



Use of universal *18SrDNA* gene and CHROMagar *Candida* medium for the identification of *Candida* species isolated from denture wearers.

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Abstract

Candida spp. are considered the most important species able to cause oral infections in denture wearers during the past two decades, from 35 swab samples were isolated from denture wearers showed only 12(34.3%) positive culture result on SDA , PCR products test (genotypic methods) to the universal *18SrDNA* fungi gene amplified for these positive culture isolated gave bands at the position 260bp when compared with standard molecular DNA ladder. In this investigation, chromogenic agar test (phenotypic methods) products showed 7(58.3%), Pink, mauve colonies were identified as *Candida krusei*, 3(25%) blue colonies were identified as *Candida tropicalis* and 2(16.7%) light green colonies considered as *Candida albicans*, and showed [*C. krusei*, *C. tropicalis*] were negative to germ tube formation test and *C. albicans* was positive to germ tube test.

Key words: *Candida* spp., denture wearer, universal *18SrDNA*, CHROMagar *Candida*.

1- Introduction

In recent years, there has been a significant increase in the incidence of human fungal infections[1].*Candida* species are found in the oral cavity of 60%–100% of denture-wearing persons[2]. Several studies on oral *Candida* colonization have shown that *Candida albicans* is the most frequently isolated species, but a growing trend toward recovery of non-*albicans* species, including *Candida glabrata*, *Candida tropicalis*, *Candida krusei*, and *Candida dubliniensis*, has been reported in the last decade [3]. These species have also emerged as etiological agents in oral candidosis, both as co-infecting organisms with *C. albicans* and as sole pathogens themselves [4]. Denture wearing is recognized as a predisposing factor for oral colonization by *Candida* species and a relationship between oral hygiene habits, denture cleanliness, yeast carriage and denture stomatitis has been proven, the species isolated in denture wearers did not significantly differ from those encountered in dentate individuals or in edentulous patients not wearing dentures, with *C. albicans* being the most prevalent species [5].

Therefore, *C. albicans* is the major species found as a commensal in healthy individuals, with four other species, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, and *C. krusei*, also found. *Candida* species, however, have an alternative lifestyle, causing opportunistic infection in hosts with altered physiological or immune response, the infections caused by *Candida* species range from self-limiting,

superficial mucosal lesions (commonly referred to as thrush), chronic and/or recurrent mucosal, skin,

and nail infections, through to life threatening invasive or disseminated infection [6,7]

Recently, universal primers common to all fungi were used as a promising approach for clinical microbiological diagnosis [8]. Thus far, a few techniques were utilized in the past to classify and recognize medically important fungi [9, 10]. In the same context, other researchers put forth a novel approach for distinguishing opportunistic fungal pathogens by employing polymerase chain reaction (PCR)-based amplification of the conserved region of the *18S rRNA* gene, followed by single-stranded conformational polymorphism (SSCP) assay, however, the applicability of this technique has never been sought in ocular infections, since SSCP was used in the past to discriminate between and to characterize medically important opportunistic fungi, we decided to look for an alternative application of the above strategy [11]. As the traditional methods are tedious and time consuming to perform in the routine laboratories numerous isolation media are available in the market that can identify pathogens within 4_72 hours, depending upon the system [12,13]. Several brands of chromogenic media are available for rapid identification of yeast, these special media yield microbial colonies with varying pigmentation secondary substrates that react with enzymes secreted by micro organisms [14]. These media are species-specific, allowing the organisms to be identified to the species level by their color and colonial



characteristics, the manufacturer of CHROMagar Candida currently advertises its product as able to detect and differentiate three species, *C. albicans* by

growth as light to medium green colonies, *C. tropicalis* by growth as steel blue colonies accompanied by purple pigmentation diffused into surrounding agar, and *C. krusei* by growth as large, fuzzy, rose colored colonies, after incubation for 48 hours at 37°C, as also reported in several studies. Detection of Candida on CHROMagar Candida from poly fungal specimen also allows direct and more rapid and specific identification of *C. albicans* and other spp. which could decrease the time required to obtain results, thus, use of chromogenic media in clinical microbiology laboratories for the isolation and presumptive identification of important Candida species is easy to perform, requires less time and is cost effective tool [15,16]. CHROM agar Candida contains enzymatic substrates which are linked to chromogenic substrates, these substrates acted upon by different enzymes produced by *Candida* species, these enzymes results in colour variations which then useful for the presumptive identification of the yeasts [17].

In this study our goal was to isolated and identification *Candida spp.* from denture wearers and evaluate the usefulness of CHROMagar Candida for detection and identification of major Candida species with accuracy to reduce the time of identification.

