

Non Detection of *Candida nivariensis* and *Candida bracarensis* Among *Candida glabrata* Sensu Lato Isolates in the West Region of Cameroon

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To cite this article:

Claude Nangwat, Aude Nguenim Dougue, Cyrille Levis Kountchou, Alfred Itor Ekpo, Thierry Kammalac Ngouana, Jean Paul Dzoyem, Christopher Bonglavnyuy Tume. Non Detection of *Candida nivariensis* and *Candida bracarensis* Among *Candida glabrata* Sensu Lato Isolates in the West Region of Cameroon. *European Journal of Clinical and Biomedical Sciences*. Vol. 6, No. 5, 2020, pp. 71-77. doi: 10.11648/j.ejcb.20200605.11

Received: August 12, 2020; Accepted: August 24, 2020; Published: September 7, 2020

Abstract: No study in Cameroon has been undertaken to check for the presence of the cryptic species belonging to the *Candida glabrata* complex, which have varying antifungal susceptibility profiles. In this regard, we analyzed a collection of 54 clinical isolates of *C. glabrata* sensu lato obtained from 1551 samples of 490 participants. These participants included 80 diabetic patients, 323 HIV-infected subjects, 73 pregnant women and 14 of some other patients who had none of the aforementioned conditions. Our study aimed at identifying *Candida glabrata* cryptic species among clinical isolates of *Candida glabrata* sensu lato from the West region of Cameroon and to evaluate their susceptibility pattern to some antifungals. Conventional tests such as culture on CHROMagar™ *Candida*, germ tube formation and chlamydospore formation tests were used for the 1st line identification of our isolates, while the matrix assisted laser desorption ionisation – time of flight mass spectrometry (MALDI-TOF) was used for second line identification and confirmation of identifications. The minimal inhibitory concentrations (MICs) of Ketoconazole (Sigma Aldrich, China), Nystatine (Sigma Aldrich, China), Amphotericin B (Dominique Dutscher), Itraconazole (Sigma Aldrich, China), Voriconazole (Acros organics, China), and Clotrimazole (Acros organics, China) vis-à-vis 43 isolates, were determined according to the protocol proposed by the Clinical and Laboratory Standards Institute (CLSI) M27-A3 and M27-S4, with slight modifications. Of the 54 isolates, none was identified as *C. nivariensis* or *C. bracarensis* by MALDI-TOF, all the 54 (100%) isolates were confirmed to be *C. glabrata* stricto sensu. CLO (MIC range: 0.25 - >4 µg/mL) was most resisted by our isolates (95.35%), while 32.56% were resistant to KET (range of MICs: 0.03 - >4 µg/mL). On the other hand, ECVs showed that AMB (MIC range: 0.03 - 4 µg/mL) and ICZ (MIC range: 0.25 - 4 µg/mL) had very good activities against our isolates, as 81.4% of the isolates were wild type for both antifungals. On the other hand, VOR (range of MICs: 0.125 - >4 µg/mL) showed a poor activity, as most of our isolates (93.02%) were non-wild type for the antifungal. The number of isolates used in this study was not enough to conclude that *C. nivariensis* and *C. bracarensis* isolates are absent in the West Region or other parts of Cameroon, so we recommend that more of such studies be carried out in Cameroon, as this could help detect the presence of any of these emerging species with varying antifungal susceptibility profiles.

Keywords: *Candida glabrata* Sensu Lato, *C. nivariensis*, *C. bracarensis*, *C. glabrata* Stricto Sensu, Antifungal Susceptibility Testing

1. Introduction

Candidiasis caused by *Candida* species is one of the most common fungal infections that lead to a range of life-threatening invasive to non-life-threatening mucocutaneous diseases [1]. Its epidemiology has changed in recent years with contributing factors such as HIV infection, diabetes and pregnancy, which have also contributed to the emergence of new species that account for more than 90% of invasive fungal infections, and 40-75% of deaths [1-3].

