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Molecular Identification of Fungal Species by Multiplex-qPCR in Candidal Vulvovaginitis Specimens and Antifungal Susceptibility

Inés Arrieta-Aguirre [1,†,*], Pilar Menéndez-Manjón [1,2,†], Giulia Carrano [2], Ander Diez [2], Íñigo Fernandez de Larrinoa [3] and María Dolores Moragues [1,4*]

[1] Department of Nursing, Faculty of Medicine and Nursing, University of the Basque Country UPV/EHU, Leioa, Bizkaia, Spain.; ines.arrieta@ehu.eus
[2] Department of Immunology, Microbiology and Parasitology, University of the Basque Country UPV/EHU, Leioa, Bizkaia, Spain.
[3] Applied Chemistry, University of the Basque Country UPV/EHU, Donostia, Spain.
[4] BioCruces Research Institute, Bizkaia, Spain
* Correspondence: ines.arrieta@ehu.eus; Tel.: +34 94 601 2906
† These two authors contributed equally to this work. Author order was determined alphabetically.

Abstract: Vulvovaginal candidiasis (VVC) is a prevalent condition affecting women worldwide. This study aimed to develop a rapid qPCR assay for accurate identification of VVC etiological agents and reduced azole susceptibility. One hundred and twenty nine vaginal samples from an outpatient clinic (Bilbao-Spain) were analyzed using culture-based methods and multiplex qPCR targeting fungal species, *Candida albicans* being the predominant species. Antifungal susceptibility tests revealed reduced azole susceptibility in three (3,48%) isolates. Molecular analysis identified several mutations in genes associated with azole resistance and novel mutations in *TAC1* and *MRR1* genes were also identified, which could contribute to drug resistance.

Keywords: Vulvovaginal Candidiasis; qPCR; *Candida* species; antifungal susceptibility; resistance-related mutations

1. Introduction

Vulvovaginal candidiasis (VVC) is a common condition characterized by vulvovaginal inflammation in combination with the detection of one or more *Candida* or fungal species [1]. Approximately 75% of women will experience at least one episode of VVC in their lifetime [2], while some of them will develop recurrent VVC (RVVC), which is arbitrarily defined as four or more episodes every year; this fact translates into disease, affecting millions of women worldwide [3,4]. Even though it is not a life-threatening infection, for some women, VVC implies a severe loss of life-quality, mainly due to the wide variety of clinical symptoms, which include vulvovaginal pruritus, soreness or dyspareunia, and also to the fact that acute recurrences often lead to depression and anxiety [5]. Although it is widely recognized that misdiagnosis of vaginal complaints is extremely common, the lack of an accurate diagnosis is still widespread [6]. This fact and the availability of the topical over-the-counter (OTC) drugs, compel patients to self-diagnose and self-medicate, ultimately leading to a bias of epidemiological data and to an increase in less drug-susceptible isolates [4]. The latter may occur due to the appearance of non-*albicans Candida* (NAC) isolates, some of which are less susceptible or intrinsically resistant to azoles, particularly *Nakaseomyces glabrata* (formerly known as *Candida glabrata*), *Candida tropicalis* or *Pichia kudriavcevii* (formerly known as *Candida krusei*) [3]. In this line, some authors have described a trend shift towards NAC species in vaginal samples [7]. Another possibility is the increase of *C. albicans* resistant isolates, and recent data show a worrying high level of resistance to various azoles in vulvovaginal isolates [7,8]. The long-term treatment with fungistatic fluconazole may entail ideal conditions for *C. albicans* strains to evolve into a resistant phenotype [4].

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The main trajectory for resistance development is up-regulation of *CDR1*, *CDR2*, or *MDR1* genes related to efflux-pumps. In addition, over-expression or mutations in the *ERG11* gene (encoding 14-α demethylase) and loss-of-function mutations in the *ERG3* gene (encoding Δ5,6 desaturase), both involved in ergosterol synthesis, are frequently reported in *C. albicans* resistant isolates [9]. Furthermore, gain-of-function (GOF) mutations in *TAC1*, *UPC2*, *MRR1* [10–12], and more recently *MRR2* genes [13], transcription regulators of some of the genes mentioned above, lead to constitutive over-expression, resulting in increased resistance. Besides, genome aneuploidies and loss of heterozygosity are also involved in azole resistance [14]. Many *C. albicans* isolates exhibit a combination of resistance mechanisms resulting in high levels of drug resistance and clinical failure [15].

Based on isolates identified in VVC, here, we report a real-time PCR assay targeting the multi-copy ITS1 gene of six yeasts species namely *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *N. glabrata*, *P. kudriavcevii*, and *Pichia guilliermondii*. We aimed to identify the etiologic agent as accurately and quickly as possible. The association of VVC/RVVC and some risk factors were also studied. Finally, we also characterized polymorphisms in genes related to azole resistance.

