African Journal of Microbiology Research Vol. 6(9), pp. 2150-2155, 9 March, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR11.1584
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Differential expression of the vacuolar aspartic proteinase (*APR1*) gene in *Candida albicans* strains isolated from Malaysian and Iranian human and mice blood

Amri Saroukolaei S.[1]*, Shokri H.[2], Aklilu E.[3], Pei Pei C.[1], Amini P.[4], Badiei A.[3], Sharifzadeh A.[5] and Amin F.[6]

[1]Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, University of Putra Malaysia, 43400 Serdang, Selangor, Malaysia.
[2]Faculty of Veterinary Medicine, University of Mazandaran, Amol, Iran.
[3] Faculty of Veterinary Medicine, University Malaysia Kelantan.
[4]Department of Pathology, Faculty of Medicine and Health Sciences, University of Putra Malaysia, 43400 Serdang, Selangor, Malaysia.
[5]Mycology Research Center, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.
[6]Public Health Discipline, Royal College of Medicine Perak, University Kuala lumpur.

Accepted 08 February, 2012

Intracellular proteinase A is an aspartic enzyme in *Candida albicans* that is expressed by *APR1* gene. The aims of this study were to evaluate and to compare *APR1* gene expression in *C. albicans* strains isolated from Malaysian and Iranian patients and mice infected by *C. albicans* obtained from Malaysian and Iranian patients. The evaluation of *APR1* gene expression was performed using reverse transcriptase-polymerase chain reaction (RT-PCR) with two-pair primers and 18S rRNA as housekeeping gene. The expression of *APR1* gene and 18S rRNA was determined on agarose gel electrophoresis. The results did not show any significant difference in *APR1* gene expression between *C. albicans* isolated from mice blood infected by *C. albicans* isolated from Malaysian and Iranian patients ($p > 0.05$). The expression of *APR1* gene in *C. albicans* strains isolated from Malaysian patients was more than Iranian patients ($p < 0.05$). In addition, the gene expression in *C. albicans* strains obtained from mice blood was significantly higher than *C. albicans* strains isolated from both Malaysian and Iranian patients ($p < 0.05$). Considering the important role of intracellular proteinase A in *C. albicans, APR1* gene as producer of this enzyme can act as an important gene in virulence of *C. albicans* in different environmental situations.

Key words: *Candida albicans*, *APR1* gene, reverse transcriptase-polymerase chain reaction (RT-PCR) method.

INTRODUCTION

Candida albicans is a member of the microflora in most healthy people, where it predominantly colonizes the mucosal surfaces of the gastrointestinal tract (Ryan and Ray, 2004). However, especially in immunocompromised patients, *C. albicans* develops from a harmless commensal to an opportunistic pathogen that can cause superficial as well as life-threatening disseminated infections (Anaissie et al., 2003). Although the immune status of the host is the major factor that determines whether *C. albicans* can become a pathogen and cause infection, the fact that *C. albicans* is by far the most

*Corresponding author. E-mail: shahla1304@yahoo.com. Tel: 0098 21 61117130. Fax: 0098 21 66933222.

frequent cause of fungal infections in such debilitated patients indicates that it must possess traits that make it a more successful colonizer and pathogen than other medically important *Candida* species. It is a general view that the pathogenicity of *C. albicans* is not caused by single dominant virulence factors (Odds, 1994; Zarin and Zarei, 2009).

Rather, it seems to be the high adaptability of *C. albicans* to many different host niches, as illustrated by the possession of many different adhesins that mediate binding to a variety of tissues, which allows the yeast colonize and infect virtually all body locations (Kumamoto, 2008). A prerequisite for this adaptability is the capacity to respond to complex environmental signals representing the different host niches by the expression of an appropriate set of virulence-related and other genes (Sundstrom, 1999). *C. albicans* possesses a gene family encoding secreted aspartic proteinase, and these enzymes have been linked with the virulence of the fungus since their discovery (Brown et al., 2007).