Studies show that *Candida glabrata* has emerged as the second most important *Candida* pathogen after *C. albicans* [4], accounting for about 26.7% of *Candida* infections. It is also the second most frequent cause of mucosal and disseminated candidiasis [2, 5, 6, 7]. It has been grouped together with two other new, rare and emerging cryptic species called, *C. nivariensis* and *C. bracarensis*, to form the *Candida glabrata* complex [8], referring to closely related but distinct species that had been erroneously classified under the species name, “*glabrata*” [9]. Although these cryptic species of the *C. glabrata* complex likely cause similar disease manifestations as *C. glabrata* sensu stricto, the knowledge of their presence or absence within a given geographical area is very important due to differences in antifungal susceptibility, whereby, both *C. nivariensis* and *C. bracarensis* have been reported to be more resistant to the azoles and amphotericin B compared to *C. glabrata* [10, 11].

Up till now, no study in Cameroon has revealed the presence of these new cryptic species, probably due to the lack of adequate equipment. To the best of our knowledge, most of the studies carried out in Cameroon so far concerning *Candida* species have used only methods based on the detection of phenotypic characteristics (except that of Ngouana in 2014 that focused principally on the *C. albicans* complex) which are non-specific and very limited, especially in the identification of cryptic species [4, 12]. With the coming of more stable, precise and specific molecular techniques like the Matrix Assisted Laser Desorption Ionisation - Time of Flight (MALDI-TOF) mass spectrometry, *C. nivariensis* and *C. bracarensis* which used to be mistaken for *C. glabrata*, have now been identified in some African countries other than Cameroon. For instance, *C. nivariensis* was recently isolated from samples of an asymptomatic Burkinabe patient, showing that this species also exists on the African continent, and possibly in Cameroon [13].

Our study aimed at identifying *Candida glabrata* cryptic species among 54 clinical isolates of *Candida glabrata* sensu lato from the west region of Cameroon and to evaluate their susceptibility pattern to some antifungals.

2. Materials and methods

2.1. Population Study and Sample Collection

2.1.1. Ethical Considerations and Enrollment of Participants

We started off with our work after receiving an ethics

clearance from the CRERSH/C (*Comité Régional d'éthique de la Recherche pour la Santé humaine du Centre*) ethics research committee, with authorization number CE58100No/CRERSCH/2016, and an authorization (number; 1503/ L/ MINSANTE/ SG/ DRSP0/ HRB/D) from the authorities of the Bafoussam Regional Hospital.

The purpose of the study and potential benefits were explained to participants, and those willing to participate in the study signed a written informed consent or assent form prior to their registration as participant. In this regard, participants enrolled for this study were pregnant women, diabetic patients (patients with both types 1 and 2 diabetes mellitus), HIV/AIDS and other patients who came for consultation and had none of these diseases. These included patients of all ages and of both sexes, who came for their follow up and who presented or not, symptoms of candidiasis and who did not receive any antifungal treatment during the last 3 months preceding their enrollment. Prior to sample collection, the clinical data of participants was registered. This included; age, gender, HIV status (including CD4+ counts) and diabetes status (type 1 or type 2, glycaemia).

2.1.2. Sample Collection

The clinical samples collected were stool (S), urine (U), oro-pharyngeal (OPS) and cervico-vaginal (CVS) swabs. The collection of samples, especially cervico-vaginal samples, was done with the assistance of qualified medical personnel. Prior to CV and urine sample collection, 0.4 to 0.5% Dakin's solution, prepared from potassium permanganate and diluted Sodium hypochlorite solution, was applied as an antiseptic on the genital openings. Sterile CV swab and speculum were used to collect CV samples, while 10 – 20 ml of mid-stream urine samples were collected by participants themselves in special sterile urine containers, as they were guided. The collection of OP swabs was also done using sterile swabs, which were inserted into the throats of the participants and then rubbed against the walls of the throats and buccal cavity, while stool samples were collected by the patients themselves in special stool containers.

2.2. Mycological and Molecular Identification of Isolates, and Antifungal Susceptibility Testing

2.2.1. Mycological Identification

The identification and isolation of *C. glabrata* sensu lato isolates was done following a series of steps. These included; culture on sabouraud chloramphenicol agar (SCA) [Oxford Lab Chem - Mumbai, India], subculture of colonies obtained on CHROMagar *Candida* (CHROMagar TM *Candida*, Paris), germ tube test using fresh sheep serum, chlamydosporulation test on rice tween agar (RTA), confirmation of isolate identification using the Matrix Assisted Laser Desorption Ionisation - Time of Flight Mass Spectrometry (MALDI-TOF), and then, amplification and conservation of isolates.