2. Patients and Methods

2.1. Vaginal Samples

One hundred and twenty nine vaginal samples were obtained from 115 women (aged >17 years) attending the outpatient clinic Bombero-Etxaniz (Bilbao, Spain) from February 2013 to July 2015. The research protocol was approved by the ethics committee of the Hospital of Basurto and the University of the Basque Country (UPV/EHU), and all subjects gave their informed consent to participate. Samples were obtained from the lateral vaginal wall with a sterile cotton-tip swab stored at 4°C and processed no later than 48 h. A case of VVC was defined as a patient with vulvar itching, vaginal discharge, and a positive culture.

2.2. Culture and Fungal Identification

Swab content was resuspended in 400 µL of sterile Phosphate Buffered Saline (PBS) and divided into two aliquots. One of the aliquots was cultured on chromogenic agar (Condalab, Spain) for 48h at 37°C, and the other one was stored at -20°C for later multiplex qPCR identification.

2.2.1. Isolates Identification by PCR or ID32C

Green colonies grown on chromogenic medium were further identified by PCR following the protocol described by Romeo and Criseo (2008), to allow discrimination between *C. albicans*, *Candida dubliniensis* and *Candida africana* [16]. Briefly, two colonies from fresh culture were added to 200 µL of PBS with glass-beads, vortexed and added directly to PCR mixture, which included 10 µL of DNA Polymerase Biomix buffer (Bioline, United Kingdom), and 0.8 µL of each Cr primer (25 µM). Not green colonies on chromogenic medium were identified using the ID32C system (bioMérieux, France). Isolates not identified by any of the above-described methods were derived to rDNA amplification (White et al, 1990) [17] and sequencing was performed at the SGIker for identification.

2.3. Molecular Identification of Fungal Species by Multiplex qPCR

2.3.1. Automated DNA Extraction

Vulvovaginal samples were thawed, 90 mg of glass-beads were added and vortexed for 5 minutes. Supernatants were transferred to a new collecting tube and 360 µL of MagNA Pure Bacteria Lysis Buffer (Roche, Germany) and 40 µL of Proteinase K (20 mg/mL) (Roche, Germany) were added. After incubating for 1 hour at 65°C and 10 min al 95°C, automated DNA extraction was performed by using 400 µL of the supernatant in the MagNA Pure Compact nucleic acid extraction instrument (Roche, Germany) using the protocol DNA_Bacteria_V3_2 and the extraction kit MagNA Pure

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Compact Nucleic Acid Isolation Kit I (Roche, Germany). The extracted DNA was eluted in 50 µL of buffer.

2.3.2. Primer and Taqman Probe Design for qPCR Assays

Sequences of the 18S-ITS1-5.8S region were aligned using the MUSCLE software [18]. All the sequences were obtained from the NCBI Entrez Nucleotide Database (https://www.ncbi.nlm.nih.gov/nucleotide/). Homologous areas of the 18S-ITS1-5.8S region were used to design Diamol-F and Diamol-R primers with the Primer Express software (Applied Biosystems). Furthermore, 12 specific Taqman probes that were designed hybridized into the ITS1 region. Six of them were labelled with 5'-FAM, four with 5'-HEX and two with 5'-JOE; all of them were labelled with 3'-BHQ1. On the other hand, an internal control (IC) of amplification and a probe (ICP) hybridizing this IC were designed to detect inhibition of qPCR. The 5' end of the IC included the same sequence of the primer Diamol-F, and the 3' end the reverse-complementary sequence of the primer Diamol-R. The ICP sequence was located four bases downstream the Diamol-F primer sequence, the rest was a chimeric sequence designed not to hybridize with any or the target species, neither with cohabitating species of the vulvovaginal niches nor with human DNA. ICP was labelled with 5'-ROX and 3'-BHQ2. Table 1 lists the sequences of primers and probes designed.

Table 1. Sequences of primers and probes used in the multiplex qPCR assays.

