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Full Length Research Paper

Numerical analysis of whole-cell and cell wall proteins' profiles of human oral cavity *Candida* isolates

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Protein electrophoresis has been of great value for the delineation of fungi taxonomy. The aim of this study was to evaluate the protein polymorphism degree among thirteen oral *Candida* strains belonging to six species and isolated from adult patients submitted to the Dental Hospital of Monastir (Tunisia) and suffering from denture stomatitis using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in order to identify the similarities in oral isolates and to establish their possible dissemination route. Proteins were extracted using two different protocols (cell wall and whole-cell proteins) and then submitted to SDS-PAGE technique. After electrophoresis, the protein bands were stained with Coomassie-blue and analyzed using the Gel Pro Analyzer (3.1). Dendrograms of each proteins gel were generated by the unweighted pair group arithmetic average (UPGMA 4.0) software package. A total of 30 major phenons (clusters) were analyzed, according to their homogeneous (species and period) and heterogeneous (distinct species and/or period) characteristics when we analyzed the cell wall proteins profiles and also 30 major phenons of whole-cell proteins patterns. The protein profile of each *Candida* isolate using two different methods was patient-specific and not associated with the date of isolation or the cell surface hydrophobicity (CSH) of the strain.

Key words: *Candida*, cell wall proteins, whole-cell proteins, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), hydrophobicity.

INTRODUCTION

Candida species are a common opportunistic pathogen fungal in humans which causes mucosal infection (Odds and Bernaerts, 1994). Human pathogenic *Candida* strains isolated from different clinical sources are increasingly responsible for hospital outbreaks in many countries around the world (de Brito et al., 2003; Saunte et al., 2005). This yeast is also the principal etiological agent of oral candidal infection (Nikawa et al., 1998; Webb et al., 1998). In fact, *Candida* strains have been isolated from 93% of patients with denture stomatitis (Budtz-Jorgensen et al., 1975) which are now considered to be the commonest form of oral candidosis. Oral cavity is a flow environment, where *Candida* cells are mixed with saliva and can adhere and grow (Cannon and Chaffin, 1999). Although, the strongest mechanism for adherence involves a mannoproteins adhesin on *C. albicans*, cell surface hydrophobicity (CSH) has been described by many investigators as involved in adherence (Hazen, 1989). Hydrophobic proteins, however, embedded in the matrix of the *C. albicans* cell wall beneath the fibrillar layer; provide the hydrophobic

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interactions needed to turn this initial attachment between the fungus and the surface into a strong bond (Hazen, 1989).

Protein electrophoresis has been of great value for the delineation of fungi taxonomy. In fact, different types of electrophoretic techniques have been used for the characterization or typing of *Candida* species including separation of chromosomes, DNA fragments, cell-wall glycoproteins and whole-cell proteins (Maiden and Tanner, 1991; Monod et al., 1990). Also, several investigators employed electrophoretic analysis of whole-cell proteins in the fungi taxonomy (Vancanneyt et al., 1992; Höfling et al., 2001).

Different physical, chemical and enzymatic techniques and a combination of them have been used to extract cell wall components of *C. albicans*. The choice of extraction techniques may affect both qualitative and quantitative solubilization of cell wall components (Fleet, 1991).

The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has also been used in the identification of oral yeasts. This technique showed high specificity in addition to the significant data for classification (Höfling et al., 2001). In fact, the comparison of electrophoretic proteins patterns offer considerable potential for typing strains of clinical interest and for taxonomic purposes, especially for the level of species, subspecies and biotype (Kersters, 1985).

Regarding the major components of cell wall of *Candida* species, mannoproteins are considered as the most important antigenic component composing 10-30% of the cell wall (Elorza et al., 1988). This group of proteins is mainly composed of carbohydrate polymannose containing more than 150 strongly bonded mannosyl units. Solubilization of cell wall materials using chemicals such as mercaptoethanol (ME) has usually been used in order to know its compounds (Chaffin and Stocco, 1983; Mormenco et al., 1996).

The aim of the present investigation was to analyze the similarity levels of whole-cell proteins and cell wall proteins profiles among *Candida* strains isolated from some adult patients frequenting the Dental Hospital of Monastir (Tunisia) during 2 years and suffering from denture stomatitis using SDS-PAGE in order to identify the similarities in oral isolates and to establish their possible dissemination route.

MATERIALS AND METHODS

Yeast isolates

A total of 30 *Candida* strains consisting of 18 *C. albicans*, 5 *C. parapsilosis*, 3 *C. kefyr*, 2 *C. glabrata*, 1 *C. holmii* and 1 *C. sake* collected from patients admitted to the Dental Hospital of Monastir (Tunisia) and Department of parasitology of Farhat Hached hospital (Sousse, Tunisia) were included in this study (Table 1). The type-strains *C. albicans* ATCC 90028, *C. albicans* SC 5314, *C. parapsilosis* ATCC 22019, *C. parapsilosis* CECT 13011 and *C. parapsilosis* J 981226 were used in this study.

