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Abstract

Non-*albicans Candida* (NAC) species have emerged as potent pathogenic yeasts among HIV-infected patients. Authors evaluated the epidemiology and antifungal susceptibility testing of non-*albicans Candida* species colonizing Yaoundé (capital of the Republic of Cameroon, Central Africa) HIV-infected patients.

The mucosal specimens were collected and submitted to the mycological diagnosis. Yeast isolates were identified by the Matrix Assisted Laser Desorption Ionisation - Time of Flight Mass Spectrometry (MALDI-TOF MS). The antifungal susceptibility testing was achieved by the CLSI-M27 protocols, and the interpretation of clinical break points (CBPs) and epidemiological cutoff values were in accordance with the CLSI-M60 and M59 recommendations.

402 patients were recruited and 1218 samples collected. The colonisation frequency was 24.1 % and 304 yeasts isolated. Yeast isolates were 113 (37.2%) *C. albicans,* 2 (0.7 %) *C. africana* and 172 (56.6 %) NAC isolates. The NAC isolates were grouped into 13 species including *C. krusei* (18.1 %)*, C. glabrata* (10.9 %)*, C. tropicalis* (8.5 %) and *C. parapsilosis* (5.9 %) as the major ones*.* All the isolates appeared to be wild-type for amphotericin B and itraconazole. One (1/33) isolate of *C. glabrata* was resistant to fluconazole. *C. parapsilosis* isolates appeared all susceptible to fluconazole. *C. tropicalis* isolates presented 50 % (13/26) resistance to fluconazole.

The achieved results bring out new insights about epidemiology of NAC species in Cameroon. The results also highlight the resistance of NAC species to current antifungal drugs.

Key words: Non-*albicans Candida* species, MALDI-TOF, Antifungal, HIV-patients, Cameroon.

1. Introduction

Yeast infections caused by *Candida* species are the most frequent opportunistic infections among HIV infected patients and extend from superficial to systemic [1-4]. Although *Candida albicans* remains the most frequently isolated agent from candidiasis, non-*albicans Candida* (NAC) species now account for a substantial part of clinical isolates collected worldwide [1,5,6]. The most current species include *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, and *Candida krusei* [7,8]. The less-frequent are *Candida guilliermondii, Candida lusitaniae, Candida kefyr, Candida famata*, *Candida inconspicua*, *Candida rugosa* and *Candida norvegensis* [7-9]. The frequency is highly region-dependent. *C. glabrata* is more frequent in North America than in Latin America, *C. tropicalis* is frequently isolated in Asia-Pacific and less in the rest of the world, whilst *C. parapsilosis* remains 3-fold more commonly recovered in North America than in Europe. *C. guilliermondii* and *C. rugosa* are more frequent in Latin America, and *C. inconspicua* and *C. norvegensis* in Europe than in the rest of the world [1,8,9].

Although biochemical profile-based and time-consuming microscopy assisted methods have dominated mycology diagnostic laboratories for many years, proper species identification is sometimes hindered by several factors, such as complex taxonomy, genetic relatedness of species, and misleading microscopic evidences. In the past few years, the MALDI-TOF mass spectrometry has revolutionized the medical microbiology [8], enabling rapid and accurate fungal species identification (within few minutes) with simple and reliable procedures [10]. Since its first application to yeast identification [11], there has been a growing body of

evidence indicating that microbial fingerprint by MALDI-TOF can represent a robust and fast tool for routine identification of clinically important fungi [12–20].

