKS1* showed only faint bands. However, subjecting these amplicons to PCR sequencing yielded sequences with decent qualities. No mutations were observed in the HS1 for *FKS2* and in *FKS1* two of the isolates contained silent mutations. The first isolate carried a homozygote and a heterozygote mutations in the residues of 628 (A1884T) and 630 (C1888Y), and the second isolate harboured a mutation in the residue of 630 (C1888T). All these mutations were silent and did not affect the amino acid sequence of the HS1 of *FKS1*.

Genotyping by AFLP

AFLP analysis clustered our isolates with the CBS reference and type strains of *C. nivariensis* and they were grouped in two discrete clusters. Three of our isolates (2 N, 5 N and 100B) from Mashhad and Tehran clustered with CBS 10161, while the other isolate from Mashhad (35 N) clustered with CBS

9983–9985 (Fig. 2). The isolates 2 N and 5 N that were isolated – with an interval of >30 days – from the same patient were found to share the same AFLP genotype.

Antifungal susceptibility testing

Based on clinical breakpoints, all of our isolates showed a susceptible phenotype for FLC (0.0625 – $1 \mu\text{g ml}^{-1}$), AND and MCF ($\leq 0.016 \mu\text{g ml}^{-1}$) and AMB (0.5 – $1 \mu\text{g ml}^{-1}$). Based on epidemiological cut-off value, our isolates were susceptible to ITC (≤ 0.016 – $0.0625 \mu\text{g ml}^{-1}$), VRC (≤ 0.016 – $0.03 \mu\text{g ml}^{-1}$), PSC (≤ 0.016 – $0.125 \mu\text{g ml}^{-1}$) and 5-FC (0.0625 – $0.125 \mu\text{g ml}^{-1}$) (Table 1).

DISCUSSION

Despite an increasing number of reported *C. nivariensis* cases from different countries, limited data is presented with regards to antifungals' susceptibility pattern, virulence factors, genuine distribution and epidemiology, and biological niches of this species. Here, we have systematically screened clinical isolates of *C. glabrata* collected from 2015 to 2018 to assess the nationwide epidemiology of *C. nivariensis* in Iran.

Table 1. MIC of our isolates of *C. nivariensis* and CBS reference and type strains of *C. bracarensis* and *C. nivariensis* determined by EUCAST methodology

Strains	MIC values ($\mu\text{g ml}^{-1}$)							
	Fluconazole	Itraconazole	Voriconazole	Posaconazole	5FC	AMB	Anidulafungin	Micafungin
CBS9983	1	0.06	0.6	0.125	0.06	0.5	≤ 0.016	≤ 0.016
CBS9984	1	0.125	0.6	0.125	0.06	1	≤ 0.016	≤ 0.016
CBS9985	1	0.06	0.03	0.125	0.06	0.5	≤ 0.016	≤ 0.016
CBS10161	0.125	≤ 0.016	≤ 0.016	≤ 0.016	0.125	1	≤ 0.016	≤ 0.016
2N	0.06	≤ 0.016	≤ 0.016	≤ 0.016	0.125	1	≤ 0.016	≤ 0.016
5N	0.125	≤ 0.016	≤ 0.016	≤ 0.016	0.125	1	≤ 0.016	≤ 0.016
35 N	0.125	≤ 0.016	≤ 0.016	≤ 0.016	0.125	0.5	≤ 0.016	≤ 0.016
81–7	1	0.06	0.03	0.125	0.06	1	≤ 0.016	≤ 0.016

***C. nivariensis* was exclusively found in blood samples**

Isolates of *C. nivariensis* were only obtained from blood samples, and we did not find this species from other clinical samples, such as urine, vaginal swabs, BAL, sputum and stool. These four isolates were recovered from three patients (two females and one male) and for one of them within 30 days two isolates were recovered, which represented two episodes. These four cases were reported from two different hospitals. The median age of infected patients was 62 years and it seems that *C. glabrata* and *C. nivariensis* tend to cause infection in the elderly [17]. All of our patients had received broad spectrum antibiotics, which is one of the highlighted risk factors for development of candidemia [18].

