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Study of Enzymatic Activity and Biofilm Formation of *candida albicans* in Oral Isolates Obtained from Group of Patients with Invasive Oral Candidiasis

Maher K. Jasim[1], Jamal N. Ahmed[2], Wifaq M. Alwatar[3], Enas A. Rashad[4]

[1]College of Dentistry –Baghdad University

[2]College of Dentistry –Baghdad University

[3]College of Medicine–Baghdad University

[4]Biotchnology Research Center–Al-Nahrine University

Abstract: ***Background: Candidiasis is one of the most common diseases of human caused by several species of Candida spp., The yeast Candida is a normal habitant of the mucous membrane of oral cavity which switch to pathogenic microorganism in immunocompromised people. Multiple virulence factors are contributing to enhance the infection in the host. Adherences to host tissues, switching from yeast to hyphae and exoenzymes secretion are important virulence factors of Candida albicans. These hydrolytic enzymes play important roles in pathogenicity of Candida infection. Objective: The present study was conducted with an aim to determine in acomparative study phospholipase, proteinase, haemolysin, lipase activities and biofilm formation in oral Candidiasis isolated from diseased group and healthy group isolates and patients group before and after treatment with Nystatine 500000IU/ml. Material and Methods: A total of 40 Candida albicans were isolated from oral cavity of patients with symptoms of oral candidiasis and from 12 healthy group. The specimens were identified by standard microbiological methods up to species level and were inspected for production of hydrolytic enzymes and biofilm formation. Results: Phospholipase activity was strong in 8.3% of healthy isolates and 25% of patients group, 40% of patients group isolates produced strong proteinase activity and 0% in healthy group, haemolysin activity was strong in 37.5% of patient group and 8, 3% of healthy group, lipase activity was strong in 0% of isolates of patients and healthy groups, 35.7% of Candida albicans of patient group showed strong biofilm formation in comparison to healthy group 8.3%. Conclusion: Candida albicans showed more extracellular hydrolytic enzyme activity more biofilm formation in patients group than control group, Both the C. albicans in patients and control groups are capable of producing extracellular hydrolytic enzymes and biofilm formation but in filmentous form more than yest form.***

Keywords: *C.albicans*, exoenzymes, filamentous form, conidial form, biofilm formation

1. Introduction

Candida spp. are normal flora of the oral cavity of healthy people, the strain candida albicans is capable of switching between the yeast form and the pseudo- hyphal form, this advantage enable it to establish diseases in immunosuppresed peoples causing diseases ranging from superficial infection to deep disseminated infection[1, 2].candida albicans has multiple virulence factors contributing to colonization and pathogenicity of candida albicans which include : adhesion, invasion, yeast –hyphal transion, biofilm formation, phenotyping switching and secretion of hydrolytic exoenzymes[3]. Exoenzymes play a major role in overgrowth of candida albicans as it pave the way to adhesion, penetration and invation of tissue host[4]. In vivo study secreted aspartyl protienase (SAP) are secreted by filamentous form of candida albicans.in vitro study enzymes SAP secreted by candida albicans when cultured in media containing bovine serum albumin protein as the nitrogen source.phospholipase enzymes is anthor extracellular hydrolytic enzymes associated with cell damage, adhesion and penetration and so invasion[5].phospholipase enzymes acting by destruction of phospholipids in epithelial cells resulting in cell membrane damage, lysis, and so invasion[6], there are four types of phospholipase (PLA, PLB, PLC, PLD).during cell destruction elemental iron stored in the cell is acquired by the candida by production of hemolysine enzymes after which chelation and transporting to fungus for metabolism and growth and enhancing infection[7].in vivo study, the ability of candida albicans to use haemoglobine in erythrocyte as a source of iron by a process called hemolysis[8].

In 2008, study by almeida[9] observed that candida albicans cause greater damage to oral epithelial cells containing elevated concentration of ferritine as compared to cells containing low iron levels. Lipase enzymes which hydrolyse triacylglycerol by acting on the ester bonds in glycerides[7]. In vitro candida albicans can induce lipase activity in media containing tween 80 [10]and considered as one of the pathogenicity factors of this yeast. Biofilm formation defined as organized strucutres involving microbials that are attached to tissue and circumvented in a matrix of exopolymeric materials causing severe damage to the tissue.