2-materials and methods:

1-sampling:

Thirty-five sample were isolated from either maxillary or mandibular complete denture wearers Patients for at least one year with or without denture stomatitis were selected from both genders aged between 25-60 years old attending the outpatient clinics of Basra Province, Iraq. All samples were collected from the contiguous denture surface by sterile cotton wool swabs before a examination with the doctor under sterile condition. Samples were directly immersed in brain heart infusion broth and incubated for 24-48 h at 37°C. then streaking on the sabouraud dextrose agar (SDA, Himidia) and incubated for (with chloramphenicol) 16 mg/mL, (Neo Química). The plates were incubated at 37°C for 48 h. [18].

2-Genotypic(molecular) diagnosis:

2-1-Extraction of DNA

DNA of *Candida spp.* was extracted by picking single colony using sterile loop and suspended into

300 µl of lysis buffer (10 mM Tris, 1mMEDTA (pH8),1% SDS, 100 mM NaCl, 2% Tween 80), 300 µl phenol-chloroform (1:1); it was shaken for 5 min and centrifuged at 10000 rpm. The supernatant was transferred to new tube and equal volume of chloroform was added, mixed and centrifuged. 500µl ethanol was added to the supernatant and centrifuged at 10000 rpm for 7 min. Dry DNA pellet was re-suspended in 100 TE buffer and stored at -20°C until use, DNA were run on 0.8% agarose gel containing 1 µl ethidium bromide, DNA bands were detected and examined under UV. Transilluminator (Cleaver, USA) and photographed [19].

2-2-PCR assay

The primers used for the amplification universal 18SrDNA gene : Forward 5'-GTG AAA TTG TTG AAA GGG AA-3'-3 and Reverse 5'-GAC TCC TTG GTC CGT GTT-3' [20]. The PCR mixture volume was amplified by thermal cycler PCR System (TC-3000 in PCR Thermal Cycler, USA) of the final volume of 25 µl containing 5 µl DNA template, 1 µl (10 pmol) forward primer, 1 µl (10 pmol) reverse primer, 12.5 µl master mix and 5.5 µl nuclease free water. The PCR program involved initial denaturation at 94°C for 15 min, 30 cycle (denaturation at 94°C for 30sec, annealing at 50°C for 1 min and extension at 72°C for 2 min) and final extension at 72°C for 15 min. The PCR products were run on 2% agarose gel containing 1 µl ethidium bromide using a 50 bp ladder (Bioneer) as molecular weight marker. The gel was examined on an ultraviolet transilluminator and photographed.

3-Phenotypic diagnosis

3-1-Chromogenic agar culture test

Each isolate was cultured on SDA at 37°C for 48 h. After this, they were seeded on CHROMagar™ Candida (CHROMagar Candida, France), and incubated at 37°C for 48 h. The CHROMagar™ allows selective yeast isolation, identifying colonies of *C. albicans*, *C. tropicalis* and *C. krusei* by morphology and color reaction [15, 16,21].

3-2- Germ tube formation test

A loopful of yeast cells suspension was inoculated into 0.5 ml of human serum and incubated at 37°C for 3 h. After incubation period, it was examined under field microscope. Germ tube was considered as a lateral tube without septum and had no constriction at initiating site, which is a positive test for *C. albicans* [22].

3-Results and Discussion

3-1-molecular diagnosis

only 12(34.3%) from 35 specimens were positive culture for *Candida* spp. on agar. The presence of a denture in the oral mucosa with the increasing time of the denture use, over time, changes occur in the surface acrylic base, such as increased surface roughness ,changes in oral microflora and alters the local environmental conditions to lead increase of microorganisms formed biofilm causing infection , systematic disease and denture stomatitis [23,24] . However, there have

been relatively few studies on non *candida albicans candida* spp. in vitro that isolated in dentures wearers in general in Iraq .

These specimens were extracted and examined by agarose gel electrophoresis as figure (1). The extracted DNA for all isolates were subjected to PCR for amplifying universal *18SrDNA* gene, PCR products for the universal *18SrDNA* primers isolated gave bands on agarose gel at the position 260bp when compared with standard molecular DNA ladder as figure (2). universal fungi primer successful in amplification of target region of *18S rRNA* genes for all fungi species preventing to lose any possible or new specie in agreement with [6].

