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Fluconazole Resistance in *Candida albicans*: basal *ERG11*, *MDR1* and *CDR1* gene expression of Clinical Isolates in Ecuador

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Fluconazole Resistance in *Candida albicans*: basal *ERG11*, *MDR1* and *CDR1* gene expression of Clinical Isolates in Ecuador

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Resumen

Candida albicans es uno de los patógenos oportunistas comúnmente asociado con infecciones fúngicas, que pueden llegar a ser potencialmente mortales y presentar altas tasas de morbilidad. Los azoles son la primera línea de tratamiento contra este hongo, pero la resistencia a estos medicamentos ha sido reportada a nivel mundial y está asociada con mecanismos moleculares tales como la sobreexpresión y las mutaciones de los genes *ERG11*, *MDR1* y *CDR1*. Una vigilancia adecuada de los mecanismos de resistencia permitiría mejores estrategias de tratamiento. El objetivo de esta investigación fue determinar la expresión basal de genes relacionados con la resistencia a los azoles (*MDR1*, *CDR1* y *ERG11*) mediante qPCR en 53 aislados clínicos de Ecuador. Los resultados muestran que la mayoría de las muestras (75,47%) expresaron los tres genes, siendo *ERG11* fue el más frecuentemente expresado (94,34%). Sin embargo, el gen *CDR1* tuvo el mayor nivel de expresión (9,37 E+04) entre los tres genes evaluados. Notablemente, solo una muestra no presentó expresión de estos genes. Aunque los análisis de correlación no muestran una relación entre el origen de la muestra y los niveles de expresión de los genes, la mayoría de aislados con alta expresión de estos genes resistentes a azoles provienen de muestras de orina. Este es el primer estudio que analiza la expresión basal de genes relacionados con la resistencia a los azoles en *Candida albicans* en aislados clínicos en Ecuador y evidencia la importancia de la vigilancia epidemiológica de este patógeno en el sistema de salud del país.

Palabras clave: *Candida albicans*, resistencia a azoles, expresión de genes, *ERG11*, *CDR1*, *MDR1*, qPCR

Abstract

Candida albicans is one of the most frequently reported opportunistic pathogens that cause life-threatening conditions with high mortality rates. Azoles are the first line of treatment for

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this fungus, but resistance to these drugs has increasingly been reported worldwide. Azoles resistance has been associated with various molecular mechanisms such as overexpression and mutations of *ERG11, MDR1* and *CDR1* genes. Adequate surveillance of resistance mechanisms would allow better treatment strategies. The aim of this study was to determine the basal expression of genes related to the azole resistance (*MDR1, CDR1* and *ERG11*) by qPCR in 53 *C. albicans* clinical isolates in Ecuador. The results showed that most samples (75,47%) expressed the three genes, with *ERG11* being the most frequently expressed (94,34%). However, the *CDR1* gene had the highest expression level (9,37E+04) among the three evaluated genes. Remarkably, only one sample presented no expression of these genes. Although correlation analyses do not show a relationship between the origin of the sample and the expression levels of the genes, most isolates with high expression of these azoles resistant genes come from urine samples. To the author's knowledge, this is the first study that analyzes the basal expression of genes related to azole resistance in *Candida albicans* and highlights the importance of epidemiological surveillance of this pathogen in the country's health system.

Keywords: *Candida albicans*, azoles drug resistance, gene expression, *ERG11*, *CDR1*, *MDR1* genes, qPCR.

1. Introduction

Fungal pathogens represent a significant global health challenge, infecting billions of individuals annually and contributing to an estimated 1.5 million deaths worldwide. In some cases, these infections can lead to mortality rates as high as 50% (1–4). Despite their impact, fungal infections have often been overlooked in hospital care (5). The rise in the immunocompromised population, including cancer patients, organ transplant recipients, HIV-Infected individuals, and the elderly, has contributed to the incidence of serious fungal-related diseases in recent years (6). Among these infections, invasive fungal infections caused by *Candida species* are the most prevalent among hospitalized patients globally (7–9).

Candida species are typically part of the normal human microbiota, existing as a commensal fungi that coexists harmlessly in the skin, mouth, genitourinary and gastrointestinal tract of most healthy individuals (2). However, they can become opportunistic pathogens and cause infections in various anatomical sites (10,11). These infections range from superficial mucocutaneous conditions, such as vulvovaginal candidiasis affecting 75% of women at least once in their lives, to life-threatening invasive candidiasis (candidemia and deep-seated tissue candidiasis) in immunocompromised individuals (12). Unfortunately, the incidence of candidemia has doubled over the last two decades, accounting for 9% of all nosocomial bloodstream infections worldwide (13–16). *Candida* stands as one of the five pathogens that are most frequently isolated in blood cultures

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(17). The heightened risk of infection arises from alterations in microbial composition or disruptions in the host immune system, facilitating fungal invasion (18,19).

C. albicans, among *Candida* spp. is the most widely prevalent and commonly associated opportunistic fungus linked to infections in humans (14,18,19). It possesses a range of virulence factors, such as germ tubes, adhesins, biofilm, hydrolytic enzymes, and the ability to switch between its both unicellular budding yeast and filamentous morphology (20,21). Furthermore, *C. albicans* exhibits a concerning rapid evolution of resistance to commonly used antifungals which include azoles (fluconazole, voriconazole, itraconazole, posaconazole), polyenes (amphotericin B), echinocandins (caspofungin, aminocandin, micafungin, anidulafungin) and antimetabolites (5-fluorocytosine) (22,23).

The primary classes of antifungal medications currently approved and used for the treatment of mycoses include azoles (fluconazole, voriconazole, itraconazole, posaconazole), polyenes (amphotericin B), echinocandins (caspofungin, aminocandin, micafungin, anidulafungin) and antimetabolites (5-fluorocytosine) (22,23). Remarkably, there have been no new classes of antifungals introduced in clinical practice since the mid-2000s (24). Azoles inhibit the lanosterol 14-alpha-sterol demethylase enzyme, encoded by the *ERG11* gene. This inhibition effectively blocks the synthesis of ergosterol, a vital component for maintaining the structural integrity of the fungal cell membrane. As a result, azoles lead to the accumulation of toxic sterol intermediates (14-a-methyl-3,6-diol) and reactive oxygen species (ROS), ultimately inhibiting the growth of the fungus (20,24,25). However, *Candida* species can develop resistance to azoles through various mechanisms, such as, chromosomal abnormalities involving to loss of heterozygosity in regions containing azole resistance genes like *ERG11*, *TAC1*, *MRR1* leading to the formation of isochromosome [i(5L)]; activation of cellular stress responses pathways including: Hsp90, Sgt1, calcineurin, KDACs, PKC; inhibition of Erg3 as well as *ERG3* mutations; up-regulation of multidrug transporters (Cdr1, Cdr2, and Mdr1); target alteration of ergosterol biosynthesis enzyme (Erg11) and the overexpression of the drug target gene *ERG11*, causing an increase in lanosterol 14-alpha-sterol demethylase enzyme in the fungal membrane and disrupting the affinity of azoles for the enzyme (26–28).

ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporters, encoded by the *CDR* and *MDR* genes respectively, play a crucial role in reducing the accumulation of azoles within yeast cells. They achieve this by actively shuttling these compounds across the cell membrane, thus constituting common mechanisms underlying acquired azole resistance. Indeed, several studies have implicated these genes with azole resistance. For instance, Shi et al. showed an elevation in the expression of efflux pump genes, which correlated with the development of fluconazole resistance in the initial phases of biofilm formation (29) . Moreover, the ABC transporters Cdr1 and Cdr2 are expressed in more than 50% of the isolates resistant to antifungal drugs (6,7,24,25,30–32). Notably, Cdr1 is the main determinant of azole resistance between the two transporters (29). Therefore, it becomes imperative to identify both the presence and the levels of expression of these resistance genes.

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The increasing rates of fluconazole resistance in *Candida* species can be primarily attributed to its widespread and prolonged use (4,20,25,33,34). This poses a direct impact on patient outcomes, as healthcare professionals are now faced with limited available and effective treatment options for one of the leading causes of potentially fatal invasive infections in hospitalized patients, creating a substantial risk to public health (3,4,35). Addressing this growing challenge requires urgent attention and innovative approaches to combat drug resistance in these opportunistic fungal pathogens.

