Journal of
Medical Microbiology & Diagnosis

Hana et al., J Med Microb Diagn 2020, 9:1

Original Paper Open Access

Characterization of the '*Candida albicans* Complex': First Report of *Candida africana* in Tunisia

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Abstract

Background: New molecular studies tools have recently recognized *Candida africana* as a new atypical strain of the '*C. albicans* complex' which consists previously of *C. albicans* and *C. dubliniensis* only. As currently available yeast identification tools fail to differentiate these species, their incidence is poorly understood in different geographical regions such as Tunisia. In this study, we analyzed the genetic diversity of the '*C. albicans complex*'.

Materiel and methods: We selected 105 isolates of *C. albicans* and *C. dubliniensis* from oral and vaginal samples. These yeasts were phenotypically identified by chlamydosporulation on Agar Tween and Sunflower medium and biochemical tests using Vitek 2 Compact. To confirm this identification, the *HWP1* gene was amplified by PCR using a single primer pair (CR-f/CR-r) for all isolates and sequenced for 16 strains.

Results: Out of 105 oral and vaginal swabs, conventional identification tools revealed 67 *C. albicans* and 38 *C. dubliniensis*. Screening of PCR products by electrophoresis gel highlights the presence of 64 *C. albicans*, 43 *C. dubliniensis* and one strain of *C. africana*. This is the first description of *this* species in Tunisia. Three cases of co-infection by *C. albicans* and *C. dubliniensis* were detected after molecular analysis.

Conclusion: *Candida albicans* is the major selected yeast in human fungal pathologies. The study highlights the need to discriminate between this species and its related subgroups which greatly facilitates early initiation of pathogen-specific therapy.

Keywords: *Candida albicans complex; Candida africana*; *HWP1* gene; PCR; Sequencing

Introduction

The frequency of fungal infections is considerably increased worldwide. This increasing is directly associated with the growing numbers of immune-compromised patients, the prolonged use of broad-spectrum antibiotics, and to the large use of invasive dispositive. These opportunistic mycoses are commonly caused by the genus *Candida spp* and *Candida albicans* is undoubtedly the most frequently reported species in clinical diagnostic laboratories [1,2]. It has been known for some time that *C. albicans* represents a 'complex' of genetically two different strains: *C. albicans* and *C. dubliniensis*. However, early work showed that species of the '*C. albicans complex*' were more genotypically heterogeneous and can be divided into three groups/strains. In 1993, "*C. africana*" was described as a new bio-variant of *C. albicans* [3-5]. Discrimination between species of the '*C. albicans* complex' needs the application of accurate and reliable tests demanding DNA analysis, such as DNA amplification by PCR with specific primers and DNA sequencing [4,6]. In Tunisia, the global epidemiology of candidiasis is still unclear and useful data on *C. albicans*, *C. dubliniensis*, and *C. africana* are scarce also adds to the misunderstanding of their impact on human pathology. In this study, we describe an essay to differentiate these three species which represent a daily challenge for the clinical diagnostic.

Materials and Methods

Phenotypic identification

We collected 105 isolates belonging to '*C. albicans* complex' and isolated from 53 vaginal swabs and 52 or pharyngeal swab in laboratory of parasitology of the Military Hospital of Tunis. All isolates were cultivated onSabouraud mediums with or without Actidione. Appearance of the colonies on CHROMagar *Candida* medium (CHROMagar, France) and assimilation profiles with the VITEK 2 compact system (bioMérieux, Marcy l'Etoile, France) were also recorded according to the manufacturer's instructions. Chlamydospors production was performed by culturing yeasts on Agar Tween 80' and on Sunflower at 27°C for 24 h both.

Molecular identification: *HWP1* gene amplification

Genomic DNA was extracted and the primer set CR-f 5′-GCTACCACTTCAGAATCATCATC-3′/CR-r 5′- GCACCTTCAGTCGTAGAGACG-3′ was selected to amplify the *HWP1* genes [4]. PCR reactions were performed in a final volume of 50 µl containing 100 ng of DNA, 10 mM dNTP, 5 ul of 10X Buffer, 25 pmol of each forward and reverse primer and 1U of Platinum Taq DNA polymerase (Invitrogen, USA). Amplification conditions were as follows: denaturation at 95°C for 5 min, 30 cycles of denaturation at 94 c for 45 s, primer annealing at 58°C for 40 s, and extension at 72°C for 55 s, followed by a final extension at 72°C for 10 min in a T personal thermal cycler (Bio-Rad, Italy). PCR products were separated and visualized on a 1.5% (wt/vol) agarose gel and visualized under UV light. A 100 bp molecular-size marker (Promega Co) was loaded to allow analysis of the PCR products.