Biofilm initiated by irreversible adherence of microbial cells to tissue followed by growth and maturation to form a mesh with altered the phenotype, growth rate, gene expression in comparison to planktonic cells[11]. Biofilm maintain the role of fungus as pathogenic by evading the host immune mechanism, resisting antifungal treatment and withstanding the competitive pressure from other organisms[12].

Commercially antifungal treatments were developed to inhibit or kill pathogenic form of candida and because of the resistance developed recently in cases of immunocompresion and suppression so new strategies targeting the virulence factors by neutralization or inhibition should be studied, our study pave the way to understand the extracellular hydrolytic enzymes and biofilm formation in hyphal and yeast form of candida albicans.

2. Methods

Specimens Collection

From july 2016 to July 2017, 40 specimens (Oral Swabs) were collected in sterilized containers from patients attending Al-yarmouk teaching hospitals having clinical symptoms of oral candidiasis and 12 specimens from healthy people matching in age and gender.

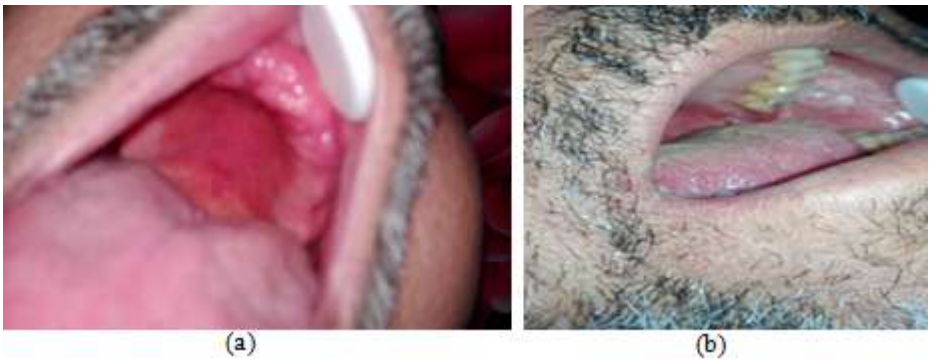

Figure 1: (a) patients with denture stomatitis (b) patients with pseudomembranous candidiasis

Isolations and Identification of *Candida* isolates

All samples were cultured on Sabouraud Dextrose agar (SDA), then was incubated aerobically at 37ºC for 24-48 hrs. *Candida* isolated were identified depending on the morphological features on culture medium, germ tube formation, Chlamydospore formation, CHROMagar (figure 2) and with the use of himedia API 20C [13, 14, 15, 16, 17].

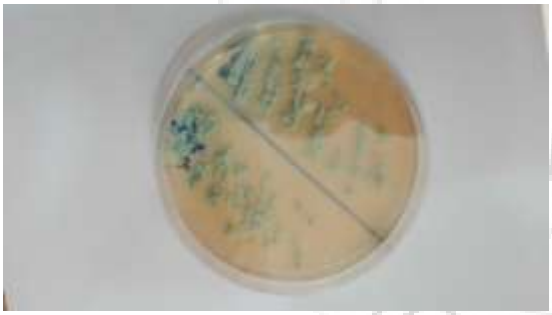

Figure 2: Isolation and identification of candida albicans on chromagar as green colony

Detection of some virulence factors

Determination of phospholipase activity[18, 19].

The test medium contained 65g Sabouraud dextrose agar, 58.4g NaCl and 5.5g CaCl2. All were dissolved in 980ml distilled water and sterilized by autoclaving at 121ºC pounds/inch2 for 12min. Egg yolk was centrifuged at 5000g for 30 minutes. The supernatant was collected and added at rate of 2%to the above medium, mixed and dispensed in plates. An aliquot (10µl) of the yeasts suspension was inoculated on the center of test medium which was then incubated at 37°C for 4 days. Then the phospholipase activity was determined as clear zone around each colony of candida due to removal of lipids by candida.

Figure 3: Phospholipase activity of candida albicans

Determination of proteinase activity[20, 21].

