Journal of Biology, Agriculture and Healthcare
ISSN 2224-3208 (Paper) ISSN 2225-093X (Online)
Vol.6, No.18, 2016
www.iiste.org

Isolation and Identification of Candida Species from the Oral Cavity of Cancer Patients Undergoing Chemotherapy in Basrah, Iraq

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Abstract
Candida species are a common normal flora in the human oral cavity , they can cause oral candidiasis especially in the immunocompromised patients like cancer patients. A total of 500 cancer patients and 200 healthy controls were included in this study. Breast cancer was the most prevalence between patients (145 cases) , followed by leukemia (114 cases) . *Candida albicans* was the most yeast species isolated from the oral cavity of the patients and control persons , followed by *C. tropicalis* and *C. glabrata* along with other non-*Candida albicans Candida* (NCAC).All the yeast isolates were identified by different phenotypic methods including germ tube and chlamydospores production, growing on chromogenic media and assimilation test.
Keywords: Yeast, oral cavity, cancer patients, *Candida* species

1. Introduction

Cancer patients have a high prospect for fungal infection especially by *Candida* species, as a result of the immunocompromised state and the effect of the chemotherapy. These yeasts are usually normal oral commensal in about 20-60% of the population and their transition to an opportunistic pathogen may be associated with the virulence of the organism and the host factors (Al-Dawairi *et al*. 2014 ; Bezerra *et al*. 2015 ; Jayachandran *et al*. 2016).

When the host immune defenses are impaired or when the normal oral microbiota balance is disarranged, *Candida* species will take the opportunity to colonize, proliferate, discourage other microorganisms and cause recurrent infections in oral mucosa depending on numerous factors such as phenotypic switching, dimorphism, adhesive properties, extracellular enzymes production and biofilm formation (Sousa *et al*. 2016 ;Kang *et al*. 2016).

Oral candidiasis is an opportunistic infection that affects cancer patients, particularly, those ones undergoing chemotherapy. This infection is usually accompanied by various symptoms including burning, painful sensation, change of taste, reduced saliva secretion and swallowing difficulty, but it can be also asymptomatic (Lone *et al*. 2014 ; Nikolic *et al*. 2016).

Although *Candida albicans* is considered the main agent of candidiasis and to be the most frequently isolated from oral cavity, but in recent two decades there has been important increase of other non-*Candida albicans* species such as *Candida glabrata, Candida tropicalis, Candida krusei, Candida parapsilosis* and *Candida dubliniensis*as a result of a different factors like immune-suppressants and prolonged use of broad-spectrum antibiotics and antifungal drugs (Martins *et al*. 2014 ; Patil *et al*. 2015 ;Jain *et al*. 2016).

The aim of this study was to identify *Candida* species and their epidemiology in oral cavity of cancer patients undergoing chemotherapy, with different age groups and various types of malignancies by using phenotypic methods and compare it with healthy controls.

2. Materials and Methods

2.1. Samples collection

Samples were taken from patients suffering from different types of cancer whore viewed “The Center of Oncology and Hematology” in Al-Sader Teaching Hospital, Basrah, Iraq for period from 3/12/2014 to 17/2/2015. Similar samples were also taken from healthy persons as a control group for comparison. It has been obtaining the approval of patients and healthy individuals around sampling and other information such as age , sex, use of antibiotics and type of cancer.

A total of 500 oral swabs were taken from patients and 200 from healthy controls with age ranging from 12-84 years. The samples were collected by rubbing sterile swabs on different mucosa areas in oral cavity. All swabs were inoculated directly on Sabouraud dextrose agar medium (SDA) supplemented with chloramphenicol (250 mg/L.). The plates were incubated at 37℃ for 24-48 hours until the creamy white yeast colonies looks clearly visible. The colonization with that color indicates to the presence of yeast cells in the oral cavity (Magare & Awasthi 2014 ; Sharifzadeh & Shokri 2016).

