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MOLECULAR IDENTIFICATION AND HEMOLYTIC ACTIVITY OF CANDIDA SPECIES ISOLATED FROM URINE OF HEALTHY AND DIABETIC WOMEN IN KURDISTAN OF IRAQ

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ABSTRACT:
A survey was carried out on the incidence of Candida yeasts in urine of healthy (non-diabetic) and diabetic women in Duhok and Zakho cities from February 2019 to April 2019. Out of 620 urine samples examined, 144 samples were found positive for Candida infection (23.2%). There wer e significant differences in incidence of Candida infection between urine of healthy and diabetic women as well as among age groups. All Candida isolates from positive samples were identified by using Chromogenic Candida Agar medium and confirmed by sequencing of the ITS1 and ITS4 region of rDNA. The identified Candida species were C.albicans, C.glabrata, C.krusei and C.tropicalis. The most common species was C.albicans (34%), followed by C.glabrata (29.9%), then C.tropicalis (13.8%), whereas, C.krusei (=Pichia kudriavzevii) displayed the least incidence (9.7%). All tested strains (n=83) for their hemolytic activity on Sabouraud's dextrose agar amended with sheep blood and glucose showed positive activity. Candida albicans and C.tropicalis displayed the highest hemolytic activity.

KEYWORDS: Hemolysin, Candida spp., Urine, Diabetic Mellitus, Sequencing, ITS.

1. INTRODUCTION

One of the most common mycobiota in human which colonizing several functional sites like skin, mouth, urinary tract, vagina is Candida wi thout causing any infection (Seneviratne et al., 2008). Modification in local environment of the host enhanced Candida growth and hence led to candidiasis (Pope and Cole, 2002). Virulence factors of Candida as well as immune system of the host switching Candida from harmless commensally to disease causing pathogen (Yang ,2003). Most common virulence factors of Candida; adherence and extracellular hy drolase enzymes which help the Candida to adapt to specific anatomical sites. The pathogenesis of Candida species enhanced by phenotypic switching, filamentation and biofilm formation (Calderone and Fonzi, 2001). Extracellular enzymes like as proteases, phospholipases, haemolysin and lipase contributed in pathogenicity of Candida species (Silva et al., 2009).

Furthermore, the capability of C. albicans to get iron by haemolysin production is essential in its persistence and capability to form infections in the human's body. Hemolytic activity is one of the virulence factors that facilitate hyphal invasion and di sseminate candidiasis. The acti vity of hemolysin by Candida is enhanced by present of glucose in the blood agar (Manns et al., 1994) . Hemolysins production by Candida albicans destroy hemoglobin in erythrocytes and obtain elemental iron. Hence, hemolysins c onsidered as essential virulence factors which make pathogens to survive and persist (Luo et al., 2004).

The most suitable med ium for study the hemolytic activity of Candida isolates is Sabouraud's dextrose agar with addition of Sheep blood. The role of $CaCl_2$ on the hemolysin activity of Candida was studied by Linares and his colleagues (Linares et al., 2007) . The addition of 2.5 % $CaCl_2$ to Sabouraud's dextrose agar contains sheep blood reduce the hemolytic activity of C.dubliniensis while the C. albicans strains were stimulated (Koga-Ito et al., 2006) Unicellular and multicellular organisms need essential elements for growth such as iron, which present in the hemoglobin within red blood cells in the human body. C.albicans binding to complement receptors of erythrocytes by mannoprotein on the cell surface of yeast then produces hemolysin which lysis of the erythrocyte (Moors et al.,1992; Watanabe et al., 1999 and Almeida et al., 2009) Various studies showed significant relationship between high incidence of Candida in urine samples and predisposing factors such as pregnancy state of woman, Diabetic mellitus, prolong used of antibiotics and used bladder catheters (Khudor et al.,2002; Falahati et al.,2016 and Lima et al.,2017) . Several studies for diagnosis of Candida species from urine of diabetic and non -diabetic women revealed that there is significant relationship between high level of glucose in blood (diabetic patients) and high prevalence of Candiduria (Pandey and Pandey,2013; and Yismaw et al.,201 3). Although, several diagnostic methods such as phenotypic methods (germ tube, Chlamydospores production and Chromogenic Candida Agar) were used, molecular analysis technique such as Multiplex PCR and Sequenced PCR are seem to be more accurate and precise methods for identification of Candida species from urine and vaginal samples (Mohammed and Said, 2015; Lima et al.,2017). Intergenic Spacer Region (ITS) is placed among the constant genes coding for 28S rRNA and 18S rRNA , while 5.8SrRNA gene is highly conserved and located between ITS1 and ITS4(White et al.,1990). The genetic diversity of ITS1 and ITS4 regions give well identification of closely related species of Candida(Ciardo et al., 2006). The aim of the present study was isolation and identification of Candida species from urine of diabetic and non -diabetic women by using molecular