The test medium composed of 60 ml of solution containing 0.04g of MgSO4.7H2O, 0.5g of K2HPO4, 1g of NaCl, 0.2g of yeast extract, 4g of glucose and 0.5g of BSA (bovine albumin serum) was prepared and the pH adjusted to 4.0. The solution was sterilized by filtration then mixed with 140ml of molten agar. An aliquot (10µl) of the yeasts suspension was inoculated on the center of test medium which was then incubated at 37ºC for 7 days. The diameter of the clear zones around the colonies was considered as a measure of protease production**.**

Figure 4: Protienase activity of candida albicans

Determination of Lipase activity[22]

The test medium contained 10g of peptone, 5g of NaCl, 0.1g of CaCl2.2H2O, 15 g of agar, and dissolved in 1, 000 ml of distilled water, with pH adjusted to be 6.5. Sterilized by autoclaving at 121ºC pounds/inch2 for 15min then, it was cooled to about 50 ºC, and 5 ml of sterilized Tween 80 was added. An aliquot (10µl) of the yeasts suspension was inoculated on the center of test medium which was then incubated at 37ºC for 5days, Then the lipases activity was determined as precipitation zone around the colonies.

Figure 5: Lipase activity of candida albicans

Determination of hemolysin activity[23].

A Sabouraud dextrose agar (SDA) was prepared according to supplied company instruction, and sterilized by autoclave. When the medium was cooled down to 50-55°C, 7 % of human blood and 3% glucose with a final pH adjusted to 5.6 was added and dispensed into sterile Petri dishes. An aliquot (10µl) of the yeasts suspension was inoculated on the center of test medium which was then incubated at 37ºC for 48 h. This medium was used to detect the ability of isolates to produce hemolysin

Figure 6: haemolysine activity of candida albicans

Enzymes assay[18, 24].

The activity was expressed according to the Pz index, i.e. colony diameter (a)/ total diameter (b) of the colony plus the precipitation halo. The following ranges of activity were established according to the Pz index: very strong (++++), Pz < 0.69; strong (+++), Pz =0.70- 0.79; mild (++), Pz = 0.80- 0.89; weak (+), Pz = 0.90- 0.99; and Negative Pz = 1.

$$Pz = \frac{Colony\ \ diameter}{Colony\ \ diameter\ +Zone\ \ of\ \ precipition}$$

Figure 7: Measuring enzyme activity diameter

Biofilm formation by *Candidaalbicans* Isolates [25, 26, 27].

To study the ability of candida isolate to produce biofilm, 40 isolates of *Candida spp*. were grown in sabouraud dextrose broth (SDB) containing 8% glucose in 96-well polystyrene tissue culture plates and incubated at 37ºC for 48 hrs under aerobic conditions. After incubation, the planktonic cells were washed ten times with deionized water, and the adhering fungi cells in each well were fixed with 200 µl of absolute methanol for 20 mins. The plates were emptied and left to dry overnight. The adhering cells were stained with 200 µl of 0.1% crystal violet for 15mins, and excess stain was rinsed off. The plates were washed with distilled water and air-dried overnight. The crystal violate dye bound to the adherent cells was dissolved with 1ml of 95% ethanol per well, and the plates were read at 490 nm using micro ELISA auto reader. The experiment was performed in triplicates, and the absorbance of wells containing sterile SDB was used as the negative control the result calculate as in table 1

Table 1: Classification of fungi adherence by tissue culture plate method [28, 29].

Optical Density values (OD)	Adherence	Biofilm formation
ODt < OD c	Non −	Non −
ODc1< ODt ≤ ODc2	Weakly +	Weak + / Moderately++
ODc 1<ODt >ODc2	Moderately++/ strong +++	strong +++

*OD c : optical density of control well.
*ODt : optical density of tested well.

Table 2: Data of biofilm formation of patients group

Study group of oral candidiasis		
Biofilm formation-Before treatment	N	%
Negative -	0	0.0
Weak - /+	12	30.0
Moderate ++	14	35.0
Strong +++	14	35.0
Median	2	
Mean Rank	30.2	

3. Results

The samples from oral cavity of forty patients were collected and included in the study during the period from July 2016 to July 2017 from patients attending Al-yarmouk teaching hospital having clinical symptoms of oral candidiasis , The patients were N= 40 in patient group before treatment (26males, 14 females), their age ranges between 30-71 years and N=38 in patient group after treatment (24 males, 14 female) with the same age range. Control group N=12 (8males, 4 females) age ranges 20-60 years. There are no significant differences between two groups according to gender (table 2.1) and age (table 2.2).

