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Candida albicans infections in a north Indian tertiary care hospital: antifungal resistance pattern and role of SDS-PAGE for characterization

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Abstract
With the increase in the number of immunocompromised individuals, there has been a consequent rise in the number of opportunistic infections, especially those due to *Candida* sp. A rise in the incidence of antifungal resistance has also been reported. The present study was undertaken to evaluate the incidence of *C. albicans* in superficial and deep-seated infections, to study its antimicrobial susceptibility profile, to analyze the protein-band profile of isolates of *C. albicans* and assess its use as a means of characterizing the yeast, especially in resistant strains. Seventy-six isolates of *C. albicans* from various clinical specimens were identified by standard mycological techniques and further subjected to SDS-PAGE. Molecular weights were calculated with reference to the marker and dendrograms were prepared using the SPSS software. Susceptibility testing of five antifungal agents (fluconazole, clotrimazole, nystatin, amphotericin-B and voriconazole) was done by the disc diffusion/colorimetric microdilution method. On cluster analysis, six types of banding patterns were observed. Maximum resistance (19.8%) was observed against fluconazole. On analysis of the dendrogram cluster groups, the fluconazole-resistant isolates of *C. albicans* formed a separate cluster distinct from those of the fluconazole-sensitive isolates. It was also observed that the specimens from a common site tended to fall close together in the dendrogram pattern. Significantly, high frequency of fluconazole resistance was noticed in this study, which is alarming. In resource-limited laboratories, SDS-PAGE could be used as an alternative method for typing.

Keywords: *Candida albicans*; fluconazole resistance; SDS-PAGE; characterization.

Introduction

With the remarkable modern advances in medicine, there has been an increase in the number of immunocompromised individuals who need extensive care in hospitals. This has resulted in a rise in the incidence of fungal infections, especially those due to *Candida* species. *C. albicans* is amongst the most common fungal causative agent in superficial and deep seated candidiasis (Gullo, 2009). However, non-albicans *Candida* species are also being implicated in recent years (Paul *et al*., 1999; Peter *et al.,* 2004; Fadda *et al*., 2008). A rise in the incidence of antifungal resistance to *Candida* spp. has also been reported over the past decade (Sojakova *et al*, 2004; Skrodeniene *et al,* 2006).

Due to the recognition of the increasing importance of *Candida* spp. as an opportunistic pathogen, an effective method is required to type the organism. Analysis of the proteins of *Candida* spp. can be used as a sensitive method of characterizing the yeast on the basis of their protein band profile. Keeping these facts in mind, the present study was undertaken to find out the incidence of *Candida albicans* in superficial and deep-seated infections, to analyze their antifungal susceptibility pattern and to characterize the isolates using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Materials and Methods

The present study was conducted in the Department of Microbiology, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh, over a period of two years. The cases selected for the study included symptomatic high-risk patients in whom no other aerobic pathogen was identified, and *Candida* spp. were isolated repeatedly in pure culture, and these infections were associated with clinical signs and symptoms of infection.

Seventy-six isolates of *C. albicans* from various clinical specimens were obtained from a total of 714 patients. The isolates were identified by standard mycological methods that included colony morphology on Sabouraud's dextrose agar (SDA), germ tube test (GTT), morphology on corn meal agar (CMA) and sugar fermentation/assimilation profile (Milne, 1996). The isolates were further characterized by SDS-PAGE and antifungal susceptibility testing was performed.

SDS-PAGE

SDS-PAGE was performed by the method described by Laemmli (1970) with some modifications.

Sample preparation

A single colony on SDA was inoculated in 10ml Brain Heart Infusion (BHI) broth and incubated at 37^{o}C for 48 hrs. After incubation, pellets were formed by centrifugation at 4000 rpm for 10 minutes. The pellets were then washed twice in normal saline and the supernatant was discarded. To the pellets, 100 µl of 10X Tris-glycine buffer [Tris-30.3g, Glycine-144.2g, SDS-10g, Distilled water-1litre: pH=8.3] and 10% SDS was added and sonication was performed in 4 bouts of 30 seconds each. After each bout, the sample was kept on ice for 15 seconds. After sonication, the material was transferred to a sterile Eppendorf tube, and 20 µl of 2X sample buffer [10% SDS-4ml, Glycerol-2ml, 1M Tris (pH=6.8)-1.2ml, Distilled water- 2.8ml, Bromophenol blue-0.01%] was added to each tube. The tubes were then boiled for 4 minutes in a water bath. Tris base was procured from SRL, Mumbai; SDS from SISCO Research Lab Pvt. Ltd., Mumbai; Bromophenol blue and Glycerine from HiMedia Laboratories, Mumbai, India.