Growth conditions

All samples were cultured on Sabouraud chloramphenicol agar (Bio-rad, Marnes-La-Coquette, France) for 48 h at 30°C. Biochemical identification of *Candida* species was tested on the base of carbohydrates assimilation using ID 32 C system (bio-Mérieux, Marcy l'Étoile, France) according to manufacture instructions.

Cell surface hydrophobicity (CSH)

The hydrophobicity of *Candida* strains were measured according the protocol described by Rosenberg et al. (1983) which consists of measuring the adherence of yeast to hydrocarbons, such as cyclohexane or xylene.

The tested strains were grown overnight in 5 ml of yeast extract peptone dextrose (YPD) or yeast nitrogen base (YNB) broth at 28°C. Cells were washed with phosphate buffer saline (PBS) and concentrated to obtain a solution corresponding to OD_{600}=1. For adhesion assays, 3 ml of the cell suspension were mixed with 150 µl of cyclohexane or xylene in an acid-washed glass tube. The sample was vigorously mixed using vortex for 1 min. After 20-60 min at room temperature, the absorbance at 600 nm of the aqueous phase (A_1) was measured and compared with that obtained prior to the mixing procedure (A_0). The percentage of cells in the cyclohexane or xylene layer (adhered cells) was used to estimate hydrophobicity using the following formula:

[Percentage of cell adhesion= $(A_1/A_0) \times 100$].

All tests were run in duplicate. The results shown represent the mean of two consecutive experiments.

Whole-cell protein extraction

All *Candida* strains were grown overnight in 50 ml of YEPD medium (2% dextrose, 2% peptone, 1% yeast extract) in a shaker table under 150 rpm, at 30°C in order to obtain 10^8 cells/ml approximately (Asakura et al., 1991; Casanova and Chaffin, 1991). After growth, cells were harvested by centrifugation at 3000 g for 5 min and the pellets were washed three times in sterile water in order to remove either culture medium traces or extra-cellular metabolites (Woontner and Jaehning, 1990). The last washed pellets were mixed with 2 ml of phosphate buffer. Cells were lysed using sonication for four times of 1 min intervals, and placed in an ice bath for 30 s. After disruption, the proteins concentrations in the supernatant were determined according to Bradford (Eze and Dumbroff, 1982). 75 µl of supernatant and 25 µl of loading buffer (5 mM Tris, 2.5% 2-ME, 1.5% SDS, 0.025% bromophenol blue, 15% glycerol) were combined and heated in a boiling water bath for 10 min.

Cell wall protein extraction

Candida cell wall extraction

All *Candida* strains were grown overnight in 500 ml of YEPD medium under a rotation at 150 rpm at 30°C in order to obtain a maximum of cells culture. After growth, cells were harvested by centrifugation at 5000 g for 10 min at 4°C and the pellets were washed twice in sterile water. The last washed pellets were washed with 1 mM phenylmethylsulphony1fluoride (PMSF) phosphate buffer. Cells were lysed using glass beads for 10 to 12 times of 1 min intervals, and placed in an ice bath for 1 min. The supernatant representing the cell wall was washed again many times with PMSF

Table 1. Clinical characteristics of *Candida* isolates, hydrophobicity, and cell wall and whole cell proteins patterns diversity.

