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Research Paper

Molecular Identification Candida isolates from HIV positive patients in India

Medical Science

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ABSTRACT

The objective of the study was to identify, characterize the Candida isolated from HIV/AIDS patients. Relevant clinical samples were collected from 200 HIV/AIDS patients. 143 yeasts were isolated and subjected to speciation by the conventional and molecular methods. We performed the Southern blot hybridization with radioactive labelled CARE-2 probes and random amplification of polymorphic DNA (RAPD). Patients ranged from17-68 years with a mean age of 33.83±9.07 years. Conventional methods identified C.albicans(82.51%), C.tropicalis(4.89%), C.krusei(4.19%), C.parapsilosis(1.49%), C.glabrata(3%), C.zeylanoids(0.69%), C.kefyr(2.1%), C.lusitaniae(0.69%), Rhodotorula(1.4%). Further C.albicans was reconfirmed by DNA fingerprinting method and Candida non-albicans by RAPD method. DNA fingerprint patterns showed that isolates collected from different patients over a period of time were different compared to some which were related; they were mostly identical only in different body locations of the same patient. DNA fingerprint patterns indicate that the source of infection is not common to all patients and suggesting that commensal or endogenous isolates might be turning into pathogens. Early accurate diagnosis of the source of Candidiasis is needed for better understanding and clinical management in HIV/AIDS patients.

1. Introduction:

Candida species are opportunistic pathogens which can cause diseases ranging from mucosal infections to systemic mycoses depending on the vulnerability of the host [1,2]. It is an important pathogen, especially in immunocompromised patients. *Candida* spp. is now considered fourth in the rank of the causative agents of nosocomial invasive infection with a high mortality and morbidity[3]**.** Overall data suggest that in most cases, the source of an infecting strain of *C. albicans* is endogenous flora, but that in certain circumstances transmission of more virulent strains may occur in the nosocomial setting. However, several reports of outbreaks of infection with *Candida* species support the hypothesis that exogenous acquisition (transmission of a strain from one patient to another) of the infecting yeast strain can occur [4].

The typing of *C. albicans* at the strain level has become crucial for medical mycology. For this species, as for all microbial pathogens, a reproducible and discriminatory strain typing system is of benefit for clinical and epidemiological studies to provide information on sources, carriage, and transmission of infection and on relations between strain types and properties, such as virulence and antimicrobial resistance [5].Thus, studying the relatedness of clinical strains is relevant in clinical management [6]. The capacity to assess genetic relatedness among strains not only provides us with insights into the population structure and mode of propagation of Candida species [7], but also with information on the epidemiology of infections [8, 9].

Over the past 20 years, a number of technologies have been used to assess the level of strain relatedness [10]. Molecular typing methods should be reproducible, discriminatory, high throughput, easy-to-use, digitally portable and amenable to standardization and library typing [8]. These methods have included multilocus enzyme electrophoresis (MLEE), restriction fragment-length polymorphism (RFLP), Southern blot hybridization of restriction fragments with repetitive or "complex" probes, random amplification of polymorphic DNA (RAPD), electrophoretic karyotyping, and a variety of sequencing methods, including multilocus sequence typing (MLST) [10]. Southern blot hybridization of restriction fragments with complex probes provides a highly reproducible method for assessing genetic relatedness . RAPD analysis also suggested being an adequate method for monitoring overall genome flexibility of medically important *Candida* species and has been extensively applied in *C. albicans* strain typing [11].

In the present study, an attempt was made to identify, characterize the *Candida* species isolated from HIV/AIDS patients. For this purpose we performed the Southern blot hybridization of restriction fragments with radioactive labelled CARE-2 probes and random amplification of polymorphic DNA (RAPD).

2. Material and Methods:

2.1. Study population and design: Two hundred symptomatic confirmed HIV-positive adult patients, of both sexes, suspected of having a fungal infection were taken as subjects. Cases were recruited from the outpatient department, wards and the Anti-Retroviral Treatment Clinic of Lok Nayak Hospital and GB Pant

Hospital, New Delhi, India. All patients were evaluated by a predesigned protocol covering the biodata, history, including high-risk behavior, mode of transmission, marital status, partner status, presenting complaints and physical examination. Depending on the clinical symptoms, relevant clinical samples were collected with complete universal precautions.

