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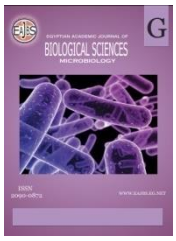

Molecular and Phenotypic Analysis as A Diagnostic Tool of Candida Spp Infections Associated with COVD19

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ABSTRACT

Several cases of oral candidiasis have recently been reported in patients with COVID-19, and this may lead to increased risks associated with morbidity and mortality. The current study aimed to evaluate the Molecular and phenotypic analysis as a diagnostic tool for Candida spp infections associated with COVD19**.** The study included 100 patients infected with the Coronavirus 19 who attended Al-Shirqat General Hospital, where yeasts were isolated from the oral cavity of those infected with the virus, by taking a swab and examining it in two ways, the KOH method and by culturing on a pre-prepared nutrient medium, the plates were incubated. The reactions of the RAPD (using 10 primers) technique were used for molecular diagnosis and analysis. The results of direct microscopic examination of a total of 100 samples showed that 35 samples were positive at a rate of 35%, while the number of negative samples was 65 samples at a rate of 65%. The results of the laboratory culture showed that there were 40 positive samples for growth, with a percentage of 40%, while the number of negative samples was 60 samples, and with a percentage of 60%, no growth.The results of RAPD-PCR indicators using 10 primers and the phase on 1% agarose gel showed 90 identification sites for the total primers, of which 89 were differentiated sites and one year site. Unique and absent, a total of 13 bundles for all prefixes, 11 unique and 2 absent bundles, and the total variance ratio was 99% for all prefixes The molecular sizes of the resulting bundles of the prefixes ranged between 100 -2000bp, where the highest number of packets was in primer P9, which gave 13 sites, while the lowest was in primer P10, which gave 3 sites, and the highest packets were in primer P5 with a total of 45 packets, and the lowest in primer P10 with a total of 12 packets, and also produced in primer P9 with 4 unique packets The lowest was in P1, P3, P5, P6, P8, with one beam for each primer, while the absent appeared only in P3, P2 primer s, respectively, with one beam for each primer. We can conclude the possibility of using the primers used in the technique of RAPD PCR as a diagnostic method for candidiasis in a more efficient and faster way than the endoscopic diagnosis in infections associated with Covid 19.

INTRODUCTION

Candida albicans (C. albicans) were naturally present within the oral cavity in approximately 40-65% of the oral cavities of healthy adults. It often involves Candida infection of the oral cavity in the immunocompromised host (Riad *et al.,* 2020). Although this infection is rarely found in target organs in healthy people, it can lead to life-threatening invasive diseases in patients with a Weak immune system(Li *et al.,* 2019)

Several cases of oral candidiasis have recently been reported in patients with COVID-19, and this may lead to increased risks associated with morbidity and mortality. Therefore, early identification of oral candidiasis in these patients is essential for successful and effective management as laboratory testing and management of this condition have been a challenge in COVID-19 patients(Prakash & Chakrabarti, 2021)

Molecular diagnosis of fungal infection is based on pure cultures. In order to start the antifungal treatment early and get better treatment results, the detection of fungal DNA that is detected by polymerase chain reaction (PCR) with its different techniques, from RAPD-PCR and others, is faster and more sensitive than traditional methods, which are time-consuming and sensitive. (Mokaddas *et al.,* 2010)

DNA-dependent detection plays an important role in the identification of fungal analyzes by providing sensitive and specific biological assays where the method relies on three reactions that depend on the heat cycles of DNA amplification. The first of these reactions is the mutagenesis that transforms the double-stranded DNA into a single-stranded template under extreme temperature conditions, (Marchetti et al., 2004) and the second is the annealing of the single-stranded DNA template at a strict annealing temperature that is suitable for a specially manufactured starter material, Polymerase enzyme (Ellepola & Morrison, 2005; Wisplinghoff *et al.,* 2004). Finally, elongation depends on the activity of the polymerase chain reaction by creating new chains complementary to the single-chain sequence (Ellepola & Morrison, 2005; Khan & Ahmad, 2017). Nucleic acid-based detection or molecular diagnosis based on hybridization and microarray techniques can be used. The sample preparation is also one of the most difficult stages of using these techniques (Ellepola & Morrison, 2005). The current study aimed to evaluate the Molecular and phenotypic analysis as a diagnostic tool of Candida spp infections associated with COVD19.

