Use of Random Amplified Polymorphic DNA to Identify *Candida* Species, Originated from Cancer Patients

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Abstract

Introduction: Recently, the incidence of fungal infections dramatically increased with an appearance of many novel species due to different criteria. Therefore, several molecular methods have been established for identification of these agents which cause human and animal disorder based on genomic DNA. Among these methods, RAPD-PCR technique is a powerful discriminative method based on amplifies target genomic DNA sequence by short random primers (arbitrary short primers) with low annealing temperature (36˚C) for discrimination and identification in the species level.

Materials and Methods: All clinical strains were originated oropharyngeal lesions of cancer patients from four Mazandaran University Hospitals in Iran. These strains were previously identified by phenotypic methods such as colony on CHROM-agar *Candida* medium, germ-tube formation in horse serum and chlamydospore formation. In this study RAPD-PCR technique was used to amplify hyper variable inter-repeat DNA sequences using oligonucleotide primers specific microsatellite (GACA)4 for identifying, clustering and take into account the genetic correlation of 30 clinical isolates.

Results: The results in this study showed that the RAPD-PCR by using of arbitrary short primer was able to amplify hyper variable inter repeated DNA sequences with classifying the isolates. RAPD patterns showed genetically inter speciation relationship in the best possible way and PCR-fingerprinting with primer (GACA)4, was able to discriminate both *C. albicans* with other *Candida* species based on size and number of bands.

Conclusion: We concluded that, regarding to the previous studies which have been reported misidentification by conventional mycological method for identifying *Candida* species, RAPD-DNA method is able to discriminate *Candida* species by using of a single primer. However, determination of differences and accurate assessment of genetic distances in the RAPD technique was generally limited.

Key words: DNA Fingerprinting, *Candida* species, Microsatellite, Identification, Cancer.

Introduction

The incidence of opportunistic fungal infections, nowadays, dramatically increased due to the species of *Aspergillus*, *Candida* and rarely other filamentous and yeast-like fungi. *Candida* spp. is ubiquitous opportunistic yeast fungi which remarkably frequently encountered in invasive candidiasis especially in immunocompromised host with high morbidity and mortality, despite the application of antifungal therapy.(1) One of the most significant issues in the routine laboratories of medical mycology is the correct identification and characterization between *Candida* species, especially discrimination of *C. albicans* from *non-Candida albicans* or *C. dubliniensis*. The identification of latter organism by conventional mycological procedures remains difficult and is not easily detectable than other *Candida* species. In addition, prevention of nosocomial infections due to latter agents are difficult to obtain and always determination of colonization sources is not possible.(2) Therefore, appropriate and early

detection and therapeutic intervention is critically important in the management of the hospital candidiasis which caused by either endogenous or exogenous origin of *C. albicans* and related species in unhealthy individuals to avoid of recurrent and disseminated infection.(3)

Since, with development and increasing of incidence of candidiasis due to *Candida* species, especially on patients with bone marrow and solid organ transplantation, patients who are on steroid therapy and patients infected with human immunodeficiency virus, correct identification by mycological procedures remains difficult, due to the high degree of phenotypic similarity between these groups of yeast recently, trends has increased remarkably for correct identification between these groups of fungi by use of various molecular methods based on genomic DNA. These novel tools are including RFLP (restriction fragments length polymorphism), DNA-Hybridization fingerprinting, the oligonucleotide probes synthetic homologous of microsatellite sequences eukaryotes, electrophoresis karyotype analysis of V3 region sequence of large ribosomal subunit gene. Although, these techniques are powerful methods for discrimination, often have some drawbacks.(4) It is now well assessed that molecular identification methods, which have driven new developments in fungal taxonomy and genetic diversity, are more reliable than classical morphological methods. Random amplified polymorphic DNA (RAPD) markers generated with single primers of arbitrary nucleotide sequence with low annealing temperature have been used in detecting intraspecific polymorphisms among the DNA of any organism.(5, 6) The latter method can generate specific DNA fragments that can be useful for typing of *Candida* species. Remarkably, *Candida* species have potential pathogenicity with different variation in susceptibility against novel and conventional antifungals, identification and differentiation in the species level might be relevant for clinical managements. Therefore, the purpose of this study was to evaluate of genomic variations of 32 *Candida* isolates originated from urine, lip, throat and cheek of cancer patients by use of microsatellite primer (GACA)4 as a single short primer for confirming the Candida species characterization based on RAPD-PCR to amplify the nuclear genomic DNA sequences inter-repeat.