Electrophoresis

Electrophoresis was performed in 8% (resolving) and 2.25% (stacking) acrylamide gel. Current was applied at the rate of 2mA/well in the stacking gel and 4mA/well in the resolving gel.

Analysis

After electrophoresis, the data was analyzed and molecular weights were calculated with reference to the marker using the gel documentation system (Vilber Lourmat, France). Dendrograms were made by the average linkage method using the SPSS software (Chicago, U.S.A.) to perform the cluster analysis.

Antifungal susceptibility testing

Susceptibility testing to nystatin (100 units), clotrimazole (10µg), fluconazole (10µg) and amphotericin-B (100 units) (all from HiMedia Laboratories, Mumbai, India) was performed by the disc diffusion method (Chakrabarti *et al*., 1995). *Candida kefyr* strainY/16 was used as control. The medium used was yeast nitrogen base glucose (YNBG) agar (HiMedia Laboratories, Mumbai, India). For susceptibility testing of azoles, 1.5% l-asparagine (HiMedia Laboratories, Mumbai, India) was added to the YNBG agar. Results were interpreted as either sensitive or resistant.

Antifungal susceptibility testing to voriconazole (Pfizer Inc.) was done by broth microdilution and Alamar blue colorimetric microdilution method. These were also used to re-test the fluconazole resistant isolates identified by the disc diffusion method. Broth microdilution was performed in accordance with the guidelines in NCCLS document M27-A (1997). Quality control isolates of *C.albicans* (ATCC 10231), *C. parapsilosis* (ATCC 22019) and *C. krusei* (ATCC 6258) were included with every test. Endpoints were determined visually by observing a colour change from blue (indicating no growth) to pink (indicating growth).

Statistical analysis

The 'Z' test for proportion was used to compare the data. A *p*-value of < 0.05 was taken as indicative of statistical significance, and a *p*-value of < 0.01 was considered highly significant.

Results

In the present study, 76 isolates of *C. albicans* (10.6%) were obtained from 714 high-risk patients, implying an isolation rate of 10.6%. The age of patients in the study group ranged from newborns to $\geq$61 years. There was a slight female preponderance in the study population (M:F ratio= 0.85:1). The maximum number of *C.albicans* isolates were from urine (30.3%), and cervical swabs (25.0%). Other specimens in the study included BAL fluid, CSF, pus, sputum etc. (Fig.1).

The antifungal susceptibility pattern of the isolates showed that all (100%) isolates were sensitive to nystatin, amphotericin-B and voriconazole in the concentrations tested. Fluconazole resistance was observed in 15 (19.8%) isolates (Table 1). The MIC values of fluconazole for the isolates ranged from 32 µg/ml (7%) to $\geq$64 µg/ml (93%). On the other hand, all (100%) isolates were sensitive to $\leq$0.06 µg/ml of voriconazole (Table 2).

The broth microdilution method and the Alamar blue colorimetric microdilution method showed good correlation (data not shown). SDS-PAGE analysis of various isolates revealed at least 6 types of banding patterns, with the number of bands ranging from 5 to 18. The maximum number of isolates (34.2%) had a banding pattern with seven bands (Table 3).

Fig. 1: Isolation of *C. albicans* from various clinical specimens.

