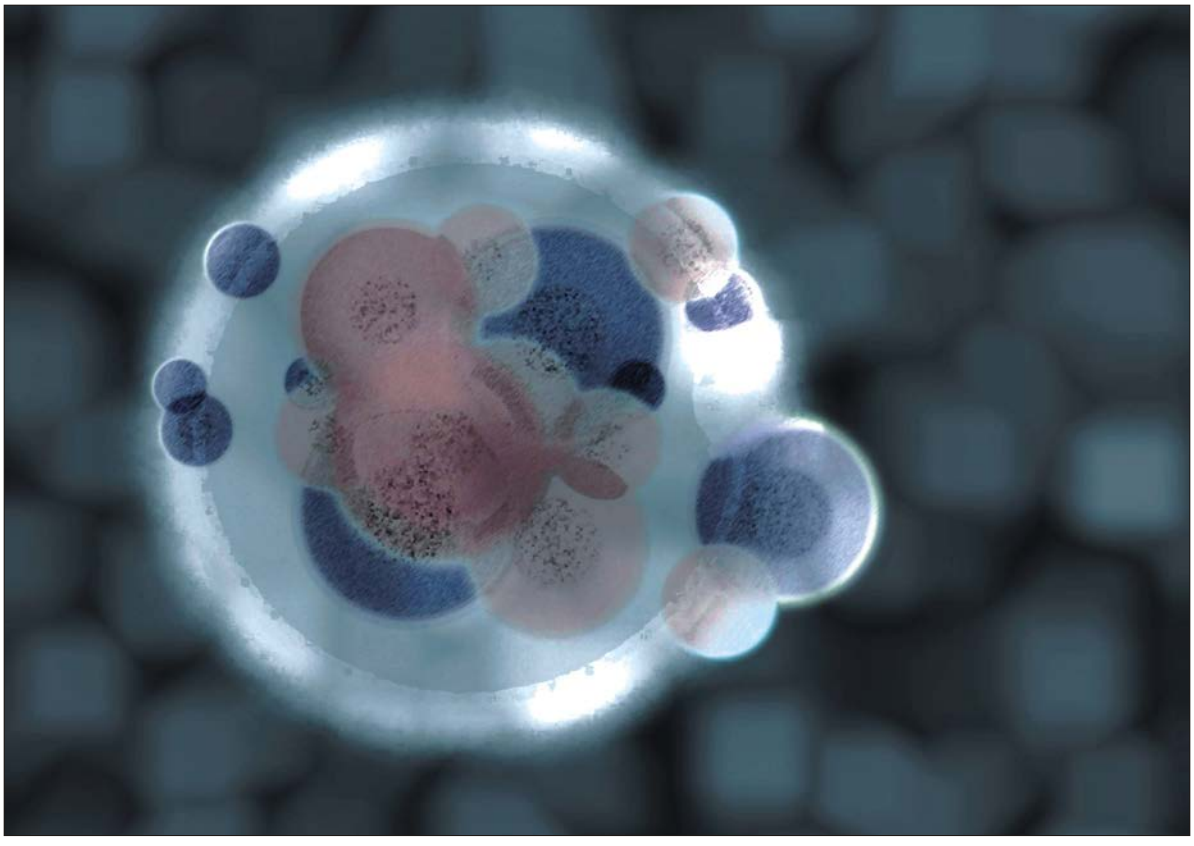

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Characterization of the Similarity of Protein Patterns and Virulence of Clinical *Candida albicans* Isolates

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Abstract: The aim of the study was to evaluate the protein similarity degree among 15 *C. albicans* isolates with different virulence obtained from healthy and infected human and animals. Yeast cells were grown in YPG medium and collected by centrifugation. After cell wall disruption, the cell wall and cytoplasmic proteins were submitted to PAGE and SDS-PAGE techniques. Similarity degree was determined using the Dice similarity coefficient (S_D). *Candida albicans* isolates showed high similarity together with average 86.2% ($78.1<S_D<100$). Regarding to the virulence and host aspects, the means S_D of high virulent and low virulent isolates as well as human and animal isolates were 85.8 and 85.6% in PAGE, respectively. Considering SDS-PAGE, electrophoregrams of *C. albicans* cytoplasmic proteins of low and high virulent isolates as well as human and animal isolates showed the similarity about 90%. The mean S_D of cell wall was 91.9% between low virulent and high virulent isolates and 92.2% between human and animal isolates. Such results suggest a highly protein similarity degree among the most isolates with low and high virulence as well as from human and animal hosts. The whole-cell protein profile obtained by these techniques could provide additional criteria for the serologic and immunologic studies of *C. albicans*.