The treatment of candidiasis due to NAC species is currently done with antifungal agents belonging to three families including polyenes, azoles, and echinocandins [21]. Nowadays, most of the NAC species exhibit particular patterns of primary resistance or reduced susceptibility toward these antifungals. In fact, a high level of resistance against azoles was observed for *C. krusei*, *C. inconspicua*, *C. rugosa*, and *C. norvegensis* [21]. In addition, a decreased susceptibility of *C. glabrata*, *C. parapsilosis* and *C. guilliermondii* to echinocandins was observed [22]. In a recent study by Castanheira et al. involving 3,557 invasive yeasts and moulds collected in 29 countries worldwide during 2014-2015, Epidemiological cutoff values (ECVs) published in the CLSI M59 document were applied for species with no clinical breakpoints. Their results stated that echinocandins susceptibility rates were 95.9 % to 100 % for the 5 most common *Candida* species except anidulafungin and *C. parapsilosis* (88.7 % susceptible/100 % wild-type). Fluconazole resistance was 8 % for *C. glabrata.* Azole resistance among *C. tropicalis* and *C. parapsilosis* and echinocandin resistance in *C. krusei* was higher in Denmark compared to other regions [23].

For a global view of *Candida* distribution and their antifungal susceptibility profile, almost all the countries should contribute by publishing their local statements. However, such information about isolates from sub-Saharan region, especially in Cameroon is scarce. Authors investigated the NAC species distribution isolated from Yaoundé HIV infected patients by MALDI-TOF MS as well as the antifungal susceptibility of these isolates to some antifungal drugs.

2. Materials and methods

2.1. Ethical considerations and enrollment of participants

The survey was carried out at the Yaoundé Central Hospital (capital of the Republic of Cameroon, Central Africa), and involved HIV-infected patients presenting or not clinical signs of any mucosal candidiasis. This study was approved by the Cameroonian National Ethical Committee (N°128/CNE/CNM/2011). The survey took place from January 2012 to October 2013. Patients enrolled for this study were HIV-infected individuals of both genders, between 18 and 66 years old, who did not receive any antifungal treatment during the last 3 months preceding their enrollment. The purpose of the study and potential benefits were explained to patients, and those willing to participate were required to sign a written inform consent form prior to their registration as participant.

2.2. Determination of clinical status of patients and collection of study samples

Prior to sample collection, the patient's information was registered including: age and HIV status (CD4+ count, type of HIV, stage of the HIV infection, antiretroviral therapy). Samples collected included: Vaginal discharge, oropharyngeal swab, stools and urine.

2.3. Mycological diagnosis

Samples were submitted to direct macroscopic and microscopic analyses using routine laboratory protocols prior to culture on Sabouraud chloramphenicol medium for 24 to 48 hours at 37°C. Colonization was identified as described by Bouchara et al. [24] in urine (culture ≥ 10^4cfu/ml), in vaginal collections (culture ≥ 10 colonies), in oropharyngeal mucosae (culture ≥ 5-10 colonies/cm^2) and in stool (culture ≥10^4cfu/g of stool).

Primary identification was assessed by combination of culture on chromogenic medium (ChromID CAN2 from Biomerieux, Marcy l'Etoile, France), germ tube test, evidence of

chlamydospores production and biochemical analysis (ID32C kit from Biomerieux, Marcy l'Etoile, France). Reference *C. albicans* ATCC90028 strain was used throughout the mycological diagnosis as quality control strain.

2.4. Identification by Mass Spectrometry

The second identification of yeasts isolates by the MALDI-TOF was achieved as described by the manufacturer of the Vitek MS (Biomerieux, Marcy l'Etoile, France). Briefly, yeast cells were grown on Sabouraud dextrose agar medium plates for 24h, at 37°C. A loopful (1µL) of yeast cells was directly transferred from the culture medium onto each position of the 48-well flex target plate, and 0.5µL of 25 % formic acid was immediately mixed with the yeast. After evaporation, 0.5µL matrix solution (75mg/mL 2,5-dihydroxybenzoic acid in ethanol/water/acetonitrile [1:1:1] with 0.03 % trifluoroacetic acid) was added and gently mixed. All sample mixtures were air dried at room temperature. Each isolate was spotted in duplicate. Analyses were performed on a Vitek MS (Biomerieux, Marcy l'Etoile, France) equipped with a nitrogen laser (337nm). 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™ software package where the final identifications were achieved. Cluster analysis of the MALDI-TOF ICMS mass spectral data was performed using the SARAMIS™ database by comparing database peak lists of individual samples with Super Spectra and/or reference spectra.

