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# ABC genotyping and putative virulence factors of *Candida albicans* clinical isolates

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## ABSTRACT

**Aims:** *Candida albicans* is a diploid yeast which interacts with the host in a complex nature involving several fungal virulence factors and host's response. In this study, we investigated the different ABC genotypes of 26 clinical *C. albicans* isolates which is based on the presence of absence of transposable intron in the 25S rDNA, and the phenotypic expression of their virulence factors: phospholipase production, esterase production, haemolytic activities, biofilm formation, and white-opaque switching.
**Methodology and results:** In this study, we investigated the ABC genotypes of 26 clinical *C. albicans* isolates, and the phenotypic expression of their virulence factors. The *C. albicans* isolates were tested for their *in vitro* abilities in exhibiting the following virulence factors: phospholipase, biofilm, esterase, hemolysin and phenotypic switching. Phospholipase activities and biofilm formation were detected in 57.7% and 65.38% of the isolates, respectively. All of the isolates showed phenotypic white-type colony, while none showed esterase and hemolytic activities. ABC genotyping revealed that 50% of the isolates were grouped under Genotype A, followed by Genotype C (42.3%), and B (7.69%).
**Conclusion, significance and impact of study:** This study provides information in regard to virulence potential and the ABC genotype of *C. albicans* from the Philippines.

*Keywords:* Phospholipase, biofilm, white-opaque switching, introns, ABC genotype

## INTRODUCTION

*Candida albicans* is one of the causative agents of candidiasis, which is considered to be one of the major healthcare-associated diseases especially among immunocompromised patients (Mishra *et al.*, 2007). Most pathogens of *C. albicans* have developed a battery of putative virulence factors to assist in their colonization, invasion, and pathogenesis. The main virulence factors reported for *C. albicans* are biofilm formation, especially on implanted devices, acid proteinases and phospholipases, which facilitate adherence by damaging extracellular proteins or degrading cell membranes, respectively. This allows the fungal cells to enter the host and to invade the immune system. Other virulence properties include the ability to switch morphology from a unicellular budding yeast cell to a filamentous hyphal form and switching from white to opaque cells (Biswas *et al.*, 2007; Chaffin 2008; Mohandas and Ballal, 2011; Jacobsen *et al.*, 2012; Mayer *et al.*, 2013).

Over the recent years, there has been an increase in the occurrence of diseases caused by *C. albicans* for which it has become recognized as the dominant human pathogenic fungus affecting patients with systemic life-threatening infections (Patel *et al.*, 2012). Hence, the identification of *C. albicans* from clinical specimens is essential. In addition, recent documented studies have presented the increase in the incidence of newly emerging species closely related to *C. albicans,* as well as the coexistence of genotypic variants during infections or among commensal isolates. Hence, application of PCR-based molecular typing methods in identifying these fungi is crucial and vital (Vrioni *et al.*, 2001; Warren *et al.*, 2010). Molecular typing methods such as genomic sequencing, multi-locus sequence typing, pulsed field gel electrophoresis, restriction fragment length polymorphism, and randomly amplified polymorphic DNA analysis have been developed for the characterization and deeper comprehension of *C. albicans* (Bretagne *et al.*, 1997; Tamura *et al.*, 2001; Luo *et al.*, 2002).

The identification of genotypes of *C. albicans* provides understanding in the dynamics of microevolution in the population of *Candida,* and its relatedness with the appearance of genetic variants with new phenotypes such as resistance to antifungal drugs and improved virulence (Samaranayake *et al.*, 2003). Several studies have evaluated the ABC genotypes of *C. albicans* isolated from clinical specimens (Dalle *et al.*, 2000; Zeng *et al.*, 2011). This method was developed on the basis of the presence or absence of a transposable group I intron in the gene

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which encodes for the 25S ribosomal RNA (Odds *et al.*, 2006). Identification using this molecular typing in *C. albicans* has provided valuable information in supplementing the growing knowledge of yeast epidemiology.