Key words: *Candida albicans*, protein profiles, SDS-PAGE, PAGE, dice similarity coefficient

INTRODUCTION

Candida albicans is an opportunistic fungal pathogen that is the principal cause of superficial and systemic candidiasis in human and animals. The antigenic composition of *C. albicans* appears to be very complex and it includes proteins, polysaccharides and glycoproteins (Chaffin *et al*., 1998). Identification of immunodominant antigens or those antigens expressed exclusively in a pathogenic situation may be of relevance for serodiagnosis of severe candidiasis (López-Ribot *et al*., 2004; Elguezabal *et al*., 2005). Different types of electrophoretic techniques have been used for the characterization or typing of *C. albicans* isolates including separation of chromosomes, DNA fragments, isoenzymes, cell wall glycoproteins and whole cell proteins (Asakura *et al*., 1991; Rustchenko, 2007). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) has been employed to analyze the constituents of soluble extracts obtained from intact fungal cells and those of isolated cell walls. This technique showed high specificity in addition to the significant data for classification (Rodrigues *et al*., 2004). Many investigators employed electrophoretic analysis of whole-cell proteins in the fungi taxonomy (Höfling *et al*., 2001). In this regard, several studies have reported the cytoplasmic and cell wall proteins of *C. albicans* (Ashman *et al*., 1990; Ishiguro *et al*., 1992). In a previous study, electrophoretic protein profiles on different slab gels gave the mean value of S_D approximately 89.7% in 75 *C. albicans* isolates from the oral cavities of subjects (Boriollo *et al*., 2003). The comparison of electrophoretic protein patterns has been considered as a method with satisfactory taxonomic resolution, which can be applicable to the level of species, subspecies and biotypes (Caugant and Sandven, 1993). In this study, we described the use of SDS-PAGE, along with PAGE techniques, to analyze the similarity of the protein profiles of the cytoplasmic extracts and cell walls of different *C. albicans* isolates obtained from different host conditions (human against animal isolates and patient against healthy subject isolates) with various virulence degrees.

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MATERIALS AND METHODS

Culture of *C. albicans* isolates: Seventeen fresh *C. albicans*, originated from infected human (7 isolates) and animals (10 isolates), were obtained from Fungal Collection of Mycology Research Center, University of Tehran between April and November, 2007. All strains were recharacterized and identified using chlamydospore formation, germ tube test, CHROM agar, β-glucosidase test, sugar fermentation and assimilation tests as major taxonomic criteria (Zaini *et al*., 2006). All chemicals used, unless otherwise stated, were obtained from Merck Company (Darmstadt, Germany).

Determination of *C. albicans* virulence: One hundred-seventy, 10-week-old male, BALB/c mice were purchased from Razi Institute (Karaj, Iran). Animals were divided into 17 equal groups, kept in cages and fed under specific pathogen-free conditions. For experimental systemic infection, yeasts were grown in YPG broth (2% glucose, 2% peptone, 1% yeast extract) at 35°C for 48 h on a shaker at 150 rpm (New Brunswick Scientific Co., Inc., UK), subsequently washed 3 times with 10 mM phosphate-buffered saline (PBS, pH 7.2), counted by a hemocytometer and adjusted to 2×10^6 cell per ml. Of these suspensions, 0.5 mL containing 1×10^6 yeast cells was injected intravenously into mice of each group. Survival was monitored over a period of 60 days and was expressed as the number of dead animals over the total number of infected animals during 10 days (mortality rate) and as mean survival time in days. Subsequently, the isolates were categorized into two groups including high virulent and low virulent isolates according to mean survival time; isolates with mean survival time lower than that of all isolates were considered as high virulent isolates and isolates with mean survival time higher than that of all isolates were considered as low virulent isolates.

