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Mannose-Binding Lectin Gene Polymorphism versus Microbial Virulence in the Pathogenesis of Vulvovaginal Candidiasis and Recurrent Vulvovaginal Candidiasis

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Abstract

BACKGROUND: Vulvovaginal candidiasis (VVC) is one of the most common infections affecting women.

AIM: This study aimed to determine MBL2 polymorphism frequency among females with VVC together with assessment of Candida virulence attributes in the pathogenesis of it.

METHODS: Fifty Egyptian patients and 50 controls were included in the study. Vaginal swabs were cultured to identify *Candida* spp. D-ARMs were used to determine *MBL2* polymorphism.

RESULTS: Cases with VVC revealed *Candida albicans* 37(74%) and non-Albicans *Candida* (NAC) 13 (26%) with a significant difference between *C. albicans* and NAC esterase and phospholipase. Thirty *C. albicans* isolates (81.1%) possessed three or more virulence factors, compared to only four NAC (30.8%) ($p = 0.002$). As regards *MBL2* polymorphism, the X allele was found to be significantly high in cases than in controls ($p \leq 0.001$). B allele on codon 54 and L allele on promoter region was more in cases. Other haplotypes were more in cases than controls with a significant difference for LXQB and HXQB. HXPB and LXQB were detected among recurrent VVC (RVVC) cases more than VVC cases.

CONCLUSION: RVVC appears to be a multi-factorial disorder; hence, treatment should be individualized. Recombinant MBL may be considered in the future treatment of RVVC in the case of associated genetic *MBL2* polymorphism.

Introduction

Vulvovaginal candidiasis (VVC) due to excessive growth of *Candida* species, especially *Candida albicans*, is the most common genital tract infection in women during their childbearing years [1]. About 75% of women experience at least one symptomatic episode during their lifetime [2].

Risk factors for VVC include the use of the intrauterine contraceptive device, recent antibiotic use, sexual activity, pregnancy, smoking, and immunosuppression [3], [4]. However, a small fraction of VVC still occurs without known risk factors suggesting an underline genetic predisposition [5].

Candida spp. was found to have many virulence factors that cause their transition from an opportunistic mucosal colonizer to a pathogenic one. Among these virulence factors come the expression of adhesins and invasins, formation of biofilms, morphologic transformation from yeast to hyphal form, and the secretion of hydrolytic enzymes [6].

Human Mannose-Binding Lectin (MBL) is a C-type lectin receptor (CLR) encoded by the MBL_2 gene which is located on chromosome 10. It binds to a wide array of different pathogens including bacteria, viruses, fungi, and parasites to initiate an immune response against them [7], [8]. Serum MBL (sMBL) levels were found to be affected by single nucleotide polymorphisms (SNPs) present in the coding and promoter regions of MBL_2 [8], [9], [10].

Allele A is the normal structural allele of MBL_2. There are three more structural alleles designated as 'O'allele [10]. They are "B:" with substitution of aspartic acid instead of glycine at codon 54, "C:" with substitution of glutamic acid instead of glycine at codon 57, and "D:" with substitution of cysteine instead of arginine at codon 52 [9], [11]. MBL expression is also controlled by three promoter variations at positions: G–550C (H/L), G–221C (Y/X), and C+4T (P/Q). There is linkage-disequilibrium

between the MBL_2 gene promoter and its coding variants resulting in different haplotypes. Seven of which have been defined, namely, HYPA, LYQA, LYPA, LXPA, HYPD, LYQC, and LYPB [12], [13].

This study aimed to determine the frequency of MBL_2 gene polymorphism in Egyptian females with VVC and to assess the role of *Candida* virulence attributes and host genetic polymorphism in the pathogenesis of VVC.

Methods

The study population included 100 Egyptian non-pregnant females in their reproductive period, 50 female patients complaining of VVC, and 50 females not suffering from VVC as a control group. Both cases and control groups attended the out-patient clinic of the Obstetrics and Gynecology Department, Kasr El Aini Hospital, Faculty of Medicine, Cairo University, during the period from January to June 2018.

The study was approved by the Ethical Committee of Cairo University, and the participants were informed about the study, and vaginal swabs together with blood samples were taken after their consent.

Cases and controls were subjected to history taking (including; name, age, gravidity, parity, method of contraception “if present”, symptoms suggestive of VVC, and associated risk factors) and gynecological examination (for signs of vulvovaginitis). Cases giving the history of four or more episodes per year were considered recurrent VVC (RVVC) [14], [15]. Two types of specimens were collected from all participants (patients and controls). First, 3 ml of blood on EDTA were collected and stored at −80°C for the molecular study. Second, vaginal swabs were obtained from the vaginal walls and fornix. The microbiological work was done at the Medical Microbiology and Immunology Department, Faculty of Medicine, Cairo University from January 2018 to July 2019.

Isolation and identification of Candida spp.

The vaginal swabs were subjected to direct Gram stain for the presence of budding yeast cells, pseudohyphae, and hyphae [16], then directly cultured on Sabouraud’s dextrose agar (SDA, Oxoid, UK), and incubated for 24–48 h at 37°C.

