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Research Article

High Virulence and Antifungal Resistance in Clinical Strains of *Candida albicans*

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Antifungal resistance and virulence properties of *Candida albicans* are a growing health problem worldwide. To study the expression of virulence and azole resistance genes in 39 clinical strains of *C. albicans*, we used a model of infection of human vaginal epithelial cells with *C. albicans* strains isolated from Mexican women with vulvovaginal candidiasis (VVC). The strains were identified by PCR amplification of the ITS1 and ITS2 regions of rRNA. The detection and expression of virulence genes and azole resistance genes *MDR1* and *CDR1* were performed using PCR and RT-PCR, respectively. All strains were sensitive to nystatin and 38 (97.4%) and 37 (94.9%) were resistant to ketoconazole and fluconazole, respectively. *ALS1*, *SAP4–SAP6*, *LIP1*, *LIP2*, *LIP4*, *LIP6*, *LIP7*, *LIP9*, *LIP10*, and *PLB1-PLB2* were present in all strains; *SAP1* was identified in 37 (94.8%) isolates, *HWP1* in 35 (89.7%), *ALS3* in 14 (35.8%), and *CDR1* in 26 (66.6%). In nearly all of the strains, *ALS1*, *HWP1*, *SAP4–SAP6*, *LIP1–LIP10*, *PLB1*, and *PLB2* were expressed, whereas *CDR1* was expressed in 20 (51.3%) and *ALS3* in 14 (35.8%). In our in vitro model of infection with *C. albicans*, the clinical strains showed different expression profiles of virulence genes in association with the azole resistance gene *CDR1*. The results indicate that the strains that infect Mexican patients suffering from VVC are highly virulent and virtually all are insensitive to azoles.

1. Introduction

Candida albicans is an opportunistic fungus that infects the mucosae of oral and vaginal cavities [1]. Vulvovaginal candidiasis (VVC) is among the most common infections that affect women of childbearing age [2]. Clinical strains of *C. albicans* possess an array of virulence genes that directly influence the pathogenesis of VVC, including genes responsible for phenotypic switching [3], *HWP1* (hyphal wall protein 1) [4], *ALS* (agglutinin-like sequence) [5], *SAP* (secreted aspartyl proteases) [6], *PL* (phospholipases) [7], and *LIP* (lipases) [8]. The aspartyl proteases, phospholipases, and lipases are involved in the breakdown of cell membranes in host epithelia, which promotes colonization and invasion [9].

The increase of *C. albicans* resistant to antifungal agents, mainly to azoles, is a serious health problem and hampers the treatment of VVC. The mechanisms responsible for resistance to azoles include overexpression of *CDR1* and *CDR2* from the ABC transporter family and *MDR1* which encodes a multidrug efflux pump. Mutations in *ERG5* and *ERG11*, which cause alterations in C22-desaturase and sterol 14α-demethylase, respectively, are also associated with azole resistance [10].

The expression of *ALS* and *SAP* gene families in *C. albicans* of vaginal origin has recently been studied [11, 12]. Likewise, the gene expression patterns of the *LIP* and *PL* family, which are associated with the formation of biofilm during infection, have been studied [13]. However, the expression patterns of *ALS*, *HWP1*, *SAP*, *LIP*, and *PLB* in association with azole resistance genetic determinants have been less studied in strains of vaginal origin. In this work, to analyse the expression patterns of *ALS1–ALS3*, *HWP1*, *SAP1*, *SAP4–SAP6*, *LIP1–LIP10*, *PLB1-PLB2*, *CDR1*, and *MDR1*, we implemented an in vitro model of infection of human vaginal cells with *C. albicans* strains isolated from women with VVC.