The samples were subjected to direct microscopy using Gram staining and KOH wet mount. Fungal culture was done on Sabouraud dextrose agar, with and without chloramphenicol (16µg/ml). Specimens were streaked in duplicate; one set of inoculated slants was incubated at 25°C and the other at 37°C, and were examined every other day for growth up to 4-6 weeks before discarding as negative. The identification and speciation of the isolates was conducted by colony morphology, Gram staining, germ tube formation, growth on corn meal agar with Tween 80, HiCrome Candida morphology agar and an enzymatic triphenyl tetrazolium chloride reduction test as per standard procedures[12]. For further characterization, each isolate was subjected to carbohydrate assimilation and carbohydrate fermentation tests as per standard recommended procedures.

2.2.Restriction Fragment-Length Polymorphism (RFLP): The genetic relatedness of *C. albicans* isolates obtained from HIV/AIDS patients was tested by Southern blot hybridization using the moderately repetitive, *C. albicans*-specific DNA fragment CARE-2 [13, 14, 15] CARE-2 (kind gift from Dr. B. A. Lasker, Centers for Diseases Control, Atlanta), To accomplish this, Chromosomal DNA was isolated from each isolate using conventional technique after lysis of the cells by Zymolyase (Seikagaku, Japan) treatment. Around 2 µg chromosomal DNA from each isolate was digested with restriction enzyme EcoRI to completion. Digested DNA samples were then separated on agarose gel (0.8%) in 1x TBE buffer (89 mM Tris-borate, 1mM EDTA) by applying a voltage gradient of 2 volts/cm for a period of 20 hours, stained with Ethidium bromide (0.5g/ml), destained, visualized under UV and photographed using gel documentation system (Alpha Innotech, USA). Immobilized fragmented DNA were hybridized with [^{32}P] dATP (Amersham Pharmacia Biotech, U. K.), labeled *C. albicans* specific probe CARE-2 (13), at the same temperature for 16 hours. The nylon membrane containing the hybridized DNA fragments were then washed several times with 2x SSC containing 0.1% SDS exposed to X-ray film at – 80°C for 16–24 hours and developed [15]. Dendrogram was generated by comparing the relatedness of DNA band pattern in the autoradiogram, by computing the similarity coefficient (S_{AB}) of the bands, using the software Dendron software package (version 3.0; Solltech, Iowa City, Iowa) as described by Pujol et al. [16].

2.3.Rapid Amplified Polymorphic DNA (RAPD) was performed using self designed twenty random oligonucleotides and obtained from Sigma SUN-1 to SUN-20), primers composed of 10 bases and containing various percent GC contents. The primer which gave the most discriminative result was SUN-12. Different PCR conditions were used to optimize conditions for better amplicons products. PCR carried out with about 50 ng of DNA; 200 µM (each) dATP, dCTP, dTTP, and dGTP; 50pmol of oligonucleotides; 0.25 U of Taq polymerase (NEB Bio-lab.); and PCR buffer. The final volume of the reaction was 30 µl. The cycling conditions were 94°C for 2 minutes, then 30 cycles of denaturation at 94°C for 1 minute, 30 cycles of annealing 42°C for 1 minute and at extension at 72° C for 2 minutes. Final extension was given for 5min at 72 ° C. Amplified products (30 µl) were resolved by agarose 1% gel electrophoresis at 100V for 1.5 hours [17].

3. Results:

A total of 314 samples comprising of oral swabs collected from all patients, while sputum, blood, CSF, and urine were collected from 39(12.4%), 32(10.2%), 23(7.32%), 09(2.87%) patients respectively depending on the organ system involved. Yeasts were isolated in one twenty patients and among these patients *Candida* species were isolated in one forty three samples while one seventy one samples were sterile.

By Conventional method the isolates were identified as *C.albicans(*82.51%), *C.tropicalis(*4.89%), *C.krusei(*4.19%), *C.parapsilosis(*1.49%), and *C.glabrata(*3%), *C.zeylanoids(*0.69%), *C.kefyr(*2.1%), *C.lusitania(*0.69%), *Rhodotorula(*1.4%). For further characterization and conformation of results *Candida* isolates were subjected to molecular techniques like RFLP (Restriction fragment length polymorphism) and RAPD (Rapid amplified polymorphic DNA). RAPD was done for species identification of *Candida non albicans* isolates.

