Molecular detection of biofilm genes and some virulence factors in *candida albicans* isolated from the oral cavity

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ABSTRACT

Background: Oral candidiasis is a common localized opportunistic infection of the oral mucosa that is susceptible to infection. For treatment, it occurs in children, the elderly, and uncles who have cellular immune deficiency. Candida yeast, which is a pest of the West, has nodal strategies that help it localize, cause disease, it can grow in a variety of forms, ranging from a single active donor yeast (spore) Budding single-celled yeast (Blastopore), pseudo hyphae, Pseudo mycelium, and this transformation in the cell is one of the most important insulating the fluid that helps it to traverse tissues and escape from phagocytic cells. **Objectives:** The aims of study was Isolation of *C. albicans* yeast from oral infections and their diagnosis using modern and accurate methods and Studying some of its vairulence factors. **Materials and Methods:** During this study, 120 swabs were collected from the oral cavity of children whose ages ranged from two weeks to six years in the consulting clinic of the Children's Teaching Hospital in Karbala. 23 isolates of Candida albicans yeast were obtained. The swabs were immediately transferred to the microbiology laboratory in the College of Science, and were The sample was grown on Sabouraud Dextrose Agar and incubated in the incubator at 37°C for 24-48 hours until the yeasts appeared. **Results:** 23 isolates of *C. albicans* yeast were obtained from 120 samples taken from children after diagnosing the yeasts and growing them on HiCrome™ Candida Differential Agar. Molecular diagnosis using primers (*ALS1*, *HWP1*, *CALB1*). The results of molecular tests showed the presence of (*ALS1, CALB1, HWP1*) genes by 100% in the twenty-three isolates tested. It turned out that 9 of the isolates produced phospholipase, i.e. 39.1% among the *C. albicans* isolates obtained in this study.It turned out that all of the above yeast isolates were hemolytic through 100% hemolysin production. Studies have shown that all isolates have the ability to produce biofilm using the plate method (MTP) by 30.4%, strong for production, 69.5% of which are medium. **Conclusion:** The isolated C. albicans yeast is present in the oral cavity at a high rate. The isolated *C. albicans* yeast contains many virulence factors that help it infect the host, such as hemolysin, phospholipase enzyme, and biofilms.

***Key Words*:** *Candida albicans, CALB1, ALS1, HWP1, PCR*

1.INTRODUCTION

Fungi are the most prevalent living organisms in most environments. They are divided into molds and yeast. Yeasts are single-celled organisms that reproduce by budding. There are several types of them, some of which are beneficial to humans and others that are diseased, such as Candida. (Nada *et al*.,2020). Oral thrush is caused by *Candida albicans*, a condition in which the yeast *Candida albicans* accumulates on the lining of the mouth. *C.albicans* are unicellular, dimorphic eukaryotes that are the most common and colonizing fungi of the oral cavity, and the most isolated type of oral cavity (Mrudula,2022). Oral thrush causes creamy white lesions, usually on the tongue or the inside of the cheeks. Oral thrush can sometimes spread to the roof of the mouth, gums, tonsils, or the back of the throat. C. albicans is characterized by its ability to transform from a yeast to a filamentous form, at which point it transforms from a normal agent into an opportunistic pathogen (Marcela, 2021). It seizes the opportunity of weakening the body's immunity to become a pathogen, so it is then called an opportunistic fungus, and it is present in about 40-65% of healthy individuals in the world (Massimo *et al*., 2023), and this can be attributed to the various factors of its virulence represented by hyphal formation, adhesion, and surface penetration. The cell and biofilms formation (Al-Saeedi, 2015). Fungal infections are becoming more prevalent especially with the increase in immunodeficiency disorders, post-transplant immunosuppression, cancers and cancer treatment. They are spread everywhere and cause infections that may be simple or more virulent and severe infection associated with death. The ability of some fungal species to cause disease is due to different virulence factors that help the fungus survive and persist in the host, which leads to tissue damage and disease. (Iyalla, 2017). *C. albicans* can grow in a variety of phenotypes ranging from budding unicellular yeast to pseudohyphae and true hyphae. This transformation in form is one of the most important virulence factors that helps it invade tissues and escape from phagocytic cells (AL-Taee *et al*., 2020). The hydrolytic enzymes described in *C. albicans* are aspartyl protease, hemolysin and phospholipase, which they are associated with *C. albicans* pathogenesis (Gharaghani *et al*.,2021). The enzyme of phospholipase increases the ability of the organisms for the destruction of immune factors of host cells and makes better opportunity for invasion and achieving nutrients . The other virulence factor that is important in the pathogenicity