2. Materials and Methods

2.1. Patients Analysed and Sampling. Our study included a group of 200 women (ages 18–57) who attended the gynaecology department of public hospitals in the State of Mexico, Mexico, and presented characteristic symptoms of VVC (itching, burning, dysuria, and curd-like discharge). In this study, adult women with/without active sexual life were included. The exclusion criteria were as follows: patients with cervical cancer, being pregnant, being under antibiotic treatment, or having ingested antifungal drugs in the last 30 days. In addition, the patients informed if they had been previously exposed to azoles at least once in the last year as treatment for VVC. All participating women signed a letter of informed consent. The ethics committee of each hospital approved the study. Two samples were taken from the cervicovaginal cavity of each patient, using sterile swabs. The first was used to observe the presence of yeast, hyphae, or pseudohyphae by light microscopy. The second was incubated for 24 h at 37°C in brain heart infusion (BHI) medium (BD Bioxon, Cuautitlan Izcalli, State of Mexico, Mexico). After 24 h, the samples were incubated at 37°C in Sabouraud agar plates (BD Bioxon) with 50 μg/mL chloramphenicol.

2.2. Identification of C. albicans. *C. albicans* strains were identified by the API 20 C Aux test (bioMerieux, Durham, NC, USA), germ tube test [11], and PCR amplification of ITS1 and ITS2 rRNA loci (the primers are shown in Table 1) [14]. The DNA of the strains was extracted by boiling, as described by Paniagua-Contreras et al. [15]. The *C. albicans* strain B311 (ATCC 32354, ATCC, Manassas, VA, USA) was used as a positive control.

2.3. Antifungal Susceptibility. To test *C. albicans* antifungal susceptibility, we used diffusion disks impregnated with 25 μg of fluconazole, 10 μg of ketoconazole, and 100 U nystatin (HiMedia Laboratories, Mumbai, India). Antifungal susceptibility was established according to the interpretation criteria previously described (Table 2) [16].

2.4. Detection of Genetic Virulence Markers and Azole Resistance Genes by PCR. The primers and PCR conditions used were previously described: ALS1 and ALS3 [17], HWP1 [18], SAP1 [19], SAP4–SAP6 [20], LIP1–LIP10 and PLB1-PLB2 [13], and CDR1 and MDR1 [21] (Table 1).

2.5. In Vitro Infection Model. To study the expression of virulence markers and azole resistance genes, we used the A431 cell line (ATCC CRL-1555), which is derived from a vulvar epidermoid carcinoma. The growth conditions of the strains were previously reported [22, 23]. The A431 cell culture was inoculated with 50 μL of phosphate-buffered saline (PBS) with 2×10^6 *C. albicans* cells. The mixed cultures were incubated for 72 h at 37°C in 5% CO_2. The culture medium was changed every 24 h. *C. albicans* (ATCC 32354) and *Staphylococcus epidermidis* (ATCC 35984) were used as positive and negative controls, respectively.

2.6. RNA Purification and cDNA Synthesis. After infection of A431 cells, the yeasts were harvested and suspended in 200 μL of buffer Y1 (Qiagen, Hilden, Germany) with lyticase (50 U/10^7 cells). Samples were incubated for 20 min at 30°C with gentle agitation to facilitate the formation of spheroplasts. Total RNA was purified on a robotic QIAcube workstation (Qiagen) using the RNeasy Mini Kit (Qiagen), following the manufacturer's instructions, including DNase treatment. The concentration and purity of total RNA were analysed on a NanoDrop spectrophotometer 2000 (Thermo Scientific, Waltham, MA, USA). cDNA was synthesized with the QuantiTect Reverse Transcription kit (Qiagen) according to manufacturer's instructions.

2.7. Amplification and Detection of the Azole Resistance and Virulence Genes. Amplification and detection of azole resistance and virulence genes were performed using real-time PCR with the Rotor-Gene SYBR Green PCR kit (Qiagen). The primer sequences used for the gene expression analysis are shown in Table 1. The final volume of each reaction was 25 μL, consisting of 12.5 μL of SYBR Green Master Mix, 1 μL of forward primer (1 μM), 1 μL of reverse primer (1 μM), 2 μL of cDNA (~20 ng), and 8.5 μL RNase-free water. Amplification conditions were 95°C for 5 min, 40 cycles at 95°C for 5 s, and combination alignment/extension at 60°C for 10 s. The amplification conditions for the positive and negative controls were the same as described above.