The capability of *Candida* spp. to invade and colonize host tissues is associated with their ability to produce virulence factors. The virulence and genotypes of *C. albicans* isolated from clinical specimens are not well studied in the Philippines. This study, in general, aims to characterize clinical *C. albicans* isolates in relation to its virulence factors and ABC genotypes. Hence, this study determined the phospholipases, biofilms, hemolytic factor, esterases and white-opaque switching that are expressed by local clinical *C. albicans* isolates. In addition, the isolates were characterized in terms of their genetic relatedness based on ABC genotyping. Results of the study provide the much-needed information on the less studied *C. albicans* in the country.

## MATERIALS AND METHODS

### *Candida albicans* isolates

This study involved 26 clinical *C. albicans* isolates obtained from different biological materials such as skin scrapings, throat swabs, sputum, and colonoscopy drain that were retrieved from two tertiary hospitals in Metro Manila, Philippines. The 26 isolates were the total number of yeast isolates obtained during the sampling period from November 2016 to January 2017. The isolates were cultured and maintained on Sabouraud dextrose agar (SDA, Merck). Identification of the *C. albicans* was performed using the following: germ tube, chlamydospore production, carbon assimilation, and internal transcribed spacer (ITS) sequencing.

### Virulence factors

*Phospholipase activity*

Fungal inoculum for each isolate was prepared by growing the isolates on SDA plates for 24 h at 37 °C. Following incubation, colonies from SDA were aseptically transferred into sterile 0.85% saline solution, and cell densities were standardized to the turbidity of 0.5 McFarland. Phospholipase activity was carried out using the egg yolk agar plate method (Price *et al.*, 1982). A base medium was prepared with the following components: 65 g SDA, 58.4 g NaCl, and 5.5 g $CaCl_2$ dissolved in 980 mL of distilled water. The prepared solution was autoclaved and after cooling down to 50 °C, the sterile base medium was mixed with 20 mL of sterile egg yolk emulsion. Egg yolk emulsion was prepared by centrifuging sterile egg yolk at 5000 × *g* for 30 min, and 2 mL of the supernatant were added to the cooled base medium. An aliquot of 10-µL yeast suspension was spot inoculated and incubated at 37 °C. Phospholipase activities were checked daily for six days and interpreted as positive when a precipitation zone was visible around the growth. Phospholipase activity (Pz) was computed as the ratio of the diameter of the colony to the total diameter of the colony plus the precipitation zone, and was scored and categorized as follows: Pz-1 (negative); 0.35-0.5 (high producers, +++); 0.51-0.74 (moderate producers, ++); and 0.75-0.9 (low producers, +) (Price *et al.*, 1982). Each test strain was tested in triplicate, and phospholipase activity was recorded as the average of the three measurements.

*Determination of biofilm formation*

Biofilm production was determined by visual methods following the methods of Vinitha and Mamata (2011) and Branchini *et al.* (1994). One hundred (100) µL standardized inoculum with turbidity equal to 0.5 McFarland were transferred into a screw-capped tube containing 10 mL of Sabouraud-dextrose broth (SDB) and supplemented with 8% (w/v) glucose. All fungal cultures were incubated at 37 °C for 48 h. Following incubation, the culture broth in the tubes was gently aseptically aspirated. The tubes were then washed with sterile distilled water twice and stained with 2% safranin for 10 min. The presence of an adherent layer in the tube was examined. Biofilm production was scored as negative (no biofilm), weak (very thin layer, just visible at the bottom), moderate (thin layer at the bottom and sides of the tube) or strong (a thick layer all over the bottom and sides of the tube (Yigit *et al.*, 2011). The test was done in triplicate and was read by two independent observers.

*Phenotypic switching*

The *C. albicans* were inoculated on yeast extract peptone dextrose (YPD, Hi-Media) agar and incubated for 3 days at 37 °C. After incubation, one to five colonies were diluted with 200 µL sterile distilled water and adjusted to 0.5 McFarland turbidity standard. The prepared yeast suspensions were inoculated on YPD medium supplemented with 50 µg/mL phloxin B. Inoculated plates were incubated for three days at room temperature. Cell morphology was confirmed using a light compound microscope after lactophenol cotton blue staining. The white cells were expected to produce regular white colonies, while opaque cells were red on solid medium. The white cells appear bright and spherical following staining, while opaque cells appear dark and oval (Hnisz *et al.*, 2011).

