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Oral *Candida* in β-thalassemia Major and Healthy Population and Their Fluconazole Susceptibility Pattern

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Abstract **Objective:** *Candida* constitutes the main yeast flora in the oral cavity. Different species are known with *C. albicans* being the principal one. In healthy individuals, the balance among the oral microbial flora is maintained. However, this equilibrium might be altered in certain diseases resulting in the predominance of yeast flora over other microorganisms and behave as pathogens. Thalassemias constitute a large group of immunocompromised patients in our country with multiple hematological, immunological and endocrine disorders making them susceptible to local and systemic opportunistic yeast infections. Fluconazole is a globally used antifungal antibiotic especially in treating recurrent infections in immunocompromised patients. Little is known about the oral yeast carriage in the healthy individuals and thalassemic patients in our population, and their susceptibility pattern to fluconazole. This study has been done to assess the frequency of oral *Candida* carriage in a group of β-thalassemia major patients and sex and age-matched healthy controls, and to evaluate the susceptibility pattern of the isolates to fluconazole. **Place of study:** Microbiology Laboratory at College of dentistry/Hawler Medical University, Erbil, Iraq. **Methodology:** Two hundred subjects were included in the present study, 100 patients with β-thalassemia major (38 females &62 males) aged 4-26 years and 100 apparently healthy subjects age and sex-matched with thalassemic patients. Oral swab specimens were collected from each individual subject, cultured on sabouraud dextrose agar plates and incubated at 37^{o}C for 48-72 hours. Identification of *Candida* species was based on colony morphology, germ tube test, biochemical reaction using API *Candida* system and growth characteristics on CHROMagar Candida. The disc diffusion method was applied to test sensitivity of the isolated strains to various concentrations of fluconazole. **Results:** All the subjects included in this study harbored only *C. albicans* in their oral cavities, with higher carriage rate observed among thalassemic patients (20%) than healthy controls (7%). *Candida albicans was recovered much frequently from* healthy children (57%) and adolescent thalassemias (65%) than other age groups within each study group. However the statistical analysis showed no significant correlation between age and oral *C. albicans* carriage rate (χ^2 *p*-value > 0.05). Healthy females showed higher carrier rate than males, meanwhile *C. albicans* was equally distributed between male and female thalassemic patients. Susceptibility of *C. albicans* isolates to a 25 µg/ml fluconazole showed that out of the 27 *C. albicans* isolates, 23 were resistant to fluconazole. The resistant *albicans* strains showed high resistance rate (>128 µg/ml) within both groups. Whereas the response of sensitive strains to fluconazole was variable ranged from >16 µg/ml in healthy subjects to 4 - >16 µg/ml in thalasemias. **Conclusion:** *C. albicans* was the only isolated species from the oral cavity of thalassemic patients and healthy subjects. However the carrier rate was higher among thalassemias than healthy controls. Most of the isolated strains were resistant to fluconazole. Further studies are required to assess the sensitivity of oral *Candida* isolates to other antifungal drugs.

Keywords: *β-thalassemia major, disk diffusion, Candida, fluconazole*

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1. Introduction

Candida species, mainly *Candida albicans* (*C. albicans*) compose the yeast commensal flora of the oral cavity, which can be isolated in healthy oral cavities with different frequencies [1,2]. A common complication implicated in immunocompromised patients is the opportunistic oral and oropharyngeal candidiasis. It is mostly caused by *C. albicans*, however other species such as *C. glabrata*, *C. krusei* and *C. tropicalis* has been also reported [3,4]. Many studies have documented the increase in number of oral *Candida* and/or the rate of candidiasis in cancer patients, diabetic patients and HIV-infected patients in comparison to healthy subjects [5,6,7,8,9]. Thalassemias, another class of compromised patients with multiple immunological disorders, constitute a heterogeneous group of chronic hemolytic anemias that arise from genetic disorders of hemoglobin synthesis. Based on the degree of anemia and their effect on the body, three types of thalassemia have been identified;

minor, intermedia and major [10]. Fluconazole, a triazole drug, has a wide spectrum of activity against yeasts and moulds. The anti-yeast activity of this drug relies on its capability to inhibit ergosterol biosynthesis pathway thereby interfering with yeast's plasma membrane synthesis [11]. Treatment of recurrent candidiasis and prophylaxis of immunocompromised patients against oral candidiasis using fluconazole drug is routinely applied in many clinical settings, since good concentrations are usually achieved in saliva and gingival crevicular fluid (GCF) [12]. Thus, the wide spread use of fluconazole makes the clinical resistance being a real problem. Therefore, periodic screening for the susceptibility of *Candida* isolates to fluconazole and other antifungal drugs is necessary to guide antifungal therapy and monitor local resistant pattern [13]. Previous studies that compared the reliability of different antifungal susceptibility including standardized NCCLS broth macrodilution and microdilution methods, E-tests and disc diffusion methods suggest a possibility of using the less expensive easy to perform disc diffusion method in clinical microbiology laboratories [14,15,16]. Much information is readily available about the characteristics of oral *Candida* in European and American healthy population from numerous studies [17,18,19,20]. However, little is known about the yeast flora in developing countries. In addition, scarce information regarding oral *Candida* species colonization in subjects with thalassemia is available [21,22]. Review of literatures demonstrated a lack of studies evaluating the rate of colonization of *Candida* species in the oral cavity of thalassemic patients coupled with their susceptibility to fluconazole. To assess the prevalence rate of oral candidal colonization in subjects with β-Thalassemia major versus healthy controls and to evaluate the susceptibility pattern of the isolated strains to fluconazole.

