ORIGINAL ARTICLE

Prevalence and antifungal susceptibility of *Candida albicans* and its related species *Candida dubliniensis* and *Candida africana* isolated from vulvovaginal samples in a hospital of Argentina

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Abstract *Candida africana* taxonomical status is controversial. It was proposed as a separate species within the *Candida albicans* species complex; however, phylogenetic analyses suggested that it is an unusual variety of *C. albicans*. The prevalence of *C. albicans*-related species (*Candida dubliniensis* and *C. africana*) as vulvovaginal pathogens is not known in Argentina. Moreover, data on antifungal susceptibility of isolates causing vulvovaginal candidiasis is scarce. The aims of this study were to establish the prevalence of *C. dubliniensis* and *C. africana* in vaginal samples and to evaluate the antifungal susceptibilities of vaginal *C. albicans* species complex strains. We used a molecular-based method coupled with a new pooled DNA extraction methodology to differentiate *C. dubliniensis* and *C. africana* in a collection of 287 strains originally identified as *C. albicans* isolated from an Argentinian hospital during 2013. Antifungal susceptibilities to fluconazole, clotrimazole, itraconazole, voriconazole, nystatin, amphotericin B and terbinafine were evaluated by using the CLSI M27-A3 and M27-S4 documents. Of the 287 isolates, 4 *C. dubliniensis* and one *C. africana* strains (1.39% and 0.35% prevalence, respectively) were identified. This is the first description of *C. africana* in Argentina and its identification was confirmed by sequencing the ITS2 region and the *hwp1* gene. *C. dubliniensis* and *C. africana* strains showed very low MIC values for all the tested antifungals. Fluconazole-reduced-susceptibility and azole cross-resistance were observed in 3.55% and 1.41% of the *C. albicans* isolates, respectively. These results demonstrate that antifungal resistance is still a rare phenomenon in this kind of isolates.

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Prevalencia y sensibilidad a los antifúngicos de *Candida albicans* y sus especies relacionadas, *Candida dubliniensis* y *Candida africana* aisladas de muestras vulvovaginales en un hospital de Argentina

Resumen   La clasificación taxonómica de *Candida africana* está en discusión, es considerada una nueva especie dentro del complejo *C. albicans* o una variedad inusual de *C. albicans*. La prevalencia de las especies relacionadas a *C. albicans* (*C. dubliniensis* y *C. africana*) como agentes de vulvovaginitis en Argentina se desconoce. Los objetivos de este trabajo fueron determinar la prevalencia de *C. dubliniensis* y *C. africana* en muestras vaginales y evaluar la sensibilidad a los antifúngicos de aislamientos vaginales de las especies del complejo *C. albicans*. Para diferenciar *C. dubliniensis* y *C. africana* utilizamos un método molecular asociado a un nuevo método de extracción de ADN. Se utilizó una colección de 287 cepas originalmente identificadas como *C. albicans* aisladas durante 2013 en un hospital de Argentina. Se evaluó la sensibilidad a fluconazol, clotrimazol, itraconazol, voriconazol, nistatina, anfotericina B y terbinafina utilizando los documentos M27-A3 y M27-S4 del CLSI. De los 287 aislamientos, se identificaron 4 *C. dubliniensis* y 1 *C. africana* (1,39 y 0,35% de prevalencia, respectivamente). Esta es la primera descripción de *C. africana* en Argentina. Su identificación fue confirmada por secuenciación de la región ITS2 y del gen *hwp1*. Las cepas identificadas como *C. dubliniensis* y *C. africana* mostraron valores de CIM muy bajos para todos los antifúngicos probados. En los aislamientos de *C. albicans*, la sensibilidad reducida al fluconazol y la resistencia cruzada a todos los azoles se observó en el 3,55% y el 1,41%, respectivamente. Estos resultados demuestran que la resistencia a los antifúngicos es todavía un fenómeno raro en este tipo de aislamientos.