Colonies suspected to be *Candida* spp. were identified by Gram stain and germ tube test. They were subcultured on: (Rice Tween- 80 agar) and interpreted according to the morphologic scheme suggested by Deorukhkar and Roushani [17] and CHROMagar *Candida* (CHROMagar Company, France). Then they were furtherly identified biochemically using sugar assimilation and fermentation tests [17].

Evaluation of virulence factors of isolated Candida spp.

10 μL saline suspensions of the isolates from the fresh cultures adjusted to 0.5 McFarland were used for further testing for virulence factors (namely, hemolytic, phospholipase, proteinase, and esterase activities) [18], [19] which then were subcultured onto the corresponding culture media and incubated at 37°C under aerobic.

Assessment of hemolytic activity

The test was performed on SDA (Oxoid, UK) supplemented with 7% sheep blood and 3% glucose and adjusted to a pH of 5.6 ± 0.2. After incubation for 48 h, isolates positive for hemolytic activity showed a transparent/semitransparent zone of alpha- or beta-hemolysis around the inoculation site [19].

Assessment of phospholipase activity

Modified egg yolk agar media (Oxoid, UK) were used and incubated at 37°C for 5 days under aerobic conditions. Phospholipase activity (Pz) was detected as a precipitation zone around the positive colonies and it was calculated as the ratio of the colony diameter to the diameter of the colony plus precipitation zone. Positive result was obtained in range (Pz = 0.64–0.99), very strong (Pz ≤ 0.63), and negative when (Pz = 1) [20].

Assessment of proteinase activity

Bovine-serum albumin agar (Sigma-Aldrich, USA) was adjusted to a pH of 4.5 and incubated for 10 days. Then, it was further fixed with 20% trichloracetic acid, stained with 1.25% amido black (Sigma-Aldrich, USA), and then decolorized with 15% acetic acid. Proteinase activity (Pz) was detected as the presence of an unstained zone around the colonies and calculated as the ratio of the colony diameter to the diameter of the colony plus the proteolytic unstained zone [21]. Positive result was obtained in range (Pz = 0.64–0.99), very strong (Pz ≤ 0.63), and negative when (Pz = 1) [18].

Assessment of esterase activity

The inoculated Tween-80 agar plates (Himedia, India) were incubated for 10 days. A halo against light around the inoculation site denoted esterase activity [20].

Assessment of biofilm formation

The formation of biofilm was assessed using the sterile 96-well flat-bottom microplates method according to Deepa *et al.* [18]. Briefly, freshly prepared isolates were inoculated into 2 mL of brain heart infusion broth

medium (BHIB) (Sigma-Aldrich, USA) supplemented with 0.25% glucose. After incubation for 24 h at 37°C, it was diluted at a ratio of 1:20 then 200 μL from the final dilution were placed into the corresponding well. The last well had sterile plain BHIB as a negative control. They were re-incubated for another 24 h. Then, the test was read using an enzyme-linked immunosorbent assay (ELISA) reader (Awareness Technology, USA) at 450 nm. The cutoff value was estimated as: mean OD of the negative control added to twice the standard deviation. Samples with an OD higher than the cutoff value were considered positive.

Detection of MBL_2 single nucleotide polymorphisms (SNPs)

Genotyping for the three SNPs in the promoter regions, namely, rs7096206 (Y/X), rs11003125 (L/H), rs7095891 (P/Q), and one SNP rs1800450 present in codon 54 of *MBL_2* was done using D-ARMS-PCR (double amplification refractory mutation system- PCR) [10].

100 μL of whole blood, collected on EDTA (1 mg/mL) was used for DNA extraction using Cinna Pure DNA extraction kit (AryoGen Complex, Tehran, Iran) according to manufacturer's instructions.

Four primer sets (specific and common) were used, as shown in Table 1.

PCR was performed in 25 μL reaction volumes containing 5 μL of extracted DNA, 10 μL of 0.5X of ViRed Taq buffer master mix (Vivantis, Malaysia), 1 μL of specific primer of a set, and 1 μL of the common primer of the same set with 8 μL of sterile nuclease-free water. Cycler conditions were run according to Kalia *et al.* [10]. Gel electrophoresis was done. The genotypes were identified by the pattern of bands obtained from PCR products where these amplicons were compared to 50 PB DNA markers (range from 50 to 1500) (Cleaver Scientific, UK) [10].

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) version 25 was used for data coding and entering (IBM Corp., Armonk, NY, USA). Data were expressed using mean, standard deviation, minimum, and maximum in quantitative data and using frequency (count) and relative frequency (percentage) for categorical data. Chi-square (χ^2) test was used for comparing categorical data, performed. An exact test was used instead when the expected frequency is <5 [22]. Genotype and allele frequencies were compared between the disease and the control groups using logistic regression. Odds ratio (OR) with 95% confidence intervals was calculated. $p < 0.05$ was considered statistically significant.

Results

Demographic data of the study population and predisposing factors

The age of the patients ranged from 19 to 48 years, where most cases were observed in the childbearing age (20–40 years of age). The gravidity of the patients ranged from 0 to 8 and the parity ranged from 0 to 5 offspring.

