

## Protein Profile of a Fluconazole-resistant *Candida albicans* Isolated from HIV-1 Infected Patient: Evaluation of Protein Extraction Methods and Development of a Simple Procedure

M. Shahid, A Malik, M.W. Rizvi and M. Singhai

Section of Mycology, Department of Microbiology,  
J.N. Medical College and Hospital, Aligarh Muslim University Aligarh-202002, UP, India

**Abstract:** A fluconazole-resistant ( $MIC > 32 \mu g\ ml^{-1}$ ) *Candida albicans* strain obtained from oral thrush lesion of an HIV-1 infected patient was studied for protein profile by Sodium dodecyl Sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Membrane, cytosolic and total proteins were analysed by respective conventional buffers. An in-house, simple, buffer was designed for effective extraction of the proteins and compared with conventional buffers. Clinical and standard strains of *Candida spp* including fluconazole-resistant and susceptible *C. albicans* isolates obtained from other clinical specimens were included to evaluate the reproducibility and discriminatory power of SDS-PAGE by using our in-house prepared buffer. Analysis of the whole cell protein extract of fluconazole-resistant *Candida albicans* strain, from HIV patient, revealed at least 23 proteins of molecular weights ranging between 13 kDa-104 kDa. Proteins between 13 kDa-64 kDa were identified as abundant proteins. Reproducible results were obtained by using in-house buffer and were able to discriminate different *Candida spp*. On analysing the protein profile of fluconazole-resistant isolates of *Candida albicans* as opposed to fluconazole-susceptible isolates, significant difference in the protein banding patterns were noticed. Cluster analysis, by using SPSS Software Inc. USA, revealed that separate clusters were formed by fluconazole-resistant isolates. Thus, we conclude that our method could be incorporated in routine mycology laboratories as a simple and reproducible method.

**Key words:** protein profile • fluconazole-resistant • *Candida albicans* • HIV-1 • simple method

### INTRODUCTION

*Candida albicans* is an important opportunistic fungal pathogen that continues to be a leading cause of infections, especially in immunocompromised hosts and the frequency of candidiasis as a major cause of morbidity and mortality among hospitalised patients has increased in recent years [1, 2]. Though, it is a commensal of healthy individuals, the interaction between fungal cell wall components and the immune system governs the occurrence of infection [1].

Several studies have described the antigenic and allergenic components of *C. albicans* but their results have been non-uniform [3]. The variations in the reported molecular mass of antigens and their isolation from various forms and parts of *C. albicans* may be because of differences in growth media and also on the antigen

extraction method used [4]. In most of the studies a complex liquid culture medium containing yeast nitrogen base and galactose was used for the growth of *Candida spp*. Hence, we planned this study to find out the protein profile of a fluconazole-resistant *C. albicans* strain isolated from an oral thrush lesion of an HIV-1 infected patient by growing in a simple glucose peptone broth. We also attempted to evaluate various antigen extraction methods and tried to develop a rapid and reproducible method of sample preparation for Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). We feel that a rapid and reproducible method of sample preparation for determining the protein profile by SDS-PAGE would be useful in determining the epidemiology of the infection and also for serodiagnostic tests for antigen and antibody detection.

## MATERIALS AND METHODS

**Fungal strains:** A clinical isolate of fluconazole-resistant *C. albicans* (MIC>32 µg ml<sup>-1</sup>) isolated from the oral thrush of an HIV-1 infected patient was used to study the protein profile and to evaluate the various protein extraction methods. The isolate was identified in our laboratory using fermentation and sugar assimilation procedures as described by Silva Hunter [5] and used throughout the study. The same strain was inoculated in multiple sets of culture tubes containing glucose peptone broth and then subjected to various protein extraction methods. Standard strains of *C. albicans*, *C. krusei*, *C. parapsilosis*, *C. tropicalis* as well as clinical strains of *C. albicans*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*, *C. guilliermondii* and *C. stellatoidea* were also used to evaluate the discriminatory power of the simple protein extraction method designed in our laboratory. Numerous fluconazole-susceptible and fluconazole-resistant isolates obtained from routine clinical specimens were also analysed to access the discrimination between the two groups by using our procedure.

**Growth condition and cell harvesting:** The yeasts were grown in 10 ml glucose-peptone broth, in screw capped polypropylene tubes, for 24-36 h at 37°C to yield a growth of late log to stationary phase. The tubes were aerated by shaking intermittently. The yeast cells were harvested by centrifugation at 6000 rpm for 10 min and washed twice with normal saline. These pelleted cells were further subjected to different protein extraction methods as described below.