Table 3: Gender difference between control and patients group

	Study group			
	Controls		Cases (Oral candidiasis)	
	N	%	N	%
Gender				
Female	4	33.3	14	35.0
Male	8	66.7	26	65.0
Total	12	100.0	40	100.0

Table 4: Mean age difference between controls and patients group

	Study group		
	Controls	Cases (Oral candidiasis)	P
Age (years)			0.02
Range	(20 to 60)	(30 to 71)	
Mean	41.4	51.8	
SD	13.3	13.3	
SE	3.8	2.1	
N	12.0	40.0	

These isolates were studied for the production of hydrolytic enzymes in patients with oral candidiasis before and after taking antifungal agents (nystatine 400, 000 IU) and in healthy control group such as phospholipase, proteinase, lipase, haemolytic activity and for the biofilm formation.Phospholipase activity was found in 40 (100%) isolates of patients group and 9 (75%) of control group. Positivity for proteinase activity was found in 38 (94%) *Candida* isolates from patient group and 6 (50%) of control group. Hemolysin activity was seen in 25 (62%) of patients isolates and 3 (25%) in control group.lipase activity was found in 24 (60%) of patients group and 7 (58.4) in control group. About 40 (100%) isolates gave positive result for biofilm formations in patient group while in control group are 9 (75%). Maximum phospholipase (100%) activity and biofilm formation activity (100%) was seen in *C. albicans* isolated from patients with oral candidiasis.

Table 5: Virulence factors difference between control and patients group

	Study group				P
	Controls		Cases (Oral candidiasis)		
	N	%	N	%	
Phospholipase-Before treatment					<0.001
Negative	3	25	0	0	
Weak (+)	7	58.3	11	27.5	
Moderate (++)	1	8.3	19	47.5	
Strong (+++)	1	8.3	10	25	
Median	1		2		
Mean Rank	14.3		30.2		
Proteinase-Before treatment					<0.001
Negative	6	50	2	5	
Weak (+)	4	33.3	7	17.5	
Moderate (++)	2	16.7	15	37.5	
Strong (+++)	0	0	16	40	
Median	0		2		
Mean Rank	11.6		31		
Haemolysin-Before treatment					0.019
Negative	9	75	15	37.5	
Weak/moderate (+)	2	16.7	10	25	
Strong activity (++)	1	8.3	15	37.5	
Median	0		1		
Mean Rank	18.2		29		
Lipase-Before treatment					0.62[NS]
Negative	5	41.7	16	40	
Weak (+)	5	41.7	24	60	
Moderate (++)	2	16.7	0	0	
Strong (+++)	0	0	0	0	
Median	1		1		
Mean Rank	28.2		26		
Biofilm formation-Before treatment					<0.001
Negative	3	25	0	0	
Weak	7	58.3	12	30	
Moderate	1	8.3	14	35	
Strong	1	8.3	14	35	
Median	1		2		
Mean Rank	14.3		30.2		

Phospholipase: phospholipase enzyme activity in patient group ranged from (weak to strong) with a median value of 2 which statistically significant with p value of >0.001when compared to control group which enzyme activity ranged from (negative to strong) with a median of 1

Proteinase: proteinase enzyme activity in patient group ranged from (negative to strong) with a median value of 2 which statistically significant with p value of >0.001when compared to control group which enzyme activity ranged from (negative to moderate) with a median of 0

Haemolysine: haemolysine enzyme activity in patient group ranged from (negative to strong) with a median value of 1 which statistically significant with p value of 0.019 when compared to control group which enzyme activity ranged from (negative to strong) with a median of 0

Lipase: lipase enzyme activity in patient group ranged from (negative to weak) with a median value of 1 which statistically non-significant with p value of 0.62when compared to control group which enzyme activity ranged from (negative to moderate) with a median of 1

Biofilm formation: biofilm formation in patients group ranged from (weak to strong) with a median value of 2 which statistically significant with a p value <0.001when compared to healthy control.

Table 6: Enzymes activity and biofilm formation percentage before and after therapy

Enzymes activity after treatment		Enzymes activity before treatment		Extracellular Enzymes and Biofilm formation
N	%	N	%	
29	76.40%	40	100%	Phospholipase
23	60.50%	38	95%	Proteinase
18	47.40%	24	60%	Lipase
19	50%	25	62.50%	Haemolysine
32	84.20%	40	100%	Biofilm formation

Table 6: Phospholipase activity after treatment

	Phospholipase-After treatment									
	Negative		Weak (+)		Moderate (++)		Strong (+++)		Total	
	N	%	N	%	N	%	N	%	N	%
Phospholipase-Before treatment										
Negative	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Weak (+)	3	30.0	5	50.0	2	20.0	0	0.0	10	100.0
Moderate (++)	4	21.1	15	78.9	0	0.0	0	0.0	19	100.0
Strong (+++)	2	22.2	1	11.1	6	66.7	0	0.0	9	100.0
Total	9	23.7	21	55.3	8	21.1	0	0.0	38	100.0

This table show the amount of changes in enzyme activity after treatment with Nystatine as 23.7% of cases have negative activity as compared to 0.0% before treatment and 55.3% of cases have weak activity as compared to 27.5% before treatment and 21.1% of cases have moderate activity as compared to 47.5% in cases before treatment, finally 0.0% strong enzyme activity as compared to 25% strong enzyme activity before treatment.

