International Scholars Journals

African Journal of Parasitology Research ISSN 2756-3391 Vol. 7 (8), pp. 001-005, August, 2020. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Genotyping and antifungal susceptibility of *CANDIDA ALBICANS* clinical isolates from Yangzhou region in China

Xiaofang Zhu[1], Naijun Dong[2], Qin Wang[1], Beibei Cheng[1], Liqian Zhu[3], Jie Tao[3], Guoqiang Zhu[3]* and Ping Bo[4]*

[1]Department of Dermatology of Clinical Medical School, Yangzhou University, Yangzhou 225001, China.
[2]Department of Obstetrics and Gynecology of Clinical Medical School, Yangzhou University, Yangzhou 225001, China.
[3]College of Veterinary Medicine, Yangzhou University, Yangzhou 225009, China.
[4]Medical College, Yangzhou University, Yangzhou 225001, China.

Accepted 03 August, 2020

In this report, 500 clinical isolates of *CANDIDA ALBICANS* originated from patients' body sites (vaginal, rectum, tongue and pharynx) and samples (stool, urine) in Yangzhou-located region were selected, screened and characterized. *C. ALBICANS* DNA fragments spanning a potential transposable group I intron-containing region in 25S r DNA were amplified by polymerase chain reaction (PCR), cloned, sequenced and analyzed. Based on the DNA band, size and sequence data, they could be classified into three genotypes: genotype A (365 strains), genotype B (83 strains) and genotype C (52 strains). Sequence alignment analysis indicated that genotype B contained the conserved sequence element P, Q, R and S of group I intron, genotype C only harbored sequence element P and Q, while genotype A lacked these characteristic element sequences. 80 isolates covering the three genotypes were further selected to perform the *IN VITRO* antibiotics susceptibility test. The results indicated that *ALBICANS.* strains of genotype A were much less susceptible to flucytosine than either strains of genotype B or genotype C. But no regularity was observed between the susceptibility of *C. ALBICANS* isolates to azoles, and to amphotericin B. To our knowledge, this is the first report about the prevalence and antibiotics susceptibility of Chinese-based *C.*

Key words: *Candida albicans*, polymerase chain reaction (PCR), genotyping, antifungal susceptibility.

INTRODUCTION

Candida albicans, is a commensal of human microflora, residing at oral cavity, gastrointestinal tract, vaginal and urinary environments (Pfaller and Diekema, 2007), but in some cases, it worked as a conditioned pathogen. Since the early 1980s, fungi including *Candida albicans* have emerged as attention causes of human disease, especially among the immunocompromised and those hospitalized patients with serious underlying disease (Fridkin, 2005; Segal et al., 2006). Fungal diseases have emerged as important public health problems (Martin et al., 2003). McNeil et al. (2001) reported that Mycoses led to a dramatic increase in multiple-cause mortality and the majority of these Mycoses-related deaths were associated with *Candida, Aspergillus* and *Cryptococcus* sp. infection. In particular imimmunocompromised individuals related to the fungi including *C. albicans* infection, whose numbers are constantly increasing due to organ transplant, chemotherapy and to the prevalence of AIDS and Hepatitis C (Pfaller and Diekema, 2007).

Genotyping is significantly important for the epidemiolog of *C. albicans*, including recognizing outbreaks of infection, detecting cross-transmission, determining the source of the infection, recognizing particularly virulent strains if any, or detecting the emergence of drug resistant strains (Garcia-Hermoso et al., 2007). Several methods have been developed to

*Corresponding author. E-mail: yzgqzhu@yzu.edu.cn, bopping@yzu.edu.cn.Tel:86-514-87972590,86-514 87978801.Fax: 86-514-87311374, 86-514-87311374.

differentiate *C. albicans* strains. Strain typing techniques such as electrophoretic karyotyping, restriction length polymorphic DNA with hybridization with a *C. albicans*-specific probe, and random amplified polymorphic DNA (RAPD) have been employed elsewhere (Soll, 2000). Recently, several new techniques have been explored to genotype the *C. albicans*, for examples: Polymerase chain reaction Melting Profile (PCR MP) (Krawczyk et al., 2009), high-resolution DNA melting (HRM) analysis (Costa et al., 2010), ultilocus sequence typing (MLST), microsatellite length polymorphism (MLP) (Garcia-Hermoso et al., 2007).