In Ecuador, the 2018 antimicrobial resistance date report only includes bacterial agents such as *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, leaving out *Candida* spp. and other fungal pathogens (33). There is a scarcity of studies related to the epidemiology of fungal infections in the country, and the lack of genomic studies on *C. albicans* limits accurate description of its current epidemiological status. Molecular characterization of pathogens is crucial to understanding the evolution of this species and developing appropriate/effective treatments. This study aims to contribute to the epidemiological monitoring of *C. albicans* by determining the basal expression levels of *MDR1, CDR1, ERG11* genes through Real-time PCR of previously isolated clinical samples from an Ecuadorian health center.

2. Materials and Methods

2.1 *Candida albicans* isolates and culture conditions:

53 samples of isolates of *C. albicans* for this study taken in Guayaquil between January 2019 and February 2020, were provided by the Mycology laboratory of the Universidad Espíritu Santo, and the ATCC MYA-3573 isolated by the American Type Culture Collection was used as reference (Supplementary Table 1). The isolates were inoculated into Sabouraud Dextrose Agar (SDA Merch, Germany) medium and CHROM agar medium (CAC, Becton Dickinson, Heidelberg, Germany). Cultures were incubated at 37ºC for 48 hours, then the colony-forming units (CFU) were evaluated. Samples were stored at -80ºC in glycerol 40% stocks in sterile water. An initial concentration of 1×10^7 viable cells/mL was established on all samples using a Neubauer Counting chamber (34). This concentration was used for future experiments of gene expression.

2.2 Primer design for *MDR1*, *CDR1*, *ERG11* gene expression.

Coding sequences of candidate genes *MDR1*, *CDR1* and *ERG11* were obtained from NCBI (https://www.ncbi.nlm.nih.gov/) enlisted in Table 1. Parameters of primers to be selected included amplicons between 70- 200 bp long, melting temperature between 55°C to 63°C and 3' residues containing C-G, avoiding primer self- formation and GC content around 50-60%. The specificity of primers was evaluated by BLAST (36) and synthesized in the company Macrogen® (South Korea). Primers for actin gene (*ACT1)* was used as a housekeeping in all qPCR reactions (35,36).

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Table 1. List of primer sequences used for amplification of *MDR1*, *CDR1* and *ERG11* genes of *C. albicans* by qPCR

Gen	Primer sequences 5´→ 3´	Product length (bp)	Gene ID/Reference
MDR1	Fw1:GTCCCCGAGAAATATTTAGCCG Rv1:CACCATCCCAAGTGACAAC	199	This study
	Fw2: CCCTCAAAATTGGCCAAC Rv2: GGGCCAACACCATAACC	189	This study
	Fw3: CACCCTCGTTGAATTGGG Rv3: CACTTCGGGGAAAACCTG	136	This study
	Fw4: AGTTGCTTGGGGTAGTTCCG Rv4: CTTGCTCTCAACTTTGGTCCG	96	Maheronnaghsh, *et al*. (35) XM_714072.2 (37)
CDR1	Fw1:CCACGGGTTTGATGCCC Rv1: CCTGCTGACGAGTCATC	90	This study
	Fw2: GGCAACCTATGGGTTATCAC Rv2: CCCTCTAGTGGCATTATCCC	151	This study
	Fw3: TGTTGGGTTGGTCTCGATG Rv3: TCATAACCTGGACCACTTGG	130	Maheronnaghsh, *et al*. (35) XM_718116.2 (38)
	Fw4: GTGGGGTCTGTTCCAGG Rv4: CCCCCTTTTGCATAGCACC	178	This study
	Fw5: CACAGCAACCATGGGTC Rv:5: CCTGGTAATACATCAGGACC	132	This study
ERG11	Fw1: CTCCAGTTTTCGGTAAAGGGG Rv1: CATAACATTGGCAACCCC	200	This study
	Fw2: ACTCATGGGGTTGCCAAT Rv2: GTTGAGCAAATGAACGGTCA	115	This study
	Fw3: GGAGACGTGATGCTGCTC Rv3: CACCACGTTCTCTTCTCAG 3'	75	This study
	Fw4: GGGATACTGCTGCTGCC Rv4: CATCTATGTCTACCACCACC	130	This study
	Fw5: TTGGTGGTGGTAGACATAGATG Rv5: AACTATAATCAGGGTCAGGCAC	132	Maheronnaghsh, *et al*. (35) XM_711668.2 (39)
ACT1	Fw:AAGTGTGACATGGATGTTAGAAAAGAAT Rv: ATGGAGCCAAAGCAGTAATTTCC	118	(Bonfim-Mendonça *et al*, 2021) (40)
HWP1	Fw: CAGCCACTGAAACACCAACT Rv: AACCTCACCAATTGCTCCAG	597	XM_704869.2 Gene ID: 3645372

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2.3 RNA isolation and cDNA synthesis of *C. albicans* isolates.

Yeast suspension was grown overnight in Yeast Extract–Peptone–Dextrose (YPD) broth medium at 37ºC. After incubation, the cells were adjusted to 2x 107 cells/mL of concentration in 5 mL of liquid YDP broth medium at 37 and 200 rpm for 20 hrs. Cells were snap–frozen in liquid nitrogen and stored at −80°C. RNA isolation of the 54 samples of *C. albicans* was performed using the Total RNA kit I (Omega Bio-Tek, Inc., Norcross, GA, USA) following the manufacturer's instructions. The quality and quantity of RNA were evaluated by spectrophotometry (Synergy HTX- biotek). All RNA samples were diluted to achieve a final concentration of 40 ng/uL.

cDNA synthesis was performed immediately after RNA isolation using the Superscript Double-Stranded cDNA synthesis kit (Invitrogen) following the manufacturer's recommendations. Briefly, a mix of 1 uL of oligo (dT)20, 1 uL of diluted RNA, 1 uL of dNTPs, and 10 uL of ultrapure water was heated to 65ºC for 5 min and then chilled on ice. This was followed by the addition of First-Strand buffer, DTT, RNaseOUT, and Reverse Transcriptase (SuperScript III RT). The reverse transcription reaction was carried out for 60 minutes at 50ºC and inactivated at 70ºC for 15 minutes. It was assumed that the efficiency of the reaction was the same for all samples.

2.4 qPCR.

Real-time PCR reactions were performed in duplicates. in a total reaction volume of 7, 5uL comprising 3.75uL of 2X FastGene Buffer, 0.4uM of each gene-specific primer, 0.5uL of template, and 2.65uL of nuclease-free water. qPCR reactions were optimized for each pair of gene-specific primers using DNA extracted from a clinical isolate as a template. Primer temperature of annealing was assessed through real-time PCR with an extension set at 66ºC. Non-specific PCR products were ruled out by the Melting curve analysis. The presence of multiple peaks in the melt curve during qPCR reactions resulted in the discarding of the corresponding primer pairs. Briefly, the qPCR conditions consisted of an initial temperature of 50ºC for 3 minutes, denaturation at 95ºC for 5 min (one cycle) and 95ºC for 15 seconds and annealing and elongation at 66ºC for 30 seconds (40 cycles). A melting curve step (from 58ºC, gradually increasing at 0.5ºC/s to 95ºC, with acquisition data every 0,05 s) was included for every reaction. The intra-assay variation of the qPCR was assessed by running duplicates for 4 clinical DNA samples. Results reflect a high level of precision (CV<10%).

2.5 *HWP1* Cloning and standard curve.

The hyphal wall protein-coding gene (*HWP1*) constantly expresses the adhesin protein Hwp1 on the germ tube and hyphal surface in *Candida* species. A 600-bp region of the *HWP1* gene was amplified from a clinical strain of *C. albicans* using the primers F1

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HWP1 and R3 *HWP1*. The fragment was then cloned into the pGEMT-Easy vector (Promega) following the manufacturer's instructions. The ligation reaction was incubated for 16 hours at 4ºC followed by transformation of the recombinant plasmid into *E. coli* DH5**α**. Constructions were verified by colony PCR and electrophoresis with 1% gel. The plasmid was extracted with PureLink Quick Plasmid Miniprep Kit (Invitrogen) following the manufacturer's protocol. Plasmid mass concentration was determined by Qubit dsDNA BR Assay kit (Invitrogen) and then converted into copy number concentration using the formula described by Whelan, 2003 (41,42).