*Hemolytic activity*

Yeast suspensions adjusted to 0.5 McFarland were prepared from 24 h old colonies of *C. albicans*. Ten (10) µL of each yeast suspension were inoculated on sheep blood agar plate (Asiagel). Inoculated plates were incubated at 37 °C for 48 h (Favero *et al.*, 2011). The presence of a translucent and/or a greenish halo surrounding the colony indicated a positive hemolytic activity. The translucent halo measurements were evaluated as: (-) for no activity, (+) for weak activity (a

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halo zone <1.0 mm), (++) for mild activity (a halo zone of 1.1-1.49 mm), and (+++) for strong activity (a halo zone of ≥ 1.5 mm) (Favero *et al.*, 2014). Triplicate independent experiments were performed.

*Esterase activity*

Test media consisting of 1% peptone, 0.5% NaCl, 0.01% $CaCl_2$, 0.5% Tween-80, and 1.5% agar were prepared. Yeast suspensions adjusted to 0.5 McFarland were prepared from 24-h old colonies of *C. albicans*. Ten (10) µL of each yeast suspension was inoculated on the Tween-80 opacity test medium and incubated at 37 °C for 10 days. Esterase activity was read as positive in the presence of a halo pervious to the light around the inoculation site. Triplicate independent experiments were done and interpreted by two independent observers.

**ABC genotyping**

DNA extraction was done following the protocol of commercially available Bio-Rad Instagene Matrix Bacterial DNA reagent. PCR amplification was performed using the primer sequences shown in Table 1 (McCullough *et al.*, 1999). The reaction mixture was consisted of 1× PCR buffer, 0.2 mM of dNTP, 1.5 mM $MgCl_2$, 0.05 unit/µL of DNA polymerase, 0.5 µM of each primer, approximately 10 ng of template DNA and deionized distilled water. PCR was conducted as follows: 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. A final extension cycle was performed at 72 °C for 5 min. The amplified products were analyzed in a 1.8% (w/v) agarose gel stained with GelRed and viewed using ultraviolet light (UV). Genotypes of *C. albicans* isolates were identified and grouped based on their amplicon size as revealed in the agarose gel (Table 1). Single DNA band was expected for genotypes A (450 bp) and B (840bp), whereas two major products (450bp and 840 bp) were revealed for genotypes C due to the partial insertion of the intron in the ribosomal gene repeats of the organism's genome.

**Table 1:** Primers for ABC genotyping used in the study (McCullough *et al.* 1999).

| Primer | Nucleotide sequence (5'-3') | Amplicon size (bp) | Genotype |
|---|---|---|---|
| Forward | ATAAGGGAAGTCGGC AAAATAGATCCGTAA | 450 | A |
| Reverse | CCTTGGCTGTGGTTT CGCTAGATAGTAGAT | 840 | B |
| | | 450 & 840 | C |

## RESULTS

**Production of virulence factors**

Production of biofilm and phospholipases was detected in the clinical yeast isolates (Table 2, Figure 1). A total of six (23.08%) isolates were strong producers of biofilm, five (19.23%) were moderate producers, six (23.08%) were weak producers, while nine (34.61%) failed to form adherent biofilm layers in the tubes following incubation. On the other hand, six (23.08%) and nine (34.61%) isolates were moderate and weak producers of the enzyme phospholipase, respectively, while 11 (42.31%) of the test isolates were not able to produce the enzyme.

**Table 2:** Virulence factors (biofilm and phospholipase activities) produced by the *Candida albicans* isolates.

| | Biofilm | | | |
|---|---|---|---|---|
| | Negative (n) | +, (n) | ++, (n) | +++, (n) |
| *C. albicans* (n=26) | 9 | 6 | 5 | 6 |
| | Phospholipase | | | |
| | Negative (n) | +, (n) | ++, (n) | +++, (n) |
| | 11 | 9 | 6 | 0 |