MATERIALS AND METHODS

The study was conducted in Salah Al-Din Governorate - Al-Shirqat District for the period from November 2021 to February 2022. The study included 100 patients infected with the Coronavirus 19 who attended Al-Shirqat General Hospital, where yeasts were isolated from the oral cavity of those infected with the virus, by taking a swab and examining it in two ways, the KOH method and by culturing on a pre-prepared nutrient medium, the plates were incubated in an incubator at 35 °C for 24 hours.

Culture of Samples:

The samples were planted on the saproide dextrose agar medium, as it was planned with cotton swabs on the surface of the nutrient medium. As for the urine samples, a sterile plant vector was immersed in it and then planned on the pre-prepared nutrient medium. The dishes were incubated at a temperature of 37 °C for a period ranging between 1-4 days According to the growth rate of candida (Atlas, 2004).

Macroscopic Features:

The external appearance of the colonies growing on the saproide medium was examined by dextrose agar, and the colony color, shape, diameter, height and odor were observed (Ellis *et al.,* 2007).

Microscopic Features:

Part of the colony was taken by the conveyor, then mixed with a drop of distilled water, then the sample was spread on a glass slide and left to dry, then fixed on a fire flame and dyed with crystal violet dye and cotton blue dye - lactophenol, then it was examined

by microscope under the power of X100 and x400 for the purpose of microscopy (Ellis *et al.,* 2007)

Molecular Study:

Preparation of Fungal Samples for Study:

Three different isolates of isolated yeasts were tested based on their initial diagnosis and virulence factors, and the concentration was 25 mg/ml for molecular study. After the samples were incubated for 24 hours on a saproide-dextrose-agar culture medium, the yeast suspension was added to it and mixed quietly, then poured into Petri dishes, and after 24 hours of growth, half a gram of colonies growing on the culture medium was taken and DNA was extracted from them.

DNA Extraction:

The genomic DNA was extracted by taking 5-10 gm of the sample from the culture media of the fungal isolate, then 700 microliters of the lysis buffer solution were added to the sample in a test tube, then the sample was shaken with the solution in a vortex vibrator, then the test tube was transferred to a water bath for a period Half an hour at a temperature of 60 ° C, then the mixture was left to cool, then 200 microliters of protein lysis solution were added, then the tube was shaken well and the mixture was transferred to the refrigerator for 5 minutes, then the tube was then transferred to the centrifuge for 4 minutes at 13000 rpm, then 700 µl of the resulting sediment was isolated to an Eppendorf tube, 500 µl of the binding solution was added and the mixture was left for 5 minutes, then 750 µl of the mixture was transferred to a separation column and then transferred to the centrifuge for one minute at a speed of 10,000 rpm, and the mentioned process was repeated twice

Washing solution No. 1 with a volume of 500 µl was added to the column and transferred to the centrifuge at 10,000 rpm for one minute. Then the process was repeated with Wash Solution No. 2 at a number of cycles of 13,000 for one minute. The separated solution resulting from this step was neglected and the separation column was left to dry in the centrifuge.

Then a DNA elusion solution with a volume of 100 µl was added, then left at laboratory temperature for 5 minutes, then centrifuged at 13,000 rpm for 2 minutes, then the separation column was neglected and the separated solution was kept for the purpose of conducting migration and PCR experiments.

Agarose Gel Electrophoresis:

The electrical relay process was conducted to detect the presence of DNA and it is carried out in two phases, each phase has a specific time, current and voltage. Also, the solutions were prepared in the electrophoresis process, the agarose gel preparation method, and the DNA electrophoresis process. The gel was prepared for all reactions according to the method (Slater, 2009).

After that, agarose gel was prepared at a concentration of 1% to migrate the genomic DNA, and then the samples were loaded into the gel pits. The samples were flown by an electric current at a voltage of 3 V/cm with the adjustment of the electrodes, and this process takes 30-35 minutes. Then the gel was examined by exposing it to ultraviolet light on a UV-Transilluminator for the purpose of visualizing the formed packets of DNA and estimating their size based on the volumetric index (Maniatis *et al.,* 2001).

