

Identification of *Candida* species from Human Immunodeficiency Virus-infected Patients in Ethiopia by Combination of CHROMagar, Tobacco agar and PCR of Amplified Internally Transcribed rRNA Spacer Region

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ABSTRACT

The purpose of this study was to determine phenotypic and genotypic identification of *Candida* species from HIV infected patients in Ethiopia. Oral rinses from 13 human

immunodeficiency virus (HIV) positive patients were inoculated on CHROMagar. Colonies were then sub cultured on Tobacco agar. DNA was extracted from 71 colonies and amplified by PCR targeting the conserved regions of 18S, 5.8S, and 28S rRNAs. The amplified DNA samples were analyzed their sequence and a part of them were examined by restriction fragment length polymorphism (RFLP). Oral *Can-*

didia colonization was detected in all of patients with or without oral candidiasis. The number of *Candida* species on the CHROMagar was more than 1000 CFU/ml in 12 of 13 patients. We showed that phenotypic and genotypic identification process was important to determine the species. These results indicated that HIV-positive patients have high risk to *C. albicans* and the other *Candida* species infection in Ethiopia.

INTRODUCTION

Infections due to *Candida* spp. and other fungi have increased dramatically in recent years and are of particular importance because of the rising number of immunocompromised patients³. *Candida albicans* accounts for approximately 90% of *Candida* spp. isolated from yeast infected patients. However, during the last 20 years a marked shift in the spectrum of *Candida* species such as *C. tropicalis*, *C. glabrata* and *C. krusei* has been noted among different immunocompromised patients, with CD4 lymphocyte counts (less than 200 cells/mm³). These *Candida* species; *Candida tropicalis*, *Candida parapsilosis*, *Candida glabrata*, and *Candida krusei* are of increasing significance as they tend to be more resistant to antifungal agents^{2,4}. In Ethiopia, there have been no reports about *Candida* spp. from oral origin. A rapid screening approach is needed for the diagnosis.

While a number of useful tests based on phenotypic characteristics, such as discrimination based on colony color on CHROMagar *Candida* medium, growth at 42 to 45°C, and assimilation profiles, have been developed⁵⁻⁸, they are not completely reliable⁹. In this study, we determined in *Candida* species by combination of CHROMagar, Tobacco agar and polymerase chain reaction (PCR) of the amplified internally transcribed rRNA spacer region. We showed that phenotypic and genotypic identification process was important to determine the species. This work is a first step to determine the clinical features in HIV-associated candidiasis in Ethiopia.

MATERIALS AND METHOD

Thirteen HIV positive patients with different clinical stages were randomly selected at the Black Lion Specialized Hospital, Addis Ababa, Ethiopia. After informed consent was obtained, patients were instructed to provide a rinse of oral cavity. Oral rinses were obtained from patients by asking them to rinse their mouths with 10 ml of sterile distilled water for 30 seconds and to expectorate the rinse in to a sterile container. Then the fluid was poured to a CHROMagar (Paris, France) plate and after 30 seconds the plates was drained. In preliminary experiment, we determined 283.6 ± 23.6 μ l of fluid remained on the plate. The plate was then incubated at 37°C for 48 h. A presumptive identification was made based on the color of the colonies.

The identities of *C. dubliniensis* and *C. albicans* isolates were further confirmed by Tobacco agar, which is a new medium for differentiating *C. dubliniensis* from *C. albicans*. In addition, seventy-one clinical isolates each of *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis*, and *Candida krusei* were also tested for colony characteristics on tobacco agar. The method used for the preparation of tobacco agar was the same as that described by Tendolkar et al.¹⁰, except that we used cigarette tobacco instead of tobacco leaves according to the method of Khan et al¹¹. Briefly, 50 g of tobacco from commercially available cigarette brands (Marlboro; tar, 8 mg; nicotine, 0.6 mg; Philip Morris Products SA, Richmond, Va.) was mixed with 1 liter of distilled water. The mixture was boiled for 30 min and then filtered through several layers of gauze. To this filtrate, 20 g of agar was added, and the volume was made up to 1 liter. It was autoclaved at 121°C for 15 min. All the test isolates were freshly sub-cultured on Sabouraud dextrose agar (Difco, Detroit, MI), and tobacco agar plates were streaked with a small amount of inoculum from the isolated colonies. The culture plates were incubated at 30°C and observed daily up to 96 h for colony characteristics, such

as surface topography (rough or smooth), formation of hyphal fringes at the periphery, and color. In addition, tobacco agar prepared from Ethiopian cigarettes (Nyala, Ethiopia) was also used for comparison.