Strain	Hospital	Body site	Service	Date	Hydrophobicity (%)		Cell wall proteins pattern	Whole- cell proteins pattern
					X	C		
				C. albicans				
ATCC 90028	Type strain				88.4	84.9	II B	II B
SC 5314	Type strain				97.4	92	II B	II B
3^1	Dental hospital	Oral cavity	Odontology	03/2006	71.8	70.5	II B	II B
4	Dental hospital	Oral cavity	Odontology	03/2006	98	96.2	II B	II B
6	Dental hospital	Oral cavity	Odontology	04/2006	97.3	97.4	II B	II B
7	Dental hospital	Oral cavity	Odontology	04/2006	98.8	93	II B	II B
9	Dental hospital	Oral cavity	Odontology	06/2006	62.8	71.1	II A	II B
10	Dental hospital	Oral cavity	Odontology	07/2006	69.9	73.2	II B	II B
11	Dental hospital	Oral cavity	Odontology	07/2006	40.2	46.5	II B	II B
13	Dental hospital	Oral cavity	Odontology	09/2006	88.1	86.9	II B	II B
14	Dental hospital	Oral cavity	Odontology	02/2007	91	81.1	II B	II B
15_B	Dental hospital	Oral cavity	Odontology	02/2007	92.4	94.8	II B	II B
16	Dental hospital	Oral cavity	Odontology	04/2007	81.4	61.6	II B	II B
17*	Dental hospital	Oral cavity	Odontology	04/2007	90.7	79	II B	II B
17_R*	Dental hospital	Oral cavity	Odontology	04/2007	66.3	82.6	I	II B
18	Dental hospital	Oral cavity	Odontology	04/2007	81.6	82.3	II B	II B
21	Dental hospital	Oral cavity	Odontology	09/2007	77	72.7	II B	II B
65	Dental hospital	Oral cavity	Odontology	10/2007	95.1	74	II B	II B
%					82.68	79.96		
				C. parapsilosis				
ATCC 22019	Type strain				98.7	94.4	II B	II B
CECT 13011	Type strain				*	*	II A	II B
				C. orthopsilosis				
J 981226	Type strain				*	*	II A	II B
H_{11}	Farhat Hached	Oral cavity	Parasitology	03/2006	84.3	61.2	II A	II A
H_{12}	Farhat Hached	Oral cavity	Parasitology	03/2006	72.7	59	II A	II B
%					85.23	71.53		
				C. glabrata				
8	Dental hospital	Oral cavity	Odontology	05/2006	72.1	63.3	II A	II B
15_T	Dental hospital	Oral cavity	Odontology	02/2007	80.8	92.2	II A	I
%					76.45	77.75		
				C. kefyr				
1	Dental hospital	Oral cavity	Odontology	03/2006	55.6	81.8	II A	II A
12	Dental hospital	Oral cavity	Odontology	08/2006	90.9	75.3	II B	II B
35	Dental hospital	Oral cavity	Odontology	09/2007	90.7	75.1	II B	II B
%					79.07	77.4		
				C. holmii				
2	Dental hospital	Oral cavity	Odontology	03/2006	74.3	53.7	II B	II A
				C. sake				
3^2	Dental hospital	Oral cavity	Odontology	03/2006	40.2	43	II B	II B

(1 mM) by centrifugation at 3000 g for 10 min. The pellet finished by weighting.

Protein extraction

Proteins on the cell wall of *Candida* species were isolates with SDS. For this, the generated pellet was added with SDS 2% and boiled for 10 min. After boiling, SDS extracts were obtained by centrifugation at 3000 g for 10 min and the pellets consist on SDS-extracts.

The proteins concentrations were determined according to Bradford protein assay procedure (Eze and Dumbroff, 1982). Twenty microlitres (20 µl) of SDS-extracts and 5 µl of loading buffer (5 mM Tris, 2.5% 2-ME, 1.5% SDS, 0.025% bromophenol blue, 15% glycerol) were combined and heated in a boiling water bath for 10 min.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) protein separation

Proteins were separated using electrophoresis with polyacrylamide vertical gels as described by Laemmli (1970). The separating gels of polyacrylamide were used at a concentration of 10% (relation of acrylamide: bisacrylamide of 30:0.2). The concentrating gels (upper part) were prepared with a concentration of acrylamide of 4%.

Protein sample (30 µg of proteins) was mixed with 10 µl of a solubilization solution (0.25 M Tris-HCl pH 6.8, 20% glycerol, 10% SDS, 4% β-mercaptoethanol, 0.4% bromophenol blue) and boiled for 10 min in order to denaturalize the proteins. The buffer used for the electrophoresis was composed by 25 mM Tris-HCl pH 8.3, 0.1% SDS and 0.2 M glycine. The migration was first let at 120 V in order to concentrate the proteins and then switched to 160 V for the separation.

After the proteins separated on polyacrylamide gels, they were colored at room temperature for 1 h with 0.2% Coomassie blue in methanol/acetic acid/sterile water (30:7.2:30; v/v) with soft shaking. The excess of dye was eliminated washing the gel several times with methanol/acetic acid/sterile water (25:7:68; v/v).

Statistical analysis

The images of the gels were captured using a gel capture system (Bio-rad, France) and the molecular weight of each protein bands was determined by Gel Pro Analyzer (3.1). Presence or absence of protein bands received the numbers 1 and 0, respectively. These data allowed building matrixes of binary values which were analyzed using the Gel Pro Analyzer (3.1). Dendrograms of each proteins gel were generated by the unweighted pair group arithmetic average (UPGMA 4.0) software package (Sneath and Johnson, 1972). The type-strains of each *Candida* species (*C. albicans* ATCC 90028 and SC 5314) and (*C. parapsilosis* ATCC 22019, CECT 13011 and J 981226) and molecular weight markers (PageRuler™ Plus Pre-stained Protein Ladder 250 kDa and Pre-stained Protein Molecular Weight Marker 120 kDa-Fermentas) were used in order to determinate the real seize of the band and to establish the degree of similarity among *Candida* strains (Vancanneyt et al., 1991).