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Received February 4, 2020; **Accepted** February 27, 2020; **Published** March 03, 2020

Citation: Hana S, Latifa M, Camilia C, Boutheina J (2020) Characterization of the '*Candida albicans* Complex': First Report of *Candida africana* in Tunisia. J Med Microb Diagn 9: 307.

HWP sequencing

Sixteen PCR products were sequenced using primers of the PCR amplification on ABI 3730 automatic sequencer (Applied Biosystems, Foster City, CA). Sequencing results were compared with *HPW1* gene sequences on the GenBank database.

Antifungal susceptibility

For one strain which was identified by PCR as *Candida africana*, antifungal susceptibility was determined in accordance with the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) using AST vitek 2 cards and E-test technique. CMI values for *C. albicans* were used as reference [7].

Results

Phenotypic identification

Out of our isolates, 104 produced chlamydospores *C. albicans* was the most common identified species (66 strains), representing 62.8% of the isolates, followed by *C. dubliniensis* (38 strains/36.2%). *C. albicans* was recovered especially from vaginal samples (42 isolates). Indeed, *C. dubliniensis* isolates were found primarily in oral specimens (27 isolates). Only one species, isolated from an oral sample, failed to produce chlamydospores but identified *C. albicans* by Vitek 2. It presents blue green colonies on the chromogenic medium as shown in Figure 1.

Molecular identification: *HWP1* gene amplification

Molecular identification, based on partial amplification of the *HWP1* gene, confirmed the genetic variability of these strains. Electrophoretic separation demonstrates the presence of different PCR profiles. *C. albicans* isolates were either homozygous at the *HWP1* locus with a single band at ~941 pb (49 strains) or heterozygous with two bands at 800 and 941 bp (15 strains). Forty three strains showed a single band of ~56 bp which correspond to *C. dubliniensis*. The chlamydospores-negative strain provided 700 bp DNA fragment specific for *C. africana* (Figure 2). Molecular analysis shows that five *C. dubliniensis* strains were misidentified as *C. albicans* by using conventional identification tools. In addition, three cases of co-infection by *C. albicans* and *C. dubliniensis* are proven in 3 oral samples by PCR amplification but weren't revealed by phenotypic identification (Figure 3). An overview of results obtained from the molecular analysis is given in Table 1 and Figures 2 and 3.

HWP1 gene sequencing

All sequenced PCR products show a high degree of similarity with other sequences of HPW 1 deposited on Genbank. The sequence of *C. africana* matches with other isolates recovered from *C. africana* and other atypical strains of *C. albicans*.

Antifungal susceptibility

The identified *C. africana* was susceptible (with low MICs) to all antifungal agents tested (Table 2).

Discussion

The '*C. albicans* complex' is one of the major fungal groups involved in human infections, which represents more than 50% of *Candidiasis* [2]. Species of this group are more often involved in vaginal and oral human infections [8,9]. Clinical routine identification of yeasts includes a series of classical tests based on phenotypic or

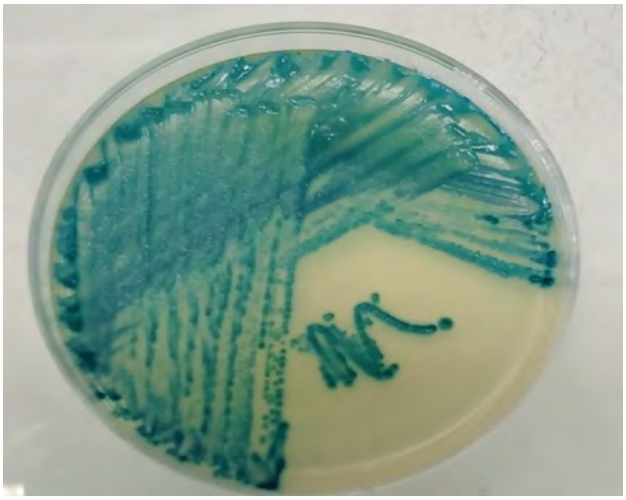

Figure 1: Culture of *Candida africana* on CHROMagar *Candida* medium.