2.5. Antifungal susceptibility testing

The antifungal susceptibility testing was assessed as described by the CLSI M27-S4 protocol [25]. Amphotericin B, fluconazole, and itraconazole provided by Sigma-Aldrich (USA) were serially diluted in a 96 wells microtiter plate with RPMI 1640 (Sigma Aldrich) broth medium. Final range concentrations were as follow: Amphotericin B (0.0313-16µg/mL), fluconazole (0.125-64µg/mL), and itraconazole (0.0313-16µg/mL). A standardized fungal inoculum (spectrophotometrically calibrated) was then added into each well to yield 0.5 to $2.5x10^3$CFU/mL in 200µL as final volume. Plates were incubated at 37°C for 24 hours and results read afterwards. *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were used as quality control strains.

3. Results

3.1. Patients characteristics

From January 2012 to October 2013, 402 patients were included in the study. They were 306 (76.1 %) females and 96 (23.9 %) males. The mean age was 40.2±9.5 years with extremes at 23 and 66 years. Only one patient was infected by two types of HIV strains (HIV 1-M and HIV 1-O), the others were all infected by the HIV 1-M strain. Among the 402 included patients, 250 (62.1 %) were colonized by yeasts on at least one of the collected sites. Non-*Candida* isolates were obtained from 16 patients and *Candida* species isolated from 232 (57.6 %) patients. The mean body mass index (BMI) of patients colonised by *Candida* was 25.0±5.2. The mean CD4+ count among patients colonised by *Candida* species was 371±222 CD4/mm^3 with extremes at 34 and 1127 CD4/mm^3. There was no relationship between CD4 counts and *Candida* colonisation (p-value >0.05), results are presented in table 2.

3.2. Mycological diagnosis and yeasts identification

Candida colonisation was observed in 88.9 % of HIV patients naïve to antiretroviral therapy and was also observed in 59. % of patients on antiretroviral therapy. Statistical analysis showed that there is no relationship between *Candida* colonisation and antiretroviral therapy (p-value >0.05).

From the 402 recruited patients, 1218 samples were collected and analysed, including 322 oropharyngeal swabs, 262 vaginal swabs, 308 stools and 326 urine samples. The table 3 contains results of colonisation at each collected site. When considering collected samples, the frequency of colonisation is 24.1 %. The intestinal tract was the most colonized site, followed by buccal mucosae.

The use of identification tools allowed identification of 304 yeast isolates, from which 113 (37.2 %) *C. albicans,* 2 (0.7 %) *C. africana,* 172 (56.6 %) NAC isolates, 15 (4.9 %) non *Candida* yeasts species, and 2 (0.7 %) non identified yeasts as presented in table 4. The most important NAC species were *C. krusei* (18.1 %)*, C. glabrata* (10.9 %)*, C. tropicalis* (8.5 %) and *C. parapsilosis* (5.9 %)*. C. krusei* and *C. parapsilosis* were mostly identified in stools. *C. glabrata* was important in vaginal collections and *C. tropicalis* had a high frequency in urine samples. There were patients with two or more species in a particular sample. *C. albicans* was associated with *C. krusei* (40 %), *C. glabrata* (13.3 %), *C. parapsilosis* (6.7 %) and *C. rugosa* (6.7 %).

3.3. Antifungal susceptibility testing

The MIC results were interpreted according to the updated clinical breakpoints (CBPs) recommended by the Clinical and Laboratory Standards Institute [26] or epidemiological cut-off values (ECVs) [27-30] as presented in table 1.

