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Original Article

Comparison between Conventional Methods and Molecular Diagnosis for *Candida albicans* and *Candida dubliniensis* isolated from Cancer Patients Infected with Oral Candidiasis

Nada Fadhil Abbas

Department of Biology, College of Science, University of Misan, Misan, Iraq

Abstract

Background: Two related pathogenic yeast species, *Candida albicans* and *Candida dubliniensis* share many phenotypic and microscopic diagnostic characteristics, in addition to the similarity in the characteristics of diagnosis. **Objectives:** The present study aimed to diagnose both yeast cells and compare them at the level of the percentage of similarities and differences according to the traditional methods and molecular approaches, which are considered more accurate and sensitive. **Materials and Methods:** The ability to form germ tubes was conducted for *C. albicans* and *C. dubliniensis*. The identification using biochemical tests, VITEK techniques, and molecular techniques was also conducted. **Results:** The results showed that both pathogenic yeast cells are similar in phenotypic and biochemical diagnosis, whereas the results of molecular diagnosis showed a difference between them, as well as the results of the drug sensitivity test, showed that the two types of yeast cells are sensitive to the antifungal drugs. The current study also showed the diagnosis of new isolates of the two species for the first time, which can be attributed to the occurrence of genetic mutations in them. **Conclusion:** Both types of pathogenic yeasts that were diagnosed in the current study are the important pathological types prevalent in cancer patients, especially in oral cavity infections, and they are similar to a large extent in terms of phenotypic and diagnostic characteristics.

Keywords: *Candida albicans*, *Candida dubliniensis*, oral candidiasis

Introduction

Oral candidiasis is one of the most prevalent opportunistic fungal illnesses, which is particularly common in immunocompromised patients, such as those with cancer, diabetes, and babies. Oral candidiasis initially presents as ulcers that progress to become white patches that may converge to form a membrane. Oral thrush is the term used for an infection. Infants and the elderly are particularly susceptible to oral candidiasis. It indicates that human immunodeficiency virus infection is progressing toward the emergence of acquired immune deficiency syndrome.[1,2] Oral candidiasis has been reported in cancer patients, with colonization of approximately 30%–50%. Among the yeasts responsible for candidiasis, *Candida albicans* is the most commonly isolated species.[3] *Candida dubliniensis* is closely related to *C. albicans*, sharing its properties of commensalism and opportunistic infection. For instance, 96% of genes are ≥60% homologous between these species, with the vast majority of genes being approximately 90% homologous.[4]

Candida species are thought to be a typical component of the flora in the mouth, gastrointestinal tract, skin, and vaginal canal of healthy people, but their occurrence and virulence appear to be elevated in immunosuppressed patients.[5,6] These species can transform into pathogenic in response to physiological changes in the host by producing pseudohyphae and true hyphae, causing oral candidiasis

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Address for correspondence: Dr. Nada Fadhil Abbas, Department of Biology, College of Science, University of Misan, Misan, Iraq.
E-mail: Nadafadhil@uomisan.edu.iq

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or invasive systemic infections.[7] Their primary oral reservoirs are mucosal surfaces, although they are also present in endodontic infections, peri-implantitis lesions, tooth plaque, and the subgingival biofilm of periodontal pockets in periodontitis, especially in cancer patients and diabetic patients.[8]

Numerous phenotypic diagnosis techniques are employed often in microbiology labs, including the growth of chlamydospores on rice-agar-tween media, the generation of germ tubes in serum at 37°C, and the absorption of carbohydrate patterns in commercial kits, which are conventional and classical to identify *C. albicans*. Often by using such biochemical tests, atypical *C. albicans* and *C. dubliniensis* strains would normally be classified as *C. albicans*. In the API ID 32C kit (bioMérieux, Craponne, France), however, these atypical isolates show sugar assimilation patterns, which were previously unknown,[9,10] but now they have been documented in the API ID database. However, these traditional methods are useful, but they have some disadvantages, such as the prolonged time they take to generate results until the identification of the microorganism is complete. Moreover, they have limited sensitivity; therefore, using nucleic acid-based assays such as polymerase chain reaction (PCR) allows rapid identification of *Candida* species.[11] Most antifungal medicines used for candidiasis have shown some serious side effects, and the development of resistance in *Candida* strains has become a serious health concern.[12]

Materials and Methods

Samples collection

Samples were taken from patients suffering from different types of cancer who visited The Center of Oncology in Al-Sader Teaching Hospital, Maysan, Iraq, for the period from December 14, 2021, to April 5, 2022. It has been obtaining the approval of patients regarding sampling and other information such as age, sex, use of antibiotics, and type of cancer. A total of 48 oral swabs were taken from patients with ages ranging from 12 to 84 years.