Predisposing factors

Risk factors that were compared between cases and controls showed contraception and antifungal use were more in cases than controls, but only antifungals showed an almost statistically significant difference between cases and controls (p-value = 0.059). As regards antibiotic use, controls were more than cases with a statistically significant difference.

Among the studied cases, 40 patients (80%) gave history suggestive of RVVC, with four episodes or more per year. Comparing risk factors of cases of RVVC with control groups showed that some risk factors were more in RVVC as contraception, immunosuppressive treatment, antifungal use, and early age of intercourse (21–25 year).

Identification of isolated Candida spp.

Based on the conventional methods of identification (germ tube, Chrom agar, rice tween 80, sugar assimilation, and fermentation), species distribution of isolated *Candida* is shown in Figure 1.

Table 1: Primer type, sequence, and product size of amplified DNA Kalia *et al.,* 2017 [10]

MBL_2 SNP	Primer type	Sequence	Product size (bp)
rs11003125	H specific forward primer	5′-GCTTACCCAGGCAAGCCTGT**G**-3′	316
	L specific forward primer	5′GCTTACCCAGGCAAGCCTGT**C**-3′	
	Reverse common primer	5′AACAAATGGGACCGTGCATTGC-3′	
rs7096206	Forward common primer	5′-CCTGCCAGAAAGTAGAGAGG-3′	440
	Y specific reverse primer	5′-CTGGAAGACTATAAACATGCTTTC**C**-3′	
	X specific reverse primer	5′ -GGAAGACTATAAACATGCTTTC**G**-3′	
rs7095891	P specific forward primer	5′CAGATTGTAGGACAGAGGGCATGCT**C**-3′	332
	Q specific forward primer	5′ -TTGTAGGACAGAGGGCATGCT**T**-3′	
	Reverse common primer	5′ -CCAGGCAGTTTCCTCTGGAAGG-3′	
rs1800450	Forward common primer	5′ -CTGCACCCAGATTGTAGGACAGAG-3′	278
	A specific reverse primer	5′ -CCCCCTTTTCTCCCTTGGTG**C**-3′	
	B specific reverse primer	5′ -CCCCCTTTTCTCCCTTGGTG**T**-3′	

The nucleotides specific for polymorphisms are written in bold.

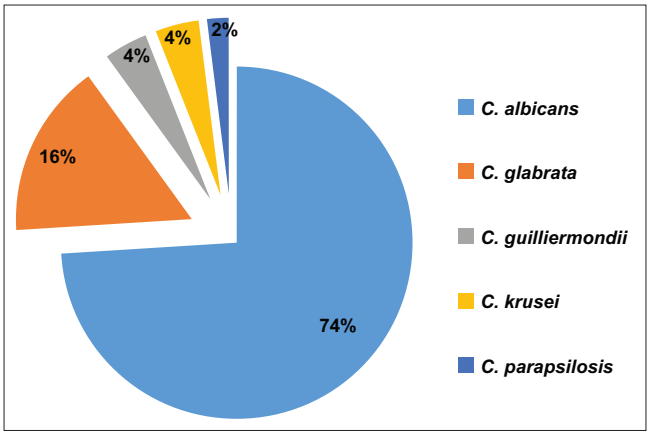

Figure 1: Species distribution of Candida isolates from 50 patients with vulvovaginal candidiasis. C. albicans (60%) was recovered from cases with recurrent vulvovaginal candidiasis more than any other species

C. albicans (60%) was recovered from cases with RVVC more than any other species. However, there was no statistically significant relationship between VVC/RVVC and *Candida* spp. (p = 0.707).

Virulence factors of isolated Candida spp.

Out of the 50 isolated *Candida* spp., the following virulence factors were detected, as shown in Table 2.

Table 2: Virulence factors produced by different *Candida* spp.

Virulence factors	No. of positive isolates	*Candida* spp.				*P*-value
		C. albicans (n = 37)		Non-albicans *Candida* (n = 13)		
		Count	%	Count	%	
Esterase						
Yes	38	34	91.9	4	30.8	*<0.001
No		3	8.1	9	69.2	
Hemolysin						
Yes	26	21	56.8	5	38.5	0.256
No		16	43.2	8	61.5	
Phospholipase						
Yes	25	24	64.9	1	7.7	*<0.001
No		13	35.1	12	92.3	
Proteinase						
Yes	20	15	40.5	5	38.5	0.895
No		22	59.5	8	61.5	
Biofilm						
Yes	13	8	21.6	5	38.5	0.281
No		29	78.4	8	61.5	

p ≤ 0.05 is considered significant.

Thirty *C. albicans* isolates (81.1%) possessed three or more virulence factors, compared to only four NAC (30.8%) with statistically significant difference (p = 0.002).

Most of the *C. albicans* isolates possessing three or more VF were isolated from RVVC; however, this was statistically insignificant.

Whereas, NAC which possessed <3 virulence factors was isolated from RVVC cases more than VVC, but the difference was insignificant.

Comparing between VVC and RVVC groups caused by *C. albicans* showed that all tested virulence factors were detected among RVVC more than VVC, but the difference was statistically insignificant.