Primers and probes	Species	Gene	Sequences (5′→ 3′)
Primers			
Diamol-F	-	18S	TAGGTGAACCTGCGGAAGGA
Diamol-R	-	5.8S	TCGCTGCGTTCTTCATCGAT
Probes			
Calb	*C. albicans*	ITS1	**FAM**-CGGTGGGCCCAGCCTGCC-**BHQ1**
Calb2	*C. albicans*		**FAM**-ATCAA[C]TTGTCACA[C][C]AGA-ZNA4-**BHQ1**
Cpar2	*C. parapsilosis*		**JOE**-AGGCC[C]CATA[T]AGAAGG[C]CTA-**BHQ1**
Cpar3	*C. parapsilosis*		**HEX**-TGGCAGGCCCCATATAGAAGGCCTAC-**BHQ1**
Cgla	*N. glabrata*		**FAM**-ATTTCTCCTGCCTGCGCTTAAGTGCG-**BHQ1**
Cgla2	*N. glabrata*		**FAM**-TTAAGTGCGCGG[T][T]GGTGG-ZNA4-**BHQ1**
Cgui3	*M. guilliermondii*		**JOE**-AA[C]CTA[T]CT[C]TA[G]GC[C]AAA-**BHQ1**
Cgui4	*M. guilliermondii*		**HEX**-CAGCGTTTAACTGCGCGGCGA-**BHQ1**
Ctro	*C. tropicalis*		**HEX**-CGGTAGGATTGCTCCCGCCA-**BHQ1**
Ctro2	*C. tropicalis*		**HEX**-CGGTAGGATTGCTCCCGCCACC-**BHQ1**
Ckru	*P. kudriavzevii*		**FAM**-TTTAGGTGTTGTTGTTTTCGTTCCGCTC-**BHQ1**
Ckru2	*P. kudriavzevii*		**FAM**-CTACACTGCGTGAGCGGAACGAAAAC-**BHQ1**
Control			
Probe-ICP	-	-	**ROX**-AACGTGCGACGTTCCGAGCA-**BHQ2**
IC	-	-	*TAGGTGAACCTGCGGAAGGA*TCGA**AACGTGCGACG CTTCCGAGCA**TGATCACTATGTCCTAATCCCATAT ATTATTCACTGTGTACTAGCCCTTCTTGGTTCTCGC*A TCGATGAAGAACGCAGCGA*

Probes were marked with different fluorescent reporters attached at the 5' end (FAM, JOE, HEX, and ROX) and Black Hole Quencher (BHQ) at the 3' end. These were supplied by Metabion International AG, Germany, IDT, and/or Sigma-Aldrich. Internal Control (IC) 5' end includes the same sequence (italic letters) of the primer Diamol-F, and the 3' end the reverse-complementary sequence of the primer Diamol-R; Probe-ICP sequence is highlighted in bold. Locked Nucleic Acids are shown in square brackets [].

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2.3.3. Multiplex qPCR

All PCR protocols followed the recommendations of Khot and Fredricks (2009) to avoid both DNA contamination and PCR inhibition [19]. Briefly, DNA extraction, PCR reaction mixes, amplification, and PCR product analysis were performed in different rooms. In addition, PCR reaction mixtures were performed in a laminar-flow biosafety cabinet, whose surfaces were cleaned with Termi-DNA-tor (Biotools, Spain).

Before proceeding with multiplex assays, singleplex assays were carried out in a 7300 Real Time Cycler (Applied Biosystem, USA). Amplification mixtures with a final volume of 25 µL contained 10 µL of Premix Ex TaqTM (Takara, Japan), 0.65 µM of each primer, 0.3 µM of each probe, and 1 pmol of the IC. Twenty nanograms of DNA extracted from microorganisms cultures or 2 µL of DNA extracted from vaginal samples were added as template. To determine the analytical sensitivity and reproducibility of the qPCR, decreasing amounts of DNA, from 20 ng to 20 fg, extracted from cultures of 6 fungal species were tested. All samples were assayed in triplicate and on 3 different days. Afterwards, probes were combined in pairs, considering the fluorophore label.

2.4. Antifungal Susceptibility Testing

Fungal isolates were tested for in vitro susceptibility to fluconazole and clotrimazole (Sigma, USA). Fluconazole testing was performed as described in the document M27-4th ed. from the Clinical Laboratory Standards Institute (CLSI) [20], and result interpretations were performed according to M27M44S document [21]. On the other hand, since there is no standardized procedure nor MIC limit ranges for microdilution test for clotrimazole, we followed the protocol proposed by Pelletier et al. (2000), which is based on CLSI M27-A3 document with some modifications [22].

The isolates showing reduced susceptibility to fluconazole or clotrimazole were also tested by Sensititre YeastOne10® (Trek Diagnostic System, UK) colorimetric method.

2.5. Gene Expression Analysis by RT-qPCR

The expression levels of *CDR1*, *CDR2* and *MDR1* genes were measured in three resistant (Be-113, Be-114 and Be-129), and three susceptible yeast strain (SC5314, Be-47 and Be-90); for normalization purposes, expression of two reference genes, *ACT1* (actin) and *PMA1* (H+-ATPase) was also measured [12,23] (Table S1 upper panel). Total RNA was extracted from mid-log cultures in YPD (1% Yeast Extract, 2% Peptone, 2% Glucose) using the MasterPure™ Yeast RNA Purification Kit (Epicentre) following manufacturer's instructions with some modifications; proteinase K treatment was carried out at 50°C for 20 minutes and DNase treatment was extended up to 30 minutes. In every mutant obtained by CRISPR-Cas9 RNA was extracted from three independent cultures and it was quantified with NanoDrop 1000 (ThermoFisher Scientific). After checking the quality and integrity of RNA the cDNA was synthesized with PrimeScript™ RT reagent Kit (Perfect Real Time; Takara) following manufacturer's instructions and negative controls (RT-minus) of the reverse-transcription were performed. RNA and cDNA samples stored at - 80°C until use. RT-qPCR was performed in an Applied Biosystems 7300 Real-Time PCR System with SYBR® Premix ExTaq™ (Tli RNaseH Plus; Takara). Technical triplicates of each sample were performed and for each gene a non-template control was included. Stability of the reference genes used for normalization was assessed with the web-based RefFinder tool (https://www.heartcure.com.au/reffinder/). The fold-change was calculated with the 2-ΔΔCt method [24]. RNA transcript levels of the resistant clinical isolates were compared to the average expression of the susceptible ones, which was set to one, and each gene was considered to be overexpressed when fold-change in expression was ≥ 2.