Seventy-one local clinical isolates and six reference strains were studied in this study. *C. albicans* IFM4009, *C. glabrata* IFM54350, *C. parapsilosis* IFM5774 and IFM5804, *C. tropicalis* IFM46821, and *C. dubliniensis* IFM54605 were kindly provided from Chiba University Research Center for Pathogenic Fungi and for Microbial Toxicoses.

The species identification of the test isolates was done by combination of PCR amplification of their rRNA genes corresponding to unique sequences within the internally transcribed spacer (ITS). Primers for PCR were designed for two separate areas of the DNA encoding the rRNA. The pair of primers, ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3'), targeting the conserved regions of 18S, 5.8S, and 28S rDNAs, have been reported in previous studies¹²⁻¹⁴. Furthermore, oligonucleotide primers were derived from rRNA genes of fungi and can be used for universal fungi PCR¹⁵. Forward primer ITS3 (5'-GCA TCG ATG AAG AAC GCA GC-3') corresponds to the 5.8S rRNA gene, and reverse primer ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') corresponds to the 28S rRNA gene of fungi.

PCR (ITS1/ITS4 and ITS3/ITS4 primer set, respectively) was performed in a total volume of 50 µl of mixture including 1 µl of extracted specimens according to the manufacture protocol (Takara Premix gamma Taq Takara, Japan). Samples were placed on Astec Program temp control system PC-800 DNA thermal cycler. After an initial step of 5 min at 94°C, 35 cycles were performed for 30 sec at 94°C, 30 sec at 55°C, and 2 min at 72°C. Finally, an additional extension was achieved for 2 min at 72°C, and samples were cooled to 4°C. These products were kept at -20°C until further processing.

PCR products were digested individually with 10 U of restriction enzyme Hae III by overnight incubation at 37°C. The resulting restriction fragments were analysed by agarose gel electrophoresis.

Gel electrophoresis with 3% agarose gel was conducted with Tris-acetate-EDTA buffer (20 mM Tris-Acetate, 0.5 mM EDTA, pH 8.3). A Novagen PCR Marker, 50-2000bp (Merk & Co, NJ, USA) was run concurrently with amplicons for sizing of the bands. Gel was stained with 0.01% (v/v) ethidium bromide solution for 20 min and then photographed.

PCR products were purified by Labo-Pass™ Products DNA Purification kit (COSMO Genetech Co., Korea) and dissolved in distilled water to a final concentration of 20 ng/µl. The PCR products with sequencing primer ITS1 or ITS4 were automatically sequenced with PRISM 3100 genetic DNA analyzer (Applied Biosystems, Weiterstadt, Germany) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany).

This study was conducted after obtained institutional ethical clearance and informed consent from the study subjects. All work was conducted in accordance with the Declaration of Helsinki (1964).

RESULTS

Phenotypic examination showed that oral *Candida* colonization was detected in all of patients. Eight patients had oral *candidiasis* such as pseudomembraneus, candidal leukoplakia and erythematous angular cheilitis. The number of *Candida* species on the CHROMagar (CFU/ml in rinse) was more than 1000 in 12 of 13 patients. There were no significant differences of number of candidal colonization between symptomatic and asymptomatic groups. The species isolated most frequently from the patients was *C. albicans* (12 of 13 patients), followed by *C. tropicalis* (11/13), *C. krusei* (9/13), *C. parapsilosis* (4/13), *C. guilliermondi* (2/13), *C. glabrata* (2/13), and *C. dubliniensis* (2/13) in phenotypic examinations. Seven patients had only *C. albicans* and 1 patient had only