Introduction

More than twenty different species of *Candida* have been reported as human pathogens[14,20]. However, *Candida albicans* is the most common human fungal pathogen and the most studied of all *Candida* species[8,10,15,17,20]. *C. albicans* has a great degree of variability and some of the firstly known as ''atypical'' *C. albicans* isolates were later considered as related species. That is the case with the well-known *Candida dubliniensis*[30]. Other ''atypical'' *C. albicans* considered as separated species are now described as varieties (*e.g. C. albicans var. stellatoidea*) and included as one of the 173 *C. albicans* synonyms[10]. The most recently described *C. albicans* related species is *Candida africana*[31]. The first *C. africana* strain was isolated from African patients suffering from vulvovaginal candidiasis (VVC) and firstly considered ''atypical'' *C. albicans* strains[32]. Later, it was proposed as a new species based on morphological, biochemical and physiological differences which include its inability to assimilate glucosamine and N-acetylglucosamine and its impossibility to form chlamydospores on corn meal agar[24,31]. Odds *et al*. in 2007 included *C. africana* in *C. albicans* clade 13 and supported a varietal status (*C. albicans* var. *africana*)[16]. One year later, the same group included several *C. africana* strains in a group highly distinct from the majority of *C. albicans* together with *Candida stellatoidea* type I and other sucrose-negative atypical *C. albicans* isolates[9]. These facts support the controversial taxonomic status of these isolates.

The prevalence of these *C. albicans* related species as agents of VVC is not known in Argentina. Moreover, data on antifungal susceptibility patterns of isolates causing VVC are scarce[12].

The aim of this study was to establish the prevalence of *C. dubliniensis* and *C. africana* in a collection of yeasts originally identified as *C. albicans* and isolated from VVC using a molecular-based method coupled with a pooled DNA extraction methodology. Additionally, we evaluated the antifungal susceptibility patterns of these strains.

Materials and methods

Yeast strains

A total of 287 strains were included in this study. All isolates were obtained from patients having vaginal infection symptoms attending the *José María Cullen Hospital* (Santa Fe – Argentina) during 2013. All the strains were identified as *C. albicans* by germ tube formation in human serum and their ability to form green color colonies on CHROMAgar Candida™ (Biomerieux – Medica-Tec SRL, Buenos Aires, Argentina). All yeast isolates were referred to the ''*Micología y Diagnóstico Molecular*'' Laboratory (CONICET Universidad Nacional del Litoral) where they were stored and identified in accordance with morphology and carbohydrate assimilation and fermentation[11] and by a molecular procedure as described below. *C. albicans* ATCC 90028 and *C. dubliniensis* NCPF 3949 were used as control strains.

Table 1 Oligonucleotide primers used in this study

Oligonucleotide	Target gene	$5' \rightarrow 3'$ sequence	Primer orientation
CR-F[a]	HPW1	GCTACCACTTCAGAATCATCATC	Sense
CR-R[a]	HPW1	GCACCTTCAGTCGTAGAGACG	Antisense
ITS1[b]	ITS	TCCGTAGGTGAACCTGCGG	Sense
ITS4[b]	ITS	TCCTCCGCTTATTGATATGC	Antisense

[a] Romeo et al.[23].
[b] White et al.[35].

Molecular identification by using *hwp1* gene amplicon length

A PCR method based on *hwp1* gene amplification to differentiate *C. albicans*, *C. africana* and *C. dubliniensis* was used. This method identified the three species through the differences in sizes of the obtained PCR fragments: 569 bp, 700 bp and 941 bp, respectively, when the DNA of *C. dubliniensis*, *C. africana* and *C. albicans* DNA was used as template[23]. Primers are displayed in Table 1.

Individual and pooled DNA extraction

The isolates were individually grown in 1 ml of YPD broth (1% yeast extract, 2% Bacto peptone, 2% dextrose) at 35 °C for 24 h and constant shaking (200 rpm). Then, 200 µl of 10 individual cultures were mixed to obtain 2 ml yeast cells pools from which the pooled DNA was extracted. The remaining 800 µl of yeast cultures were kept at −20 °C and used later to obtain individual genomic DNAs if necessary. Yeast DNAs (individual and pooled) were extracted using a phenol-based procedure[26].

High-throughput screening to rapidly identify *C. africana* and *C. dubliniensis* in a strain collection

Originally, Romeo *et al.*[23] designed their method to identify strains using individual DNAs. In an effort to establish a high throughput screening method to study fungal collections we decided to use the same PCR technique but using pooled DNAs (containing DNAs isolated from ten different strains). The presence of *C. africana* or *C. dubliniensis* DNA was suspected when more than one PCR band was observed. To uncover *C. dubliniensis* and *C. africana*, we extracted individual DNA from all the pools showing multiple bands (941 bp plus 569 bp and/or 700 bp) and the *hwp1* PCR was repeated using individual DNAs as templates. Additionally, we extracted individual DNA for 10 randomly chosen pools showing one 941 bp band (100 DNAs in total) and repeated the *hwp1* PCR to confirm the specificity of the technique.