of Candida spp is the hemolysin enzyme that facilitates the pathogen to extract iron from molecules with hemoglobin or hemin (Nouraei *et al*.,2020). In view of the above and the importance of *C. albicans* yeast, we aim through this study to Isolation of *C. albicans* yeast from oral infections and their diagnosis using modern and accurate methods and Studying some of its vairulence factors.

2. MATERIALS AND METHODS

2.1 Study setting

During this study, 120 swabs were collected from the oral cavity of children whose ages ranged from two weeks to six years in the consulting clinic of the Children's Teaching Hospital in Karbala Within two months .and the study was cross-sectional. The swabs were immediately transferred to the microbiology laboratory in the College of Science, and the sample was cultured on Sabouraud Dextrose Agar and incubated in the incubator. at 37°C for 24-48 hours until yeast appears.

2.2 HiCrome™ Candida Differential Agar medium

The medium was prepared according to the company's instructions, 7.42 gm of the culture medium powder in 1 liter of distilled water, after which the solution was heated to the boiling point to completely dissolve the medium, and it was poured into Petri dishes directly. Yeast isolates were grown on this medium by spreading a whole loop of yeast on this medium, and development took place at a temperature of 37 °C for 48 hours after incubation (Hospenthal *et al*.,2006).

2.3 Germ Tube Formation (GT)

This test was conducted using human blood serum by placing 1 ml of the serum in a test tube and inoculated with a pure colony of yeast and incubated at 37 °C for 2-3 hours after that a drop of the suspension was taken and placed on a glass slide and examined microscopically (to see the germ tube that emerges From one side of the cell in the form of a bud and it is three to four times longer than the mother cell (Maysa Khazali, 2022).

2.4 Detection of virulence factors in the yeast isolates under study

2.4.1 Detection of hemolysin production

The effectiveness of the isolates in blood analysis and hemolysin production was tested using the plate assay method. (18-24) hours, at a concentration of 108 cells / milliliter, and placed in the form of drops to form spots on the surface of SDA medium rich in sugar, to which fresh human blood was added, and incubated at 37 °C for a period of 48 hours. The positive result is indicated by the appearance of a semi-translucent halo around the colony. which can be seen using Transmitted light (Levinson and Jawetz,1996).

2.4.2 Detection of phospholipase enzyme formation

The activity of phospholipase in Candida isolates was evaluated using egg yolk agar, and then placed The suspension (0.5 McFarland) on the surface of nutrient agar and incubated at 37 °C for 48 hours (AL-khalidi, 2015).

2.4.3 Detection of biofilm forming Candida spp .

The microtitre plate assay described by (Millsap *et al*.,2001) is the most widely used method and was considered as standard test for the detection of biofilm formation. In the present study, 23 Candida isolates were screened for their ability to form biofilm. Individual wells of sterile, polystyrene, 96 well-flat bottom microtitre plates were filled with 100 µl aliquots of the cell suspension and broth served as control to check sterility and non-specific binding of media. The microtitre plates were incubated for 72 h at 37 °C. After incubation content of each well was removed by tapping the plates. The wells were washed four times with 200 µl of PBS (pH 7.2) to remove free-floating planktonic organism. Biofilms formed by adherent organisms in plate were stained with crystal violet (0.1% w/v). Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying). Adherent Candida cells usually formed a biofilm at the bottom of the wells and were uniformly stained with crystal violet. Optical density (OD) of stained adherent Candida biofilm was determined with a micro ELISA auto reader at wavelength of 492 nm. The OD values were considered as an index of Candida adhering to surface and biofilms forming (Table 2.1).

Table 2.1 Classification the adherence of candida by the MTP method.

Mean values of OD	Adherence	Formatting of biofilm
>0.320	Strong	High
0.120–0.320	Moderately	Moderate
<0.120	Non	Non/weak

2.5 Molecular characterization of *C. albicans* yeast

DNA was extracted from *C. albicans* isolates by using kit diagnoses manufactured by Intron biotechnology/Korea using the primers that are described in the following below (Table 2.2).