Appropriate identification tool is a prerequisite

As a preliminary screening tool, all of the presumptive isolates of *C. glabrata* were cultured on CHROMagar and isolates of *C. glabrata* and *C. nivariensis* showed pink and white coloration, respectively, while a 21-plex PCR [12] identified them all as *C. glabrata* species complex. MALDI-TOF MS and nine-plex PCR, unequivocally identified four isolates of *C. nivariensis* in agreement with rDNA sequencing. In contrast, Vitek MS does not have the ability to identify isolates of *C. nivariensis* and *C. bracarensis* [19]. Hence, it is recommended that laboratories equipped with this device reidentify isolates of *C. glabrata* with inexpensive alternative means of identification such as CHROMagar and/or PCR-based approaches. In our study, 4.41% (3/68) of candidemia cases caused by members of the cryptic complex species of *C. glabrata* were attributable to *C. nivariensis*. Although, most of studies have shown a low clinical prevalence of *C. bracarensis* and *C. nivariensis* [10], the prevalence we reported here is in agreement with the study conducted in Poland, where they did not find *C. bracarensis*, while they showed that *C. nivariensis* constituted 6% of clinical isolates of *C. glabrata* [20]. The lower clinical prevalence of *C. nivariensis* and *C. bracarensis* is partially explained by genomic studies, where it was shown that these two species are more closely related to the non-pathogenic members of the *Nakaseomyces* clades than to *C. glabrata* [8]. However, accelerated evolution of their genome enable them to become more virulent [8] and with an increase in the population of immunocompromised patients might lead to a higher prevalence of infections pertained to these two species. Moreover, all pathogenic attributes of *C. glabrata* are present even in environmental (non-pathogenic) species of *Nakaseomyces* clade, for example *N. delphensis* [8]. Furthermore, *N. delphensis* compared to clinical isolates of *C. bracarensis* was found to be pan-azole-resistant (FLC=128, ITC>16, PSC>8, VRC>4 µg ml⁻¹) and its MIC values for echinocandins and AMB were higher or equal to that of *C. bracarensis* [21]. As a result, expanding appropriate and accurate means of identifications will result in a more comprehensive view on the distribution and epidemiology, antifungal susceptibility patterns, and even pathogenicity of species within the *Nakaseomyces* clade.

Various genotypes of *C. glabrata* proved to differ in their antifungal susceptibility and mortality rates [22], highlighting

the importance of using highly resolutive genotyping techniques, such as AFLP, in microbiological investigations. Using AFLP, our isolates clustered into two distinct genotypes of *C. nivariensis*, where three of our isolates (one from Tehran and two from Mashhad from the same patient) clustered with CBS 10161 and the fourth isolate (from Mashhad) clustered with CBS 9983, CBS 9984 and CBS 9985.

