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Isolation and Identification of *Candida dubliniensis* in Patient with Cervical-Vulvovaginal Candidiasis in Erbil City

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ABSTRACT

Vulvovaginal Candidiasis (VVC) is a fungal infection caused by *Candida* spp. of the genital mucosa that is still a health concern for women. This is a first-of-its-kind investigation on the isolation of *Candida dubliniensis*, from cervical-vulvovaginal patients in Erbil hospitals. The study was carried out for isolation and identification which performed by using phenotypic and molecular approaches. A total of one hundred samples of vaginal swabs (Copan transport swabs dry (in a cover)), were taken from patients with cervical vulvovaginal candidiasis in order to test for the presence of Candida species. According to macroscopic and microscopic examination, *Candida* spp. was discovered in 60 of the 100 samples (60%). On CHROM agar medium, eleven non-duplicated *C. dubliniensis* were identified depending on color of colony (dark green colonies-blue colony). Then the species were confirmed using API 20C assay. Finally, a multiplex PCR technique was performed depending on two types of primers, which are universal and specific for identifying *C. dubliniensis* and the results of the CHROM agar and API 20C assays were identical with molecular method.

Keywords: *Candida dubliniensis*, API20C, CHROMagar candida, molecular identification, VVC.

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INTRODUCTION

Candida species are regarded to be part of the human body's normal commensal microbiome. It can, however, act as an opportunistic pathogen in immunocompromised patients and in hospitals (nosocomial infection). For candidiasis control and management, quick, precise, reliable and accurate Candida identification to the species level is critical (Hemaid *et al.,* 2021). Candida is a major Ascomycetes yeast genus with over 150 species, of which more than 20 are clinically important. They live on the skin and mucous membranes of the tracheal, gastrointestinal, and genitourinary systems (Fotos *et al.,* 1992, Martins *et al.,* 2014).

Vulvovaginal candidiasis (VVC) is a genital mucosa infection caused by a variety of Candida species (Brandolt *et al.,* 2017). It is a prevalent and unpleasant condition that affects up to 75% of women of reproductive age and it is a common infection among pregnant women (Pietrella *et al.*, 2011, Mustafa, 2021). Because many people are self-treat, it is difficult to determine the exact incidence of VVC (Dovnik *et al.,* 2015).

Sullivan *et al.* discovered *C. dubliniensis* in oral specimens from HIV-infected and AIDS patients in Ireland who had recurrent oral candidiasis (Sullivan *et al.,* 1995). It is a novel species that is closely connected to *C. albicans* phylogenetically (Pincus *et al.,* 1999). *C. dubliniensis* shares diagnostic traits with *C. albicans,* such as the ability to produce germ tubes and chlamydospores, and is frequently misclassified as such by traditional diagnostic processes. A number of researches have sought to identify important morphological and genotypic features for distinguishing the two species. A wide range of techniques has been developed to aid in the speedy and precise identification of this species. Chromogenic culture medium and biochemical methods have been among them (Marian *et al.,* 2003).

Chromogenic Candida agar is a new medium that can be used to isolate and identify Candida species, as well as detect polyfungal communities in clinical sample. It appears to be well suited for the rapid isolation and identification of *Candida* species (Borman *et al.,* 2021). CHROM agar Candida has higher detection rates than standard medium and can be used to presumptively identify *C. dubliniensis* based on colony color. On CHROM agar Candida, it has been found to generate a unique dark green color (Schoofs *et al.,* 1997).

Most *C. dubliniensis* isolates are accurately identified by the API 20C AUX system. Many writers, however, believe that traditional procedures for *Candida* spp. differentiation are time consuming, typically insensitive, and can fail to differentiate *C. dubliniensis*. To circumvent these limitations, molecular methods for identifying *C. dubliniensis*, notably those based on the polymerase chain reaction, have been developed. For identifying *Candida* spp., commercial systems and molecular diagnostic techniques are quick and accurate (Gutierrez *et al.,* 2002; Deorukhkar and Roushani, 2018,).