The samples were collected by rubbing sterile swabs on different mucosal areas of the oral cavity. All swabs were inoculated directly on swab containers that contained normal saline and later, examined microscopically using methylene blue or lactophenol cotton blue to detect the presence of pseudohyphae and true hyphae. Then, the swabs were streaked on potato dextrose agar (PDA) plates supplemented with chloramphenicol, and CHROMagar *Candida* medium, and then, incubated at 37°C for 24–48 h until the creamy white yeast colonies were clearly visible.[13]

Germ tube test

Yeast colonies were incubated with 0.5 mL of human serum and incubated at 37°C for 3 h, then, slides were prepared and examined to diagnose the germ tube. Germ tube was indicative of *C. albicans* and *C. dubliniensis*.

Biochemical tests

Using the KB006 HiCandida Identification Kit (HiMedia Laboratories, Mumbai, India) for identification and differentiation of *Candida* species, the test was conducted according to the company's instructions. In addition, the isolates were identified using VITEK (bioMérieux) techniques to compare identification and antifungal drug susceptibility.

Molecular diagnosis

The yeast colonies were sub-cultured on Sabouraud dextrose agar and incubated at 37°C for 48 h suspected to Genomic DNA extraction was performed using Presto™ Mini gDNA Yeast Kit (Geneaid, New Taipei City, Taiwan). according to the manufacturer's instructions.

PCR was performed using Maxime PCR PreMix Kit (i-Taq) according to the manufacturer's instructions (iNtRON Biotechnology, Seongnam, Kyonggi-do, South Korea) [Maxime PCR PreMix Kit (i-Taq)] by mixing [template DNA 1–2 µL, primer (F: 10 pmol/µL) 1 µL, primer (R: 10 pmol/µL) 1 µL, distilled water 16–17 µL]. The PCR was conducted in a thermal cycle with the following settings: one cycle of initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 20°s, 30 cycles of annealing at 50°C–65°C for10 s, and 30 cycles of extension at 72°C for 30 s, with a final extension at 72°C for 5 min. DNA samples were sequenced by (Psomagen, Rockville, MD, USA).

Results

Clinical diagnosis of samples collected from cancer patients (of both genders and different ages) showed clinical signs such as light and severe ulcers and the presence of areas of white patches as an indicator of *Candida* yeast growth [Figure 1].

Microscopic examination and culture results

Results of wet slide smears stained with methylene blue or lactophenol cotton blue stain appeared pseudohyphae and true hyphae under the light microscope as indicators for pathogenic isolate [Figure 2].

The result of culturing on PDA showed that out of 48 isolates, only 11 positive isolates of *Candida* spp. grew a smooth, sticky texture with entire margin colonies and creamy in color on PDA Figure 3 with a specific yeast odor. Whereas the growth results on CHROMagar media plates through (24–48) h according to enzymatic activity appeared that *C. albicans* and *C. dubliniensis* grew in bright green colonies.

All positive isolates were tested for the ability to produce germ tubes, which showed that both *C. albicans* and *C. dubliniensis* were able to form germ tubes, which seem without constriction after a 3 h incubation period in human serum [Table 1].