3. Results

3.1. Identification of C. albicans and Antifungal Resistance Phenotypes. *C. albicans* was identified in 19.5% ($n = 39$) of the samples and, in the remaining ones (80.5%, $n = 161$), bacteria such as *Escherichia coli*, *Klebsiella* spp., *Staphylococcus aureus*, or *Staphylococcus epidermidis* were found.

All 39 isolates were sensitive to nystatin, whereas 97.4% ($n = 38$) and 94.9% ($n = 37$) were resistant to fluconazole and ketoconazole, respectively (Table 3). Resistance to both azoles was found in 94.9% ($n = 37$) of the strains.

3.2. Detection of Virulence Markers and Azole Resistance Genes. All of the isolated strains ($n = 39$) were carriers of the *ALS1, SAP4-SAP6*, *LIP1*, *LIP2*, *LIP4*, *LIP6*, *LIP7*, *LIP9*, *LIP10,* and *PLB1–PLB2* genes. Other genes detected were *SAP1* in 94.8% ($n = 37$), *HWP1* (89.7%, $n = 35$), *ALS3* (35.8%, $n = 14$), and *CDR1* (66.6%, $n = 26$) of the strains (Table 4).

3.3. Expression Patterns of the Virulence Genes and CDR1. In nearly all of the isolates, *ALS1*, *HWP1*, *SAP4–SAP6*, *LIP1–LIP10*, *PLB1*, and *PLB2* were expressed after infection of the cell line A431, whereas *CDR1* was expressed in 20 (51.3%) and *ALS3* in 14 (35.8%) (Table 4).

Fourteen distinct expression patterns of *ALS*, *HWP1*, *SAP*, *PLB*, *LIP*, and *CDR1* were identified (Table 5). The most prevalent expression profile, Pattern 1, composed of 18 genes, was found in 25.6% ($n = 10$) of the strains. Pattern 2 included 20 expressed genes and was found in 18% ($n = 7$); Patterns 3 and 4, both expressing 19 genes, were detected in 12.8% (5

Table 1: Primers used in PCR and real-time PCR assays.

Gene	Orientation	Sequence 5′ to 3′
rRNA	FW	TTTATCAACTTGTCACACCAGA
	RV	ATCCCGCCTTACCACTACCG
ALS1	FW	CCATCACTGAAGATATCACCACA
	RV	TGGAGCTTCTGTAGGACTGGTT
ALS3	FW	CCAAGTGTTCCAACAACTGAAA
	RV	GAACCGGTTGTTGCTATGGT
HWP1	FW	CCATGTGATGATTACCCACA
	RV	GCTGGAACAGAAGATTCAGG
SAP1	FW	TCAATCAATTTACTCTTCCATTTCTAACA
	RV	CCAGTAGCATTAACAGGAGTTTTAATGACA
SAP4	FW	TTATTTTTAGATATTGAGCCCACAGAAA
	RV	GCCAGTGTCAACAATAACGCTAAGTT
SAP5	FW	AGAATTTCCCGTCGATGAGACTGG
	RV	CAAATTTTGGGAAGTGCGGGAAGA
SAP6	FW	CCCGTTTTGAAATTAAATATGCTGATGG
	RV	GTCGTAAGGAGTTCTGGTAGCTTCG
LIP1	FW	AGCCCAACCAGAAGCTAATGAA
	RV	TGATGCAAAAGTCGCCATGT
LIP2	FW	GGCCTGGATTGATGCAAGAT
	RV	TTGTGTGCAGACATCCTTGGA
LIP3	FW	TCTCACCGAGATTGTTGTTGGA
	RV	GTTGGCCATCAAATCTTGCA
LIP4	FW	GCGTCCTGTTGCTTTCACT
	RV	ACACGGTTTGTTTTCCATTGAA
LIP5	FW	TGGTTCCAAAAATACCGTGTT
	RV	CGACAATAGGGACGATTTGATCA
LIP6	FW	AAGAATCTTCCGACCTGACCAA
	RV	ATATGCACCTGTTGACGTTCAAA
LIP7	FW	AACTGATATTTGCCATGCATTAGAAA
	RV	CCATTCCCGGTAACTAGCATGT
LIP8	FW	CAACAATTGCTAAAATCGTTGAAGA
	RV	AGGGATTTTTGGCACTAATTGTTT
LIP9	FW	CGCAAGTTTGAAGTCAGGAAAA
	RV	CCCACATTACAATTTGGCATCT
LIP10	FW	CACCTGGCTTAGCAGTTGCA
	RV	CCCAGCAAAGACTCATTTTATTCA
PLB1	FW	GGTGGAGAAAGATGGCCAAAA
	RV	AGCACTTACGTTACGATGCAACA
PLB2	FW	TGAAACCTTTGGGCGACAACT
	RV	GCCGCGCTCGTTGTTAA
CDR1	FW	AAGAGAACCATTACCAGG
	RV	AGGAATCGACGGATCAC
MDR1	FW	GGAGTTTAGGTGCTGT
	RV	CGGTGATGGCTCTCAA