Preparation of antigenic extracts: All strains were grown in 200 mL of YPG medium at 30°C for 48 h on a shaker at 140 rpm. After growth, the cells were harvested by centrifugation at 3000 g for 5 min and the pellets were washed 3 times with sterile distilled water in order to remove either culture medium traces or extra-cellular metabolites. The yeast cells, breaking buffer (62.5 mM Tris-HCl, 15% Glycerol, 1 mM Dithioteritol and 0.2 mg mL^{-1} PMSF, pH 7.2) and glass beads (1 mm in diameter) in proportions of 1:2:2 were transferred to glass tubes, shook at 2800 rpm (15 times of 1 min at 5 min intervals) and placed in an ice bath until yeast cells were disrupted about 80-90%. After cell disruption, the cell walls were separated from other cell components by centrifugation at 3000 g for 20 min, washed 3 times with sterile distilled water containing 0.2 mg mL^{-1} PMSF and stored at -20°C until used. For the isolation of cytoplasmic extract, disrupted cells extract was centrifuged at 105000 g for 60 min, subsequently the supernatant was isolated and stored at -20°C until used.

Determination of protein: Protein concentrations of obtained samples of the cell wall and cytoplasmic extracts were determined by Lowry *et al.* (1951) method.

PAGE and SDS-PAGE techniques: PAGE and SDS-PAGE protein profiles were obtained using the method of Laemmili (Laemmili, 1970). In PAGE, the yeast cell wall and cytoplasmic extracts were separated in a 5-15% gradient separation gel with a 5% stacking gel, whereas in SDS-PAGE, the above samples (containing 15 μg proteins) were loaded onto poly acrylamide slab gel with SDS in a discontinuous buffer system with a 8-20% (for cytoplasmic extract) and 8-15% (for cell wall) gradient separating gel and 5% stacking gel and subjected to electrophoresis at 120 volts. The gels were stained with silver nitrate (Sigma, St. Louis, USA). Protein standards (Sigma, St. Louis, USA) used for estimation of molecular weight was: Bovine Albumin: 66 kDa, Egg Albumin: 45 kDa, Bovine Pancreas Trypsinogen: 24 kDa, β-Lactoglobulin: 18.4 kDa, Lysozyme: 14.3 kDa. In this research, the Dice similarity coefficient (S_D) was used to obtain the degree of similarity.

Statistical analysis: The Chi-square (χ^2) and t-test were used to assess statistical relationship between the groups. Probabilities of 5% were taken to be statistically significant.

RESULTS

Virulence of *C. albicans* isolates: The comparative results revealed that there were considerable variations within the *C. albicans* isolates in relation to mean survival time and the mortality rate. The survival time ranged from 1 to 45 days, mean time 13.4 days. Of 17 isolates, 15 were virulent due to the mortality more than 50%, 5 in low and 10 in high virulent groups. The mean survival times of low virulent and high virulent isolates were calculated approximately 23.52 and 8.17 days, respectively, representing significant difference between two groups ($p<0.0001$). In addition, there were no significant differences between the virulence of human and animal isolates as well as between commensal and patient isolates, although both high virulent and low virulent isolates were found within human and animal isolates.