RESULTS AND DISCUSSION

The results of measuring the adherence of *Candida* cells to liquid hydrocarbons (Xylene and cyclohexane) are summarized in Table 1. The two tested hydrocarbons were able to bind *Candida* cells with different degrees function of the species. In fact, the optimal adherence was observed in the presence of xylene: 82.68% for *C. albicans*; 85.23% for *C. parapsilosis*; 76.45% for *C. glabrata*; 79.07% for *C. kefyr*; 74.3% for *C. holmii* and 40.2% for *C. sake*. Adherence to cyclohexane was lower than that observed for xylene. This sequence of affinity for the tested hydrocarbons has been observed with the six tested *Candida* species especially for *C. holmii* (74.3% for xylene and 53.7% with cyclohexane) and may be related to the relatively high viscosity of cyclohexane. Our results showed that *C. albicans* is the most hydrophobic specie using xylene and cyclohexane as liquid hydrocarbons. In fact, the hydrophobicity is an important virulence factor associated to the pathogenicity of *C. albicans* strains (Odds, 1994). The evaluation of hydrophobicity potency is correlated to the increase of the adhesion to tissues and biomaterials and may contribute to the resistance to antifungal agents (De Repentigny et al., 2000). The CSH of *C. albicans* depends on the growth phase, some environmental factors such as the temperature and the nutriments in the growth medium (Hazen and Hazen, 1988). Some studies reported the differences in CSH of different *Candida* isolates (Radford et al., 1999). The CSH of *C. albicans* strains can affect its “cellular behavior” and the hote interactions. In fact, metabolic activity, the colonization and response to cells change with the CSH. The CSH has also an important role in adhesion to tissues, to buccal epithelium cells (BEC) and to acrylic resines (Radford et al., 1999). Raut et al. (2010) studied the hydrophobicity of 50 clinical *C. albicans* isolates and the correlation with biofilm formation on polystyrene. The results proved that CSH varied between 2 and 41%. They demonstrated also the absence of correlation between CSH and adhesion to polystyrene.

The analysis of electrophoretic profiles of proteins has allowed the identification and the classification of numerous strains, species and genera of yeasts in taxonomic and epidemiological studies (Al-Rawi and Kavanagh, 1998).

The whole-cell protein profile of the thirteen human pathogenic *Candida* strains, obtained by one-dimensional denaturing gel electrophoresis is shown in Figure 1. The protein profiles of all *Candida* tested species (*C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. kefyr*, *C. holmii* and *C. sake*) were inspected visually and compared with each others. This method of whole-cell protein extraction was also used to distinguish between the four *Candida* species obtained from clinical specimens.

The numerical analysis of the whole-cell protein profiles used for average linkage and correlation coefficient distance yielded a dendrogram, consisting of two basic clusters (I and II) at similarity levels between 75 and 100% (Figure 2). Cluster I is represented by the oral isolate of *C. albicans* (H_{12}) and the cluster II comprises