The plasmid DNA sample was serially diluted (from $1x10^9$, $1x10^8$, $1x10^7$, $1x10^6$, $1x10^5$, $1x10^4$, $1x10^3$, $1x10^2$, $1x10^1$, $1x10^0$ copy/µL) with ultrapure water and used to prepare the standard curve. The software Bio-Rad CFX provided by the CFX96 thermocycler (Bio-Rad) calculates the standard curve for each run based on the cycle threshold and plots a linear regression line. The resulting equation is used for the absolute quantification of transcripts for unknown samples. (Supplementary figure 1)

2.6 RT-qPCR: Quantitative real-time PCR with BioRad Maestro CFX (software)

Once the cDNA of each sample was obtained, the FastGene® IC Green 2x qPCR Universal Mix (Nippon Genetics Europe Gmbh) was used to perform the qPCR using the designed primers for each gene (*CDR1*, *MDR1*, *ERG11*); as inner control, *ACT1 primer* was used as housekeeping to verify retro-transcribed cDNA samples viability and the standard curve previously described. DNA sample of a *C. albicans* strand (DCA49) was used as a positive control after confirming its resistance to azoles by VITEK Cassette. Each reaction was made with a total volume of 7,5 µL as mentioned before with optimization conditions of qPCR. Finally, the measured expression level of the genes by qPCR were shown in Sq (number of copies sequenced) units.

2.7 Ethics statements:

This study was approved by the Ethics Committee of the University Especialidades Espíritu Santo (protocol 2022-001A), certified by the Ministry of Public Health from Ecuador. All samples were anonymized, and no data of the patients were made available.

2.8 Statistic analysis

qPCR results were analyzed to identify if there were gene expression and to determine significant differences in the expression levels of the *ERG11*, *MDR1*, and *CDR1* genes between the *C. albicans* isolates to suspect or not azoles drug resistance. In the statistical analysis of the data, the Fisher exact test paired by the program R Studio was used. The value of $p<0.05$ was considered statistically significant. GraphPrim (USA) software was used to compare the expression of the genes between the isolates and the ATCC by creating some of the graphics published in this study.

3. Results

A total of 53 clinical isolates of *C. albicans* were collected from various sources among patients admitted in the public health center of Guayaquil. Isolates were most frequently sourced from urine (n=16, 30.19%), followed by respiratory tract secretion (n=14, 26.42%), vaginal secretion (n=8, 15.09%), blood cultures (n=7, 13.21%), catheters (n=4, 7.55%), wounds (n=3, 5.66%), and pleural effusion (n=1, 1.89%). Data of in vitro antifungal susceptibility fluconazole testing by the clinical laboratory of a public health center yield data on 36 isolates. Of these, 95% exhibit susceptibility, while 5% were resistant. However, the remaining 17 isolates do not register results (Supplementary table 1).

In selecting the primers used for expression analysis, it was considered as optimal annealing temperatures and produced unique PCR products by melting curve analysis. The primer sets Fw4/Rv4, Fw3/Rv3, and Fw5/Rv5 were used to quantify the expression levels of the genes *MDR1*, *CDR1*, and *ERG11*. Subsequently, the Coefficient of variability (CV) was evaluated for each primer in duplicate samples, for *MDR1* gene CV=9.59, *CDR1* gene CV=3.47 *ERG11* gene CV= 4.12. This shows the low variability of expression levels in the qPCR assays. The amplification efficiency (E) was validated by generating serial dilutions ranging from 83 to 107% using a DNA template. Efficiency of the MDR1 primer was 107.1%, the *CDR1* primer was 83.4% and the ERG11 primer was 95.5%. This allows us to ensure reproducibility accuracy.

Considering the entire optimization process for amplification, the expression frequency was analyzed. The gene frequency expression of the above-mentioned genes (*MDR1*, *CDR1*, and *ERG11)* is shown in Table 2. *ERG11* exhibited the highest frequency of expression, followed by *CDR1* and *MDR1*. Notably, the vast majority of the samples (73.58%) expressed higher levels than ATCC in all three resistance genes, while only one isolate #19 (DCA#42) did not surpass the ATCC expression in any of the three genes (Figure 1).

Table 2. *Frequency in gene expression of clinical isolates.*

Gene expression		***Count***	***Percentage***	***>ATCC****	***Count***	***Percentage***
MDR1	*YES*	*50*	*94,34%*	*YES*	*45*	*84.91%*
	NO	*3*	*5,66%*	*NO*	*8*	*15,09%*
CDR1	*YES*	*46*	*86,79%*	*YES*	*45*	*84.91%*
	NO	*7*	*13,21%*	*NO*	*8*	*15.09%*
ERG11	*YES*	*53*	*100,00%*	*YES*	*50*	*94.34%*

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	NO	0	0,00%	NO	3	7.55%

**: Basal expression higher than reference ATCC*

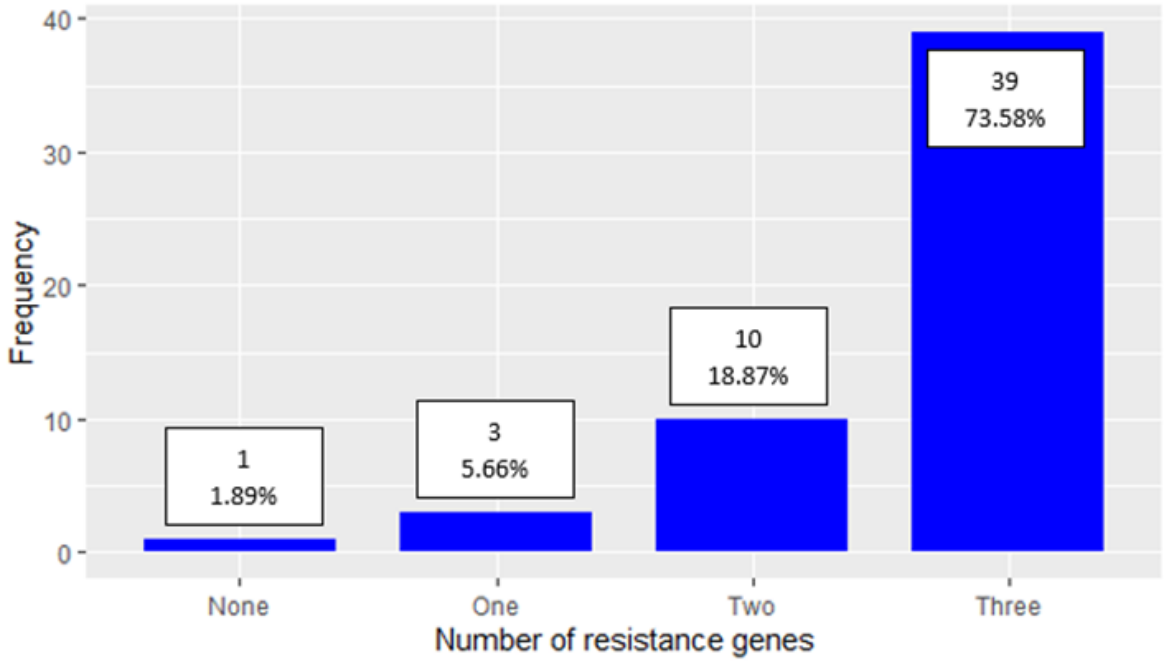

Figure 1. *Frequency of isolates that expressed higher levels than ATCC respectively for each gene.*

The basal gene expression analysis by RT-qPCR revealed significant differences in the average number of sequence copies among the three genes (Table 3). The *CDR1* gene showed the highest average number of copies (9,37E+04), while comparatively lower average numbers of copies were observed for the *ERG11* (4,93E+04) and *MDR1* genes (1,76E+04). It is important to mention the elevated expression (9,72E+05) in isolate #31 (DCA60) influenced the mean of the absolute quantification of the *CDR1* gene. Although, *ERG11* has a higher frequency of expression (Table 2). The basal expression of *MDR1*, *CDR1*, *ERG11* on the clinical isolates is useful to identify the behavior of *C. albicans* in absence of stimuli such as fluconazole. This shows a high variance of basal expression in genes related to azole resistance.

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Table 3. Mean, standard deviation of the absolute quantification of transcripts of clinical isolates of *MDR1*, *CDR1*, *ERG11* genes.