All the NAC isolates appeared to be wild-type for amphotericin B and itraconazole, where ECVs have been established. CBPs for fluconazole have been published only for *C. glabrata, C. parapsilosis* and *C. tropicalis* amongst NAC species [31]. Isolates of *C. krusei* are assumed to be intrinsically resistant to fluconazole [25]. One (1/33; 3.0 %) isolate of *C. glabrata* was resistant to fluconazole. *C. parapsilosis* isolates appeared all susceptible to fluconazole. *C. tropicalis* isolates presented 50 % (13/26) resistances to fluconazole (table 5).

4. Discussion

The very nature of infectious diseases has undergone profound changes in the past few decades. Hitherto unknown microorganisms with no pathogenic role have emerged as important causes of morbidity and mortality worldwide. In recent years, *Candida* spp. have emerged as principal pathogens of a variety of human infections [28-31]. In the present study, data for the population characteristics are close to those published for HIV infected patients in sub-Saharan region in general and particularly in Cameroon [32-38].

The study of mucosal *Candida* infection among HIV infected patients is mostly limited to oropharyngeal location as it is a major indicator of the AIDS state [32-38]. However, all the human mucosae can be affected by *Candida* species. We found that the intestinal mucosa is more affected than others (41.5 %) and this result was different from findings by Khan et al. [39] who obtained 5 % of gastro-intestinal colonization in their study. These later also obtained 65 % of oral colonization [39] whilst we found 28.6 % of oro-pharyngeal colonization in our study. Merenstein et al. [33] obtained 18.8 % of vaginal colonization among HIV infected patients, which is not far from our own findings (14.5 %). Mukasa et al [40] obtained 45.4 % of vaginal candidiasis in Uganda. Another study in Ethiopia stated 41 % of vaginal candidiasis in their study population [41]. In a recent study in South Africa, authors

obtained following features; candiduria 46.5 %, vaginal colonization 30.6 %, oral colonization 11 % [42]. In Cameroon, Lohoue et al. [43] identified candiduria in 36.2 % of HIV infected patients. A team in Buea found 18.2 % of *Candida* urinary tract colonization among 207 HIV positive patients [44].

From the 1218 analyzed samples, we identified 304 yeast isolates. NAC species represented 56.7 % of *Candida* isolates with one of the most important being *C. krusei* (18.1 %). This is of particular interest since it is rare to observe *C. krusei* at a percentage in humans. A study in South America established the presence of *C. krusei* among 30 % of the population [45]. Of the total 132 neonates included in a study by Juyal et al. [46], NAC species were responsible for 80.3 % candidemia cases with *C. parapsilosis* (25.0 %) and *C. tropicalis* (22 %) as the most predominant species. Mohandas and Ballal [47] when studying a total of 111 isolates of *Candida* species found NAC species with 60.4 %; they also observed *C. krusei* (50.0 %) being commonly isolated in urinary tract of hospitalized patients, followed by *C. albicans* (25.0 %). When evaluating the prevalence of *Candida* species among patients with vulvo-vaginal candidiasis, Kumari et al. [48] obtained 32.4% *C. albicans*, 45.1 % *C. parapsilosis* and 22.5 % of *C. glabrata.* Another study obtained 41.4 % of NAC species from vaginal isolates [41]. Although *C. albicans* is the most prevalent species involved in fungal infections, the incidence of infections due to NAC species is increasing, as we observed in the present work. This changing in epidemiology could be associated with severe immunosuppression or illness, exposure to broad-spectrum antibiotics and intensive use of azoles that are less effective against NAC species [49,50].

The Clinical and Laboratory Standards Institute (CLSI) developed new *Candida* species-specific clinical breakpoints (CBPs) for fluconazole, voriconazole, and echinocandins [25,51].

A recent report indicated that resistance to azoles and echinocandins of *Candida* species may be increased using the new CLSI CBPs [52,53]. Therefore, the new CBPs of the CLSI may be applied to antifungal susceptibility studies as sensitive tools for detecting emerging resistance in *Candida* isolates [25]. We applied new species-specific CLSI CBPs associated to epidemiological cut-off values to determine the antifungal susceptibility of *Candida* species isolates in Cameroon.