Table 2.2 primers sequence and product size used in the present study

Primer name	primer sequences	A mplicon size (bp)	references
ALS1-F	5'-GAC TAG TGA ACC AAC AAA TAC CAG A -3'	**318 bp**	İnci M *et al* .,(2013)
ALS1-R	5'-CCA GAA GAA ACA GCA GGT GA -3'		
HWP1-F	5'-TCAGTTCCACTCATGCAACCA-3'	**572 bp**	Kaminska *et al*.,(2019)
HWP1-R	5'-AGCACCGAAAGTCAATCTCATGT-3'		
CALB1-F	5'-TTTATCAACTTGTCACACCAGA-3'	**273 bp**	Orçun *et al* .,(2022)
CALB1-R	5'-ATCCCGCCTTACCACTACCG-3'		

at a concentration of 10 picomoles / µL the primer solution was prepared and The PCR device programmed as shown in the following (Table 2.3).

Table 2.3 The PCR protocol in the present study

The steps	The process
1	One 5-minute cycle at 95 ° C is for the initial mutagenesis of template DNA.
2	30 cycles included: A 1 min at 96° C for template DNA denaturation. B 30 seconds at 52°C for primers binding to template DNA C 1 minute at a temperature of 72 ° C for elongation of associated primroses.
3	in 10-minute One cycle at 72 ° C is for the final elongation of a strand of replicated DNA.

3. RESULTS AND DISCUSSION

After culturing the samples on SDA medium and incubating them at a temperature of 37 ° C for a period of 24-48 hours, different types of yeasts were obtained, however, Candida albicans was selected, as 23 isolates were obtained after conducting phenotypic and molecular tests to confirm the diagnosis of the isolates, as the percentage of isolates obtained reached It has 19.2%. This result is close to what was reached by (Samah al-naserat, 2017), and in a study conducted in Brazil indicated by (Lívia *et al*., 2015), that the percentage of Candida yeast isolates reached 15.52%. While the percentage recorded in this study is lower than what was achieved by (Anmar and Fadhil,2023), as the positive percentage in their study was 76%. The low percentage of yeasts isolated in this study may be due to the fact that there are many diseases that are similar to candidiasis with symptoms such as viral and bacterial stomatitis, or it may be due to the infected people taking antifungals without consulting a doctor. In addition to the isolation site, the clinical condition, the immunological status, and the patient's treatment regimen (Birinci *et al*., 2005).

3.1 Germ tube formation

One colony of cells was inoculated with human serum and incubated at 37 °C for 2-4 hours, then examined under a microscope to detect the germ tube. It gave a positive result for all C. albicans yeast isolates, as the results indicated that all the isolates formed germ tubes in the form of bumps. Long tube-like extending from yeast cells (Rakan,2019). The results of this examination supported the findings of (Abdullah *et al*., 2022), as the percentage of germ tube formation of the isolates was 100%. (Matare *et al*.,2017) indicated that all isolates of Candida albicans can form germ tubes upon colony examination. The development of germ tubes in human serum allows rapid detection of C. albicans, and a conversion occurs from its normal form to filamentous growth or mycelial growth due to its production of these tubes (Mayer *et al*., 2016). Rapid identification of this organism in vitro and clinically is critical and relevant because C. albicans is a major public health issue worldwide (Matare *et al*., 2017).

3.2 Results of the diagnosis of Candida isolates using HiCrome™ Candida Differential Agar media

It is a selective differential medium that promotes rapid isolation of Candida from mixed culture that can differentiate Candida species from others based on colony appearance. Candida agar contains a unique cross-reactive substrate with a specific enzyme belonging to Candida. Specifically, *C. albicans*, as the results of the current study agree with the previously published study (Al-Dabbagh *et al*., 2023). The color of the colonies is light green, as shown in (Figure 3.1).

