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 VVC. 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 ≤ 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 underlie 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 invasions, 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 MBL2 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 MBL2 [8], [9], [10].

Allele A is the normal structural allele of MBL2. 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 MBL2 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 MBL2 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 MBL2 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 MBL2, 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].

### 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]

<table>
<thead>
<tr>
<th>MBL_SNPs</th>
<th>Primer type</th>
<th>Sequence</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs11003125</td>
<td>H specific forward primer</td>
<td>5′-GCTTACCCAGGGCAACGTCTTG-3′</td>
<td>316</td>
</tr>
<tr>
<td>rs7096206</td>
<td>L specific forward primer</td>
<td>5′GTTACCCAGGGCAACGTCTTG-3′</td>
<td></td>
</tr>
<tr>
<td>rs7095891</td>
<td>Reverse common primer</td>
<td>5′AAACAAATAGGGACGCTAATGG-3′</td>
<td>440</td>
</tr>
<tr>
<td>rs1800450</td>
<td>Y specific reverse primer</td>
<td>5′-GCTTACCCAGGGCAACGTCTTG-3′</td>
<td>332</td>
</tr>
<tr>
<td>rs7096206</td>
<td>X specific reverse primer</td>
<td>5′GTTACCCAGGGCAACGTCTTG-3′</td>
<td></td>
</tr>
<tr>
<td>rs7095891</td>
<td>P specific forward primer</td>
<td>5′GGAAAGACTATAAACATGCTTTC-3′</td>
<td>278</td>
</tr>
<tr>
<td>rs1800450</td>
<td>Q specific forward primer</td>
<td>5′GGAAAGACTATAAACATGCTTTC-3′</td>
<td></td>
</tr>
<tr>
<td>rs7096206</td>
<td>Reverse common primer</td>
<td>5′TTGTTAGGAGCAGAGGACGATCT-3′</td>
<td></td>
</tr>
<tr>
<td>rs7095891</td>
<td>A specific reverse primer</td>
<td>5′-CCAGCGATTTCCCTTGAG-3′</td>
<td></td>
</tr>
<tr>
<td>rs1800450</td>
<td>B specific reverse primer</td>
<td>5′-CCAGCGATTTCCCTTGAG-3′</td>
<td></td>
</tr>
</tbody>
</table>

The nucleotides specific for polymorphisms are written in bold.
C. albicans (60%) was recovered from cases with recurrent vulvovaginal candidiasis 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>
<thead>
<tr>
<th>Virulence factors</th>
<th>C. albicans (n = 37)</th>
<th>Non-albicans Candida (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esterase</td>
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<td></td>
</tr>
<tr>
<td>Yes</td>
<td>38 (91.9%)</td>
<td>4 (30.8%)</td>
</tr>
<tr>
<td>No</td>
<td>3 (8.1%)</td>
<td>9 (69.2%)</td>
</tr>
<tr>
<td>Phospholipase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>26 (56.8%)</td>
<td>5 (38.5%)</td>
</tr>
<tr>
<td>No</td>
<td>16 (43.2%)</td>
<td>8 (61.5%)</td>
</tr>
<tr>
<td>Proteinase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>25 (64.9%)</td>
<td>1 (7.7%)</td>
</tr>
<tr>
<td>No</td>
<td>13 (35.1%)</td>
<td>12 (92.3%)</td>
</tr>
<tr>
<td>Biofilm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>20 (50.5%)</td>
<td>5 (38.5%)</td>
</tr>
<tr>
<td>No</td>
<td>22 (49.5%)</td>
<td>8 (61.5%)</td>
</tr>
</tbody>
</table>

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.

MBL2 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 MBL2 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 MBL2 haplotypes were detected. LXQA, LXPO, and LXQO were more in cases than controls with statistically significant difference LXQO, as shown in Table 5.

Comparing MBL2, 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 vulvar swelling (72%). This agrees with El Feky et al. [23] and is also similar to Mitbaa 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...
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 Mtbbaa et al. [32] stated that RVV 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 MBL2 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 Eifeky 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], Eifeky 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.

