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Phenotypic and Molecular Characterization of *Candida albicans* Isolated from Milk and Buccal Cavity of Child at Kaliobeya, Egypt

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ABSTRACT

Candida albicans is an opportunistic pathogen causing invasive and noninvasive diseases. So, this study aimed to estimate the occurrence and phenotypic characterization of *C. albicans* in 175 specimens (150 samples from milk and 25 buccal cavity swabs of candidiasis patients) collected from different dairy shops and hospitals at Kaliobeya Governorate, Egypt, beside genotypic identification of some isolated strains. This study cleared that *C. albicans* isolates were isolated from 29 samples, 11 (7.3%) from milk samples and 18 (72%) from buccal cavity swabs. Moreover, all *C. albicans* isolates were sensitive for antifungals, and they were highly sensitive to fluconazole followed by voriconazole then itraconazole, amphotericin-B, nystatin and finally clotrimazole. In addition, PCR results demonstrated that, *itS* and *al sI* genes were detected in all seven studied *C. albicans* strains giving products of 109 bp and 318 bp, respectively. So, this study concluded that marketed milk could serve as a pathogenic *C. albicans'* transmission vehicle and could be controlled by using fluconazole, voriconazole, nystatin, clotrimazole, itraconazole and amphotericin-B. Also, *itS* and *al sI* genes are good tools for *C. albicans* isolates' identification.

1. INTRODUCTION

Candida species especially *Candida albicans* are one of the major etiologies of invasive fungal infections broadly (Mbuk *et al*., 2017). A fungal condition known as candidiasis is induced by yeasts belonging to the almost 200 species-strong genus Candida, of which six are most commonly isolated. Although *C. albicans* is the most prevalent and major species, other species involving *C. tropicalis, C. glabrata, C. krusei, C. parapsilosis, and C. lusitaniae* have also been linked to the infections (Singh *et al*., 2014; Muadcheingka and Tantivitayakul, 2015; Zaidi *et al*., 2018; Mba and Nweze, 2020). An asporogenous, pseudomycelial, dimorphic yeast with the capacity to ferment is known as *Candida albicans.* It thrives on common medium in a variety of pH and temperature conditions. Budding yeast formations (blastospores) are 3-4 µm in diameter on epithelial surfaces, while pseudo-hyphae or branching septate hyphae are 3-5 µm in diameter in deeper tissues. It can use ammonia but not nitrate. Most strains require to have additional biotin and nitrogen for growth (Novak *et al*., 2003; Gümrü*et al*., 2006; Dhama *et al*., 2013).

Yeasts represent an important component of the microflora of lactic products, being usually detected in milk and derivates. The species of the genus Candida are the yeasts more commonly isolated from milk. They can cause biochemical alterations in milk and endanger the consumer's health. Yeasts can be related to cases of mycotic mastitis in goats and cows, being responsible for economic losses due to the reduction of milk production and augmentation of costs of the production (Spanamberg *et al*., 2009).

C. albicans has established numerous virulence tools for evading and colonize the host immune system involving the invasins and adhesins' expression which relate to the cell wall, polymorphism, the biofilms' formation, phenotypic switching and the hydrolytic enzymes' secretion as proteases, chitin, neuraminidase, manno-protein and lipids are considered virulence parameters (Dhama *et al*., 2013; Abirami *et al*., 2020).

The clinic has encountered significant difficulties due to the inability to effectively treat candidiasis due to antifungal resistance. The selective therapies' application at insufficient doses or the popular applications of the medication in the prevention of fungal infections in both humans and animals may be to blame for the rise in antifungal resistance (Galle and Gianinni, 2004; Colombo and Guimarães, 2007).

Invasive fungal infections and resistance to antifungal treatments are becoming more common despite the

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availability of improved antifungal medications for the infections' medication caused by Candida species (Pfaller, 2012). As a result, testing for Candida species' susceptibility to *in-vitro* antifungal drugs has become crucial for both identifying drug resistance and managing patients effectively (Barry *et al*., 2003; El-Mashad and Mahmoud, 2011; Pfaller, 2012).