Preparation of RAPD-PCR Reactions:

The reactions of the RAPD technique were carried out using the main reaction mixture Premix prepared by the company Bioneer U.S.A according to the instructions. Each tube contains the main components important for the polymerase chain reaction, which includes one unit of Taq M enzyme, DNA polymerase 250, a mixture of Nitrogenous bases Tris-Hcl (pH9) 10 dNTPs 1.5MgCL, 30Mm KCL, Mm 13 primers were used in this study. These primers were prepared from Operon technologies U.S.A and their sequences are shown in Table 3-6. The PCR was performed with a volume of Eat 20 µl of the ingredients (5Unit/ µl i-Taq DNA Polymerase, 2.5Mm DNTPs, Reaction buffer (10X), MgCl2, Tris-HCl, KCl and Gel loading buffer).

The Primers of The Study:

The primers from Biolab Company were used for the polymerization reaction shown in Table 1, where the ten primers shown in the table were used to assess the genetic dimension among Candida species.

Table 1: Primers of the study.

Cod	Sequence	Primer name
1P	CAGGCCCTTC	OPA-01
2P	GATGACCGCC	OPC-05
3P	ACCCGGTCAC	OPD-20
4P	CTCTCCGCCA	OPG-13
5P	ACGACCGACA	OPG-17
6P	CATTCGAGCC	OPK-01
7P	CTGGGCAACT	OPM-06
8P	AGGGTCGTTC	OPM-14
9P	GAGCGCCTTG	OPQ-06
10P	GGGTGTGCAG	OP-V19

RESULTS AND DISCUSSION

The results of direct microscopic examination of a total of 100 samples showed that 35 samples were positive at a rate of 35%, while the number of negative samples was 65 samples at a rate of 65%.

The results of the laboratory culture showed that there were 40 positive samples for growth, with a percentage of 40%, while the number of negative samples was 60 samples, and with a percentage of 60%, no growth appeared as shown in Table (2) which shows the results of the microscopic and cultured laboratory examination of the study samples.

Table 2: Numbers and percentages of laboratory and microscopic examination of samples

Test type	Total no.	Positive (%)	Negative (%)
Cultures	100	35(35%)	65(65%)
Microscopic examination	100	40(40%)	60(60%)

Types of Candida spp Isolates:

The results in Table 3 showed that Candida albicans outperformed albicans by 60%, followed by tropical albicans by 30%, while the remaining percentage (10%) represented other types

Table 3: candida spp isolates.

Candida spp	%
Candida albicans	%60
Candida tropicalis	%30
Others	%10

The results of RAPD-PCR indicators using 10 primers and the phase on 1% agarose gel showed 90 identification sites for the total primers, of which 89 were differentiated sites and one year site. Unique and absent, a total of 13 bundles for all prefixes, 11 unique and 2 absent bundles, and the total variance ratio was 99% for all prefixes, Table (3). The molecular sizes of the resulting bundles of the prefixes ranged between 100 -2000bp, where the highest number of packets was in primer P9, which gave 13 sites, while the lowest was in primer P10, which gave 3 sites, and the highest packets were in primer P5 with a total of 45 packets, and the lowest in primer P10 with a

total of 12 packets, and also produced in primer P9 with 4 unique packets The lowest was in P1, P3, P5, P6, P8, with one beam for each primer, while the absent appeared only in P3, P2 primer s, respectively, with one beam for each primer, as shown in Table (4).

Table 3: results of the primers used in detecting the genetic relationship of candidiasis.

No.	Primer	No. of locus	General locus	Hetero locus	Total bands	General bands	Hetero bands	Unique bands	Absent bands	Diversity %
1	P1	9	0	9	27	0	27	1	0	%100
2	P2	10	1	9	44	8	36	0	1	%90
3	P3	8	0	8	27	0	27	1	1	%100
4	P4	6	0	6	17	0	17	0	0	%100
5	P5	12	0	12	45	0	45	1	0	%100
6	P6	7	0	7	16	0	16	1	0	%100
7	P7	11	0	11	29	0	29	2	0	%100
8	P8	11	0	11	33	0	33	1	0	%100
9	P9	13	0	13	30	0	30	4	0	%100
10	P10	3	0	3	12	0	12	0	0	%100
Total		90	1	89	280	8	272	11	2	% 99

As for the unique and absent distinct bands, where the total of these prefixes produced 13 distinct bands, which were divided into 11 unique and 2 absent bands. The starter 9 was distinguished by showing the greatest number of bands, and this corresponds to what most researchers who used RAPD indicators to detect the genetic relationship where unique and absent distinct bands appear.

Table 4: unique and absent characteristic bands, efficiency and discriminatory ability of candidiasis samples for the study samples.