| Table 3: Different MBL2 alleles among cases and controls |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Alleles         | Cases two alleles/case | Controls two alleles/control | p-value | OR  |
|                 | Count | %      | Count | %      |       |     |
| Allele X (more in cases than control) | 52    | 52.0%  | 23    | 23.0%  | <0.001 | 3.627 |
| Allele Y        | 48    | 48.0%  | 77    | 77.0%  | 1      | 1.034 |
| Allele P        | 9     | 9.0%   | 3     | 3.0%   | 0.089  | 3.198 |
| Allele Q        | 91    | 91.0%  | 97    | 97.0%  | 1      | 0.839 |
| Allele H        | 50    | 50.0%  | 52    | 52.0%  | 1      | 12.183 |
| Allele L        | 50    | 50.0%  | 48    | 48.0%  | 0.777  | 1.083 |
| Allele A        | 25    | 25.0%  | 29    | 29.0%  | 1      | 0.626 |
| Allele B        | 34    | 34.0%  | 25    | 25.0%  | 0.229  | 1.578 |
| Allele O        | 41    | 41.0%  | 46    | 46.0%  | 0.923  | 1.034 |

*p ≤ 0.05 is considered significant.

| Table 4: Different MBL2 genotypes frequencies among cases and controls |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Genotypes       | Cases (n = 50)  | Controls (n = 50) | p-value | OR  |
|                 | Count | %      | Count | %      |     |     |
| XX (more in cases than control) | 20    | 40.0%  | 6     | 12.0%  | *0.001 | 6.111 |
| YY (more in cases than control)  | 12    | 24.0%  | 11    | 22.0%  | 0.174  | 2.000 |
| PQ genotypes    |        |        |       |        |     |     |
| PP              | 2     | 4.0%   | 0     | 0.0%   | 0.999  | --   |
| PQ              | 5     | 10.0%  | 3     | 6.0%   | 0.430  | 1.822 |
| QQ              | 43    | 86.0%  | 47    | 94.0%  | 1    | 8.062 |
| HL genotypes    |        |        |       |        |     |     |
| HH              | 22    | 44.0%  | 17    | 34.0%  | 1    | --   |
| LL (more in controls than cases) | 6    | 12.0%  | 18    | 36.0%  | *0.018 | 0.258 |
| LL (more in cases than control)  | 22    | 44.0%  | 15    | 30.0%  | 0.788  | 1.133 |
| AB genotypes    |        |        |       |        |     |     |
| AD              | 13    | 26.0%  | 17    | 34.0%  | 1    | --   |
| AB              | 12    | 24.0%  | 12    | 24.0%  | 0.626  | 1.308 |
| BO              | 22    | 44.0%  | 13    | 26.0%  | 0.118  | 2.213 |
| OO              | 3     | 6.0%   | 8     | 16.0%  | 0.355  | 0.490 |

*p ≤ 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].

Table 5: Different *MBL*<sub>2</sub> secretor haplotypes among cases and control groups

<table>
<thead>
<tr>
<th>Haplotypes</th>
<th>Cases, N = 100</th>
<th>Controls, N = 100</th>
<th>p-value</th>
<th>OR</th>
<th>95% CI Lower</th>
<th>Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Count</td>
<td>%</td>
<td>Count</td>
<td>%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High secretor</td>
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<td></td>
<td></td>
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<tr>
<td>LYQA</td>
<td>2</td>
<td>2.0</td>
<td>3</td>
<td>3.0</td>
<td>0.964</td>
<td>1.048</td>
</tr>
<tr>
<td>HYQA</td>
<td>7</td>
<td>7.0</td>
<td>11</td>
<td>11.0</td>
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<tr>
<td>HYPQA</td>
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<td>0.0</td>
<td>1</td>
<td>1.0</td>
<td>1.000</td>
<td>---</td>
</tr>
<tr>
<td>Low secretor</td>
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<td></td>
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<tr>
<td>LXPA</td>
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<td>0</td>
<td>0.0</td>
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<td>---</td>
</tr>
<tr>
<td>HXPA</td>
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<td>0</td>
<td>0.0</td>
<td>0.999</td>
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<tr>
<td>HXPB</td>
<td>1</td>
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<td>1</td>
<td>1.0</td>
<td>0.762</td>
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<tr>
<td>LXQB</td>
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<td>4.0</td>
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<td>13.0</td>
<td>0.332</td>
<td>0.484</td>
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<tr>
<td>LXQB</td>
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<td>1.0</td>
<td>1</td>
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<td>17.286</td>
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<td>0.030</td>
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</tr>
<tr>
<td>LXQB</td>
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<td>New non reported haplotypes</td>
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<td>0.448</td>
<td>0.629</td>
</tr>
</tbody>
</table>

*p ≤ 0.05 is considered significant.*

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 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*<sub>2</sub> secretor haplotypes among VVC and RVVC

