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1 ***Candida nivariensis*: identification strategy in mycological laboratories.**

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31 **ABSTRACT**

32 *Candida nivariensis* is a cryptic fungal species classified within the *Candida glabrata* complex. It was described for the first time in 2005 by the
33 means of DNA sequencing. We report a rare case of *C. nivariensis* deep-seated infection occurring in a 77-year-old man hospitalized for cysto-
34 prostatectomy. Phenotypic testing based on the direct examination and the macroscopic features of the *in vitro* culture initially suggested *C.*
35 *glabrata* species, while MALDI-TOF mass spectrometry enables correct identification. The isolate was found resistant to fluconazole, like in
36 almost 20% of the reported cases. Herein, we present our practical strategy to reliably characterize this rare cryptic species. To date, MALDI-
37 TOF mass spectrometry-based analysis showed very good results for such a purpose.

38

39 **Keywords:** *Candida nivariensis*; *Candida glabrata*; candidemia; candiduria; *Candida bracariensis*; antifungal resistance

40 INTRODUCTION

41 Candidiasis is worldwide the most frequent deep-seated fungal infection. For example in France, 15,500 new cases occurred during the 2001-10
42 period, which represented 43.4% of all the recorded cases of fungal infection [1]. With the emergence of new antifungal drugs and the extensive
43 practice of prophylaxis and empirical treatments, the epidemiology of candidiasis has been slightly changing. While *Candida albicans* remains
44 the major species, minor or new species have progressively emerged and have become isolated more and more frequently in medical laboratories
45 [2]. For instance in a French cohort focusing on candidemia (*i.e.* cases of bloodstream infection due to *Candida* species), the proportion of *C.*
46 *krusei* moved from 3 to 8%, and this of *C. glabrata* from 18 to 29%, following pre-exposure to fluconazole [3].

47 However, such results should be interpreted with caution, because of possible species misidentifications in former studies. Through the recent
48 advent of DNA sequencing techniques and MALDI-TOF mass spectrometry, a few new species have been described within large species
49 complexes. These species are referred to as cryptic species because they do exhibit phenotypic features that are similar to those of the major
50 species of the complex. Therefore, *Candida nivariensis*, which is very close to *C. glabrata*, was genetically identified as a new distinct species in
51 2005. *C. nivariensis* was first described in bronchial-alveolar lavage fluid, blood and urine from three distinct patients in a Spanish hospital [4].

52 Regarding the choice of the adequate antifungal drug, correct species identification of *C. nivariensis* is needed in order to thereafter decide to
53 conduct antifungal susceptibility tests. Indeed, it was showed that *C. nivariensis* frequently expresses high minimal inhibitory concentrations
54 (MICs) to azole drugs, like *C. glabrata* [5].

55 The present article describes a rare case of *C. nivariensis* candidemia occurring in a cancer patient of a French university hospital. It also
56 underscores the technical limitations for its identification in mycological laboratories. Moreover, an updated overview of the literature about *C.*
57 *nivariensis* is presented.

58

59 **CASE REPORT**

60 A 77-year-old man was hospitalized on day 0 for cysto-prostatectomy with Bricker trans-ileal bypass in a context of urothelial carcinoma relapse.
61 The patient also suffered from a primary pulmonary adenocarcinoma with lymph node involvement that was treated with pembrolizumab. On
62 day 5, the patient was febrile at 38.5°C. He was empirically given an antibacterial treatment based on piperacillin/tazobactam, and blood cultures
63 (BD Bactec™ Plus Aerobic/F vial, BD, Franklin Lakes, United States) were collected from his central catheter. One culture vial was detected
64 positive after four days and six hours in the incubator. Its direct microscopic examination showed small budding yeasts that could rightfully
65 evocate *C. glabrata*. After 48 hours of incubation at 35°C, subculture onto chromID® Candida agar plates (BioMérieux, Marcy-l'Etoile, France)
66 exhibited small white smooth and shiny colonies looking like *C. glabrata*. The Glabrata R.T.T. Fumouze® test (Biosynex, Illkirch-
67 Graffenstaden, France), based on the ability of *C. glabrata* to hydrolyze trehalose but not maltose [6], was revealed positive after eight minutes.
68 An Api 20C® auxanogram gallery (BioMérieux, Marcy-l'Etoile, France) suggested *C. glabrata* species with an identification score of 99.4%.
69 The MALDI-TOF mass spectrometry (Bruker Daltonics, Bremen, Germany) identified *C. nivariensis* with a Log-score of 1.72, although the
70 profile was very close to *C. glabrata* (the software MBT Compass IVD® v.4.2 was used with the database MSP 7171, year 2018). Strain