+: weak activity, ++: moderate activity, +++: strong activity

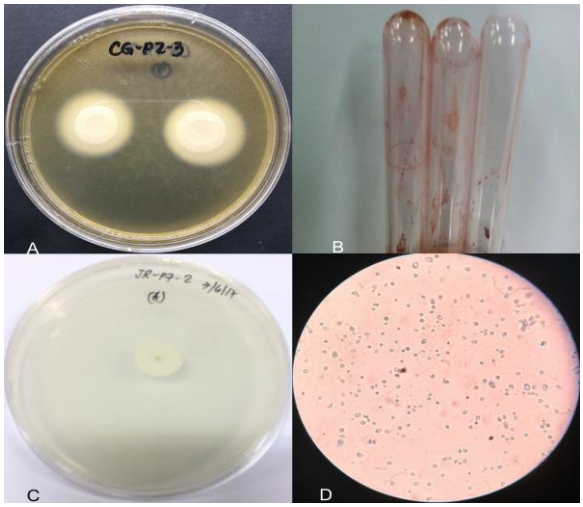


**Figure 1:** Phenotypic expression of virulence factors in *Candida albicans*. (A) Moderate phospholipase activity by strain CG12; (B) Strong biofilm formation by strain CG21 (from left to right: first two tubes with inoculated strain and rightmost is uninoculated control tube); (C) *Candida albicans* strain J8 grown on Tween 80 – opacity medium (negative esterase result), and (D) *Candida albicans* appear as bright circular cells after lactophenol staining and viewed under 1,000× magnification showing absence of white-opaque switching activity.

**Hemolytic activity, esterase and phenotypic switching**

All of the isolates did not produce esterases and failed to display hemolytic activities. White to opaque phenotypic transition was not observed in the clinical isolates tested.

***C. albicans* genotypes**

ABC genotyping was used to determine the distribution of genotypes among our *C. albicans* clinical isolates. Table

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3 shows that the most predominant genotype was A, where 13 (50%) isolates were identified to be belonging to this group. Eleven (42.31%) of the isolates, on the other hand, were identified belonging to genotype C, and two isolates (7.69%) were grouped under genotype B following PCR amplification and gel electrophoresis (Figure 2).

## DISCUSSION

The isolates in the study were evaluated for the production of virulence factors. Among the 26 *C. albicans* isolates tested, 57.69% were either moderate or weak producers of the enzyme phospholipase, the remaining were negative for the enzyme. Haynes (2001) confirmed that extracellular hydrolytic enzymes such as phospholipase play a significant role in the overgrowth of *Candida*. This enzyme targets the membrane phospholipids and digests the membrane's components, resulting to disruption of the cell membrane leading to host cell lysis (Souza *et al.*, 2014). As such, the presence of phospholipase in our isolates can be a parameter in distinguishing virulent invasive isolates from non-invasive colonizers.

At the same time, 65.39% of the isolates were determined to be either strong (23.08%), moderate (19.23%) or weak (23.08%) biofilm producers. Biofilms are sessile microbial communities, which can strongly adhere to contact surfaces and to each other, and are protected by an extracellular matrix composed of extracellular polymeric substance, polysaccharides like α-1,3-glucans, melanin, hydrophobins, galactomannan, monosaccharides, polyols, and antigens (Martinez *et al.*, 2010; Ramage *et al*., 2011). Biofilm formation shown by the test isolates likewise implies their ability to cause clinical infections. *Candida albicans* has previously been reported to produce biofilms on implanted synthetic materials that might serve as reservoirs on infectious particles, eventually releasing cells into the bloodstream where the yeast can access distant sites. The yeast then becomes a potential agent for infection of deep organs leading to invasive systemic infections (Uppuluri *et al.*, 2010). The production of biofilms of *Candida albicans* is also related to its phenotypic resistance to antifungals and the elements of the immune system since the extracellular matrix in the biofilm provides a protective physical barrier for the microbial cells. The specific binding of antifungals to β-1,3-glucans in the matrix hampers the agents from reaching their targets (Mitchell *et al.*, 2016). In addition, other factors such as inactivation of the antibiotic by high metal concentration and low pH, tolerance and cell density (quorum sensing), and presence of metabolically-inactive persister cells, which can persist during antibiotic treatment, can also promote fungal biofilm resistance (Costerton *et al*., 2007; Lewis 2008; Kostakioti *et al.*, 2013).