Materials and Methods

Fungal strains: Table- 1 summarizes and characterizes the all information of 32 isolates of *Candida* spp, previously obtained from patients which suffering from cancer at four Mazandaran University Hospitals in Iran under regulations of research ethic committee of Mazandaran University of Medical Sciences.(7;8) As well as, reference strains were used as type controls which are *C. albicans*, *C. tropicalis*, *C. dubliniensis*, *C. glabrata* and *C. krusei* which kindly obtained from CBS-KNAW, fungal biodiversity centre, Utrecht, Netherlands.

Phenotypic identification: Briefly, all specimens initially were examined by direct examination with KOH (10%) and were cultured by standard techniques on routine media on Sabouraud Dextrose Agar (LAB M, Bury, UK) supplemented with chloramphenicol (0.5 µg/ml) for recovery of fungi at 20-30°C for 24-48 h. For determination and identification of multiple yeast species used CHROM-agar *Candida* also (CHROM-agar Company, Paris, France) and reading results according to the colony color within 48h at 37°C. In addition the *Candida* isolates were identified by other discriminative tools including germ-tube formation in horse serum, and chlamydospore formation on Cornmeal Agar (DIFCO laboratories, Detroit, Michigan, USA) with 1% Tween 80 (CMA-T_{80}). Stock cultures for transient working collections were initially grown on Sabouraoud's dextrose agar (SDA; Difco) at 32°C for 2 days, and the organisms were identified to the species level and evaluated for molecular relatedness by using of RAPD-PCR technique.

DNA Extraction: DNA was extracted using an method of glass bead disruption.(9) Briefly, a loopful of fresh cultured yeasts was suspended in 300µl of lysis buffer (10mM Tris , 1mM EDTA pH 8, 1% SDS , 100mM NaCl, 2% Triton X-100), then 300 µl of phenol-chloroform (1:1) solution and 300mg of 0.5 diameter glass bead were added to samples. For disrupting cell, samples were vortexed vigorously for 5min. Then, samples were centrifuged at 10,000 rpm for 5 min, supernatant was separated and transferred to a new micro tube and equal volume of chloroform was added, centrifuged 10,000 rpm for 5 min and supernatant was separated and transferred to a new micro tube again. For alcohol precipitation, 2.5 ml volume of cold absolute ethanol were added and frozen in -20º C for 10 min. After freezing, samples were centrifuged 12,000 for 12 min. The precipitate was centrifuged and washed with 70% ethanol, air-dried, re-suspended in 100µl of TE (10mM Tris, 1Mm EDTA) and was preserved at -20ºC until

Table- 1: Summarized the isolation data of examined strains originated from cancer individuals from four Mazandaran University Hospitals in Iran.