Researchers are investigating more dependable methods in order to differentiate properly of the Candida isolates due to variations in their ability to distinguish the two species based on their morphological traits. This necessitates the use of a combination of biochemical, mycological and molecular techniques for identification of *C. dubliniensis* unequivocally (Schoofs *et al.*,1997, Sullivan and Coleman, 1998)

The aim of the present study is to isolate *C. dubliniensis* from Cervical-Vulvovaginal patients then identify by using conventional and molecular-based method in Erbil hospitals/Iraq.

MATERIAL AND METHODS

Samples Collection and Direct Examination:

Gynecologists analyzed patients with genital tract problems based on the indications and symptoms of vaginal abnormalities. Vaginal swabs of participants with vaginal discharge, discomfort, or vulvar pruritus were obtained. All swabs were subsequently sent to mycology laboratory in Salahaddin University's Department of Biology's in the College of Sciences. Sabouraud Dextrose Agar (SDA) with chloramphenicol was inoculated with each swab (Oxoid, Basingstoke, UK) and aerobically incubated for 48 hours at 37°C. (Yonis *et al.,* 2019).

Identification of Yeast

1. Phenotypic identification: After incubation, cultures were separated into positive (yeast growth) or negative cultures (no yeast growth). For all positive cultures, the colony was inspected macroscopically, then microscopically by placing a piece of it on a clean glass slide and adding a drop of Lactophenol cotton blue, then covered with a cover slip. A light microscope was performed (40X) to examine the slide. This enables for a clear view of pseudohyphae as well as blooming oval yeast cells, which belong to different Candida species (Babic and Hukic, 2010). All positive cultures were grown on CHROM agar candida (bioMérieux, France), identification of Candida was preformed based on a colony color (Nadeem *et al.,* 2010), The colonies were subsequently cultured at 30°C for up to 5 days, with daily inspections for light green colonies (typical of *C. albicans*) and dark green colonies (typical of *C. dubliniensis*) (Mariano *et al*., 2003).

2. Biotyping: API 20C Aux strips were used to do biochemical identification of the Candida isolates. In an API 20C suspension tube, a single colony was submerged from a young Candida isolate culture taken from an SDA plate. After filling the cupules of the strip with the suspension from the API 20C medium tube, the strips were incubated for (24, 48, and 72) hours at 37°C. After incubation, the turbidity of cupules was evaluated and recorded, and a profile number was established (Hemaid *et al*., 2021).

MOLECULAR METHOD

1. DNA extraction

Candida isolates were cultured for 48 hours on SDA at 37°C, then DNA was extracted using a Genomic DNA Extraction kit (Fungi/Yeast Genomic DNA isolation kit. Norgen Biotek/Canada), based on the guidelines.

2. PCR amplification

- In two rounds of amplification, multiplex nested PCR was used: First round was done by Universal primer (*UNI1* and *UNI2*) to amplify the large target sequence. The total of 25 µl PCR master mix reaction volume was performed containing 3µl of genomic DNA, 12.5 µl of 2X GoTaqGreen Master Mix (Promega/ USA) and 1µl was added for each of the forward and reverse primer for both *UNI1* and *UNI2*, forward (*UNI1*, F´5- GTCAAACTTGGTCATTTA-´3), reverse UNI2, R-5´ TTCTTTTCCTCCGCTTATTG-´3 (Trost *et al*., 2004, Carvalho *et al.*, 2007).
- The mixture was completed by adding 7.5µl of nuclease free water. The program cycle for *UNI1* and *UNI2* as follow: initial denaturation of DNA at 94°C for 3min. The total cycle number 30, each cycle consisted of a denaturation step at 94°C for 30s, an annealing step at 50°C for 30 s and an extension step at 72°C for 1min, with a final extension at 72°C for 10min then 10µl was used as a template for the second-round amplification for species level identification.
- The master mix preparation for the specific primer was done in 50µl as a total volume, 25µl of 2X GoTaqGreen Master Mix (Promega/USA) and 2µl was added for each of the primers, Forward: *Calb*: AGCTGCCGCCAGAGGTCTAA(583/446bp), Forward: *Ckru*: CTGGCCGAGCGAACTAGACT(590/169bp), Forward: Cpar: GTCAACCGATTATTTAATAG (570/370bp), Forward: *Cdub*: CTCAAACCCCTAGGGTTTGG(591/217bp) and Reverse: Clus: TTCGGAGCAACGCCTAACCG (Carvalho *et al.*, 2007), then completed by adding 5µl of Dnase, Rnase free water.
- The program file for the above specific primers with minor modification (cycling conditions): Initial denaturation 94°C for 5min, 40 cycles of 15s at 94°C, 30 s at 55°C, and 45s at 65°C, following a 10-minute period at 94°C for DNA denaturation and enzyme activation. The *ITS* region was amplified via PCR amplification process with a Techne/UK thermocycler cycler under the following conditions: an initial denaturation cycles at 95°C for 5min., followed by 35 cycles at 94°C for 1min., 55°C for 1min., 72°C for 1min. and a last extension for 7min. at 72°C. The 505bp