Gene	*MDR1*	*CDR1*	*ERG11*
Mean	1,76E+04	9,37E+04	4,93E+04
SD	8,67E+04	1,52E+05	4,39E+04
Q1	6,54E+03	1,80E+04	2,03E+04
Q2	2,84E+03	4,79E+04	3,97E+04
Q3	8,35E+03	1,18E+05	5,97E+04
Min	0,00E+00	0,00E+00	6,93E-01
Max	6,34E+05	9,72E+05	2,29E+05

SD: standard deviation, Q1: quartile 1, Q2: quartile 2, Q3: quartile 3, Min: minimum value of gene expression, Max: maximum value of gene expression

The difference in basal expression between the *C. albicans* clinical isolates compared to ATCC expression for *MDR1*, *CDR1*, *ERG11* genes are presented in Figure 2. The absolute quantification (copies/1 000 000) of ATCC was *MDR1*= 6,56E+01, *CDR1*=3,67E+02, *ERG11*= 9,09E+02, respectively. The basal expression of the *MDR1* gene in the isolate #30 (DCA45) records the highest value. The majority of isolates (45 isolates) exceed the level of expression of ATCC (6,56E+01). However, the levels of expression show variability. The level of expression in 8 isolates (#3, #5, #13, #18, #19, #20, #22, #26) did not express the gene or their level of expression is lower than the ATCC. This information suggests great variability in basal expression of *MDR1* gene in all isolates.

From the 46 isolates that expressed *CDR1* gene, 45 isolates surpass the ATCC basal expression on this gene. Isolate #11 had a very low expression (4,18E+00) which is imperceptible in Figure 2.B. Isolate#31 (DCA60) shows the most elevated basal expression in this gene. Despite these isolates, the distribution of the samples that expressed a higher number of copies than the ATCC (3,67E+02), does not vary as much as *MDR1* gene.

All the isolates expressed *ERG11* gene. However, isolate#7 (DCA 66), #17 (DCA20), #19 (DCA 42) did not exceed ATCC basal expression. *ER11* gene basal expression is similar to *CDR1* gene, although they contribute to azole resistance through different mechanisms.

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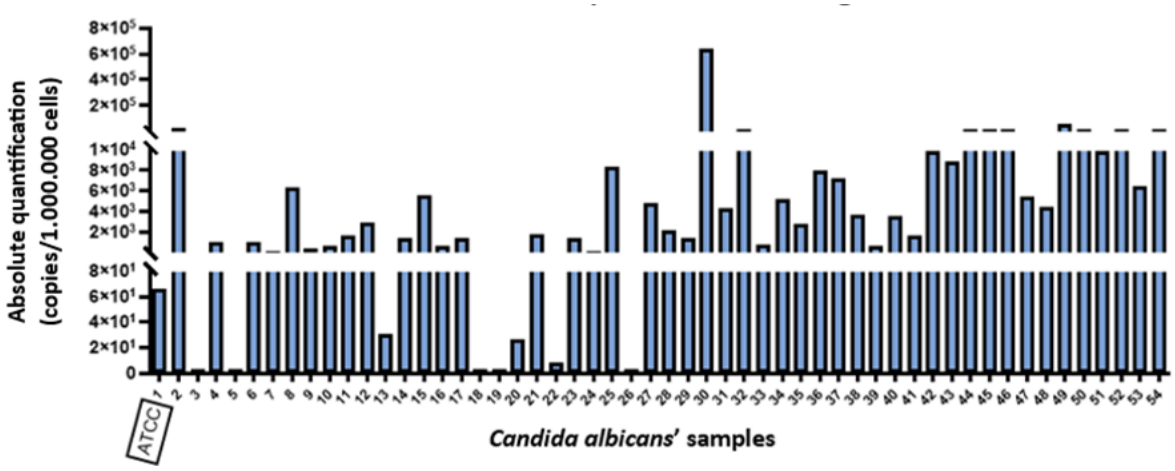
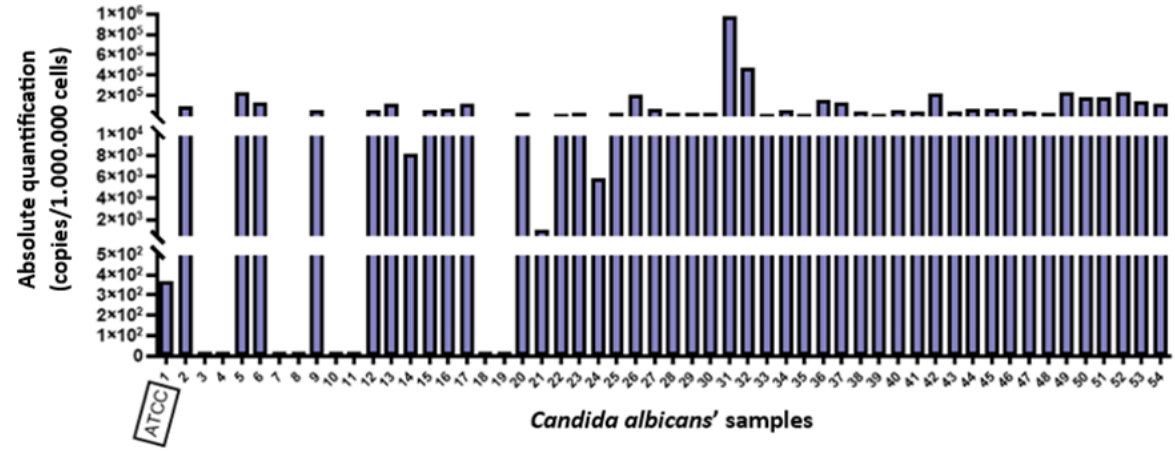
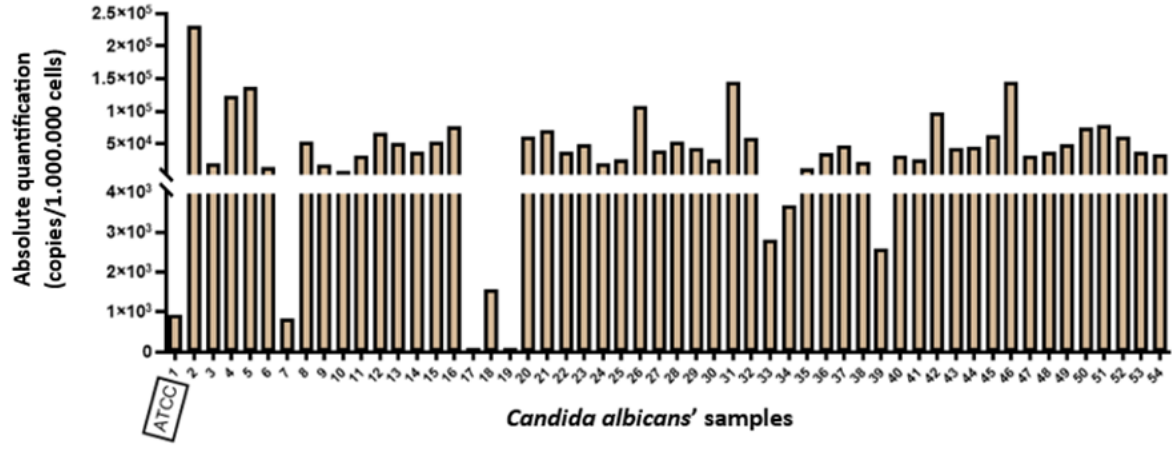
A.


Absolute quantification (copies/1.000.000 cells)
8×10⁵
6×10⁵
4×10⁵
2×10⁵
1×10⁴
8×10³
6×10³
4×10³
2×10³
8×10¹
6×10¹
4×10¹
2×10¹
0
ATCC
Candida albicans' samples

B.


Absolute quantification (copies/1.000.000 cells)
1×10⁶
8×10⁵
6×10⁵
4×10⁵
2×10⁵
1×10⁴
8×10³
6×10³
4×10³
2×10³
5×10²
4×10²
3×10²
2×10²
1×10²
0
ATCC
Candida albicans' samples

C.