Table 2: Interpretive criteria of susceptibility and resistance to antifungals used in this study.

Antifungal drugs	Zone diameter (mm)		
	Sensitive	Dose-dependent	Resistant
Ketoconazole	≥30	23–29	≤22
Fluconazole	≥19	15–18	≤14
Nystatin	≥25	17–24	≤14

Table 3: Susceptibility and resistance of *Candida albicans* strains to antifungal drugs.

Antifungal drugs	Resistant Number (%)	Sensitive Number (%)
Nystatin	0 (0)	39 (100)
Fluconazole	37 (94.9)	2 (5.1)
Ketoconazole	38 (97.4)	1 (2.6)

Table 4: Percentages of detection and expression of virulence markers and azole resistance genes in *Candida albicans* strains.

Gene	Detection Number (%)	Expression Number (%)
ALS1	39 (100)	39 (100)
ALS3	14 (35.8)	14 (35.8)
HWP1	35 (89.7)	35 (89.7)
SAP1	37 (94.8)	36 (92.3)
SAP4	39 (100)	39 (100)
SAP5	39 (100)	39 (100)
SAP6	39 (100)	39 (100)
LIP1	39 (100)	39 (100)
LIP2	39 (100)	39 (100)
LIP3	38 (97.4)	38 (97.4)
LIP4	39 (100)	39 (100)
LIP5	35 (89.7)	35 (89.7)
LIP6	39 (100)	39 (100)
LIP7	39 (100)	39 (100)
LIP8	38 (97.4)	38 (97.4)
LIP9	39 (100)	39 (100)
LIP10	39 (100)	39 (100)
PLB1	39 (100)	39 (100)
PLB2	39 (100)	39 (100)
CDR1	26 (66.6)	20 (51.3)
MDR1	0 (0)	0 (0)

strains); Patterns 5 (18 genes) and 6 (19 genes) were detected in 5.1% ($n = 2$) (Table 5). The following eight expression patterns (Patterns 7–14) were represented by a single strain. *CDR1* was found in eight different patterns ($n = 20$).

4. Discussion

In this study, we isolated and identified the virulence gene expression patterns of *C. albicans* strains in 19.5% of 200 vaginal samples. VVC is a condition that affects a quarter of young women [2, 24]. The presence of *E. coli*, *Klebsiella* spp., *S. aureus*, or *S. epidermidis* in most vaginal samples ($n = 161$) agrees with previous reports showing bacterial vaginitis as the first cause of vaginal infections [24].

The chronicity of VVC or recurrent episodes by *C. albicans* is due to prolonged use of antifungals, which select for resistant strains, and due to the numerous virulence genotypes that promote adhesion to epithelial cells, production of hydrolytic enzymes, colonization, and invasion [9]. We found that each strain isolated that carried *ALS1* (39/39) and *ALS3* (14/14) was able to express these genes in the in vitro model of infection (Table 4). The frequency of expression of the *ALS* genes reported here is similar to those reported in vaginal candidiasis models [25]. The ALS proteins are extracellular components of the cell membrane which mediate adhesion and colonization of host cells [26, 27]. Widespread expression of these proteins in the isolated strains supports their role in infection.