The yeast vacuole contains a variety of hydrolytic enzymes, which are acquired for many cellular functions such as the turnover of cellular proteins and the ability of cells to survive in nitrogen and carbon starvation (Correia et al., 2010). The action of vacuolar proteolysis is very important when yeast is under nutritional stress conditions and sporulation (Van Den Hazel et al., 1996). Intracellular proteinase A as a vacuolar aspartic protease is produced by *APR1* gene expression in *C. albicans*. The contribution of this gene for pathogenesis has been clearly demonstrated using *APR1* deficient mutants and proteinase inhibitors (Komeda et al., 2002). These studies demonstrated that *APR1* gene expression appears to be crucial for mucosal and systemic infections, and are involved in *C*. *albicans* adherence, tissue damage and evasion of host immune responses. Therefore, the *APR1* isoenzymes have a variety of functions *in vivo*, which are probably called upon at different stages and in different types of *C*. *albicans* infections (Niimi et al., 1997). This study aims to evaluate and to compare *APR1* gene expression in *C. albicans* strains isolated from Malaysian and Iranian patients and mice infected by *C. albicans* strains obtained from Malaysian and Iranian patients.

MATERIALS AND METHODS

Yeast strains and media

Sixteen Malaysian and Iranian clinical strains of *C. albicans* isolated from patients with systemic candidiasis and 16 control strains of *C. albicans* obtained from healthy humans (non-systemic candidiasis) were used for evaluating the *APR1* gene expression. The yeast cells were cultured on sabouraud dextrose agar (SDA, *Merck Co., Darmastdt, Germany*) at 37°C for 24 h. In order to identify the yeast species, germ tube test, CHROM agar, β-glucosidase test, urease test, cornmeal agar-Tween 80 for chlamydospore production, sugar fermentation and assimilation tests were carried out by RapID Yeast Plus System (Remel Inc., USA).

In vivo evaluation of *C. albicans APR1* gene expression in mice model

A total number of 48 six-week-old specific-pathogen-free female BALB/C mice (weighing 25 to 30 g) were purchased from Razi Institute, Karaj, Iran. Sixteen strains of *C. albicans* (4 Malaysian and 4 Iranian clinical isolates, 4 Malaysian and 4 Iranian control isolates) were used and 3 mice were considered for each strain. All experiments were conducted in accordance with standard ethical guidelines. *C. albicans* strains were intravenously administered into the mice and then the changes of *APR1* gene expression were evaluated. The yeasts were cultured on SDA media and incubated at 37°C for 24 h. The colonies were removed from SDA media and washed twice with phosphate-buffered saline (PBS). The yeast suspension was adjusted approximately 500 × 10^3 cells/ml using hemocytometer slide. The mice were infected by inoculation of 0.1 mL of *C. albicans* suspension in the caudal vein in each experiment. Five hours after injection, the animal blood was collected in Ethylenediaminetetraacetic acid (EDTA)-coated tubes. A volume of 0.2 mL of the blood was directly cultured on SDA medium and incubated at 37°C for 4 days. *Candida* cells harvested from SDA media were used for evaluating the *APR1* gene expression in reverse transcriptase-polymerase chain reaction (RT-PCR) process.

Preparation of cell extract and RT-PCR reaction

The *Candida* colonies was heated at 100°C for 10 min and then centrifuged at 4500 × g at 4°C for 15 min. The supernatants were used in RT-PCR test in three steps as follows:

A) RNA extraction

RNA was extracted from *Candida* cell extract by using the RNX™-Plus kit (RNX™-Plus from Cinnagen kit, Iran) and then was stored at 2 to 8°C. RNA extraction was done in order to complete separation of RNA from DNA and production of DNA-free RNA. A final reaction volume of 20 µL containing dH_2O, RNA (0.1 ng), 10X reaction buffer with $MgCl_2$ and Deoxyribonuclease 1(0.2 units, Fermentas) was added to the RNase-free tube. This mixture was incubated at 37°C for 30 min and then 1 µL 25 mM EDTA was added and incubated at 65°C for 10 min to stop the reaction. This extract had pure RNA without any DNA.

