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Detection of yeast species associated with respiratory tract infections using internal transcript spacer

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Abstract:
Fungal infections have increased during last two years to become a grown threat due to the prevalence of COVID-19, which is the first factor to inhibit the immune system. Therefore, this study aimed to screen fungal species associated with respiratory infections using internal transcript spacer as diagnostic marker.
The total samples collected in this study were 160 samples of sputum, which included filamentous fungi and yeasts. The total number of positive samples was 124 (77.5%) which included 62 yeast positive samples, and 62 filamentous positive samples. Yeast samples included different genera and the highest percentage belonged to the genus Candida. Five species of the genus *Candida, C. tropical. C. albicans, C. dubliniensis, C. krusei and C. glabrata* were identified based on phenotypic characteristics and biochemical tests as well as using chromogenic Candida agar medium. The highest percentage of isolated yeasts was Candida yeast 53, with a rate of (85.48%) out of 62 samples of yeasts.The highest percentage of yeasts was *Candida albicanis*, which amounted to 26 (41.93) positive samples for examination according to the genetic diagnosis of yeasts.
Keywords: Candida, respiratory system, biochemical diagnosis, DNA sequencing,

Introduction

The increase in the spread of diseases is closely related to the decrease in immunity in most patients, especially pathogenic fungi and bacteria, and the spread of viral diseases that cause a significant decrease in immunity and a decline in the immune system in the vast majority of patients has spread, such as the recent spread of Covid 19 [(1).] Mycoses are caused by fungi infection play an important role in causing human diseases or causing various injuries. They range from superficial skin infections to organ invasions and internal organs in the host's body [(2)]. Fungi have several virulence factors to infect humans such as the growth at a temperature close to the human body 37 °C and the production of fungal spores that are easy to enter and its adhesion to the different cells of the host's tissues .They produce harmful substances like toxins and enzymes that can overcoming the host body's immune defense mechanism [(3).] Opportunistic fungal diseases have increased recently. One of the most important and most common yeasts is *Candida*, which constitute a greater proportion of disease-causing that may be considered a significant source of infection and death in patients with severe immune deficiency [(4).] this study aimed to identification of pathogenic yeast associated with respiratory tract infection using internal transcript spacer gene.

Methods

Samples Collection and classical identification

Sputum samples were collected during the period from July 2020 to June 2021. A total (160) sputum samples were collected from patients attending the Respiratory Center and Chest Diseases Center - Al-Thawra Center and Heart Hospital in Nasiriyah city at southern of Iraq. The samples were cultured on Sabouraud dextrose agar (SDA) and incubated at 37 C˚ for 5 days.

Fungal growth was firstly diagnosed by relying on phenotypic characteristics to identify both filamentous fungi and yeast [(5).] However, yeast growth was subcultured on the chromogenic agar and incubated at 37 C˚ for 48 hrs. This medium was used to diagnose *Candida* yeast species. The mechanism of action of this medium depends on the method of separating the basic materials in the medium (Gleavage Chromogenic Substrate). This leads to the intervention of the color indicator that works on the appearance of each species belonging to the genus *Candida* in different and distinct colors for that species, due to the secretion of enzymes by each type of yeast which makes them appear in distinct and different colors from each other, for example, *C. albicans* produces green color (Table 1.). The colors of the developing colonies on this medium were used to distinguish yeast species compared to the standard key [(6).]

DNA extraction

Yeasts isolates (54 isolates) were subcultured on Sabouraud dextrose agar (india) and incubated at 37°C for 24 to 48 h. The genomic DNA was extracted by using manually method in accordance with the

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manufacturer's instructions. The extracted DNA was migrated by 2% agarose to check out the presence of DNA molecules.

Amplification and ITS sequencing

The fungus-specific universal primers ITS1F (5-TCCGTAGGTGAACCTGCGG-3) and ITS4R (5-GCATATCAATAAGCGGAGGA-3) were used to amplify the ITS1 and ITS2 regions as well as 5.8S region. The amplification was carried out according to Kit amplification and by using Thermo cycler (35) and PCR conditions as following: initial denaturation at 94°C for 3 min; 30 cycles of denaturation (94°C for 1 min), annealing (60°C for 1 min), and extension (72°C for 1 min); and a final extension step at 72°C for 3 min. The amplification products of the polymerase chain reaction were migrated by 2%agarose gel electrophoresis to confirm the amplified DNA regions. After validation of the migration process, the PCR product of 54 isolates was sent to Macrogen Company in South Korea for sequencing the partial ITS genes. Nucleotide sequences were assembled and analyzed using DNASTAR lasergene SeqMan Pro software (v. 7.1.0).

Yeast identification

Yeast species were identified using BLAST sequence analysis tools (http://www.ncbi.nlm.nih.gov/BLAST/) and compared by using nucleotide-nucleotide blast tool. Species identification was determined from identity percent with the standard strain sequences as registered in GenBank.