All *C.albicans* were reconfirmed by RFLP method using middle repetitive (1.06 kbp) DNA probe CARE-2 giving 10-14 bands while *Candida non albicans* isolates showed no bands the results are presented in Figure 1. All the *Candida non albicans* were subjected to RAPD- PCR by using self designed twenty random oligonucleotides. The primer which gave the most discriminative result was SUN-12. The isolates were identified on the basis of the band sizes of the different species on comparison with those of the corresponding ATCC strains *Candida non albicans* were identified as, *C.tropicalis*(6.29%), *C.krusei*(4.89%), *C.parapsilosis*(3.49%), and *C.glabrata*(2.1%) the results were presented in Figure 3. The identification of *Candida non albicans* from conventional and RAPD method were presented table 1

Molecular typing of *C.albicans* isolates by RFLP revealed a complex set of hybridization profiles with CARE-2 probe (Figure1&2). Computer generated dendrogram by Southern blot hybridization provide useful information, whether all the strains were identical, highly related, related or different. SAB of 1.00 representing identical banding patterns, 0.00 to <0.80 representing patterns with no matching i.e different, 0.80-0.89 representing related banding patterns and 0.90-0.99 representing highly related banding patterns (Figure 4).

In five of our patients *C.albicans* isolated both in oral and sputum samples of the same patients were identified to be genetically identical while *C.albicans* in two patients isolated from oral and sputum samples were identified as genetically highly related and related respectively thereby suggesting that the origin of the isolates was probably endogenous (Table 2).

Eight different patients had four identical sets of *C.albicans* isolated from different samples namely two of (oral swab –oral swab) and one each of (blood culture – oral swab) and (sputum – oral swab) while fourteen patients had six sets of highly related isolates namely three of (oral swab- oral swab), two of (sputum – oral swab) and one each of (blood culture- oral swab - oral swab) from different samples. Nineteen patients had nine related sets of isolates namely three sets of (oral swab – sputum – oral swab) and (oral swab- oral swab) and single sets of (sputum – oral swab), (oral swab – oral swab – oral swab – oral swab) and (sputum – oral swab – oral swab). Among hundred isolates ninety single isolates were isolated from ninety different patients and ten isolates in sets of two were isolated from five different patients. The organisms isolated from different sites might not be responsible for causation of disease in an endogenous fashion and could be due to cross contamination between *C.albicans* strains.

4. Discussion:

Candida isolates from HIV/AIDS patients in our study include, 82.51% *C.albicans*, 4.89% *C.tropicalis*, 4.19% *C.krusei*, 1.49% *C.parapsilosis*, and 3% *C.glabrata*, 0.69% *C.zeylanoids*, 2.1% *C.kefyr*, 0.69% *C.lusitaniae*, 1.4% *Rhodotorula spp.* There is not much change observed in predominant species of *Candida* reported in the present study versus the report in 2007 from the

same institution, where *C.albicans* was also heading the list of all the isolates [18] in spite of the fact that the presently studied patient population was much larger and the duration of the study was much longer. *C.albicans* was also the most frequently observed species in a study in USA in 2001 [19]. This is a surprising observation compared to other from developed world, where *Candida non albicans* is a major problem in HIV/AIDS [20,21,22].

A study in Nigeria in 2011 on HIV patients showed a relatively lower percentage of *C.albicans* (45%), *C.tropicalis* (18.3%), *C.parapsilosis* (15%), and *C.guilliermondii* (9.2%) [23]. Again a study from Iran in 2010 [24] also reported the most abundant species isolated from HIV/AIDS patients (total 234 isolates) to be *C.albicans* (50%) followed by *C.glabrata* (21.4%), *C.dubliniensis* (13.3%), *C.krusei* (9.8%), C.*kefyr* (3.1%), *C.parapsilosis* (1.6%), and *C.tropicalis* (0.8%). Workers from Ethopia in 2013 also reported *C.albicans* species to be predominant in 78.5% HIV/AIDS patients with *C.glabrata* (22.5%) being predominant among *Candida nonalbicans* species followed by *C.tropicalis* (14.1%)[25].