of PCR products were confirmed by using agarose gel electrophoresis (2%) in 1XTBE buffer (Carvalho *et al.*, 2007).

RESULTS AND DISCUSSION

A total of 11 *C. dubliniensis* isolates were recovered from cervical-vulvovaginal candidiasis patients in various hospitals in Erbil, Iraq. Each isolate was identified macroscopically and microscopically, the positive culture (*Candida* sp.), identified on CHROM Agar following a 48hr incubation period at 37°C based on colony color (Table 1), Fig. (1).

After completing the API 20C AUX assay on a subset of isolates (according to colored colonies grown on CHROM agar), all 11 isolates with dark green colored colonies revealed *C. dubliniensis* when tested with API 20C Fig. (2). Candida isolates were subjected for the molecular confirmation depending on Multiplex-PCR. The result of the molecular identification, by using universal and specific primers gene amplification were consistent with the phenotypic analysis, which includes *C. dubliniensis.* The specific species primers were successfully applied for identification of *C. dubliniensis* Fig. (3).

According to some of the findings, using CHROM agar Candida for quick identification of Candida species from clinical specimens could be highly useful in creating appropriate therapeutic strategies and patient management (Nadeem *et al.*, 2010). Visualization of pseudohyphae and budding yeast cells are typical of many Candida species (Alghnam and AL-Dabbagh, 2012).

In the identification of *Candida* spp. for diagnostic purposes, multiplex PCR may be more reliable and time-saving than phenotypic techniques (Liguori *et al.*, 2010). Multiplex-PCR can be recommended as a sensitive and specific technique for *Candida* spp. identification because it is significantly more sensitive than culture (Tarini *et al.*, 2010). Rad *et al.*, isolate Candida and use multiplex PCR to identify the species and show that the most common cause of vulvovaginal candidiasis is *C. albicans*(Rad *et al.*, 2012). Carvalho and his colleagues, show that a multiplex PCR allows the detection of eight therapeutically important yeasts, such as: *C. albicans, C. lusitaniae, C. krusei, C. dubliniensis, C. glabrata,* and *C. parapsilosis* (Carvalho *et al.*, 2007).

Research findings are consistent with those of Mohsin and Ali, who cultured swabs on (SDA), then identified all isolates macroscopically and microscopically following incubation (Mohsin and Ali, 2021) Habib and his colleagues., who used CHROM agar to identify different species of *Candida* causing vulvovaginal Candidiasis (Habib *et al.*, 2016). Othman *et al*., who identifying species of Candida after culturing samples on Chromogenic agar Candida (Othman *et al.*, 2018). Kirkpatrick et al., demonstrates that due to the phenotypic similarities between the two species, *C. dubliniensis* isolates may have been mistaken for atypical *C. albicans,* leading to the discovery of anomalous dark green isolates on CHROM agar Candida, as opposed to light green *C. albicans* (Kirkpatrick *et al.,* 1998). Our findings are consistent with those of. Carvalho *et al* who used a multiplex PCR to identify therapeutically significant yeasts, including, such as: *C. albicans, C. krusei, C. dubliniensis, C. tropicalis, C. glabrata, C. guilliermondii, C. lusitaniae and C. parapsilosis* (Carvalho *et al.*, 2007).