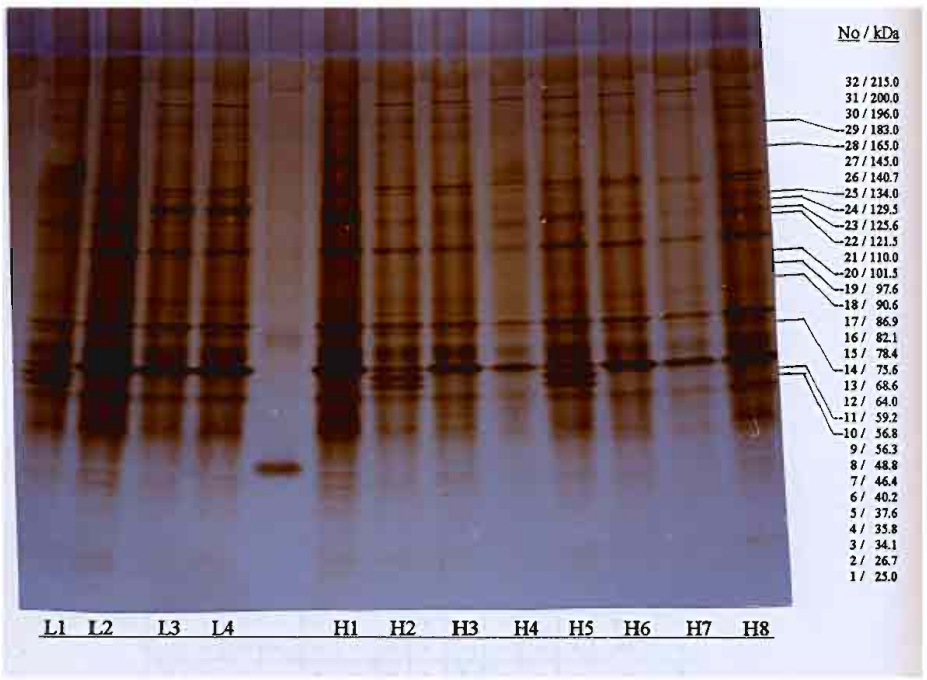

Fig. 1: Protein pattern of cytoplasmic extracts of *Candida albicans* isolates in gradient PAGE (5-15%, silver nitrate staining). Low virulent isolates (L_1-L_4), High virulent isolates (H_1-H_8), Human isolates (L_1, L_4, H_2-H_3, H_5), Animal isolates (L_2-L_3, H_1, H_4, H_6-H_8), Healthy host isolates (L_4, H_6, H_8), Infected host isolates (L_1-L_3, H_1-H_5, H_7)

Table 1: Dice similarity coefficient for electrophoretic pattern of cytoplasmic extracts of different *Candida albicans* isolates in gradient PAGE (5-15%)

Isolate	Similarity mean	Similarity range	Difference mean	Difference range
	(%)			
All isolates	86.2	78.1-100	13.8	0-21.9
Low virulent isolates	83.3	81.2-87.5	16.7	12.5-18.8
High virulent isolates	87.4	78.1-100	12.6	0-21.9
Low and high virulent isolates	85.8	78.1-100	14.2	0-21.9
Human isolates	88.1	81.2-100	11.9	0-18.8
Animal isolates	86.3	78.1-100	13.7	0-21.9
Human and animal isolates	85.6	78.1-100	14.4	0-21.9

Electrophoregrams of cytoplasmic extract and cell wall of *C. albicans*: In electrophoresis performed, no protein band related to the cell wall was observed in PAGE, whereas the cytoplasmic extracts showed 32 major bands, within a range molecular weight varying between 25 and 215 kDa (Fig. 1). The relative mobility of proteins on gel was identified as numbers 1 to 32. The apparent differences were observed within low and high virulent isolates, which were associated with bands of 10, 11 and 12 with approximate weights of 56.8, 59.2 and 64 kDa, respectively. Overall, the isolates were different in 12 bands, whereas the other bands were common in all isolates. According to Fig. 1, no specific band was observed in high virulent and low virulent isolates. Dice similarity coefficient (S_D) was used for calculation of the similarity degree of protein patterns of isolates. If S_D for two isolates were more than 95%, the patterns would be considered as similar pattern. Regarding to the results of Table 1, the tested isolates had the S_D approximately 86.2% together with the range of 78.1 to 100 %. The mean similarity of electrophoregrams of high virulent with low virulent isolates was 85.8%, representing no significant difference with all isolates (86.2%) in PAGE electrophoretic patterns. Also, the mean S_D of low virulent (83.3%) and high virulent isolates (87.4%) was to some extent similar in electrophoregrams. In addition, some isolates with different virulence had completely similarly patterns (L_4 with H_1 and H_8). As shown in Fig. 1, human and animals isolates of *C. albicans* showed different electrophoregrams, but there was not specific band in each group to differentiate human isolates from animal isolates. The results showed that the S_D of electrophoregrams of human and animal isolates varied from 78.1 to 100% (median 85.6%). Also, the mean