**Lysis buffers used:** Membrane lysis buffer (LBM): Membrane lytic buffer having a composition of 150 mM NaCl, 1%, NP-40, 0.5% deoxycholate, 0.1% SDS and 50 mM Tris HCl, pH 7.4, was used to yield membrane proteins of *Candida* spp.

**Cytosole Lysis Buffer (LBC):** Cytosolic lytic buffer with a composition of 50 mM Tris, 5 mM MgCl<sub>2</sub> and 1 mM EDTA, pH 7.5, was used to yield cytosolic proteins.

**In-house Buffer System (TGBS):** In-house developed lysis buffer containing 10% SDS in 50 mM Tris glycine buffer (which was used as an electrophoretic buffer in SDS-PAGE), was tried in our laboratory as a cheaper substitute to yield the proteins from *Candida* spp.

### Sample preparation methods

**Whole Cell Protein Extracts (WCPE):** For extraction of whole cell proteins, cells were re-suspended in 200 µl of LBM, LBC and TGBS respectively. The re-suspended cells were sonicated giving 3 bouts each of 30 sec. The sample was centrifuged at 5,000 rpm for 10 min and the supernatant was collected.

**Membrane proteins:** Yeast cells were resuspended in 200 µl of LBM and placed in a 37°C shaking water bath for 45 min, centrifuged at 5,000 rpm for 10 min and the supernatant was collected.

**Cytosolic proteins:** Yeast cells were resuspended in 200 µl of LBC and processed in the way as for membrane proteins.

**Protein extraction by TGBS without sonication:** Pelleted yeast cells were re-suspended in 200 µl of TGBS and placed in 60°C shaking water bath for 45 min, centrifuged at 5,000 rpm for 10 min and the supernatant was collected.

**SDS-PAGE:** SDS-PAGE of the protein extracts was performed according to the method of Laemmli *et al.* [6] with some modifications as described by us elsewhere [7-9]. For electrophoresis, 10% acrylamide gel was used.

**Evaluation of proteins extraction by TGBS without sonication:** Since various clinical laboratories do not have facilities for sonication, we tried to evaluate the results obtained by using TGBS without sonication. Standard as well as clinical strains of various *Candida* spp., as described previously, were subjected to protein extraction by TGBS and SDS-PAGE was performed on 10% acrylamide gel. The stained gels (by Comassie brilliant blue R250) were examined for presence and absence of bands. Dice index of similarity [8, 10] was determined and dendrogram was prepared. Based on the dendrogram results, discriminatory power of this protein extraction method was determined.

### Statistical analysis

**Dice index of similarity:** Dice index of similarity coefficient, as described previously [8], was calculated between the groups formed by banding patterns in SDS-PAGE by using the formula:  $S(\%) = \frac{2X}{a+b} \times 100$  where a is the total number of bands in lane 1, b is the total number of bands in lane 2, X is the total number of similar

Table1: Protein profile of the *C. albicans* strain (isolated from HIV-1 patient) by various protein extraction methods

Total proteins analysed by whole cell protein extraction (WCPE)			Membrane proteins obtained by membrane lysis buffer			Cytosolic proteins obtained by cytosole lysis buffer		
104	101	95	73	62	60.5	73	62	60.5
91.5	89	82	51	42	38.5	48	38.5	13
79	76	73	20	13				
64	62	60.5						
54	51	48						
42	38.5	35.5						
32.5	29.5	26.5						
20	13							

Table2: Protein profile of the *C. albicans* by using TGBS with and without sonication

Whole cell Proteins obtained by TGBS with sonication			Proteins obtained by TGBS without sonication			Abundant proteins noticed in WCPE		
104	101	95	76	64	54	64	62	54
91.5	89	82	51	48	35	48	35.5	26.5
79	76	73	29.5	26.5	20	20	13	
64	62	60.5	13					
54	51	48						
42	38.5	35.5						
32.5	29.5	26.5						
20	13							

bands in both the lanes. Dendrogram was made by single linkage method (Jaccord's method) using the SPSS Software (SPSS Inc. USA).

**Reproducibility:** The reproducibility [8] was determined by using formula:  $R = Nr/N$  where Nr is the number of isolates assigned the same type on repeat testing and N is the number of isolates tested.

**Discriminatory power:** Discriminatory indices were calculated for all the three above-mentioned methods using the Simpson's discriminatory index.

$$DI = 1 - \frac{1}{N(N-1)} \sum_j^s n_j(n_j - 1)$$

Where; N is the total number of strains under study, s is the total number of groups described and n is the number of strains belonging to j<sup>th</sup> group.