MBL_2 polymorphism

The mutant B allele on codon 54 and mutant L allele on promoter region were more among cases than controls, but the difference was not statistically significant. On the other hand, the normal wild alleles; Y, H, and A were more among controls than cases (77.0%, 52.0%, and 29.0%, respectively) but without statistical significance, as shown in Table 3.

On comparing the distribution of MBL_2 alleles among VVC and RVVC cases, mutant allele X, allele Q, allele L, and allele O were more among cases of RVVC than VVC (55.0%, 92.5%, 55.0%, and 41.3%, respectively) without statistical significance.

At codon 54, it was noted that neither homozygous AA nor BB were found in our study and the A genotype was more among controls (58%) than cases (50%). Abnormal BO genotype was also more among cases than controls, but the difference was statistically insignificant.

On comparing the genotypic frequency between VVC and RVVC cases, it was found that abnormal XX, QQ, LL, and AB genotypes appeared to be more among RVVC. Meanwhile, abnormal AO and BO were more among VVC cases but all without a statistically significant difference, as shown in Table 4.

Independent analysis of each individual haplotype indicated that LYPA, LXPA, HXPA, LXQB, HXQB, and HYQB were more in cases than controls with statistically difference for LXQB and HXQB haplotypes (*P*-value = 0.013, OR = 17.286, CI: 1.811-164.958), (*P*-value = 0.030, OR = 12.571, CI: 1.280-123.480), respectively. Apart from the well-known *MBL2* haplotypes, eight additional MBL_2 haplotypes were detected. LXQA, LXPO, and LXQO were more in cases than controls with statistically significant difference LXQO, as shown in Table 5.

Comparing MBL_2 haplotypes present in cases with VVC and RVVC is shown in Table 6.

Discussion

In the present study, accurate diagnosis of VVC necessitates the presence of symptoms as well as evidence of fungi in the diagnosis [16]. In the present study, all of the patients complained of increased whitish vaginal discharge. Other symptoms were present as itching (86%), dyspareunia (82%), dysuria (76%), and vulval swelling (72%). This agrees with El Feky *et al.* [23] and is also similar to Mtibaa *et al.* [24] but, unlike Sangaré *et al*. [25]. Ghaddar *et al*. [26] also reported that 82% of pregnant women experienced asymptomatic VVC.

In the present study, the most common predisposing factors associated with candidiasis were the

Table 3: Different *MBL*$_2$ alleles among cases and controls

Alleles	Cases two alleles/case		Controls two alleles/control		p-value	OR	95% CI	
	Count	%	Count	%			Lower	Upper
Allele X (more in cases than control)	52	52.0%	23	23.0%	*<0.001	3.627	1.973	6.668
Allele Y	48	48.0%	77	77.0%	1			
Allele P	9	9.0%	3	3.0%	0.089	3.198	0.839	12.183
Allele Q	91	91.0%	97	97.0%	1			
Allele H	50	50.0%	52	52.0%	1			
Allele L	50	50.0%	48	48.0%	0.777	1.083	0.622	1.886
Allele A	25	25.0%	29	29.0%	1			
Allele B	34	34.0%	25	25.0%	0.229	1.578	0.750	3.318
Allele *O	41	41.0%	46	46.0%	0.923	1.034	0.523	2.042

*O stands for C or D alleles ($p \leq 0.05$ is considered significant)

use of contraceptives, antifungals, immunosuppressive treatment, and early onset of sexual activity. The same risk factors have been reported in other studies [23], [27], [28].

In the present study, 40 patients (80%) gave a history suggestive of RVVC. A similar study in Egypt by Ismail *et al.* [29] showed that RVVC accounted for 75%; however, Gonçalves *et al.* [30] and Brandolt *et al.* [31] reported that only 5–10% developed RVVC and Mtibaa *et al.* [24] stated that RVVC was encountered in 14.3% of cases. The high rate of recurrence in the present study could be due to different predisposing risk factors and/or the virulence attributes of isolates and/or associated *MBL*$_2$ genetic polymorphism as VVC is a multifactorial problem.

In the present study, *C. albicans* (74%) was the most predominant *Candida* species, while NAC spp. constituted 26% of isolates causing VVC. Similar results which corroborate these findings were reported worldwide [20], [32], [33], [34].

However, a dramatic increase of VVC caused by NAC species has been reported by others [35], [36].

In the present study, *C. glabrata* (16%) was the most common isolated NAC species. This corresponds to the results of other studies from different regions of the world [37], [38].

It has been reported that some virulence factors (VF) such as biofilm formation and the synthesis of hydrolytic enzymes can play a key role in aggravating the pathogenesis of candidiasis. They were suggested to be used as targets for new anti-*Candida* agents for the treatment of candidiasis [39]. Both *C. albicans* and NAC species express esterase activity which appears to be a common feature of *Candida* species isolated from clinical specimens [18].

In the present study, most of the isolated *Candida* spp. (38/50.76%) demonstrated esterase activity, *C. albicans* strains exhibited the highest esterase activity (92%), and there was a statistically significant difference between *C. albicans* and NAC ($p < 0.001$) (Figure 5). Our results are similar to the results of ElFeky and Gohar [6], Kumar *et al.* [40], and Pakshir *et al.* [41]. Noori *et al.* [39] found that 63% of the *Candida* species had esterase activity, with the highest esterase activity reported in *C. krusei*, followed by *C. albicans* and *C. glabrata*.

