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Genetic diversity, antifungal susceptibility and enzymatic characterisation of Malaysian clinical isolates of *Candida glabrata*

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Abstract. *Candida glabrata* has been reported as the second or third most common yeast species isolated from patients with vaginitis and invasive candidiasis. This study was aimed to determine the genetic diversity, antifungal susceptibility and enzymatic profiles of *C. glabrata* isolated from vaginal and blood samples in the Medical Microbiology Diagnostic Laboratory, University Malaya Medical Centre. A random amplified polymorphic DNA (RAPD) analysis method, using M13 and $(GTG)_5$ primers, was used for strain differentiation of *C. glabrata* isolates. Antifungal susceptibility testing of *C. glabrata* isolates was determined using E-test against amphotericin B, caspofungin, fluconazole and voriconazole and microbroth dilution method against clotrimazole. The enzymic profiles of *C. glabrata* were determined using APIZYM semi-quantitation kit and egg-yolk agar method. A total of 14 RAPD patterns were identified amongst *C. glabrata* isolates investigated this study. Susceptibility to amphotericin B, caspofungin, fluconazole and voriconazole was noted. Approximately one third of the isolates demonstrated resistance to clotrimazole (MIC≥1 µg/ml). A single isolate of *C. glabrata* was resistant to caspofungin (MIC:1.5 µg/ml). Enzymatic activities of acid and alkaline phosphatase, aminopeptidases, esterase and lipase and phospholipase were detected in the *C. glabrata* isolates. The genetic diversity and antifungal susceptibility profiles of *C. glabrata* isolates were presented in this study. Continued surveillance and monitoring of the incidence and antifungal resistance in *C. glabrata* isolates is necessary.

INTRODUCTION

Candida glabrata has been frequently reported as an etiological agent of vaginal candidiasis and bloodstream infections (Rodrigues *et al.*, 2014). The organism has been recently recognized as a species complex consisting of *C. glabrata*, *C. nivariensis* (Alcoba-Flórez *et al.*, 2005) and *C. bracarensis* (Correia *et al.*, 2006). Invasive candidiasis is usually treated using amphotericin B, fluconazole, voriconazole and caspofungin, while azole drugs (including clotrimazole) are used to treat *Candida* vaginitis. Resistance of *C. glabrata* isolates to fluconazole, voriconazole, clotrimazole and caspofungin has been reported (Pfaller *et al.*, 2015; Wisplinghoff *et al.*, 2014).

Limited data is available on the genetic diversity of *C. glabrata* isolated from different clinical sources. Strain differentiation of yeast isolates can be achieved using random amplified polymorphic DNA analysis (RAPD) method (Soll, 2000). Information is scarce regarding virulence properties and hydrolytic enzymes of *C. glabrata*. *C. glabrata* has been reported to cause less host tissue damage and induce low immune response (Brunke and Hube, 2013). Little information is available on enzymes which facilitate growth and survival of *C. glabrata*. Evaluation of yeast enzymatic activity can be performed using APIZYM test

(Staniszewska *et al.*, 2015). Most studies reported phospholipase production in *C. albicans* (Ghannoum, 2000; Samaranayake *et al.*, 1984; Tay *et al.*, 2011). About 41% of *C. glabrata* isolates have been reported to produce small amounts of phospholipase (Ghannoum *et al.*, 2000).

This study was designed to investigate the genetic heterogeneity of blood and vaginal isolates of *C. glabrata*. The antifungal susceptibility and enzymic profiles of *C. glabrata* isolates were also determined using established procedures.

MATERIALS AND METHODS

Yeast isolates

A total of 50 *C. glabrata* isolates obtained from the Medical Microbiology Diagnostic Laboratory, University Malaya Medical Centre from 2008 to 2012 were investigated in this study. The isolates were recovered from vaginal swabs (n= 34) and blood culture (n=16). The identity of *C. glabrata* was confirmed using a singleplex PCR assay as described by Enache-Angoulvant *et al.* (2011). The isolates were subcultured on Chromogenic CHROMagar Candida agar (Difco, USA) for up to 2 days and the colony growth was recorded.