Absolute quantification (copies/1.000.000 cells)
2.5×10⁵
2×10⁵
1.5×10⁵
1×10⁵
5×10⁴
4×10³
3×10³
2×10³
1×10³
0
ATCC
Candida albicans' samples

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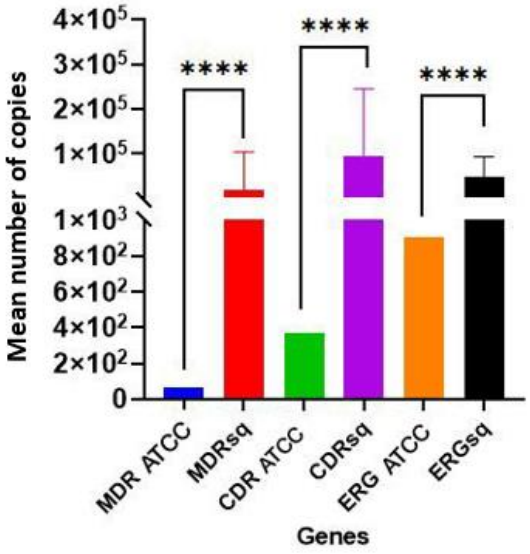

Figure 2. Absolute quantification (copies/ 1 000 000 cells) genes involved in fungal resistance to azoles in *C. albicans* for each isolate. A. Absolute quantification of *MDR1* gene. B. Absolute quantification of *CDR1* gene. C. Absolute quantification of *ERG11* gene. Wild type strain ATCC= MYA-3573

We compared the difference between the mean basal expression ATCC and strains from clinical isolates on *MDR1*, *CDR1*, and *ERG11* genes. Figure 3 shows the mean number of the gene's transcripts to demonstrate the statistical significance among the expression levels on the different genes. The mean number of the gene transcripts from all the clinical isolates is higher than the expression in the reference ATCC *C. albicans* strain for the genes studied. (Figure 3). There is a difference in the mean basal expression within the clinical isolates analyzed in this study.

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Figure 3. *Mean number of copies (gene transcripts) of all clinical isolates for each gene (MDRsq, CDRsq, ERGsq) compared to ATCC C. albicans expression respectively. **** p value < 0.0001.*

The higher gene expression of isolated compared to the ATCC expression level in relation to the origin of the samples was evaluated. It was observed that from all the *C. albicans* isolates, the majority (31,71%) that surpassed the ATCC expression of *MDR1* gene came from urine samples, followed by respiratory tract secretions (21.95%). The isolates that expressed *CDR1* gene in higher levels than the ATCC were more frequent in urine (26,67%), as well as respiratory tract samples (26,67%). Regarding the *ERG11* gene, 30,61% of isolates expressed this gene at a higher level than the ATCC and came from urine samples. However, these associations did not reach statistical significance, as indicated by the p-value exceeding p<0,05. The percentage of isolates that surpass or not the expression of the ATCC of *MDR1*, *CDR1* and ERG11 according to the sample's origin are presented in Table 4. Our results did not reveal any significant association between the higher expression of the isolates compared to the ATCC in resistance-related genes and the specific origin of the samples.

Table 4. Relation between isolates with higher expression than ATCC and sample origin.

Origin	***n***	***Frequency***	***MDR1***		**p value ***	***CDR1***		**p value ***	***ERG11***		**p value ***
			< ATCC (%)	**> ATCC (%)**		**< ATCC (%)**	**> ATCC (%)**		**< ATCC (%)**	**> ATCC (%)**	
Catheter	**4**	**7.55%**	8,33	7,32	0,88	0,00	8,89	0,95	0,00	8,16	0,87
Blood culture	**7**	**13.21%**	8,33	14,63		12,50	13,33		0,00	14,29	
Wound	**3**	**5.66%**	0,00	7,32		0,00	6,67		0,00	6,12	
Pleural effusion	**1**	**1.89%**	0,00	2,44		0,00	2,22		0,00	2,04	

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Urine	**16**	**30.19%**	25,00	31,71		50,00	26,67		25,00	30,61	
Respiratory tract secretions	**14**	**26.42%**	41,67	21,95		25,00	26,67		50,00	24,49	
Vaginal secretion	**8**	**15.09%**	16,67	14,63		12,50	15,56		25,00	14,29	

Note:
n= number of samples
< ATCC%= frequency of isolates that did not surpass the ATCC expression.
> ATCC%= frequency of isolates that surpass the ATCC expression
p value was calculated by Fisher exact test. **p <0,05,*

To analyze the distribution of genes associated with resistance compared to the absolute quantification (copies/1 000 000), the results were divided into the origin of the sample from which the isolates were obtained (Figure 4).

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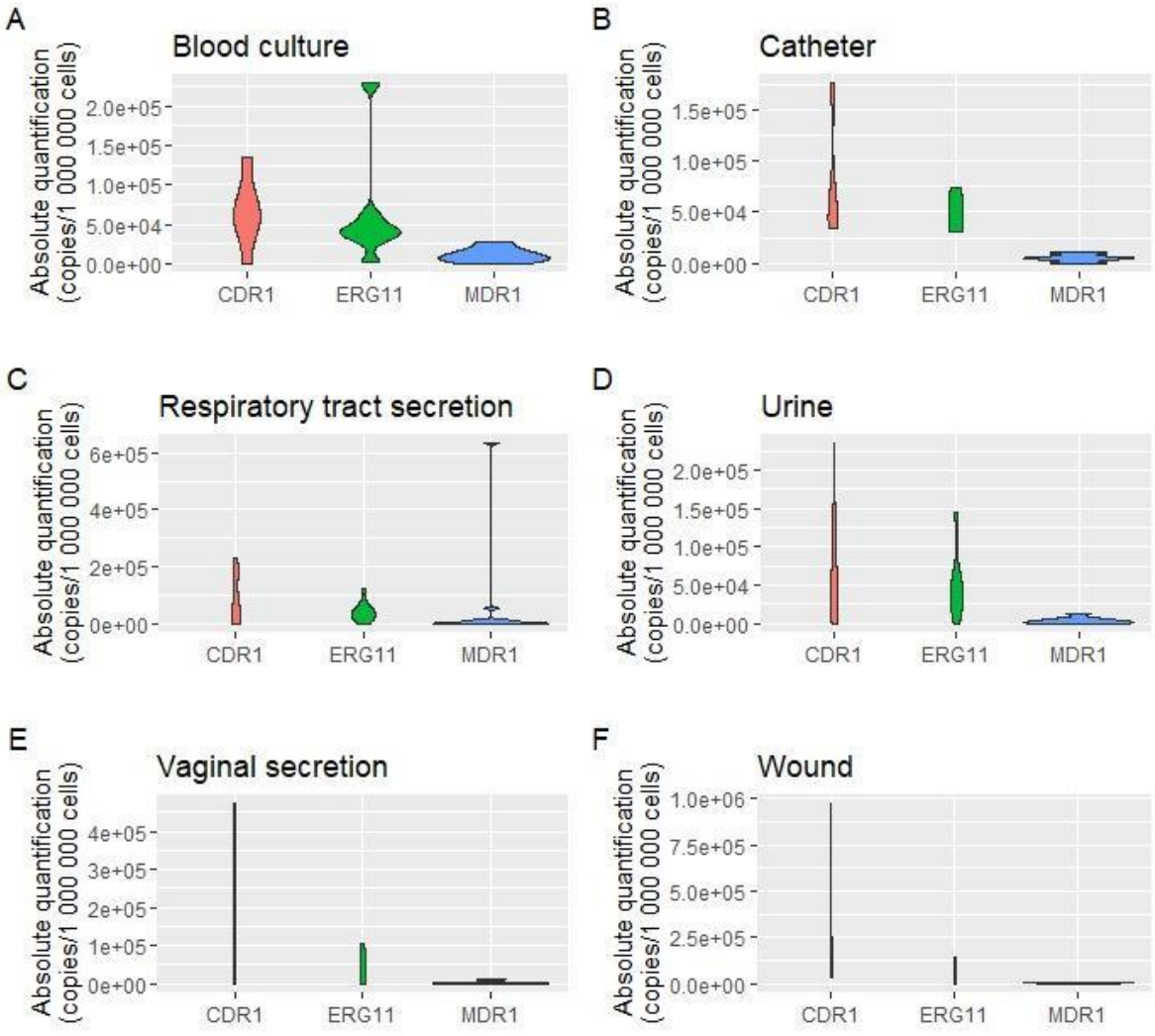

Figure 4. *Distribution of the absolute quantification (copies/ 1000 000 cells) of the clinical isolates for CDR1, ERG11, MDR1 genes.*

Isolates taken from blood culture show a high level of expression in *CDR1* (mean=6.48e+04), *ERG11* (mean= 6.38e+04), and *MDR1* (mean=1.12e+04) genes. Isolate #1 (KCA4) shows a different behavior on *ERG11* gene with the highest expression level (2,29E+05) driving it away from the mean. Isolates from catheter, urine, vaginal secretion and wound share similar distributions of the expression of the clinical isolates on each gene. However, on the *CDR1* gene, the highest level of expression (9,72E+05) was found on a wound sample: isolate #31 (DCA60). Isolates from respiratory tract secretions conserve an expression level not to variable within the same gene. Only isolate#30 (DCA45) deviated away from the average level of expression of *MDR* gene with an expression level of 6,34E+05 copies/1 000 000 cells. It cannot be determined if there is variability of expression in pleural effusion because we only have 1 isolate from that origin.