In the present study, hemolytic activity was observed in 26/50 (52%) of the isolates, more in *C. albicans* 21/37 (56.8%) than NAC 5/13 (38.5%) (Figure 2). This is similar to Malcok *et al.* [42] and Noori *et al.* [39]. In contrary to Deepa *et al.* [18], ElFeky and Gohar [6] reported that NAC was more than *C. albicans* as regards hemolytic activity. Whereas, Rossoni *et al.* [43] found that hemolytic activity was equally present among *C. albicans* and NAC spp.

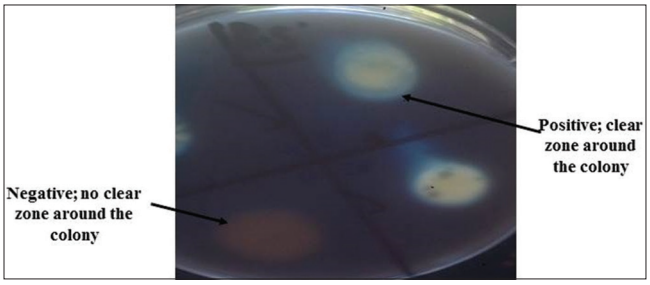

Figure 3: Proteinase activity of different Candida spp.

Table 4: Different *MBL*$_2$ genotypes frequencies among cases and controls

Genotypes	Cases (n = 50)		Controls (n = 50)		p-value	OR	95% CI	
	Count	%	Count	%			Lower	Upper
YX genotypes								
XX (more in cases than control)	20	40.0	6	12.0	***0.001**	**6.111**	**2.079**	**17.960**
YX (more in cases than control)	12	24.0	11	22.0	0.174	2.000	0.736	5.434
YY	18	36.0	33	66.0	1			
PQ genotypes								
PP	2	4.0	0	0.0	0.999	--	--	--
PQ	5	10.0	3	6.0	0.430	1.822	0.411	8.082
QQ	43	86.0	47	94.0	1			
HL genotypes								
HH	22	44.0	17	34.0	1			
HL (more in controls than cases)	6	12.0	18	36.0	***0.018**	**0.258**	**0.084**	**0.789**
LL (more in cases than control)	22	44.0	15	30.0	0.788	1.133	0.455	2.820
AB genotypes								
AO	13	26.0	17	34.0	1			
AB	12	24.0	12	24.0	0.626	1.308	0.445	3.842
BO	22	44.0	13	26.0	0.118	2.213	0.818	5.990
OO	3	6.0	8	16.0	0.355	0.490	0.108	2.221

*p ≤ 0.05 is considered significant

Table 5: Different *MBL*$_2$ secretor haplotypes among cases and control groups

Haplotypes	Cases, N = 100		Controls, N = 100		p-value	OR	95% CI	
	Count	%	Count	%			Lower	Upper
High secretor								
LYQA	2	2.0	3	3.0	0.964	1.048	0.138	7.934
HYQA	7	7.0	11	11.0	1			
HYPA	0	0	1	1.0	1.000	---	---	---
Intermediate secretor								
LYPA	2	2.0	1	1.0	0.384	3.143	0.238	41.507
Low secretor								
LXPA	2	2.0	0	0.0	0.999	---	---	---
HXPA	2	2.0	0	0.0	0.999	---	---	---
HXPB	1	1.0	1	1.0	0.762	1.571	0.084	29.409
LYQB	4	4.0	13	13.0	0.332	0.484	0.111	2.098
LXQB	11	11.0	1	1.0	***0.013**	**17.286**	**1.811**	**164.958**
HXQB	8	8.0	1	1.0	***0.030**	**12.571**	**1.280**	**123.480**
HYQB	10	10.0	9	9.0	0.403	1.746	0.472	6.454
New non reported haplotypes								
HXQA	4	4.0	9	9.0	0.642	0.698	0.154	3.167
LXQA	6	6.0	4	4.0	0.288	2.357	0.485	11.452
Others								
HXQO	6	6.0	6	6.0	0.548	1.571	0.359	6.875
LXPO	1	1.0	0	0.0	1.000	---	---	---
LXQO	11	11.0	1	1.0	***0.013**	**17.286**	**1.811**	**164.958**
LYPO	1	1.0	0	0.0	1.000	---	---	---
HYQO	12	12.0	14	14.0	0.633	1.347	0.397	4.570
LYQO	10	10.0	25	25.0	0.448	0.629	0.190	2.083

$p \leq 0.05$ is considered significant

It is generally known that the active role of phospholipase is important in the host tissues invasion seen in lesions produced by *Candida* spp. [44].

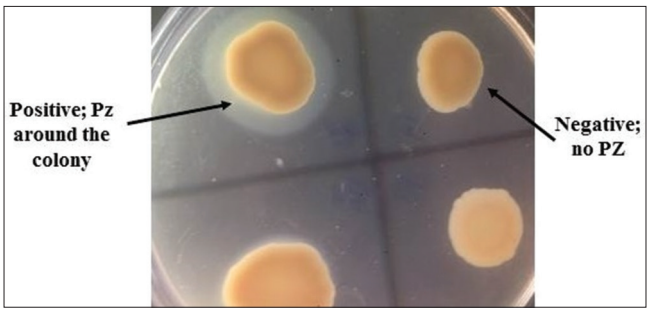

Figure 4: Phospholipase activity of different Candida spp.