Table 7: Phospholipase enzyme activity changes after treatment

Phospholipase-Changes after treatment	N	%
-3	2	5.3
-2	5	13.2
-1	24	63.2
0	5	13.2
1	2	5.3
Total	38	100.0
P (Wilcoxon Signed Ranks Test)		<0.001

This table explain the degree of shifting toward increase (+) or decrease (−) in enzymes activity, two cases their enzymes activity after treatment had been reduced by three degree from strong – moderate- weak (or) moderate – weak – negative. Five cases their enzyme activity reduced by two degree strong—moderate or moderate —weak or weak—negative. twenty four cases their enzymes activity have not been changed reduced or increased and only two cases their enzyme activity increased by one degree moderate —strong or weak—moderate or negative —weak.

Table 8: Proteinase enzyme activity after treatment

	Proteinase-After treatment									
	Negative		Weak (+)		Moderate (++)		Strong (+++)		Total	
	N	%	N	%	N	%	N	%	N	%
Proteinase-Before treatment										
Negative	2	100.0	0	0.0	0	0.0	0	0.0	2	100.0
Weak (+)	3	42.9	4	57.1	0	0.0	0	0.0	7	100.0
Moderate (++)	6	40.0	9	60.0	0	0.0	0	0.0	15	100.0
Strong (+++)	4	28.6	6	42.9	4	28.6	0	0.0	14	100.0
Total	15	39.5	19	50.0	4	10.5	0	0.0	38	100.0

This table show the amount of changes in enzyme activity after treatment with Nystatine as 39.5% of cases have negative activity as compared to 5.0% before treatment and 50% of cases have weak activity as compared to 17.5% before treatment and 10.5% of cases have moderate activity as compared to 37.5% in cases before treatment, finally 0.0% strong enzyme activity as compared to 40% strong enzyme activity before treatment

Table 9: Proteinase enzyme activity changes after treatment

Proteinase-Changes after treatment	N	%
-3	4	10.5
-2	12	31.6
-1	16	42.1
0	6	15.8
Total	38	100.0
P (Wilcoxon Signed Ranks Test)		<0.001

This table explain the degree of shifting towared increase (+) or decrease (−) in enzymes activity, four cases their enzymes activity after treatment had been reduced by three degree from strong – moderate- weak (or) moderate – weak – negative. Twelve cases their enzyme activity reduced by two degree strong—moderate or moderate —weak or weak—negative. six cases their enzymes activity have not been changed reduced or increased and only six cases their enzyme activity decreased by one degree strong — moderate or moderate —weak or weak —negative.

Table 10: Haemolysin enzyme activity after treatment

	Haemolysin-After treatment							
	Negative		Weak/ moderate (+)		Strong activity (++)		Total	
	N	%	N	%	N	%	N	%
Haemolysin-Before treatment								
Negative	14	93.3	1	6.7	0	0	15	100
Weak/moderate (+)	4	40	6	60	0	0	10	100
Strong activity (++)	1	7.7	6	46.2	6	46.2	13	100
Total	19	50	13	34.2	6	15.8	38	100

This table show the amount of changes in enzyme activity after treatment with Nystatine as 50% of cases have negative activity as compared to 37.5% before treatment and 34.2% of cases have weak/moderate activity as compared to 25% before treatment, finally 15.8% of cases have strong enzyme activity as compared to 37.5% strong enzyme activity before treatment

Table 11: Haemolysine enzyme activity changes after treatment

Haemolysin-Changes after treatment	N	%
-2	1	2.6
-1	10	26.3
0	26	68.4
1	1	2.6
Total	38	100.0
P (Wilcoxon Signed Ranks Test)		0.005

This table explain the degree of shifting toward increase (+)

or decrease (−) in enzymes activity, one caseits enzyme activity reduced by two degree strong—moderate or moderate —weak or weak—negative and only Ten cases their enzyme activity decreased by one degree strong — moderate or moderate/weak – negative. twenty six cases their enzymes activity have not been changed reduced or increased and only one case their enzyme activity increased by one degree moderate/weak —strong or negative — weak/moderate.