B) RT reaction

Reverse transcriptase test was performed for the production of cDNA from RNA. A final volume of 12.5 µL solution containing RNA, random hexamer primer (Fermentas) and dH_2O was prepared. In addition, 7.5 µL of another solution containing reaction buffer 10X, dNTP (10X), Ribolock™ (RNase Inhibitor) and RevertAid M-MuLV was prepared. Twenty µL of each solution were obtained and maintained in the room temperature for 10 min. These solutions were incubated at 42°C for 60 min in heat block (Clifton, USA) and then at 70°C for 10 min.

C) PCR reaction

Two-pair primers, including APR1-f: 5´-TCCACCAATCTACAATGCCA-3´ and APR1-r: 5´-ATTTCAGCCAATGAGGATGG-3´ as proteinase A gene, and 18S rRNA-f: 5´-GCCAGCGAGTATTAACCTTG-3´ and 18S rRNA-r: 5´-ATTTCAGCCAATGAGGATGG-3´ as housekeeping gene, were selected for this study (Rogers and Barker, 2002). PCR

Table 1. The ratio of *APR1* gene to 18S rRNA in *Candida albicans* strains isolated from Malaysian and Iranian patients.

Subjects	Mean ratio of *APR1*/18S rRNA		
	All samples (Systemic and Non-systemic)	Systemic	Non-systemic
Malaysian isolates	0.5531 ± 0.24934	0.7256 ± 0.18820	0.3806 ± 0.17405
Malaysian controls	0.6764 ± 0.23264	0.8359 ± 0.16619	0.5168 ± 0.17541
Iranian isolates	0.2957 ± 0 .22586	0.4454 ± 0.23491	0.1460 ± 0.06401
Iranian controls	0.3805 ± 0.23511	0.5475 ± 0.20585	0.2134 ± 0.11039

Table 2. The ratio of *APR1* gene to 18S rRNA in *Candia albicans* strains isolated from mice blood infected by Malaysian and Iranian strains.

Subjects	Mean ratio of *APR1*/18S rRNA		
	All samples (Systemic and Non-systemic)	Systemic	Non-systemic
Malaysian isolates	0.7237 ± 0.28350	0.8671 ± 0.33535	0.5804 ± 0.14246
Malaysian controls	0.8484 ± 0.32187	1.0070 ± 0.36034	0.6899 ± 0.21170
Iranian isolates	0.5816 ± 0.19137	0.6341 ± 0.22326	0.5292 ± 0.16812
Iranian controls	0.7427 ± 0.29261	0.8389 ± 0.34639	0.6465 ± 0.23473

amplification was carried out in a final volume of 25 µL containing PCR buffer, $MgCl_2$, dNTP, reverse primer, forward primer, template cDNA (0.1 ng), Taq polymerase (0.2 units) and distilled water. The best annealing temperature was considered 55.5°C for both *APR1* and 18S rRNA genes.

Agarose gel electrophoresis

In order to show the cDNA bands of *APR1* and 18S rRNA genes, the PCR products were run on 1.5% (w/v) agarose gel. A 1 kb DNA ladder (Fermentas) was used as marker at this stage.

Statistical analysis

The analysis of data was carried out using the statistical package for social sciences (SPSS, version 16). Differences were considered significant at $p < 0.05$. The independent t-test was performed to determine the difference in the mean of *APR1* gene expression between the groups studied.

RESULTS

APR1 gene expression was evaluated in both clinical and control strains of *C. albicans* using RT-PCR method. The "Lab Works 4.6 Acquisition and Analysis Program CD" software program was used for determining the density of gene band on agarose gel and then the ratio of the *APR1* gene to 18S rRNA gene expression was calculated (Tables 1 and 2). The statistical results showed significant differences in *APR1* gene expression in *C. albicans* isolates between Malaysian and Iranian isolates as well as between Malaysian and Iranian controls ($p < 0.05$), while there was no significant difference in gene expression of *APR1* in *C. albicans* strains isolated from mice blood infected by *C. albicans* obtained from Malaysian and Iranian patients ($p > 0.05$).