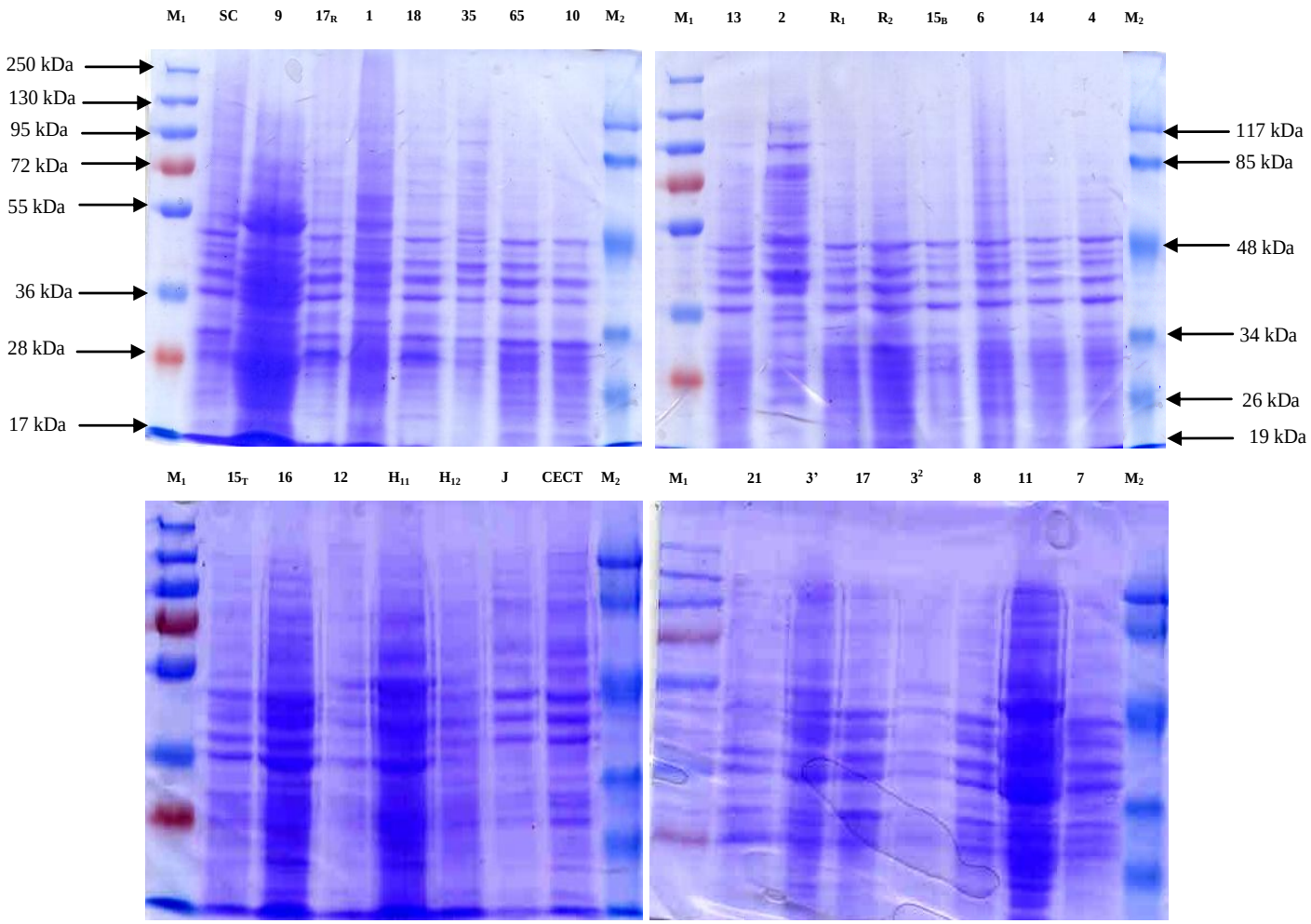

Figure 1. Representative protein band profiles of different *Candida* species. M_1, PageRuler™ Plus Pre-stained Protein Ladder (Fermentas, Madrid, Spain); M_2, Pre-stained Protein Molecular Weight Marker (Fermentas, Madrid, Spain).

the other 29 *Candida* strains. Moreover, the SDS-PAGE analyses indicated that there are major similarities between all *C. albicans* strains in their high-molecular mass range (>40 kDa). However, the minor distinctive proteins were observed both in the low (<30 kDa) and high-molecular mass range (>60 kDa).

In fact, SDS-PAGE plays a major role in the experimental analysis of proteins and protein mixtures. One-dimensional polyacrylamide gel electrophoresis is still the most widespread form of the technique (Hames, 1990) and has been used to separate proteins from cell extracts after treatment with SDS and β-mercaptoethanol.

The Figure 1 revealed that the whole-cell protein patterns of each one of the *Candida* strains had seven major protein-bands. The tested strains had 85 different protein bands (molecular weights between 16-324 kDa). Similarities in the profiles of all *Candida* strains were manifested by the existence of some bands.

In fact, *C. kefyr* isolate (strain 1), *C. orthopsilosis* (H_{11}) and *C. holmii* (2) were clustered in the same cluster (IIA), whereas all *C. albicans* isolates were grouped in the same cluster (IIB). The cluster (I) was represented by only the strain "H_{12}" of *C. orthopsilosis*. The strains 6 and 7 of *C. albicans* isolated at the same period (04/2006) have the same proteins patterns (IIB). It was the same case for the strains 16 and 18 (04/2007). The strains 3^1 (*C. albicans*) and 3^2 (*C. sake*), 15_B (*C. albicans*) and 15_T (*C. glabrata*), 17 (*C. albicans*) and 17_R (*C. albicans*) isolated from the same patient each two of them and at the same period have the same proteins patterns (Table 1). In addition, the two isolates of *C. glabrata* (8, isolated in 07/2006) and (15_T, isolated in 02/2007) were grouped in the same cluster (IIB). It was the same observation for the three type strains of *C. parapsilosis* (Figure 2).