On SCA, *C. glabrata* sensu lato isolates formed shining, smooth, cream coloured and small colonies, which were fairly indistinguishable from those of other yeasts [14]. All yeast

isolates obtained on SCA were subcultured and incubated for at least 48 hrs on CHROMagar *Candida* medium. Colonies which appeared either white, pink, purple or mauve-brown, were isolated, and submitted to germ tube and chlamydospore formation tests. Isolates that were negative for germ tube and chlamydospore formation tests, were presumptively identified as *C. glabrata* sensu lato [1, 14]. Pure colonies were then analysed with the MALDI-TOF mass spectrometry

2.2.2. Molecular Identification

The molecular identification of our yeast isolates was done by MALDI-TOF MS, following the protocol provided by the manufacturer of the Vitek MS (Biomérieux, Marcy l'Etoile, France). Based on that protocol, yeast cells were grown on Sabouraud dextrose agar (SDA) medium plates for 24 hrs, at 37°C. A loopful (1 mL) of yeast cells was then directly transferred from the culture medium onto each position of the 48-well flex target plate, and 0.5 mL of 25% formic acid was immediately mixed with the yeast. After evaporation, 0.5 mL of matrix solution (75 mg/mL 2, 5-dihydroxybenzoic acid in ethanol/water/acetonitrile [1:1:1] with 0.03% trifluoroacetic acid) was added and gently mixed. This was followed by the air drying of all sample mixtures at room temperature. Isolates were then smeared in duplicates on a target plate, and analyses were performed on a Vitek MS (Biomérieux, Marcy l'Etoile, France) equipped with a nitrogen laser (337 nm). The mass range from 2,000 to 20,000 Da was recorded by using the linear mode.

An *Escherichia coli* ATCC 8739 strain was used for external calibration of the spectra. *C. albicans* ATCC 90028, *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were used as quality control strains. Spectra were exported to the SARAMIS TM software package where the final identifications were achieved. Cluster analysis of the MALDI-TOF ICMS mass spectral data was performed using the SARAMIS TM database by comparing database peak lists of individual samples with Super Spectra and/or reference spectra.

2.2.3. Antifungal Susceptibility Testing

The antifungals used for susceptibility testing were: Ketoconazole (Sigma Aldrich, China), Nystatine (Sigma Aldrich, China), Amphotericin B (Dominique Dutscher), Itraconazole (Sigma Aldrich, China), Voriconazole (Acros organics, China), Clotrimazole (Acros organics, China).

In vitro antifungal susceptibility testing was performed according to the protocol proposed by the Clinical and Laboratory Standards Institute (CLSI) M27-A3 and M27-S4, with slight modifications. A volume of 100 µL of SDB was introduced in each well of a 96-well microtitre plate, followed by 100 µL of the stock solutions of the samples in the first wells of each column. By use of a 12-channel pipette and sterile tips, two-fold serial dilutions were made in the other wells while maintaining their volumes at 100 µL. This was done in order to attain concentrations of the antifungals ranging from 4 – 0.03 µg / mL. A volume of 100 µL of inoculum with a concentration of 1.5×10^4 CFU / mL was

again introduced into each well, followed by the incubation of the microtitre plates at 35°C for 48 hrs. Positive and negative control wells were also set apart; the negative control wells contained a mixture of SDB and DMSO (9: 1 V / V) only, while the positive control wells contained the culture medium and the test sample. After incubation, fungal growth was revealed by observing the turbidity at the bottom of the wells and comparing it to that of the negative controls. The tests were performed in duplicates and the MIC was defined as the lowest concentration of antifungal, for which we had no growth visible to the naked eye.