## RESULTS AND DISCUSSION

Oropharyngeal Candidiasis is the most common opportunistic infection in patients infected with HIV and *C. albicans* is the species most frequently isolated from patients with AIDS who have oral thrush [11]. Fluconazole is an orally active, triazole antifungal agent

that is less toxic, better tolerated and has greater *in vivo* activity against *C. albicans* than ketoconazole [12]. However, in recent years there are reports that an increased number of patients have had oral thrush caused by *C. albicans* strains that are clinically and microbiologically resistant to fluconazole [11, 12].

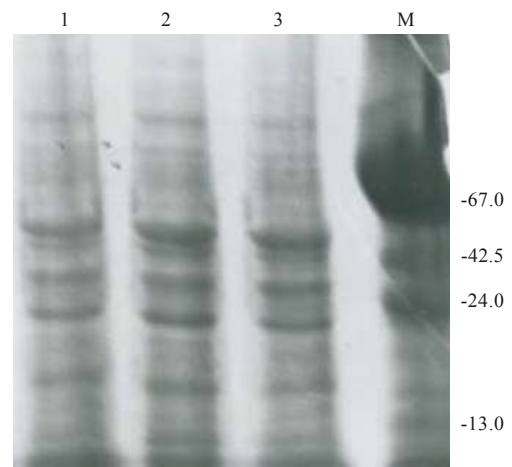


Fig. 1: Acrylamide gel electrophoretogram of *Candida albicans* showing the protein bands obtained by in-house (Lane 1), membrane (Lane 2) and cytosolic buffers (Lane 3). Lane M shows the molecular weight markers

