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Research Paper

High diversity of yeast species and strains responsible for vulvovaginal candidiasis in South-East Gabon

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ABSTRACT

Objectives: Candida albicans generally remains the principal pathogenic yeast responsible for vulvovaginal candidiasis (VVC), although with variable prevalence. In this study, we evaluated the evolution of the prevalence of the non-*Candida albicans Candida* (NCAC) species and investigated the genotypic diversity and the population genetic structure of the circulating *C. albicans* strains associated with VVC in the vicinity of Franceville (Gabon).

Methods: A total of 110 independent isolates were identified using both MALDI-TOF MS and conventional techniques. The population genetic structure of the *C. albicans* strains was determined by multiple locus variable-number tandem repeat analysis using 4 microsatellite markers.

Results: The mean and median age of the patients was 31 years. Seven patients had a mixed infection. *C. albicans* accounted for 62 % (n=68) of the total isolates. NCAC were dominated by *C. glabrata*, followed by *P. kudriavzevii, C. parapsilosis, C. tropicalis, M. guilliermondii*, and *C. nivariensis*. The cluster analysis revealed a high diversity, with a total of 50 different genotypes. The most represented genotype was shared by only four strains, while the vast majority (39 strains) had a unique MLVA pattern. Geographic clusters were not detected.

Conclusion: The study provides information on species distribution and possible changing epidemiology while reporting for the first time *C. nivariensis* in VVC in Africa. This study is also the first to investigate the genotypic diversity of the circulating *C. albicans* strains associated with VVC in Central Africa. Such analyses would help understand the molecular epidemiology of *C. albicans.*

Introduction

Vulvovaginal candidiasis (VVC) is a gynecological condition that is frequently diagnosed in women with vaginal complaints in the primary care setting [1,30]. It affects mainly women during their childbearing years and is considered the second most common cause of vaginitis after bacterial vaginosis [8]. The clinical symptoms of VVC are nonspecific and include generally pruritus and vaginal discharge accompanied by soreness, irritation, and burning that lead to dyspareunia and dysuria [33,36,38]. VVC is also a source of mental distress and can be classified into uncomplicated and complicated cases. Uncomplicated VVC is defined by fewer than four episodes per year with mild to moderate severity, caused by *Candida albicans* in healthy women. Complicated VVC comprises notably infections due to non-*albicans* species, recurrent VVC, and VVC in patients with risk factors [1,8]. The latter include mainly pregnancy, uncontrolled diabetes, immunosuppression, antibiotic treatments, hormone replacement therapy, and genetic predispositions [8,33,38].

Diagnosis of VVC requires a combination of clinical findings and laboratory confirmation, preferably direct examination. Moreover, vaginal pH is normal in the case of VVC while it is usually higher in the case of bacterial or trichomonas infections [1,33,36]. Vaginal culture is also of interest, especially to identify the yeast. However, a positive culture associated with negative microscopic examination can indicate colonization and does not necessarily mean that the identified yeast is responsible for the vaginal symptoms [38].

VVC is predominantly caused by *Candida albicans*, which accounts for the majority of the episodes in European and American countries as well as in Australia and China [1]. However, infections caused by non-*Candida albicans Candida* (NCAC) are reported worldwide and outnumber *C. albicans* in some Asian and African countries [8, 14]. Some of these species, especially *Candida glabrata* and *Pichia kudriavzevii* (formerly *Candida krusei*), are more resistant to commonly used

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triazole antifungal drugs. Moreover, NCAC are more implicated in the occurrence of recurrent VVC and their incidence is believed to increase [24]. This increase results presumably from the widespread or inappropriate utilization of antifungals such as single-dose treatments, prolonged antifungal therapy, or self-medication with over-the-counter antimycotics [37,42]. Estimating the distribution of the species causing VVC is thus important in order to guide appropriate therapy and predict the possibility of infection recurrence.

In Africa, *C. albicans* generally remains the principal pathogenic yeast responsible for VVC, although with variable prevalence. In Burkina Faso, it was isolated in only 43.6 % of symptomatic pregnant women from three primary health centers, while in Benin, *C. albicans* was identified in 96.1 % of cases in a single-center study [26,32]. NCAC can thus represent a large proportion of the species distribution, especially *C. glabrata* which is usually the second most encountered species, as well as *P. kudriavzevii* and *C. tropicalis* [23]. *C. glabrata* was even reported as the primary agent in some centers in Nigeria and Ghana, reaching a prevalence of 33.7 % and 57.4 %, respectively [27,41].