2.6. Sequencing Analysis of ERG11, TAC1, UPC2, MRR1 and MRR2 Genes

Those *C. albicans* isolates that showed reduced or dose dependent azole susceptibility were selected for sequencing of resistance related genes. DNA was extracted using the DNeasy® Plant Mini Kit (QIAGEN) following manufacturer's instructions with modifications. Briefly, 4-5 yeast colonies grown in Sabouraud agar O/N at 37°C were suspended in 400 µL of sterile PBS, 90 mg of

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sterile glass beads (SIGMA) were added and vortexed for 5 minutes. The lysate was transferred to another tube, and 300 µL of Tris buffer (50 mM Tris-HCl pH8, 25 mM Na-EDTA, 75 mM NaCl) and 15 U of lyticase were added. After an incubation at 30°C for 20 minutes, 4 µL of RNAse (QIAGEN) was added and incubated at 37°C for 20 minutes. Then 100 µL of 10% SDS and 40 µL of Proteinase K (ROCHE) were added and incubated at 55°C for 20 minutes. At this point, the manufacturer's kit protocol was followed from the fourth step. The DNA sample was quantified in NanoDrop 1000 (ThermoFisher Scientific) and stored at -20°C until use.

The primers used for sequencing *TAC1*, *ERG11*, *UPC2*, *MRR1* and *MRR2* genes were designed to amplify the regions where mutations related to resistance to azoles had been previously identified in the literature and are listed in Table S1 (lower panel) [25,26]. The sequences of the amplicons were provided by the Sequencing and Genotyping Service SGIker of the UPV/EHU. The analysis was done with Chromas 2.5.1 software (Technelysium) to identify heterozygous mutations and, in order to identify the homozygous ones, BioEdit Sequence Alignment Editor 7.2.5 was used to align the sequences obtained with reference sequences for *TAC1* (GeneBank accession no. DQ393587), for *UPC2* (GeneBank accession no. EU583451), for *ERG11* (GeneBank accession no. X13296), for *MRR1* (GeneBank accession no. XM_711520) from Morio and collaborators (2013) [15], while for *MRR2* the sequence with the GeneBank accession no. XM_705846 was used.

2.7. Statistical Analysis

We used the 2x2 contingency table to calculate sensitivity, specificity, and predictive values of the multiplex qPCR, and X2 test for the association between risk factors and VVC. P values ≤ 0.05 were considered statistically significant.

3. Results

Of the 115 patients enrolled in this study, VVC was confirmed in 86 (66.67%) cases and, among them, 65 (75.58%) were classified as RVVC. The median age of the patients with VVC/RVVC was 30 years (range 17 to 51 years), slightly lower than the total population analyzed, 32 years (range 17 to 57 years). Among positive patients, 46 (53.49%) were pregnant, 17 (19.77%) had received antibiotic treatment in the last month, 13 (15.12%) had a history of allergic disorders, including allergic rhinitis, asthma, or sinusitis, 11 (12.8%) were taking oral contraceptives, 3 (3.49%) male partners of the patients with VVC had symptoms of *Candida* infection, and 1 (1.16%) was diabetic. Pregnancy was the only risk factor statistically associated to VVC/RVVC.

A total of 86 isolates were obtained from 129 vaginal specimens, including 81 (94.2%) *C. albicans*, 2 (2.32%) *C. parapsilosis*, 2 (2.32%) *N. glabrata*, and 1 (1.16%) *C. tropicalis*. Only one specimen registered a mixed infection with *C. albicans* and *N. glabrata*.

3.1. Yeast Identification by Multiplex qPCR

3.1.1. Collection of Microorganisms

The Diamol primers pair and the combinations of Calb-Cpar3, Cgla-Cgui4, and Ctro2-Ckru2 probes allowed the specific detection of *C. albicans*-*C. parapsilosis*, *N. glabrata*-*M. guilliermondii* and *C. tropicalis*-*P. kudriavzevii*, respectively (Table 2). Almost all the other species in our collection analyzed were negative for all the probes mentioned above, with the exception of the Cgla probe, which hybridized with *Saccharomyces cerevisiae* DNA. We decided to remove the IC, excluding the possibility of detecting qPCR inhibition, since probe-ICP had to be labelled with ROX; and this impeded the detection of any signal when three probe pairs were combined (data not shown).