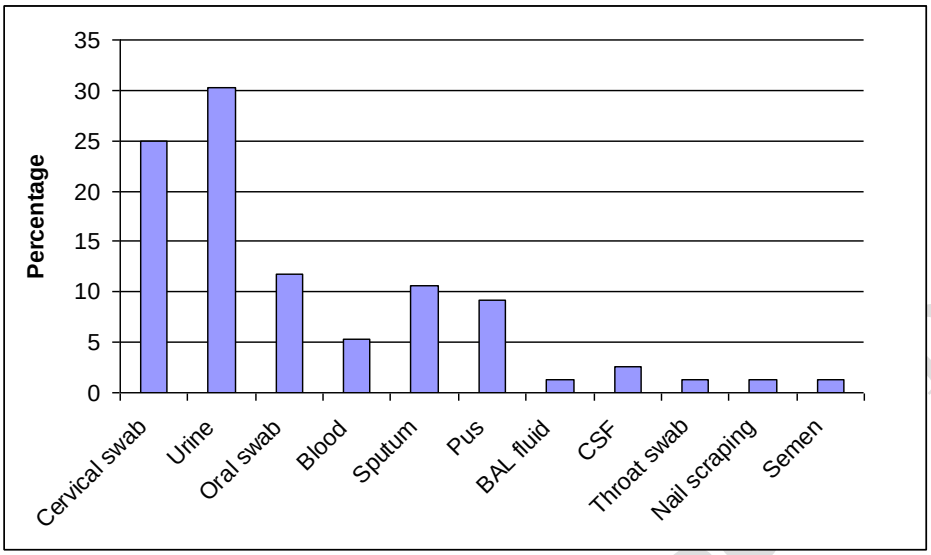

Table 1. Susceptibility pattern of the isolates.

Antifungal agent	Disc potency	No. of strains*	
		Sensitive	Resistant
Clotrimazole	10 µg	73(96)	3(4)
Nystatin	100 units	76(100)	-
Fluconazole	10 µg	61(80.2)	15(19.8)
Amphotericin-B	100 units	76 (100)	-
Voriconazole	-	76(100)	-

* Figures in parentheses indicate percentage

Table 2. MIC values of fluconazole and voriconazole.

Antifungal agent	MIC value (µg/ml)								No. of isolates
Fluconazole	0.5	1	2	4	8	16	32	≥64	15 (100%)
	-	-	-	-	-	-	1 (7%)	14 (93%)	
Voriconazole	<0.06	0.125	0.25	0.5	1	2	4	8	76 (100%)
	76 (100%)	-	-	-	-	-	-	-	

Table 3. SDS-PAGE analysis of *C. albicans* isolates.

Total no. of isolates	SDS-PAGE pattern	No. of bands	No. of isolates*
76	1	5	18(23.8)
	2	6	7(9.2)
	3	7	26(34.2)
	4	8	11(14.5)
	5	10	6(7.9)
	6	18	8(10.4)

* Figures in parentheses indicate percentage

Proteins with molecular weights 22.5 kDa, 44.0 kDa, 60.5 kDa and 117.5 kDa were consistently present in the fluconazole-resistant isolates. The dendrogram of *Candida albicans* isolates showed that all the 15 fluconazole-resistant isolates were grouped in two closely related clusters. The clusters of fluconazole-resistant isolates were significantly different from and unrelated to that of the fluconazole-susceptible isolates (Fig 2). It was also seen that isolates from a common site tended to fall close together on cluster analysis.

Discussion

A significant increase in the incidence of fungal infections, especially those due to yeasts, particularly *Candida* species is being seen in recent times. This is mostly due to rising numbers of immunosuppressed patients and widespread use of broad-spectrum antibiotics and steroids. *Candida albicans* is considered the most pathogenic member of the genus *Candida* and is the species most commonly isolated from clinical materials, although infections with other species of *Candida* have been described in recent times (Pfaller, 1996; Fadda *et al.,* 2008).

Due to the recognized importance of these agents as nosocomial and opportunistic pathogens, it is important to have a simple, effective and relevant typing method for the characterization of these yeasts. One such method could be the generation of protein profiles, which involves solubilisation of microbial proteins and electrophoresis in gel matrices such as polyacrylamide. The use of SDS-PAGE in epidemiological typing of nosocomial infection in neonates by *Klebsiella* spp. has been reported (Malik *et al*., 2003). The efficacy of use of SDS-PAGE has also been described in aspergillosis in bronchogenic carcinoma (Malik *et al.,* 2003a). Rodrigues *et al.* (2004) have also reported the use of SDS-PAGE for typing of *C. albicans* isolates. They found this method to be important for characterizing the yeast for epidemiological and taxonomic purposes. Based on these findings, we evaluated the use of SDS-PAGE in *Candida albicans* isolates, and performed analysis of the whole cell lysate to characterize the yeast.

In the present study, we found that there was considerable variation among *C.albicans* isolates from different sites and from different patients. Merz *et al*. (1988) who performed orthogonal field alteration gel electrophoresis (OFAGE) also reported a high degree of variability among their isolates. In a study similar to ours, Khosravi *et al.* (2008) have also demonstrated that the use of SDS-PAGE showed a high degree of protein similarity among isolates of *C.albicans* with low and high virulence. They have further concluded that these techniques could provide additional criteria for serologic and immunological studies of *C.albicans*.