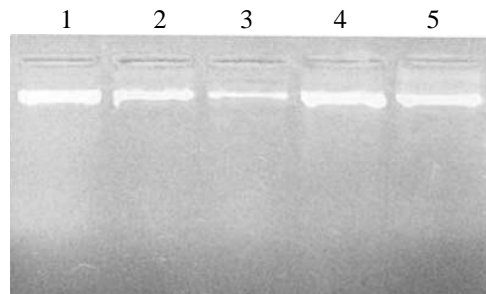


Figure (1): Agarose (0.8%) gel electrophoresis for DNA bands (1-5) of *Candida* spp. isolates from dentures under UV transilluminator .

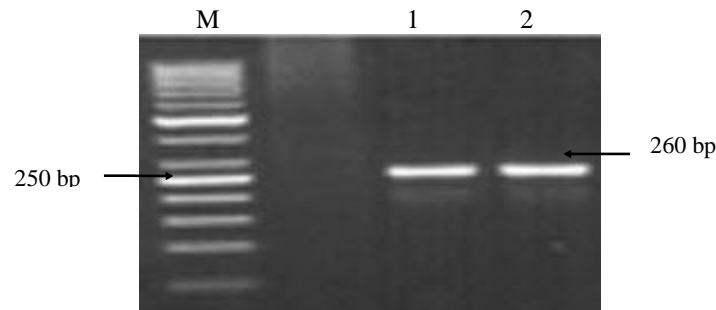


Figure (2):Agarose (2%) gel electrophoresis of universal *18SrDNA* PCR products for *Candida* spp. isolates from denture under UV transilluminator.(M): (50 bp – 1000bp) DNA ladder. Lane 1-2: *18SrDNA* bands(260bp) for *candida* spp.

3-2-phenotypic diagnosis

Total of 12(34.3%) isolates from *Candida* spp. showed 10(83.3%) isolates they failed to produce germ tube these are 7(58.3%) isolates of *C. krusei* appear as fuzzy, rose colonies belong to and 3(25%) isolates of *C. tropicalis* appear as blue colonies on chromogenic medium but 2(16.7%) isolates of *c. albicans* have the ability to form germ tube and showed green colonies on chromogenic medium. Commensal existence of oral *Candida* species varies from 20% to 50% in dentulous population ,as growth on surfaces is a natural part of

the *Candida* lifestyle [25, 26, 27]. Thus one can expect that *Candida* spp. colonized denture wearers. At the first time in Iraq especially in Basra Governorate isolation and identification of *C. krusei* 7(58.3%) and fuzzy ,pink colored on chrome agar , *C. tropicalis* 3(25%) and blue colony colored an chrome agar and *C. albicans* 2(16.7%) and green colored an chrome agar with germ tube formation were cultivated from patients with and without DRS this agreement with previously published reports [28,29,30,31] . The germ-tube production test has the advantage to be simple and efficient in the



economical and fast identification of *C. albicans* [32, 33].

CHROMagar Candida had allowed the growth of most clinically relevant yeasts and also allowed presumptive identification of *C. albicans*, *C. tropicalis*, *C. krusei*, CHROMagar Candida also facilitated recognition of specimens containing mixtures of yeast species, and was also suitable for primary isolation of yeasts from blood cultures [17]. The ability of adherence of *Candida* spp. to saliva-coated acrylic resin may play a role in establishing itself on the denture surface [34]. However, *Candida* biofilms formation has been described on polymethylmethacrylate strips which occur essentially in three overlapping phases: early (0-11h), intermediate (12-30h), and maturation (38-72 h) phases, the early stage is characterized by adherence and development of blastospores into distinct microcolonies, by 18 to 24 h, the *Candida* biofilm community can be seen as a bilayered structure comprising a mixture of yeasts, germ tubes, and young hyphae; this intermediate phase is distinguished by the production of extracellular polymeric substance (EPS), during maturation, the biofilms becomes a thick EPS layer in which a dense network of yeasts, pseudohyphae, and hyphae are embedded [25,35].

Hence, we conclude isolation and identification *candida* spp. at the first time in Iraq like *C. krusei*, *C. tropicalis*, and *C. albicans* adherence on the denture surface, and showed the specificity and sensitivity of CHROMagar Candida for *C. tropicalis* calculated as 99%, and *C. krusei* it is 100% for *C. albicans* calculated as 98%. Furthermore CHROMagar Candida (CHROMagar Candida, France) can be used as culture medium for the primary isolation and presumptive identification of organisms in cases where early diagnosis of infections is needed without doing PCR and biochemical test also the CHROM agar is a simple, rapid and inexpensive method with good sensitivity and specificity for identification of such species.

Reference

1- Lass-Flörl, C." The changing face of epidemiology of invasive fungal disease in Europe" [Mycoses](#), 52(3):197-205,2009.