In our study, we used a simple method for the extraction of whole-cell proteins from *Candida* species. This technique has been adopted for *Saccharomyces cerevisiae* described by Horvath and Riezman (1994). Also, the protein electrophoretic fingerprinting showed that protein bands greater than 45 kDa are repeated in

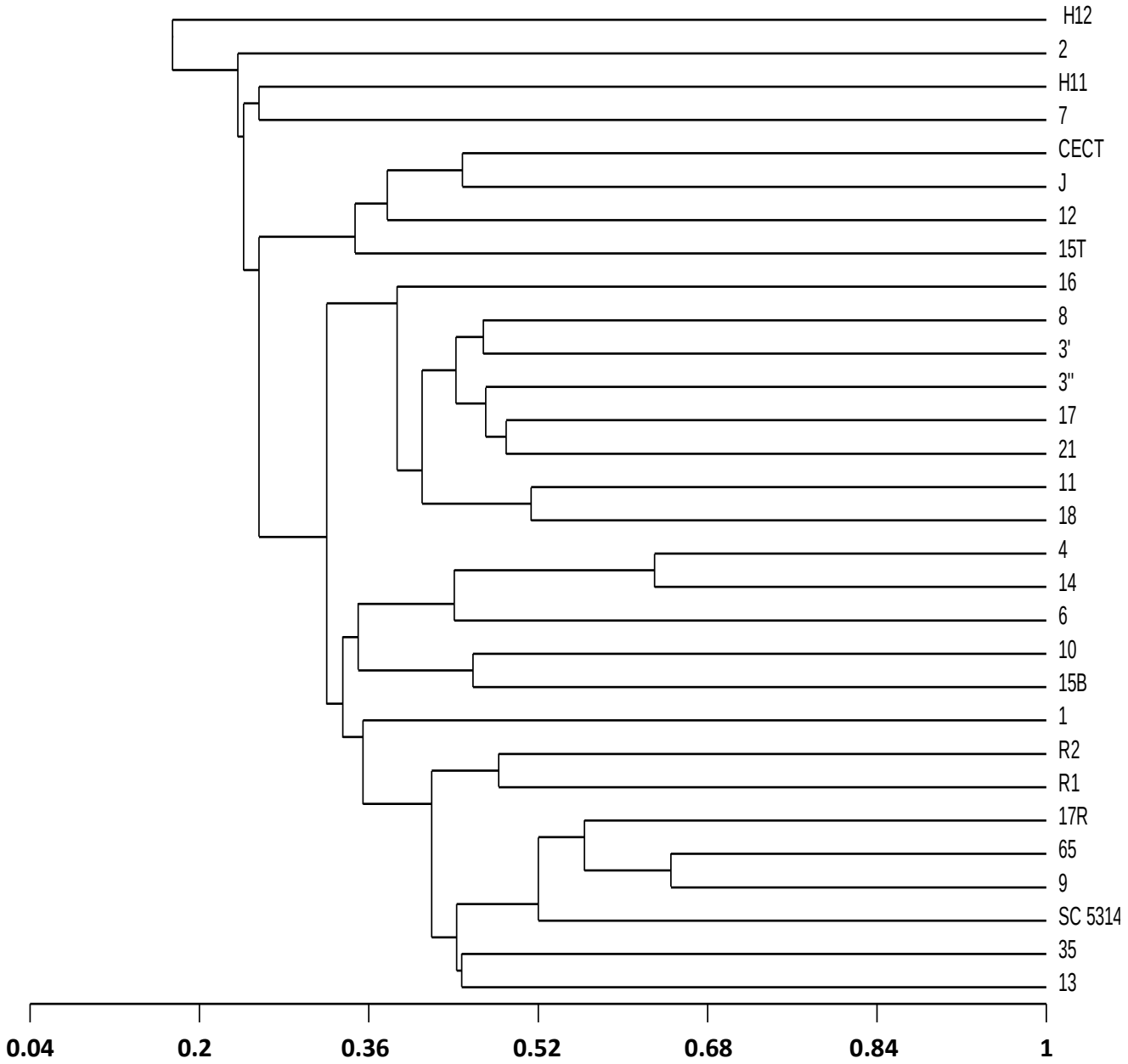

Figure 2. Dendogram (% similarity) based on whole proteins profiles of 30 *Candida* isolates from oral cavity infections. The dendogram was constructed by using UPGMA.

the majority of *Candida* species, suggesting that it may be representative of the genus (Rosa et al., 2000).

The similarity of the electrophoretic whole-cell protein patterns among *Candida* strains samples observed in UPGMA dendrograms showed values between 0 and 100%. The data obtained from grouping of *Candida* strains based on their electrophoretic profiles showed high level of agreement with the inter-specific classification established by conventional methods. Moreover, the isolates of each species showed similar profiles (Figure 2). This suggests that these protein profiles obtained by SDS-PAGE are relatively stable taxonomic characteristics.