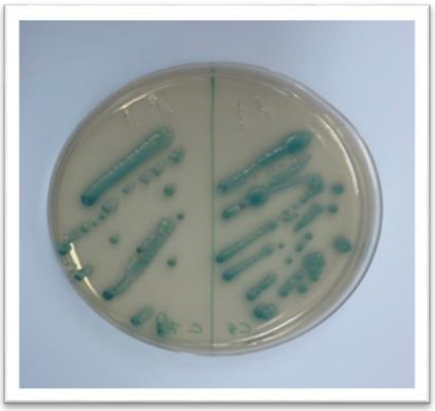

Figure 3.1 Appearance of Candida Colonies Developing on HiCrome™ Candida Differential Agar Medium

3.4 Investigation results for virulence factors of Candida albicans

3.4.1 Haemolysin Production

The hemolytic activity was expressed by observing the complete decomposition of the blood, as it appeared in the form of a semi-transparent area around the growing colonies on the solid SDA medium containing human blood and fortified with glucose at a concentration of 3%. The diameter of the areola of the colony was measured using a ruler (Rakan and Hussein, 2019). The results of this test showed that all C. albicans isolates in our current study were effective in blood analysis, and they all produced hemolysin (100%), as isolate C17 recorded the highest diameter, while C4 recorded the lowest diameter. The results of the study came in agreement with the study carried out by (AL-Taee *et al*., 2020) by 100%. In another study conducted in Malaysia (Chin *et al*., 2013), the percentage of *C.albicans* isolates producing hemolysin was 100%, and this is consistent with the results Our current study.(Jacob

et al., 2014) mentioned in a study that hemolysin production rate was 97.5%. Hemolysis is not present when glucose is not available in the culture medium of the colony (Manns *et al*., 1994).

Table 3.1 Efficacy of *C. albicans* isolates in producing hemolysin

No.	Number of isolate	The result
1	*C.albicans1*	6.25
2	*C.albicans2*	11
3	*C.albicans3*	4.5
4	*C.albicans4*	11.5
5	*C.albicans5*	12.5
6	*C.albicans6*	13.5
7	*C.albicans7*	15
8	*C.albicans8*	14.5
9	*C.albicans9*	12
10	*C.albicans10*	13.5
11	*C.albicans11*	11.5
12	*C.albicans12*	13.5
13	*C.albicans13*	12
14	*C.albicans14*	14.5
15	*C.albicans15*	13
16	*C.albicans16*	13.5
17	*C.albicans17*	20.5
18	*C.albicans18*	13.5
19	*C.albicans19*	13
20	*C.albicans20*	12.5
21	*C.albicans21*	14.5
22	*C.albicans22*	16
23	*C.albicans23*	11.5

3.4.2 phosphplipase production

The results of this test showed 9 positive isolates out of a total of 23 *C. albicans* isolates , accounting for about 39.1%, as shown in Table 6, as it showed the effectiveness of phospholipase production through the appearance of a white semi-transparent area around the colonies growing on medium Hard egg yolk ,This is not consistent with the results of the study conducted (Tsang *et al*., 2007), when the percentage of yeast production of the phosphplipase enzyme was 100%, as this percentage is high compared to the percentages of isolates. positive that was included in the study

Table 3.2 Efficacy of *C. albicans* isolates in producing hemolysin

No.	Number of isolate	The result
1	*C.albicans 1*	+
2	*C.albicans2*	-
3	*C.albicans3*	-
4	*C.albicans4*	-
5	*C.albicans5*	-
6	*C.albicans6*	-
7	*C.albicans7*	-
8	*C.albicans8*	+
9	*C.albicans9*	+
10	*C.albicans10*	+
11	*C.albicans11*	+
12	*C.albicans12*	+
13	*C.albicans13*	+
14	*C.albicans14*	+
15	*C.albicans15*	+
16	*C.albicans16*	-
17	*C.albicans17*	-
18	*C.albicans18*	-
19	*C.albicans19*	-
20	*C.albicans20*	-
21	*C.albicans21*	-
22	*C.albicans22*	-
23	*C.albicans23*	-

Molecular detection of *C. albicans*

The *CALB1* gene was used to detect this yeast, and it is clear from the results in Figure 1 and 2 that the primer for this gene successfully amplified a PCR product with a size of 273 bp.
and this confirms that all Yeast isolates belong to the genus C. albicans and thus match the traditional diagnosis with the molecular diagnosis by 100%. As this gene was used in many studies in diagnosing the yeast *C. albicans*, in a study conducted by (Abdul-Lateef et al., 2015), the percentage of this gene was 100%, and in another study conducted in the United States, the percentage was also consistent with the results of our study 100% (Luo & Mitchell, 2002).