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analysis techniques and study the hemolytic activity of some Candida isolates.

2. MATERIALS AND METHODS

2.1. Samples Collection

Urines samples (n= 620) were collected from healthy and diabetic women attended laboratory of Maternity hospital and Diabetes Center in Zakho and Duhok city. The study was achieved between February 2019 and April 2019. Ten ml of urine samples were kept in sterilized universal containers. Patient information was noted such as age, types of therapy (antibiotic and antifungal) and other possible risk factors. All urine samples were transported to mycology laboratory at Zakho University for analysis.

2.2. Isolation and phenotypic Identification of Candida Species

Samples were cultured within few hours on Sabouraud'sdextrose agar (SDA) amended with 0.05% (Weight/Volume) chloramphenicol for inhibiting bacteria growth by using streak plate method (Bhavan et al., 2010). Cultures Incubated at 37ºC for two days after that pure Candida colonies were sub-cultured on Harlequin TM Candida Chromogenic agar (CHROM agar Company,Acumedia, Neogen,UK) at 35ºC for 48 hours. Presumptive identification of Candida strains was done depend on color of the colonies (Pfaller et al.,1996).

2.3. Determination of Hemolysin activity

Eighty three isolates of Candida recovered from urine specimens were checked for hemolytic activity according to Manns et al. (Manns et al., 1994).To determine the hemolytic activity; Sabouraud'sdextrose agar enriched with 7 ml of sheep blood was inoculated with 108 cells/ml suspension of each isolated Candida species and incubated for one day at 37°C. To estimate the effect of glucose on the production of hemolysis, 10 µl of each sample's suspension inoculated on two SDA medium containing blood; one enriched with 3% glucose and other without glucose within two days at 37 ° C in the present of 5% CO2 atmosphere. The activity of Hemolysis by different Candida species was determined by measure hemolytic index (Hz value) as seen in the following equation:

$$Hz = \frac{translucent\ zone\ of\ haemolysis\ (mm)}{Colony\ diameter\ (mm)}$$

A reference strain of Staphylococcus aureus was used as a positive control for beta hemolytic activity (Luo et al., 2001).

2.4. Molecular Analysis

2.4.1. DNA Extraction: A loop-full from each Candida isolate was taken and cultured on disposable sterile tube (2 ml) containing 1 ml Yeast Extract Peptone Dextrose (YPD) Broth which had been prepared previously and then incubated in shaking incubator at 37ºC for 24 hours. The genomic DNA extract using a method based on spin column genomic DNA purification using yeast DNA preparation kits (Jena Bioscience- Germany). All extracted genomic DNA was suspended in 50 µl of Elution Buffer (EB) and freeze at 4 °C until used. Purity and concentration of DNA, was measured by the Nanodrop.