C. tropicalis. Two or more *Candida* species were isolated from the other 5 patients.

The colony color and morphology of 71 isolates on CHROMagar were examined and *Candida* species were suspected. *C. albicans* and *C. dubliniensis* showed mint green colony. *C. albicans* showed slightly light color but it is difficult to differentiate *C. dubliniensis* from *C. albicans*. Forty-three isolates were considered to be *C. albicans* or *C. dubliniensis*. Purple to pink or purple 11 colonies were determined as *C. tropicalis*. Nine large rough colonies with whitish pink color were determined as *C. krusei*. Two isolates showed small smooth pink or red-purple colonies. These were determined as *C. glabrata*. Six non-colored isolates were suspected to be *C. parapsilosis* or the other species.

Fig. 1 shows flowchart for detection of yeast isolates from HIV-infected patients in Ethiopia. On Marlboro tobacco agar, 69 of 71 isolates showed white-to-cream colored colonies (Fig. 1). Two isolates produced rough, yellowish-brown colonies with peripheral hyphal fringes after incubation for 72 hours at 30°C and determined to *C. dubliniensis*. Thus, it is easy to differentiate *C. dubliniensis* from *C. albicans*. On

this medium, major isolates (*C. albicans*) showed smooth, white-to-cream-colored colonies without hyphal fringes after extended incubation for up to 10 days. Like *C. albicans*, none of the isolates of *C. tropicalis* and others formed yellowish-brown colonies on the Tobacco agar and thus were indistinguishable from each other. Similar observations were made when Nyala Tobacco agar prepared from Ethiopian cigarettes were used.

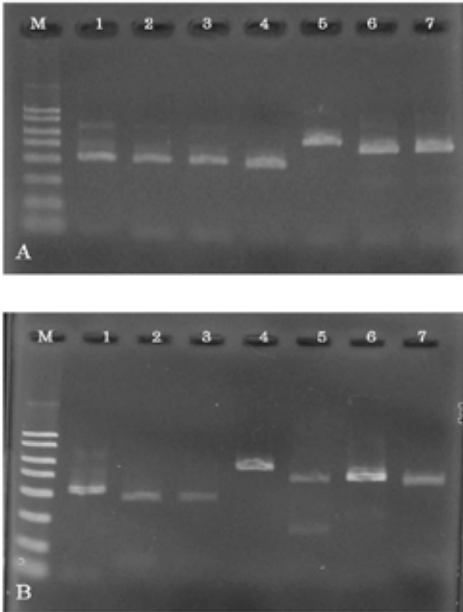
After determination of colony color, PCR was done by using primer sets as shown in Fig.1. All isolates were amplified using universal fungal primer ITS3-4. Most of isolates yielding a products as *C. albicans*, *C. dubliniensis*, *C. tropicalis*, *C. krusei* is approximately from 330 to 340 bp. Size of PCR products was 330 and 420 bp in six non-colored isolates. Two isolates of them were suspected as *C. parapsilosis* or the other species (Their species were identified as *C. tropicalis* after analyzing of DNA sequence). *C. glabrata* can be differentiated by the approximately 420 bp size of the PCR product. However, PCR products of former non-colored isolates also showed similar size. Size of PCR products was from 330 to 340 bp in reference strain of *C. albicans* IFM4009, *C. parapsilosis* IFM5774,

Fig. 1 Detection of yeast isolates from HIV-infected patients in Ethiopia.

