Detection of pulmonary candidiasis in immunocopromasied Iraq patients by conventional polymerase chain reaction technique

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الكشف عن داء المبيضات الرئوي في عينات المرضى العراقيين المنقوصي المناعة بواسطة تقنية تفاعل البلمره المتسلسل التقليدي

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المستخلص

المبيضات هي من المسببات المحتملة لداء المبيضات الرئوي والتي تحتاج الى عزل وتنميتها على الأوساط الزرعية يحدث هذا المرض عادةً في المرضى الذين يعانون من نقص المناعة، وهدف هذه الدراسة هو عزل وتشخيص داء المبيضات الرئوي باستخدام الطرق التقليدية والجزيئية، شملت هذه الدراسة ما مجموعه مئة وخمسون (150) عينة من غسيل القصبات تم جمعها من 100 مريض منقوص المناعة يعانون من أمراض مختلفة وشملت مجموعة السيطرة 50 عينة من الأفراد الأصحاء من المرضى الراقدين والمراجعين ومن كلا الجنسين في مدينة الإمامين الكاظمين الطبية ومستشفى بغداد التعليمي للفترة من أيار 2014 وحتى شهر آذار 2015. وتم تنمية العينات على كروم أكار وبفحص API 20 Candida لتحديد ألأولي لعزلات المبيضات. ,واستخدم أيضا اختبارالأنبوب الجرثومي ، وتكوين المتدثرة أيضا لتأكيد التشخيص. تم استخلاص الحمض النووي للعزلات لغرض التفاعل البلمرة المتسلسل (PCR).أظهرت الدراسة أن جميع الأفراد الأصحاء كانت النتائج سالبة بالطرق التقليدية والجزيئية. وفيما يتعلق بمرضى منقوصي المناعة الذين يعانون من أمراض مختلفة 60 عينة من مجموع 100عينة أظهرت نتائج سالبة بواسطة الطرق التقليدية والجزيئية. فقط 38 (38٪) من مرضى منقوصي المناعة حققت نتائج إيجابية بواسطة الطرق التقليدية والجزيئية وعينتان(2%) من خلال الطرق الجزيئية , وأظهرت النتائج أيضا أن كروم أكار كان وسيلة جيدة لتحديد أولي لعزلات المبيضات. وتخلص هذه الدراسة إلى أن داء المبيضات قد تكون العدوى الانتهازية في المرضى الذين يعانون من نقص المناعة وان فحوصات تفاعل البلمره المتسلسل أكثر تأكيد لتشخيص عزلات المبيضات مقارنه بوسط كروم أكار وغيرها من الاختبارات المظهرية.

Abstract

Candida species was possible to cause pulmonary candidiasis, this infection commonly occurred in" immunocompromised patients. This study aimed to isolate and diagnose of pulmonary candidiasis by using molecular method (Conventional PCR). This study was included a total of hundred and fifty (n=150) samples of bronchoalveolar lavage were collected from 100 immunocompromised patients with underlying diverse diseases and control group included 50 bronchoalveolar lavage from immunocompetent individuals were composed from in-and out patients who attended of Al-Imammian Al-Kadhmain Medical City, Baghdad teaching Hospital, Baghdad, during the period from May 2014 to March 2015. Samples were cultured on CHROMagar and after that detected by using API 20 Candida stripe for initial identification of Candida isolates. Germ tube test, chlamydospore formation were also performed to reinforce identification them DNA isolates was extracted for conventional polymerase chain reaction (PCR) method. Results showed that all apparently healthy individuals samples were negative by conventional PCR. Concerning immunocompromised patients with underlying different diseases, 60 out of 100 samples revealed negative results by each of conventional and molecular methods Only 38 (38%) immunocompromised patients had positive results by molecular and conventional methods and two samples (2%) positive result by molecular methods also "show that CHROMagar was a good tool for initial identification of Candida isolates. This study concludes that candidasis may be as opportunistic infection among immunocompromised patients and PCR method confirmed more suitable diagnosed other than culture on CHROMagar medium and other phenotypic technique".