2.4.2. PCR Amplification Technique: The non-specific fungal primers ITS1 (5′- TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATAT GC-3′) were used to amplify the Internal Transcribed Spacer (ITS) of ribosomal DNA region (rDNA)(20). The PCR was performed in 30 µl reaction mixture consisting of approximately 15 µl of master mix, 2 µl of template DNA, 1 µl of forward primer ITS1 and 1 µl of reverse primers and 11 µl of PCR-grade water all of these kept in a single tube. The PCR cycle parameters were as follows: an initial denaturation step was 95°C for 5 min. followed by 10 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 45seconds, and extension at 72°C for 90 seconds and for 25 cyclers as followed; denaturation at 94°C for 30 seconds, annealing at 55°C for 45seconds, and extension at 72°C for 90seconds then final extension at 72°C for 10 minutes. PCR products were analyzed by agarose gel electrophoresis in 1X TBE buffer at 100 V for 120 min in gel composed of 1.5% agarose. 100bp ladder DNA Marker was run with PCR products for sizing of the bands. Gels were stained with Red Safe DNA loading dye, then visualized with a UV Transilluminator and photographed.

2.4.3. Sequencing of DNA: PCR products of fifteen Candida species isolates from the samples were sequenced using primers (ITS1 and ITS4) by Macrogen Company (Seoul, Korea). Basic Local Alignment Search Tool (BLAST) for sequence analysis databases was used to identify the obtained DNA sequences (http:// www.ncbinlm.nih.gov/BLAST). All sequences were clean up and align by BioEdite application.

2.5. Data Analysis

The data were analyzed using the SPSS software (Statistical Package for the Social Sciences, version (Kauffman et al., 2000). The relative proportions were calculated with a confidence interval of 95%. To determine the association between variables, the chi-squared (x2) test was used; a p – value< 0.05 was considered significant and more than that considered not significant.

3. RESULTS AND DISCUSSION

A total of 620 urine samples were analyzed for presence of Candida yeast, 144 samples showed positive results. In diabetic patients, the highest percentage was recorded for the age group 15-25years (35.7%), followed by (32.0%) for age group 26-36 and (25%) for age group 48-60 years, whereas 12% was recorded for age group 37-47. The least ratio (4.5%) was recorded for the age group above 60 years. For non-diabetic women, the highest incidence of Candida infection (18%) was recorded for the age group 26-36 years, followed by (14.2%) for the age group 15-25 years and (4%) for the age group 37-47 year; p=0.01. We observed that there was significant association between age groups and the positive percentage of diabetes and non-diabetes women (Table 1).

The detection of Candida species in urine of diabetic and non-diabetic women could be contamination or reflection of colonization or infection of the urinary tract by Candida yeast (Kauffman et al., 2000 and Mohammed et al.,2017). In general, the incidence of Candida yeast isolated form urine of patients with diabetes were higher than non-diabetes patient and this can be explained due to the high level of glucose in urine of diabetic patients as well as the suppression of the immune system in diabetic women (Falahati et al., 2016).The highest incidence of Candida yeasts (35.7% and 32%) was found in the age groups of 15-25 years and 26-36 years in diabetic patients respectively. Higher incidence (14.2% and 18%) was also reported for the age groups 18-25 years and 26-36 years respectively. This finding was in agreement with other studies (Navin et al., 2004; Geerlings et al., 2014; Falahati et al., 2016).

Table 1. Incidence percentage of Candida in urine samples from diabetic and healthy women

Age	Number of samples examined	% of positive samples in diabetes women	% of positive samples in non-diabetes women	% of Positive samples
15-25	70	25/70 (35.7 %)	10/70 (14.2 %)	35/70 (50 %)
26-36	100	32/100 (32 %)	18/100 (18 %)	50/100 (50 %)
37 - 47	150	18/150 (12 %)	6/150 (4 %)	24/150 (16 %)
48-60	100	25/100 (25 %)	0 (0%)	25/100 (25 %)
60 <	200	9/200 (4.5%)	1/200 (0.5%)	10/200 (5 %)
Total	620 (100 %)	109/620 (17.5 %)	35/620 (5.6 %)	144/620 (23.2 %)
P value = 0.010 analyzed using the SPSS software using the chi-squared (x2) test				

All positive Candida isolates were cultured (n=144) on chromogenic Candida agar for presumptive identification of Candida species. Chromogenic Candida agar medium detected 131 isolated out of 144; including 49 isolates of C. albican (green), 43 isolates of C. glabrata (white shinny), and 14 isolates of C. krusei (purple to pink) while 20 isolates were C. tropicalis (blue). Mix infection with C. albicans and C. glabrata were observed in urine of our diabetes patients while only one non diabetes patient harbored two Candida species, the p value was 0.096 which is more than p value = 0.05 hence, we did not observe any significant associations among different species of Candida variables with Diabetes and Non diabetes and other disease (Table2).