<table>
<thead>
<tr>
<th><em>MBL</em>&lt;sub&gt;2&lt;/sub&gt; secretor haplotypes</th>
<th>VVC, N = 20</th>
<th>RVVC, N = 30</th>
<th>p-value</th>
<th>OR</th>
<th>95% CI Lower</th>
<th>Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Count</td>
<td>%</td>
<td>Count</td>
<td>%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High secretor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LYQA</td>
<td>0</td>
<td>0.0%</td>
<td>2</td>
<td>2.5%</td>
<td>0.999</td>
<td>---</td>
</tr>
<tr>
<td>HYQA</td>
<td>3</td>
<td>15.0%</td>
<td>4</td>
<td>5.0%</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Low secretor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LXPA</td>
<td>1</td>
<td>5.0%</td>
<td>1</td>
<td>1.3%</td>
<td>0.858</td>
<td>0.750</td>
</tr>
<tr>
<td>HXPA</td>
<td>1</td>
<td>5.0%</td>
<td>1</td>
<td>1.3%</td>
<td>0.858</td>
<td>0.750</td>
</tr>
<tr>
<td>HXPB</td>
<td>0</td>
<td>0.0%</td>
<td>1</td>
<td>1.3%</td>
<td>1.000</td>
<td>---</td>
</tr>
<tr>
<td>LXQB</td>
<td>1</td>
<td>5.0%</td>
<td>3</td>
<td>3.8%</td>
<td>0.558</td>
<td>2.250</td>
</tr>
<tr>
<td>LXQB</td>
<td>1</td>
<td>5.0%</td>
<td>10</td>
<td>12.5%</td>
<td>0.120</td>
<td>7.500</td>
</tr>
<tr>
<td>LXQB</td>
<td>2</td>
<td>10.0%</td>
<td>6</td>
<td>7.5%</td>
<td>0.468</td>
<td>2.250</td>
</tr>
<tr>
<td>LXQB</td>
<td>3</td>
<td>15.0%</td>
<td>7</td>
<td>8.8%</td>
<td>0.587</td>
<td>1.750</td>
</tr>
<tr>
<td>New non reported haplotypes</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>LXQA</td>
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<td>0.0%</td>
<td>4</td>
<td>5.0%</td>
<td>0.999</td>
<td>---</td>
</tr>
<tr>
<td>LXQA</td>
<td>0</td>
<td>0.0%</td>
<td>6</td>
<td>7.5%</td>
<td>0.999</td>
<td>---</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HXQA</td>
<td>1</td>
<td>5.0%</td>
<td>5</td>
<td>6.3%</td>
<td>0.322</td>
<td>3.750</td>
</tr>
<tr>
<td>LXQA</td>
<td>1</td>
<td>5.0%</td>
<td>0</td>
<td>0.0%</td>
<td>1.000</td>
<td>0.000</td>
</tr>
<tr>
<td>LXQA</td>
<td>1</td>
<td>5.0%</td>
<td>10</td>
<td>12.5%</td>
<td>0.120</td>
<td>7.500</td>
</tr>
<tr>
<td>LXQA</td>
<td>0</td>
<td>0.0%</td>
<td>1</td>
<td>1.3%</td>
<td>1.000</td>
<td>---</td>
</tr>
<tr>
<td>HXQA</td>
<td>4</td>
<td>20.0%</td>
<td>8</td>
<td>10.0%</td>
<td>0.679</td>
<td>1.500</td>
</tr>
<tr>
<td>LXQA</td>
<td>1</td>
<td>5.0%</td>
<td>9</td>
<td>11.3%</td>
<td>0.142</td>
<td>6.750</td>
</tr>
</tbody>
</table>

*p ≤ 0.05 is considered significant.*

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,
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 MBL2 gene has been proposed as one of such genetic factors [53]. Such a proposal has been supported by the finding that an individual’s MBL2 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 MBL2 genes in different populations with different medical conditions [8], [53], [55], [56].

In the present study, as regards MBL2 alleles, the frequency of the mutant X allele was found to be significantly higher in cases as compared to controls (p ≤ 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 MBL2 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 MBL2 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 MBL2 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, 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<sub>2</sub> 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<sub>2</sub> codon 54 polymorphisms. Moreover, the wild-type allele (A) for the MBL<sub>2</sub> 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<sub>2</sub> 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 (LX/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 LXRQ 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 LYP, 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<sub>2</sub> haplotypes, eight additional MBL<sub>2</sub> haplotypes were detected. Six of them, HXPA, HXPB, HXQB, HYQB, LXQB, and LXQB, 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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 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, 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 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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PMid:21655939
PMid:26166955
PMid:23026768
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PMid:14764589
PMid:28197138
PMid:16395391
PMid:25900955
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