2- [Loster, B.W.](#), [Loster, J.](#), [Wieczorek, A.](#), [Ryniewicz, W.](#) "Mycological analysis of the oral cavity of patients using acrylic removable dentures" [Gastroenterol Res Pract](#), 951572:1-9, 2012.

3-Reichert, P.A., Schmidtberg, W., Samaranyake, L.P., Scheifele, B. C."quid-associated oral lesions and oral *Candida* species in a female Cambodian cohort" *J Oral Pathol Med*, 31: 468–472, 2002.

4-Redding, S.W "The role of yeasts other than *Candida albicans* in oropharyngeal candidiasis. " *Curr Opin Infect Dis*, 14: 673–677, 2001.

5-Budtz-Jørgensen, E., Mojon, P., Rentsch, A., Deslauriers, N. "Effects of an oral health program on the occurrence of oral candidosis in a longterm care facility" *Community Dent Oral Epidemiol*, 28: 141– 149, 2000.

6-Jaeger, E.E., Carroll, N.M., Choudhury, S., *et al.* "Rapid detection and identification of *Candida*, *Aspergillus*, and *Fusarium* species in ocular samples using nested PCR." *J Clin Microbiol*, 38:2902-2908, 2000.

7-Kirkpatrick, C. H., "Chronic mucocutaneous candidiasis" *Eur. J. Clin. Microbiol Infect Dis*, 8(5): 448–456, 1989.

8-White, T.J., Bruns, T., Lee, S., Taylor, J. "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics In: PCR Protocols: a guide to methods and applications. (Innis M.A., Gelfand D.H., Sninsky J.J., White T.J.(eds)" Academic Press San Diego, 315-322, 1990.

9-Hopfer, R.L., Walden, P., Setterquist, S., Highsmith, W.E "Detection and differentiation of fungi in clinical specimens using polymerase chain reaction (PCR) amplification and restriction enzyme analysis" *J Med Vet Mycol*, 31:65–75, 1993.

10-Maiwald, M., Kappe, R., Sonntag, H.G. "Rapid presumptive identification of medically relevant yeasts to the species level by polymerase chain reaction and restriction enzyme analysis" *J Med Vet Mycol*, 32:115–22, 1994.

11-Walsh, T.J., Francesconi, A., Kasai, M., Chanock S.J. "PCR and single-strand conformational polymorphism for recognition of medically important