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Table 2. Results obtained by multiplex qPCR with some species from our microorganism collection including Ct mean values ± SD.

		Probes (Ct ± SD)[b]					
Species	**Strain[a]**	**Calb**	**Cgla**	**Cpar3**	**Cgui4**	**Ctro2**	**Ckru2**
C. albicans	NCPF 3153	14.26 ± 0.056	-	-	-	-	-
N. glabrata	NCPF 3203	-	13.34 ± 0.056	-	-	-	-
C. parapsilosis	NCPF 3104	-	-	14.4 ± 0.106	-	-	-
M. guilliermondii	NCPF 3099	-	-	-	14.33 ± 0.891	-	-
C. tropicalis	NCPF 3111	-	-	-	-	14.31 ± 0.993	-
P. kudriavzevii	ATCC 6258	-	-	-	-	-	13.64 ± 0.007
Other yeasts							
Saccharomyces cerevisiae	CECT 1678	-	18.37	-	-	-	-
Saprochaete capitata	IHEM 5666	-	-	-	-	-	-
Yarrowia lipolytica	UPV 12-097	-	-	-	-	-	-
Rhodotorula mucilaginosa	CECT 11016	-	-	-	-	-	-
Filamentous fungi							
Aspergillus fumigatus	Af-293	-	-	-	-	-	-
Lomentospora prolificans	ATCC 64913	-	-	-	-	-	-
Cryptococcus neoformans	ATCC 90113	-	-	-	-	-	-
Bacteria							
Staphylococcus aureus	CECT 435	-	-	-	-	-	-
Streptococcus pyogenes	CECT 985	-	-	-	-	-	-
Streptococcus viridans	CECT 804	-	-	-	-	-	-
Streptococcus pneumoniae	CECT 993	-	-	-	-	-	-
Escherichia coli	CECT 434	-	-	-	-	-	-
Klebsiella pneumoniae	CECT 144	-	-	-	-	-	-
Pseudomonas aeruginosa	CECT 108	-	-	-	-	-	-
Proteus mirabilis	CECT 4168	-	-	-	-	-	-
Gardnerella vaginalis	ATCC 14018	-	-	-	-	-	-

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Human DNA							
Human Genomic DNA (Promega, Spain)	G304A*	-	-	-	-	-	-

On the other hand, Calb, Cpar3 and Cgla probes were tested in order to evaluate their specificity within the *C. albicans*, *C. parapsilosis* and *N. glabrata* complexes, respectively (Table S3). Cpar3 did not recognized *C. metapsilosis* nor *C. orthopsilosis* cryptic species, while Calb and Cgla probes hybridized with all species of their respective complexes. New Calb2 and Cgla2 new probes were designed, Calb2 did not hybridize with *C. dubliniensis*, while Cgla2 did not improve its specificity with respect to the Cgla probe.

3.1.2. Analytical Sensitivity or Limit of Detection (LOD) of the Multiplex qPCR

Three independent standard curves ranging 20 fg to 20 ng of DNA for *C. albicans*, *N. glabrata*, *C. parapsilosis*, *M. guilliermondii*, *C. tropicalis*, and *P. kudriavzevii* showed LOD of 20 fg for all the probes (Figure 1).

3.1.3. Validation of the Multiplex qPCR with Vaginal Swab Samples/Analytical Sensitivity and Specificity of Multiplex qPCR Compared with Conventional Culture

The multiplex qPCR developed was applied to 129 human vulvovaginal samples. Along with these samples, DNA extraction controls were performed, and multiplex qPCR was applied to these controls in order to monitor fungal/DNA contamination and detect false positives.

Of the 129 vaginal swabs, 123 (96.09%) were PCR-positive for *C. albicans*, 3 (2.325%) for *N. glabrata*, 2 (1.55%) for *C parapsilosis* and *M. guilliermondii*, 3 (2.325%) for *C. tropicalis*, and 4 (3.1%) *P. kudriavzevii*. Given such a high rate of positivity, probably because of the non-sterile origin of the samples, a Ct cut-off value of 30 cycles was stablished, so that samples with Ct values above 30 were considered negative. Notably, PCR contamination could be excluded, as evidenced by the lack of amplification in Non Template Controls (NTC) for the 6 probes used (data not shown). The majority of species identified in vaginal swabs by fungal culture and multiplex qPCR were *C. albicans*, therefore, it was only determined the analytical sensitivity of the qPCR for Calb probe. For that purpose, we built a contingency table (Table S4); the analytical sensitivity of the qPCR for Calb probe using fungal culture as the gold standard was 91.35%, the analytical specificity was 89.6%, the positive predictive value was 93.67%, and the negative predictive value 86%.