71 identification was finally confirmed as *C. nivariensis* by sequencing the D1/D2 region of the 28S rDNA (GenBank accession number No.
72 MN306567) [7]. The antifungal susceptibility of the isolate showed elevated MICs to fluconazole, but not to other azoles, neither to
73 echinocandins nor to amphotericin B (Table 1).

74 Thereafter, a second blood culture collected from the central catheter on day 6 was detected as positive for *C. nivariensis*, after four days and 14
75 hours in the incubator. The urines collected on Bricker's system on day 8 were also positive for *C. nivariensis*. The central catheter was removed
76 on day 9, but its *in vitro* culture remained sterile after 48 hours of incubation. On day 12, the concentration of β -D-glucan antigen was measured
77 at 218 pg/mL in blood, with the Fungitell assay® serum test (Cape Cod, East Falmouth, United States). *Candida* antigen was negative with
78 mannan concentration measured at 40 pg/mL in blood (BioRad, Hercules, United States).

79 On day 9, piperacillin/tazobactam antibiotics were withdrawn, and antifungal therapy was initiated with caspofungin. Fever then decreased, and
80 all subsequent blood cultures remained sterile thereafter. Ultrasound examination didn't show any sign suggestive for endocarditis, and the
81 dilated fundus exam was normal. After two weeks of treatment, caspofungin was switched to oral voriconazole for one month in order to
82 complete the expected overall treatment duration for six weeks (as recommended for the intravascular infection, as a partial thrombosis was
83 herein highlighted by a Doppler ultrasound examination in the right internal jugular vein after the removal of the catheter) [9]. After one year of
84 follow up, the patient was still alive.

85

86 **DISCUSSION**

87 *Candida nivariensis* is an emergent yeast belonging to the *Candida glabrata* complex. Since its first description, 181 human cases (ranging from
88 simple colonization to true infection) have been reported (Table 2 and Suppl. table 1): 112 in Europe, 51 in Asia, 11 in Oceania, six in America
89 and one in Africa. The actual prevalence of *C. nivariensis* infection is thought to be probably underestimated in view of the theoretical worldwide
90 distribution of this yeast species. This hypothesis has been raised from several previous studies [10]–[12], because at that times laboratories
91 didn't have access to modern diagnostics tools for distinguishing between *C. nivariensis* and *C. glabrata* (e.g. auxanogram galleries, like the
92 API® yeast identification, didn't permit the identification of cryptic species like *C. nivariensis* and both are able to assimilate glucose and
93 trehalose [4], [13], that can mislead the interpretation of the Glabrata R.T.T. Fumouze® test). In our mycology laboratory, it was the first time
94 that *C. nivariensis* was unambiguously isolated, whereas the annual incidence of *C. glabrata* has been meanwhile estimated at 41.1 *per* 1,000
95 samples for the last ten years. There are however big needs to accurately identify *C. nivariensis*, mostly for epidemiological purposes. Until
96 recently, genomic analysis was the only reliable method for *C. nivariensis* characterization. Many PCR methods have been developed to
97 distinguish between *C. nivariensis* and *C. glabrata*, and some have showed good results [14], [15]. Most target the internal transcribed spacer
98 (*ITS*) sequences. A PCR-based assay using a single primer pair targeting the *RPL31* gene allowed correct discrimination between *C. glabrata*, *C.*
99 *nivariensis* and *C. bracarensis* (*i.e.* the latter is another cryptic species belonging to the *C. glabrata* complex), according to the size of the
100 amplicon [16]. In contrast, a home-made multiplexed PCR specifically-developed for identification of clinically-important yeast species didn't
101 permit the identification of *C. nivariensis* [17]. Unfortunately, no marketed commercial kit for such a purpose is available for now. Hopefully for
102 a few years, MALDI-TOF technology has been introduced into diagnostic laboratories and now enables the reliable identification of various