The highest incidence displayed by C.albicans (34%) followed by C.glabrata (29.9%) and C.tropicalis (13.8%), whereas, the least incidence was recorded for C.krusei (9.7%), 3.5% of Mix infection with C. albicans and C. glabrata. Thirteen samples were not detected on chromogenic candida agar (Table 2).

This is in line with other different studies that reported a prevalence for C.albicans, C.glabrata and C.tropicalis (Achkar and Fries 2010; Ozhak-Baysan et al., 2012 and Ortiz et al.,2018). The three Candida species have been reported as pathogens in urinary tract infections (Papon et al., 2013). In contrast, C.krusei was found with high prevalence (26.31%) in urine samples collected from patients with urinary tract infection from Duhok (Mohammed et al., 2017). A mixed Candida infection was found in four urine samples from diabetic women and one sample from non-diabetic patient. Our result is in agreement with other studies that reported more than one Candida species (Kauffman et al.,2000; Mohammed et al., 2017 and Ortiz et al., 2018).

Table 2. Detection of Candida species among Diabetic and non-diabetic patients by using Chromogenic Candida Agar

Species	NDM No (%)	DM No (%)	DM& other disease*No (%)	Total positive No (%)
C.albican	16/50 (32%)	10/50 (20%)	23/50 (46%)	49/144 (34)
C. glabrata	9/43 (20.9%)	14/43 (32.6%)	20/43 (46.5%)	43/14 (29.9 %)
C. tropicalis	5/20 (25%)	5/20 (25%)	10/20 (50%)	20/144 (13.8%)
C. krusei	4/14 (28.5%)	6/14 (42.8%)	4/14 (28.5%)	14/144 (9.7%)
C.albican& C.glabrata	1/4 (25%)	4/4 (100%)	0/4 (0%)	5/144 (3.5%)
Not detected on chromo agar	3/13 (23%)	4/13 (30.7%)	6/13 (46.1%)	13/144 (9%)
Candida spp. identificatio n	35/131 (26.7%)	39/131 (29.7%)	57/131 (43.5%)	131/144 (90.9%)
Total	38/144 (26.4)	43/144 (29.8)	63/144 (43.8)	144/144 (100%)
DM: Diabetes mellitus NDM: Non Diabetes mellitus *Other diseases = hypertension and cholesterol P value = 0.096analyzed using the SPSS software using the chi-squared (x2) test				

All tested isolates of Candida have beta hemolytic activity, the hemolytic index of Candida albicans and Candida tropicalis were (1.80 ± 0.1) and (1.48 ± 0.159) respectively, which were higher than hemolytic index of C. glabrata (1.36 ± 0.129) and C. krusei (1.11 ± 0.101) as shown in Table3.

Table 3. Hemolytic Activity of Candida species on Blood Sheep Agar

Species	No. of isolates with hemolysis (Hemolysis Index, mean ± SD)
	Beta
Candida albicans	20 (1.80 ± 0.1)
Candida glabrata	32 (1.36 ± 0.129)
Candida tropicalis	18 (1.48 ± 0.159)
Candida krusei	13 (1.11 ± 0.101)
All Data analyzed by using the SPSS software using the Standard Division SD P value = 0.091	