The identification of Candida species phenotypically by traditional microscopic, cultural using chromogenic medium and metabolic characteristics are laborious, time-consuming, demand extensive technological know-how and are occasionally ineffective due to the unusual characteristics of specific isolates (David *et al*., 2007; Moron *et al*., 2017). Consequently, compared to conventional phenotypic approaches, molecular procedures have been established to enable more quick and accurate detection of pathogenic Candida species (Liguori *et al*., 2010; Moron *et al*., 2017; Liu *et al*., 2018).

Candida albicans is one of the most fungi infect human and animal causing serious disease. So, this study was designed for estimation the *C. albicans*' incidence in milk samples and buccal cavity swabs of candidiasis patients gathered from various dairy shops and hospitals at Kaliobeya Governorate and phenotypic characterization of *C. albicans* isolates beside genotypic identification of some isolated strains and effect of anti-fungal sensitivity on it.

2. MATERIAL AND METHODS

2.1. Isolates swaps and milk sampling

An overall of 175 specimens (150 specimens from row milk and 25 buccal cavity swabs of candidiasis patients) collected from different dairy shops and hospitals at Kaliobeya Governorate. Each specimen was taken separately, placed in sterile plastic bags, maintained in an icebox, and transported as quickly as possible to the lab for mycological analysis to investigate the phenotypic and genotypic characterization of isolated *C. albicans* strains.

2.2. Preparation and pre-enrichment of samples

A total of 100 µl of each milk sample and swab of each buccal cavity ones were cultured on modified Sabeurand's dextrose broth (CONDA) (amended with chloramphenicol (50 µg/ml) (CONDA) under aseptic condition and incubated in air conditions at 37 °C for 24 h (Murray et al., 1995).

2.3. Isolation and phenotypic identification of isolates:

Sabeurand's dextrose agar chloramphenicol media were streaked with a total of 50 µl from the incubated modified Sabeurand's dextrose broth and incubated for one to three days at 37 °C. The sub-cultured yeast growth colonies were placed into the Dichloran Rose Bengal chloramphenicol agar (Lab M) and Dichloran Glycerol agar medium (Oxoid), and they were then incubated at 37 °C for one to three days. The yeast growth colonies were removed, maintained in Sabouraud dextrose agar gradients, and incubated for one to three days at 37°C. The macroscopically and biochemically feature of the yeast colonies were carefully observed and measured to identify the pure colonies David et al. (2007), ISO (2008), and Markey et al. (2013).

2.4. In-Vitro antifungal sensitivity test for Candida albicans isolates

In-Vitro sensitivity test was performed on each derived *C. albicans* strain to study its antifungal sensitivity (CLSI M44 series) using disc diffusion test on modified Mueller Hinton agar (HIMEDIA- M 173) by adding 2% glucose to increase the number of *C. albicans* and 0.5 mg /liter methylene blue to trigger the yeast development and supply inhibition's sharp zones for the drugs' azole group. According to Barry *et al.* (2003) and CL SI (2009a&b). The following antifungal standardized disks (Oxoid) used were (amphotericin-B (AP/100); clotrimazole (CC/10); fluconazole (FLC/10); itraconazole (IT/10); nystatin (NS/100) and voriconazole (VRC / 1). as showen in (Fig 5).