Based on statistical analysis concerning the clinical and control strains of *C. albicans* isolated from both Malaysian and Iranian patients, there was a significant difference in the ratio of *APR1* gene to 18S rRNA between *Candida* cells obtained from systemic and non-systemic samples ($p < 0.05$), but this difference was not found in *C. albicans* isolated from mice blood ($p > 0.05$). The results of *APR1* and 18S rRNA gene expressions from clinical isolates were illustrated in Figures 1 and 2, respectively. As observed, the *APR1* and 18S rRNA bands were appeared on the agarose gel after electrophoresis of the clinical isolates of *C. albicans* obtained from human patients and mice blood. The locations of the *APR1* gene and the 18S rRNA gene bands were detected about 220 and 302 bp, respectively.

DISCUSSION

Vacuole aspartic proteinase A enzyme is expressed by *APR1* gene and it is one of the important factors in the development of systemic candidiasis caused by *C. albicans* (Niimi et al., 1997). In this study, *APR1* gene expression was evaluated using RT-PCR in clinical and control strains of *C. albicans* isolated from Malaysian and Iranian patients and mice blood.

Our results showed that *APR1* gene expression in clinical and control strains of *C. albicans* isolated from human patients in Malaysians was higher than that of Iranians. In addition, *APR1* gene expression in systemic samples was significantly higher than non-systemic samples ($P < 0.05$). Little is known about vacuolar aspartic proteinase from opportunistic *C. albicans*. However, a gene *CAP-RA* encoding an aspartic