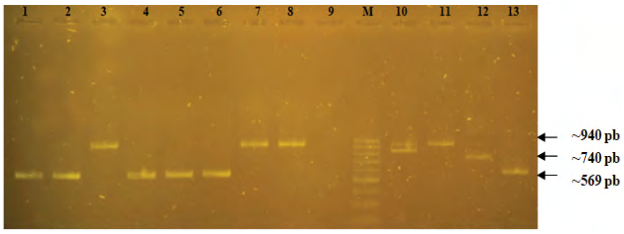

Figure 2: Agarose gel electrophoresis of amplified *PCR* products of '*Complex C. albicans' yeasts* by CR-f and CR-r primers. **Lanes: M**, 100 bp DNA size marker; **1, 2, 4, 5, 6, et 13:** *C. dubliniensis*; **3,7 et 8 et 11:** homozygous profiles of *C. albicans*; **10:** heterozygous profile of *C. albicans*; **12:** *C. africana*; **9:** negatif control.

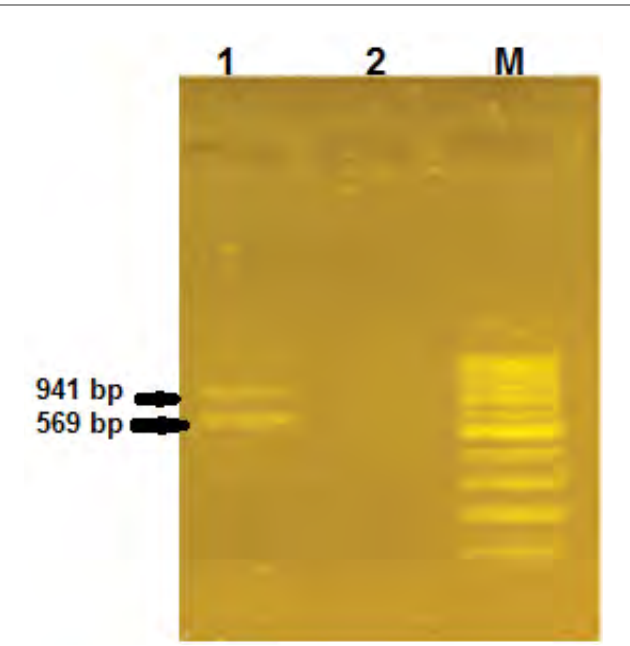

Figure 3: Agarose gel electrophoresis of amplified *HWP I gene showing the simultaneous presence of C. albicans and C. dubliniensis.* **Lanes: M**, 100 bp DNA size marker; **1:** PCR products of *C. albicans* and *C. dubliniensis*; **2:** negatif control.

Species	CSP (+/-)	Vitek 2	Conventional PCR	PCR product size (bp)	PCR sequencing
C. albicans	66 (+)	67	61	~ 941 (49)* ~ 800+941 (15)**	10
C. dubliniensis	38(+)	38	40	~ 569	5
C. africana	1(-)	0	1	~ 700	1
Association *C. albicans*+*C. dubliniensis*	0	0	3		0
Total	105	105	105	105	16
CSP: Chlamydospore; *C. albicans* homozygote ; **C. albicans* heterozygote					

Table 1: Results of identification techniques of *Candida* isolates examined in this study.

Technique	Vitek 2 compact	E test (MIC µg/ml)
Fluconazole	S	S (0.25)
Voriconazole	S	-
Amphotericin B	S	-
Flucytosin	S	S (0.094)
Caspofungin	S	S (0.064)
Micafungin	S	-
Anidulafungin	-	S (0.002)
S: Sensitive		

Table 2: Antifungal susceptibility of *Candida africana.*

Region (Total of strains)	Country	Source	Number of isolated *C. africana* strains	References
Africa (13)	Senegal	Vaginal	3	[14]
	Nigeria	Vaginal	2	[15]
	Cameroon	Vaginal	2	[1]
	Algeria	Vaginal	2	[13]
		Blood	1	
		Cerebral liquid	1	
		Buccal	1	
		Urine	1	
Europe (15)	United Kingdom	Vaginal	15	[10]
America (35)	Argentine	Vaginal	1	[2]
		Vaginal	1	[16]
	Colombia	Nosocomial	11	[3]
	USA	Vaginal	22	[17]
Asia (13)	Iran	Vaginal	5	[12]
		Vaginal	10	[18]
		Vaginal	4	[19]
	China	Balanoposthitis	5	[8]
	Turkey	Vaginal	3	[20]

Table 3: Overview of *C. africana* cases reported from 2010 to 2019.

biochemical criteria. *C. dubliniensis and C. africana* represent a high degree of genetic similarity to *C. albicans*. However, *C. africana* shows remarkable distinctive phenotypic traits. This new isolates have lost the ability to produce chlamydospores as described here. Borman *et al.,* linked this lower hyphal formation capacity to the reduced virulence of *C. africana* and its narrow anatomical niche (Table 2) [9,10]. Despite those differences, the identification of *C. africana* still remains a problem in routine laboratories. This specie was isolated firstly in Madagascar and Angola [5]. To date, its distribution continues to increase worldwide. An overview of the global epidemiological status of this yeast since 2010 is given in Table 3. According to this table, the majority of strains of *C. africana* were identified in America (35/90), in 38.8% of cases, followed by Asia (27/90), Europe (15/90) and Africa (13/90).