2.5. Genotypic identification and detection of virulence genes of Candida albicans isolates:

PCR utilizing fungus-specific universal primer pairs (*itS*) was utilized for *C. albicans* isolates' identification and genotypic characters and one virulence agglutinin-like sequence gene (*al sI*). It was applied on 7 random isolated *C. albicans* isolates (4 from buccal cavity and 3 from milk samples), which had the characteristic biochemical features as that of *C. albicans* and were positive for germ tube test, subsequent QIAamp® DNA Mini Kit instructions (Qiagen, Germany, GmbH), Emerald Amp GT PCR mastermix (Takara, Japan) with Code No. RR310A and 1. 5% agarose gel electrophoreses (Sambrook *et al.,* 1989) utilizing the Primers sequences, amplicons sizes, target genes, and cycling circumstances demonstrated in table (1)

3. RESULTS

The mycological examination's results of 175 specimens (150 samples from milk and 25 buccal cavity swabs of candidiasis patients), revealed that, *C. albicans* isolates were isolated from 29 positive samples, represented as 11 positive samples (7.3%) from milk samples and 18 positive samples (72%) from buccal cavity swabs (Table 2).

Table 2 Positive specimens' incidence for *Candida albicans* isolation among analyzed specimens

Samples	No. of samples	No. of Positive specimens	Percentage of positive specimens %
Milk	150	11	7.3
Buccal cavity swabs of candidiasis patients	25	18	72

% Percentage is no. of positive specimens divided by its no. of samples

Phenotypic characterization

The recovered *C. albicans* isolates in this study were Gram-positive (purple color), large spherical, unicellular budding yeast cells and they appeared as a spherical thick wall chlamydospores, appeared with cluster of blastopores on Corn Meal with Tween 80 agar media (Fig. 1). Meanwhile, they grow well and showed off-white to cream in color, pasty, shiny, raised, smooth, high -convex and have a pleasant beery smell colonies on Sabouraud Dextrose agar (Fig. 2), creamy, smooth, dump, circular in shape with entire margins on Dichloran Glycerol (DG) Agar and pinkish, smooth convex colonies on Rose Bengal agar (Fig. 3).

Moreover, the germ tube test of isolated Candida using human serum revealed that, all of them with pseudomycelium, pseudogerm tubes appeared as small tubes projecting from yeast cells with no constriction at the origin's point, characteristic feature for *C. albicans* (Fig. 4).

The recovered outcomes for the antifungal sensitivity of 29 *C. albicans* isolates (Table 3) revealed that, all of them were sensitive for antifungals studied with various percentages, and they were highly sensitive to Fluconazole followed by voriconazole then itraconazole, amphotericin-B, nystatin and finally clotrimazole

Table 1 Primers sequences, amplicons sizes, target genes, and cycling circumstances

Target gene	Primer sequence (5'-3')	Amplified segment (bp.)	Amplification (35 cycles) Primary denaturation	Secondary denaturation	Annealing	Extension	Final extension	References
itS	F GGTTTGCTTGAAAG ACGGTAG	109 bp.	94˚C	94˚C	50˚C	72˚C	72˚C	Tarini et al. (2010)
	R AGTTTGAAGATATA CGTGGTAG		5 min	30sec	30 sec.	30 sec.	7min.	
al sI	F GAC TAG TGA ACC AAC AAA TACCAGA	318 bp.	94˚C	94˚C	50˚C	72˚C	72˚C 10	İnci et al. (2013)
			5 min	40 sec	40 sec.	40 sec.	min.	

Biochemically all the 29 isolates were positive for germ tube test for glucose, maltose, sucrose, and galactose sugar fermentation and assimilation of glucose, maltose, sucrose, galactose, D-Mannitol, and soluble starch. Meanwhile, they were negative for lactose fermentation and assimilation, nitrate assimilation and urease tests.

Fig. 1 *Candida albicans* on Corn Meal with Tween 80 agar

Fig. 2 *C. albicans* on Sabouraud dextrose agar

Fig. (3): *C. albicans* on Rose Bengal agar

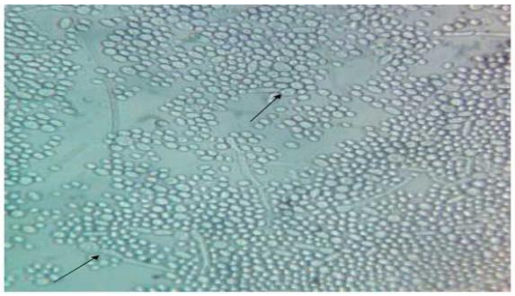

Fig. (4): Microscopy Germ tube test by using human serum after incubation 1-3 hours at 37 ºC.