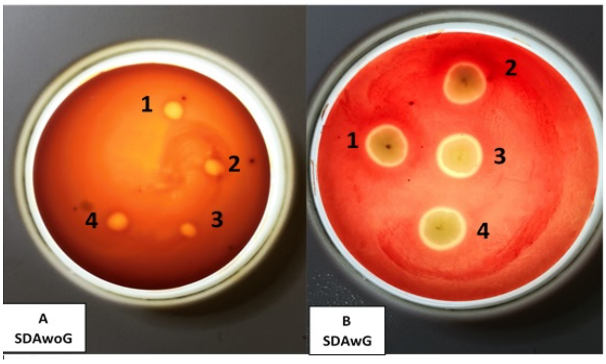

Figure 1. Hemolysis of Sheep blood agar induced by different species of Candida. A: Candida species on (SDAwoG) Sabouraud 's dextrose agar without glucose; B: Candida species on (SDAwG) Sabouraud 's dextrose agar with glucose. 1. C. albicans; 2. C. glabrata; 3. C. tropicalis; 4.C. krusei.

Figure (1) showed the hemolytic activity of different species of Candida after 24hours of inoculation. In the present study C. albicans, C. glabrata, C. tropicalis and C. krusei on Sabouraud's Dextrose blood supplemented with glucose (SDBwG) have high hemolytic activities compared to same strains cultured on Sabouraud Dextrose blood without glucose SDBwoG that showed negative hemolytic activity (Figure1).

One of the virulence factors of Candida is hemolysins. Species of Candida; C. albicans and C. tropicalis showed significantly beta-hemolytic activities higher than those of C. glabrata, and

C. krusei . In the current study, the hemolysis degree of all isolated Candida species was totally high in the medium containing glucose. The hemolytic activity of Candida was different regarding the present of glucose in the medium. Candida albicans is dimorphic microorganism which exist in two form hyphal and blastoconidial phases and that relay on the growth medium and condition. Some previous studies reported that Candida produced hemolysin only in the hyphal phases, our result do not agree with this finding as C. glabrata which is considered a hyphae-negative species exhibited both alpha and beta hemolysis (Paul et al., 1999).

Based on PCR results, universal primer ITS1 and ITS4 amplified DNA fragment from DNA template, PCR products size ranged from 500 to 1000 base pair. The results of DNA sequencing were aligned by BioEdite program and all edited sequences were submitted to Gene bank and compared with other references strains from same host which have similarity ranged from 96% to 100% (Table 4). Data of DNA sequencing of Candida species were analyzed by Neighbor-Joining method using MEGA 7 (Tree View software) to construct phylogenetic and compared with references strain (Figure 2).

In the present study, specimens of Candida spp. were characterized by sequencing of the ITS-1 and ITS-4 rDNA, compared with references from different country such as china, Iran, Japan, Saudi Arabia, Kuwait and Egypt. The analysis revealed that the similarity ranged from 96% to 100%. The phylogenetic tree showed high relatedness among isolates of the same species of Candiada, they were generally assigned into two main clusters; most of them were belonged to the first cluster except Candida glabrata (MN50788) was clustered as monophyletic. In addition, this isolate has also a relationship with Candida glabrata (MN521700) with 76% bootstrap.

Table 4. List of internal transcribed spacers (ITS) sequences accession number and their similarities with our Candida isolates at Gen bank.

Spp.	Accession number	Strain no.	Source	Location	Similarity
C.tropicalis	MN504645		Urine DM	IRAQ	100 %
C.tropicalis	LT837794	Kw3-15	Urine	Kuwait	100%
C. topicalis	MN508369		Urine NDM	IRAQ	100 %
C.tropicalis	KP674700	h24b	Oral	China	100%
C.tropicalis	KX015889	AUMC 10251	Gut	Egypt	100%
C. albicans	MN519554	29	Urine DM	IRAQ	100 %
C. albican	MK793255	Y94	Clinical isolated	Iran	99 %
C. albican	MK568486	CA06.1	Vaginal swab	Saudi Arabia	99%
C. albicans	MN336231		Vulvovagina l	Iran	100 %
C. albican	MN519597	44	Urine NDM	IRAQ	100 %
C. albican	MK564528	CA-18G	Human/urine	Iran	99%
C. glabrata	MN521700	H35	Urine DM	IRAQ	98 %
C. glabrata	LC389242	H7	Vagina	Iran	98%
C. glabrata	LC388879	IFM 64903	Clinical	Japan	98%
C. glabrata	LC389246	H35	Vaginal	Iran	99%
C. glabrata	MN507880		Urine NDM	IRAQ	100%
C. glabrata	LC389256	H108	Vagina	Iran	98%
C. krusei	MN515434	m80b	Urine DM	IRAQ	100 %
C. krusei	KP675284	m80b	Oral	China	97%
C. krusei	KP765013	121A	Gastric mucosa	China	96%
C. krusei	KP675284	m80b	Oral	China	97 %