Available data on the distribution of the species causing VVC in Central Africa is very limited. A previous study performed in 2016-2017 in Franceville, Gabon, showed that 28.5 % of symptomatic women were culture-positive for *Candida* spp. with *C. albicans* accounting for more than 80 % of the isolates [4]. In the present study, a new sampling campaign was conducted in the same area to estimate the evolution of the NCAC prevalence. Moreover, the genotypic diversity of the circulating *C. albicans* strains associated with VVC was investigated by microsatellite typing. The goals of the typing analysis were to evaluate the extent of this diversity, to determine the population genetic structure, and to detect possible clusters based on the local geographic origin of the patients.

Material & methods

Sampling

Sampling was performed through a single-center cross-sectional study carried out between June 2018 and June 2019 at the International Center for Medical Research of Franceville (CIRMF), Gabon, which houses a secondary care center. Franceville is the chief town of the Haut-Ogooué province, the second most populated province of Gabon, situated at the border of the Republic of the Congo.

After obtaining written consent, patients referring to the CIRMF for symptoms of VVC were recruited. The age and the place of residence of the participants were recorded and a vaginal swab sample was taken. On each sample, a direct examination was carried out to confirm the diagnosis. Swabs were inoculated on a chromogenic medium (CHROMID Candida Agar, bioMérieux, France) and incubated at 37°C for 24 h under aerobic conditions. This isolation allowed the direct identification of *C. albicans* isolates and the detection of mixed cultures. Isolates were then subcultured on Sabouraud dextrose agar with chloramphenicol (bioMérieux, France) to obtain pure colonies for further identification using the Vitek 2 Compact system (bioMérieux, France). Strains were finally stored in a 20 % glycerol solution at −80°C until subsequent analyses. A selection of these strains was deposited at the BCCM/IHEM collection under accession numbers IHEM 28304 to IHEM 28367.

Identification of isolates by MALDI-TOF mass spectrometry

Strains were re-identified by MALDI-TOF MS. They were first grown on Sabouraud dextrose agar plates supplemented with chloramphenicol and incubated for 48 h at 25°C. Five to ten colonies were then taken using a sterile loop and transferred to a 1.5 ml microcentrifuge tube containing 300 μl of sterile water. After the addition of 900 μl of absolute ethanol, the samples were centrifuged for 5 min at 13,000 g. The supernatant was discarded and the pellet was air-dried before incubation for 5 min in 50 μl of 70 % formic acid. An equal volume of acetonitrile was then added, and the samples were submitted to another 5 min-incubation before final centrifugation for 2 min at 13,000 g. For the acquisition of the spectra, 1 μl of the supernatant of each extract was deposited in quadruplicate on a polished steel target plate and allowed to dry. Each spot was covered with 1 μl of a cyano-4-hydroxycinnamic acid matrix solution (Bruker Daltonics). The spectra were obtained after 240 shots in positive linear mode with a Microflex LT MALDI-TOF MS instrument (Bruker Daltonics), with an m/z range of 2-20 kDa and an accelerating voltage of 20 kV. A bacterial test standard (Bruker Daltonics) was used for calibration. The data were automatically acquired by the FlexControl version 3.4 software using the AutoExecute default settings. Identification of the spectra was performed using the MSI-2 online application (https://msi.happy-dev.fr) and the Bruker Fungi Library. Identifications with scores >20 and >1.70 were considered reliable for the MSI-2 and Bruker databases, respectively.

Genotyping

The population genetic structure of the *C. albicans* strains was determined by multiple locus variable-number tandem repeat analysis (MLVA) using 4 microsatellite markers, namely CAI, CAIII, CDC3, and HIS3 [5,31]. For DNA extraction, cultures were first freeze-dried and mechanically broken by bead-beating. DNA was then extracted using the ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research) following the manufacturer's instructions. Genotyping was performed by Genoscreen (Lille, France) using the PCR conditions described in the literature [5,31]. The size of the amplicons was determined with an ABI 3730XL genetic analyzer using the GeneScan 500 LIZ size standard (ABI) and the GeneMapper v5.0 software. The relatedness of the strains was analyzed by a minimum spanning tree analysis in Bionumerics 8.0. The discriminatory power of the microsatellite markers was calculated using the Simpson index of diversity [12].