Table12: lipase enzyme activity after treatment

	Lipase-After treatment									
	Negative		Weak (+)		Moderate (++)		Strong (+++)		Total	
	N	%	N	%	N	%	N	%	N	%
Lipase-Before treatment										
Negative	15	93.8	1	6.3	0	0	0	0	16	100
Weak (+)	5	22.7	17	77.3	0	0	0	0	22	100
Moderate (++)	0	0	0	0	0	0	0	0	0	0
Strong (+++)	0	0	0	0	0	0	0	0	0	0
Total	20	52.6	18	47.4	0	0	0	0	38	100

This table show the amount of changes in enzyme activity after treatment with nystatine as 52.6% of cases have negative activity as compared to 40% before treatment and 47.4% of cases have weak activity as compared to 60% before treatment and 0.0% of cases have moderate activity the same 0.0% in cases before treatment, finally 0.0% strong enzyme activity as well as 0.0 % strong enzyme activity before treatment

Table 13: lipase enzyme activity changes after treatment

Lipase-Changes after treatment	N	%
-1	5	13.2
0	32	84.2
1	1	2.6
Total	38	100.0
P (Wilcoxon Signed Ranks Test)		0.1[NS]

This table explain the degree of shifting towared increase (+) or decrease (−) in enzymes activity, only five cases their enzyme activity decreased by one degree strong — moderate or moderate/weak – negative. Thirty two cases their enzymes activity have not been changed reduced or increased and only one case their enzyme activity increased by one degree moderate/weak —strong or negative — weak/moderate.

Table 14: Biofilm formation ability after treatment

	Biofilm formation-After treatment									
	Negative		Weak		Moderate		Strong		Total	
	N	%	N	%	N	%	N	%	N	%
Biofilm formation-Before treatment										
Negative	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Weak	3	25.0	9	75.0	0	0.0	0	0.0	12	100.0
Moderate	3	21.4	6	42.9	5	35.7	0	0.0	14	100.0
Strong	0	0.0	2	16.7	10	83.3	0	0.0	12	100.0
Total	6	15.8	17	44.7	15	39.5	0	0.0	38	100.0

This table show the amount of changes in biofilm formation ability after treatment with nystatine as 15.8% of cases have negative activity as compared to 0.0% before treatment and 44.7% of cases have weak activity as compared to 30% before treatment and 39.5% of cases have moderate activity as compared to 35% in cases before treatment, finally 0.0% strong enzyme activity as compared to 35% strong enzyme activity before treatment

Table15: Degree of changes in biofilm formation ability after treatment

Biofilm formation-Changes after treatment	N	%
-2	5	13.2
-1	19	50.0
0	14	36.8
Total	38	100.0
P (Wilcoxon Signed Ranks Test)		<0.001

This table explain the degree of shifting toward increase (+) or decrease (−) in biofilm formation ability, Five cases their biofilm formation ability reduced by two degree strong—moderate —weak or moderate —weak—negative. nineteen case their biofilm formation ability have been decreased by one degree strong — moderate or moderate —weak or weak —negative and only fourteen case their biofilm formation ability have not been changed reduced or increased.

4. Discussion

Virulence factors in control and patients group

Infections caused by Candida spp. increase as a result of the increase of immune compromised patients in the community, thus, oral candidiasis is one of the most common oral opportunistic infection in this group of patients (Venkatesan et al. 2015; Mushi et al. 2016). Little is known about the epidemiology of oral Candida colonization and infection in immunocompromised patients in developing countries. As show in the result, all patients have a high of *Candida* colonization in their oral cavity, about (77.8%), compared with (37.5%) the colonization of control individuals. This finding may be a result of the using of chemotherapy, radiation, high doses of oral and systemic corticosteroids, and underlying diseases such as diabetes mellitus, which inhibit the immune system and contributed to this phenomenon (Magare & Awasthi 2014; Teoh & Pavelka 2016).