On certain isolates with various colored colonies, Hemaid et al. employed the API 20C assay. and found that the results were identical to those of CHROMagar. When the isolates were PCR-assayed with ITS1 and ITS4 primers, 8 of the 14 PCR result bands seemed to be between 510 and 535 bp, making it difficult to distinguish between them (Hemaid *et al.*, 2021). The colony color on CHROM agar Candida medium and the results of a sugar assimilation test with the API 20C AUX yeast identification system was used to screen for *C. dubliniensis* by Jabra-eizk, *et al*., who isolate *Candida* spp. from patients who were presumptively diagnosed as *C. albicans.* Five isolates from 25 HIV-seropositive patients and one isolation from a patient whose HIV status was unknown were found to be compatible with the European reference strain of *C. dubliniensis* after phenotyping and electrophoretic karyotyping (Jabra-rizk *et al.*, 1999). From 1994 to 2000, Mariano *et al.* looked for *C. dubliniensis* among isolates previously classified as *C. albicans* and kept in a yeast stock collection. After performing a screening test for *C. dubliniensis* isolates using three different

primers and molecular analysis. Molecular analysis identified eleven isolates as *C. dubliniensis* (Mariano *et al*., 2003). Musa *et al*., were collected urine samples from pregnancy, he employed Chromogenic Agar medium to distinguish between *Candida* spp. 17 (8.67%) were positive for *Candida* species, these were *C. albicans* (23.52%), *C. dubliniensis* (11.76%), *C. glabrata* (41.18%), *C. krusei* (17.66%), and *C. tropicalis* (5.88%) (Musa *et al.*, 2020). According to Raut and Varaiya, *C. dubliniensis* isolates have been mistaken for *C. albicans*. To distinguish these two Candida species, phenotypic tests have been applied, such as colony color development on differential media CHROM agar Candida medium. Nine isolates were identified as *C. albicans* on CHROM agar, while 19 were identified as *C. dubliniensis* (Raut and Varaiya, 2009).

The API20C method was used to confirm the diagnosis of Candida species (Malla Obaeda, 2020). It was compared to polymerase chain reaction amplification in a study by Xu *et al.* When the API method identified the isolate as *C. albicans*, but a molecular test revealed it to be *C. dubliniensis*, the phenotypic and genotypic identifications conflicted (Xu *et al.*, 2002).

Because of its phenotypic similarities to *C. albicans*, Yu *et al.* show that identifying *C. dubliniensis* in ordinary clinical practice can be difficult. They report the first instance of candidemia caused by *C. dubliniensis* in Korea. On Sabouraud agar plates, white colonies were identified, indicating *Candida* spp. Chromogenic agar and multiplex PCR were used to identify *C. dubliniensis* (Yu *et al.*, 2012).

Table1: Phenotypic and molecular test of *C. dubliniensis*

Phenotypic method	
Microscopic identification	Positive
Color on CHROM agar	Dark green colonies
API20C	Positive
Molecular method	Positive