Results

A total of 110 independent isolates were obtained from 103 patients with symptomatic VVC, including 7 with a mixed infection. The latter always combined a *C. albicans* strain with another yeast species, mainly *C. tropicalis* (in 4 patients). The age of the patients ranged from 12 to 57 years, with a mean and median age of 31 years. Eighty percent of the affected women were between 20 and 40 years (Table 1, Fig. 1). There was no difference in age between patients infected by *C. albicans* and those with VVC caused by NCAC.

The isolates were identified by MALDI-TOF mass spectrometry using the Bruker Fungi Library and the MSI-2 databank. Both databases provided the same identifications (Supplementary Table 1). Fig. 2 shows the distribution of the seven identified species. The majority of the isolates (n=68) belonged to *C. albicans*, representing 62% of the total strains. NCAC thus accounted for the remaining 38% and were dominated by *C. glabrata* (n=16), followed by *P. kudriavzevii* (n=10), *C. parapsilosis* (n=8), *C. tropicalis* (n=6), *M. guilliermondii* (n=1) and *C. nivariensis* (n=1).

The population genetic structure of 64 *C. albicans* strains (the genotype could not be determined for 4 isolates) was estimated by MLVA genotyping using 4 microsatellite markers. The measured length of these markers is provided for each strain in Supplementary Table 2. They appeared to be polymorphic with 26 distinct alleles for CAI, 7 for CAIII, 4 for CDC3, and 16 for HIS3. Considering that *C. albicans* is diploid, the number of different allelic combinations reached 36 for CAI, 8 for CAIII, 7 for CDC3, and 17 for HIS3. Taken together, these four loci resulted in a total of 50 different genotypes with a calculated discriminatory power of 99.1. One genotype was shared by four strains while another was common to three strains. Nine

Table 1
Age and place of residence of the 103 patients with VVC included in the study.

Patient	Age	Place of residence	Patient	Age	Place of residence
1	17	Franceville	53	38	Franceville
2	29	Franceville	54	25	Franceville
3	36	Franceville	55	28	Leconi
4	27	Franceville	56	23	Franceville
5	38	Franceville	57	41	Franceville
6	36	Franceville	58	24	Franceville
7	12	Okondja	59	22	Boumango
8	44	Moanda	60	36	Okondja
9	46	Franceville	61	48	Franceville
10	41	Franceville	62	23	Franceville
11	29	Franceville	63	47	Franceville
12	23	Franceville	64	18	Franceville
13	44	Franceville	65	35	Franceville
14	29	Franceville	66	33	Ouelle
15	21	Franceville	67	24	Franceville
16	28	Franceville	68	32	Franceville
17	51	Franceville	69	18	Boumango
18	28	Franceville	70	29	Franceville
19	33	Franceville	71	35	Franceville
20	34	Franceville	72	30	Franceville
21	36	Franceville	73	22	Franceville
22	27	Franceville	74	20	Bakoumba
23	30	Franceville	75	23	Okondja
24	25	Franceville	76	44	Franceville
25	31	Mounana	77	40	Franceville
26	22	Franceville	78	29	Moanda
27	24	Franceville	79	36	Franceville
28	27	Franceville	80	36	Moanda
29	27	Franceville	81	22	Franceville
30	27	Franceville	82	37	Okondja
31	20	Franceville	83	39	Franceville
32	53	Okondja	84	41	Mvengue
33	27	Moanda	85	27	Franceville
34	40	Franceville	86	40	Franceville
35	31	Franceville	87	42	Moanda
36	29	Franceville	88	34	Moanda
37	23	Moanda	89	23	Leconi
38	33	Franceville	90	31	Franceville
39	24	Moanda	91	36	Franceville
40	36	Bongoville	92	17	Franceville
41	28	Moanda	93	16	Franceville
42	20	Franceville	94	27	Franceville
43	31	Franceville	95	26	Franceville
44	33	Moanda	96	43	Franceville
45	33	Moanda	97	38	Ouelle
46	36	Franceville	98	31	Franceville
47	27	Franceville	99	26	Franceville
48	37	Franceville	100	43	Moanda
49	25	Franceville	101	57	Moanda
50	35	Franceville	102	35	Franceville
51	34	Moanda	103	35	Franceville
52	21	Franceville			

genotypes were represented by two strains and the remaining 39 strains had a unique MLVA pattern. The genetic distance between all isolates is shown by a minimum-spanning tree (Fig. 3). By mapping the place of residence, cities appeared to be scattered across the tree and geographic clusters were not detected. Noteworthy, the most represented genotype occurred in four strains isolated from patients living in four different cities. Moreover, 4 out of the 9 genotypes grouping two strains were found in separate places of residence. Some clustering can be observed among strains originating from patients living in Franceville but this results rather from the overrepresentation of this city in the sampled population (72% of the total isolates).