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4. Discussion

Opportunistic pathogen infection rates have risen in recent years, with *C. albicans* being the most frequently infectious fungus reported (29). The World Health Organization recognizes *C. albicans* as a "critical Priority Group" in the fungal priority pathogens list, highlighting the urgent need for research, development, and public health actions to address this concern (43). Azoles are the primary line of treatment for *Candida spp.* infections. However, the escalating resistance to this class of drug is becoming a global challenge in recent years (44–46). Antifungal resistance can either be intrinsic (primary) or acquired (secondary) resistance (47). Prolonged exposure to antifungals, results in acquired resistance. *Candida* usually turns into multidrug resistant by acquired resistance in species with intrinsic resistance. *C. albicans* commonly acquire azole resistance after prolonged azole therapy, as demonstrated after 6 months therapy by Bhattachary (48). One of the key contributors to azole drug resistance is the overexpression of genes such as *ERG11*, *MDR1,* and *CDR1*, which trigger cellular mechanisms leading to resistance (49). Remarkably, a study by Dhasarathan, P. et al, revealed that a hundred *C. albicans* isolates from chronic tuberculosis patients' sputum were extremely resistant to all azole medications, except for nystatin, which belongs to the polyene class (50).

The prevalence and antifungal susceptibility of *C. albicans* in the Ecuadorian population have been scarcely described, and the available studies are limited to specific groups of individuals (51–56). To the best of our knowledge, our study represents the first comprehensive analysis of gene expression related to azole resistance *C. albicans*, encompassing 53 isolates from different origins (catheter, blood samples, wounds, pleural fluid, urine, respiratory tract secretions, vaginal discharge) from a health center in Guayaquil, Ecuador. Using qPCR, we have gained insights into the gene expression basal patterns of *ERG11*, *MDR1,* and *CDR1* genes in these clinical isolates. Considering that RT-qPCR is the most accurate approach for determining the expression of genes and understanding the mechanisms of resistance, our results are particularly valuable because studying the basal expression of underlying genes that contributes to antifungal resistance is essential to understand the development of resistance at the molecular level in local outbreaks.

Overall, the results of our study of the absolute expression levels revealed that most isolates (73,58%, n=40) express all 3 genes (*ERG11, CDR1, MDR1*) and only 1 isolate #18 (DCA42) did not express these genes. Even though the expression of the genes cannot absolutely confirm the resistance without in vitro testing as MIC, studies have demonstrated that the overexpression of these 3 genes are important factors for azole drug resistance (50). The most frequently expressed gene was *ERG11* (53 out of 53 isolates), followed by *MDR1* (50/53) and finally *CDR1* (46/53). These findings contrast with those from the study by Maheronnaghsh, M. *et al*., who analyzed 12 sensible and resistant isolates. Among the resistant isolates, *MDR1* gene was the most commonly expressed gene, followed by *CDR1* gene (35). Additionally, Rosana et al. found that isolates that exhibited single-fluconazole resistance expressed the highest level of *ERG11* gene overexpression (57). Meanwhile, Monroy-Pérez et al., identified 94.9% of azole resistance phenotypes in their *C. albicans*

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isolates with *CDR1* expression identified in 66.6% of these isolates. They attributed this detection gap to the gene *ERG11,* which results in an overproduction of an azole target, 14-demethylase (58). Collectively, these studies show the complexity of gene expression patterns related to azole resistance in *C. albicans* and underscore the importance of research and monitoring different genes linked to azole-resistant *C. albicans* across global populations.

The overexpression of *CDR1* gene has emerged as one of the predominant mechanisms in azole-resistant *Candida albicans* clinical isolates (35,59) . In our study, *CDR1* reached the highest mean expression level (9,37E+04), based on the mean number of copies detected in 46 *C. albicans* isolates. Isolate #31 (DCA60) had the greatest expression of the *CDR1* gene (Figure. 2B), contributing significantly to this elevated mean expression level. Isolate#31 (DCA60) was isolated from a wound. Wounds are listed as a risk factor of candida infections. C. *albicans* is frequently identified in wounds, is prone to form biofilm there, and can lead to osteoarticular mycoses (60). Alteration or loss of the host natural barriers like wounds and intravascular catheters are predisposing factors for *C. albicans* infections (61). In our study, isolates from wound and catheter share similar distribution and levels of expression. Monroy-Pérez *et al.*, reported that the combined expression of *CDR1* gene with virulence markers are indicators of high pathogenicity in *C. albicans* and also recommended non-azole medication for treating such infections (58). Additionally, Zhang *et al*., reported that isolates resistant to fluconazole increased *CDR1* gene expression in clinical isolates with minimum inhibitory concentrations (MICs) of ≥64 µg/mL, the relative quantification analysis revealed heightened expression levels of *CDR1*, *CDR2*, and *MDR1* genes. Moreover, inhibiting these genes resulted in increased sensitivity to azoles (62). These results suggest a critical role of the *CDR1* gene in azole resistance.

Azole resistance to *C. albicans* has been related to overexpression and/or mutations in *ERG11* gene. In this study, the quantified expression of the *ERG11* gene showed that 3 isolates did not express a higher number of copies compared to ATCC, but the rest of the 50 isolates (94,34%) did. *ERG11* gene was the second gene with the highest mean expression (4,93E+04) and isolate #2 (KCA4) expressed the greatest number of copies (2,29E+05) for this gene, as observed in Figure 2C. These results are consistent with various publications mentioning that the high expression of *ERG11* decreases susceptibility to azole treatment and is commonly expressed in azoles-resistant *C. albicans* (6,7,24,25). Rojas, A., found *ERG11* gene to be the most frequent gene that is overexpressed in resistant strains (63), which is important to consider because *ERG11* gene is also the one which is most frequently expressed as basal expression among our isolates. Vasconcelos, JM, et al., assessed the relative expression level of *ERG11* gene in *C. albicans* isolates exposed to 1/4, 1/2 of MIC and the MIC for fluconazole and in the absence of this drug. They reported by analyzing the relative level of mRNA, that *ERG11* gene expression was significantly increased in all isolates exposed to fluconazole compared to its absence. They observed a wide range of *ERG11* gene expression in isolates exposed to MIC for fluconazole, 1.915 was the minimum value and 126.105 the maximum value expressed (64). Corroborating this, in our study a wide range of

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ERG11 gene expression was present, being 6,93E-01 the minimum value and 2,29E+05 the maximum value in absolute expression. Different results were obtained by Zare-Bidaki, M., et al. who found that *ERG11* gene was upregulated in 77% of *C. albicans* susceptible to fluconazole isolated from vulvovaginal candidiasis (65). However, this study is limited to compare and corroborate the level of gene expression with the resistant phenotype.

One of the fundamental causes of azole resistance in *C. albicans* is the overexpression of the *MDR1* gene. Under normal circumstances, fluconazole-susceptible strains typically do not express the *MDR1* gene and the inactivation of this gene leads to a reduction in the virulence of *C. albicans* (66,67). Hiller, D. states that the overexpression of the *MDR1* gene is enough to confer resistance to drugs like fluconazole and toxic compounds in *C. albicans.* The overexpression of *MDR1* showed a strong correlation with drug resistance (68). In this study, it was found that (n=45) 84.91% of the isolates expressed the *MDR1* gene at a higher level than the ATCC. Isolate #30 (DCA45) shows the highest expression of 6,34E+05 for this gene (Figure 2A.) Jahanshiri *et al*., reported that clinical isolates from patients diagnosed with cancer and having a MIC>32 μg/mL showed increased relative expression of resistance genes *MDR1*, *ERG11*, *CDR1*. Interestingly, isolates with decreased fluconazole sensitivity had higher expression levels of *MDR1* gene compared to *ERG11* gene (22). In our results, *ERG11* gene has a higher expression mean (4,93E+04) than *MDR1* gene (1,76E+04), but *MDR1* gene has a maximum expression of (6,34E+05), which is greater than the maximum of *ERG11* gene (2,29E+05). Rojas AE, et al. mentioned that *MDR1* gene generally overexpresses along with other genes related to azole resistance (69). However, in this study, *MDR1* gene shows a different pattern of the isolate's expression compared to *CDR1* and *ERG11* genes (Figure 2A, 2B, 2C.). El-Kholy, et al analyzed *MDR1* and *CDR1* gene expression in clinical *Candida tropicalis* isolates. They found higher relative expression of the *CDR1* gene in fluconazole-non susceptible isolates than in the ones susceptible to fluconazole. While the relative expression of *MDR1* gene did not differ significantly between the fluconazole-non-susceptible isolates and the ones susceptible to fluconazole (70). The gene expression between *MDR1* and *CDR1* gene can adopt different patterns, despite sharing similar mechanisms in azole resistance. Discrepancies in the gene expression levels, as noted in multiple studies, might be attributed to the different fluconazole concentrations to which the isolates were exposed (22,63,69,70). Our isolates demonstrated basal expression of clinical samples, which could contribute to these variations.