In the present study, phospholipase activity was found in 25/50 (50%) *Candida* isolates and there was a statistically significant difference between *C. albicans* and NAC (*P-value* < 0.001) being more in *C. albicans* (Figure 4). This is similar to studies elsewhere [6], [20], [45]. On the other hand, Moharram *et al.* [46] reported higher phospholipase activity in *C. tropicalis,* more than *C. albicans*.

In the present study, proteinase activity was found in 20/50 (40%) *Candida* isolates, and the highest proteinase expression was seen in *C. albicans*, more than NAC species (Figure 3). This is similar to others [6], [47], [48].

However, Pinto *et al.* [49] showed that *C. albicans* and *C. parapsilosis* exhibited 90% and 100% proteinase activity, respectively. Furthermore,

Table 6: Different *MBL*$_2$ secretor haplotypes among VVC and RVVC

MBL2 secretor haplotypes	VVC, N = 20		RVVC, N = 80		p-value	OR	95% CI	
	Count	%	Count	%			Lower	Upper
High secretor								
LYQA	0	0.0%	2	2.5%	0.999	---	---	---
HYQA	3	15.0%	4	5.0%	1			
Intermediate secretor								
LYPA	0	0.0%	2	2.5%	0.999	---	---	---
Low secretor								
HXPA	1	5.0%	1	1.3%	0.858	0.750	0.032	17.506
LXPA	1	5.0%	1	1.3%	0.858	0.750	0.032	17.506
HXPB	0	0.0%	1	1.3%	1.000	---	---	---
LYQB	1	5.0%	3	3.8%	0.558	2.250	0.149	33.933
LXQB	1	5.0%	10	12.5%	0.120	7.500	0.590	95.376
HXQB	2	10.0%	6	7.5%	0.468	2.250	0.251	20.131
HYQB	3	15.0%	7	8.8%	0.587	1.750	0.233	13.159
New non reported haplotypes								
HXQA	0	0.0%	4	5.0%	0.999	---	---	---
LXQA	0	0.0%	6	7.5%	0.999	---	---	---
Others								
HXQO	1	5.0%	5	6.3%	0.322	3.750	0.274	51.373
LXPO	1	5.0%	0	0.0%	1.000	0.000	0.000	.
LXQO	1	5.0%	10	12.5%	0.120	7.500	0.590	95.376
LYPO	0	0.0%	1	1.3%	1.000	---	---	---
HYQO	4	20.0%	8	10.0%	0.679	1.500	0.220	10.218
LYQO	1	5.0%	9	11.3%	0.142	6.750	0.526	86.561

$p \leq 0.05$ is considered significant.

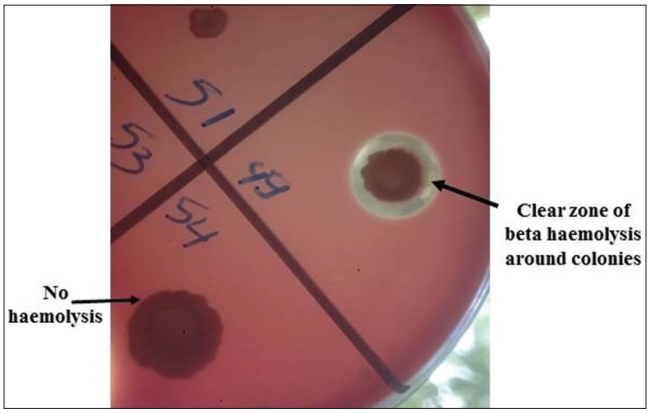

Figure 2: Hemolytic activity of different Candida spp.

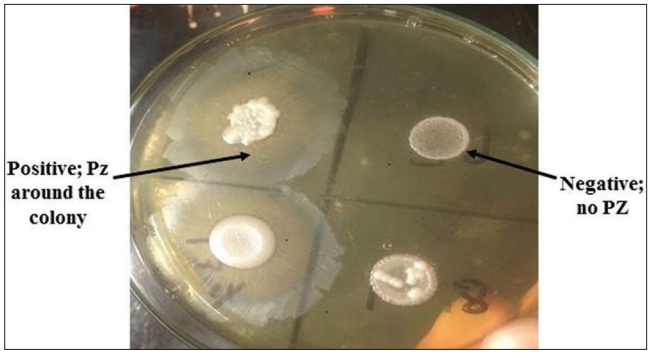

Figure 5: Esterase activity of different Candida spp.

Moharram *et al.* [46] found that NAC produced the highest levels of proteinase activity.

In the present study, only 13/50 (26%) of *Candida* isolates were biofilm-forming and biofilm formation was detected more in NAC 5/13(38.5%) than *C. albicans* 8/37 (21.6%). This is similar to Demirbilek *et al.* [50], Deepa *et al.* [18], and ElFeky and Gohar [6]. Contrary to our results, Dabiri *et al.* [51] reported low ability to form biofilm among NAC isolates, but there was a good biofilm-forming ability among *C. albicans* isolates.