Genotypical methods are known to provide significant advantages in typing microorganisms like *Candida* that are difficult to be typed with phenotypical methods. Species identification based on conventional phenotypic methods is often time-consuming and laborious and is hindered by the unstable and subjective nature of phenotypic characteristics, which are readily influenced by culture conditions. Conversely, molecular methods involving gene sequencing are objective, yield results that are uninfluenced by growth conditions, are frequently more rapid than phenotypic approaches and are capable of discriminating between fungi that fail to produce distinctive morphological features[28]. In our study *C.albicans (*82.51%), *C.tropicalis (*6.29%), *C.krusei (*4.89%), *C.parapsilosis (*3.49%), and *C.glabrata (*2.79%) were identified by molecular techniques.

Melo et al in 1998 observed RAPD results for identification of *C.albicans, C.tropicalis, C.parapsilosis, C.glabrata and C.krusei* were 100 % consistent with the results obtained by conventional diagnostic methods[26]. Reef et al. (1998) also analyzed 49 *C.albicans* isolates to determine the mode of acquisition of infection in hospitalized patients by restriction fragment length polymorphism analysis using genomic blots hybridized with the CARE-2 probe. But found that the method suffered from certain limitations such as they are more expensive and time consuming as well as needs skilled workers trained in such techniques to interpret the data [27].

In coming years, RAPD might prove to be a promising tool in determining the relationship between genotype and drug susceptibility of *Candida* species simultaneously. Unfortunately, there is lack of research work focused on establishing the RAPD tools for simultaneous discrimination of resistant isolates from the sensitive ones along with species identification.

In recent years, because of the increasing numbers of immunocompromised patients, molecular typing techniques have become fundamental for studying the epidemiology of *Candida* species. Molecular epidemiology methods are required for the demonstration of clonal relationship among isolates. DNA fingerprinting of the infectious fungi has become an important subdiscipline of medical mycology. As DNA fingerprinting is more frequently applied to a variety of epidemiological problems. Species typing is a necessary first step in all epidemiological studies, but one must have a way of assessing the relatedness of isolates within a species if one wants to understand many of the epidemiological questions that are posed [8].

The purpose of using molecular technique was not only to identify the species but to have the knowledge about the commensal status of the OPD/Ward patients surveyed; it was not possible to speculate about the primary source of infection and totally to rule out the possibility of a commensal organism becoming a pathogen. Southern blot hybridization of restriction fragments with complex probes provides a highly reproducible method for assessing genetic relatedness. Dendrograms based on banding patterns generated by Southern blot hybridization of moderately repetitive sequences have been shown to provide a particularly useful representation of the relationship observed between strains and groups of strains, SAB of 1.00 representing identical banding patterns, 0.00 representing patterns with no matching bands, and 0.01–0.99 representing banding patterns of increasing similarity[29].

In our study in five of our patients, *C.albicans* were isolated from oral swabs and sputum samples of the same patients. They were identified as genetically identical while in two patients *C.albicans* strains from oral swabs and sputum sample were identified as genetically highly related and related respectively thereby suggesting the origin of the isolates to be probably endogenous. Whereas *C.albicans* isolated from (four different patients) in different samples like two sets of (oral swab- oral swab) (oral swab- sputum) and (blood culture and oral swab) were also identified as identical, leading to the possibility of such a result being due to cross contamination between *C.albicans* isolates like Marco et al., 1999 [30]reported in a study on blood cultures of ICU patients finding cross contamination between *C.albicans* isolates, Arif et al., 1996 [31]also reported cross-contamination between *C.glabrata* isolates of their ICU patients with no clonal relationships being found between *C.albicans* isolates of ICU patients and very few genotypically identical isolates being observed [32,33]. Similar results were found by Gulay et al. in 2002 in Turkey for *C.albicans* strains isolated from urine of Anesthesia ICU patients, and reported clonally-related isolates were obtained from different patients over a short-time interval, and the origin of most of the isolates was endogenous [34]. Like wise for highly related and related *C.albicans* isolates isolated from different patients in our study, the result could be due to cross contamination between *C.albicans* isolates and not being transmitted to the patients from hospital environment as the patients belonged to different setups in the hospital and not to the same ward or area. Genotypic methods of typing have increased in importance in recent years, as increasing incidence of nosocomial infections have begun to attract attention, but are still less prevalent than endogenous infections [29].

At the time of our study we were unable to carry out screening from hospital environment and identify *Candida* isolates at hospital admission so it was difficult to determine whether these patients became infected from a common source within the hospital environment or if the yeast was transmitted from first patients to the others.