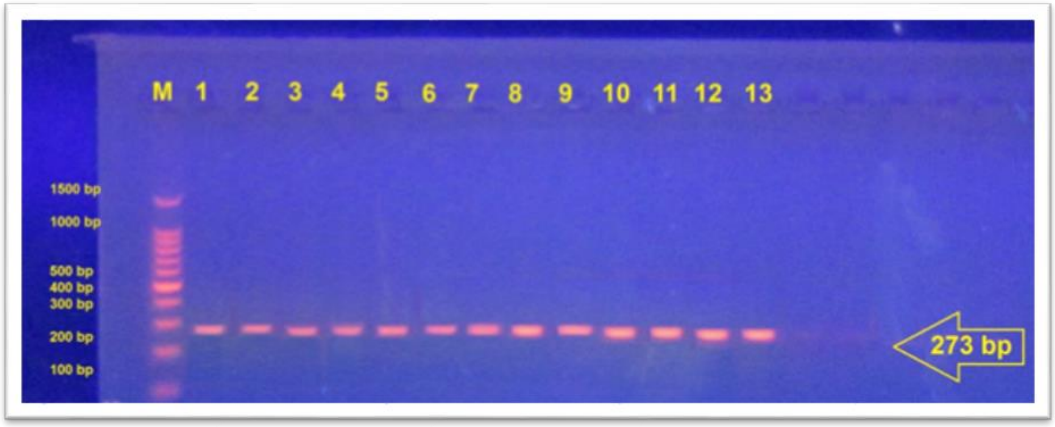

Figure .4.1 the Agarose gel electrophoresis of PCR product using specific primers to detect *CALM1* gene in *C. albicans* (C1-C13) that isolated from oral cavity .

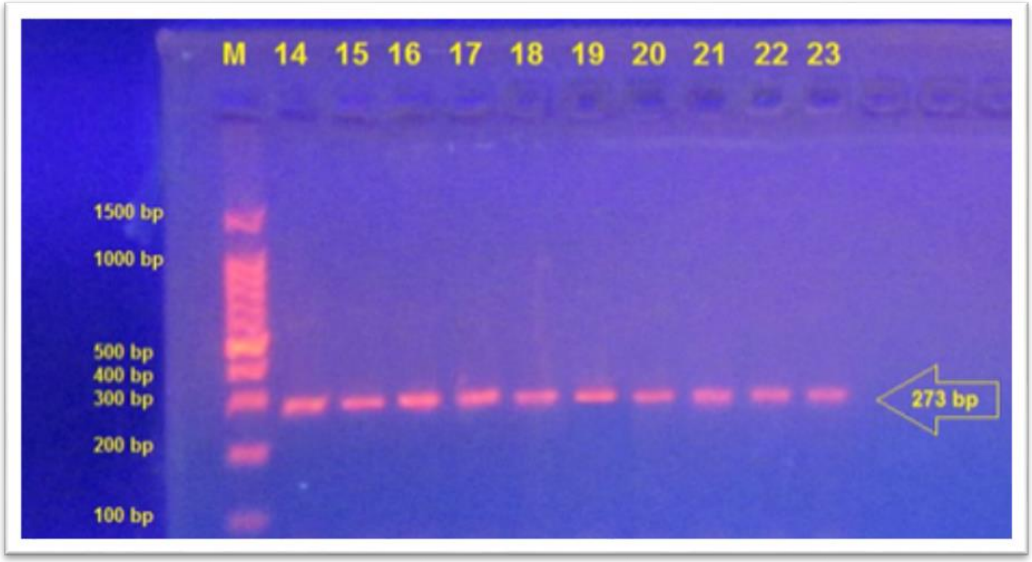

Figure .4.2 the (Agarose gel electrophoresis of PCR product using specific primers to detect *CALM1* gene in C. albicans (C14-C23) that isolated from oral cavity .