To assess whether the origin of the samples influenced the expression being higher than the ATCC in *MDR1*, *CDR1*, and *ERG11* genes, and found no significant correlation between the level of gene expression and the source of the samples. In this study, urine is the origin in which isolates' expression exceeds ATCC more frequently in every gene: *MDR1* (31.71%), *CDR1* (26,67%), *ERG11* (30,61%). Respiratory tract secretion takes the second place, in every gene, as the origin which isolates' expression exceeds ATCC: *MDR1* (21.95%), *CDR1* (26,67%), *ERG11* (24.49%). The third place in *ERG11* gene is shared by blood culture and vaginal secretion (14,29%). A study conducted by Avcioglu et al, in the Intensive Care Unit in Turkey found that bloodstream (50%), catheter-associated infections, and urinary tract infections (38%) were the most common types of infections. Interestingly, isolates from urine samples had a higher mortality rate (76%) compared to other sites (71). The main genes

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related to azole resistance in *C. albicans* are the most frequently found overexpressed in urine samples, which is related to high mortality rates. This information urges to gather complete clinical data on patients with *Candida* infections in order to evaluate the severity of the clinical isolates.

Azoles share structural similarities, and their widespread use has led to cross-resistance among different members of this drug family. Recent findings by Zhang H, indicate that most strains resistant to fluconazole also demonstrate resistance to itraconazole and ketoconazole (62). In line with this, in Ecuador a descriptive cross-sectional study on 136 isolates from vaginal samples conducted by Orellana and Pacheco reported an incidence of resistance to miconazole (19.9%), itraconazole (16.9%), and fluconazole (14%) (72). Similarly, Rodriguez analyzed the sensitivity of *C. albicans* and non-albicans species from vaginal secretions using the disk diffusion test. The study revealed that the majority of *C. albicans* isolates were susceptible to fluconazole (53%), voriconazole (80%), and clotrimazole (80%), while showing resistance to fluconazole (23.4%) and voriconazole (10%), with no resistance observed to clotrimazole (53). However, it is important to note that these results may not fully align with the international panorama, as Feng *et al*. reported higher resistance rates of 70.59% for itraconazole, 50.00% for voriconazole, and 52.94% for fluconazole in their study on *Candida* isolates (73). While this observation is noteworthy, further investigation is required to fully understand the implications of these findings.

Nystatin has shown a high level of sensitivity, reported at 97.4% in *C. albicans* strains in Latacunga-Ecuador. Research conducted by Núñez on *C. albicans* resistance to fluconazole and nystatin from vaginal secretions indicated a high percentage of women with candidiasis being resistant to fluconazole (72%) and nystatin (55%) (74). These findings suggest that despite a smaller percentage of drug resistance to nystatin in Ecuador, further investigation is required.

This study has several limitations. The number of samples used provides a general view of the behavior (basal expression) of the clinical isolates, however, it is not representative of Ecuador. Moreover, we only assessed the basal expression of 3 genes, without considering other variables. For instance, there are additional genes that have been associated with azole resistance in *C. albicans*, such as, *CDR2, MDR2, ERG3, UPC2*. Mutations such as LOH, SNPs, INDELs, CNVs in *TAC1* and *MRR1* which have also been found to play an important role in acquiring azole resistance in *C. albican*s. In future studies, microbiology analysis along with gene sequencing, and gene expression analysis could be performed to determine the correlation between the *Candida* spp. genetics and azole resistance. In addition to this, in hospital centers in Ecuador susceptibility tests to candida isolates are not always performed. This limitation did not allow us to carry out a more in-depth analysis of the microbiological characteristics and their relationship with molecular studies.

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Overall, our study sheds light on the growing concern of azole resistance in *C. albicans* in an Ecuadorian population and emphasizes the necessity of continuous research and vigilance to effectively combat this health challenge. Understanding the local gene expression profiles provides valuable information for enhancing treatment strategies and ensuring better patient outcomes in the face of emerging drug resistance. As the landscape of antifungal resistance continues to evolve, ongoing efforts in monitoring and studying the genetic mechanisms underlying resistance will be critical for effective management and control of *Candida* infections in the region.

5. Conclusions

The higher expression of *ERG11*, *MDR1*, *CDR1* is present in most isolates and could be correlated with azole resistance. These findings had not been reported previously in isolates of *C. albicans* in Ecuador, which allows an understanding of the resistance mechanisms to azoles being generated locally. *ERG11* was overexpressed in the great majority of the isolates. 73.58% of the isolates expressed more copies/ 1 000 000 cells than the ATCC of each gene respectively, which suggests that azole drug resistance in Ecuador is multifactorial and can involve various molecular mechanisms. This information is important to notice and consider as clinicians, for the treatment of candidiasis in patients with history of azoles usage and in high risk of *C. albicans* infections.

In addition to some limitations, the results obtained in this study provide valuable insights into the molecular epidemiology and potential consequence of widespread azole exposure to the development of resistance in *C. albicans* isolates. These findings reveal the importance of suggesting restrictions on the unjustified and inadequate prescription of this drug family without a confirmed diagnosed and fungal susceptibility test.

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Supplementary information

Supplementary Table 1. Details of clinical isolates of *C. albicans* from Ecuador

No. isolate	Code	Origin of the sample	Resistance (Fluconazole)
1	ATCC	Wild type strain ATCC= MYA-3573	
2	KCA4	Blood culture	NO
3	DCA17	Urine	ND
4	DCA29	Respiratory tract secretion	ND
5	DCA38	Urine	ND
6	DCA 52	Respiratory tract secretion	NO
7	DCA 66	Blood culture	NO
8	DCA 76	Urine	ND
9	KCA6	Respiratory tract secretion	NO
10	DCA18	Urine	ND
11	DCA30	Urine	NO

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12	DCA39	Urine	NO
13	DCA54	Urine	NO
14	DCA 67	Vaginal secretion	NO
15	DCA 78	Urine	ND
16	FCA 3	Urine	NO
17	DCA20	Urine	ND
18	DCA 31	Respiratory tract secretion	ND
19	DCA 42	Vaginal secretion	NO
20	DCA 58	Catheter	NO
21	DCA 68	Vaginal secretion	NO
22	FCA 2	Respiratory tract secretion	NO
23	DCA 21	Respiratory tract secretion	NO
24	DCA 33	Pleural effusion	ND
25	DCA 43	Respiratory tract secretion	ND
26	DCA 59	Vaginal secretion	NO
27	DCA69	Catheter	NO
28	JCA 1	Respiratory tract secretion	ND
29	DCA 22	Wound	NO
30	DCA45	Respiratory tract secretion	NO
31	DCA60	Wound	NO
32	DCA70	Vaginal secretion	YES
33	DCA10	Vaginal secretion	ND
34	DCA23	Wound	NO
35	DCA34	Urine	NO
36	DCA48	Respiratory tract secretion	NO
37	DCA62	Urine	NO
38	DCA71	Urine	NO
39	DCA11	Respiratory tract secretion	ND
40	DCA25	Blood culture	NO
41	DCA35	Vaginal secretion	NO