We were able to detect the two *N. glabrata* culture-positive samples with Cgla probe, but we obtained a discordant result negative-culture positive-PCR in one sample. Ctro2 probe detected the only sample culture positive for *C. tropicalis*, but we were not able to detect the two *C. parapsilosis* culture-positive samples with Cpar3 probe. Finally, Cgui4 and Ckru2 probes did not hybridized with any sample, so no false-positives were obtained for these probes.

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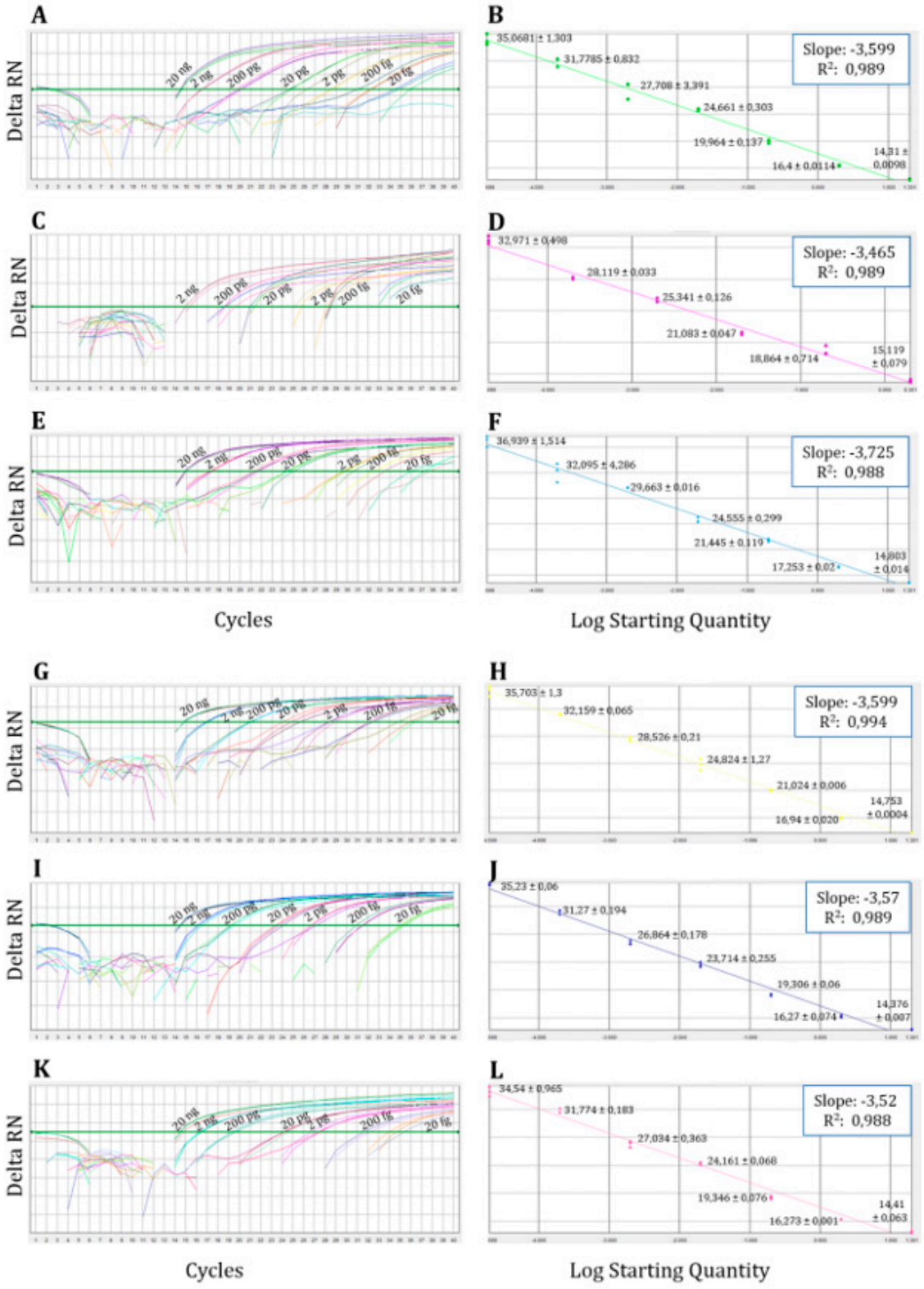

Figure 1. Results of the representative standard curves with de singleplex qPCR assay: Logarithmic representation of fluorescence detected with (A) Calb, (C) Cgla, (E) Cpar3, (G) Cgui4, (I) Ctro2, and (K) Ckru2 probes with the C. albicans, N. glabrata, C. parapsilosis, M. guilliermondii, C. tropicalis, and P. kudriavzevii (respectively) DNA serial dilutions from 20 fg to 20 ng. (B, D, F, H, J, and L) Plot of the Ct values versus the logarithmic of the genomic DNA starting concentration. Numbers next to replicates indicate the detection threshold cycles mean values obtained for each DNA concentration ± SD.