Colony color on CHROMagar (No. of isolates)	Mint green (43)		Purple to pink, Purple (11)	Large rough whitish pink (9)	Small smooth pink, red purple (2)	Non-color (6)	
Colony color on Tobacco agar	White to cream	Yellow to brown (2)	White to cream	White to cream	White to cream	White-to-cream	
Species estimated	<i>C. albicans</i>	<i>C. dubliniensis</i>	<i>C. tropicalis</i>	<i>C. krusei</i>	<i>C. glabrata</i>	<i>C. parapsilosis</i> <i>C. tropicalis</i> <i>C. guilliermondii</i> <i>C. kefyr</i>	
PCR product (bps)	330-340		320-330	330-340	410-420	520-530	420-430
ITS3-4	330-340		320-330	330-340	410-420	520-530	420-430
ITS1-4	530-540		520-530	500-510	870-880	520-530	720-730
Fragment size after digestion of <i>Hae</i> III	90/440		80/440	40/60/370	200/670	80/440	20/70/630*
Results from sequence	<i>C. albicans</i>	<i>C. dubliniensis</i>	<i>C. tropicalis</i>	<i>C. krusei</i>	<i>C. glabrata</i>	<i>C. tropicalis</i>	<i>C. kefyr</i>
Identification of species	<i>C. albicans</i>	<i>C. dubliniensis</i>	<i>C. tropicalis</i>	<i>C. krusei</i>	<i>C. glabrata</i>	<i>C. tropicalis</i>	<i>C. kefyr</i>

* Small fragment 20-bp was not visual in agarose gel electrophoresis.

Fig. 2 Ethidium bromide-stained agarose gel of (A) PCR products obtained with primers ITS1 and ITS4. Lanes: M, molecular weight marker (2000, 1500, 1000, 750, 500, 350, 150, 50-bp, respectively), 1, *C. albicans* 2B3; 2, *C. tropicalis* 6W1; 3, *C. tropicalis* 6B02; 4, *C. krusei* 11R01; 5, *C. glabrata* 11V02; 6, *C. kefyfyr* 13V03; 7, *C. kefyfyr* 13V05 and (B) restriction digestion of PCR products obtained with primer ITS1 and ITS4 with *Hae*III, Lanes: M, molecular weight marker (2000, 1500, 1000, 750, 500, 300, 150, 50-bp ladder, respectively), 1, *C. albicans* 2B1; 2, *C. albicans* 2B1 (digestion); 3, *C. albicans* 2B3 (digestion); 4, *C. glabrata* 11V02; 5, *C. glabrata* 11V02 (digestion); 6, *C. kefyfyr* 13V03; 7, *C. kefyfyr* 13V03 (digestion). PCR products are obtained from clinical isolates.



C. tropicalis IFM46821, and *C. dubliniensis* IFM54605. The size was approximately 420 bp in *C. glabrata* IFM54350.

The intergenic spacer region (primer ITS1-4) was successfully amplified from all tested isolates (Fig. 1, Fig.2A). A distinct product size was obtained before and after *Hae*III enzyme treatment (Fig. 1, Fig.2B). A product of approximately 500-530 bp was obtained from *C. albicans*, *C. dublini-*

ensis, *C. tropicalis* including 4 non-colored isolates on CHROMagar and *C. krusei*. A product of approximately 870 bp was obtained from *C. glabrata*. A product of approximately 720 bp was obtained from 2 isolates. These 2 isolates were determined as *C. parapsilosis* on CHROMagar and size of PCR products using primer ITS 1 and ITS4 was near to *C. glabrata*. The product of 720 bp could not be obtained from *C. parapsilosis* IFM5774 and *C. glabrata* IFM54350. These isolates were determined as *C. kefyfyr* after sequencing.

The isolates were studied further by RFLP analysis following digestion of the PCR products by the *Hae*III. After *Hae*III digestion, *C. albicans*, *C. dubliniensis* and *C. tropicalis* yielded 80-90 and 440 bp. *C. krusei* yielded 40, 90 and 370 bp. *C. glabrata* yielded 200 and 670 bp. *C. kefyfyr* yielded 20, 70 and 630 bp.

Size of PCR products (ITS1-4) was from 520 to 540 bp in reference strain of *C. albicans* IFM4009, *C. parapsilosis* IFM5774, *C. tropicalis* IFM46821, and *C. dubliniensis* IFM54605. The size was approximately 870 bp in *C. glabrata* IFM54350. After *Hae*III digestion, *C. albicans* IFM4009, *C. tropicalis* IFM46821, and *C. dubliniensis* IFM54605 yielded 90 and 430-440 bp. *C. glabrata* IFM54350 yielded 200 and 670 bp after the digestion. A product of approximately 340 bp (ITS3-4) or 540 bp (ITS1-4) was obtained from *C. parapsilosis* IFM 5774. After *Hae*III digestion, the fragment size was 40, 110 and 390 bp, respectively, and such RFLP pattern was not observed in clinical isolates.