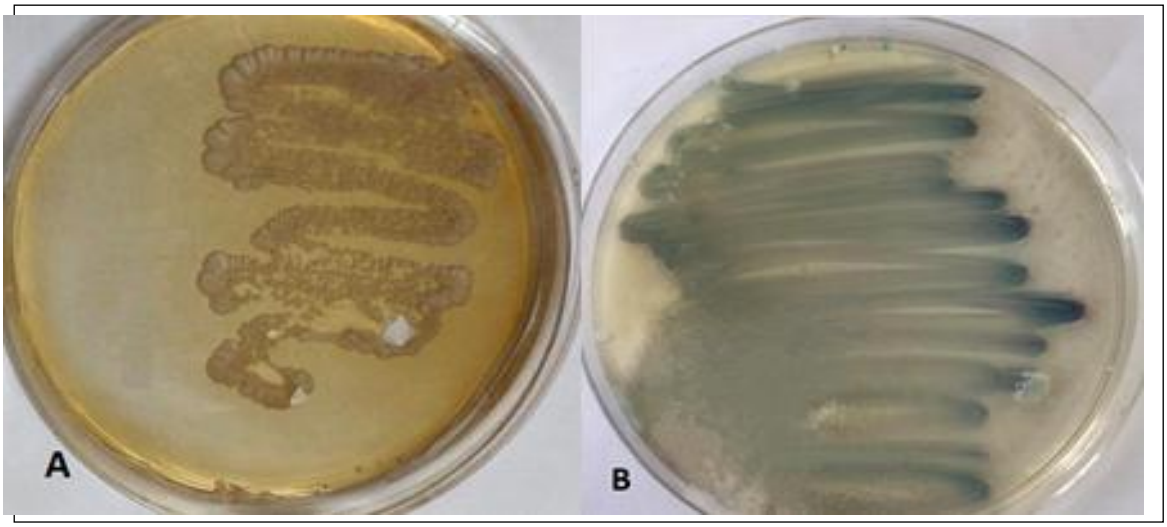

Fig. 1: *C. dubliniensis* on A. Sabouraud Dextrose Agar (SDA) B. CHROM agar medium

Fig. 2: Identification of *C. dubliniensis* by API 20C AUX

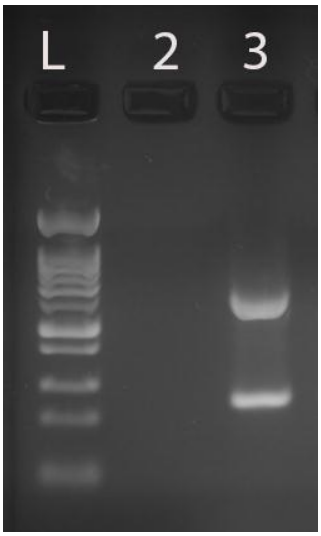

Fig. 3: Multiplex PCR amplification. Lane 1: DNA ladder 1kb, Lane 2: Negative control, Lane 3: *Candida dubliniensis* (591/217bp).

CONCLUSIONS

This study indicated that C. *dubliniensis* were isolate from Cervical Vulvovaginal Candidiasis patients, by API 20C assay. Its colony is dark green on Chromogenic agar. Multiplex-PCR is more recommended as a sensitive and specific technique for *Candida* spp. identification.

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عزل وتحديد *Candida dubliniensis* في المرضى المصابات بداء المبيضات عنق الرحم والفرج والمهبل في مدينة أربيل

الملخص

داء المبيضات الفرجي المهبلي (VVC) عبارة عن عدوى فطرية تصيب الغشاء المخاطي للأعضاء التناسلية والتي تسببها عدة أنواع من *Candida*، حيث لا تزال تشكل مصدر قلق صحي للنساء. هذه الدراسة تعد الاولى من نوعها حول عزل *Candida dubliniensis*، من مرضى عنق الرحم والفرج والمهبل في مستشفيات أربيل، لقد أجريت هذه الدراسة على العزل وتم التعرف عليها باستخدام الأساليب المظهرية والجزيئية. حيث تم أخذ مائة عينة من مسحات المهبل (مسحات نقل كوبان الجافة) (في غطاء)، من مرضى داء المبيضات الفرجي المهبلي العنقي من أجل اختبار وجود أنواع المبيضات. ووفقا للفحص المظهري والميكروسكوبي تم تحديد 60 عينة *Candida* spp. من أصل 100. وقد تم التعرف على 11 مستعمرة غير مكررة من *C. dubliniensis* على وسط CHROMagar والتي تعتمد على لون المستعمرة (خضراء داكنة–زرقاء). ثم تم تأكيد الأنواع بإجراء اختبار API 20C أخيرًا تم إجراء تقنية multiplex PCR اعتمادًا على نوعين من البادئات، وهما universal و specific لتحديد *C. dubliniensis* وكانت نتائج فحوصات CHROM agar و API 20C متطابقة مع الطريقة الجزيئية.

الكلمات الدالة: *Candida dubliniensis*، API20C، CHROMagar candida، التشخيص الجزيئي، داء المبيضات الفرجي المهبلي.