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3.2. Antifungal Susceptibility

A total of 78 isolates were tested for susceptibility to fluconazole and clotrimazole (8 isolates lost viability), MIC ranges, MIC50s and MIC90s of C. albicans isolates are summarized in Table S5. Most isolates were susceptible to both azoles. Three *C. albicans* isolates (Be-113, Be-114 and Be-129), showing high MICs of fluconazole or clotrimazole, were also tested with caspofungin, micafungin, anidulafungin, amphotericin B, 5-flucytosine, voriconazole, posaconazole, itraconazole using the Sensititre YeastOne method (Table S6). Be-113 was resistant to posaconazole and itraconazole, and susceptible-dose-dependent to fluconazole, voriconazole and clotrimazole; Be-114 was susceptible-dose-dependent to fluconazole and clotrimazole. Be-113 and Be-114 showed a MIC of 0.25 μg/ml for clotrimazole, a notably higher value than the rest of the vulvovaginal isolates, therefore they were considered susceptible-dose-dependent. Finally, Be-129 was resistant to fluconazole and voriconazole, and itraconazole susceptible-dose-dependent. Related to NAC strains, the two *N. glabrata* isolates showed dose dependent-susceptibility to fluconazole and clotrimazole, while *C. parapsilosis* and *C. tropicalis* strains were susceptible to both azoles.

3.3. Expression of CDR1, CDR2 and MDR1 Genes of Azole Resistant Isolates

We measured the expression levels of these genes, prior to sequencing, in order to identify which isolates could harbor GOF mutations in Tac1, Mrr1 and Mrr2 transcription factors. Be-113 overexpressed *CDR1* and *CDR2* (expression level>x2), while Be-114 reached an overexpression close to 2 and Be-129 did not overexpress any of the genes analyzed.

3.4. Amino Acid Substitutions in Erg11, Tac1, Upc2, Mrr1 and Mrr2

Be-113 and Be-129 isolates harbored mutations leading to amino acid substitutions in Erg11 (A114S, Y132H, Y257H and G450E) and Upc2 (G648S, in heterozigosis) that have been previously described and related to azole resistance (Table 3) [12,15,27–30]. Moreover, Be-113 displayed a homozygous genetic alteration in Tac1, S758F, which is described for the first time in this study. Finally, Be-114 isolate did not harbor any of the known resistance-related mutations that could be related to reduced azole susceptibility, but it had a new mutation, A311V.

Table 3. Amino acid substitutions in the transcription factors Tac1p, Upc2p, Mrr1p and Mrr2p and in Erg11p.

Isolate	Erg11p[a]	Tac1p[a]	Upc2p[a]	Mrr1[a]	Mrr2[a]
Be-113	**A114S**; **Y257H**	N396S[h]; S758F	-	V341E[h]; L592F[h]; E1020Q[h]	-
Be-114	-	A337V[h]; N396S; N772K; D776N; E829Q[h]; S941P[h]	-	V341E; E1020Q	A311V; A451A; V582L
Be-129	**Y132H**; **G450E**	N396S[h]; N772K[h]; D776N[h]; E829Q[h];S935L[h]; S941P[h]	**G648S[h]**	-	-

4. Discussion

We have developed a multiplex-qPCR assay to identify several *Candida* and fungal species simultaneously from vaginal swabs. Assay sensitivity and specificity for the *C. albicans* probe (Calb) and cut-off Ct value of 30 were 91.35% and 89.6%, respectively. Our results improve those obtained by Giraldo and colleagues (S=29.4%) [31], Weissenbacher and colleagues (S=42%) [32] or Mårdh and colleagues (E~50%) [33] and are similar to the excellent of Cartwright and colleagues [34] or, more recently, of Tardif and Schlaberg or Gaydos and colleagues [1,35]. Non-*albicans Candida* isolates were scarce so we were unable to validate the qPCR assay for the other probes, but Cgla and Ctro2 probes

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served to detect two infections caused by *N. glabrata* and one by *C. tropicalis*, respectively. The Cpar3 probe could not detect two samples of *C. parapsilosis*, probably because of the low fungal load of these samples (1-2 CFU). Cgui4 and Ckru2 probes, designed to detect *P. guilliermondii* and *P. kudriavzevii*, did not cross-react with any of the clinical samples, highlighting the specificity of these two probes.

Differentiation of *Candida*/fungal species is clinically relevant, not only for epidemiological reasons but also for differences in virulence and antifungal susceptibility among species [36]. Accurate diagnosis is, therefore, of utmost importance to provide optimal treatment. We were able to detect cryptic species of the *Candida albicans* and *Candida parapsilosis* complexes since Calb2 and Cpar3 probes only hybridized with their respective *C. albicans* and *C. parapsilosis* strains from our collection. We could not distinguish *C. albicans* and *C. africana* using the Calb probe, but both species are highly similar and several studies have also failed to achieve this milestone [36]. Furthermore, some authors do not even consider them different species [37]. Due to differences in virulence and antifungal susceptibility patterns among species within the same complex, the differentiation of *Candida* cryptic species has also become clinically relevant [36]. Finally, regarding the specificity of the multiplex qPCR assay, none of our probes hybridized with any of the tested microorganisms that can be present in human microbiota, not even with human DNA. An exception came from Cgla probe, which cross-reacted with DNA from *S. cerevisiae*, a species very much closer to *N. glabrata* than other species of *Candida* are [38]. This mismatch implies that this probe must be optimized, and designing a new probe with locked nucleic acid (LNA) technology could be a good option.