SDS–PAGE analysis revealed that the cell wall protein patterns of *Candida* isolates had a total of seventy protein-bands with a molecular weight between 16-127 kDa. Drawing a vertical line across the dendrograms at a similarity value of 75 and 100% (average similarity), 30 phenons for both whole-cell and cell wall proteins were detected. The similarity has been employed in several epidemiological studies of *Candida* species using proteins techniques associated to numerical analysis,

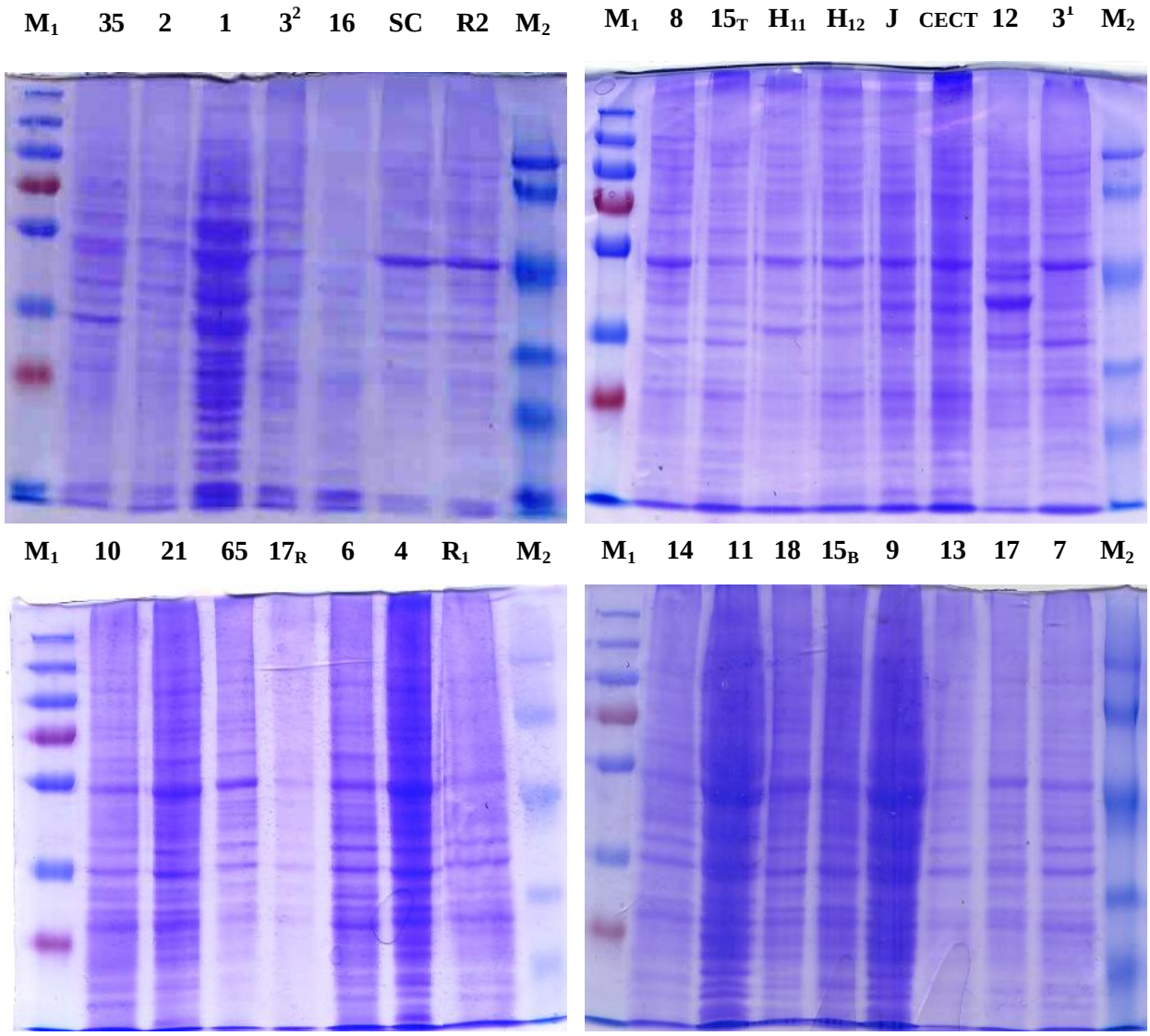

Figure 3. Representative protein band profiles of different *Candida* species. M_1: PageRuler^{TM} Plus Prestained Protein Ladder (Fermentas, Madrid, Spain), M_2: Prestained Protein Molecular Weight Marker (Fermentas, Madrid, Spain).

allowing better discriminatory power during analysis of the compositions of clusters and their relationships (Pujol et al., 1997; Rosa et al., 1999).

The strains *C. parapsilosis* CECT 13011, J 981226, H_{11} and H_{12} (*C. orthopsilosis*), *C. kefyr* (1), *C. glabrata* (8 and 15_T) and *C. albicans* (9) were clustered in the same cluster (IIA). The cluster (I) is represented by only the *C. albicans* strain 17_R (Figure 3). The cell wall proteins patterns showed that the isolates 15_B of *C. albicans* and 15_T of *C. glabrata* isolated from the same patient and t the same period have different proteins patterns. In addition, the proteins profiles of the strains (3^1 and 3^2) isolated from same patient were similar. The *C. albicans* reference strains (SC 5314 and ATCC 90028) were very similar to other *C. albicans* strains isolated from the oral cavities. Finally, oral strains of *C. albicans* 9 (IIA) and 17_R (I) were different from other *C. albicans* isolates (Table 1 and Figure 4). The results of numerical analysis confirmed that each cluster had characteristic and distinctive protein profiles.