The minimum inhibitory concentration (MIC) results for amphotericin B (AMB), itraconazole (ICZ) and voriconazole (VOR) were interpreted according to the updated epidemiological cut-off values (ECVs) recommended by the Clinical and Laboratory Standards Institute (Table 2) [15]. The interpretative clinical breakpoint values for ketoconazole (KET), clotrimazole (CLT) and nystatin (NYS) have not been defined by the CLSI, thus, the breakpoints stated by Costa *et al*, Mulu *et al*, and Nenof *et al*. (table 1) [16-18], were used.

Table 1. Clinical breakpoints used for interpretation of MICs.

Antifungal agents	MIC breakpoint (µg/mL)	
	S	R
Ketoconazole (KET)	---	≥4
Clotrimazole (CLO)	≤ 0.5	≥1
Nystatin (NYS)	≤ 1.25	---

---: non-published.

When the MIC was less than or equal to the susceptibility breakpoint, the yeasts were considered susceptible to the antifungal, and if the MIC was greater than the susceptibility breakpoint, they were resistant [19].

Table 2. ECVs for in vitro susceptibility testing of *C. glabrata* isolates [15].

Antifungal agent	ECV (µg/mL)	
	WT	NWT
Amphotericin B (AMB)	≤ 2	> 2
Itraconazole (ICZ)	≤ 4	> 4
Voriconazole (VOR)	≤ 0.25	> 0.25

WT: Wild type NWT: Non-Wild Type.

Strains were considered to be wild type (lack mutational or acquired resistance mechanisms) if they had MIC values for AMB, ICZ, VOR to be ≤ 2, ≤ 4 and ≤ 0.25 respectively, and non-wild type (have mutational or acquired resistance mechanisms) if their MICs were >2, >4 and >0.25 respectively.

3. Results

3.1. Patients' Socio-demographic Characteristics

From March 2017 to September 2019, a total of 490 participants were recruited into our study. This included 323 (65.92%) HIV-infected patients, 80 (16.33%) diabetic patients, 73 (14.90%) pregnant women, and 14 (2.86%) participants who had none of the aforementioned conditions. Most

(81.43%) of the participants were of the female gender, while only 90 participants (18.37%) represented the male gender. The HIV-infected participants' group was the most (65.92%) represented in our study compared to other groups. This was followed by the group of diabetic patients with a percentage of 16.33% (80 participants). The least represented group was that of those who had neither HIV, diabetes nor pregnancy. They constituted just 2.86% (14 participants) of our study population. Participants' ages ranged from 5 to 81 years, with a mean age of 45.12 ± 13.41 . Among HIV-infected participants, 6.07% of them had CD4 counts lower than 200 counts/ μ l (Severe immune depression), 44.39% had CD4 counts ranging between 200 and 499 counts/ μ l (Advanced or moderate immune depression), while 49.53% had CD4 counts equal to or higher than 500 counts/ μ l (normal). Among the participants who were diabetic in our study (80), a greater percentage (64.86%) were hyperglycemic (glycemia > 1.27 g/l), while 4.05% were hypoglycemic (glycemia < 0.6 g/l). The

rest of the patients (17.57%) had a normal glycemia ($0.6 \leq$ glycemia ≤ 0.6 g/l). Most diabetic participants (53.75%) suffered from type 2 diabetes, while the rest (53.75%) suffered from type 1 diabetes.

3.2. Distribution of *Candida glabrata* Sensu Lato Isolates

From our study population, a total of 1551 samples were collected. These included 460 (29.66%) urine samples, 266 (17.15%) stool samples, 335 (21.60%) cervico-vaginal swabs (CVS) and 490 (30.78%) oro-pharyngeal swabs (OPS). The combined use of conventional identification tools permitted us to identify 54 *C. glabrata* sensu lato isolates, among which 20 (37.04%) were from cervico-vaginal swabs (CVS), 9 (16.67%) from oropharyngeal swabs (OPS), 16 (29.63%) from stool (S), and 9 (16.67%) from urine (U). This distribution was as seen in table 3 below.

Table 3. Distribution of *Candida glabrata* sensu lato isolates.

	CVS	OPS	S	U	Total	Percentage (%)
HIV-infected patients	9	0	9	4	22	40.74
Diabetic patients	1	5	2	3	11	20.37
Prenant women	9	4	2	2	17	31.48
Others	1	0	3	0	4	7.41
TOTAL	20	9	16	9	54	100.00
Percentage (%)	37.04	16.67	29.63	16.67	100.00	

C. glabrata sensu lato isolates were more abundant in cervico-vaginal swabs (37.04%), followed by stool samples (29.63% of all isolates).