**Table 3:** Genotypes and virulence activities of *Candida albicans* isolates in the study.

| Isolate no. | Hospital | Specimen Type | ABC Genotype | Biofilm production | Phospholipase activity |
|---|---|---|---|---|---|
| J1 | PB | Skin | C | Weak | - |
| J2 | PB | Skin | C | Moderate | Weak |
| J3 | PB | Skin | C | - | - |
| J4 | PB | Skin | C | Moderate | - |
| J5 | PB | Skin | C | Weak | Weak |
| J6 | PB | Skin | C | Moderate | - |
| J7 | PB | Skin | C | Strong | Weak |
| J8 | PB | Skin | C | - | Weak |
| J9 | PB | Skin | C | Weak | Weak |
| CG10 | PR | Sputum | A | - | Moderate |
| CG11 | PR | Sputum | A | - | Moderate |
| CG12 | PR | Sputum | A | - | Moderate |
| CG13 | PR | Sputum | C | Strong | Moderate |
| CG14 | PR | Sputum | A | Strong | Weak |
| CG15 | PR | Colonoscopy drain | A | Weak | Weak |
| CG16 | PR | Sputum | C | Moderate | Weak |
| CG17 | PR | Sputum | A | Weak | - |
| CG18 | PR | Sputum | A | Strong | - |
| CG19 | PR | Sputum | A | - | - |
| CG20 | PR | Sputum | A | - | - |
| CG21 | PR | Sputum | B | Strong | - |
| CG22 | PR | Sputum | A | - | Moderate |
| CG23 | PR | Sputum | B | Strong | Weak |
| CG24 | PR | Sputum | A | - | Moderate |
| CG25 | PR | Throat swab | A | Moderate | - |
| CG26 | PR | Throat swab | A | Weak | - |

- : no activity detected, PR: specimen obtained from public hospital, PR: specimen obtained from private hospital
Hemolytic activity, esterase production and white-opaque switching were not detected in all isolates

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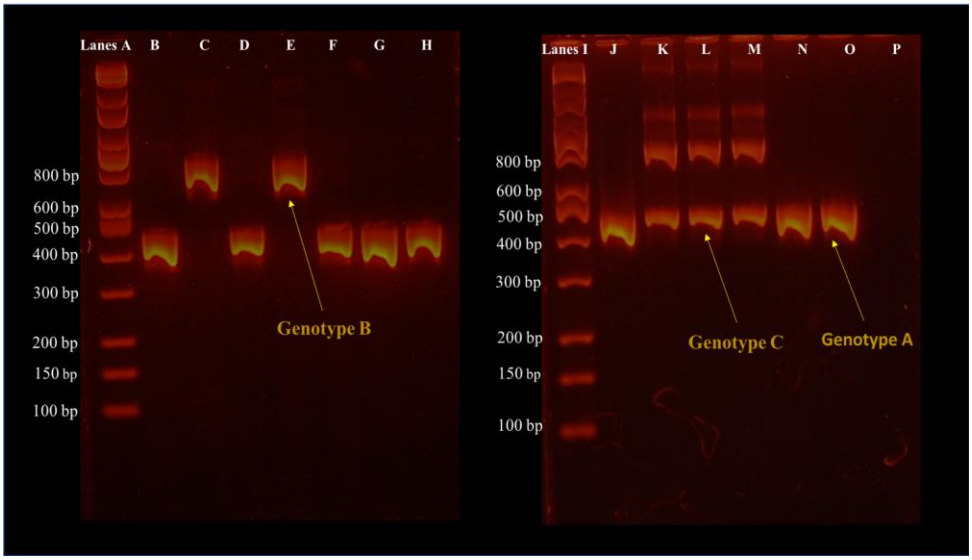


**Figure 2:** DNA bands following PCR amplification using primers for ABC genotyping. Lanes A and I, 100 bp DNA ladder; Lane J, Genotype A, *C. albicans* ATCC 14053 reference strain; Lanes C and E- B, genotype test isolates with transposable intron of 379-nucleotide insert in the 25S rDNA; Lanes A, D, F, G, H, N, and O, A genotype test isolates with no transposable intron in the 25S rDNA; Lanes K, L, and M, Genotype C isolates with two major bands due to the incomplete insertion of the transposable intron throughout the ribosomal repeats in the *Candida* genome; and Lane P, DEPC water as control negative.