2.2. Identification of *Candida* species :-

2.2.1. Germ tube formation:-

This test is a rapid method for identifying *Candida albicans* and *Candida dubliniensis* by its capability to produce short, slender, tube like structure called germ tubes when it is incubated in human blood serum at 37˚C for 2-3 hours. This structure differs from pseudohyphae because it is elongation daughter cells from the mother cell without constriction at the origin (Deorukhkar & Saini 2014 ; Aryal 2015).

2.2.2. Chlamydospores formation:-

The formation of chlamydospores aids identification of *Candida albicans* and *Candida dubliniensis*. For this purpose we used the corn meal tween-80 agar medium who encourage chlamydospores formation. Subcultures of yeasts on this medium was made by streak method and incubated at 28℃ for 2-5 days. After which they were examined by light microscope after staining with lactophenol cotton blue to detect the thick-walled chlamydospores (Hasan & Al-Jubouri 2015 ; Padmapriya et al. 2015).

2.2.3. Culturing on CHROMagar*Candida* medium:-

This is a selective and differential medium, which aids rapid isolation and presumptive identification of *Candida* species on the bases of the colonies color. CHROMagar medium contain chromogenic substrate which react with the enzymes secreted by *Candida* species to yield colonies of different color. The plates of culture were prepared according to instructions of CHROMagar company, Paris, France, and then separate colonies from *Candida* isolates on SDA were sub cultured onto CHROMagar *Candida* and incubated at 37˚C for48 hours after which color changes were noted (Imran & Ali 2015; Mathavi et al. 2016).

2.2.4. CHROMagar *Candida* supplemented with Pal's agar medium:-

This medium is used for differentiation between *Candida albicans* and *Candida dubliniensis*. In this medium *Candida dubliniensis* forms rough colonies and chlamydospores, while *Candida albicans* shows smooth colonies and does not produce chlamydospores.

Pal's agar was prepared by adding 50g unsalted powdered sunflower seeds to 1 liter of distilled water, boiling for 30 min., next the seeds extract was cooled, filtered and supplemented with glucose (1 g) KH2PO4 (1 g) and creatinine (1g), the pH was adjusted to 5.5, the volume was readjusted to 1 L. and 15 g of agar was added before the mixture was autoclaved.

Then the CHROMagar *Candida* medium supplemented with Pal's medium was prepared by mixing equal volumes of prepared CHROMagar *Candida* medium and Pal's agar . Each isolate was cultured on SDA for 48 hours at 37℃ then they were sub cultured on this medium and incubated at 37℃ for 24-72hours, and colony characteristics were recorded , chlamydospores formation was detected microscopically by using lactophenol cotton blue (Himedia) as a stain (Sahand *et al*. 2005 ; Raut & Varaiya 2009).

2.2.5. Carbohydrates assimilation and urease production test:-

The Carbohydrates assimilation test determines the ability of yeast isolate to use a particular carbohydrate as its sole carbon source in a medium, this test was done by using (KB006 HiCandida Identification Kit), this kit is a standardized test system that can be used for identification and differentiation of *Candida* species according to the colorimetric identification system utilizing 12 conventional biochemical test. This kit contains sterile media for urease production and11 different carbohydrates utilization tests: melibiose, lactose, maltose, sucrose, galactose, cellobiose, inositol, xylose, dulcitol,raffinose and trehalose as a sole carbon source. Yeasts were cultured on (SDA) for at37℃ for 24 – 48 hours. Then the inoculum prepared by picking 2-4 well isolated colonies and make homogenous suspension in 2-3 ml sterile saline, the density of the suspension should be adjusted to 0.5D at 620 nm . Then 50 µl of the suspension of each yeast isolate inoculate in each well of the carbohydrate assimilation kit and incubate at 22-25˚C for 24-48 hours, after that the results were recorded according to the colorimetric identification sheet (Daef *et al*. 2014 ; Fule, *et al*. 2015).