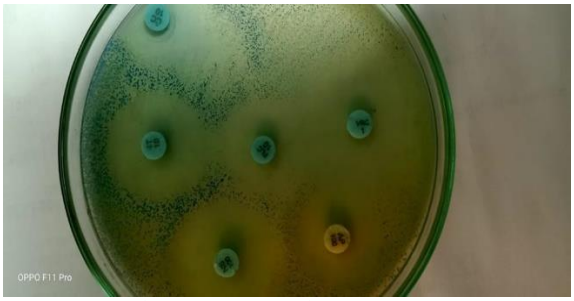

Fig 5 In-Vitro antifungal sensitivity test for isolated *Candida albicans*

Genotypic characterization

The results of identification and genotypic characterization of *C. albicans* isolates) appeared that, fungus-specific universal primer pairs (itS) and virulence agglutinin-like sequence gene (al sI) genes were detected in all seven studied *C. albicans* isolates giving products of 109 bp. And 318 bp., respectively (Fig. 6 and7).

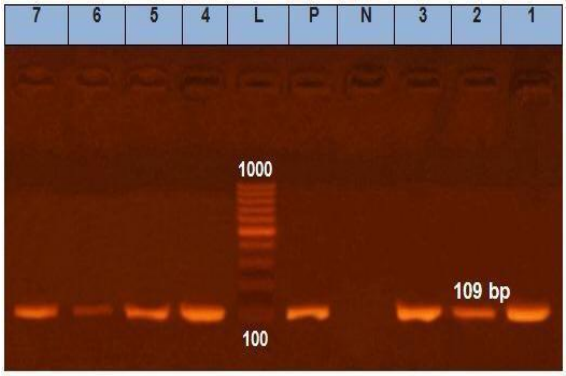

Fig 6 Agarose gel electrophoresis of (*itS)* gene. L: 100-1000 bp. DNA Ladder. N.: Negative control. P.: Positive control (*C. albicans* form Ahri. at 109 bp.) Lane 1-7: *C. albicans* (Positive at 109 bp.)

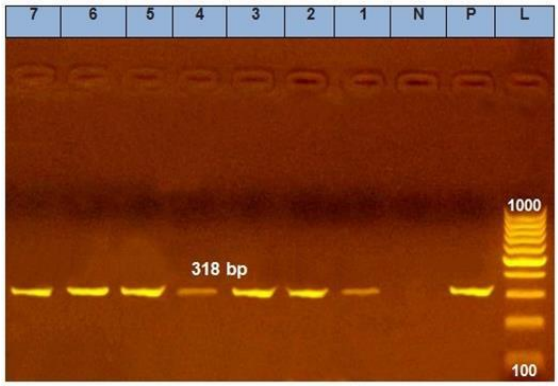

Fig. 7 Agarose gel electrophoresis of (*al sI*) gene. L: 100-1000 bp. DNA Ladder. N.: Negative control. P.: Positive control (*C. albicans* form Ahri. at 318 bp.) Lane 1-7: *C. albicans* (Positive at 318 bp.)

Table 3 In-Vitro antifungal Sensitivity test for 29 isolated *Candida albicans* strains

Antifungal agents	Disk concentrations	Sensitive		Intermediate		Resistant		
		No.	%	No.	%	No.	%	AA
Fluconazole	10 µg	21	72.4	6	20.7	2	6.9	S
Voriconazole	1 µg	20	69.0	7	24.1	2	6.9	S
Itraconazole	10 µg	18	62.1	8	27.6	3	10.3	S
Amphotericin-B	100 Unit	16	55.2	9	31.0	4	13.8	S
Nystatin	100 Unit	15	51.7	6	20.7	8	27.6	S
Clotrimazole	10 µg	14	48.3	10	34.5	5	17.2	S