			1		2		3		4		5		6		7		8			
No.	Primer name	Pb	Unique	Absents	Unique	Absents	Unique	Absents	Unique	Absents	Unique	Absents	Unique	Absents	Unique	Absents	Unique	Absents	Adequacy	Discriminating ability
1	P1	2000pb	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	9.6	9.9
2	P2	200pb	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	15.7	13.2
3	P3	700pb 600pb	-	-	-	-	-	-	-	-	-	-	-	-	1	1	-	-	9.6	9.9
4	P4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.07	6.25
5	P5	100pb	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	16.07	16.5
6	P6	500pb	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	5.7	5.8
7	P7	800pb 300pb	-	-	-	-	-	-	1	-	-	-	-	-	-	-	1	-	10.3	10.6
8	P8	300pb	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	11.8	12.1
9	P9	900pb 600pb 500pb 200pb	-	-	-	-	-	-	2	-	-	-	-	-	1	-	1	-	10.7	11
10	P10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4.28	4.4
			-	-	-	-	1	-	5	-	-	-	1	1	2	1	2	-		
Total			-		-		1		5		-		2		3		2		13	

The presence of a difference between the results of the direct microscopy examination and the results of the laboratory examination is consistent with other studies that indicated the existence of differences between the results of the two examinations (Katz, 2021; Riad *et al.*, 2021).

The clinical diagnosis does not give great accuracy unless it is supported by other types of diagnosis for several reasons, including that this diagnosis depends on the experience and diligence of doctors and the presence of diseases similar to oral candidiasis in symptoms, as is the case in viral and bacterial stomatitis. In addition, the clinical examination is supported by direct microscopic examination He gave a more accurate result, as this examination depends on the presence of pathological yeast cells, fungal hyphae, or other part of the pathogen, so it is more accurate than the clinical examination (Salman & Al-haddad, 2021). More accurate results, as this test, are one of the most accurate laboratory tests (Rai *et al.*, 2021).

With regard to obtaining some negative results, it may be attributed to the decomposition of yeast cells as a result of the host's resistance, or the appearance of negative results in the two examinations may be due to the insufficiency of the sample collected, or the pathogen is other than fungi (Satyanarayana et al., 2019) or the cause may be As a result of patients randomly using some topical treatments without consulting a specialist before conducting laboratory tests (Berkowitz & Jerris, 2016). Or it may be due to the method of culture and the culture medium, or because of a mistake in the method of storing the sample until transplantation, as it is stored in containers that retain moisture, which helps in the growth of saponified fungi (Abraham et al., 1998) and the appearance of a negative result may be due to the presence of another pathogen, such as being a causative bacterial ((Basmaciyan *et al.,* 2019) or viral (Tran *et al.,* 2013).

The use of RAPD-PCR indicators in detecting the effect of chemical mutagenic from plant extracts, whether water, alcohol, or oil, is one of the high-power indicators in detecting the effect at the level of one nitrogen base, and this makes it accurate in detection and analysis. Indicators of RAPD-PCR are important indicators for such analyzes as they are general indicators that reveal changes at the level of the regions encoding genes and non-coding regions. As for specialized indicators, they target a specific gene or a number of genes, and they target only 0.00001 of the total genomes of mushrooms, and the percentage of the genome encoding genes is 15-20% of the total genes, meaning that the non-coding value of the genes is -85% 80%, which makes it high for detection and analysis (Bardakci, 2001). One of the advantages of RAPD-PCR indicators is that it is easy, quick to analyze, inexpensive, and can be applied in most molecular laboratories, and do not require any prior knowledge of the target genome, as well as being characterized by showing high variances. (Wu *et al.*, 2008)

The change that occurred in the genome of the isolates of mushrooms treated with the large fungal extract could be due to the accumulation of molecules, proteins and materials needed for the process of DNA replication, or due to the effect on the activity of the enzymes related to replication, including DNA helicases and DNA polymerase by the action of the active compounds present in the alcoholic extract of the large fungus, as high concentrations of The extract will disrupt the process of replication and cell cycle during candida growth and thus lead to a change in the sequence of nitrogenous bases in the candida genome (Pfliegler *et al.*, 2014).

Conclusion:

We can conclude the possibility of using the primers used in the technique of RAPD PCR as a diagnostic method for candidiasis in a more efficient and faster way than the endoscopic diagnosis in infections associated with Covid 19.

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