All the isolates appeared to be wild-type for amphotericin B. This is in accordance with previous studies [53,54]. Till date, very few species have developed reduced susceptibility to amphotericin B [55,23]. Khotari et al. [56] from North India reported the susceptibility profile of *Candida* isolates as 92% were sensitive to amphotericin B, 36 % to fluconazole, and 24 % to itraconazole. *C. tropicalis* isolates presented 50 % (13/26) resistance to fluconazole. Xiao et al. [57] obtained 11.2 % of resistance by *C. tropicalis* isolates to fluconazole in China. We obtained 3.0 % resistance to fluconazole by *C. glabrata,* while Castanheira et al. [23] observed 8.0 %.

In the present study, we established the *Candida* species distribution amongst Yaoundé HIV infected patients. The achieved results bring out new insights about epidemiology of *Candida* in Cameroon as well as their antifungal susceptibility profiles. The susceptibility profiles of those isolates to other antifungal drugs need to be carried out to give a global idea about the behavior of these isolates to antifungal drugs.

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6. Conflict of interest

No conflict of interest to be mentioned

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Table 1: Epidemiological cut-off values and clinical breakpoints for antifungal agents and non-*Candida albicans Candida* species determined by 24 hours CLSI broth microdilution method [25-30]

Organism	Antifungals	CBPs (µg/mL)				ECVs (µg/mL)	
		S	SDD	I	R	WT	non-WT
C. glabrata	Fluconazole		≤32		>32	≤32	>32
	Itraconazole					≤4	>4
	Amphotericin B					≤2	>2
C. parapsilosis	Fluconazole	≤2	4		≥8	≤2	>2
	Itraconazole					≤0.5	>0.5
	Amphotericin B					≤2	>2
C. tropicalis	Fluconazole	≤2	4		≥8	≤2	>2
	Itraconazole					≤0.5	>0.5
	Amphotericin B					≤2	>2
C. krusei	Fluconazole					≤64	>64
	Itraconazole					≤1	>1
	Amphotericin B					≤2	>2
C. lusitaniae	Fluconazole					≤2	>2
	Itraconazole					≤1	>1
	Amphotericin B					≤2	>2
C. guilliermondii	Fluconazole					≤8	>8
	Itraconazole					≤1	>1
	Amphotericin B					≤2	>2
C. kefyr	Fluconazole					≤1	>1
	Itraconazole					NP	NP

	Amphotericin B	NP	NP

ECVs, epidemiological cut-off values; CBPs, clinical breakpoints; WT, wild type; non-WT, non-wild type; S, susceptible; SDD, susceptible, dose dependent; I, intermediate; R, resistant; NP, non-published.

Table 2: Distribution of *Candida* colonisation according to the CD4+ count as defined by the WHO classification [35].

	CD4+ count (/mm^3)			
	<200 (severe ID)	200-349 (advanced ID)	350-499 (moderated ID)	≥500 (no ID)
Candida + (%)	53.8	65.8	65.6	57.5
Candida - (%)	46.2	34.2	34.4	42.5

ID = immuno-depression; *Candida* +, positive to colonisation by *Candida; Candida*-, negative to *Candida* colonisation.

Table 3: Distribution of *Candida* colonisation in function of collected site.

	Mouth	Vagina	Stool	Urine	Total
Number of collected samples	322	262	308	326	1218
Number of colonised samples	92	38	128	36	294
Frequency of colonisation (%)	28.56	14.5	41.5	11.0	24.1