3. Results

A total of 500 patients with various malignancies , along with 200 healthy controls were included in this study . The patients divided into 195 males and 305 females, while the control group divided into 78 males and 122 females. Amongst the cancer patients, breast cancer was the most prevalence between them (145 females), followed by leukemia(59 males +55 females) (table 1).

The total prevalence percentage of *Candida* in the oral cavity of cancer patients was 77.8%, these percentage divided into 75.89% males and 79.01% females. The higher percentage of oral *Candida* was in breast cancer 124 (85.5 %) patients from 145 total, followed by leukemia patients 92 (80.7 %) cases from 114 (table 2), however in control individuals the total prevalence percentage of *Candida* was37.5% (39.74% males and 36.06% females) (table 3).

A total of 389 *Candida* isolates (77.8%) were recorded from 500 cancer patients which included148 from males, 241 isolates from females, *C. albicans* represented the highest percentage of isolates (208) followed by *C. tropicalis* (72) and *C. glabrata* (42) isolates. Some species appeared only one time like *C. pintolopesii* (table 4).In control group a total of 75 isolates (37.5 %) were recovered from 200 persons,31 isolates from

malesand 44 from females. Also in this group *C. albicans* represented the highest percentageofisolates (54), followed by *C. tropicalis* and *C. glabrata* 7 isolates for each species, were as *C. dubliniensis* appeared only one time (tables3,6,8).

In patients, from the total of 208 isolates of *C. albicans*, 199 (95.67%) of them were able to form germ tubes, but all *C. dubliniensis* isolates were able to produce germ tubes8 (100%) (table 5).However in control group from 54 isolates of *C. albicans*, 52 (96.29 %) were able to form germ tubes and 1 (100%) isolate of *C. dubliniesis* was able to form that structure (table6).

Based on the colony color developed on CHROMagar *Candida* medium, 389 isolates of *Candida* were differentiated as: *C. albicans* 200, *C. dubliniensis*7,*C. glabrata*41, *C. krusei*31, *C. parapsilosis*17,C. *tropicalis*70, *C. lusitaniae* 1,*C. stellatoidae*2 and *C. kefyr*2 (table7).As for the control group75 isolates have been diagnosed on CHROMagar *Candida* medium as follows:*C. albicans* 51, *C. dubliniensis* 1, *C. glabrata* 6, *C. krusei*3, *C. parapsilosis* 2 and *C. tropicalis* 7 (table 8).

Found through the use of CHROMagar *Candida* with pal's medium test that all *C. dubliniensis* which isolated from patients possess the ability to develop fringes and rough colonies with chlamydospores, whereas none of the *C. albicans* developed that on the same medium (table 5).

In control group only one isolate of *C. dubliniensis* appear and this isolate was able to develop fringes and rough colonies on CHROMagar *Candida* with pal's medium , whereas none of the *C. albicans* developed fringes on the same medium (table 6).

Out of 208 *C. albicans* isolates from patients have been cultured on CHROMagar medium, 192 (92.3%) appeared positive in this test while other species showed a percentage 75%-100% (table 7), however in control group the isolates appeared a confirmation percentage between 71.4% -100% .Finally, to confirm the identification, the carbohydrates assimilation test has been conducted for each species isolated during this study by using KB006 *Candida* Identification Kit. (table 8).

Table (1): Types of cancer according to the patients gender.

Gender / cancer	male	female
Breast	-	145
Ovaries/Uterus	-	24
Prostate	25	-
Testes	5	-
Kidney	3	2
Bladder	10	1
Brain	4	2
Nose	1	-
Tongue	1	-
Larynx	1	1
Pharynx	-	3
Bronchi	4	2
Lung	9	6
Esophagus	1	-
Stomach	19	1
Colon	16	9
Rectum	6	2
Liver	3	8
Pancreas	3	2
Skin	1	1
Bone	11	12
Spleen	2	5
Multiple myeloma	7	10
Lymphoma	4	14
Leukemia	59	55
Total	195	305

Table(2): Total *Candida* spp. isolates from the oral cavity of cancer patients according to the type of cancer and patient gender.