Identification confirmation

All the strains identified as *C. dubliniensis* or *C. africana* by *hwp1* gene amplification were subsequently identified by sequencing the 5.8S RNA gene and adjacent internal transcribed spacer 1 and 2 regions (ITS1 and ITS2) and the *hwp1* gene[2,35]. Primers are displayed in Table 1.

Antifungal susceptibility testing

Antifungal drugs tested were fluconazole (FLC), itraconazole (ITC), voriconazole (VRC), clotrimazole (CLT), terbinafine (TRB), anfotericin B (AMB) and nystatin (NYS) (all purchased from Sigma–Aldrich Química – Buenos Aires, Argentina). Drug selection was performed based on the treatment options (topical, vaginal ovules and systemic presentations) available at *José María Cullen Hospital* (Santa Fe, Argentina). Although AMB is not used to treat vaginal infections, it was added to the list of tested drugs to assess if it was possible to use it as an *in vitro* surrogate marker for NYS resistance since AMB susceptibility testing is standardized by CLSI and both are polyenes[5,6]. Inoculums of all the isolates were obtained according to CLSI document M27-A3. Result interpretations were performed according to CLSI documents M27-A3 and M27-S4. FLC, ITC and AMB MIC microtitration plates were produced following CLSI M27-A3 guidelines[5,6]. In addition, TRB, CLT and NYS were diluted in dimethyl sulfoxide and the final concentrations ranged from 8 to 0.015 µg/ml. Since there are no MIC limit ranges for microdilution tests for these antifungal agents, the quality control strains *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were subjected to twenty MIC repetitions on different days (results are displayed in Table 2). Afterwards, this MIC data was added to earlier MIC results obtained by our group[8] and were used as a control of the produced plates.

Data collection and statistical analysis

The MIC data presented here are expressed as geometric means (GMs) of three experiments performed on different days. The off-scale MICs were converted to the next concentration up or down in order to be included in the analysis. MIC values were approximated to a normal distribution by transforming them into $\log_2$ values. GMs were used to statistically compare MIC values and MIC $\log_2$ values were used to establish susceptibility differences between strains. To establish significant levels of MIC differences, a one-way ANOVA test with Bonferroni's correction for multiple comparisons was used. A *P* value ≤ 0.05 was considered significant. This study was approved by the School of Biochemistry (Universidad Nacional del Litoral) and JM Cullen ethics committees.

Nucleotide sequence accession numbers

The *C. africana* strain *hwp1* gene sequence was deposited in GenBank under accession number KR704898.

Table 2 Terbinafine, clotrimazole and nystatin MIC ranges for control strains obtained following CLSI M27-A3 and M27-S4 document recommendations

	MIC range (µg/ml)[a]		
	Terbinafine	Clotrimazole	Nystatin
C. parapsilosis ATCC 22019	0.03–0.12	0.015–0.03	0.12–0.25
C. krusei ATCC 6258	>–4	0.015–0.06	0.50–1.00

[a] 24 h-reading. MIC ranges include 20 repetitions performed in this work plus 20 repetitions performed in a previous work conducted by our group[8].

Results

### Identification and prevalence of *C. dubliniensis* and *C. africana*

With the intention of evaluating the prevalence and potential clinical significance of *C. dubliniensis* and *C. africana* in vaginal samples from Santa Fe city, we molecularly identify the 287 isolates received (previously identified by phenotypic methods as *C. albicans*) by using the amplification of the *hwp1* gene using a single primer pair[23]. We employed a higher throughput methodology using DNA pools obtained from ten different isolates as templates. When pooled DNAs were used, the presence of non-*C. albicans* was suspected when two or more bands appeared in the electrophoresis. A 941 bp fragment along with a 569 bp fragment demonstrated the presence of at least one *C. albicans* and one *C. dubliniensis* strain in the DNA pool while the presence of a 700 bp fragment proved the existence of at least one *C. africana* strain in the pool (Fig. 1). The presence of two PCR bands was observed in five out of the 29 DNA pools. Four showed a 941–569 bp-double band and the fifth showed a 941–700 bp-band pair representing the presence of at least four *C. dubliniensis* and one *C. africana* strains. These five DNA pools were subsequently studied to confirm the results. Individual DNAs were used as PCR template plate to amplify the *hwp1* gene. Four *C. dubliniensis* and one *C. africana* were identified. Altogether, we obtained the same results using pooled and individual DNA extraction: four *C. dubliniensis* and one *C. africana* strains out of