5. Detection of the ability of *C. albicans* yeast to produce biofilms by molecular and conventional methods

5.1 Detection of the ability of *C. albicans* yeast to produce biofilms by molecular methods

The *CALB1* and *ALS1* genes were used in our study to molecularly detection the biofilm-forming yeast *C. albicans*. DNA was extracted from 23 isolates, and the results showed that the primers for these genes successfully amplified PCR products with a size of 132 bp, as shown in Figures 1, 2, 3, and 4. This confirms that all *C. albicans* isolates have the ability to form biofilms , and thus the conventional diagnosis matches the molecular diagnosis by 100%. The *ALS1* gene is considered one of the important factors in organism adhesion and biofilm formation, which plays a role in the production of glycoproteins on the cell surface, which leads to increased adhesion to host cells (Nailis *et al*., 2009). Large in adhesion and plays an important role in the formation of biofilms in *C. albicans* called (the hyphal wall protein) (Nobile *et al*., 2006).

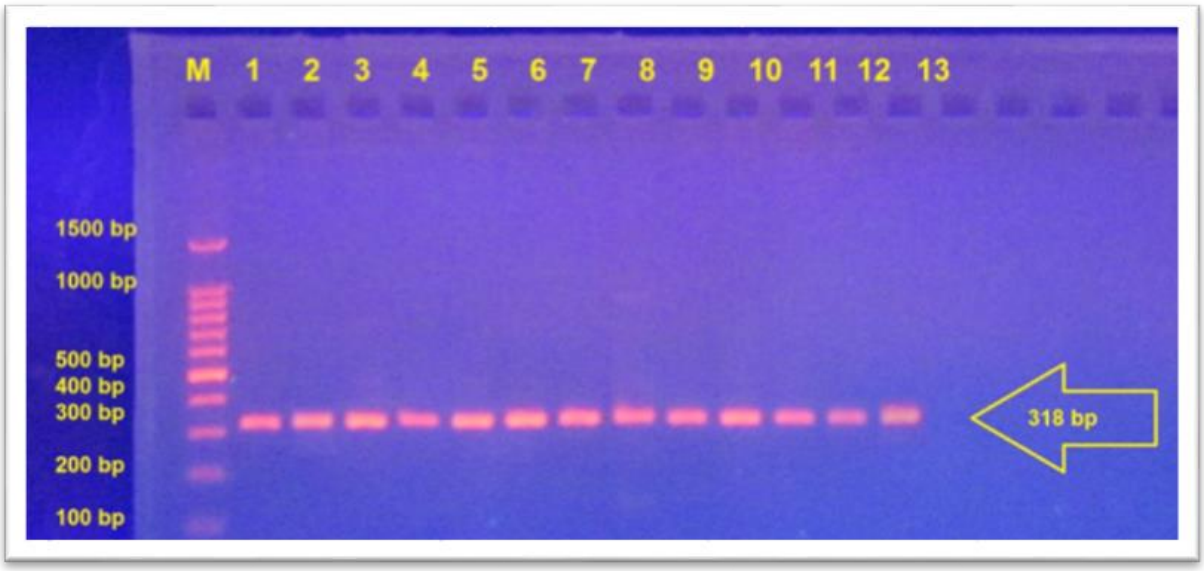

Figure 5.1.the Agarose gel electrophoresis of PCR product using specific primers to detect *ALS1* gene in *C. albicans* (C1-C13) that isolated from oral cavity .