Gender / cancer	male	Female
Breast	-	124
Ovaries/Uterus	-	14
Prostate	21	-
Testes	4	-
Kidney	2	1
Bladder	10	-
Brain	1	1
Nose	1	-
Tongue	1	-
Larynx	1	1
Pharynx	-	2
Bronchi	3	2
Lung	8	4
Esophagus	1	-
Stomach	12	1
Colon	11	7
Rectum	5	1
Liver	2	6
Pancreas	3	1
Skin	1	-
Bone	7	9
Spleen	-	6
Multiple myloma	4	7
Lymphoma	2	10
Leukemia	48	44
Total	148	241

Total appearance in patients = 77.8 from 500
Total appearance in males = 75.89 from 195

Total appearance in females = 79.01 from 305

Table (3): Appearance of different *Candida* species in the oral cavity of control persons according to their gender.

Male							Female						
C. albicans	*C. Dubliniensis*	*C. glabrata*	*C. krusei*	*C. parapsilosis*	*C. tropicalis*	Total	*C. albicans*	*C. Dubliniensis*	*C. glabrata*	*C. krusei*	*C. parapsilosis*	*C. tropicalis*	Total
20	1	4	2	1	3	31	34	/	3	2	1	4	44

Total appearance percentage = 37.5 % from 200 persons
Total appearance percentage = 39.74% from 78 males
Total appearance percentage = 36.06% from 122 females

Table(4): Appearance of different species in the oral cavity of cancer patients according to their cancer type and gender

Sex	Male									Female										
Species / Cancer.	C. alb.	C. dub.	C. glab.	C. kru.	C. para.	C. trop.	C. lusi.	C. stel.	Total	C. alb.	C. dub.	C. glab.	C. krus.	C. para.	C. trop.	C. lusi.	C. stel.	C. kefyr	C. pinto.	Total
Breast										68	2	12	9	5	26		1	1		124
Ovaries/Uterus										6		2	1	1	4					14
Prostate	7		5	4	4	1			21											
Testes	2					2			4											
Kidney	2								2	1										1
Bladder	6			1		2		1	10											
Brain	1								1	1										1
Nose	1								1											
Tongue	1								1											
Larynx	1								1	1										1
Pharynx										1					1					2
Bronchi	2		1						3				1		1					2
Lung	6			1	1				8	2		1			1					4
Esophagus	1								1											
Stomach	8		1			3			12						1					1
Colon	8	1	1			1			11	5	1		1							7
Rectum	3			1		1			5	1										1
Liver	2								2	4	1				1					6
Pancreas	1		1			1			3	1										1
Skin	1								1											
Bone	5				1	1			7	5			1		3					9
Spleen										4		1	1							6
Multiple Myeloma	3					1			4	4		1			2					7
Lymphoma	1					1			2	6		2	1		1					10
Leukemia	20	1	6	7	4	9	1		48	16	2	8	4	4	8			1	1	44
Total	82	2	15	14	10	23	1	1	148	126	6	27	19	10	49		1	2	1	241

Table (5): Chlamydospores , Germ tube production of *C. albicans* and *C. dubliniensis* isolated from cancer patients and their appearance on CHROMagar *Candida* with Pal's Medium.

Candida species	CHROMagar *Candida* with Pal's Medium				Germ tube		
	Chlamydospores	Colonies	+	%	Total	+	%
C. albicans	not produce	Smooth	0	-	208	199	95.67%
C. dubliniensis	Produce	Rough	8	100%	8	8	100%

Table (6): Chlamydospores , Germ tube production of *C. albicans* and *C. dubliniensis* isolated from control persons and their appearance on CHROMagar *Candida* with Pal's Medium

Candida species	CHROMagar *Candida* with Pal's Medium				Germ tube		
	Chlamydospores	Colonies	+	%	Total	+	%
C. albicans	not produce	smooth	0	-	54	52	96.29%
C. dubliniensis	Produce	rough	1	100%	1	1	100%

Table (7): *Candida* species from cancer patients which isolated on CHROMagar *Candida* Medium and KB006 *Candida* Identification Kit.