Random amplified polymorphic DNA (RAPD) analysis of *C. glabrata* isolates

Genomic DNA was extracted using a MasterPure™ Yeast DNA Purification Kit (EpiCenter, Madison, WI). Amplification was performed in volumes of 50 µl containing 1 µl (25 ng) yeast DNA, 0.25 µl *Taq* polymerase (5 U/µl) (Fermentas, Lithuania), 1 µl deoxyribonucleoside triphosphate mix (10 mM of each nucleotide), 5 µl of 10X PCR buffer, 6 µl $MgCl_2$ (25 mM) and 0.25 µl 100 pmol/µl of each primer, M13 (5'- GAGGGT GGCGGTTCT -3') or $(GTG)_5$ (5'-GTGGTGG TGGTGGTG-3') in a Veriti thermal cycler (Applied Biosystems, USA) (Meyer *et al.*, 1993). The PCR condition used were as follows: an initial denaturation step at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 20 secs, annealing at 50°C for 1 min and extension at 72°C for 20 sec. The PCR assay was continued with a final extension at 72°C for 6 min. A volume of 10 µl of PCR product was mixed with 2 µl of 6X loading dye solution (Fermentas, Lithuania) prior to electrophoresis on a 2% agarose gel at 100V for 2.5 h. A 100 bp DNA marker (Vivantis, Malaysia) was included to enable size estimation of the PCR products. The RAPD patterns were then visualized and image was captured for clustering analysis using unweighted pair group method with arithmetic mean (UPGMA) method of the Gel Compar II software (Version 4.0, Applied Maths, Kortjik, Belgium). Isolates sharing a similarity index of 60% were grouped into a RAPD cluster. By combining RAPD patterns generated from M13 and $(GTG)_5$ dendrograms, an isolate was assigned to a specific RAPD type. For instance, RAPD type A was designated to isolates belonged to cluster 1 of M13 dendrogram and cluster 1 of $(GTG)_5$ dendrogram; while RAPD type B was designated to isolates belonged to cluster 1 of M13 dendrogram and cluster 2 of $(GTG)_5$ dendrogram and etc.

Antifungal susceptibility testing of *C. glabrata* isolates

E-tests were performed for 41 *C. glabrata* (representing more than 75% of vaginal and blood isolates) using amphotericin B, fluconazole, caspofungin and voriconazole strips (bioMe´rieux, Marcy l'Etoile, France). Categorization of *C. glabrata* as susceptible or resistant strains was based on published interpretative criteria. Amphotericin B resistance was defined as MIC ≥2 µg/ml (Pfaller, 2012; CLSI, 2012). Breakpoints for fluconazole were as follows: susceptible (S), ≤8 µg/ml; susceptible dose dependent (SDD), 16 to 32 µg/ml; resistant (R), ≥64 µg/ml (Pfaller *et al.*, 2006). *C. glabrata* isolates with voriconazole MIC >1 µg/ml and caspofungin MIC of ≥0.5 µg/ml were considered resistant (Farmakiotis *et al.*, 2014).

As Etest for clotrimazole was not available at the time of testing, clotrimazole MICs were determined using a microbroth dilution method, as described by Pelletier *et*

al. (2000). Final drug concentrations ranging from 0.03 and 16 µg/ml were prepared for clotrimazole (Calbiochem, Germany). The growth of the isolates was inspected with a reading mirror and scored. The MIC was defined as the lowest concentration resulting with less than 50% growth (approximately 80% inhibition), as compared with the growth of the control wells after 24 hours of incubation at ±35ºC. MIC of ≥1 µg/ml is considered as a possible interpretive resistance breakpoint (Costa *et al.*, 2016).

Determination of enzymic profiles of *C. glabrata* isolates

APIZYM system (bioMerieux, Marcy l'Etoile, France) was used to define the enzymatic profiles of eight randomly selected clinical isolates of *C. glabrata.* Briefly, a loopful of *C. glabrata* colony was inoculated into 2 ml of API suspension medium to a density of McFarland 5. The cell suspension (65 microliters) were then dispensed into the specified cupules and incubated at 37ºC for 4 h. A drop of Zym-A and Zym-B reagents were added and the intensity of colour developed at the end of the testing period was graded to high (4 to 5+), moderate (2 to 3+) and weak (<2+) in accordance with the manufacturer's instruction.

Extracellular phospholipase activity was determined using the egg-yolk agar plate method of Price *et al.* (1982). To prepare the agar, ten millilitres of egg yolk added with an equal volume of sterile distilled water were incorporated into Sabouraud's dextrose agar (SDA) medium which had been supplemented with 1 M sodium chloride, and 0.005 M calcium chloride. A loopful of an overnight yeast culture was inoculated onto the egg-yolk agar plate using a sterilized flamed loop, and incubated at 37°C for 7 days. The agar plate was examined daily for formation of white precipitation zone around the colony. The diameter of colony (a) and the diameter of precipitation zone around the colony (b) were measured. Phospholipase production of isolates were calculated as P_z=a/b, as described by Price *et al.* (1982). *C. albicans* (ATCC 90028) was used as a positive control.