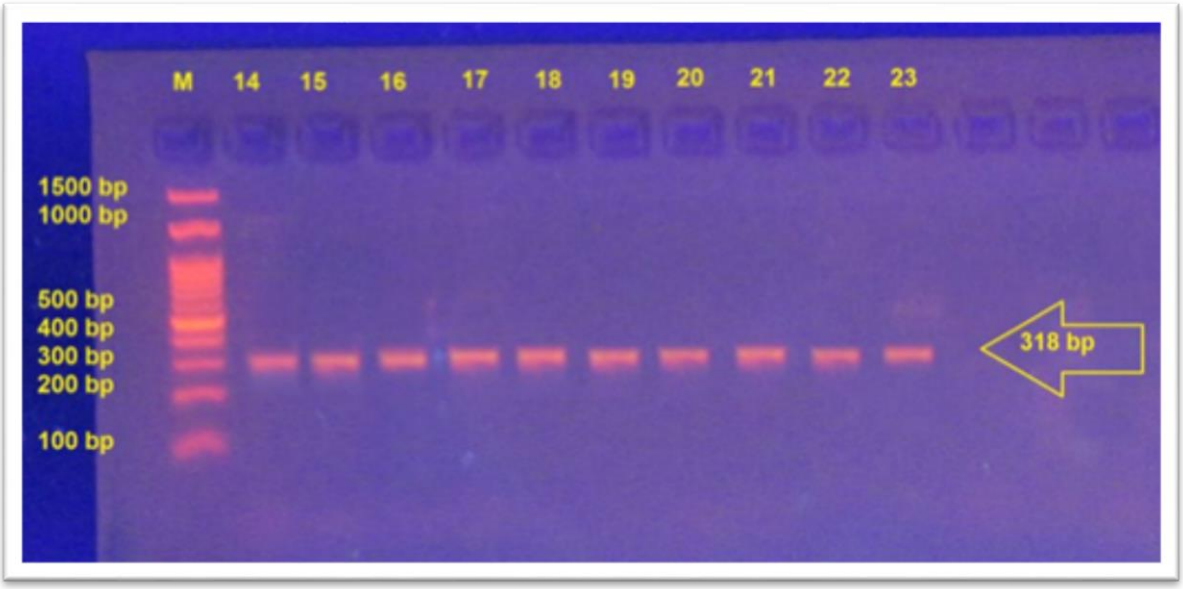

Figure 5.2 the Agarose gel electrophoresis of PCR product using specific primers to detect *ALS1* gene in *C. albicans* (C14-C23) that isolated from oral cavity .

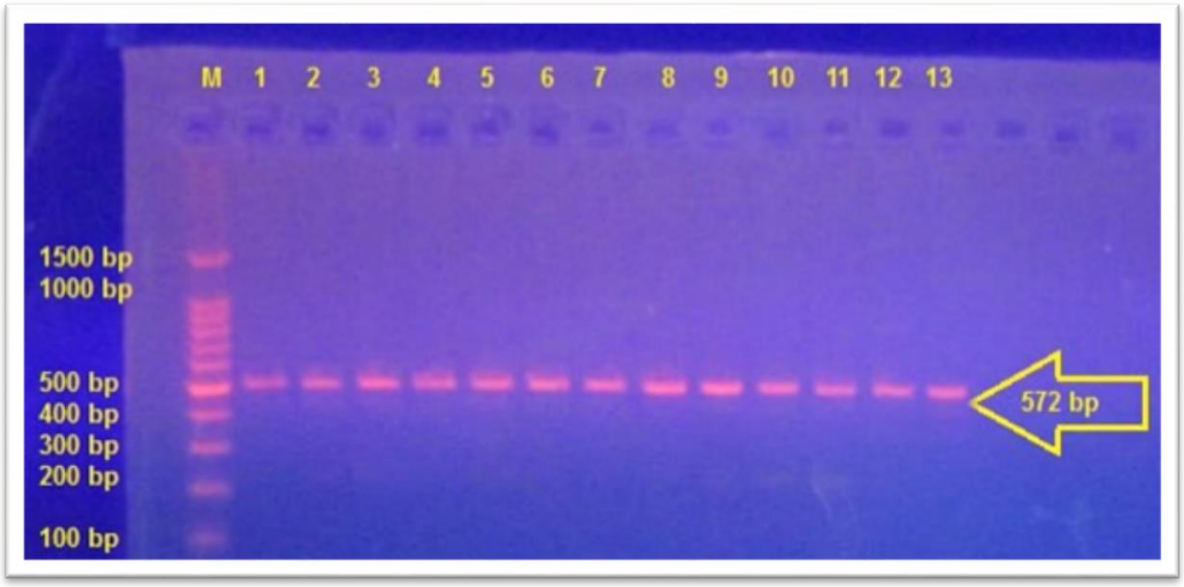

Figure. 5.3. the Agarose gel electrophoresis of PCR product using specific primers to detect *HWP1* gene in *C. albicans* (C1-C13) that isolated from oral .