C. albicans is a well known opportunistic pathogen will well developed virulence factors responsible for tissue invasion of host tissues (scheller et al, 2005). But it can be part of normal flora healthy individuals mouthand in certain conditions turned to virulent. This is particularly important, where the organism has to resist the washing action of saliva flow (sitheeque and samaranayake, 2003).virulence factors including the expression of adhesinsand invasins on the cell surface (canon and chaffin, 1999). Many different hydrolytic enzymes are identified in *Candida* sp. including secreted aspartyl proteinase, phospholipase, lipase as the production of these enzymes helps in host cell colonization.

in our study many hydrolytic enzymes were tested in order to help *C.albicans* to colonize the host mucus membrane and establish infection as a part of complicated process to overcome host defense mechanism (calderone et al, 2002) As Out of 40 isolate obtained from diseased patients in medical wards complaining from various systematic diseases

in association with oral invasive candidiasis, shown a variable enzymatic activity as 100% of them had a possible phospholipase activity, a similar finding noticed by T-sang at 2007 when all his oral candidiasis cases showed phospholipase activity. Price in 1982, wu *et al* in 1996, reported that this enzyme digesting the host membrane phospholipase to end up in cell lysis and attain a sustained adhesion to achieve subsequent infection with *Candida albicans* so that it was one of the strongest virulence factors for the measure of the degree of invasion or non-invasion in various candidal strains.

Regarding hemolysine activity it also notably elevated in our sample of patients as it was seen in 62% of cases and only seen in 25% of control group with a statistically significant difference at a p value < 0.01 the administration of any oral antifungl agents as a mouth wash to reduce local infection it was seen also by other coworkers as (mann 1994, watanbe 1999) as hemolytic activity greatly related to the invasive strains that are utilizing iron from oral cavity by hemolysis of red blood cell, and as iron bind to a protein found in the saliva called lactoferrin, to supply candida albicans with its demands of iron to survive and multiply in the oral cavity of diseased patients with symptomatic oral candidiasis, so this finding was mainly related to the mannoprotein released from yeast to bind band 3 protein on red blood cell promoting their distribution and hemolysis providing iron for candida albicans (almemdia 2008, watanabe 1999).

About 94% of *C. albicans* showed proteolytic activity in patient group in the present study This observation was similar to the reports given by previous workers[46].

As for the other extracellular enzymes Lipase are enzyme that hydrolyse the ester bonds of mono-, di-, and triglycerols to produce free fatty acids like monoaclyglycerols and glycerols (Tsai *et al.*, 2013).Lipase play a role in the adhesion and penetration of infection process in murine model of haemetogenously disseminated candidiasis and supporting a role for these extracellular hydrolases in *Candida albicans* pathogenicity (Stiniszewska *et al.*, 2012 ;Mayer *et al.*, 2013). Lipase were seen in 60% of yeast as compared to 41, 7% in the control group as we noticed a similar result by pakshir, 2008 and aktash 2002.

Rudek et al. (1978) demonstrated that lipase activity would appear to be equal between patients and control groups as common feature of *Candida* species that are frequently isolated from clinical specimens. Kumar et al. (2006)[50] reported that lipase detection methods cannot be used as the sole phenotypic identification of *C.albicans* when adding Tween 80 to the test medium but the test appears to be simple, economical and easy method to perform for use in small clinical laboratories. Melak et al. (2012)[51] detected that C. *albicans* showed lipase activity increase in aerobic conditions like oral cavity or skin but not in anaerobic conditions

For the biofilm formation, it is a vast important virulence factor of candida species especially candida albicans as it was greatly reffered to the aggressiveness of strains during tissue invasion and mucosal barrier distribution, some researchers were unable to notice any growth on microtiter plate in vitro (gultekin, 2011), but in our study the growth was noticed clearly in microtiter plate stained in vitro and was seen in all candida albicans stains 100% and in 75% of candida albicans of the control group as well, it was the opposite to the finding of demirbilek.2007, and the difference between both control and the patients group was statistically significant at p value ≤ 0.001 referencing to a clear relationship between oral candidiasis and invitro biofilm formation for candida albicans strain, linked to more sever clinical form of the diseases.