RESULTS

Of 50 *C. glabrata* isolates cultured on CHROMagar, 40 (76%) *C. glabrata* isolates including all 16 blood isolates and 24 (70.6%) of 34 vaginal isolates produced purple colonies. Ten (20%) isolates produced white or creamy colonies. Table 1 shows the strain differentiation of *C. glabrata* based on RAPD patterns generated by M13 and $(GTG)_5$ primers, respectively. *C. glabrata* isolates were differentiated into six and five clusters by referring to M13 and $(GTG)_5$ dendrograms, while *C. nivariensis* isolates were differentiated into a single cluster in each dendrogram. By combining the clustering results obtained from the dendrogram, a total of 14 RAPD types (A-N) were assigned to each *C. glabrata* isolate. Five RAPD types (A, B, C, D, E) were identified among the blood isolates, while ten RAPD types (D, F, G, H, I, J, K, L, M, and N) were identified for the vaginal isolates. Only one RAPD type (D) was shared among one blood culture isolate and three vaginal isolates. No specific association was observed between RAPD types with the source of isolates and the colour of *C. glabrata* colony on CHROMagar.

Antifungal susceptibility testing for 41 *C. glabrata* (including 26 vaginal and 15 blood isolates) showed that all were susceptible to amphotericin B (MIC_{50}: 0.094 µg/ml, MIC_{90}:0.38 µg/ml, range: 0.004 – 0.75 µg/ml), fluconazole (MIC_{50}: 0.094 µg/ml, MIC_{90}:0.75 µg/ml, range: 0.023 – 1 µg/ml), and voriconazole (MIC_{50}: 0.006 µg/ml, MIC_{90}:0.016, range: 0.002 – 0.016 µg/ml). The caspofungin MIC ranged from 0.003 – 1.5 µg/ml (MIC_{50}: 0.016 µg/ml, MIC_{90}:0.019 µg/ml). Resistance to caspofungin was suspected in a blood isolate with the MIC of 1.5 µg/ml. The clotrimazole MICs for 41 isolates ranged from 0.1 – 4 µg/ml (MIC_{50}: 0.5 µg/ml, MIC_{90}:4 µg/ml). A total of 15 (30.0%) isolates tested including 6 vaginal and 9 blood isolates were regarded as resistant to clotrimazole (MICs ≥ 1 µg/ml).

Table 1. Colony colour variants and RAPD patterns of *C. glabrata* (Cg) blood and vaginal isolates

Strain label	Colony colour	Source	M13 dendrogram	$(GTG)_5$ dendrogram	RAPD pattern
Cg 85	purple	B/C	1	1	A
Cg 78	purple	B/C	1	1	A
Cg 70	purple	B/C	1	2	B
Cg 82	purple	B/C	1	2	B
Cg 68	purple	B/C	1	2	B
Cg 83	purple	B/C	1	3	C
Cg 91	purple	B/C	1	3	C
Cg 92	purple	B/C	1	3	C
Cg 88	purple	B/C	1	3	C
Cg 90	purple	B/C	1	3	C
Cg 72	purple	B/C	1	3	C
Cg 169	purple	B/C	3	3	D
Cg 74	purple	B/C	5	4	E
Cg 69	purple	B/C	5	4	E
Cg 67	purple	B/C	5	4	E
Cg 71	purple	B/C	5	4	E
Cg 132	white	HVS	1	4	F
Cg 137	white	HVS	1	4	F
Cg 136	white	HVS	1	4	F
Cg 135	white	HVS	1	4	F
Cg 56	white	HVS	2	1	G
Cg 58	purple	HVS	2	1	G
Cg 60	purple	HVS	2	2	H
Cg 61	white	HVS	2	4	I
Cg 170	purple	HVS	3	1	J
Cg 94	purple	HVS	3	3	D
Cg 99	purple	HVS	3	3	D
Cg 167	purple	HVS	3	3	D
Cg 52	purple	HVS	4	4	K
Cg 53	purple	HVS	4	4	K
Cg 51	purple	HVS	4	4	K
Cg 50	purple	HVS	4	4	K
Cg 38	purple	HVS	4	5	L
Cg 34	purple	HVS	4	5	L
Cg 37	white	HVS	4	5	L
Cg 42	white	HVS	4	5	L
Cg 41	purple	HVS	4	5	L
Cg 35	purple	HVS	4	5	L
Cg 39	purple	HVS	4	5	L
Cg 36	purple	HVS	4	5	L
Cg 45	purple	HVS	6	4	M
Cg43	purple	HVS	6	4	M
Cg 47	purple	HVS	6	4	M
Cg 44	purple	HVS	6	4	M
Cg 46	purple	HVS	6	4	M
Cg 48	purple	HVS	6	4	M
Cg 49	purple	HVS	6	4	M
Cg 40	purple	HVS	6	5	N
Cg 176	white	LVS	3	1	J
Cg 175	white	LVS	3	1	J

Abbreviation: B/C: blood culture; HVS, high vaginal swab; LVS, low vaginal swab.