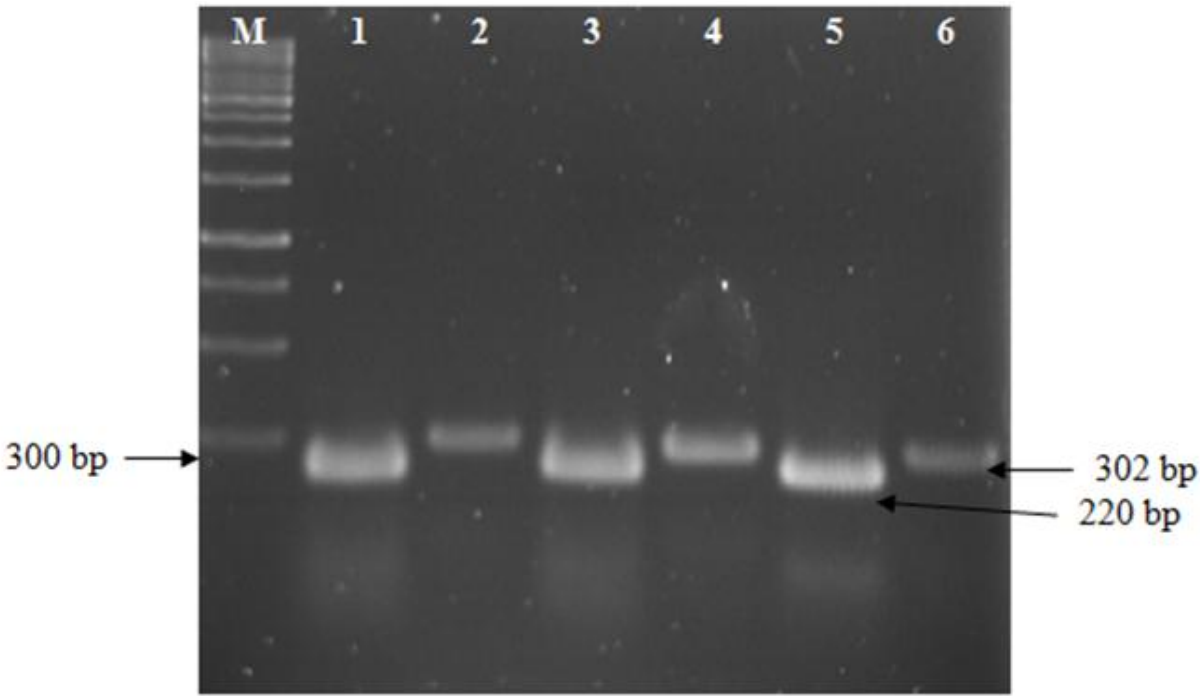

Figure 1. Gel electrophoresis of *APR1* and 18S rRNA genes in clinical and control strains of C*. albicans* obtained from Malaysian and Iranian individuals. - M: 1kb DNA Marker. - 1, 3 and 5 strains: *C. albicans APR1* gene. 1-CLA 101, Malaysian clinical strain *APR1* gene. 3- CLA 109, Malaysian clinical strain *APR1* gene. 5-CLA 204, Malaysian clinical strain *APR1* gene. - 2, 4 and 6 strains: *C. albicans* 18S rRNA gene. 1-CLA 101, Malaysian clinical strain 18S rRNA gene. 3-CLA 109, Malaysian clinical strain 18S rRNA gene. 5-CLA 204, Malaysian clinical strain 18S rRNA gene.