Discussion

VVC is associated with significant morbidity since it is the second leading cause of vaginitis worldwide, affecting millions of women every year regardless of their social class [8]. In the current investigation, the mean age of the patients was 31 years, which is similar to several studies in Africa where the mean age ranged from 26 in Burkina Faso to 33 years in Tunisia [3,13,18,22,26,28,32,41]. The infection is influenced by the hormonal state of the patient and affects mostly women of childbearing age. It is rare before menarche and after menopause unless women undergo hormone replacement therapy [42].

In comparison with a previous study performed in the same region in 2016-2017 [4], *C. albicans* remained the most frequently isolated species but showed a decreased prevalence from 82% to 62%. The predominance of *C. albicans* results from its capacity to better adhere to mucosal epithelial cells compared to other yeast species, favoring its potential for colonization and infection [8]. Its ability to germinate and produce hyphae is another virulence factor that facilitates tissue invasion [38]. NCAC thus accounted for the remaining 38% in 2019, a twofold increase since the previous reported period, with *C. glabrata* becoming the second most common pathogen. This increase could result from the relatively small sampling size of the current study and more extended, multi-centric, sampling campaigns should be performed in the future to confirm whether it reflects a real epidemiological trend. Such studies should also record patients' data in order to identify risk factors associated with NCAC infections. Our results are however consistent with several studies performed in Africa on VVC where a predominance of *C. albicans* was observed together with a growing recovery of NCAC with *C. glabrata* as the second most frequently isolated species. It was notably the case in Tunisia [22], Morocco [3], Benin [26], Burkina Faso [32], Ivory Coast [18], Cameroon [13], and Kenya [25].

The NCAC species distribution between the two reported periods in Franceville also showed differences, which can be partly attributed

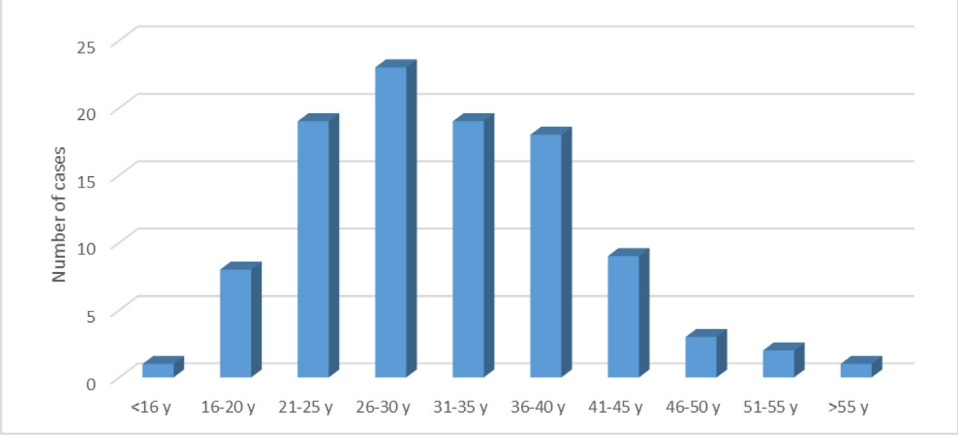

Fig. 1. Distribution of patients with VVC according to age range.