Table 4: Distribution of isolates at different collection sites

Species	Collection site					
	Mouth	Vagina	Stool	Urine	Total	Percentage (%)
C. africana	0	2	0	0	2	0.7
C. albicans	44	22	40	7	113	37.2
C. famata	0	0	1	0	1	0.3
C. glabrata	3	10	14	6	33	10.9
C. guilliermondii	1	0	0	0	1	0.3
C. intermedia	0	0	0	2	2	0.7
C. kefyr	2	2	2	2	8	2.6
C. krusei	7	7	37	4	55	18.1
C. lusitaniae	0	0	3	0	3	1.0
C. norvegensis	0	0	3	0	3	1.0
C. parapsilosis	5	2	8	3	18	5.9
C. rugosa	3	0	9	2	14	4.6
C. sake	4	0	0	2	6	2.0
C. tropicalis	3	7	9	7	26	8.6
C. valida	0	0	2	0	2	0.7
Cryptococcus sp.	0	0	2	0	2	0.7
Kodamaea ohmeri	1	0	0	0	1	0.3
Debaryomyces	0	0	2	0	2	0.7

etchelsii						
S. cerevisiae	0	0	4	0	4	1.3
Trichosporon asahii	0	0	2	0	2	0.7
Trichosporon inkin	0	0	3	1	4	1.3
non identified	0	0	2	0	2	0.7
Total					304	100.0

Table 5: *In vitro* activities of antifungal agents against non-*albicans Candida* isolates collected from mucosae of HIV infected patients at the Yaoundé Central Hospital

Species (Number of isolates)	Antifungals	MIC range (µg/mL)	Percentage (Number) of isolates				
			CBPs[a]			ECVs[a]	
			S[b]	SDD[b]	R[b]	WT[b]	NWT[b]
C. famata (1)	Fluconazole	0.25					
	Itraconazole	0.03					
	Amphotericin B	0.25					
C. glabrata (33)	Fluconazole	0.25-64	97.0 (32)		3.0 (1)		
	Itraconazole	0.03-8				97(32)	3.07 (1)
	Amphotericin B	0.5-2				100 (33)	0 (0)
C. guilliermondii (1)	Fluconazole	1				100 (1)	0 (0)
	Itraconazole	0.125				100 (1)	0 (0)
	Amphotericin B	1				100 (2)	0 (0)
C. intermedia (2)	Fluconazole	2					
	Itraconazole	0.06					
	Amphotericin B	2					
C. kefyr (8)	Fluconazole	0.25-0.5				100 (8)	0 (0)
	Itraconazole	0.03-0.125					
	Amphotericin B	0.25-2					
C. krusei (55)	Fluconazole	4-32				100 (55)	0 (0)
	Itraconazole	0.03-0.06				100 (55)	0 (0)
	Amphotericin B	1-2				100 (55)	0 (0)
C. lusitaniae (3)	Fluconazole	1				100 (3)	0 (0)

	Itraconazole	0.06				100 (3)	0 (0)
	Amphotericin B	0.5				100 (3)	0 (0)
C. norvegensis (3)	Fluconazole	16					
	Itraconazole	0.03					
	Amphotericin B	1					
C. parapsilosis (18)	Fluconazole	0.25-1	100 (18)	0 (0)	0 (0)		
	Itraconazole	0.03-0.125				100 (18)	0 (0)
	Amphotericin B	0.125-1				100 (18)	0 (0)
C. rugosa (14)	Fluconazole	0.25-2					
	Itraconazole	0.03-16					
	Amphotericin B	1-2					
C. sake (6)	Fluconazole	0.25-1					
	Itraconazole	0.03					
	Amphotericin B	0.5-1					
C. tropicalis (26)	Fluconazole	0.25->64	46.17 (12)	3.87 (1)	50 (13)		
	Itraconazole	0.25->16				15.4 (4)	84.6 (22)
	Amphotericin B	1				100 (26)	0 (0)
C. valida (2)	Fluconazole	16					
	Itraconazole	0.25					
	Amphotericin B	2					

ECVs, epidemiological cut-off values; CBPs, clinical breakpoints; WT, wild type; non-WT, non-wild type; S, susceptible; SDD, susceptible, dose-dependent; R, resistant; [a]MICs are categorised according to published date; [b]percentages (number of isolates).