103 microorganisms, including some cryptic fungal species. As mass spectrometry databases of spectra libraries are continuously improving,
104 MALDI-TOF-based identification has become the first-line technique in routine practice for most of the microbiological laboratories. Many
105 articles showed very good results for the identification of *C. nivariensis* and *C. bracarensis*, particularly with recent Bruker MS® Biotyper
106 databases [18], [19], [20], or with the user-made MSI platform [21]. For example, in the updated library Biotyper® MSP 7171, six reference
107 spectra are available for *C. nivariensis*. In contrast in a recent study focusing on the identification of *Candida* sp from blood culture, the
108 BioMérieux Vitek MS® system failed to identify the two isolates of *Candida nivariensis* [22], [23]. Thus, the BioMérieux Vitek MS® v3.2
109 library has been incremented in 2018 with *C. nivariensis* spectra. In the present case, the Bruker MS® system (Bruker, Billerica, United States)
110 correctly identified *C. nivariensis* in 100% of isolates tested (two from blood cultures, one from urines). The mass spectrometry Log-score for *C.*
111 *nivariensis* identification was 1.72 for the first blood culture isolate, 1.85 for the urine isolate and 1.93 for the second blood culture isolate (when
112 considering only the score of the top-ranked proposition for “*C. nivariensis*” amongst the whole listing of suggestions for species names). As the
113 three isolates obviously corresponded to a unique strain, the difference between the three scores was probably due to slight insignificant technical
114 variations (e.g. quality of the deposit on the MALDI-TOF plate). Noteworthy, the spectra of *C. nivariensis* and *C. glabrata* remained very
115 similar, and it’s the reason why caution is still warranted to interpret the results (Suppl. figure 1). Similar diagnostic challenges can be
116 encountered with other emergent yeasts: for instance, some diagnostic hindrance has been reported with *Candida auris* for which identification
117 issues could complicate the management of infection [24].

118 A correct identification of the yeast species name is useful for the management of patients infected with *Candida sp.*, especially because of the
119 potentially high MICs to azole drugs for *C. nivariensis* [5], [25], [26]. Most reports indicate variable profiles of antifungal susceptibility which is
120 quite similar to *C. glabrata*. When summarizing all the cases that were reported in the literature, fluconazole MICs ranged from 0.06 µg/mL to >
121 256 µg/mL (Table 2 and Suppl. table 1), so that 17.2% *C. nivariensis* strains (5/ 29 tested) should be probably considered as resistant [8]. The
122 variation in fluconazole MIC values for *C. nivariensis* (as well as for *C. glabrata*) warrants antifungal susceptibility testing, as species
123 identification alone cannot predict resistance profile in this particular context. Since invasive candidiasis are globally known to generate high
124 mortality rate about 30% [27], [28], [29], ignoring potential resistance could be particularly pejorative for the clinical outcome, worsening
125 consequently the overall mortality. By collating all the data of reported cases (Table 2 and Suppl. table 1), the mortality rate of invasive *C.*
126 *nivariensis* candidiasis is estimated at 42%. In the near future, more information will be needed to adjudicate the true clinical virulence of *C.*
127 *nivariensis*.

128 Considering the frequent misidentification of *C. nivariensis*, it therefore seems essential to optimize the techniques available for the *in vitro*
129 diagnosis of *C. nivariensis* infections. For this purpose, identification by MALDI-TOF mass spectrometry appears to be the best option available
130 to date, combining speed, simplicity and reliable results. We also largely encourage systematic antifungal susceptibility testing when isolating
131 such strains.

132

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141

142 **DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST**

143 The authors declare no conflict of interest. They have submitted the ICMJE form. The data was obtained as a part of routine work at the University
144 Hospital in Tours, France.