287 studied isolates (1.39% and 0.35% prevalence, respectively). The identification of these strains was confirmed by ITS and *hwp1* sequencing. The ITS obtained sequences were compared with the published ITS signature sequences from *C. albicans* (variants I to IV) (Gene bank aa. numbers: KP675686.1, HQ876051.1, KP675529.1 and KP675666.1, respectively), *C. dubliniensis* (variants I and II) (aa. numbers: AB035589.1 and KP131696.1, respectively), *C. albicans* var. *stellatoidea* (aa. numbers: AJ853768.1) and *C. africana* (aa. numbers: AY342214.1)[2] (Fig. 2A). The *hwp1* gene sequences of our non-*albicans* strains showed the characteristic nt. gaps (372 bp and 241 bp difference for *C. dubliniensis* and *C. africana*, respectively) (Fig. 2B).

Phenotypically, our *C. africana* strain differed from the *C. albicans* control strains in its inability to form chlamydospores on corn meal agar, to assimilate trehalose and to grow at 42 °C. However, it developed green colonies on CHROMagar and formed germ tubes under regular conditions (2 h at 37 °C). Our *C. africana* showed a lower rate of germ tube formation when compared to *C. albicans* and *C. dubliniensis* control strains.

Antifungal susceptibility testing

The *in vitro* activities of the tested antifungal drugs are summarized in Table 3. All non-*C. albicans* strains included in this study (4 *C. dubliniensis* and the *C. africana*) showed very low MIC values for all the tested antifungal agents. Similarly, antifungal drugs showed good *in vitro* activity against the majority of the remaining 282 isolates
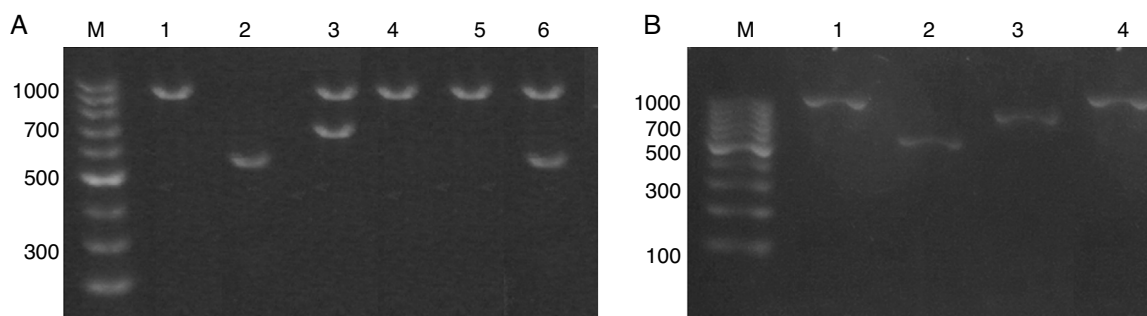
Figure 1 (A) Electrophoresis of the *hwp1* gene PCR using DNA pools resolved on 1 % agarose gel. Lane M, 100 bp size marker. Lane 1, *C. albicans* control strain (ATCC 90028). Lane 2, *C. dubliniensis* control strain (NCPF 3949). Lanes 3–6, DNA pools showing *hwp1* bands. Lane 3: 941 bp and 700 bp (*C. albicans* and *C. africana*). Lanes 4 and 5: 941 bp (*C. albicans* alone). Lane 6: 941 and 569 bp (*C. albicans* and *C. dubliniensis*). (B) Electrophoresis on 1 % agarose gel of the *hwp1* PCR using individual DNAs (from the DNA pool displayed in lane 3 – (A)). Lane M, 100 bp size marker. Lane 1, *C. albicans* control strain (ATCC 90028). Lane 2, *C. dubliniensis* control strain (NCPF 3949). Lane 3, *C. africana* (Strain LMDM 678). Lane 4, *C. albicans* (Strain LMDM 679).