All strains positive for *HWP1* (35/35) expressed this gene during infection of A431 cells (Table 4). The frequency of expression is higher than results reported in *C. albicans* strains isolated from women with vaginal infection in Turkey [28]. The high frequency of expression of *HWP1* and the *ALS* genes found in this study (Table 4) reveals the capacity of the isolated strains to cause chronic vaginal infections. This result is consistent with reports showing that the combined expression of the adhesion proteins HWP1 and ALS1–ALS3 significantly facilitates biofilm formation [29] and can participate in the resistance to antifungals by limiting their penetrance to the biofilm [30].

Likewise, all isolated strains expressed *SAP4–SAP6* (39/39) and almost all expressed *SAP1* (92.3%, (36/37)) (Table 4). These results are similar to those previously described for *SAP* expression in VVC in humans [31]. The expression of *SAP4–SAP6* has a relevant role in pathogenesis by promoting hyphae formation [32] and by the inhibition of phagocytosis [33].

In this study, most strains expressed genes of the *LIP* family, except *LIP3*, *LIP5*, and *LIP8* (Table 4). These data are consistent with those reported using an in vitro infection model of oral epithelial cells and in patient samples [34]. The specific role of LIP proteins in infection is unknown. However, it has been described that *LIP* family members are differentially expressed during infection in specific areas of affected mucosae [35].

All strains of *C. albicans* expressed *PLB1* and *PLB2* (39/39) (Table 4). Recently, phospholipase activity was reported in strains of *C. albicans* and in nonalbicans strains from patients with vulvovaginal infections [36], as well as in different *Candida* species isolated from patients with type 2 diabetes mellitus suffering from VVC [37]. The elevated expression of phospholipase genes in the *C. albicans* strains isolated in this study (Table 4) suggests that these genes are essential for vaginal infection by participating in the breakdown of the cell membrane, which allows the hyphae to penetrate the epithelial cell cytoplasm [7].

The use of azoles for the treatment of VVC over time has resulted in the selection of strains resistant to these

Table 5: Expression patterns of virulence genes and *CDR1* in *Candida albicans* strains.