Although some genotyping techniques show high discriminatory potential at strain level, they are time consuming or highly expensive. A PCR-based method with specific primers to amplify the regions of the transposable group I intron of 25S rDNA gene was developed by MuCullough et al. (1999). This technique is easy and quick to be performed with low cost. In this study, 500 clinical *C*. *albicans* isolates originated from Yangzhou, Jiangsu Province, China were genetically subtyped with this method, and the antifungal susceptibility test was performed with a broth microdilution method performed according to the NCCLS (1997) recommendations.

MATERIALS AND METHODS

Isolates of *C. ALBICANS*

640 clinical specimens were collected in Gynecological Department and Dermatology Department of Clinical Medical School, Yangzhou University from June 2009 to June 2010. Sterile Dacron swabs were used to pass several times across the surface of the vaginal canal, rectum, back of tongue, and pharynx. The samples were first cultured on Sabouraud agar for 48 h at 30°C. After first screening, they were further grown on CHROMagar Candida. In total 500 isolates of them were identified as *C. albicans* on the basis of their cultural and morphological characteristics such as colony color on CHROMagar *Candida*, chlamydospore formation and character on cornmeal agar and API20C system. Among them 220 isolates were from the patients with vulvovaginal Candidasis, the other 280 isolates came from the sputum, stool, urine of the inpatients and outpatients in hospitals. Single colony of all the isolates were selected from Sabouraud agar plates, and incubated at 37°C for 48 h, and then stored at 4°C before DNA extraction.

DNA extraction

For each isolate, the DNA genome was extracted with the following protocol. *C. albicans* of single colony was inoculated into 3 ml liquid YPD (1% yeast extract, 2% dextrose, 2% Bacto Peptone) and incubated at 30°C overnight with shaking. The cells were collected by centrifugation at 13000 × g for 1 min. After washing twice with sterile water, the cells were resuspended in 0.4 ml DNA extracting buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris-HCl, pH 8.0), 100 µl lyticase and incubated at 37°C for 2 h. Then 50 µl 10% SDS and 5 ul Proteinase K were added, and then the mixtures were incubated at 55°C for 3 h. During the incubation period, vortexes were needed for several times. 200 µl 5 M NaCl and 750 µl chloroform-isoamyl alcohol (24:1) were added. After violently vortex the solution was centrifuged at 13000 × g for 10 min and the supernatant containing the genome was collected. The genome were precipitated with 0.5 ml ice-cold isopropanol at -20°C for 30 min and centrifuged at 13000 × *g* for 5 min. The DNA was washed with 70% ethanol, vacuum dried, resuspended in 50 µl sterile ddH_2O, and stored at −20°C.

PCR and genotyping the isolates

All *C. albicans* isolates were typed by amplifying a specific DNA fragment located in the 25S rDNA bearing the potential Group I introns. According to the criterion, the PCR fragments of the strains with a band of 450bp were designated as genotype A, with a band of 840bp as genotype B, and with bands of both 450 and 840 bp as genotype C. The sequences of PCR primers, described previously (MuCullough et al., 1999), were showed as follows, CA-INT-L (forward): 5'-ATAAGGGAAGTCGGCAAAATAGATCCGTAA-3' and CA-INT-R (reversr): 5'-CCTTGGCTGTGGTTTCGCTAGATAGTAGAT-3'. Amplification reactions were performed in a volume of 25 µl containing 2 µl (~1ng) diluted DNA genome template, 2.5 µl 10x PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$), 0.2 mM (each of the four) dNTP, 0.5 mM (each) primers and 0.5 U Taq DNA polymerase (Takara Biotechnology (Dalian) Co., Ltd.). Amplification conditions of PCR were denaturated at 96°C for 5 min, followed by 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and then a final extension step of 72°C for 10 min. An aliquot of 5 µl of the PCR product was analyzed with 1% agarose gel to visualize the amplicons after staining with ethidium bromide.