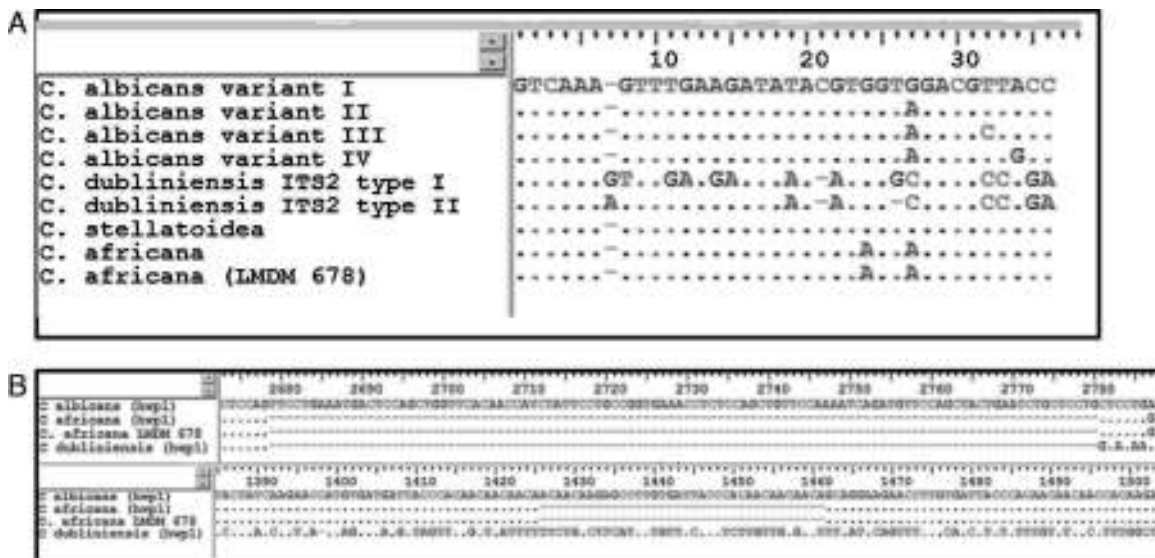
Figure 2 (A) Sequence alignments of the 35-bp portion of the ITS2 region allowing to differentiate the four *C. albicans* variants, the two *C. dubliniensis* types, *C. albicans* var. *stellatoidea* and *C. africana*[2]. *C. africana* strain LMDM 678 showed no differences with the *C. africana* type strain. Dots indicate conserved nucleotides; gaps are represented as hyphens and nucleotide differences are displayed as letters. (B) Sequence alignments of regions of the *hwp1* gene where gaps were encountered. The upper line displays *C. albicans hwp1* (GenBank accession number U64206.1); second line: *C. africana hwp1* (EU477610.1); third line: *C. africana* LMDM 678 (KR704898) and fourth line: *C. dubliniensis* (AJ632273.1).

(*C. albicans*). The most potent azole was CLT showing the lowest MIC values (GM = 0.02 μg/ml, MIC90 = 0.03 μg/ml) and only thirteen strains showed CLT MIC value ≥0.25 μg/ml. FLC was the tested drug with the widest range of MIC values (0.06–64.00 μg/ml). Reduced susceptibility to FLC was observed in ten *C. albicans* strains out of the 282 studied (3.55 %). Of these ten strains, three were FLC susceptible-dose dependent while seven were resistant to FLC (three strains were exclusively resistant to FLC and four strains were azole-cross-resistant). Concerning the oral triazoles, ITC and VRC showed very good *in vitro* activity (GM = 0.03 μg/ml and a MIC90 = 0.06 μg/ml for ITC and GM = 0.021 μg/ml and a MIC90 = 0.03 μg/ml). There were only four (1.41 %) *C. albicans* strains showing high ITC and VRC MIC values (≥4.00 μg/ml). These strains showed FLC cross-resistance (FLC MIC ≥16 μg/ml) and high CLT MIC values (≥0.25 μg/ml).

Both tested polyenes showed a narrow range of MIC values (0.03–0.25 μg/ml for both AMB and NYS) and showed no differences in *in vitro* activity (p = 0.43). None of the 287 strains showed high MIC values for NYSor for AMB. Furthermore, TRB was the least active drug against the isolates tested (GM = 6.25 μg/ml and MIC90 = 8.00 μg/ml). However, the five non-albicans isolates including four *C. dubliniensis* and one *C. africana* showed slightly lower TRB MIC values, ranging from 1.00 to 2.00 μg/ml (p = 0.42).