Conclusion

The whole-cell and cell wall protein profiles based on SDS-PAGE associated with numerical analysis, showed to be an important criterion for taxonomic and epidemiological studies of *Candida* species. Besides this, the proteins patterns similarity among *Candida* strains isolated from the patients suffering from denturestomatitis

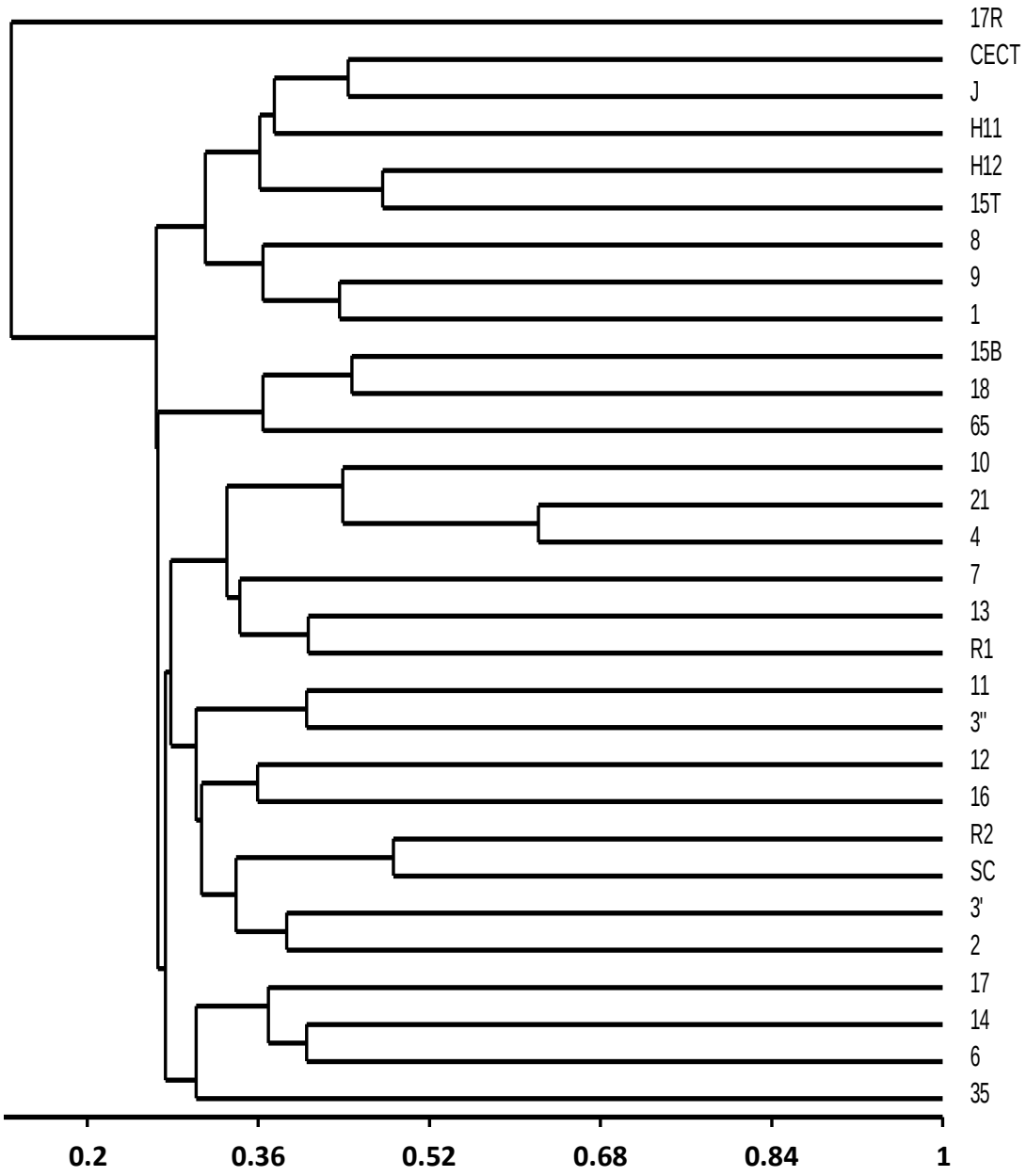

Figure 4. Dendogram (% similarity) based on cell wall proteins profiles of 30 *Candida* isolates from oral cavity infections. The dendogram was constructed by using UPGMA.

was observed in our study.

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