Sequencing of amplicons and analysis of sequence data

Each of the 10 representative isolates of genotype A, B and C were randomly chosen to amplifiy the specific PCR fragments, and the fragments were purified with a purification kit (Tiangen), ligated into pMD-19 simple T vector (TaKaRa) according to the manufacturer's instructions and then transformed into *Escherichia coli* DH5α and screened by a standard procedure. Two recombinant clones for each isolate were selected for sequencing by commercial service (Shanghai Sangon Biological Engineering Technology and Services). The obtained DNA sequences were analyzed by alignment compared to the reference strains through BLAST.

Antifungal susceptibility testing

To provide a spectrum of medical fungi related to the target clinical species, a total of 80 *C. albicans* isolates were analyzed, including 32 isolates of genotype A and 24 isolates of genotype B and Genotype C, respectively.

The susceptibilities *in vitro* to fluconazole, itraconazole, ketoconazole, flucytosine and amphotericin B were performed by the NCCLS M27-A. Testing in RPMI 1640 (Gibcobrl, USA) buffered to pH 7.0 with 0.165 mol/l 3-(N-Morpholino) Propane Sulfuric Acid. Stock solutions of drugs were prepared in domethul sulphoxide (itraconazole, ketoconazole and amphotericin B), and water (fluconazole and flucytosine). Serial dilutions of each antifungal agent and yeast inoculation were prepared exactly as outlined in the NCCLS document (NCCLS, 1997). The final concentrations of the antifungal agents ranged from 64 to 0.125 ug/ml for fluconazole and flucytosine, from 32 to 0.0625 ug/ml for itraconazole, ketoconazole and amphotericin B. Minimum inhibitory concentrations (MICs) for fluconazole, itraconazole, ketoconozole and flucytosine were defined as the first concentration at which growth inhibition was ≥80% compared to that in the control (drug–free) well. MIC for amphotericin B was defined as the first

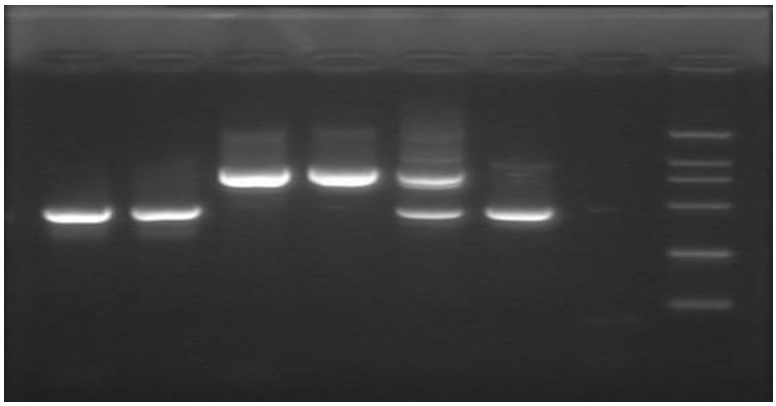

Figure 1. Profiles of the PCR-amplified DNA band for the criterion of genotyping clinical isolates of *C. albicans.*

Table 1. Genotyping of 500 clinical *C. albicans* isolates.

Genotypes	Sources of samples						
	Vaginal	Rectum	Tongue	Stool	Urine	Sputum	Pharynx
Genotype A	109 (75.7%)[§]	72 (86.7%)	17 (60.7%)	65 (74.7%)	36 (70.6%)	64 (71.9%)	12 (66.6%)
Genotype B	35 (24.3%)	11 (13.3%)	5 (17.8%)	14 (16.1%)	11 (21.6%)	16 (18.0%)	3 (16.7%)
Genotype C	0 (0%)	0 (0%)	6 (21.5%)	8 (9.2%)	4(7.8%)	9 (10.1%)	3 (16.7%)
Total	144	83	28	87	51	89	18

[§]ratio of isolation.

concentration at which no growth was detected. Azole susceptibility patterns were defined according to the NCCLS criteria (NCCLS, 1997). *Candida albicans* ATCC 09, and *Candida albicans* ATCC 19 were used as quality controlled strain in each run of the experiment (kindly provided by Professors Jiajun Wang, Qiangqiang Zhang, Shanghai Huashan Hospital, China).