- opportunistic fungi" J. Clin. Microbiol ,33:3216–20, 1995.
- 12-Schuffenecker, I., Freydiere, A., de Montclos, H., Gille, Y "Evaluation of four commercial systems for identification of medically important yeasts" Eur J Clin Microbiol Infect Dis, 12: 255_60, 1993.
- 13-Fenn, J.P., Segal, H., Barland, B., Denton, D., Whisenant, J., Chun, H., *et al.* "Comparison of updated Vitek yeast biochemical card and API 20C yeast identification systems" J Clin Microbiol, 32: 1184_7 1994.
- 14-<http://www.chromagar.com>
- 15-Pfaller ,M. A., Houston, A. & Coffmann, S. "Application of CHROMagar Candida for rapid screening of clinical specimens for *Candida albicans*, *Candida tropicalis*, *Candida krusei*, and *Candida (Torulopsis) glabrata*" J Clin Microbiol, 34:58–61, 1996.
- 16-Willinger, B., Hillowoth, C., Selitsch, B. , Manafi, M. "Performance of *Candida ID*, a new chromogenic medium for presumptive identification of *Candida* species in comparison to CHROMagar *Candida*" J Clin Microbiol ,39: 3793–3795, 2001.
- 17-Reddy, P. S., Athuluri, V. "Definite differentiation of *Candida Albicans* from other species by using chrom agar " J Microbiol Biotech Res, 2 (6):936-941 ,2012.
- 18-Milan, E.P.; Zaror, L. "Leveduras: identificação laboratorial. In: Sidrim, J.J.C. & Rocha, M.F.G. (eds) *Micologia Médica à luz de autores contemporâneos*" Rio de J G Koogan, 89-101 ,2004 .
- 19-Mousavi, S.A., Khalesi, E., Shahid, B.G.H., Aghighi, S., Sharifi, F. , Aram, F. "Rapid molecular Diagnosis for *Candida* species using PCR-RFLP" Biotech., 6:583-587, 2007.
- 20- Anand, A.R., Madhavan, H.N., Neelam, V., Therese, L.K. "Use of polymerase chain reaction in the diagnosis of endophthalmitis" Ophthalmology, 108:326–30, 2001.
- 21-Pereira-Cenci, T., Cury, A.A., Cenci, M.S., Rodrigues-Garcia, R.C. "*In vitro Candida* colonization on acrylic resins and denture liners: influence of surface free energy, roughness, saliva, and adhering bacteria" Int J Prosthodont ,20:308-10, 2007.
- 22-Marinho, S.A, Teixeira, A.B, Santos O.S, Ricardo, F. C., Ferreira, C.A.S, Cherubini, K., de Oliveira, I.S.D. "Identification of *Candida* spp. by phenotypic tests and PCR" Braz J Microbiol, 41:286-294, 2010 .
- 23-Hospenthal, D.R., Beckius, M.L., Floyd, K.L., Horvath, L.L., Murray, C.K. "Presumptive identification of *Candida* species other than *C. albicans*, *C. krusei* and *C. tropicalis* with the chromogenic medium CHROMagar *Candida*" A Clin Microbiol Antimicrob, 5: 1-5, 2006 .
- 24-Tay , L. Y . , Herrera , D.R . , Gomes , B .P., dos Santos, F.A. ,Jorge, J.H. "Identification of *Candida* Spp. In Patients with Denture Stomatitis: Relationship with Gender, Age, Time of Denture use and Newton’s Classification" J Dent App,1(3): 46-50, 2014.
- 25-Quiryneen, M., Marechal, M., Busscher, H.J., Weerkamp, A.H., Darius, P.L., van Steenberghe, D. "The influence of surface free energy and surface roughness on early plaque formation. An *in vivo* study in man" J Clin Periodonto, 17:138-44,1990.
- 26-Samaranayake, L.P. "Oral mycoses in HIV infection" Oral Surg Oral Med Oral Pathol,73:171-80,1992.
- 27-Kumamoto, C.A., Vinces, M.D. "Alternative *Candida albicans* lifestyles: growth on surfaces" Annu Rev Microbiol,59:113–33, 2005.
- 28-Beighton, D., Ludford, R., Clark, D. T., Brailsford, S. R., Pankhurst, C. L., Tinsley, G. F.,Fiske, J., Lewis, D. ,Daly, B "Use of CHROMagar *Candida* medium for isolation of yeasts from dental samples" J Clin Microbiol, 33: 3025–3027 , 1995.
- 29-Hospenthal, D. R., Murray, C. K., Beckius, M. L., Green, J. A. , Dooley, D.P. "Persistence of pigment production by yeast isolates grown on CHROMagar *Candida* media" J Clin Microbiol, 40: 4768–4770 , 2002.



30-Gasparoto, T. H., Dionísio, T. J., de Oliveira, C. E., Porto, V. C., Gelani, V., Santos, C. F.,

Campanelli, A. P., Lara, V. S. "Isolation of *Candida dubliniensis* from denture wearers." *J Med Microb*, 58: 959–962, 2009.

31-Pathak, A. K, Sharma, S., Shrivastva, P. "Multi-species biofilm of *Candida albicans* and non-*Candida albicans* *Candida* species on acrylic substrate" *J Appl Oral Sci*, 20(1):70-5, 2012.

32- Fisher, F., Cook, N. "Reagents, stains, media and methods, In: Fisher, F. & Cook, N. (eds), *Fundamentals of diagnostic mycology*" Saunders, 320-340, 1998.

33-Lacaz, C.S., Porto, E., Martins, J.E.C. *et al.* "Leveduras de interesse médico. In: Lacaz, C.S., Porto, E., Martins, J.E.C, Vaccari-Heins, E.M., De Melo, N.T. (eds) *Tratado de Micologia Médica*." São Paulo: Sarvier, 123-73, 2002.

34-Budtz-Jorgensen, E. "Ecology of *Candida*-associated denture stomatitis" *Microb Ecol Health Dis*, 12: 170-185, 2000.

35-Chandra, J., Kuhn, D.M., Mukherjee, P.K., Hoyer, L.L., McCormick, T., Ghannoum, M.A. "Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance" *J Bacteriol*, 183:5385-94, 2001.

36-Thein, Z. M., Samaranayake, Y. H., Samaranayake, L. P. "Characteristics of dual species *Candida* biofilms on denture acrylic surfaces" *Arch. Oral. Biol*, 52: 1200–1208, 2007b.



Al-Kufa University Journal for Biology
Print ISSN: 2073-8854 & Online ISSN: 2311-6544
Special Second International Scientific Conference for the Life Sciences
Faculty of Education for Women / University of Kufa / 2016 Issue