Candida Species	Total	CHROMagar *Candida* Medium			KB006 *Candida* Identification Kit	
		Color	+	Sensitivity	+	%
C. albicans	208	light green	200	96.1%	192	92.3%
C. dubliniensis	8	dark green	7	87.5%	6	75%
C. glabrata	42	pink, purple	41	97.6%	37	88.09%
C. krusei	33	pink fuzzy	31	93.9%	29	87.8%
C. parapsilosis	20	white to pale pink	17	85%	18	90%
C. tropicalis	72	metallic blue	70	97.2%	61	84.7%
C. lusitaniae	1	pink, gray, purple	1	100%	1	100%
C. stellatoidae	2	bright green	2	100%	2	100%
C. kefyr	2	pink, purple	2	100%	2	100%
C.pintolopesii	1	Creamy white	1	100%	1	100%

Table (8): *Candida* species from control persons which isolated on CHROMagar *Candida* Medium and KB006 *Candida* Identification Kit.

Candida Species	Total	CHROMagar *Candida* Medium			KB006 *Candida* Identification Kit	
		Color	+	Sensitivity	+	%
C.albicans	54	light green	51	94.4%	49	90.7%
C.dubliniensis	1	dark green	1	100%	1	100%
C. glabrata	7	pink, purple	6	85.7%	5	71.4%
C.krusei	4	pink fuzzy	3	75%	4	100%
C.parapsilosis	2	white to pale pink	2	100%	2	100%
C. tropicalis	7	metallic blue	7	100%	6	85.7%

4. Discussion

Infections caused by *Candida* spp. increase as a result of the increase of immunocompromised 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. One of the targets of this study was the compare of the epidemiology of oral *Candida* colonization in different cancer groups in Basrah.

As show in the result, all cancer 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).

Breast cancer patients were represented the higher group among different types of cancer in Basrah, this agree with the fact that this type of cancer is a worldwide distribution in different countries. *Candida* spp. appeared in (85.5%) of breast cancer patients, this owing to the fact that all of those patients had received radiotherapy in addition to chemotherapy, or they undergo surgical operation in addition to chemotherapy, and this which compounded the problem (Lone *et al*. 2014 ; Jain *et al*. 2016).

It is well known that cancer and chemotherapy results in immunosuppression which gives opportunity for emergence of *Candida* infection. The outcome is the immune dysfunction and mucosal damage which promote yeast infections such asmucositis, xerostomia and candidiasis (Singh *et al*. 2015 ; Caira *et al*. 2015).

As leukemia is widespread globally, also in this study it has emerged the second type of cancer among the different malignancies patients in Basrah., and the high percentage of this type in recent years may be due to in part to the accumulation of radioactive material from the recent wars, (80.7%) of leukemia patients showed oral *Candida* colonization this may be due to the intensive use of chemotherapy, it is well known that prolonged use of chemotherapy may lead to neutropenia, disruption of mucosal barrier and overall damage to cell mediated immunity which increases the risk of infection (Schelenz *et al*. 2011 ; Kang *et al*. 2016).This result was agreed with previous studies of Dahiya *et al*. (2003), Jham, *et al*. (2008) and Hassan and Al-Juboury (2016) who pointed that the fungal infections are more frequent in leukemic patients.

In our study a total number of 464 positive isolates from patients and control persons have been studied, and we have identified 10 species of *Candida*. *Candida albicans* was found to be the predominant, 208 isolates from patients and 54 isolates from control, followed by *C. tropicals* and *C. glabrata.* Similar results were seen in

other studies (Samaranyake, 1991; Tekeli *et al*. 2002; Kumar *et al*. 2005; Back-Brito *et al* 2009; Manikandan & Amsath 2013).