Antifungal susceptibility testing of our isolates revealed that all isolates were sensitive to amphotericin-B, nystatin and voriconazole. However, significant resistance (19.8%) was observed for fluconazole. Our findings are in accordance with those of Fadda *et al*. (2008) who also found that there was decreased susceptibility to the older azoles among *Candida albicans* isolates. Similar susceptibility of *C.albicans* isolates was also reported by Mokaddas *et al.* (2007).

In our study, the SDS-PAGE pattern revealed several characteristic bands common to all isolates of *C. albicans*. A similar finding has also been reported by other workers (Kobayashi and Suginaka, 1984). It was also noted that specimens from a common site tended to fall close together on cluster analysis.

Another interesting finding in our study was that proteins with molecular weights 22.5 kDa, 44.0 kDa, 60.5 kDa and 117.5 kDa were present in only the fluconazole-resistant isolates. As a result, all the 15 fluconazole-resistant strains formed two closely related clusters on dendrogram analysis.

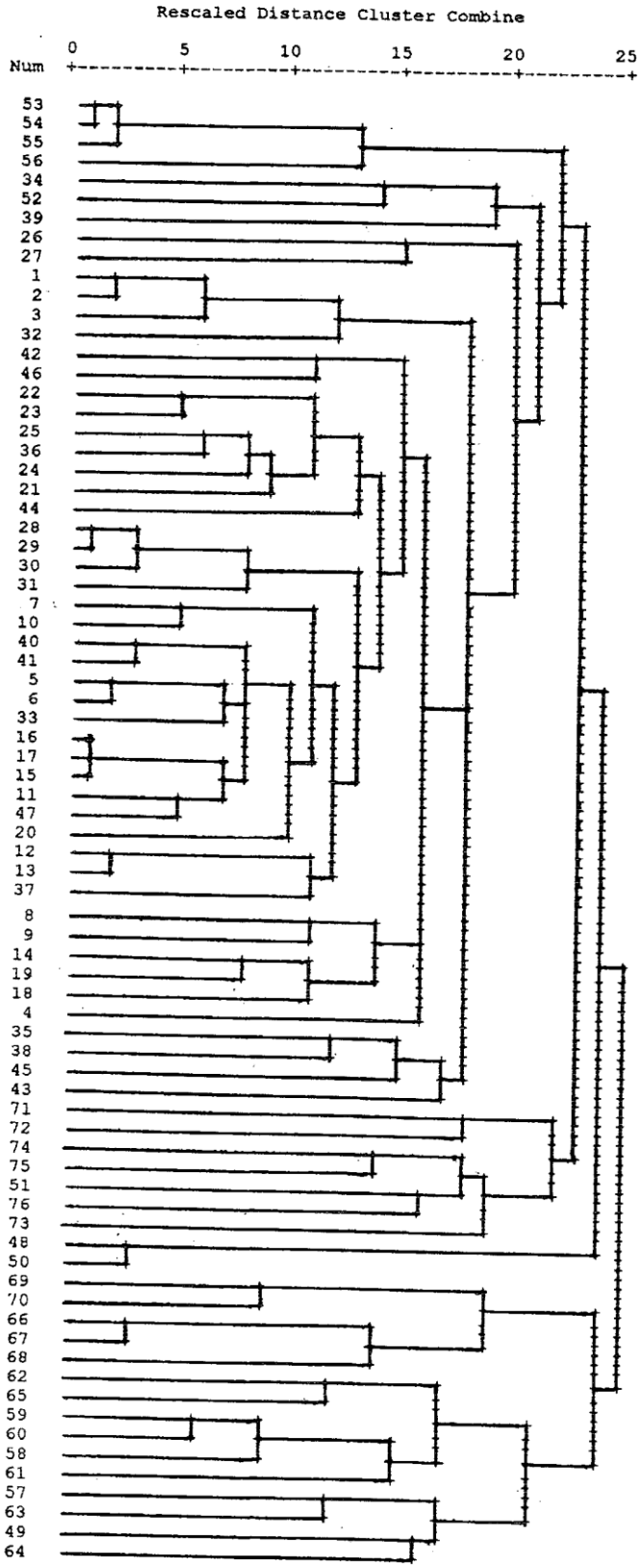

Fig. 2: Dendrogram of *C. albicans* isolates.
(Isolates marked with * represent the fluconazole-resistant strains)

Conclusion

Although SDS-PAGE is not a very good typing method when employed alone, it can be used as typing tool to supplement other typing methods especially in laboratories where genomic based molecular typing is not available. This method can also be used in an outbreak situation or to compare different isolates to establish identity or non-identity.

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