RESULTS

The clinical isolates of *C. ALBICANS* containing three genotypes

From a total of 640 samples, 500 isolates of *C. albicans* were identified and kept. According to the criterion for genotype in, strains with only one band of 450bp were designated as genotype A, with one band of 840bp as genotype B, and with bands of both 450bp and 840bp as genotype C. Examples of these profiles in agrose gel electrophoresis are illustrated in Figure 1. All the clinical isolates could be separated into three genotypes, and other DNA types were not observed in this study. In addition, *C. albicans* of the three genotypes could be isolated from the samples of different origins with various frequencies. Genotype A was the most common type with a high ratio of isolation of 86.7 and 75.7% from the rectum and vaginal, respectively. The percentage for the isolation of genotype C was lower than the other two types, and it could not be found from the rectum and vaginal. But it could be isolated from tongue, pharynx and sputum with a higher frequency than genotype B. The distribution of genotypes was showed in Table 1.

Amplified sequences of genotype A, genotype B and genotype C

The amplified products selected from the three genotypes of *C. albicans* were sequenced and compared with each other. The results showed that genotype B contained conserved elements of group I introns, including P region (GGTACGGGGAAG), Q region (AATCCCGTGG). R region (TCGTAGAGACGGA) and S region (AAGGTACGTG), genotype C only contained P and Q

Table 2. Antifungal susceptibilities of the selected *C. albicans* isolates.

Antifungal agent	Genotype	MIC (µg/ml)													Mean MIC
		≥64	≥32	32	16	8	4	2	1	0.5	0.25	0.125	≤0.125	≤0.0625	
Fluconzole	Genotype A (32)*	2	N	1	2	3	0	3	7	7	7	N	0	N	7.32
	Genotype B (24)	0	N	0	0	0	4	4	4	4	8	N	3	N	0.85
	Genotype C (24)	2	N	0	0	1	2	4	6	5	4	N	0	N	6.73
5-flucytosine	Genotype A (32)	2	N	1	3	1	7	3	5	1	3	N	5	N	8.03
	Genotype B (24)	0	N	0	0	0	0	0	2	4	3	N	14	N	0.27
	Genotype C (24)	0	N	0	0	0	0	0	3	2	9	N	10	N	0.31
Itraconazole	Genotype A (32)	N	1	N	0	0	3	0	2	23	1	2	N	0	1.81
	Genotype B (24)	N	0	N	0	0	0	3	2	9	5	4	N	1	0.60
	Genotype C (24)	N	1	N	0	0	0	3	3	7	6	3	N	1	1.94
Ketoconazole	Genotype A (32)	N	2	N	0	1	5	4	3	2	0	11	N	4	2.68
	Genotype B (24)	N	1	N	0	2	0	1	3	4	6	3	N	4	2.84
	Genotype C (24)	N	0	N	1	0	2	0	1	4	6	5	N	5	1.23
Amphotericin B	Genotype A (32)	N	0	N	1	0	2	2	5	2	12	5	N	2	1.08
	Genotype B (24)	N	0	N	0	0	0	1	4	11	2	6	N	0	0.53
	Genotype C (24)	N	1	N	0	0	0	0	3	8	6	4	N	2	1.71

*Number of selected isolate for the test, N: Not used criterion.

regions, not contained R and S regions, indicating that intron was partially present throughout genotype C genome. Genotype A lacked these characteristic element sequences described earlier.

Relationship between genotypes and antifungal susceptibility

According to the analysis with a method of Multiway ANOVA, among the five antibiotics dugs of fluconazole, flucytosine, ketoconazole, itraconazole amphotericin B, the susceptibility of the tested isolates to flucytosine has a significant difference ($P<0.05$). Namely, there are differences among the susceptibilities of the three genotypes of *C. albicans* to flucytosine. Analysis by a two-sample t test indicated that the *C. albicans* genotype A was significantly less susceptible to flucytosine than genotype B and C (both $P<0.05$). However, to the susceptibility of genotype B and genotype C to flucytosine, there was no statistical difference ($P>0.05$). The distribution of the MICs of the isolated *C. albicans* to the five antifungal agents was presented in Table 2.