Submission of Nucleotide sequences

A total of 54 nucleotide sequences were submitted to GenBank website for assigning the accession numbers using https://submit.ncbi.nlm.nih.gov/subs/genbank/ website.

Result

Fungal isolates and Classical identification

The total samples collected in this study were 160 samples of sputum. The total number of positive samples was 118 (73.75%) which included 59 positive yeast samples, and 59 positive filamentous samples.

Yeast samples appeared on SDA medium at 37°C in the form of smooth white to cream colored colonies, and the shape of the colonies is circular. Five *Candida* species, *C. tropical C. albicans, C. dubliniensis, C. krusei* and *C. glabrata* were identified based on phenotypic characteristics and chromogenic *Candida* agar medium.

Table 1 Identification of *Candida* species according to colony color on Chromogenic agar

Spices of *Candida*	*C. albicans*	*C. dubinienisi*	*C. tropicalis*	*C. kruesi*	*C. glabrate*
Colors on Chromogenic agar	green	dark green	purple	pink	white

Identification by ITS regions

The total 54 yeast isolates that were sequenced using ITS1 and ITS4 primers, distributed into eight species belonging to different genera as following: 26 isolates of *Candida albicans, C. dubliniensis* (10), *C. glabrata* (1), *C. tropicalis* (2) , *Magnusiomyces capitatus* (5), *Pichia kudriavzevii* (4), *Naganishia diffluens* (4)and *Clavispora lusitaniae* (2) (Table 3).A BLAST search revealed that the identity percentage with the reference gene sequences in the GenBank database was ranged from 93 to 100%. There was no misidentification overall the isolates. The names of fungal genera were reported according to genus name from GenBank.

The highest percentage of isolated yeasts was *Candida,* 45 isolates (83.33%) out of 59 positive samples.The highest percentage of *Candida* species was *Candida albicans*, 26 isolates (41.93) (Table 2 and 3)

Table 2 Percentage of *Candida* species according to their growth on Chromogenic agar

No	*Candida* spices	Percentage of identification
1	*Candida albicans*	49.05%
2	*Candida krusei*	26.41%
3	*Candida dubliniensis*	18.86%
4	*Candida tropicalis*	3.77%

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5	*Candida glabrata*	1.88%

Table 3 yeast identification using ITS sequencing

No	Organism	Number	Percentage
1	*Candida albicans*	26	48.15
2	*Candida dubliniensis*	10	18.52
3	*Magnusiomyces capitatus*	5	9.26
4	*Pichia kudriavzevii*	4	7.41
5	*Naganishia diffluens*	4	7.41
6	*Candida tropicalis*	2	3.70
7	*Clavispora lusitaniae*	2	3.70
8	*Candida glabrata*	1	1.85
	Candida	45	83.33
	Non-Candida	9	16.67

DNA sequencing and phylogenetic tree

According to the yeast DNA sequencing results obtained from the nested polymerase chain reaction after species identification as mentioned earlier, the tree sequences for these species are shown as shown in Figure (1), where the analysis is. Based on the partial sequence of the gene itsu used for confirmatory genetic detection The phylogenetic tree was generated using the method of unweight pairwise groups by the arithmetic mean method (UPGMA) and molecular genetics (MEGA version 6.0). Local Candida yeasts were shown as a heterogeneous group and as new closed variants associated with NCBI-Blast. Nitrogen-base sequence analysis of the PCR products of the studied yeast gene from Candida yeast showed that all studied samples were consistent with those registered with the National Center for Biotechnology Information (NCBI). The phylogenetic tree was generated using the method of unweight pairwise groups by the arithmetic mean method (UPGMA) and molecular genetics (MEGA version 6.0). Local *Candida albicans*. Viewed closed in connection with NCBI-Blast c. white (KY101873.1), with Show matching 97-100% . They looked more dissimilar and came out of the tree with overall genetic variance. The phylogenetic tree was generated using the unweight pairwise groups method by the arithmetic mean method (UPGMA) and Molecular Genetics (MEGA version 6.0). DNA sequencing results obtained from PCR results for the studied yeast samples and genetically determined species (37) samples of Candida, Magnusiomyces capitatus (5), *Pichia kudriavzevli* (4), *Naganishia difffluens* (4), *Clavispora*(2)The results of nitrogen-base sequence analysis of fungal yeasts showed that most of the expected results for the studied samples were in agreement with the results recorded by the National Center for Biotechnology Information (NCBI) by up to 100%. Confirmative DNA sequence detection of locally isolated yeast isolates using local bases and alignment analysis (NCBI-Blast) and phylogenetic tree analysis of yeasts and their comparison with the species registry recorded in the GenBank. Because of the great differences in epidemiology and diseases and the difference in demographic and spatial nature between different regions of the world, the distinction between Candida yeast species and other obtained species is difficult and important, and among the most important methods used for this purpose in identifying fungal species and yeasts is the use of analytical tools For molecular distinction between different types of yeasts, the present study used molecular methods and tools for the purpose of characterizing strains of Candida yeasts and other yeasts that included *Magnusiomyces capitatus*, *Pichia kudriavzevli, Naganishia difffluens* and *Clavispora . lusitaniae* and its genetic relationships, in which these types of patients with various respiratory diseases were isolated from Thi- Qar Governorate based on DNA analysis and nitrogen base infiltration of those yeasts. Yeast species were shown and compared with the number of reference accessions in NCBI. Appendix (3).