Patients	Age	Sex	Type of malignancy	Direct exam	Color in CHROMagar	Chlamydospore formation	Germ tube formation	Classical identification	RAPD-PCR identification
1	67	M	Larynx tumor	Budding yeast & hypha	Blue-grey	+	-	*C. tropicalis*	*C. tropicalis*
2	75	M	Lung tumor	Budding yeast & hypha	violate	-	-	*C. tropicalis*	*C. tropicalis*
3	53	F	nasopharyngeal carcinoma	Budding yeast & hypha	Blue-grey	+	-	*C. tropicalis*	*C. tropicalis*
4	35	M	Stomach tumor	Budding yeast	Light to Dark red	-	-	*C. glabrata*	*C. glabrata*
5	49	M	Esophagus tumor	Budding yeast & hypha	brown	-	-	*Candida spp*	*C. glabrata*
6	4	M	ALL	Budding yeast	green	+	+	*C. albicans*	*C. glabrata*
7	44	F	Esophagus tumor	Budding yeast	pink	-	-	*C. glabrata*	*C. glabrata*
8	44	F	Esophagus tumor	negative	Light red	_	-	*C. glabrata*	*C. krusei*
9	55	M	brain tumor	Budding yeast	Light to Dark red	_	-	*C. glabrata*	*C. krusei*
10	72	M	brain tumor	Budding yeast & hypha	Light red	+	-	*C. glabrata*	*C. krusei*
11	57	M	brain tumor	Budding yeast & hypha	Violet- grey	_	-	*Candida. spp*	*C. krusei*
12	70	F	Breast cancer	Budding yeast & hypha	Light red	_	-	*Candida. spp*	*Candida. spp*
13	68	M	Cheek tumor	Budding yeast & hypha	light red	+	-	*Candida. spp*	*Candida. spp*
14	70	M	Lung cancer	Budding yeast & hypha	white	_	-	*Candida. spp*	*Candida. spp*
15	65	F	Breast cancer	negative	white	_	-	*Candida. spp*	*Candida. spp*
16	45	F	Breast cancer	Budding yeast	white	+	-	*Candida. spp*	*Candida. spp*
17	75	F	Breast cancer	pseudohyphae	white	+	-	*Candida. spp*	*Candida. spp*
18	80	M	Esophagus tumor	Abundant Yeast pseudohyphae	Light red	_	-	*Candida. spp*	*Candida. spp*
19	60		Liver tumor	Abundant Yeast Abundant hyphae	green	+	+	*C. albicans*	*C. albicans*
20	37	F	Esophagus tumor	Budding yeast & hypha	green	+	+	*C. albicans*	*C. albicans*
21	75	F	Liver tumor	Budding yeast & hypha	green	+	+	*C. albicans*	*C. albicans*
22	57	M	Brain tumor	Budding yeast & hypha	green	+	+	*C. albicans*	*C. albicans*
23	75	F	liver tumor	Budding yeast & hypha	green	+	+	*C. albicans*	*C. albicans*
24	68	M	non-Hodgkin lymphoma	Budding yeast & hypha pseudohyphae	green	+	+	*C. albicans*	*C. albicans*
25	44	M	brain tumor	Budding yeast, hypha & pseudohyphae	Light green	+	+	*C. albicans*	*C. albicans*
26	80	M	Esophagus tumor	Abundant Yeast+ pseudohyphae	green	+	+	*C. albicans*	*C. albicans*
27	27	M	Lymphoma	Budding yeast & hypha	Light green	+	+	*C. albicans*	*C. albicans*
28	70	M	Larynx tumor	Budding yeast & hypha	green	+	+	*C. albicans*	*C. albicans*
29	56	F	Esophagus tumor	Budding yeast & hypha	green	+	+	*C. albicans*	*C. albicans*
30	9	M	Non -Hodgkin lymphoma	negative	green	+	+	*C. albicans*	*C. albicans*
31	44	F	Esophagus tumor	negative	green	+	+	*C. albicans*	*C. albicans*
32	3	F	AML	Budding yeast	green	+	+	*C. albicans*	*C. albicans*

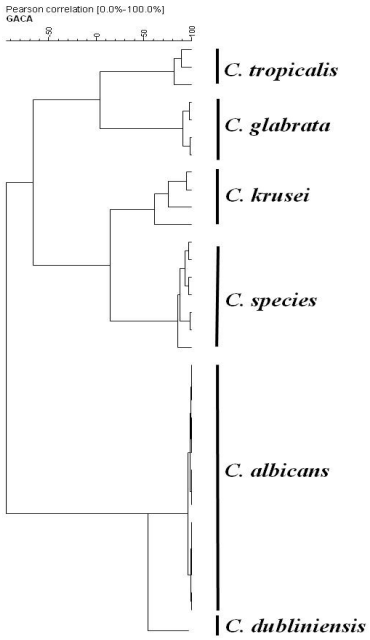

Figure- 1. The dendrogram drawing using UPGMA (Unweighted pair group method) software representing the cluster of similarity which classified based on genetic relatedness.

used. DNA was visualized by electrophoresis on 1% agarose gel stained with ethidium bromide to verify its quality and a spectrophotometer reading was performed at 260 and 280 nm to check the quantity. Nucleic acid purity was confirmed by an optical density value between 1.8 and 2.0.