145

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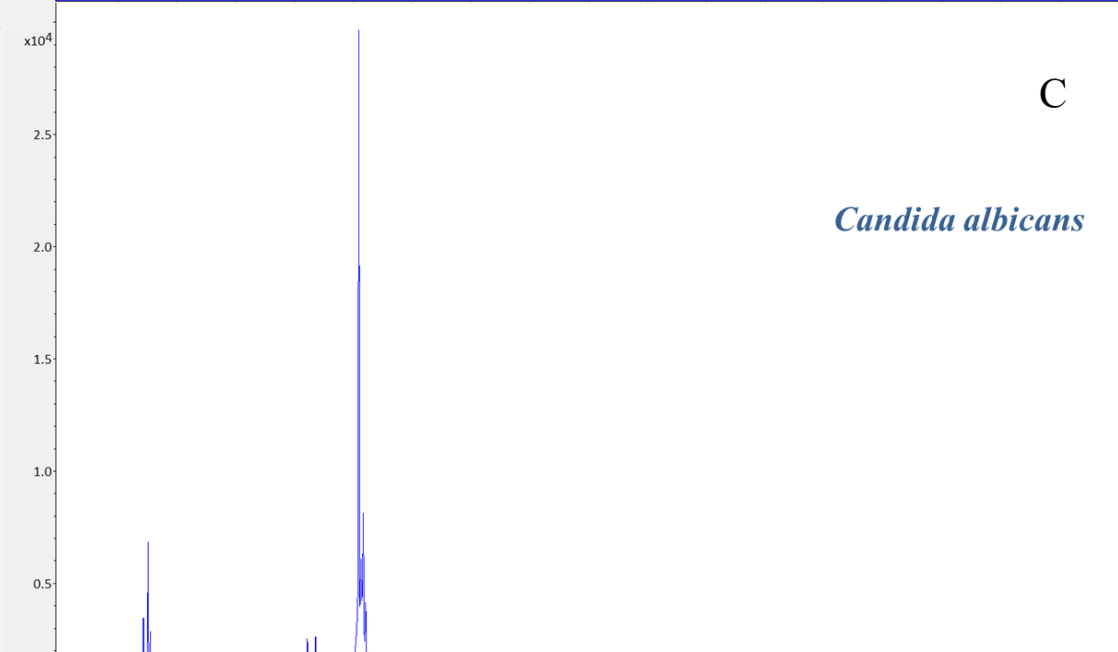
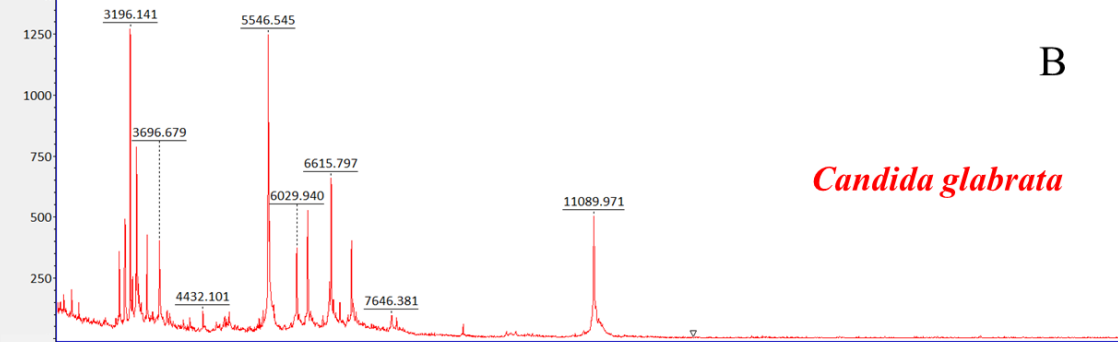
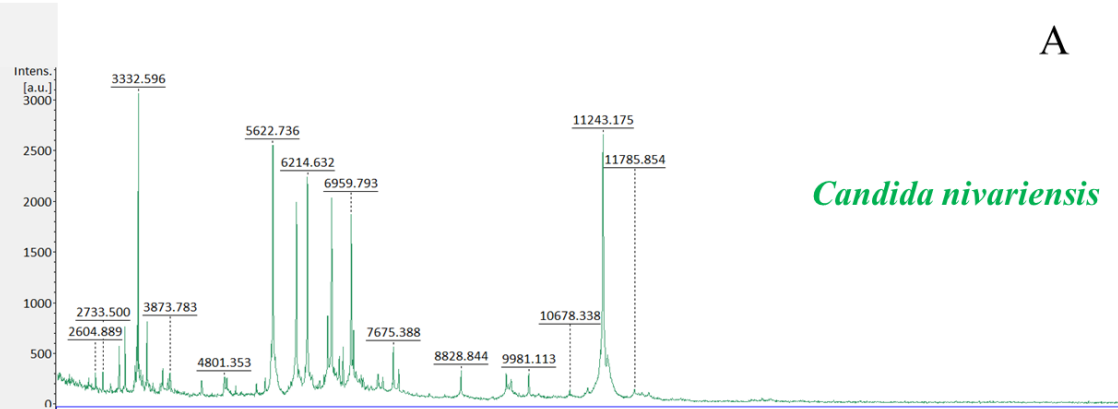
263 **LEGENDS OF SUPPLEMENTARY MATERIAL**