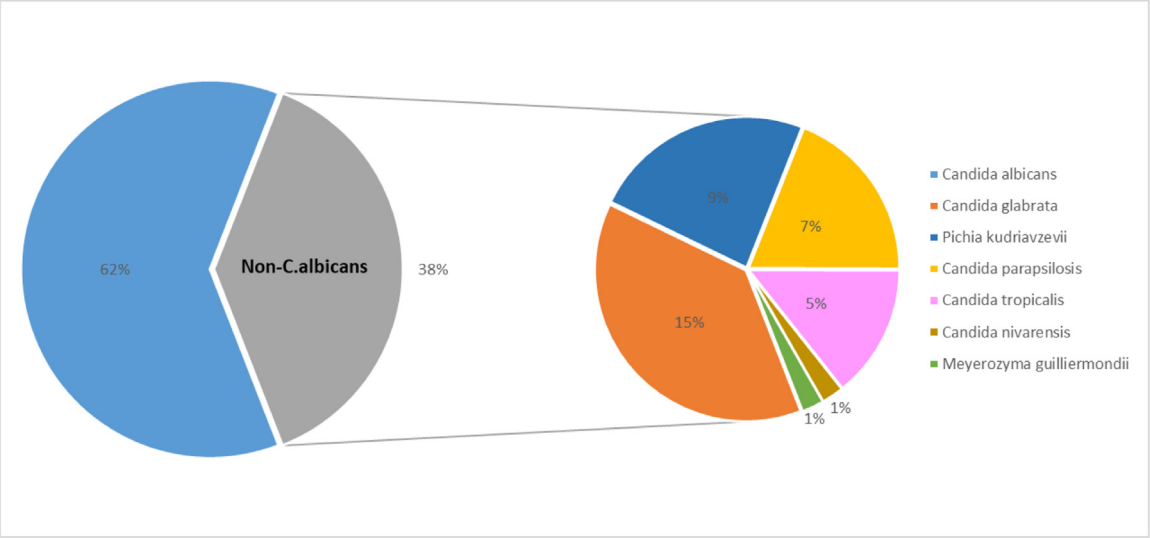

Fig. 2. Prevalence of the isolated yeast species.