RAPD-DNA analysis: PCR amplification of hyper variable inter-repeat sequences was achieved by using of (GACA) 4: 5′-GAC AGA CAG ACA GAC A-3′ (MWG-Biotech AG, Germany). Amplification reactions were performed on in a total volume of 50 µl, containing 2µl of template DNA from each train, 0.5 µl of primer at 25 µM, 1.25 µl of dNTP at 5mM, 0.5U Taq DNA polymerase and 5µl 10× PCR buffer. Amplification was performed with cycles of 1 min at 94°C for primary denaturation, followed by 35 cycles at 94°C (45 s), 50°C (60 s) and 72°C (60 s), with a final 7 min extension step at 72°C. The PCR products were electrophoretically visualized by 2% agarose gel in TBE buffer (20 mmol/l EDTA, 10 mmol Tris boric pH 8) stained ethidium bromide (0.5 µg/ml) and photographed under UV light.
Data were inspected visually and were also imported into BioNumerics (version 4.6) software (Applied Maths, Sint-Martens-Latem, Belgium) and analyzed by the unweighted-pair group method using average linkages (UPGMA), to generate dendrograms representing cluster of similarities with analysing of similarity correlation coefficient by Pearson's correlation coefficient test based on the same software.

Results

A set of 32 yeast strains, isolated from cancer patients from four Mazandaran University Hospitals in Iran, was preliminary identified by classical morphological and subsequently by molecular biological methods. The phenotypic methods were based on CHROMagar *Candida*, chlamydospore production, and germ tube formation. The results have revealed that *C. albicans* was the most prevalent species, followed by many Candida unknown species, *C. glabrata*, *C. tropicalis*, and *C. krusei* (Table- 1). Thereafter, RAPD analysis have re-confirmed and revealed that *C. albicans* is the predominant species which originated from target patients. Figure- 1 showed the dendrogram of the RAPD-DNA analysis by using the primer (GACA) 4 for all *Candida* species with involving of their type strains, represented 5 clusters of similarity which ranging from 70 to 100 percent. Our results showed that the short random primer sequence was able to amplify the hyper variable inter-repeat DNA of all studied yeast isolates successfully. RAPD pattern analysis showed the genetic relationship between *Candida* species. Fingerprinting with this primer was able to differentiate the *C. albicans* from non *C. albicans* based on size and the number of bands, which was 7 Bands for *C. albicans* and more for others species. Five large main clusters were created especially after drawing dendrogram which are *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei* and *Candida dubliniensis* were divided into groups in each cluster. Strains which were identified as *C. albicans* had >98% similarity to each other with comparison to the type strain and genetically distance to other *Candida* species. Techniques in this study were unable to assess intervals and determine the phylogenetic relationship. In this study, compatibility profiles were generated for the same species using RAPD *C. albicans*, *C. dubliniensis* and *C. glabrata* profiles were compared with standard forms and similarity between the profiles was observed. Discrepancies were found in all species groups, but mainly in *C. glabrata* and *C. krusei* (Table- 1). In some cases *C. glabrata* isolates were misidentified as *C. albicans* or *C. krusei*. In addition we have seen an obvious mismatch between phenotypic and molecular identification. (Table-1).