Introduction

In current decades, there had been a noticeable increase in the occurrence of pulmonary candidiasis com from to four factors: more forceful treatment for other conditions (use of immunosuppressant’s, transplantations, employ and abuse antibiotics); the increased occurrence of leukemia, lymphoma and other immunocompromised patients (1-2). Candidiasis is an infection caused by the yeast C. albicans or other Candida species.. "Candida is a polymorphic fungus. It is a Gram positive, oval, budding yeast produces pseudohyphae both in culture and tissues and exudates", C. albicans is the foremost fungal infectious agent in human

infection (3). Pulmonary candidiasis could can be acquired by either hematogenous dissemination caused spread pneumonia or by bronchial extension in patients with oropharyngeal candidiasis, aspiration of yeast form to oral cavity, pulmonary candidiasis is difficult to diagnosed due to non-specific radiological in most patient (4). "The number of fungal infections caused by yeasts were radically increased over the past several decades. Among them, the imperfect yeast C. albicans and several linked Candida species are of foremost importance as opportunistic pathogens in immunocompromised patients and may caused life threatening infections. Their incidence was greatly increased with increased used of broad-spectrum antibiotics, immunosuppressive corticosteroids, and antitumor agents (5)". Candidiasis is a primary or secondary fungal infection caused by members of the genus Candida. The genus Candida includes around 150 species, among these, six (Candida albicans, C. tropicalis C. glabrata, C. parapsilosis, C. krusei, C. guilleromondii) are the most frequently isolated from human and animals infection (6), associated with C. albicans in culture; on the other hand these non-albicans species are often present in low number and their pathogenic role is unsure and rarely proven. Candida dubliniensis may be mistaken for C. albicans; previously been isolated from the oral cavity in patients suffered from recurrent episodes infection (7).

In this study: Isolation and detection of Candida albicans and other Candida species in a samples of immunocompromised Iraqi patients and superior knowledge of clinical mycology and the greater correctness diagnostic technique.

Materials and methods

Patients selection

This study included a total of One hundred and fifty (n=150) clinical samples from 100 immunocompromised patients (43 men, 57 women; average age 16-90 years) as Bronchoalveolar lavage (BAL) with underlying diseases; 22 leukemia, 17solid tumor, 15 lymphomas, 12 chronic pulmonary obstruction disease(heavy smoker), 10 asthma(steroid therapy) 10 rheumatoid arthritis(cytotoxic therapy), 8 solid-organ transplantation and 6 Multiple myeloma with clinical suspicion of pulmonary candidiasis, other 50 samples was control from Bronchoalveolar lavage (BAL) all samples collected from admitted and out patients from both gender "attended

of Medical AL- Imammian AL- Kadhmain City teaching Hospital; Baghdad teaching Hospital". during the period from May-2014 to March-2015. Ethical aspects of this study had been approved by the ethical council in Medical college, Al- Nahrain University.

Samples collection

Bronchoalveolar lavage (BAL): Was done by bronchofibroscope (STORZ, Germany) wedged in segmental orifice of sedated spontaneous breathing patients or intubated patients, in most cases,20-50 ml warmed saline was infused into targeted segment followed by gentle suction by specialist physician. BAL fluids were directly collected by sterile syringe. About 10-15 ml were dispensed into sterile screw test tube and immediately placed on ice then transmitted to laboratory for processing.

Samples processing

Bronchoalveolar lavage, contain mucous martial were added to a two-fold volume of (0.9%) NaCl and mixed forcefully by vortex for 5 minutes after that centrifuged at (3000 rpm) for 5 minutes, supernatants were discarded and the precipitated pellets were directly engrossed in Sabouraud agar medium and incubated at 30°C for 24-48 h.

Germ tube test

A loopful of "Candida cells suspension was inoculated into 0.5 ml of human serum and incubated for 3 h at 37°C. After incubation period microscope examination, germ tube was lateral tube devoid of septum and had no constriction at initiating site, (8,9)."