DISCUSSION

C. albicans is the most frequent isolated causative agent of *Candida* infection in humans and is generally accepted as the main pathogenic species of the genus *Candida* (Coleman et al., 1998). With the increasing numbers of candidiasis, molecular typing provide fundamental techniques for studying the epidemiology of *C. albicans* and developing rational therapeutic strategies (Lischewski et al., 1999; Redding et al., 1999). Analysis of group I intron region in 25S rDNA gene is a rapid and simple typing method with high discriminating power and reproducibility, requiring little material (Millar et al., 2002).

In this study, the prevalence of *C. albicans* in Yangzhou area of China from 2009 to 2010 was investigated. Totally, 500 isolates of *C. albicans* in Yangzhou area, China was obtained from clinical specimen with a standard method. And a pair of PCR primers designed to amplify a region of 25S rDNA gene bearing a potential group I intron-containing was used to genotype the isolates. Based on the previous criterion reported (McCullough et al., 1999), the 500 isolates could be differentiated into three genotypes (genotype A,B and C) on the basis of the length of the amplified PCR product. No atypical profile types were found including the genotype E which was found in Japan (Tamura et al., 2001). Our data showed that 365 of the 500 isolates were characterized into genotype A (accounting for 75.0%), while genotype B and C were accounting for 19 and 6%, respectively. The distribution in the three genotypes was similar with those reported by Tamura et al. (2001) and

McCullough et al. (1999). Our study also found that isolates of genotype C were mainly isolated from mouth and pharynx, but further study was required to elucidate the relationship between genotype and body site.

Sequence analysis indicated that the 450 bp in 25S rDNA gene of *C. albicans* was very conserved. There was only one base difference between the genotype A and C. Genotype B contained the conserved sequence elements (P, Q, R, S), but genotype A completely lacked these conserved elements, and genotype C only harbored part sequence, the conserved elements (P, Q). It has been postulated that there was a possible horizontal transfer of the group I intron from genotype C to genotype B or from genotype C to genotype A (McCllough et al., 1999).

Many genotyping methods mainly focused on the study of the cloning origin of *C. albicans* and the patterns of transmission. Little is focused on the relationship between the genotyping and virulence. Jain used RAPD analysis technique to differentiate the fluconazole-resistant strains of *C. albicans* from the fluconazole-sensitive stains (Jain et al., 2001). Early reports demonstrated that there was correlation between the group I intron in the 25S rDNA of *C. albicans* and the susceptibility to 5-fluorocytosine (Mercure et al., 1997). Our results of the antifungal susceptibility testing showed there were differences among the antifungal susceptibilities of genotype A, B and C to 5-fluorocytosine. And *C. albicans* of genotype B and C were more susceptible to flucytosine than genotype A, but no differences were found in the antifungal susceptibilities of genotype A, B and C to other four antifungal agents.

The classification of genotypes employing this PCR relies on the presence of group I intron of varying size in the 25S rDNA, and group I intron has self-splicing capability which is necessary for the formation of mature 25S rRNA (Mercure et al., 1993). The self-splicing capability can be inhibited by base analogs (Mercure et al., 1997), so the strains harboring group I intron will be more susceptible to base analogs, such as flucytosine. The antifungal mechanisms of azole drugs and amphotericin B are different from flucytosine, therefore there was no association between the presences of group I intron in the 25 S rDNA of *C. albicans* and strains susceptibility to azoles and amphotericin B, and reliable markers of drug sensitivity or resistance should be sought in the future. It would be beneficial in selecting a proper antifungal drug for therapy.

ACKNOWLEDGEMENT

This study was supported by a grant for Scientific Research from Ministry of Health of Jiangsu Province (Number H200555). We thank Professors Jiajun Wang, Qiangqiang Zhang from Shanghai Huashan Hospital for providing quality controlled strains and giving instructions to this project.

REFERENCES

Coleman DC, Rinaldi MG, Haynes KA, Rex JH, Summerbell RC, Anaissie EJ, Li A, Sullivan DJ (1998). Importance of Candida species other than *Candida albicans* as opportunistic pathogens. Med. Mycol., 36(Suppl.1): 156-165.

Costa JM, Garcia-Hermoso D, Olivi M, Cabaret O, Farrugia C, Lecellier G, Dromer F, Bretagne S (2010). Genotyping of *Candida albicans* using length fragment and high-resolution melting analyses together with minisequencing of a polymorphic microsatellite locus. J. Microbiol. Methods, 80(3): 306-309.