DNA fingerprinting of all *C.albicans* isolates with CARE-2 probe was possible. Visual analysis of DNA fingerprint patterns showed that although isolates collected from different patients over a period of time were different and some were related: they were mostly identical only in different body locations of the same patients. This indicates the source of infection is not common to all patients and relatedness in different patients may be due to cross contamination of *Candida* isolates, suggesting that commensal or endogenous isolates might be turning into pathogens. Further quantitative analysis (Cluster analysis) of these fingerprint patterns was done using the software. The dendrogram generated, divided the isolates into unrelated groups. Similar to visual analysis dendrogram also revealed that most of the isolates collected from different patients were unrelated and though some of them showed relatedness but only isolates collected from different body locations of same patient were mostly highly related or identical. In our present work, we successfully

evaluated RAPD PCR as a diagnostic tool for typing of *Candida* species. The ideal typing method should be able to identify the same strain through independent isolates, group the related isolates and distinguish the completely unrelated isolates [35, 8, 36]. No method used currently meets all these criteria.

The RAPD or arbitrarily primed PCR (AP-PCR) analysis is technically simple and often detects variations among *Candida spp*. It has a good discriminatory capacity, low cost and is easy to use unlike, morphotyping which has low reproducibility. We could successfully utilize RAPD and RFLP analysis in our study, to investigate the molecular epidemiology of *Candida* isolates recovered from the patients hospitalized in a tertiary care hospital. RAPD fingerprinting is fast emerging as a method for diagnosing the species responsible for fungal diseases leading to the emergence and recognition of new and atypical varieties and species of opportunistic fungal pathogens as a consequence of the improved rate of survival of many immunocompromised patients due to rapid improvements in medical care. RAPD fingerprints along with definitive DNA probes can be rapidly developed and used as these microbes appear in many clinical situations.

5. Conclusion:

Candida albicans was the most predominant species isolated from HIV/AIDS patients, followed by *C.tropicalis* and *C.krusei*. Although different individuals can share the same or a very similar genotype, the data obtained appear to confirm with the majority of studies previously cited, which demonstrate that strains from different locations on the body of the same individual normally possess greater similarity than those derived from different hosts suggesting the role of endogenous organisms in the pathogenecity of the organisms. The mechanisms involved in the pathogenecity of these species however need to be better elucidated. This will likely be possible from more diversified research, which permits a greater understanding of the relations of these opportunistic parasites with their hosts.

Taken together, our results suggest that more clinical awareness is needed for the understanding of Candidiasis in HIV/AIDS patients, posing a serious problem, often unnoticed in Indian hospitals. Thus, this study is an attempt, to lead to a better clinical management of HIV/AIDS patients.

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Species	Conventional Methods	Molecular Methods RFLP	RAPD
C.albicans	118(82.5%)	118(82.5%)	--
C.tropicalis	7(4.89%)	NA	9(6.29%)
C.krusei	6(4.19%)	NA	7(4.89%)
C.parapsilosis	2(1.4%)	NA	5(3.49%)
C.glabrata	3(2.1%)	NA	4(2.1%)
C.zeylanoids	1(0.69%)	----	--
C.kefyr	3(2.1%)	---	--
C.lusitania	1(0.69%)	---	--
Rhodotorula spp	2(1.4%)	---	--

Table 1: ***Candida*** **species identified by conventional and molecular methods.**

	Genetic relatedness	Candida albicans in Same patients		Candida albicans in Different patients	
		No of patients	(Nos of isolates)	No of patients	(Nos of isolates)
SAB- 1.00	Identical	5	(1,2) (35,36) (65,66) (96,97) (123.124)	8	(89,141) (110,117) (121,122) (80,139)
SAB- 0.90- .99	Highly related	1	(20,21)	14	(101,143) (91,138) (129,140) (110,117,135) (107,131) (121,122,130)
SAB-0.80-0.89	Related	1	(79,80)	19	(65,66,133) (103,104) (79,80,139) (96,97,113) (55,57) (43,48) (30,38) (121,122,130,142) (101,143,108)
<0.80	Different			95	(90 single isolates from 90 different patients + 5 pairs of isolates (2 each) from five patients)

Table 2: Representing the genetic relatedness between ***Candida albicans*** **strains.**