Overall, 30/37 *C. albicans* isolates (81.1%) possessed three or more virulence factors, compared to only 4/13 NAC (30.8%), with a statistically significant difference (p-value = 0.002). Interestingly, most of the *C. albicans* isolates possessing three or more VF were isolated from RVVC cases, but this was statistically insignificant; however, it might suggest a role for VF in recurrence.

The understanding of the genetic factors which play an important role in RVVC, irrespective of the presence or absence of recognizable risk factors, can be of great help in guiding future therapeutic modalities in such patients [52]. Polymorphism in the MBL_2 gene has been proposed as one of such genetic factors [53]. Such a proposal has been supported by the finding that an individual's MBL_2 genotype is the guiding factor for variation of MBL concentration both in serum and cervicovaginal fluid. The strong binding of *Candida* spp. to MBL protein also supported this hypothesis denoting the importance of such protein in host defense against VVC [54].

Different studies have been carried out on variations of MBL concentration and corresponding MBL_2 genes in different populations with different medical conditions [8], [53], [55], [56].

In the present study, as regards MBL_2 alleles, the frequency of the mutant X allele was found to be significantly higher in cases as compared to controls ($p \leq 0.001$), and cases were three times more prone to VVC who carried variant allele "X" in comparison to those who did not; OR = 3.627, CI: 1.973–6.668) similar to Kalia *et al.* [10]. This may signify that the X allele can carry a higher risk for the occurrence of VVC.

Furthermore, in the present study, mutant L allele on promoter region was more among cases (50%) than controls (48.0%), while the normal wild allele H and allele Y were more among controls (52.0% and 77.0%, respectively), but the difference was statistically insignificant, this was also similar to Kalia *et al.* [10]. People with abnormal L/X alleles were found to be more prone to different infections than those having the wild corresponding alleles [57], [58].

In the present study, on comparing the distribution of MBL_2 alleles in VVC and RVVC, mutant allele X, allele Q, allele L, and allele O were more among cases of RVVC than VVC (55.0%, 92.5%, 55.0%, and 41.3%, respectively), but the difference was statistically insignificant.

Kalia *et al.* [10] showed structural and promoter gene polymorphisms in RVVC with similar results to the present study as regards allele X and allele L, but different results as regards allele Q and allele B. The difference in the association may be attributed to genetic heterogeneity between different ethnicities necessitating further evaluation.

Different studies discussed the importance of codon 54 in VVC [2], [59], [60] or RVVC [2], [59], [60], [61].

Liu *et al.* [60] and Giraldo *et al.* [61] found that the frequency of MBL_2 codon 54 polymorphism was more in RVVC than VVC. They assumed that women with polymorphism in codon 54 are more susceptible, as a result of immune deficiency in the vagina, to develop RVVC.

In the present study, as regards codon 54 polymorphism, normal allele A was more among controls (29%) than cases (25%) and the mutant B allele was more among cases (34%) than controls (25%), but there was no significant difference.

These results were similar to a study in Egypt by Hammad *et al.* [54], where allele A and allele B were present in 83.9% and 16.1% of RVVC cases and 94% and 6% of controls, respectively. Furthermore, Wojitani *et al*. [2] found that the frequency of allele B was more in women suffering from RVVC in contrast to the control group. Kalia *et al.* [10] showed that allele A was slightly more in cases than controls.

Collectively, the MBL plasma concentration is under the control of the individual's MBL_2 genotype. Moreover, different combinations of haplotypes result in a wide range of MBL concentrations [62].

In the present study, on comparing genotypic frequency between cases and controls, both homozygous XX and heterozygous XY genotypes were more among cases than controls with a statistically significant difference for the XX genotype (p = 0.001, OR = 6.111, CI: 2.079–17.96), demonstrating a six times higher risk for VVC.

This is in concordance with Kalia *et al.* [10] who found that the frequency of XY and XX genotypes was correlated to generally increased susceptibility to RVV infections, and it was significantly higher among RVVC cases compared to the control group.

Furthermore, in the present study, abnormal homozygous LL genotype was more among the cases than controls, but the difference was statistically insignificant. However, the heterozygous HL genotype was more among controls than cases with statistically significant difference (p-value = 0.018, OR = 0.258, CI: 0.084-0.789). This is unlike Kalia *et al.* [10] who found no difference in the genotypic frequencies of HL between RVVI cases and their controls. However, similar to our results, though in a different infection, in a study of

neonatal infections, there was a significant difference in heterozygous HL genotype between patients and controls being more among controls [63].

Concerning codon 54, studies showed that polymorphism in codon 54 was significantly associated with an increased risk of VVC [2], [59], [64]. Hammad *et al.* [54] have the first to study MBL_2 codon 54 polymorphism among Egyptian women in the childbearing period suffering from RVVC. In the present study, we explored such polymorphism in addition to the three promoter polymorphisms.

In the present study, it was noted that neither homozygous AA nor BB were found either among cases or controls. Similarly, Liu *et al.* [60] and Kalia *et al.* [10] observed that BB genotype was not found in VVC cases, while homozygous genotype (BB) was found among controls in Kalia *et al.* [10] study.