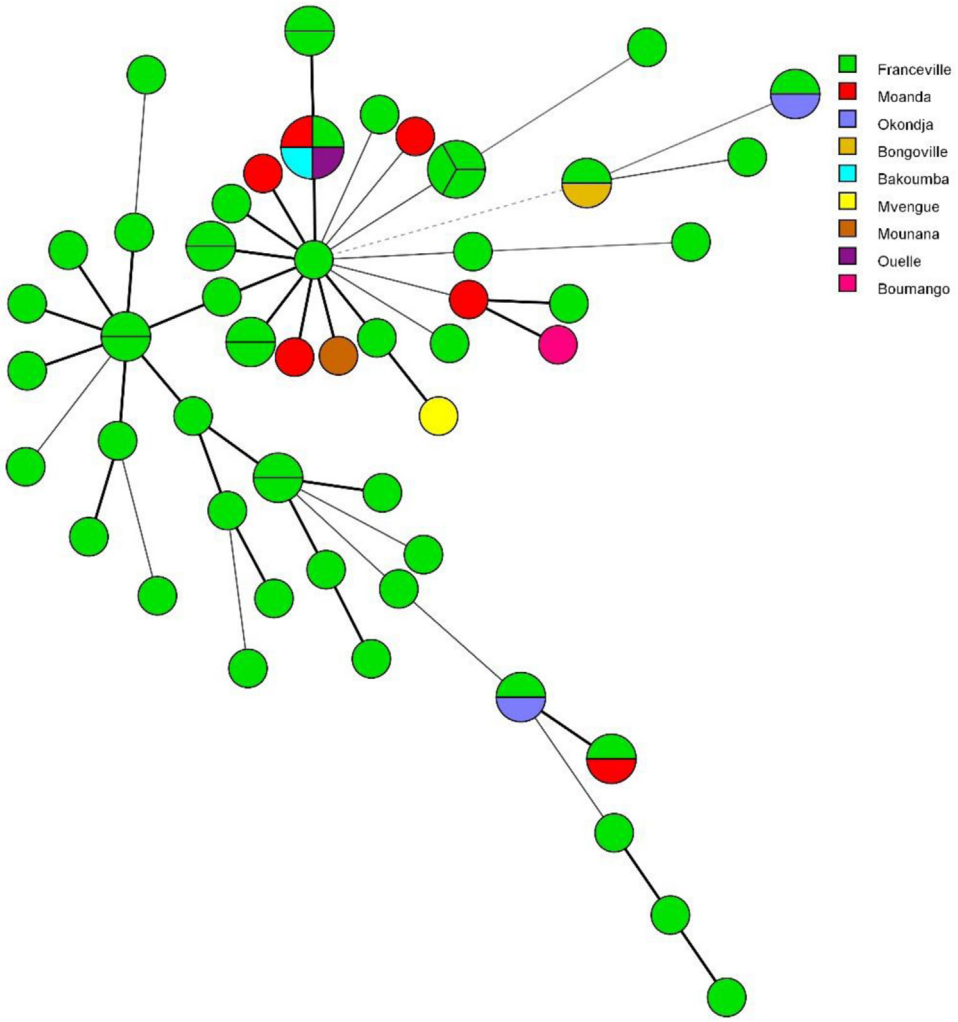

Fig. 3. Minimum spanning tree displaying the 50 different genotypes obtained from 64 *Candida albicans* VVC isolates based on 4 variable-number tandem-repeat loci. The genotypes are represented by circles. The length and thickness of the connecting lines between the circles show the similarity between the profiles. The place of residence is mapped on the tree.

to the method of identification. Bignoumba et al. [4] identified the isolates using the assimilation-based Vitek 2 system and found that *C. famata* was the most common NCAC species. Moreover, some strains were not identified at the species level and were reported as *Candida* sp. However, misidentifications of isolates as *C. famata* using the Vitek 2 were frequently reported [6,15–17], and were also observed in the present study. Noteworthy, these errors do not account for the decrease in *C. albicans* prevalence, since the latter was also 62% of the identified species using the Vitek 2.

The use of MALDI-TOF MS thus improved the accuracy of the identification. Precise identification of *Candida* species is increasingly considered relevant because some species are less sensitive or even resistant to triazole antifungal agents [14]. It is notably the case of *C. glabrata* and *P. kudriavzevii* that accounted together for almost a quarter of the total isolates in this study. The use of MALDI-TOF MS as a powerful and rapid tool for the identification of yeast pathogens was proven in numerous studies that reported correct species identification rates up to 99 % among collections of clinical yeast isolates [21,29,40]. Moreover, it appears to be cost-effective since the protocol requires inexpensive and easy-to-store consumables. It should therefore be more implemented in resource-poor settings [39].

Interestingly, our study described for the first time *C. nivariensis* as the etiological agent of VVC in Africa. The identification was confirmed by DNA sequencing (the ITS sequence was deposited at the European Nucleotide Archive under accession number OW988758). *C. nivariensis* was first reported in VVC in India in 2012 [35]. It was also identified in seven cases of VVC in a retrospective study performed in a single center in Beijing, China, using isolates collected between 2003 and 2012 [20]. In Europe, VVC caused by *C. nivariensis* was reported in four patients from a Spanish hospital [2]. This yeast pathogen is a member of the *C. glabrata* species complex. It is phenotypically indistinguishable from *C. glabrata* based on conventional chromogenic media or biochemical panels [11] and its identification in the present study was made possible by using MALDI-TOF MS. The occurrence of *C. nivariensis* is thus probably overlooked, although its differentiation from C. *glabrata* may be of importance in understanding the clinical and epidemiological roles of this species in VVC [35].

The MLVA analysis of the *C. albicans* isolates revealed a total of 50 different genotypes with 61% of the strains represented by a unique molecular profile. These results confirm the high genetic variability reported in previous similar studies [9,10,34]. However, this diversity could not be explained by the geographic origin of the strains. Other factors that remain to be investigated would thus contribute to the observed diversity. To the best of our knowledge, this study is however the first to evaluate the genotypic diversity and the population genetic structure of the circulating *C. albicans* strains associated with VVC in Central Africa. Such analyses would help understand the molecular epidemiology of *C. albicans*. In particular, it represents a fundamental work for future comparisons with strains from other countries or continents and with isolates from healthy patients.

Our study has several limitations including the lack of control in the healthy population, preventing the detection of possible genotypes related to VVC like in China [7, 19]. Moreover, this is a single-center study that cannot estimate the prevalence of VVC in symptomatic women or the general population.

Conclusion

The present study showed the species and strain diversity of yeasts responsible for VVC in South-East Gabon, with a possible changing epidemiology towards non-*albicans* species. It also emphasized the importance of using modern tools like MALDI-TOF mass spectrometry for more precise identification of the isolates. This would result in a better estimation of the species distribution, as exemplified by the detection of *C. nivariensis* in our population.

Supplementary material

Supplementary Table 1: detailed results of the MALDI-TOF mass spectrometry identifications

Supplementary Table 2: detailed results of the MLVA analyses on the Candida albicans strains

Declaration of Competing Interest

The authors declare no conflicts of interest

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mycmed.2022.101354.

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