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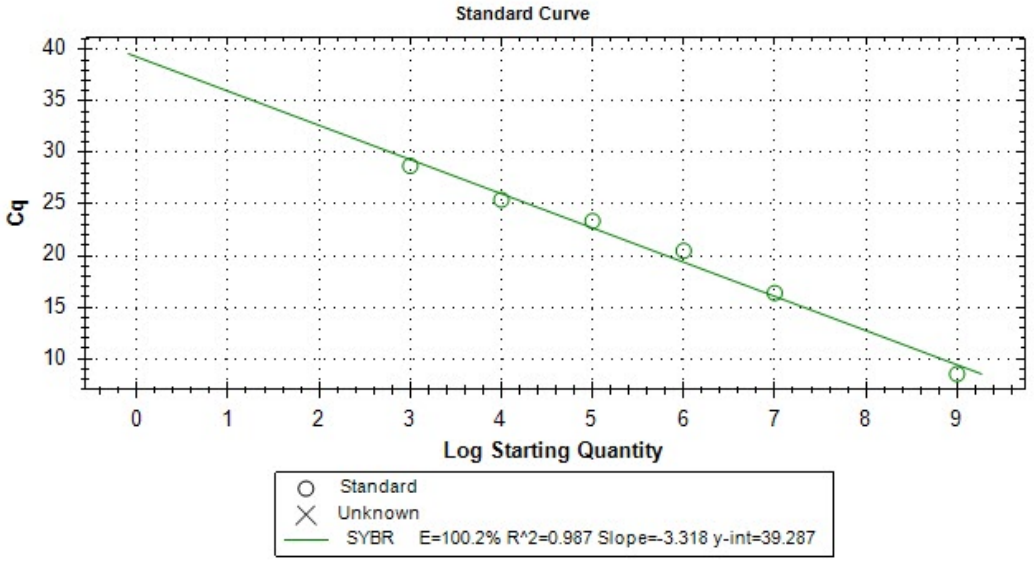
42	DCA49	Vaginal secretion	YES
43	DCA63	Blood culture	NO
44	DCA72	Blood culture	NO
45	DCA13	Blood culture	ND
46	DCA26	Urine	NO
47	DCA50	Catheter	NO
48	DCA64	Urine	NO
49	DCA14	Respiratory tract secretion	ND
50	DCA 27	Catheter	NO
51	DCA 37	Respiratory tract secretion	ND
52	DCA 51	Respiratory tract secretion	NO
53	DCA 65	Blood culture	NO
54	DCA 75	Urine	ND

ND: Non determined

.



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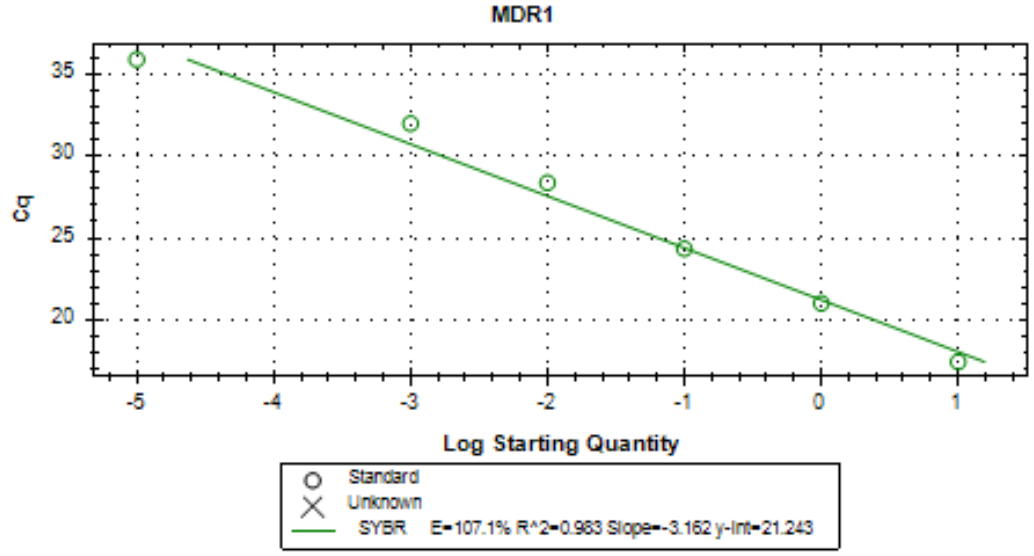
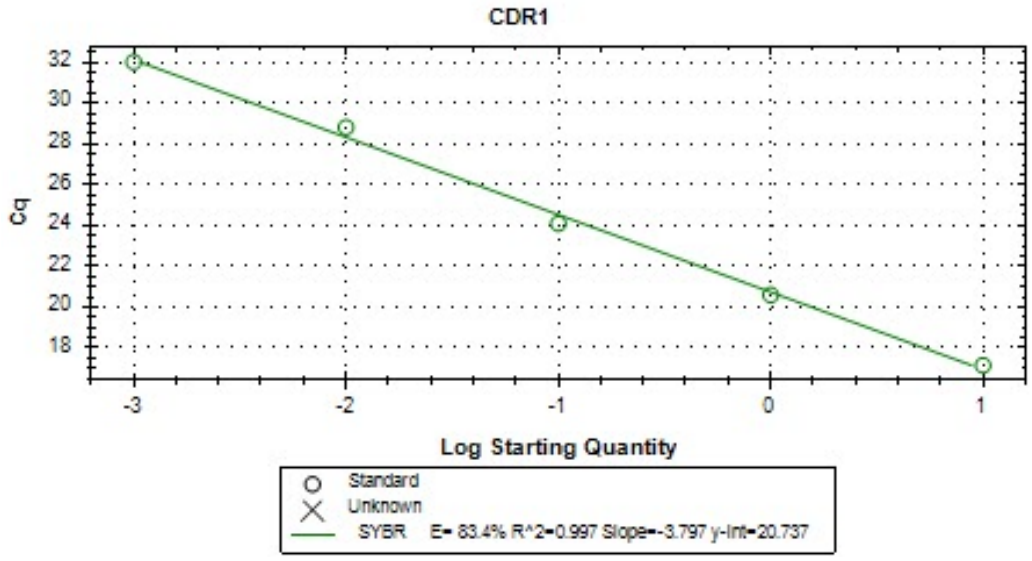
B.


MDR1
Cq
35
30
25
20
-5
-4
-3
-2
-1
0
1
Log Starting Quantity
Standard
Unknown
SYBR E=107.1% R^2=0.983 Slope=-3.162 y-Int=21.243

C.


CDR1
Cq
32
30
28
26
24
22
20
18
-3
-2
-1
0
1
Log Starting Quantity
Standard
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SYBR E= 83.4% R^2=0.997 Slope=-3.797 y-Int=20.737

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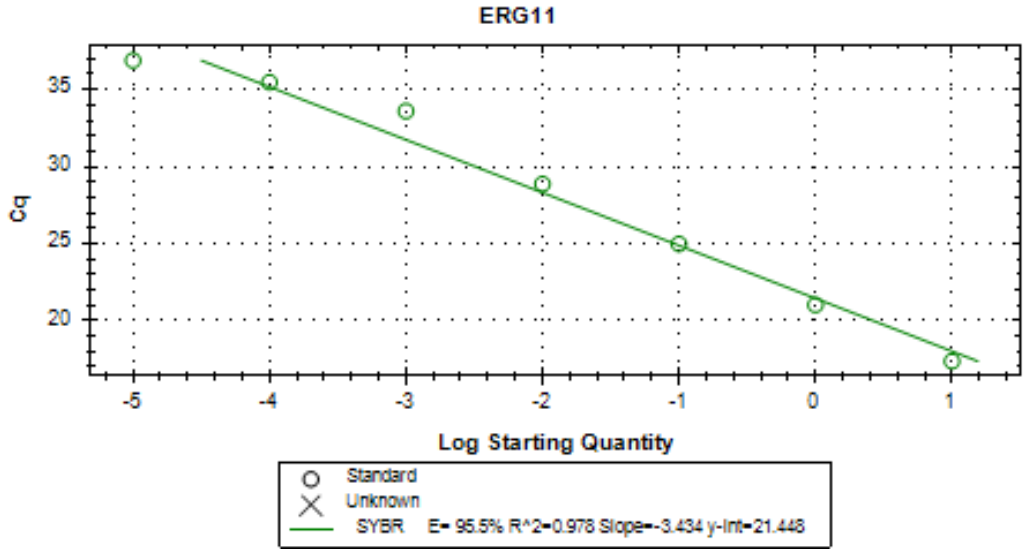
D.



Supplementary figure 1: Standard curve of optimal primers from genes related to fluconazole resistance and Serial dilution of *HWP1* cloning. A. Standard curve of the primer set of *HWP1*. B. Standard curve of the primer set of *MDR1* gene. *C.* Efficiency curve of the primer set of *CDR1* gene. *D.* Efficiency curve of primer set of *ERG11* gene.

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