Heterozygous A genotype was more among controls (58%) than cases (50%) in the present study, while abnormal BO genotype was more among cases than controls with no statistically significant difference in both genotypes. This is similar to Kalia *et al.* [10].

On comparing the genotypic frequency between VVC and RVVC cases, it was found that abnormal XX, QQ, LL, and AB genotypes appeared to be more among RVVC. Meanwhile, abnormal AO and BO were more among VVC cases but all without statistically significant difference. Kalia *et al.* [10] noticed that among all the MBL_2 genotypes, only Y/X genotypes were significantly more in cases of RVVC concluding that it was one of the mechanisms leading to RVVC.

Two studies found that MBL concentrations were higher in women with VVC than in controls, but not in women with RVVC whether it was a reflection of the wild (AA) or the heterozygous (AB) genetic types. They suggested that MBL may increase during the first attack of *Candida* vaginitis rather than in the recurrent attacks [60], [65].

Diminished binding of the MBL protein to *Candida* cells through its lectin domain, at the early phase of defense against *Candida,* increases the risk of vaginal infection by the fungus. One of the causes for such low-quality binding could be the MBL_2 codon 54 polymorphisms. Moreover, the wild-type allele (A) for the MBL_2 codon 54 can have a lot of promoter combinations, some of which like the LX haplotype, for example, can down-regulate the expression of MBL protein, resulting in expressing low levels of the functional protein [56], [65].

This is supported by results from other studies which discussed MBL_2 polymorphism with different infectious diseases. Tao *et al.* [63] found the frequency of the haplotype HYPA, which leads to high MBL protein levels, statistically higher in healthy controls than in patients, while the frequency of the haplotype LYPB, which leads to low MBL protein levels, was significantly higher in patients than in the controls. Ocejo-Vinyals *et al.* [58], in their study on pulmonary tuberculous patients, intermediate LYPA and low secretor LXPA haplotypes were more among cases and high secretor HYPA was more among controls, but the difference was statistically insignificant. Furthermore, Mashaly *et al.* [8] in Egypt found that low secretor haplotypes (LXA/O and O/O) were significantly higher in neonatal sepsis patients than in their controls.

This supports the finding in the present study which showed that the intermediate secretor LYPA and low secretor haplotypes: LXPA, HXPA, LXQB, HXQB, and HYQB were more among cases than controls with statistically significant difference for the low secretors; LXQB, HXQB, and LXQO p = 0.013, OR = 17.286, CI: 1.811–164.958, p = 0.030, OR = 12.571, CI: 1.280-123.480, and p = 0.013, OR = 17.286, CI: 1.811–164.985, respectively. However, our results are in partial agreement with Kalia *et al.* [10] who found that LYPA, LXQB, and HXQB were more among cases of VVC, but HYQB, LXPA, and HXPA were more among controls with an insignificant difference for all findings.

In the present study, apart from the seven well-known MBL_2 haplotypes, eight additional MBL_2 haplotypes were detected. Six of them, HXPA, HXPB, HXQB, HYQB, LXQB, and LYQB, were previously reported [10], [66], [67], [68] and two haplotypes were, to the best of our knowledge, not reported previously; HXQA and LXQA. The detection of these new haplotypes could be explained by the positive selection pressure exerted by (R)VVC on the MBL_2 gene, leading to increased frequency of novel haplotypes Kalia *et al.* [10]. The present study did not estimate the serum or vaginal MBL concentration, so we are as yet unable to ascertain the effect of newly non-reported MBL_2 haplotypes on MBL concentration.

Vaginal *Candida* proliferation in women suffering from RVVC has been attributed to continuous or intermittent failure to induce a Th1 immune response against *Candida* in the vagina. Low vaginal levels of MBL protein, due to differences in individuals' MBL_2 haplotypes, can aggravate such conditions [59].

In the present study, on comparing haplotypes associated with VVC and RVVC, most of the haplotypes were detected in RVVC more than VVC with no statistically significant difference. Haplotypes, HXPB and LXQB, were detected among RVVC cases more than VVC cases (1.3% and 12.5%, respectively), and most of the newly detected haplotypes were more among RVVC than VVC, but all these differences were insignificant.

Conclusion

The present study concluded that *Candida* spp. exhibited different virulence attributes with *C. albicans* generally more virulent than NAC spp. Our findings

support the concept that RVVC is a multifactorial disorder with *the* demonstration that MBL_2 polymorphism has not been the only predisposing factor, whether in protecting or favoring RVVC. However, mutant X, L, Q, O, and B alleles were found to be higher in VVC cases as compared to controls indicating the importance of MBL_2 gene polymorphism in an increased risk of VVC and RVVC in Egypt. Low secretor haplotypes such as LXPA, HYQB, HXPA, and LXQB were more in cases than controls confirming the importance of MBL protein vaginal level in the protection against VVC.

Assessment of MBL_2 polymorphism in larger populations for better achievement of statistically significant associations is recommended with exploring the possibility of recombinant human MBL or gene therapy as potential therapeutic agents against *Candida* infection.

Data Availability

The data used to support the findings of this study are available from the corresponding author on request.

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