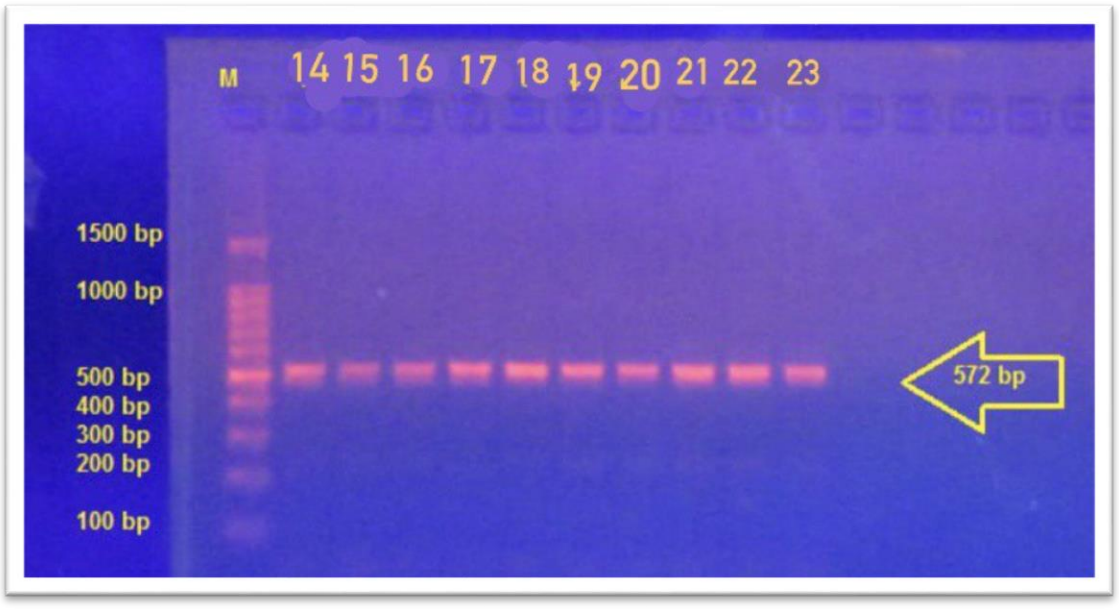

Figure 5.4 the Agarose gel electrophoresis of PCR product using specific primers to detect *HWP1* gene in *C. albicans* (C14-C23) that isolated from oral cavity (amplified size 318 bp and 1.5 percent agarose, 70volt for 1 hrs) .

5.2 Detection of the ability of *C. albicans* yeast to produce biofilm by MTP method

In this study, the micro titter plate assay was used to estimate the biofilm in the yeast C. albicans, as it is clear from Table 7 that all isolates are biofilm-forming at a rate of 100%, as there is no isolate that is not biofilm-forming or has poor production of it in view of to achieve absorbance values higher than 0.120. Where the membrane production strength in the isolates was distributed between strong and medium production, the results showed that there are 7 isolates with a strong production of 34.6% of the enzyme, namely C2, C7, C8, C11, C17, C20, while two-thirds of the isolates had medium production of the enzyme at a rate of 66.6% by 16 isolates. They are C1, C3, C4, C5, C6, C9, C10, C12, C13, C14, C15, C16, C18, C19, C21, and C23. The results obtained from the current study do not agree with what was stated by (Dhanasekaran *et al*., 2014) in a study conducted in Saudi Arabia, where the percentage of strong enzyme-producing isolates was 16.66%, medium-producing isolates 50%, while weak enzyme-producing isolates 33.33%.

Table 5.1 shows the absorbance values of *C. albican* isolates

A	0.0753	0.328	0.164	0.145	0.344	0.291	0.283	0.246	0.267	0.262	0.308	0.3
B	0.0326	0.367	0.277	0.301	0.356	0.343	0.349	0.295	0.334	0.218	0.367	0.3
C	0.0425	0.356	0.251	0.284	0.357	0.142	0.207	0.179	0.193	0.265	0.328	0.3
D	0.0331	0.369	0.297	0.275	0.326	0.201	0.23	0.144	0.218	0.263	0.338	0.3
E	0.162	0.282	0.138	0.302	0.269	0.349	0.359	0.234	0.348	0.164	0.318	0.1
F	0.217	0.158	0.193	0.333	0.259	0.352	0.135	0.229	0.356	0.291	0.197	0.2
G	0.258	0.16	0.183	0.314	0.314	0.341	0.226	0.184	0.326	0.215	0.122	0.1

CONCLUSION

Virulence of the *C. albicans* isolates isolated in this study through the strength of their biofilm production, Hemolysin and phospholipase enzyme formation.

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