Cornmeal Agar (CMA)

This medium used for chlamydospores formation test (Dalmau plate culture) performed by: flamed sterilized wire dipped into alight inoculums and then scratched into the surface of the cornmeal agar added tween-80, plate a flamed cover slip placed onto the agar surface covering the scratch's. The plates were incubated at 30 °C for 3 to 5 days then examined in situ using the high power microscope to see pseudohyphae and chlamydospore of Candida albicans (10).

CHROMagar culture

" Purified single colonies on Sabouraud agar were streaked on Chromoagar (Biomerieux, France) and incubated for 24-48 h. isolates of *Candida* were classified according to the colors on Chromoagar and based to colored key designed (11,12)."

API 20 C AUX Diagnostic Strips

It used for the confirmatory identification of C. albicans and other species. All tests were performed "according to the manufacturer's instructions. The API 20 C AUX system(Biomérieux, France) consists single-use disposable plastic strip with 20 wells (cupules) containing dehydrated substrates" to perform 19 assimilation tests"(Glucose, Glycerol , 2-Keto-D- Gluconate, L-Arabinose, D- xylose, Adonitol, Xylitol, Galactose, Inositol, Sorbitol, α-Methyl-D-Glucoside, N-Acetyl-D-Glucosamine, D-CeLlobiose, Lactose, Maltose, Sucrose, Trehalose, Melezitose and Raffinose)" in addition to hyphael or pseudohyphae formation test which was performed separately on the rice extract tween 80 agar. The inoculation of the wells was performed by added a semi solid minimal medium containing yeast suspension adjusted to (McFarland #2 Turbidity) to the dehydrated substrates. After incubation at 30°C for 48 to 72 hours, the results were read A Seven-digit numerical profile was generated for each isolated depending on the reactions produced, identifications were made by referring to the list of the numerical profiles and a computer program provided by the manufacturer.

Extraction of DNA

Deoxyribose nucleic acid "of Candida spp. was extracted by picking single of colony using sterile loop and suspended into (300 µl) of lysis buffer [10 mM Tris, 1mMEDTA (pH=8),1% SDS, 100 mM NaCl, 2% Tween 80], 300 µl phenol-chloroform (1:1); it was shaken for 5 minutes and centrifuged at (1000 rpm), supernatant was transferred to the new tube and equal volume of chloroform were added, mixed and centrifuged. (500 µl) 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" (13).

Polymerase chain reaction PCR

The universal primer pair sequences were used in conventional PCR to detect the presence of the 18S rRNA gene of Candida spp.(14). And a specific primer pair sequences used to detect presence of C. albicans based on the sequence data at (ITS) region, selected (15) and synthesized in Alpha DNA® (Canada) as shown in table 1. DNA template of Candida was prepared. The primers (Candida spp and C. albicans) were diluted by adding nuclease free water according to the manufacturer instructions. The master mix contents were thawed at

room temperature before use, and the PCR master mix was made on a separate biohazard safety cabinet with wearing hand gloves at all times to avoid contamination. For each reaction within each single pre-mixed PCR reaction tube, 2µl from each forward primer and reverse primer were added. Three microliter of DNA template was added for each reaction tube. Twelve

and a half microliters of GoTaq® Green Master Mix (Promega,USA) was added for each reaction tube, the volume was completed to 25µl with Deionized Nuclease –Free as shown in table 2, tubes were then spun down with a mini centrifuge to ensure adequate mixing of the reaction components. PCR mixture without DNA template (non-template negative control) were used as negative control. The tubes were placed in PCR machine, and the right cycling conditions pre-installed, and started. Cleaver Scientific Thermal Cycler TC32/80 was used for all PCR amplification reactions. PCR thermocycler program used with 18S rRNA gene of Candida spp and (ITS) region of C. albicans genes were designed on the basis of published paper as shown in table 3. The PCR products were run on (1.2%) agarose gel (Promega, USA) and electrophoreses were performed at 100 V in TBE buffer. The gel was pre-stained with 0.05% ethidium bromide (Promega, USA). The gel was exposed to UV using UV light transilluminator and then photographed using digital camera (Sony-Japan).