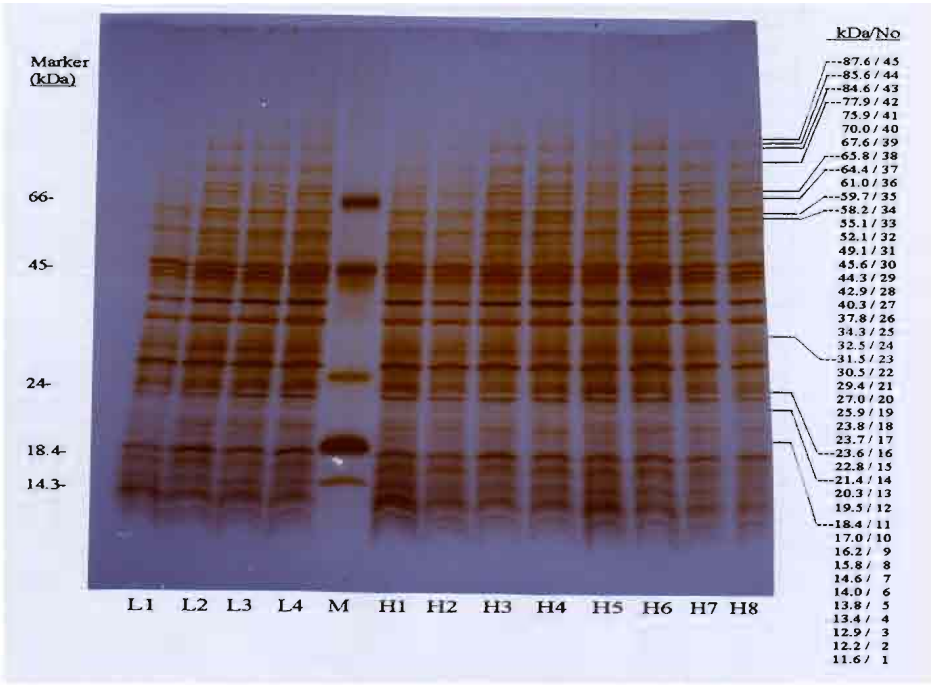

Fig. 2: Protein pattern of cytoplasmic extracts of *Candida albicans* isolates in gradient PAGE (8-20%, silver nitrate staining). Low virulent isolates (L_1-L_4), High virulent isolates (H_1-H_8), Human isolates (L_1, L_4, H_2-H_3, H_5), Animal isolates (L_2-L_3, H_1, H_4, H_6-H_8), Healthy host isolates (L_4, H_6, H_8), Infected host isolates (L_1-L_3, H_1-H_5, H_7), Marker (M)

Table 2: Dice similarity coefficient for electrophoretic pattern of cytoplasmic extracts of different *Candida albicans* isolates in gradient SDS-PAGE (8-20%)

Isolate	Similarity mean	Similarity range	Difference mean	Difference range
	(%)			
All isolates	90.2	75.6-100	9.8	0-24.4
Low virulent isolates	86.7	75.8-97.8	13.3	2.2-24.2
High virulent isolates	91.2	84.4-100	8.8	0-15.6
Low and high virulent isolates	90.0	75.6-100	10.0	0-24.4
Human isolates	86.2	77.8-100	13.8	0-22.2
Animal isolates	92.4	84.4-97.8	7.6	2.2-15.6
Human and animal isolates	90.0	75.6-100	10.0	0-24.4

similarity of human (88.1%) and animal isolates (86.3%) was similar in electrophoretic patterns. There were no specific bands in electrophoregrams of *C. albicans* isolates originating from healthy and infected hosts as well.

In SDS-PAGE electrophoregrams, at least 45 protein bands (with No. 1-45) were observed in cytoplasmic extract of isolates with molecular weights ranging 11.6 and 87.6 kDa (Fig. 2). Some isolates showed the S_D more than 95%. The mean S_D of low virulent (86.7%) and high virulent isolates (91.2%) was nearly similar (S_D between two groups: 90). Human and animal isolates of *C. albicans* showed different electrophoregrams and there was no specific band in each group. The gel showed a high degree of the similarity between the protein banding patterns of the animal and human isolates as well as healthy and patient isolates (Table 2). Concerning cell walls of *C. albicans*, SDS-PAGE analysis revealed the presence of proximately 42 distinct protein bands (with No. 1-42) with molecular weights ranging in size from 12.6 to 102 kDa (Fig. 3). The S_D of low virulent and high virulent isolates was 91.9% and that of human and animal isolates was 92.2%. Some high virulent isolates had similarity more than 95% against low virulent isolates. Electrophoregrams of human and animal isolates of *C. albicans* cell wall had the similarity about 92.6% with ranging from 81 to 100%. Some human isolates were completely similar to animal isolates (S_D >95%). The mean similarity of animal and human isolates was 93 and 92.8%, respectively. In summary, PAGE and SDS-PAGE patterns showed relative similarity degrees within *C. albicans* with different sources and host conditions (Table 3).