% Percentage in association to overall number of isolated *C. albicans* (29)

4. DISCUSSION

Candida albicans is one of the most important causes of oral candidiasis "thrush" (Magare and Awasthi, 2014; Abharianet al., 2020) and mastitis in cows (Sonmez and Erbas, 2017). Therefore, this study was prepared for estimation the *C. albicans*' occurrence in milk samples and buccal cavity swabs of candidiasis patients gathered from various dairy shops and hospitals at Kaliobeya Governorate for the phenotypic and genotypic identification of *C. albicans* isolated strains.

The obtained results for mycological examination cleared that, 29 samples were positive for *C. albicans* isolation (11 from milk samples and 18 from buccal cavity swabs). These results for milk samples came in line with that gathered by Sophia et al. (2014) who isolated *C. albicans* from the market milk and may be confirmed the results of Ksouri et al. (2015) and Mohammed et al. (2020), who reported that *C. albicans* is the predominant Candida species in the evaluated milk specimens of mycotic mastitis worldwide. Meanwhile, for buccal cavity swabs of candidiasis patients were closely similar findings gathered by Back-Brito et al. (2009), Manikandan and Amsath (2013), Sharma et al. (2019), Tata et al. (2019) and Abharian et al. (2020), who recorded that *C. albicans* is the most popularly derived yeast from the cases' oral cavity suffered from Oral thrush.

The main phenotypic features of all 29 isolated Candida in this study, morphological features of the colonies, the biochemical profile, Gram staining, germ tubes and chlamydospores' production (hyphal formation) are regarded as confirmatory for identification of them as *C. albicans* and conceded with the description of Yang, (2003), Zaini et al. (2006), Mbuk et al. (2017) and Moron et al. (2017). The recovered outcomes for the antifungal sensitivity of 29 *C. albicans* isolates (Table 3) revealed that, all of them were sensitive for antifungals studied with various percentages, and they were highly sensitive to Fluconazole followed by voriconazole then itraconazole, amphotericin-B, nystatin and finally clotrimazole. These results were in consistent with those recorded by Song et al. (2015), Shokohi et al. (2016), Mbuk et al. (2017) Sonmez and Erbas (2017); Omran et al. (2018); Zaidi et al. (2018) and Ismail et al. (2020).

The results of PCR showed that itS and al sI gene were detected in all seven studied *C. albicans* isolates. Regarding to the fungus-specific universal primer pairs (itS) gene amplification, it was amplified in all seven studied *C. albicans* isolates giving product of 109 bp. (Fig. 6) that came in accordance with those calculated by Tarini et al. (2010), Kumar et al. (2016), Sampath et al. (2017), Ali et al. (2018) and Rahadiyanto et al. (2019). The germ tube test is a simple and affordable presumptive test for *C. albicans* used to identify *C. albicans* from other species because all Candida strains identified as *C. albicans* utilizing itS sequencing were positive for the test. However, to prevent subjective interpretation of outcomes, this test involves clinical professional training and experience in separating germ-tube formations from pseudohyphae (Moron et al., 2017). Moreover, for agglutinin-like sequence (al sI) gene (Fig. 7) cleared that, it was also amplified in all seven *C. albicans* isolates giving product of 318 bp. same outcomes were obtained by İnci et al. (2013) and Soliman et al. (2020).

5. CONCLUSION

The ongoing study concluded that; c.albicans one of the most commonly isolated yeast from milk. They can cause biochemical alterations in milk and endanger the consumer's health and it is also one of the most etiologies of oral candidiasis (thrush). the isolated *C. albicans* were sensitive to Clotrimazole, Fluconazole, Voriconazole, Nystatin, Amphotericin-B, and Itraconazole, that can be used for treatment of these cases. In addition, itS and al sI genes are good tools for identification of *C. albicans* isolates.

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