Hemolytic activities, esterases, and phenotypic switching were not detected among the clinical isolates. Our results are in contrast with those in other studies (Luo *et al.*, 2001; Si *et al.*, 2013), where hemolytic and phenotypic switching were described. It is reported that hemolytic activity is related with the ability of the pathogen to acquire iron from host cells, which is a critical stage in the establishment of an infection. However, the relationship between virulence and hemolysin production still needs to be further investigated and evaluated (Watanabe, 1999). On the other hand, all of the isolates in our study have revealed primarily the white-type colonies, which are reported to be more virulent than the opaque phenotype (Huang 2012; Mandelblat *et al.*, 2017). White and opaque cells are different in terms of mating abilities and in the expression of many metabolic genes, indicating that these two types are adapted to various environments within a host (Tuch *et al.*, 2010). Though opaque cells are better colonizers of the skin, these cells do not readily form filaments under most conditions that usually induce hyphal formation in white cells, which can result in reduced ability to invade tissues. Also, in contrast to white cells, opaque cells were previously reported to be more sensitive to killing by the polymorphonuclear neutrophils. An increased susceptibility to killing by the neutrophils is a factor to the reduced virulence of opaque cells during systemic infections, since neutrophils are the first-line of host defense cells (Kvaal *et al.*, 1997; Lilic, 2012). However, it has also been reported that under certain environments, white cells are known to release a certain chemoattractant for polymorphonuclear neutrophils in contrast to opaque cells, implying that opaque cells can actually avoid being killed by neutrophils. In addition, it has also been found that white cells are more efficiently phagocytosed by other types of innate immune cells. It was concluded that depending on the environment, white-opaque switching can allow *C. albicans* to escape the defense mechanisms of the host (Geiger *et al.*, 2004; Lohse and Johnson, 2008; Sasse *et al.*, 2013).

Molecular typing methods are widely used in epidemiological studies to demonstrate control measures for clinical infections. ABC genotyping is one of the many molecular typing methods being used to differentiate *C. albicans* strains into genotypic subgroups due to its reliability and reproducibility. This method detects the presence and the size of a transposable insert in the 25S rDNA. Following PCR and electrophoresis, A genotypes will have a band size of 450 bp, B genotypes with 840 bp band size, while C genotypes will have two bands of 450 bp and 840 bp.

Our results showed that genotype A was predominant among the isolates. This finding is also similar with other studies, where genotype A was also predominant in clinical samples obtained from infections in the different anatomic sites, including systemic infections. This observation supports the theory that genotype A *C. albicans* is the most prevalent genotype worldwide (Millar *et al.*, 2002; Emmanuel *et al.*, 2012). In this study, we looked into establishing a relationship between the virulence factors and the genotypes. However, no association could be clearly established between genotypes and the virulence factors. The different virulence factors tested were not able to predict whether a specific genotype will be more likely to cause an infection

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or not. The same results were obtained in the study of da Silva-Rocha *et al.* (2014), wherein the production of phospholipase was not correlated with a particular genotype. In relation to specimen type, it was observed that all test isolates from skin scrapings samples were classified as genotype C, whereas isolates from sputum samples showed all A, B, and C genotypes. McCullough *et al. (*1999) hypothesized that genotype C strains might represent genotype A isolates that acquired the intron occurring in genotype B strains, converting these to genotype C strains. Perhaps genotype C strains are the offspring of the sexual union between genotype A with a genotype B, although the mechanism of the intron acquisition in *Candida* still remains unclear. On the other hand, the presence of all genotypes from sputum samples obtained from a private hospital suggests the existence of microevolution of *C. albicans*, supporting the hypothesis where sub-strains can be established from clones, which can dominate in a random manner during the formation of infection (Lockhart *et al.*, 1996; Chong *et al.*, 2007).

## CONCLUSION

In conclusion, our study showed the expression of the virulence factors phospholipase and biofilm formation among some of our isolates. In addition, the white-type colony of most of the isolates suggests their virulence compared to the opaque-type colonies. ABC genotyping revealed that the predominating genotype is A among our *C. albicans* isolates. The expression of the hemolytic activity, esterase production and white-opaque switching under different conditions such as temperature and nutrition can be further studied.

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