In our study, specimens were cultivated on Sabouraud Agar and on CHROMagar ® *Candida* medium. Despite chromogenic medium allowsthe detection of mixed species, it failed to unequivocally identify and differentiate these closely related species [2]. Strains of *C. africana* could also be distinguished from *C. albicans* on CHROMagar, with the producing smaller and deeper turquoise-green colonies than *C. albicans [10].* Accordingly, various molecular methodologies, including pyrosequencing, sequencing, Amplified Fragment Length Polymorphism (AFLP) have been described to tackle this problem [6-10]. The first molecular identification method was developed by Romeo et al., in which they have targeted the Hyphal Wall Protein 1 (*HWPI*) gene. This gene, codes for *C. albicans* adhesins, is developmentally regulated and expressed in germ tubes and true hyphae. This protein acts as a substrate for mammalian transglutaminases, which cross-link *C. albicans* to epithelial cells [4,9]. Romeo and Criseo's notion that *HWP1* is a useful marker to separates cryptic species of *C. albicans.* Since there, several research works have been based on the characterization of this gene; However, additional methodologies are needed to differentiate atypical species belonging to this complex [4,3].

The amplification of the HPWI shows that the majority (49/64) of our *C. albicans* strains were homozygous. In China, the distribution of heterozygous and homozygous *C. albicans* isolates at the *HWP1*

locus was very similar among patients suffering from vulvo-vaginal candidiasis and asymptomatic patients (p=0.897) [6]. In this study, the majority of *C. dubliniensis* was isolated in the oral cavity. Its identification by biochemical profiles is available since the yeast has the inability to assimilate α-Methyl-D-Glucoside (MDG), Lactate or Xylose (XYL). Clinical microbiology laboratories could use lack of growth at 45°C and a negative XYL test with either the API 20C AUX or Vitek yeast identification system to provide a presumptive identification of *C. dubliniensis*. However, The XYL and MDG tests contained in the Vitek system showed a performance slightly inferior to that of the API 20C AUX tests for detection of *C. dubliniensis* isolates. Similarly in another study of Carmen et al., it has been shown that the API ID 32 °C system obtained better overall results than the VITEK-2 system. In addition, Matrix-Assisted Laser Desorption Ionization–Time-Of-Flight Mass Spectrometry (MALDI-TOF-MS) using the direct formic acid extraction method was reliable to differentiate between the closely related species of *C. albicans* and *C. dubliniensis* [11]. Among our *C. dubliniensis* strains confirmed by PCR, five were misidentified as *C. albicans* by phenotypic assays.

In the literature, the majority of *C. africana* isolates have been recovered from vulvovaginal specimens in 72.2% of cases (65/90) (Table 3). Although, several studies conducted in Malaysia and China in which no *C. africana* was identified among, 98 and 87 *Candida* strains respectively using molecular identification [8,12]. This species was isolated from others clinical sites as blood, cerebrospinal fluid and urine. Our strain was isolated from buccal swab. A similar result has been described in Algeria in 2016. In our study, molecular identification demonstrates three co-infections by *C. dubliniensis* and *C. albicans.* Accordingly, Racha and collaborators have reported the simultaneous presence of *C. africana* and *C. albicans* [13-20]. In agreement with previous published evaluation, our isolate *C. africana* was susceptible to current antifungal drugs that would be appropriate for treating candidiasis. However, a case of resistance to ketoconazole and reduced susceptibility to amphotericin B was categorized. This funding underlines the need for detailed investigations to elucidate the mechanisms of the observed resistance.

Conclusion

The described *C. africana* is the first Tunisian isolate reported so far. The widespread distribution of this yeast throughout the world and its apparent involvement in human pathology are reasons sufficiently interesting to warrant in-depth investigations in future. We described here amplification of *HPW1* gene which was simple, cost-effective, and offers a high throughput in the correct identification of *C. africana*, *C. albicans*, and *C. dubliniensis.*

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