Difference in virulence factors (enzymes activity and biofilm formation) in patient group before and after therapy with Nystatine 400, 000 IU

Phospholipase activity in patient group before treatment were reduced as 23.7% of cases have negative activity as compared to 0.0% before treatment and 55.3% of cases have weak activity as compared to 27.5% before treatment and 21.1% of cases have moderate activity as compared to 47.5% in cases before treatment, finally 0.0% strong enzyme activity as compared to 25% strong enzyme activity before treatment Phospholipase activity was detected in 100 % of the C.albicans isolates in this study. Previous studies have reported phospholipase activity in 30 to 100 % of candida isolates from various groups of patients and from various sites (Price et al., 1982; Wu et al., 1996) Some host factors, such as salivary flow rate, salivary pH, wearing of dentures, alcohol use and smoking habits, are associated with an increased oral carriage rate of Candida species (Kadir et al., 2002). Phospholipase gene expression has been shown to be affected by growth conditions (Samaranayake et al., 2006). It has also been hypothesized that the presence of a high concentration of salivary glucose combined and reduced salivary secretion rate enhances the growth of yeasts and their adherence to epithelial oral cells of type 2 DM patients (Darwazeh et al., 1991), by increasing phospholipase activity.

Proteinase activity after treatment with Nystatine as 39.5% of cases have negative activity as compared to 5.0% before treatment and 50% of cases have weak activity as compared to 17.5% before treatment and 10.5% of cases have moderate activity as compared to 37.5% in cases before treatment, finally 0.0% strong enzyme activity as compared to 40% strong enzyme activity before treatment. Koga-Ito et al. (2006) showed that Sap activity is significantly higher in denture wearers with signs of candidiasis. A number of constituents in the saliva may contribute to the higher levels of oral proteinase observed in oral candidiasis patients (Manfredi et al., 2006). Higher salivary levels of glucose, IgA, and other salivary enzymes such as matrix metalloproteinase (MMP-8), gelatinase (MMP-9) and lysozyme, may all influence salivary proteinase activity and concentration in a direct or indirect fashion (Rayfield et al., 1982; Stevens et al., 1990; Collin et al., 2000)

Lipase enzyme activity after treatment with nystatine as 52.6% of cases have negative activity as compared to 40% before treatment and 47.4% of cases have weak activity as compared to 60% before treatment and 0.0% of cases have moderate activity the same 0.0% in cases before treatment, finally 0.0% strong enzyme activity as well as 0.0 % strong

enzyme activity before treatment. Anees etal, 2011 reported that increased lipase activity of candida albicans in oral cavity of patients with renal transplantation. Study by Kurnatowska AJ in 1998 showed that greatest lipase activity in atrophic candidiasis is for candida albicans strain.

Haemolysine enzyme activity after treatment with nystatine as 50% of cases have negative activity as compared to 37.5% before treatment and 34.2% of cases have weak/moderate activity as compared to 25% before treatment, finally 15.8% of cases have strong enzyme activity as compared to 37.5% strong enzyme activity before treatment. Yenișehirli *et al.* (2010) and França *et al.* (2011) reported that all *C. albicans* strains isolated from various clinical samples showed beta hemolysis. In another study, İncİ *et al.* (2012) reported that 91.1% of the *C. albicans* and 88% of the non-*albicans Candida* species showed beta haemolysin activity. Tsang *et al.* (2007) reported that haemolysin production by *C. albicans* is higher in diabetic patients than in non-diabetic individuals, and suggested that increased blood glucose concentration may directly or indirectly influence the haemolysin production by *C. albicans* and this study is in agreement with our study as strong activity occur in diabetic patients

Finally, All these findings (enzymes and biofilm) show difference after two to four weeks of treatment which may be affected by hydration which increase salivary flow rate reduce colony forming unit in saliva and this finding is in agreement with a study by (Torres *et al*, 2002, Nadig et al, 2017.) as well as increasing salivary flow rate also shift PH of saliva toward alkalinity by increasing bicarbonate (HCO3-) concentration which is the buffering system of unstimulated whole saliva (UWS) this finding is in agreement with a study by (Bardow *et al*, 2000). also study by Bikandi et al in (2000) reported that at a neutral PH of saliva expression of manoprotein of candida cell wall increase so Salivary secretory IgA reacts with it and ensure clearance, The low reactivity of salivary IgA with C. albicans cells grown at acidic pH values may help to explain the association between acidic saliva and the carriage of Candida in the oral cavity, as well as with oral candidiasis.

5. Conclusion

Candida albicans showed more extracellular hydrolytic enzyme activity more biofilm formation in patients group than control group, Both the C. albicans in patients and control groups are capable of producing extracellular hydrolytic enzymes and biofilm formation.

So it is necessary to understand the pathogenicity mechanisms of the *Candida* sp. for the development of new antifungal strategy. Because of the multi-drug resistance developed by *Candida* sp. The study of virulence factors of *Candida* sp. Helps for the better understanding of the various virulence factors exhibited by *Candida* sp.

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