Pattern number	Pattern	Number of strains	%
1	*ALS1/HWP1/SAP1/SAP4/SAP5/SAP6/LIP1/LIP2/LIP3/LIP4/LIP5/LIP6/LIP7/LIP8/LIP9/LIP10/PLB1/PLB2*	10	25.6
2	*ALS1/ALS3/HWP1/SAP1/SAP4/SAP5/SAP6/LIP1/LIP2/LIP3/LIP4/LIP5/LIP6/LIP7/LIP8/LIP9/LIP10/PLB1/PLB2/CDR1*	7	18.0
3	*ALS1/ALS3/HWP1/SAP1/SAP4/SAP5/SAP6/LIP1/LIP2/LIP3/LIP4/LIP5/LIP6/LIP7/LIP8/LIP9/LIP10/PLB1/PLB2*	5	12.8
4	*ALS1/HWP1/SAP1/SAP4/SAP5/SAP6/LIP1/LIP2/LIP3/LIP4/LIP5/LIP6/LIP7/LIP8/LIP9/LIP10/PLB1/PLB2/CDR1*	5	12.8
5	*ALS1/SAP1/SAP4/SAP5/SAP6/LIP1/LIP2/LIP3/LIP4/LIP5/LIP6/LIP7/LIP8/LIP9/LIP10/PLB1/PLB2/CDR1*	2	5.1
6	*ALS1/HWP1/SAP1/SAP4/SAP5/SAP6/LIP1/LIP2/LIP3/LIP4/LIP5/LIP6/LIP7/LIP8/LIP9/LIP10/PLB1/PLB2/CDR1*	2	5.1
7	*ALS1/HWP1/SAP4/SAP5/SAP6/LIP1/LIP2/LIP3/LIP4/LIP6/LIP7/LIP8/LIP9/LIP10/PLB1/PLB2*	1	2.5
8	*ALS1/ALS3/HWP1/SAP1/SAP4/SAP5/SAP6/LIP1/LIP2/LIP3/LIP4/LIP6/LIP7/LIP8/LIP9/LIP10/PLB1/PLB2*	1	2.5
9	*ALS1/SAP1/SAP4/SAP5/SAP6/LIP1/LIP2/LIP3/LIP4/LIP5/LIP6/LIP7/LIP8/LIP9/LIP10/PLB1/PLB2*	1	2.5
10	*ALS1/HWP1/SAP1/SAP4/SAP5/SAP6/LIP1/LIP2/LIP3/LIP4/LIP6/LIP7/LIP8/LIP9/LIP10/PLB1/PLB2/CDR1*	1	2.5
11	*ALS1/HWP1/SAP1/SAP4/SAP5/SAP6/LIP1/LIP2/LIP3/LIP4/LIP6/LIP7/LIP9/LIP10/PLB1/PLB2*	1	2.5
12	*ALS1/HWP1/SAP1/SAP4/SAP5/SAP6/LIP1/LIP2/LIP3/LIP4/LIP5/LIP6/LIP7/LIP8/LIP9/LIP10/PLB1/PLB2/CDR1*	1	2.5
13	*ALS1/ALS3/HWP1/SAP4/SAP5/SAP6/LIP1/LIP2/LIP3/LIP4/LIP5/LIP6/LIP7/LIP8/LIP9/LIP10/PLB1/PLB2/CDR1*	1	2.5
14	*ALS1/HWP1/SAP4/SAP5/SAP6/LIP1/LIP2/LIP3/LIP4/LIP5/LIP6/LIP7/LIP8/LIP9/LIP10/PLB1/PLB2/CDR1*	1	2.5

compounds [38]. In this study, the association of azole resistance phenotypes (fluconazole/ketoconazole) was identified in 94.9% ($n = 37$) of the strains (Table 3), whereas *CDR1* was found in only 66.6% ($n = 26$) (Table 4). This gap in detection can be explained by the idea that azole resistance is not only conferred by *CDR1*, an ABC transporter, but may also be caused by the overproduction of an azole target, 14α-demethylase, which is encoded by *ERG11*. Other mechanisms of azole resistance are caused by mutations in *ERG11* which decrease azole affinity to this enzyme [10, 39].

Interestingly, all strains described in this study were sensitive to nystatin ($n = 39$, Table 3), as also reported in other studies [40, 41]. This result suggests that nystatin can be an alternative for the treatment of vaginal infections caused by strains of *C. albicans* which are resistant to azoles [42]. The high frequencies of strains resistant to fluconazole and ketoconazole (Table 3) can be explained by the high use of these two drugs (and clotrimazole) in Mexico, given that this is the most commonly used therapy against VVC. Our results are consistent with the observation that *Candida* species isolated in different geographical regions differ in their sensitivity to fluconazole [43].

In this study, we identified 14 distinct expression patterns of *ALS*, *HWP1*, *SAP*, *LIP*, *PLB*, and *CDR1* in A431 cells infected in vitro with strains of *C. albicans* (Table 5). Pattern 1, comprising 18 genes, was expressed by most strains (25.6%). These findings show that, during the pathogenesis of infection, most virulence markers are expressed (in 8 of the 14 patterns). The combined expression of the azole resistance gene *CDR1* with the virulence markers indicates that these strains are highly virulent and should be treated with a nonazole therapy.

5. Conclusion

This is the first work done in Mexico on global expression of different markers of virulence and resistance to azoles in clinical strains of *C. albicans*. The results show that the strains that infect Mexican patients suffering from VVC are highly virulent and virtually all are insensitive to azoles.

Competing Interests

The authors declare that they have no competing interests.

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