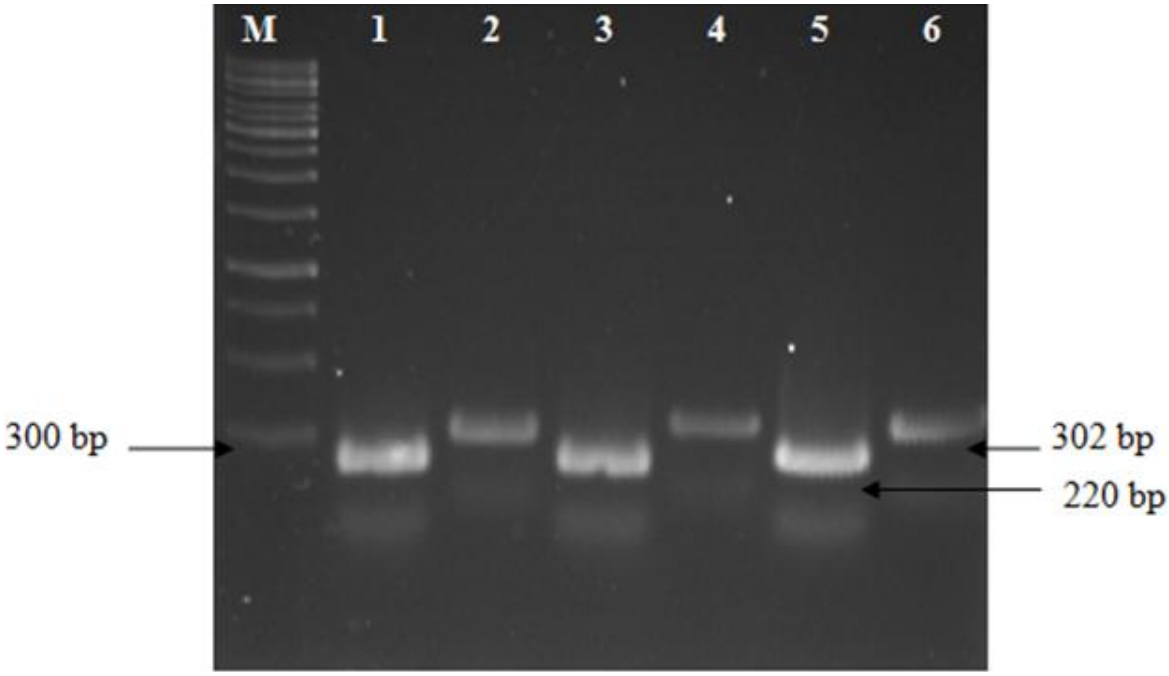

Figure 2. Gel electrophoresis of *APR1* and 18S rRNA genes in clinical and control strains of *C. albicans* obtained from mice blood infected by Malaysian and Iranian strains. - M: 1 kb DNA Marker. - 1, 3, 5 strains: *C. albicans APR1* gene 1- CLA 101, Malaysian clinical strain *APR1* gene. 1- CLA 109, Malaysian clinical strain *APR1* gene. 3- CLA 204, Malaysian clinical strain *APR1* gene. - 2, 4 and 6 strains: *C. albicans* 18S rRNA gene. 1-CLA 101, Malaysian clinical strain 18S rRNA gene. 1- CLA 109, Malaysian clinical strain 18S rRNA gene. 3- CLA 204, Malaysian clinical strain 18S rRNA gene.

proteinase has been cloned by Lott et al. (1989) and a similar gene, *APR1*, has been cloned by Cannon et al. (1992). *APR1* in *C. albicans* is unlike the secreted aspartic proteinase (SAP) family (Monod et al., 1994), and encodes a vacuolar aspartic proteinase. As the deduced mature amino acid sequence of *APR1* shares a high identity to proteinase A (Woolford et al., 1986; Vilanova et al., 2004), *APR1* could play a proteinase A-like function in *C. albicans*. All microorganisms, including pathogenic fungi, have to express the necessary genes for optimal growth or viability in order to adapt to changes in environmental conditions. So the range of differential gene expression depends on environmental conditions such as accessible nutrients, external pH, oxygen supply or the challenge of particular host defenses such as antimicrobial peptides or leukocytes (Fradin et al., 2003; Inan et al., 2004). Enjalbert et al. (2003) described that each type of environmental stresses, including thermal, osmotic, and oxidative stresses, were responded to about 100 specific genes in *C. albicans*. Some of these transcriptional induction genes are responsible for the production of enzymatic proteins that have an important role in the protection of cells.

Our results demonstrated an increase in *APR1* gene expression of *C. albicans* from mice blood when compared to strains isolated from human patients. In contrast, there was no significant difference in *APR1* gene expression between Malaysian and Iranian strains isolated from mice blood. Fradin et al. (2003) expressed that when an infection was created by microorganisms injected into the animal model, an increase of expression of genes involved in protein synthesis was observed. They described that the increase of protein synthesis is due to adaptation of cells in new conditions. Access to an energy source and nutrients are two important factors of protein synthesis and cell growth in blood. In reference to this point, *C. albicans* is an aerobe, so after entering the blood under microaerobic or semi-aerobic environments, it is involved with oxidative stress in gene expression. It was observed that four kinds of genes were involved in the presence of *C. albicans* in the blood including protein biosynthesis, stress response, hyphal production and carbohydrate metabolism (Heitman et al., 2006).

In the present study the clinical and control strains of *C. albicans* showed an increase in amounts of proteinase A and enzyme activity upon entering a new environment. The amount of this enzyme increased in all samples after injection into the mice. This showed that the *Candida* cells were adapted to the new condition to survive. It was also found that the intracellular proteinase A enzyme is necessary to adapt the new environments so that reduction in this enzyme and other enzyme disorders can lead to cell death (Noble and Johnson, 2007). The amount of the proteinase A enzyme increased when *C. albicans* cells were under nutritional or oxygen conditions in the blood. This showed the importance of the proteinase A enzyme in *C. albicans* survival and focused the level of *APR1* gene expression in *C. albicans* in the environmental situation (D'Enfert and Hube, 2007).

In conclusion, the clinical and control strains of *C. albicans* isolated from Malaysian and Iranian subjects with different geographical situation indicated some changes in the levels of *APR1* gene expression and virulence. In addition, differential *APR1* gene expression was observed between systemic and non-systemic strains as well as between mice blood infected by Malaysian and Iranian *C. albicans* isolates.

ACKNOWLEDGMENTS

This study was funded by Research Councils of University of Putra, Malaysia and University of Tehran, Iran.

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