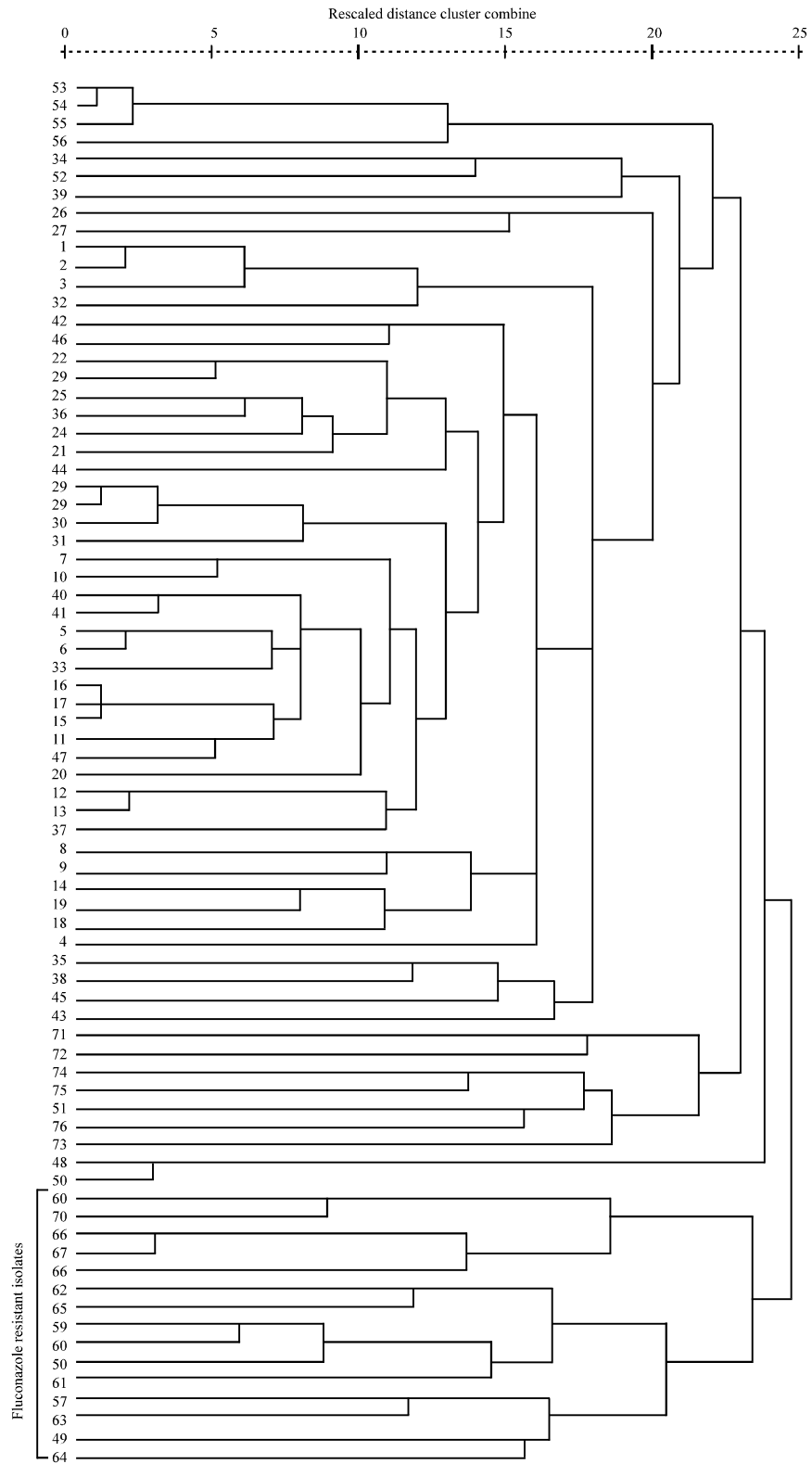


Fig. 2: Dendrogram showing the clusters formed by fluconazole-susceptible *C. albicans* isolates and separate cluster formed by fluconazole-resistant isolates

There are numerous published reports regarding the characterization of the proteins of *C. albicans* worldwide [13-15]. However the studies are elementary in our country regarding the proteins profile and characterization of proteins, especially in fluconazole-resistant *C. albicans* strains. The results of protein profile of the clinical strain of *C. albicans*, used in the present study, are shown in Table 1 and 2. Analysis of the whole cell protein extract by SDS-PAGE demonstrated at least 23 proteins of the molecular weights ranging between 13 to 104 kDa. Proteins of the molecular weights of 73, 62, 60.5, 51, 42, 38.5, 20 and 13 kilo Daltons were obtained as membrane proteins whereas, proteins of 73, 62, 60.5, 48, 38.5 and 13 kilo Daltons were obtained as cytosolic proteins (Table 1). It was interesting to note that proteins of molecular weights 73, 62, 60.5, 38.5 and 13 kilo Daltons were simultaneously isolated as the membrane and cytosolic proteins and suggest that these proteins are present in the cell membrane as well as the cytosol of the *C. albicans*. The proteins of the molecular weights 51, 42 and 20 kDa were noticed only as membrane proteins whereas; the protein of 48 kDa was noticed only as a cytosolic protein (Table 1). This 48 kDa protein isolated in the electrophoretic gel has already been described as an abundant, immunodominant, glycolytic enzyme "Enolase" in numerous studies [13]. The protein of 64 kDa, isolated in the present study, could be a mannoprotein, reported to be of 65 kDa molecular weight and target for cell mediated immune response, similarly, the protein of 42 kDa isolated in the present study, could be a immunosuppressive protein reported to be of 43kDa and the antibodies specific against this protein are protective in nature [14]. The variations in the reported molecular mass may be because of differences in growth media used, antigen extraction method and also on the environmental and gel running conditions. Most of the studies have used liquid media containing 0.68% yeast nitrogen, as a nitrogen source and 10% (555Mm) galactose as carbon source, for growing the yeasts. In the present study, we have used glucose peptone broth, the liquid media used to prepare Sabouraud's dextrose agar in most of the mycological laboratories, as a cheaper substitute for the growth of the yeasts. Similarly we have used 10% SDS in Tris glycine buffer (Electrophoretic buffer) as a lytic agent instead of complicated composition of lytic buffers. Whole cell protein extract derived in this lytic buffer yielded similar proteins as extracted by other buffer compositions (Fig. 1) Significant number of proteins were derived by this lytic buffer, when

used without sonication, including the 48 kDa protein (Table 2). The lytic buffer we designed gave reproducible and comparable results to other buffer compositions and thus we suggest that it could be used as a users-friendlier and cheaper substitute to other buffers. The study was further elaborated to find out the discriminatory power of this buffer, when used without sonication, on different *Candida* species. We found that by using our buffer composition different *Candida* species can easily be discriminated based on the protein banding patterns and the dendrogram drawn by SPSS Software formed different clusters for different *Candida* species (data not shown). On analysing the protein profiles of fluconazole-resistant and fluconazole-susceptible isolates of *Candida albicans*, significant difference in the protein banding patterns were noticed and cluster analysis revealed that separate clusters were found by fluconazole-resistant isolates (Fig. 2).

Concluding, it may be stated that the protein profile of fluconazole-resistant clinical isolate of *C. albicans* obtained from HIV-1 patient revealed at least 23 proteins ranging between 13 to 104 kDa. The growth medium and conditions, which we used and the simple lytic buffer designed in our laboratories, gave comparable results with other buffer compositions. Hence we suggest that the laboratories not having facilities for sonication could easily incorporate our method as a simple and reproducible method of protein profiling of *Candida* species.

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