Discussion

In the present study, we used the amplification of the *hwp1* gene to evaluate the presence of *C. africana* and *C. dubliniensis* among vaginal isolates identified as *C. albicans* in accordance with their phenotypic characteristics. We

demonstrated that the amplification of the *hwp1* gene using pooled DNAs was as efficient as using individual DNAs to differentiate *C. dubliniensis* and *C. africana* from *C. albicans*. Thus, this methodology can be used as a high throughput screening method to study fungal collections.

Earlier reports from Europe and Africa demonstrated that *C. africana* represented more than 5% of the *C. albicans* species complex isolated from female genital specimens and that it was more prevalent than *C. dubliniensis* in this type of samples[2,13,25,31]. Conversely, in our patients, *C. africana* was less prevalent than *C. dubliniensis* (0.35% and 1.39%, respectively).

To the best of our knowledge, the described *C. africana* strain is the first autochthonous Argentinian isolate reported so far. The strain was identified as *C. africana* by two different sequencing-based methods (ITS and *hwp1* gene sequencing). Phenotypically, our *C. africana* strain showed characteristics that coincide with previous reports[2,25,33]. We observed a lower filamentation rate in serum for the *C. africana* isolate. This fact was reported also by Borman *et al*[2]. These authors linked this lower hyphal formation capacity to the reduced virulence of *C. africana* and its narrow anatomical niche.

When antifungal susceptibility was analyzed, our *C. africana* and *C. dubliniensis* strains showed low MIC values to all the antifungal tested. Moreover, reduced susceptibility was rare in all the *Candida* strains studied, reinforcing the idea that *C. albicans* and its related species isolated from VVC patients are usually susceptible to antifungal agents[7,8,12,21,22,29]. In the case of azoles, CLT and ITC showed the highest potency and FLC MIC GMs values were similar to those obtained in previous reports for yeast causing VVC[1,3,22]. However, 3.55 % (10/282) of the *C. albicans* strains showed FLC reduced susceptibility (three susceptible-dose

Table 3 *In vitro* susceptibilities of the strains included in the study. MIC values are expressed in µg/ml.

Species	N	AMB		NYS		FLC		ITC		VRC		CLT		TRB	
		MIC[a]	Range	MIC[a]	Range	MIC[a]	Range	MIC[a]	Range	MIC[a]	Range	MIC[a]	Range	MIC[a]	Range
C. albicans	282	0.10 (0.25)	0.03–0.25	0.12 (0.25)	0.03–0.25	0.34 (1.00)	0.06–64.00	0.03 (0.06)	0.015–4.00	0.021 (0.03)	0.015–4.00	0.02 (0.03)	0.015–0.12	6.25 (8.00)	2.00–16.00
C. dubliniensis	4	0.04	0.03–0.06	0.06	0.06	1.00	0.50–1.00	0.03	0.015–0.03	0.015	0.015	0.02	0.015–0.03	0.50	0.05
C. africana	1	0.12		0.03		0.06		0.03		0.015		0.03		1.00	

AMB: amphotericin B. NYS: nystatin. FLC: fluconazole. ITC: itraconazole. VRC: voriconazole. CLT: clotrimazole. TRB: terbinafine.

[a] Geometric means expressed in µg/ml. Between parenthesis MIC_{90} for *C. albicans* strains.

dependent), three FLC-resistant and four of these strains were azole cross-resistant. This FLC resistance frequency in *C. albicans* isolated from VVC was higher than that mentioned in other published reports, where there was no FLC resistance[12,21,29].

These susceptibility patterns and the wide range of FLC MIC values observed in our strain collection allow us to infer that in Santa Fe city several molecular mechanism of azole resistance may coexist and antifungal resistance could become a problem in the future[4,18,19,27,28,34].

Here we report the first Argentinian *C. africana* isolate and a high-throughput screening method to uncover the presence of this newly described species variety in a collection of yeasts isolated from VVC. We also demonstrate that *C. albicans* related species have low prevalence in our region. Moreover, antifungal resistance seems not to be a serious concern in Santa Fe city region (Argentina). However, VVC treatment should be supported by laboratory results.

Ethical disclosures

Protection of people and animals subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that this article does not appear patient data.

Right to privacy and informed consent. The authors declare that this article does not appear patient data.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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