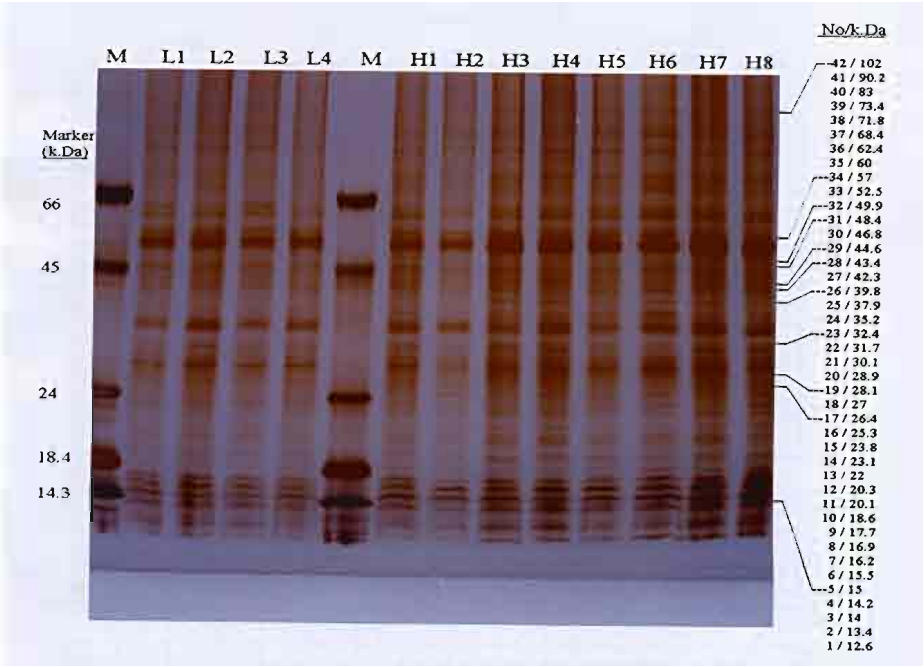

Fig. 3: Protein pattern of cytoplasmic extracts of *Candida albicans* isolates in gradient PAGE (5-15%, silver nitrate staining). Low virulent isolates (L_1-L_4), High virulent isolates (H_1-H_8), Human isolates (L_1, L_4, H_2-H_3, H_5), Animal isolates (L_2-L_3, H_1, H_4, H_6-H_8), Healthy host isolates (L_4, H_6, H_8), Infected host isolates (L_1-L_3, H_1-H_5, H_7), Marker (M)

Table 3: Dice similarity coefficient for electrophoretic pattern of cell walls of different *Candida albicans* isolates in gradient SDS-PAGE (8-15%)

Isolate	Similarity mean	Similarity range	Difference mean	Difference range
		(%)		
All isolates	92.6	81-97.6	7.4	2.4-19
Low virulent isolates	95.2	90.4-100	4.8	0-9.6
High virulent isolates	92.7	85.7-97.6	7.3	2.4 -14.3
Low and high virulent isolates	91.9	81-97.6	8.1	2.4 -19
Human isolates	92.8	85.7-97.6	7.2	2.4 -14.3
Animal isolates	93	88.1-100	7	0-11.9
Human and animal isolates	92.2	81-100	7.8	0-19