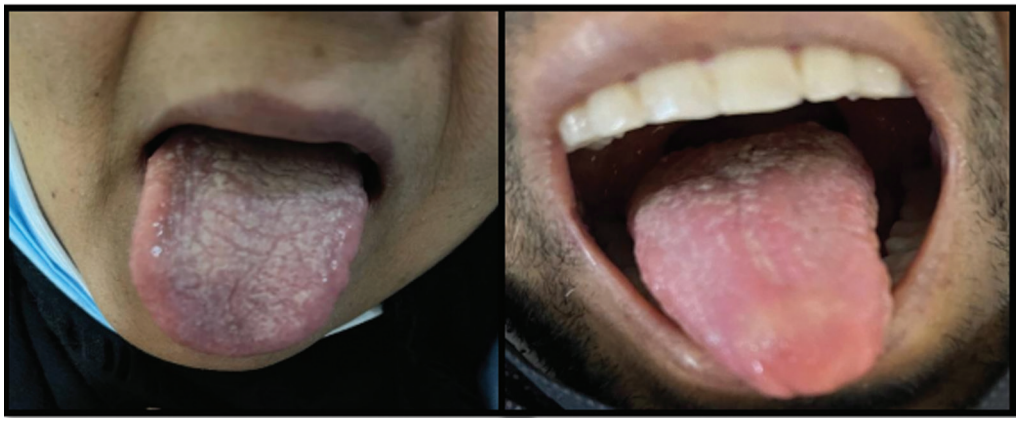

Figure 1: White patches as a result of oral candidiasis in cancer patients

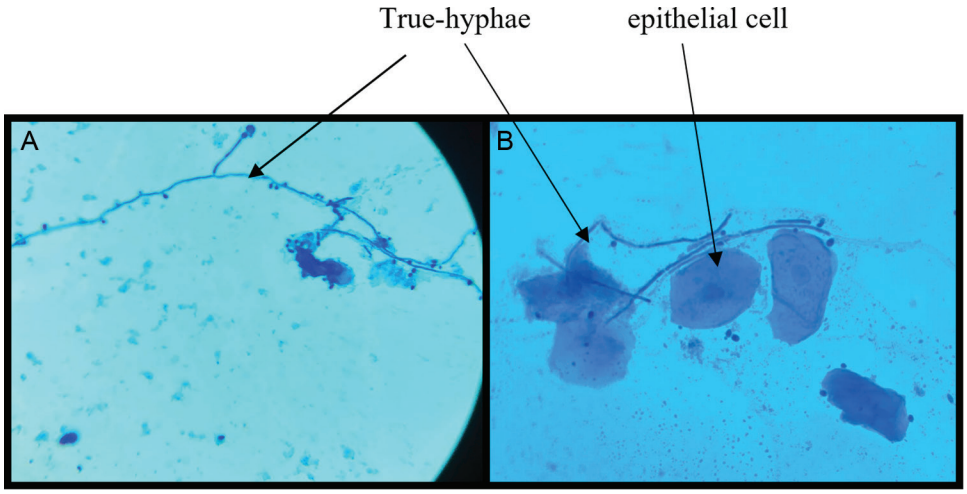

Figure 2: (A) True hyphae were shown on slide smear stained with methylene blue. (B) Slide smear stained with lactophenol cotton blue

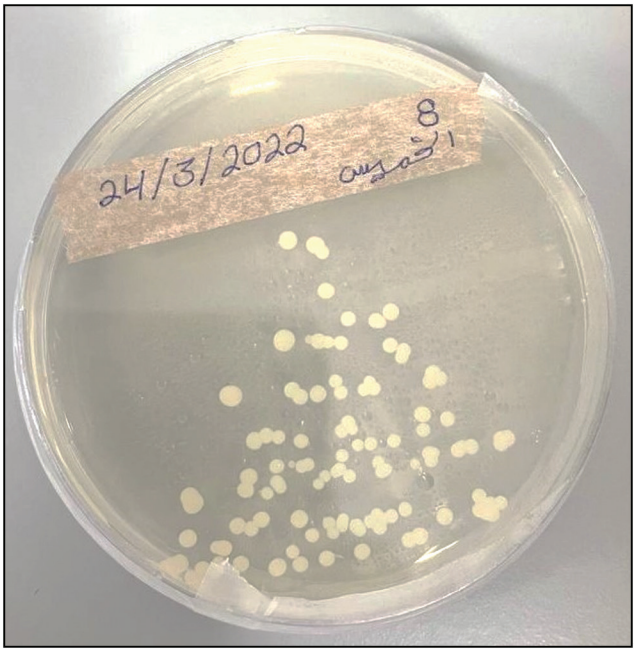

Figure 3: Colonies of *Candida* spp. on PDA

Biochemical diagnosis

Currently, the results of the biochemical tests (KB006 HiCandida Identification Kit) showed that both species consume the same carbohydrate sugars [Figure 4].

Antifungal susceptibility evaluation by VITEK technique

All positive isolates identified as *C. albicans* were sensitive to all antifungal antibiotics listed in Tables 2 and 3.

Molecular diagnosis

Eleven isolates of *Candida* spp. have been selected to confirm their identification by molecular analysis. According to the conventional PCR by using two primers Internal Transcribe Sequences (ITS)1 and ITS4, the gene bands in Figure 5 confirmed the size of the gene was 500 bp.