Variability in antifungal susceptibility patterns and clinical outcomes

Our *C. nivariensis* isolates were susceptible to all tested drugs, including AMB, AND, FLC, ITC, MCF, PSC, VRC and 5FC (Table 1). On the other hand, some clinical isolates of *C. nivariensis* recovered from blood [23] and vaginal swabs [24] showed high MIC values for FLC and infected patients were not responsive to this drug. Data derived from the literature review revealed that there is a strain and geographical variability for antifungal susceptibility patterns of isolates of *C. nivariensis* and it seems that this phenomenon is common for clinical isolates of *C. glabrata*, as well [15, 25]. Although, one of our patients that was treated with CSP plus FLC and AMB deoxycholate survived, unfortunately no data was available on his follow-up to ensure that he is alive. Moreover, two of our patients died despite treatment with FLC plus AMB deoxycholate and AMB plus CSP. Considering that there is limited information with regards to treatment options and outcomes of patients infected with *C. nivariensis*, it appears that the sole reliance on FLZ and CSP does not seem enough to resolve candidemia and vaginitis caused by this species [24, 26, 27], while a combination of VRC plus MCF followed by micafungin alone [23] or CSP plus catheter removal [27] for candidemia cases and oral VCZ [24] for recurrent vaginitis cases showed successful treatment outcomes. A higher efficacy of MCF for clearance of infections caused by *C. glabrata* might be behind the fact that this drug causes mutations in HS1 of *FKS1* and *FKS2* approximately 11 times less than CSP and five times AND [28]. Possible misidentification cases of *C. nivariensis* and *C. bracarensis* as *C. glabrata* and high MIC values for azole drugs might prompt clinicians to use echinocandins for treatment of candidemia cases caused by *C. nivariensis*. On the other hand, mutations in HS1 of *FKS1* and *FKS2* are a predictor of therapeutic failure of echinocandins against *C. glabrata* [29], hence, screening of mutations in HS1 of *FKS1* and *FKS2* could be an imperative initiative to detect resistance isolates of *C. nivariensis* and *C. bracarensis* to echinocandins. Lack of amino acid substitution in the HS1 of *FKS1* and *FKS2* was in agreement with low MIC values obtained for anilulafungin and micafungin.

Where is the original biological niche of *C. nivariensis*?

Comparative genomic studies have disclosed that *C. glabrata* is well-adapted to the human gut as its main biological niche, while *C. nivariensis* might have adapted to other environmental niches [8]. This is in line with the findings of environmental

sampling studies from Thailand that isolated *C. nivariensis* from leaves of sugarcane [30], barks and soil [10], while *C. glabrata* was not cultured. So far, all *C. bracarensis* isolates are from clinical sources, and the majority of *C. nivariensis* isolates has a clinical source. This might raise the question if these two species are part of human mycobiota and they will invade the bloodstream, once there is an opportunity. This might be further reinforced by the fact that one of the patients that had a duodenal perforation [26] developed *C. nivariensis* candidemia. Hence, it could be speculated that this species occur in the environment as the main biological niche and subsequently it undergoes adaptation to the human host. In order to address this question, development and application of sensitive and accurate metagenomics platforms might be a useful solution. Once established, subjecting human and environmental-derived samples might elucidate the real biological niches of these species.

Conclusion

In summary, we report four clinical isolates of *C. nivariensis* among a set that was presumptively identified as *C. glabrata* and that were all recovered from blood samples. Taking advantage of accurate means of identification, such as molecular assays and MALDI-TOF MS will aid in unravelling the epidemiology of these cryptic yeast species. Utilization of these techniques will even have a significant impact on our general knowledge of these species. For instance, although it was thought that *C. nivariensis* was isolated in 2005 in Spain for the first time [6], with the aid of accurate means of identification, the first documented isolates of this species dated back to 1996 in Argentina [31]. Once isolated, we will have the possibility to take a global initiative to study the lacking pieces of the puzzles of genotyping, antifungal susceptibility patterns, and virulence of members of this cryptic complex species.

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Author contributions

Authors declared that they have actively participated in this study and they are fully aware of the contents expressed in this manuscript. A.A. and A.F. have participated in study design, performing experiments, data analysis, and drafting and revising the manuscript. M.R.S., H.Z., S.K., M.R., M.J.N., K.Z. and A.C. participated in performing experiments, collection of isolates and clinical data, and revised the manuscript. P.J.H. and C.B. helped with the antifungal susceptibility testing, data analysis, and revision of the manuscript. F.H., P.W. and T.B. have participated in study design, data analysis, drafting and revision of the manuscript, and funded the project.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

This study was approved by the ethical committees of Tehran, Mashhad, Isfahan, and Shiraz Universities of Medical Sciences (IR MUMS fm REC.1397.268, and IR. TUMS. .SPH.REC.1396.4195). Consent forms

were signed by patients and in order to prevent exposing their information, clinical data and isolates derived from them were designated with specific codes.

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