DISCUSSION

Polyacrylamide gel electrophoresis plays a major role in the experimental analysis of proteins and protein mixtures. This method is still the most widespread form of the technique and has been used to separate protein profiles from different yeast species in order to facilitate identification (Vancanneyt *et al*., 1991). To our knowledge, this was the first description of the utilization of the above method for determining the similarity degree of electrophoretic protein patterns of different *C. albicans* clinical isolates, a total of 15 isolates including 5 low virulent and 10 high virulent isolates, collected from the infected human and animals. At first, we determined the virulence degree of some clinical *C. albicans* isolates in BALB/c mice with systemic candidiasis. The mean survival times of infected mice were 5 and 26.2 days in the highest and lowest virulent isolates, respectively. Also, the mortality rates were recorded approximately 91% in high virulent isolates and 12% in low virulent isolates. These results showed that there were many differences in pathogenicity of clinical *C. albicans* isolates. Some evidences indicated differences in virulence within various strains of *C. albicans* using different models of systemic candidiasis (Antley and Hazen, 1988; Andaluz *et al*., 2001). Overall, studies on the virulence and experimental pathogenicity of *C. albicans* are few and somewhat controversial. These discrepancies are related to genetic variations of strains used due to long-term storage, medium type, temperature and pH of medium, animal model, patient hosts and site of infection, which could affect on virulence and pathogenicity of *C. albicans* (Schmidt and Geschke, 1996). In the next stage, the S_D of protein patterns of different *C. albicans*

isolates was determined. The electrophoregrams of *C. albicans* isolates showed several protein profiles in cytoplasmic extracts within a range of molecular weights varying between 11.6 -87.6 kDa in PAGE and 25-215 kDa in SDS-PAGE, showing the relative similarity when visually compared. The protein electrophoretic fingerprinting showed that protein bands between 25-86.9 kDa in PAGE and 11.6-55.1 kDa in SDS-PAGE are repeated in the majority of *C. albicans* isolates, suggesting that they may be representative of the genus. These results are in relative agreement with the values reported in previous studies (Vancanneyt *et al*., 1999; Rosa *et al*., 2000). Identification of a protein in a complex antigenic extract on the basis of its molecular weight is difficult and sometimes the same antigen may have small differences in the molecular weight in different studies. These differences observed in the molecular weights of different studies may be related to differences in the calculation of the molecular weight, medium composition and incubation temperature or to the presence of several antigens with the same molecular weight (Burt *et al*., 1999). Present findings demonstrated that *C. albicans* isolates had the S_D approximately 86.2% ($78.1<S_D<100$). The mean S_D of high virulent and low virulent isolates as well as human and animal isolates was 85.8 and 85.6%, respectively, representing no significant difference with all isolates (86.2%) in PAGE. In addition, the mean S_D of cell wall and cytoplasmic extracts was 90% in both low virulent and high virulent isolates and in human and animal isolates in SDS-PAGE. Electrophoregrams of *C. albicans* cell walls of low virulent and high virulent isolates and human and animal isolates had the similarity about 91.9 and 92.2%, respectively. Boriollo *et al.* (2003) revealed that electrophoretic protein profiles on different slab gels gave the mean value of S_D approximately 89.7% in 75 *C. albicans* isolates from the oral cavities of subjects. In similar study by Rodrigues *et al.* (2004), the similarity of the electrophoretic whole cell protein patterns among *C. albicans* samples showed values among 80% and 100%. Common and frequent mechanisms involved in the diversity of *Candida* species could explain this similarity. These include chromosomal rearrangements, chromosomal alteration and complex and unknown gene regulations (Rustchenko-Bulgac *et al*., 1990; Rustchenko *et al*., 1994). In the present study, electrophoregrams obtained from healthy and infected subjects showed identical protein patterns. *Candida albicans* can be carried as commensal organisms and it has been showed that at least two-thirds of healthy subjects carry this microorganism in their natural microflora (Caugant and Sandven, 1993). Serotyping, electrophoretic karyotyping and DNA restriction fragment length polymorphism studies have shown that isolates recovered from hosts with different conditions are usually identical (Asakura *et al*., 1991; Brawner *et al*., 1992). Interestingly, no protein band related to the cell wall was observed in PAGE. Several researchers have suggested that nearly all proteins in the outer layers of the *C. albicans* cell wall are attached to large mannan molecules and that disulfide bonds within or between some of these proteins or both are important in maintaining the structure of this latticework. Therefore, this complex array of proteins cannot be extracted in PAGE method and not be appeared in gel. In summary, differential analysis of *C. albicans* based on electrophoretic protein patterns may provide preliminary criteria for taxonomic and epidemiological studies of such yeast.

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