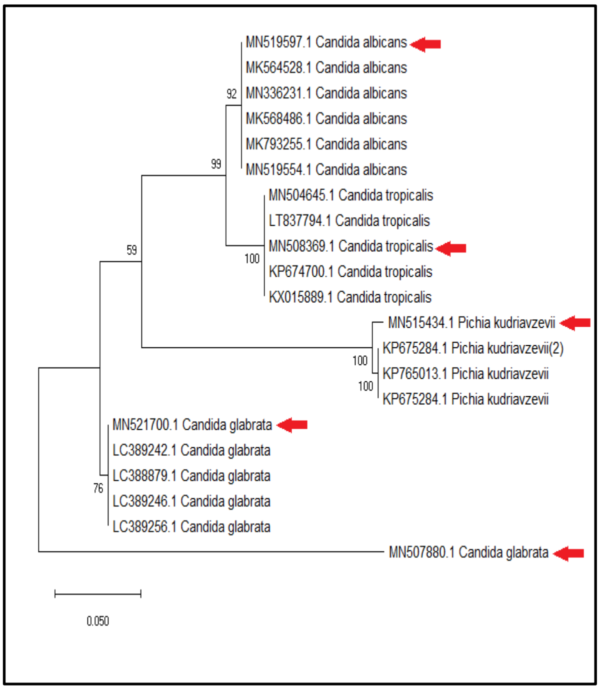

Figure 2. Phylogenetic tree of Candida spp. based on the ITS – rDNA sequencing.

Concerning the phylogenetic analysis for Candida albicans revealed that the obtained sequences shares 100% homology to Candida albicans strain: Iraqi isolate (MN519554), Iranian isolate (MN336231), 99% to both Iranian strains(MK793255 and MN564528)and Saudi Arabia isolate (MK568486).The phylogenetic analysis for Candida tropicalis showed that the obtained sequence shares 100% homology to Candida tropicalis strain: Iraqi isolate (MN504645), Kuwaiti isolate (LT837794), Chinese isolate (KP674700) and Egyptian isolate (KX015889). The morphological and molecular identification showed that the Candida isolates was Candida tropicalis with GeneBank accession number (MN508369).

The results from Table (4) and Figure (2) showed that the obtained sequence of Pichia kudoriavzevii shares 97% homology to Candida krusei strain from China (KP675284 and PK675284) and 96% homology to the Chinese strain (KP765013). The morphological and molecular identification showed that the Candida isolates was Candida krusei with GeneBank accession number (MN515434). The phylogenetic tree analysis for the species Candida glabrata (GeneBank accession number: MN521700) showed that the obtained sequence shared 99% homology to Candida glabrata strain: Iranian isolate (LC389246), 98% homology to Japanese strain (LC388879) and Iranian strain (LC389242). While the isolate Candida glabrata (GeneBank accession number: MN507880) was clustered as monophyletic.

4. CONCLUSION

Candida albicans and non-albicans species were detected in urine of diabetic and non-diabetic women. All the isolated Candida species are important opportunistic pathogens. Our clinically relevant findings indicate that presence of glucose can increase the activity of hemolysins and all Candida species showed the beta hemolysis on blood agar and both Candida albicans and Candida tropicalis showed maximum hemolysis activity compared to C. glabrata and C. krusei.

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