Discussion

Although, with developments of molecular tools which are rapid and accurate, identification and differentiation down to the species level can be easy, molecular technique have some variations as

drawback such as the quality and concentrations of DNA, amplification procedures and the type of thermal cycler. In addition, however, RAPD-fingerprinting is one of a series of methods for phylogenetic studies, genetic mapping, and genotyping, moreover is well suited for distinguishing closely related organisms at the species to strain level, it cannot be used for identification of *Candida* species without prior culture isolation. According to the molecular era, RAPD fingerprinting can be used as a method for studying of *Candida* strains that are similar phenotypically and isolates among a species in a collection that has expanded geographically and as well as a method for identification of new non-typical variety.(10) Discrete RAPD patterns were produced from *C. albicans*, *C. Lusitania*, *C. tropicalis*, *C. glabrata* and *C. krusei*.(2, 4, and 10) Rocha et al, 2008 have shown that RAPD analysis is an accurate and rapid technique for identifying of *Candida* species.(11) Moreover, Valerio et al, have differentiated *Candida* species obtained from nosocomial candidemia by using of RAPD-PCR.(12) Genotyping and identification of *Candida* can be very important to understand and to control of infections due to latter organisms.(13) The identification of strain balance has been removed the need to prophylaxis and experimental treatment, it reduces the drug resistance progression and it creates a vision about virulence quality that can be affiliated with clones and specific clusters of strains.(14) Determination of different population quality that is genetically distinct is useful for understanding the epidemiology of infectious diseases. Developed techniques allow evaluation of genotypic strain relationship in the levels of genetic and facilitating of their epidemiological analysis.(15) Interestingly, most papers have been already published in the older literatures reported, incorrect and unreliable identification by mycological techniques, specially distinguishing of *C. albicans* with *C. dubliniensis*.(4) To assess this problem, combined diagnostic procedures based on conventional and novel tools are essential. In the present study 32 Candida isolates originated from cancer patients initially were identified by conventional mycological methods (germ tube, chlamydospore production and CHROMagar), after that those strains have been identified by RAPD-PCR by using of (GACA)4 primer to amplify genomic DNA sequences. Meyer et al, 2001 successfully distinguished *C. albicans* from *C. dubliniensis* based on PCR-fingerprinting by using of two primers (GACA)4, and M13, they announced that *C. albicans* and *C. dubliniensis* created 7 and 13 bands, respectively through primer (GACA)4 (4). Surprisingly, in this study based on RAPD-PCR by using of primer (GACA) 4 showed that *C. albicans* with the created profile were distinct from all other *Candida* species (Figure-1).

Moreover, Bautista et al, and Mariano et al, 2003 have been published identification of *Candida* species RAPD-DNA analysis and differentiated between *C. albicans* and *C. dubliniensis* by direct PCR.(16, 17) Melo et al, assessed the ability of RAPD for genetic correlation and results showed that RAPD can be useful for determining the differentiation between those isolates, but accurate assessment of genetic and related distance between different species in the RAPD technique has a general limitation.(18) Meyer et al,2001 noted that PCR-fingerprinting data don't show the definite phylogeny connection between the collection of isolates(4) In the present study the RAPD-PCR was unable to evaluate distances and determine the phylogeny relationship. Francesco et al, 1999 showed that the Karyotype electrophoresis methods and RAPD, show compatible profiles for similar species with comparing of determination of species methods for clinical isolates of *C. glabrata* and when they were compared with standard isolated strains, all them were the same and similar standard strains.(19) However, RAPD-PCR method as a tool to determine the phylogeny relationship and reliable detection of species has disadvantages, including the use of RAPD profile for early identification is needed to evaluate further. Also the cost goes up if multiple primers for species identification are required for each isolated.(2)

Scoring to the bands produced by RAPD technique is hard and is associated with error and often requires subjective operator intervention. Lack of repeatability(15, 18) is another important problem of this technique.

Conclusion

Interestingly, *Candida glabrata* and *C. krusei* isolates are known for their intrinsic resistance to fluconazole, misidentification of these species may thus lead to inadequate antifungal treatment. This shows that it is important to take into account the lower discriminatory ability of the classical phenotypical identification approaches when compared to those based on molecular tools. Therefore, in the present study we successfully prove that RAPD-PCR is able to discriminates and identify Candida species by using of a single primer. However, determination of differences and

accurate assessment of genetic distances in the RAPD technique was generally limited.

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