The enzymes detected in *C. glabrata* isolates in this study include alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and β-glucosidase. Based on the amounts of APIZYM substrate metabolized by the isolates, three enzymes i.e., alkaline phosphatase, leucine arylamidase and acid phosphatase were produced more abundantly by the *C. glabrata* isolates. Trypsin activity was not detected in any of the isolates, instead, high activity of acid and alkaline phosphatase and moderate activity of esterase (C4), esterase lipase (C8), and naphthol-AS-BI-phosphohydrolase were detected. Strain to strain difference in the production of α- and β-glucosidase, and N-acetyl-β-glucosaminidase were detected amongst the isolates. Faint haziness zones (Pz values ranging from 0.40-0.68) suggesting of weak phospholipase activities were observed for 94% of the isolates. None of the *C. glabrata* isolates produced strong white precipitation zone surrounding yeast colonies (as shown by the positive control, *C. albicans* ATCC 90028).

DISCUSSION

Different colour variants (white or purple) have been described for *C. glabrata* cultured on chromogenic agar, as reported in a previous study (Bishop *et al.*, 2008) as well as in this study. As white colony-producing yeasts such as *C. bracarensis* and *C. nivariensis* may be encountered in the clinical specimens in a low frequency (Alcoba-Flórez *et al.*, 2005; Correia *et al.*, 2006, Tay *et al.*, 2014), molecular method is essential for accurate identification of *C. glabrata* complex (Lockhart *et al.*, 2009). In this study, the identity of each *C. glabrata* isolate was confirmed using a singleplex PCR as described by Enache-Angoulvant *et al.* (2011).

The genetic heterogeneity of *C. glabrata* has been documented in several studies using RAPD method. In Poland, high genetic diversity has been reported for *C. glabrata* isolates whereby RAPD typing of 17 *C. glabrata* isolates recovered from intensive care unit patients revealed 16 different genotypes (Paluchowska *et al.*, 2014). High degree of genetic diversity was also demonstrated amongst oral and vaginal isolates of *C. glabrata* in Tunisia (Noumi *et al.*, 2009). Based on the M13 and $(GTG)_5$ dendrograms generated in this study, our isolates were assigned to 14 RAPD types, with the vaginal isolates exhibiting more genotypes (n=10) than the blood culture isolates (n=5). Bloodstream isolates were also found to be less diverse compared to digestive tract isolates in a previous study (Enache-Angoulvant *et al.*, 2010). The sharing of one RAPD pattern amongst isolates from vagina and blood specimens suggests possible dissemination of isolates originated from different anatomic sites. Evidence is also available that *C. glabrata* could penetrate deeper body site with *C. albicans* co-infection during vaginal infection (Alves *et al.*, 2014; Fidel *et al.*, 1999).

None of the *C. glabrata* isolates in this study were resistant to amphotericin B, fluconazole, and voriconazole. Fluconazole-resistant *C. glabrata* has rarely been reported in Malaysia (Amran *et al.*, 2011; Santhanam *et al.*, 2013; Tay *et al.*, 2011). Clotrimazole resistance was noted in 5 (19.2%) vaginal and 9 (60.0%) blood isolates in this study. Clotrimazole is one of the most commonly prescribed drugs for treatment of vaginitis in the gynaecological clinics. The occurrence of clotrimazole resistance in 19.2% *C. glabrata* vaginal isolates may be responsible for treatment failure in recurrent vaginitis (Sobel *et al.*, 2003), hence; monitoring susceptibility trends of Malaysian isolates of *C. glabrata* is necessary. Additionally, caspofungin nonsusceptibility in *C. glabrata* isolates was reported for the first time in this study.

Despite of the lack of proteinase activity (Tay *et al.*, 2011), aminopeptidases such as leucine, acid and alkaline phosphatase have been identified in *C. glabrata* isolates by APIZYM analysis in this study. Leucine arylamidase removes N-terminal L-leucine from peptide substrates, and may facilitate the adhesion and penetration of *C. albicans*

into the host tissues (Staniszewska *et al.*, 2015). The growth of *C. glabrata* in the host may be promoted by acid phosphatase which suppresses neutrophil respiratory burst and superoxide anion production (Baca *et al.*, 1993; Reilly *et al.*, 1996), and alkaline phosphatase which facilitates nutrient uptake in the yeast (Tamás *et al.*, 2002). The identification of phospholipase in *C. glabrata* isolates is consistent with the detection of esterase (C4), esterase lipase (C8) and lipase (C14) in the APIZYM analysis. The low level production of phospholipase as compared to *C. albicans* has been noted in previous studies (Samaranayake *et al.*, 1984; Tay *et al.*, 2011).

In conclusion, the finding in this study provides evidence on the genetic diversity and emergence of antifungal resistance in *C. glabrata* isolates from blood culture and vaginal samples. In view of the emergence of clotrimazole and caspofungin resistance noted in this study, there is a need for continuing monitoring and surveillance of the incidence and antifungal susceptibility of *C. glabrata* in our setting.

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