Table (1): Primers sequences with their relevant product size.

Target species	Gene target	Primer name		Primer sequence (5' ⟶ 3')	Product Size(bp)
Candida spp	18S rRNA	CANIA	F	GAGGGCAAGTCTGGTG	273-280 bp
		CANIB	R	CTGCTTTGAACACTCTAA	
C. albicans	(ITS) region	CALB1	F	TTTATCAACTTGTTGTCACACCAGA	210 bp
		CALB2	R	ATCCCGCCTTACCACTACCG	

Table (2): Composition of PCR reaction mixture used for amplification of each 18S rRNA and (ITS) region genes.

Components		Volume /µl	Final concentration
Green Master Mix 2x		12.5 µl	1x
18S rRNA (ITS) region	Forward primer, 10µM	2 µl	0.2 µM
	Reverse primer, 10µM	2 µl	0.2 µM
DNA template		3 µl	
(DNAse free) water		5.5 µl	
Total volume		25 µl	

Table (3): The PCR thermo cycler program for each 18S rRNA and (ITS) region genes.

Steps	Temperature	Time	No. Cycles
Initial denaturation	95°C	5 min	
Denaturation Annealing Extension	95°c 56°c 72°c	30 sec 1.5 min 1 min	30
Final extension Hold	72°c 4°c	10 min	

Results and Discussion

Germ tube and chlamydospore formation

Out of 100 Bronchoalveolar lavage (BAL) samples, only thirty eight shown positive pulmonary candidiasis. Most C. albicans isolates produced germ tubes and chlamydospores on Cornmeal Agar as shown in table.4. In this study show that 93.75% and 87.5% "of C. albicans isolates created germ tubes and chlamydospores respectively. These results are in line with those of Beheshti et al. (16)".

CHROMagar culture and API 20 C AUX Diagnostic Strips

Out of 38 Candida isolates of pulmonary candidiasis, 16 (42%) that appeared as light

Table(4): Germ tube, Chlamydospore formation and colony color of pulmonary *Candida* spp.

Species	No. of isolates	Germ tube N0 (%)	Chlamydospore NO(%)	API 20 C AUX Diagnostic Strips NO(%)	Colony color on CHROMagar and texture
C. albicans	16(42%)	15(93.75)	14(87.5)	16(100)	Light green and smooth
C. parapsilosis	11(28.9%)	0	0	12(100)	White cream
C. tropicails	5(13.1%)	0	0	5(100)	Blue-pink
C. krusei	3(7.9%)	0	0	3(100)	White-pink with white border
C. glabrata	2(5.3%)	0	0	2(100)	Pink to cream
C.dubleniensis	1(2.6%)	0	0	1(100)	Dark green and rough

green and smooth colonies diagnosed as C. albicans, followed by C. parapsilosis 11 (28.9%) , C. tropicails 5(13.1%), C. krusei 3(7.9%), C. glabrata 2 (5.3%) and C. dubleniensis 1(2.6%), table 4. Based on the description of Campbell et al. (11) and Nadeem et al. (12). The results showed that chromoagar contributed to differentiation of Candida spp. into two groups, C. albicans and non-albicans species. API 20 was regard as a confirmatory identification test of the yeasts isolates. The positive result is read after 72 hr depending on the turbidity test these results corresponding with chromoagar culture.