Sequencing of the ITS region in *Candida groups* was aligned with the recorded references in the database by analysis of the Basic Local Alignment Search Tool

Table 1: Types of sugars assimilated by *C. albicans* and *C. dubliniensis*

Tests	Unease	Melibiose	Lactose	Maltose	Sucrose	Galactose	Cellobiose	Inositol	Xylose	Dulcitol	Raffinose	Trehalose
C. albicans	−	−	−	+	+	+	−	−	+	−	−	+
C. dubliniensis	−	−	−	+	+	+	−	−	+*	−	−	+

Figure 4: Results of KB006 HiCandida Identification Kit: (A) control. (B) *C. albicans*. (C) C. *albicans* or *C. dubliniensis*

Table 2: Comparison results of sequencing and VITEK (identification and antifungal susceptibility) for 11 isolates

Code. of isolate	Results of sequencing	Strain and match ratio with reference strain	Results of VITEK and probability	MIC (μg/mL)						
				Fluco	Vorico	Ampho. B	Caspo	Micaf	Flucy	Interpretation
ON1	*C. albicans*	**C98 (99%)**	*C. albicans* 96%	≤0.5	≤0.12	0.5	≤0.12	≤0.06	≤1	S
ON2	*C. albicans*	**C89 (97%)**	*C. albicans* 94%	≤0.5	≤0.12	1	≤0.12	≤0.06	≤1	S
ON3	*C. dubliniensis*	**M130013635-2B (99%)**	*C. albicans* 95%	≤0.5	≤0.12	0.5	≤0.12	≤0.06	≤1	S
ON4	*C. dubliniensis*	**Cd1 (99%)**	*C. albicans* 94%	≤0.5	≤0.12	0.5	≤0.12	≤0.06	≤1	S
ON5	*C. albicans*	VoucherNM39 (100%)	*C. albicans* 94%	≤0.5	≤0.12	0.5	≤0.12	≤0.06	≤1	S
ON6	*C. albicans*	CBS:2720 (100%)	*C. albicans* 94%	≤0.5	≤0.12	0.5	≤0.12	≤0.06	≤1	S
ON7	*C. albicans*	S229 (100%)	*C. albicans* 93%	≤0.5	≤0.12	0.5	≤0.12	≤0.06	≤1	S
ON8	*C. albicans*	MADB8–64 (100%)	*C. albicans* 94%	≤0.5	≤0.12	0.5	≤0.12	≤0.06	≤1	S
ON9	*C. dubliniensis*	KAM-90G (100%)	*C. albicans* 93%	≤0.5	≤0.12	0.5	≤0.12	≤0.06	≤1	S
ON10	*C. albicans*	**HILZK2 (99%)**	*C. albicans* 94%	≤0.5	≤0.12	0.5	≤0.12	≤0.06	≤1	S
ON11	*C. albicans*	VoucherNM39 (100%)	*C. albicans* 94%	≤0.5	≤0.12	0.5	≤0.12	≤0.06	≤1	S

Table 3: The new strains that were deposited in NCBI GenBank database

Code of isolate	Name of new strain in gene bank	Accession number
ON1	*C. albicans* strain ND1	OP480809
ON2	*C. albicans* strain ND2	OP480823
ON3	*C. dubliniensis* strain ND3	OP480831
ON4	*C. dubliniensis* strain ND4	OP480850
ON10	*C. albicans* strain ND10	OP480879

sequence "https://www.ncbi.nlm.gov/BLAST" from the National Center of Biotechnology. Depending on the sequencing of the ITS4 region, the results confirmed that three isolates were identified as *C. dubliniensis*, and eight isolates were identified as *C. albicans*.

Phylogenetic relationship analysis in Figure 6 showed the convergent and divergent evolutionary relation between the strains of *C. albicans* and *C. dublininesis* isolated from patients.

Discussion

The occurrence of *Candida* species in the oral cavity of immunosuppressed individuals is higher than that of the healthy population, especially in hospitalized patients.[14] The results of the current study showed that the yeasts of *C.*