Compared with culture, the gold standard for VVC [39], this multiplex qPCR assay might imply an improvement in diagnosing of VVC, especially including accuracy, reliability, and time needed to finish the tests. In this sense, our assay provided an answer for clinical swabs in 5 hours compared to 24-96 hours required for culture detection [6]. Given this delay, many women do not seek medical diagnosis resulting in underdiagnoses of VVC, making it impossible to perform epidemiologic studies of this infection [5]. In this study, 15/115 (13,04%) of patients suspecting fungal infection that attended the outpatient clinic were finally diagnosed with *Gardnerella vaginalis*, a bacteria responsible for bacterial vaginosis. The later and VVC are common infections that frequently are misdiagnosed [40]. Additionally, over-the-counter (OTC) availability of antifungal agents is convenient for a rapid symptom relief, but they have also worsened the misdiagnosis of candidiasis. Furthermore, azole overuse and overexpose might be associated with a high resistance rate in *C. albicans* isolates and several authors have reported a trend towards less susceptible vulvovaginal *Candida* spp. isolates [41–43]. Regarding antifungal susceptibility we found three (3,48%) susceptible dose-dependent or azole-resistant *C. albicans* isolates. All of them were isolated from patients suffering from RVVC for a long time. Two of these isolates harbored mutations related to azole resistance: A114S and Y257H in Erg11p for Be-113 isolate; and Y132H and G450E in Erg11p and G648S in Upc2p for Be-129 [12,15,27–30]. The aforementioned mutations could explain the high MIC values of Be-113 and Be-129 isolates and the treatment failure for these patients. On the contrary, the Be-114 isolate did not harbor any of the already known resistance-related mutations; firstly classified as clotrimazole SDD, it was reinterpreted with new CLSI cut-off values [21] and categorized as an azole susceptible isolate. Nevertheless, since it was obtained from a patient who did not respond to azole treatment, we would expect Be-114 to be a resistant strain. Bearing in mind that CLSI M27-A4 antifungal drug susceptibility test (AST) does not consider vulvovaginal pH and drug pharmacokinetics in this anatomical niche, Sobel and Akins compared MIC values for 125 *C. albicans* vaginal isolates at pH 7.0 and pH 4.5 and observed that the lower the pH, the higher the MIC values, as a result of reduction in drug activity Consequently, they proposed that AST for vulvovaginal isolates should be performed at pH 4.5 [8]. This could explain why Be-114 isolate was reported by the lab as sensible, although it behaved as clinically resistant isolate in situ.

To summarize, molecular characterization of resistance-related polymorphisms is plausible for rapid diagnosis of resistance in *C. albicans* isolates. We used Sanger sequencing to identify mutations associated with resistance, which is laborious and does not permit the analysis of complete genes. However, probe-based real-time PCR assays are easier to perform, and when Next Generation Sequencing (NGS) platforms become cheaper, such broad analyses could replace or encompass

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culture-based diagnosis [44]. Here we described two new mutations, S758F in Tac1p and A311V in Mrr2p, in two isolates with reduced susceptibility and/or treatment failure, whose putative involvement in fluconazole resistance must be empirically tested, and, to this end, CRISPR-Cas9 seems a promising technology. In addition, vulvovaginal *C. albicans* isolates may be considered a model to detect new mutations and/or mechanisms related to azole resistance or tolerance, since they are faced to repeated short courses of low antifungal concentration, favoring their adaptation to azole pressure [45].

5. Conclusions

In conclusion, our multiplex-qPCR assay provides a rapid method to accurately diagnose yeast vulvovaginal infections, a condition usually underdiagnosed. Furthermore, the existence of resistance-related mutations emphasizes the need for precise resistance diagnosis, and molecular characterization holds promise for rapid reporting.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Table S1: Primers for gene expression analysis by RT-qPCR and primers for sequencing of *C. albicans* resistance-associated genes; Table S2: Collections of microorganisms tested by multiplex qPCR; Table S3: Amplifications results of the probes designed for cryptic species of *C. albicans*, *N. glabrata* and *C. parapsilosis*. Table S4: Comparison of multiplex qPCR assay and culture. Table S5: In vitro antifungal susceptibility of vulvovaginal isolates to several azoles; Table S6: Sensititre YeastOne microdilution method results of 3 resistant *C. albicans* isolates.

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