264 **Supplementary figure 1.** Examples of mass spectra for the *Candida nivariensis* isolate of this study (A), and for *C. glabrata* (B) and *C.*

265 *albicans* strains (C). Data were obtained through the Microflex LT® instrument (Bruker Daltonics, Bremen, Germany), with the MALDI

266 Biotyper Compass Explorer v4.2 (Build(80))® software (Bruker Daltonics, Bremen, Germany) and the FlexControl® v3.4 software for spectral

267 analysis. Noteworthy, the mass spectra are quite close to each other between *C. nivariensis* and *C. glabrata*, as they both belong to the same
268 species complex. In comparison, spectral differences appear largely more obvious with *C. albicans*.



270 **Table 1. Minimal inhibition concentrations (MICs) for the *Candida nivariensis* strain isolated in this study**

MIC (µg/mL)	Amphotericin	Micafungin	Fluconazole	Voriconazole	Posaconazole
B					
E-test method [♦] , RPMI culture medium	0.750	0.012	6	0.047	0.380
Broth microdilution method*	0.06	≤ 0.008	4	0.25	0.125

271 [♦] According to the manufacturer's recommendations (BioMérieux, Marcy-l'Etoile, France)

272 *According to EUCAST [8]

273

274 **Table 2. Overview of the published reports about *Candida nivariensis* isolates (2014-2020)**

275 The previous cases have already been reviewed by Angoulvant et al. (2015) [30]. See supplementary table 1.

Number of isolates	Source	Patient's sex	Patient's age	Fluconazole MIC (µg/mL)	Country	References
5	No data	No data	No data	No data	Spain	Galan et al., 2014 [32]
1	Toenails	No data	No data	4	China	Feng et al., 2015 [33]
2	No data	No data	No data	No data	India	Ghosh et al., 2015 [34]
2	No data	No data	No data	No data	France	Cassagne et al., 2015 [35]
4	Vagina	F	20-33	4	Spain	Aznar-Marín et al., 2016 [19]
1	Nasal secretion	No data	No data	≥ 64	Brazil	Figueiredo-Carvalho et al., 2016 [25]

12	Blood	M	38	1	China	Hou et al., 2017 [22]
	Cerebrospinal fluid	F	47	1		
	Blood	M	24	0,5		
	Blood	M	No data	0,5		
	Ascitic fluid	F	82	1		
	Blood	F	82	0,5		
	Ascitic fluid	F	79	2		
	Blood	M	55	1		
	Blood	F	0	1		
	Blood	F	0	1		
	Blood	M	0	1		
	Blood	M	73	4		
5	Throat, renal catheter, urine,	No data	No data	4	Argentina	Morales-Lopez et al, 2017 [36]

continuous peritoneal dialysis bag, and 1 isolate with no isolation data available						
4				0,06	Iran	Arastehfar et al., 2019 [18]
	Blood	M	67	0,125 after fluconazole therapy		
	Blood	F	14	0,125		
	Blood	F	62	1		
56	No data	No data	No data	0,25- > 64	UK	Borman et al., 2019 [37]
(16 already report in 2008)						
3	Urine	No data	No data	16	India	Mashaly et al.,

						2019 [38]
9	Vagina	F	26,25 +/- 4,5 (average +/- SD)	0,125-1	China	Shi et al., 2019 [39]
24	No data	No data	No data	0,25-256	Poland	Sikora et al., 2019 [40]
1	Oral swab	No data	No data	No data	India	Gupta et al., 2020 [41]
1	Blood	M	77	6	France	Present study

276 Abbreviations: F, female; M, male; UK, United Kingdom; USA, United States of America

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