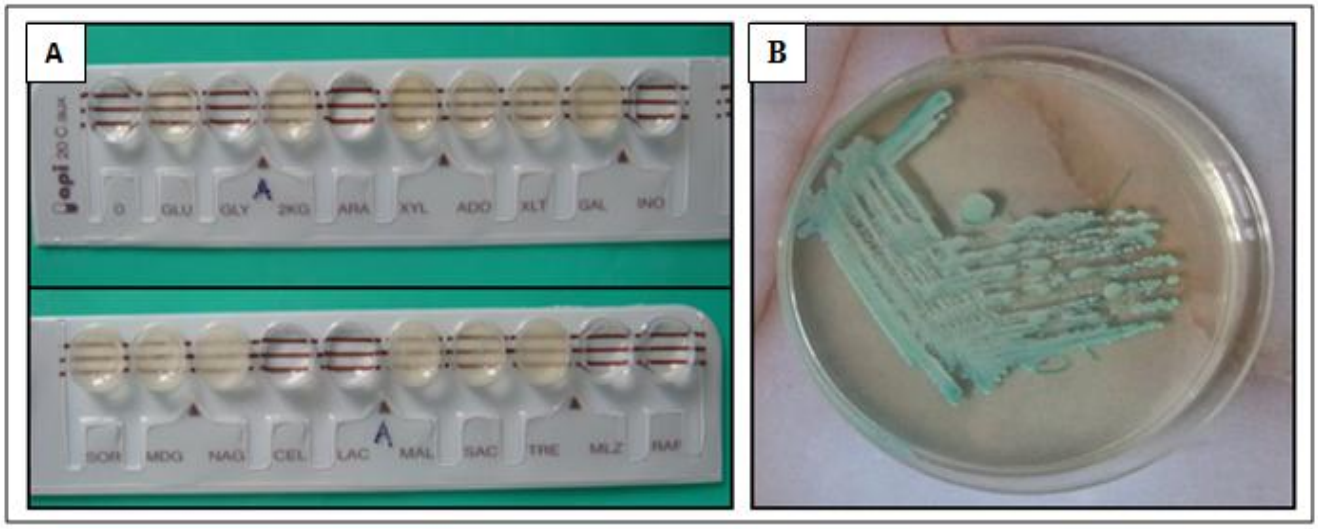

Figure (1):A- Identification of *Candida albicans* by Api 20 AUX diagnostic strips.

B- Identification of *Candida albicans* by CHROMagar.

Molecular diagnosis by PCR

Results showed that all apparently healthy individuals samples were negative by molecular methods, only 38 (38%) of immunocompromised patients had positive results by all techniques and two samples (2%) positive by molecular methods. In this study, it has been found that " primer pair CANIA and CANIB were successful in amplification of target region of 18S rRNA gene for Candida spp. with a PCR product size approximately 210 bp (Figure 2),concerning specific primer pair for C. albicans CALB1 and CALB2, it yielded approximately amplification size of 273-280 bp, but it did not give in amplification products with non-albicans species like lane B and lane I (Figure 3). The amplification sizes of this study are alike with the results of subsequent studies that" was 18S rRNA gene generated amplification size of 210 bp while (ITS) region gene produced amplification size of approximately 273 bp. These results were in line with that of Luo and Mitchell (14) and Metwally et al. (15), "the PCR results agreed with phenotypic patterns in table 4; the specific gene don't amplify the target DNA of non-albicans isolates; at the same time, these specific gene amplified the target DNA of C. tropicalis which revealed a blue" color. "This type of contradictory diagnosis by CHROMagar when compared with molecular diagnosis may show that CHROMagar was not always essential for

presumptive diagnosis of Candida species. So there was required a arrangement of some phenotypic and molecular methods for presumptive identification of most Candida isolates. These results are same with the ones obtained by Ahmed et al. (17) who stated the limitation of phenotypic tests and the molecular methods, especially PCR which was being increasingly used for rapid detection of Candida than conventional phenotypic methods".

CHROMagar medium and other phenotypic technique .Candidiasis may be a opportunistic infection among immunocopromasied patients, has become an emerging problem that deserves more clinical attention.

Figure (2). Agarose gel electrophoresis of amplified PCR products for *Candida* isolates by universal primer CANIA and CANIB, Lane A 100 bp. molecular size marker, lanes B-M represent *Candida* isolates.

Figure (3). Agarose gel electrophoresis of amplified PCR products for *Candida* isolates by specific primer CAIB1 and CALB2, Lane A 100 bp molecular size marker, lanes C-H, J-M represent *C. albicans*, lane B and lane I non-*albicans* isolates.

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