3.3. Non-Detection of *C. nivariensis* and *C. bracarensis* Within the *C. glabrata* Sensu Lato Isolates

The molecular analysis of our *C. glabrata* sensu lato isolates by MALDI-TOF, permitted us to identify 54 (100%) *C. glabrata*, 0 (0%) *C. nivariensis*, and 0 (0%) *C. bracarensis* isolates as shown in table 4 below.

Table 4. MALDI-TOF identification of isolates.

	CVS	OPS	S	U	Total	Percentage
<i>C. nivariensis</i>	0	0	0	0	0	0%
<i>C. bracarensis</i>	0	0	0	0	0	0%
<i>C. glabrata</i>	20	9	16	9	54	100%

3.4. Antifungal Susceptibility Profile of *Candida glabrata* Sensu Stricto Isolates

The susceptibility profile of our isolates was as seen in table 5. 95.35% of our isolates were resistant to CLO (range of MICs: 0.25 - >4 μ g/mL), while 32.56% were resistant to KET (range of MICs: 0.03 - >4 μ g/mL). 39.53% susceptibility was also noticed for NYS (range of MICs: 0.06 - 4 μ g/mL). On the other hand, ECVs showed that AMB (range of MICs: 0.03 - 4 μ g/mL) and ICZ (range of MICs: 0.25 - 4 μ g/mL) had very good activities against our isolates, as 81.4% of the isolates were wild type for both antifungals. On the other hand, VOR (range of MICs: 0.125 - >4 μ g/mL) showed a poor activity, as most of our isolates (93.02%) were non-wild type for the antifungal.

Table 5. Susceptibility profile of isolates.

Antifungals	MIC range (μ g/mL)	Geometric Mean	Number (percentage) of isolates N (%)			
			CBPs		ECVs	
			S	R	WT	NWT
AMB	0.03 - 4	1.05	-----	-----	35 (81.4%)	8 (18.60%)
ICZ	0.25 - 4	3.11	-----	-----	35 (81.4%)	8 (18.60%)
VOR	0.125 - >4	1.65	-----	-----	3 (6.98%)	40 (93.02%)
KET	0.03 - >4	0.87	-----	14 (32.56%)	-----	-----
CLO	0.25 - >4	2.30	2 (4.65%)	41 (95.35%)	-----	-----
NYS	0.06 - 4	1.46	17 (39.53%)	-----	-----	-----

ECVs: Epidemiological cut-off values; CBPs: clinical breakpoints; WT: wild type; NWT: non-wild type; S: susceptible; R: resistant; -----: non-published.

4. Discussion

The treatment of infections due to *Candida glabrata* sensu lato species is very challenging to health personnel who work in low-income countries. This has partly been attributed to wrong identifications due to the lack of appropriate diagnostic tools. Most health centers depend on conventional methods based on phenotypic or biochemical characteristics, which are often times insufficient to provide an accurate identification of the *Candida* species responsible for infection. Epidemiological updates on new or emerging *Candida* species within a geographical area could be clinically relevant, as they could help to enhance treatment strategies, since different *Candida* species often differ in both virulence activity, and in the spectrum of antifungal resistance. Consequently, the lack of specific microbiological data could force physicians to empirically treat life-threatening mycoses with broad-spectrum antifungal medications, which would impact existing issues with antifungal resistance. In this light, this study aimed at identifying *Candida glabrata* cryptic species among 54 clinical isolates of *Candida glabrata* sensu lato from the west region of Cameroon and to evaluate their susceptibility pattern to some antifungals.

The MALDI-TOF mass spectrometry has emerged in the last decade, as a powerful tool for the identification of cryptic species, including *C. glabrata* cryptic species [7]. The use of this technique to identify the various *C. glabrata* cryptic species involved in *C. glabrata* sensu lato infections in the West Region of Cameroon, showed that all the 54 isolates taken into consideration were all *C. glabrata* stricto sensu isolates.