The genus *Candida* is composed of heterogeneous group of organisms and more than 17 different *Candida* spp. are implicated in human infection. Although *C. albicans* is the most prevalent species involved in infections, but currently, the shift towards non-*Candida albicans Candida* (NCAC) spp. is documented in several studies (Deorukhkar & saini 2012 b; Deorukhkar & saini 2014 ;Rathod *et al*. 2015).

Formation of germ tube is associated with increased synthesis of protein and ribonucleic acid (Aryal 2015). The germ tube test provides a simple, reliable and economical procedure for the presumptive identification of *C. albicans* and *C. dubliniensis*. A number of studies show that to these two species about 95% of the clinical isolates produce germ tube. This finding become similar to our study in which 100% of *C. dubliniensis* isolates from patients and control persons were able to produce germ tube while 95.67% of *C. albicans* from patients and 96.29% from control persons were able to produce germ tube, these result similar to studies of Gatica *et al*. (2002), Maninho *et al*. (2010), Deorukhkar *et al*. (2012c) and Padmapriya *et al*. (2015), whose find results between 73-100% .

CHROMagar *Candida* medium had allowed the growth of most clinically relevant yeasts and also aids to presumptive identification of *C. albicans* and other (NCAC) spp., this medium also facilitated recognition of specimens containing mix of yeast species (Reddy & Athuluri 2012 ; Deepa *et al*. 2014 ; Metha & Wyawhare 2016).

In this work we were able to identify 9*Candida* species from the oral cavity of cancer patients, were as 6 species were identified from control persons by aCHROMagar *Candida* medium. This medium used by Latha *et al*. (2011), Reddy & Athuluri (2012) Manikandan & Amsath (2013) and Devi & Maheshwari (2014). and other as a good tool to distinguish different *Candida* species isolated from clinical specimens (Mathavi *et al*. 2016 ; Gupta *et al*. 2016) .

Similarity in morphological and physiological characteristics between *C. albicans* and *C. dubliniensis* leads to misidentification between them. Molecular-based methods are the most reliable techniques for *C. albicans* and *C. dubliniensis* differentiation. However, accurate, quick and cheap tests are needed to discrimination in many laboratories (Gamarra *et al*. 2015). In this study, colony aspects and color on CHROMagar medium supplemented with pal's agar was evaluated as a tool for differentiation between these species. (Sahand *et al*. 2005).

In our study all isolates of *C. dubliniensis* from patients and control persons develop fringes rough colonies and chlamydospores on this medium, were as all of the *C. albicans* isolates on this medium produce smooth colonies without chlamydospores. Sahand *et al*.(2005) found that 96% of *C. dubliniensis* isolates formed rough colonies were as *C.albicans* isolates produced smooth colonies, also they found that all the *C. dubliniensis* exhibited hyphal fringes on pal's agar, our results also similar to (Al-Mosaid *et al*. 2003;Raut&Varaiya 2009 ; Reddy & Athuluri 2012).

The pattern of carbohydrates assimilation is considered a reliable test for the correct identification of yeasts of clinical interest (Gatica *et al*. 2002). The assimilation results confirmed the identification of *Candida* spp. recovered on CHROMagar, the assimilation of carbohydrates used by Graf *et al*. (2000), Gatica *et al*. (2002) and Swastika *et al*. (2013) as a good tool for yeasts identification.

5. Conclusion

In the present study we demonstrate that *C. albicans* and other Non- *Candida albicans* species are associated commonly with the oral cavity in immounocompromised patients. The incidence of these yeast vary depending on cancer type and age of the patients, the frequent occurrence of *Candida* species in oral cavity of immounocompromised patients indicates a need for effective management for the isolation and identification of these yeast prior to any treatment, in this field its important to use different phenotypic tests for the identification of different yeast isolate especially the use of chromogenic media which found to be a useful media for the isolation and direct identification of *Candida* species especially the medically important one.

6. Acknowledgment

The authors represent deep thanks for each patient was graciously allowed us to take oral swab from him, despite suffering from the disease, also we deep thanks to everyone help us during the study.

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