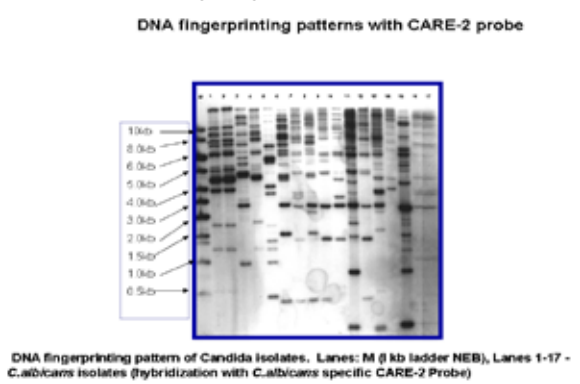


Figure 1: Representing DNA fingerprinting patterns of ***Candida albicans***

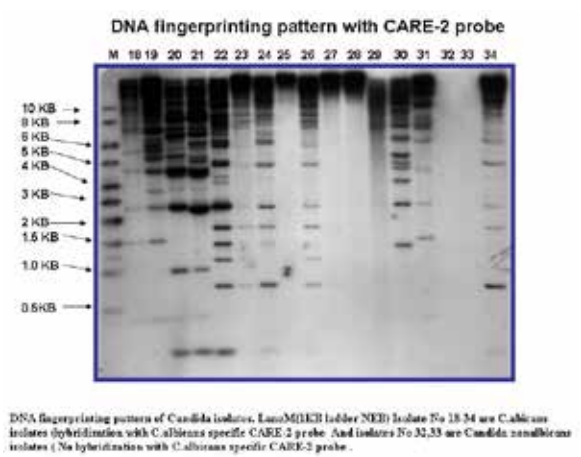


Figure 2: Representing Candida nonalbicans showing no banding pattern

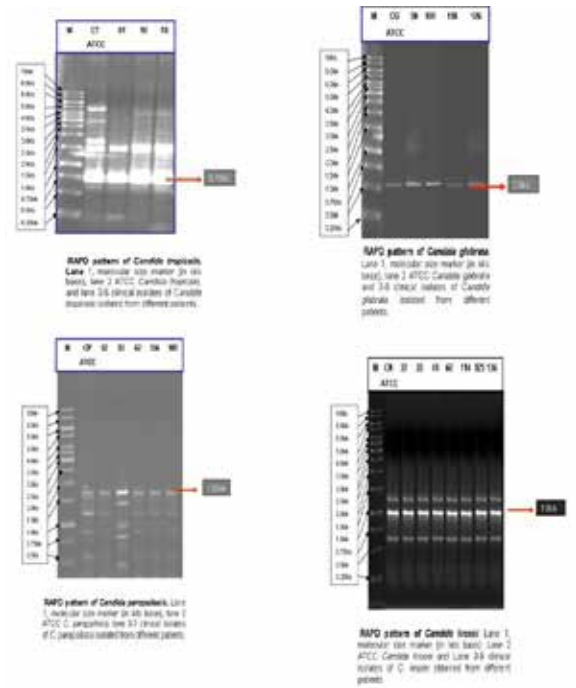
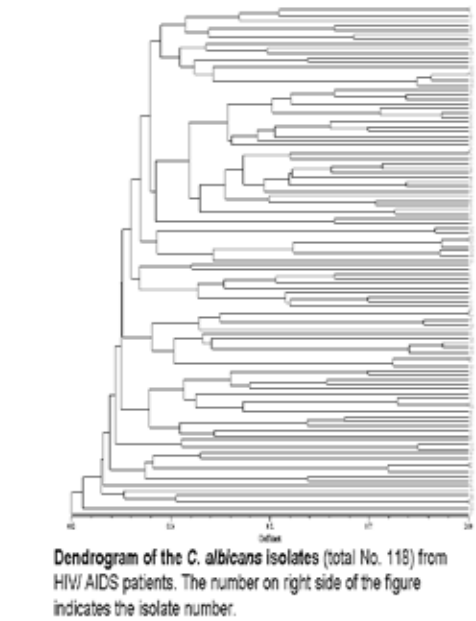


Figure 3: RAPD pattern of *Candida* Species



Dendrogram of the *C. albicans* isolates (total No. 118) from HIV/AIDS patients. The number on right side of the figure indicates the isolate number.

Figure 4: Dendrogram of the Candida albicans isolates from HIV/AIDS patients. The number on the right side of the figure indicates the isolate number.

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