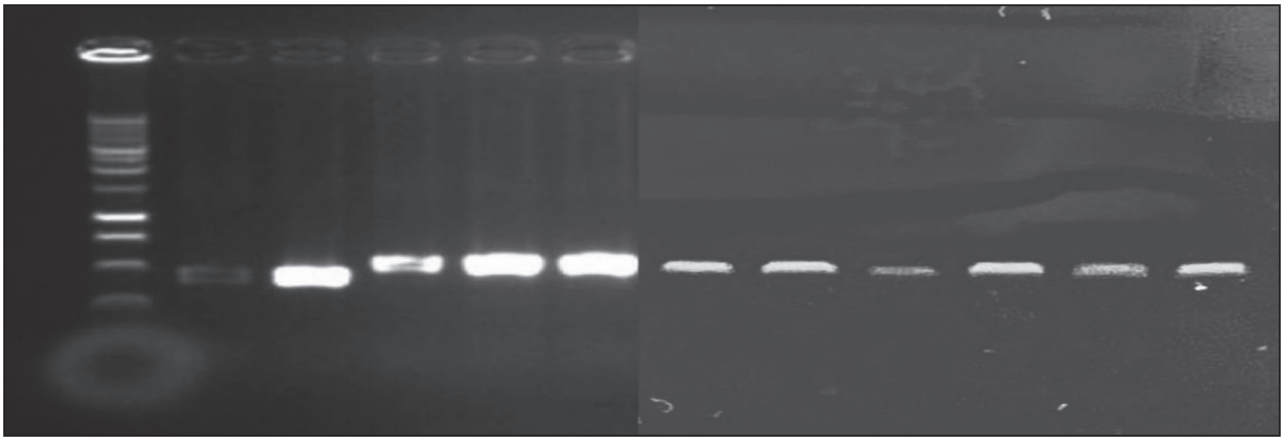

Figure 5: 2% Agarose gel electrophoresis analysis for PCR assay with two primers ITS1 and ITS4

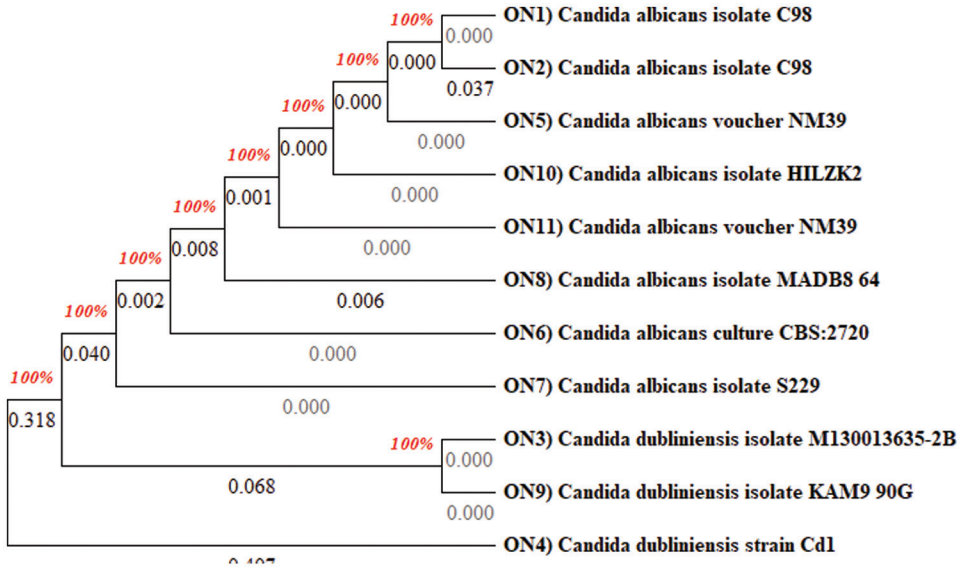

Figure 6: Collective phylogenetic tree generated with the ITS sequences of the 11 yeast isolates and the evolutionary relationship between them

albicans and *C. dubliniensis* share the same characteristics of diagnosis in culture methods in the primary isolation, where the two species appear in green color on the CHROMagar plate.[15]

The results of the microscopic examination also showed the ability of the two species to produce germ tubes without constriction in human serum, and the biochemical diagnosis results using the VITEK technology and the sugars assimilation of KB006 HiCandida Identification Kit showed that they can utilize multiple types of carbohydrates, but these techniques do not give us a 100% accurate diagnosis to determine the pathological causative species. These results were consistent with many studies conducted by several authors worldwide.[16-19]

The diagnostic results of the traditional methods indicate that it is difficult to differentiate between the two species in the medical microbiological laboratories, but the important point is that both showed sensitivity to all types of antifungal for which the samples were tested. While the results of genetic diagnosis identified three isolates of *C. dubliniensis*, it is considered more accurate in current studies and research to differentiate between them and to identify the types of mutations that may occur in the genome that could develop in the future to resist types of antifungal. It is important to detect the diversity of *Candida*, including phenotypic and genotypic features of these pathogens at the species level.

In the current study, three strains of *C. albicans* and two strains of *C. dubliniensis* were identified for the first time, and these yeasts have never been isolated from the oral cavity through the previous studies.

Conclusion

Both types of pathogenic yeasts that were diagnosed in the current study are the important pathological types prevalent in cancer patients, especially in oral cavity

infections, and they are similar to a large extent in terms of phenotypic and diagnostic characteristics.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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