After sequence, the homology score between 18S ribosomal RNA gene from *C. albicans* (GenBank/EMBL/DBJ accession No. AY342214), *C. tropicalis* (EF196807), *C. krusei* (EF198013), *C. glabrata* (AY939794), *C. kefyfyr* (AF543841) was 99% in isolates as *C. albicans*, *C. tropicalis* and *C. kefyfyr* and 96% in isolates as *C. krusei* and *C. glabrata*, respectively.

DISCUSSION

The results presented here indicate that ma-

For species of *Candida* from clinical isolates in Ethiopia should be assigned to the species *C. albicans*. PCR can be useful for the differentiation of isolates to the species level by using RFLP. The method that detects the presence and the size of the intron in the 25S rDNA is particularly easily adapted for use in reference laboratories for the rapid identification of large numbers of isolates. The results obtained in this study demonstrated that by using the restriction enzyme *Hae*III the differentiation of *C. albicans* and non-*albicans* strains could be easy. Our isolates as *C. albicans* showed light mint color in CHROMagar. All of them showed two fragments of 90 and 430 bps after the enzyme treatment.

In this study, CHROMagar allowed the presumptive identification of *C. albicans*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*. Some authors have observed that *C. albicans* and *C. dubliniensis* may show colonies with different shade of green^{16,17}. The color intensity, however, is not restrictive for each isolates including *C. albicans* and *C. dubliniensis*. It is also difficult to determine them by using PCR and RFLP. Khan et al. reported that Tobacco agar provides a simple tool for presumptive differentiation of *C. dubliniensis* from *C. albicans*¹¹. Interestingly, we could confirm the usefulness of Tobacco agar for the aforementioned purpose.

There have been various molecular approaches for detection of fungi from clinical isolates. Targets of PCR amplification included 18S rDNA, 5.8S rDNA, intergenic spacer regions and 28S rDNA regions. The size of the 18S, 5.8S and 26S rDNA genes are essentially identical in all *Candida* species, while the lengths of the ITS regions depend on the species. The ITS region is located between the 18S and 26S rDNA genes and is subdivided into the ITS1 and ITS2 regions. The differences of sequences in these regions have been used to detect and identify fungi. We used the differences in the length of both ITS1 and ITS2 regions to determine *Candida* species. *C. glabrata*

and *C. kefyr* could be easily identified from *C. albicans*, *C. tropicalis* and *C. krusei*, because the PCR products from these 2 species were significantly larger than those of *C. albicans* and other species. However, molecular weights of PCR products were near among *C. albicans*, *C. tropicalis*, *C. dubliniensis*, *parapsilosis* and *C. krusei*. There was the risk for miss identification when species were determined by only a PCR amplification method.

Phenotypic characteristics on the selective agar medium such as CHROMagar and Tobacco agar are useful to determine *Candida* species with support of molecular diagnosis. The identification of *Candida* species with the amplification and sequencing of intergenic spacer regions and RFLP analysis is a practical and reliable method. It is useful for identification a part of clinically isolated *Candida* species, such as *C. guiliermondii* and *C. parapsilosis* identified by CHROMagar in this study. Tobacco agar is useful tool for identifying *C. dubliniensis*. The composition of this medium is very simple, brands of cigarette is not concerned and easy to make. The combination of these methods is useful for diagnosis oral candidiasis and risk judgment of the disease in patients with HIV.

CONCLUSIONS

Phenotypic and genotypic identification process was important to determine *Candida* species. *C. albicans*, *C. tropicalis* and *C. krusei* were major species. In Ethiopia, HIV-positive patients have high risk to *C. albicans* and the other *Candida* species infection.

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