Fridkin SK (2005). The changing face of fungal infections in health care settings. Clin. Infect. Dis., 41(10): 1455-1460.

Garcia-Hermoso D, Cabaret O, Lecellier G, Densnos-Ollivier M, Hoinard D, Raoux D, Costa JM, Dromer F, Bretagne S (2007). Comparison of Microsatellite Length Polymorphism and Multilocus Sequence Typing for DNA-Based Typing of *Candida albicans*. J. Clin. Microbiol., 45(12): 3958-3963.

Jain P, Khan ZK, Bhattacharya E, Ranade SA (2001).Variation in random amplified polymorphic DNA (RAPD) profiles specific to fluconazole-resistant and –sensitive strains of *Candida albicans*.Diagnostic Diagn. Microbiol. Infect. Dis., 41(3): 113-119.

Krawczyk B, Leibner-Ciszak J, Mielech A, Nowak M, Kur J (2009) PCR melting profile (PCR MP)--A new tool for differentiation of Candida albicans strains. BMC Infect. Dis., 9: 177-184.

Lischewski A, Harmsen D, Wilms K, Baier G, Gunzer U, Klinker H (1999). Molecular epidemiology of *Candida albicans* isolates from AIDS and cancer patients using a novel standardized CARE-2 DNA fingerprinting technique. Mycoses.,42: 371-384.

Martin GS, Mannino DM, Eaton S, Moss M (2003). The epidemiology of sepsis in the United States from 1979 through 2000. N. Engl. J. Med., 348(16): 1546-1554.

McCullough MJ,Clemons KV, Stevensw DA (1999). Molecular and phenotypic characterization of genotypic *Candida albicans* subgroups and comparison with *Candida dubliniensis* and *Candida stellatoidea.* J. Clin. Microbiol., 37(2): 417-421.

McNeil MM, Nash SL, Hajjeh RA, Phelan MA, Conn LA, Plikaytis BD, Warnock DW (2001). Trends in mortality due to invasive mycotic diseases in the United States, 1980-1997. Clin. Infect. Dis., 33(5): 641-647.

Mercure S, Cousineau L, Montplaisir S, Belhumeur P, Lemay G (1997).Expression of a reporter gene interrupted by the *Candida albicans* group I intron is inhibited by base analogs. Nucleic Acids Res., 25(2):431-437.

Mercure S, Montplaisir S, Lemay G (1993).Correlation between the presence of a self-splicing intron in the 25S rDNA of *C. albicans* and isolate susceptibility to 5-fluorocytosine. Nucleic Acids Res., 21(25): 6020-6027.

Millar BC, Moore JE, Xu J, Walker MJ, Hedderwick S, McMullan R (2002).Genotypic subgrouping of clinical isolates of *Candida albicans* and *Candida dubliniensis* by 25S intron analysis. Lett. Appl. Microbiol., 35(2): 102-106.

National Committee for Clinical Laboratory Standards (1997). Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard. NCCLS document M27-A.Villanova, PA.

Pfaller MA, Diekema DJ (2007). Epidemiology of invasive candidiasis: A persistent public health problem. Clin. Microbiol. Rev., 20(1): 133-163.

Redding SW, Zellars RC, Kirkpatrick WR, McAtee RK, Caceres MA, Fothergill AW (1999).Epidemiology of oropharyngedl Candida colonization and infection in patients receiving radiation for head and neck cancer. J. Clin. Microbiol., 37(12): 3896-3900.

Segal BH, Kwon-Chung J, Walsh TJ, Klein BS, Batiwalla M, Almyroudi NG, Holland SM, and Romani L (2006). Immunotherapy for fungal infections. Clin. Infect. Dis., 42(4): 507-515.

Soll DR (2000).The ins and outs of DNA fingerprinting the infectious fungi. Clin. Microbiol. Rev., 13(2): 332–370.

Tamura M, Watanabe K, Mikami Y, Yazawa K, Nishimura K (2001). Molecular characterized of new clinical isolates of *Candida albicans* and *C. dubliniensis* in Japan: Analysis reveals a new genotype of *C. albicans* with group I intron. J. Clin. Microbiol., 39(12): 4301-4315.