It was found that they are identical by up to 97-100% . The nucleotide sequence data reported here were submitted to the database in addition to the entry numbers. Domestic yeasts of the genus *Candida albicans*

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were shown as new closed genetic variants related to (MN263159.1) *C.albican* NCBI-Blast and in a 99% ratio, C. MH545916.1 was shown.) 99% and local tropical ovaries were closed with NCBI-Blast *Candida tropis* (MK300694.1), 100% , While *Candida glabrata* appears to be related to NCBI-Blast (KP068737.1), Candida glabrata. The local yeast *Dipodascus capitatus* was closed using NCBI-Blast *D. capitatus* (KJ830978.1) 99%. Access numbers were obtained for the registered genes, which included loci, samples registered in the genebank and as recorded at (https://www.ncbi..nlm.nih.gov/nuccore/?term=MZ536223:MZ536276[accn])

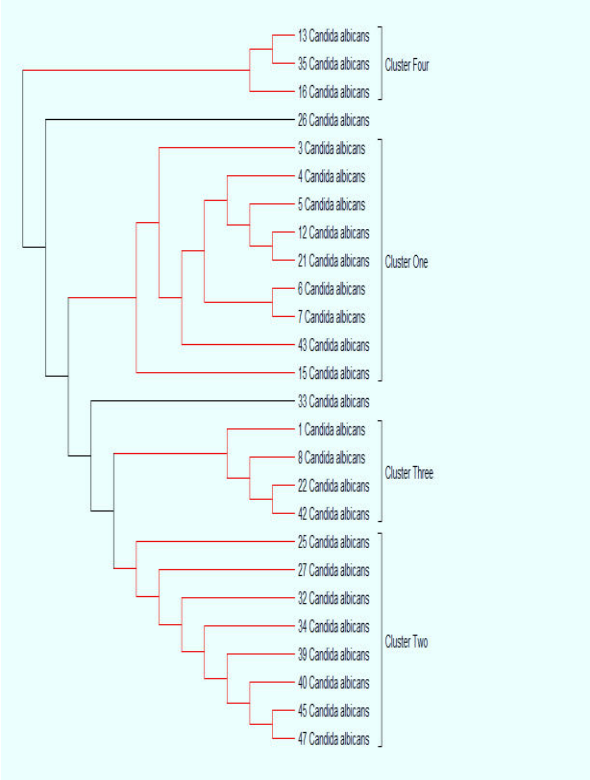

Figure (1) Phylogenetic tree analysis based on the partial sequence of the(itsu) gene, which is used to analyze the genetic relationship of local Candida yeasts. The phylogenetic tree was generated using the unweighted pairs ensemble method with arithmetic mean (UPGMA tree) in (MEGA version 6.0).

Discussion

Identification by ITS regions and alignment analysis (NCBI-Blast):

It is clear from the results obtained from the genetic sequence of the yeast samples that were isolated from the sputum of patients who were referred to hospitals affiliated with respiratory diseases in Thi-Qar Governorate, which were mentioned in the results, that these results agreed with what the researcher [(7),] who identified pathogenic yeasts by nucleotide sequencing using PCR primers. With what is in the GenBank at 99-100%. The output of the PCR for yeast genes in this study was that all those samples were identical and consistent with what was recorded in NCBI-GenBank, and the input numbers for the recorded genes were obtained which included .

Phylogenetic tree

By analyzing the phylogenetic tree based on the partial sequence of its gene, which is used to analyze the genetic relationship of local Candida yeasts. The phylogenetic tree was constructed using the arithmetic mean unweighted pairs ensemble method (UPGMA tree) in MEGA version 6.0. It was found that they are identical by up to 97-100% . The nucleotide sequence data reported here were submitted to the database in addition to the entry numbers, displaying local yeasts of the genus Candida albicans as new closed genetic variants associated with (MN263159.1) *calbican* NCBI-Blast and at 99%, C. MH545916.1.) 99% of the local tropical ovaries were closed with NCBI-Blast *Candida Tropicallis* (MK300694.1), 100% . While *Candida glabrata* appears to be related to NCBI-Blast (KP068737.1), *Candida glabrata*. The local yeast

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Dipodascus capitatus was closed using NCBI-Blast *D. capitatus* (KJ830978.1) 99%. Access numbers of the recorded genes were obtained, and these results are consistent with those reported by the researcher [(7),] which identified pathogenic yeasts by sequencing of nucleotides using specific PCR primers.

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