After analysing a diverse collection of clinical *C. glabrata* sensu lato isolates, *C. nivariensis* and *C. bracarensis* were not detected, showing that these two cryptic species may not be clinically important in the West Region of Cameroon. This finding is consistent with recent studies including that of Asadzadeh *et al.* [11], which found only *C. glabrata* sensu stricto isolates among a large collection of 440 clinical *C. glabrata* sensu lato isolates in Kuwait. It is also different from findings in other two recent studies conducted in Africa, which revealed the presence of *C. nivariensis* in Burkina Faso and both *C. nivariensis* and *C. bracarensis* in Egypt [13, 20]. In many studies conducted to determine the prevalence of *C. glabrata* cryptic species among a total of 2560 *C. glabrata* sensu lato isolates in eight countries or geographical locations, failed to identify either *C. nivariensis* or *C. bracarensis*, showing that these species are rare yeast pathogens in many countries or geographical locations [11]. Similar to the findings of other studies carried out in Cameroon, *C. glabrata* sensu stricto remains the only cryptic species of the *C. glabrata* complex identified from a collection of clinical isolates [21-25].

With the continuous variation in the antifungal susceptibility profile of *Candida* isolates, in vitro antifungal susceptibility testing has been often used to select antifungal agents with a good activity against a given *Candida* infection. It has also been prominently used in the identification of antifungal agents that would not be effective in treatment [26].

Our antifungal susceptibility test conducted in this study reveals a very high resistance profile of our isolates to clotrimazole (95.35%), and a 93.02% non-wild type phenotype to voriconazole, with varying MIC ranges of 0.25 - >4 µg/mL and 0.125 - >4 µg/mL respectively. These results accord with many other Cameroonian studies and non-Cameroonian studies which have revealed a high resistance rate of clinical *C. glabrata* sensu stricto isolates to the azoles in general, especially fluconazole [21-24]. Pfaller *et al.* revealed that cross-resistance of *C. glabrata* sensu stricto isolates to azoles, especially voriconazole, is also very common [26]. Despite the introduction of new antifungal agents to fight *Candida* infections, antifungal resistance continues to grow among *C. glabrata* isolates, thereby complicating patient management [26]. Our study equally reveals an 18.60% non-wild type profile for amphotericin B. This finding is in accordance with many other recent findings, which have described resistance of clinical *C. glabrata* isolates to polyenes, including amphotericin B [27-29].

Eventhough very high resistance rates and non-wild type phenotypes have been observed among our isolates, our study equally reveals very good activities of amphotericin B (range of MICs: 0.03 - 4 µg/mL) and itraconazole (range of MICs: 0.25 - 4 µg/mL) against our isolates, as 81.4% of the isolates were wild type for both antifungals. These results are similar to results obtained in a recent study in Yaoundé (Cameroon) by Ngouana *et al.* [25], which showed a high susceptibility rate of *C. glabrata* isolates to itraconazole and amphotericin B.

5. Conclusion

Our study highlights the fact that *C. nivariensis* and *C. bracarensis* remain very rare cryptic species, and that *C. glabrata* sensu stricto remains the most isolated species in the *C. glabrata* complex in the West region of Cameroon. Also, the high susceptibility rate of *C. glabrata* sensu stricto isolates to amphotericin B and itraconazole, suggest that these 2 antifungals could serve as potential drugs for the treatment of *C. glabrata* sensu stricto infections in the west region of Cameroon. The number of isolates used in this study was not enough to conclude that *C. nivariensis* and *C. bracarensis* isolates are absent in the West Region or other parts of Cameroon, so we recommend that other similar studies be carried out on a larger number of isolates to increase chances of detecting any of these emerging species that could possibly be circulating in Cameroon.

Contributions of the Authors

JPD and CBT participated in the design of the study. CN, AND, CLK, AIE collected the data. JPD and CN contributed to manuscript design. All authors contributed to the review and approved the final version.

Conflicts of Interest

The authors declare that they have no competing interests.

Acknowledgements

Dzoyem J. P. is thankful to "The World Academy of Sciences (TWAS) for funding this work through the TWAS Research Grant Agreement N° 17-380 RG/BIO/AF/AC_I-FR3240297751.

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