2. Materials and Methods

Source of specimens: This Study was carried out on 100 β-thalassemia major patients attending Thalassemia Center in Erbil City, and 100 apparently healthy individuals that constitute the control group. The subjects in both thalassemic patients and healthy controls were comparable in age (4-26 years) and gender (38 females &62 males). None of the subjects had a history of antifungal treatment in the weeks before sample collection, or fixed orthodontic appliances that may change the quality of oral microbiota. The study was explained for the participants and who signed an informed consent was accepted into the study. Sample collection has started in October 2012 through September 2013.

2.1. Specimen Collection and Processing

Oral swab specimens were collected from subjects of both groups by rubbing a sterile swab on gum surface, teeth, dorsum of the tongue, hard palate and cheek mucosa. The collected samples were immediately transported to the laboratory and cultured onto sabouraud dextrose agar (SDA) (……) plates and incubated aerobically for 48-72 hours at 37°C.

2.2. Microbiological Investigation

Colony morphology matching the characteristic feature of *Candida* colonies was the first step in the identification process. This was followed by microscopical examination of gram-stained smear from a well-defined colony obtained from each individual sample. For further identification of *Candida* species the standardized method described by Cheesbrough (2006) and Forbes et al. (2007) was followed that includes germ tube test, biochemical profile using API *Candida* system (bioMe'rieux Sa., France) and culturing on a specific chromogenic medium (CHROMagar™ Candida, CHROMagar, France). The latter is a differential medium for different *Candida* species with high specificity and sensitivity for a presumptive identification of the suspected *Candida* strains. *Candida* isolates were maintained on SDA slants at 4°C, and subcultured monthly.

2.3. Fluconazole Susceptibility Testing

The identified *Candida* isolates were examined for their susceptibility to Fluconazole (standard powder from Macleods Pharmaceuticals Ltd., Mumbai, India), using simple disc diffusion technique. Sterile 6mm filter paper discs (WHATMAN no.3) were used to prepare Fluconazole discs. Different concentrations of Fluconazole were prepared by serially diluting the standard powder in sterile distilled water, as described by the manufacture, to obtain the following concentrations: 1,2,4,8…..128 µg/ml in addition to 25µg/ml. Tens of discs were prepared from each dilution by adding 10µl each disc. Then the discs were dried and stored at 4°C.

All the isolates were examined for their susceptibility to a 25µg/ml fluconazole disc, as an initial step for determining the susceptible and resistant strains, by measuring the diameter of inhibition zone and comparing it with standard inhibition zone mentioned by Cronvall and Carlsson (2001). Following the identification of the resistant and sensitive strains to fluconazole among *Candida* isolates, the exact inhibitory concentration of fluconazol against each isolate was determine by testing the sensitivity of the resistant and sensitive strains to the prepared fluconazol discs with concentration higher and lower than 25µg/ml, respectively. Breifly, the inoculum from each *Candida isolate* was prepared from a fresh 24 hour culture through emulsifying 2-3 discreate colonies grown on SDA agar plate in 5 ml of sterile normal saline (0.9%) and adjusted to a 0.5McFarland turbidity standard, using spectrophotometer (BIO-TEK Instrument, Milan, Italy) at 450nm wavelength. Then, a sterile swab soaked in prepared inoculum and spread on the entire surface of sabouraud dextrose agar plate in different directions to ensure the uniform spread of the inoculums. The inoculated plate was left to dry at room temperature. Later, previously prepared fluconazole disks of desired concentrations were placed on the surface of the inoculated medium and incubated for 24 hours at 37°C, after which the diameter of inhibition zones were recorded.

2.4. Statistical Analysis

SPSS (Statistical Package for Science Services) version 20.0 under windows 2008 was used to analyze data and Excel 2010 under windows 2008 for figures. Pearson Chi-square test (χ^2) was applied to find the correlation between the oral carrier rate of *C. albicans* and age of the subjects

of both study groups. A *P-value* less than 0.05 was considered statistically significant**.**

3. Results

One hundred β-thalassemia major and one hundred age and sex-matched healthy subjects were included in this study. Each group was composed of 62 males (62%) and 38 (38%) females. The age range of both groups was 4-26 years. The mean age ± SD for thalassemic patients was 11.89 ± 4.94 years, and that of the healthy controls was 11.92 ± 5.37 years. In the present study, *C. albicans* wasl the only isolated *Candida species* from the oral cavity of subjects of both studied groups. The frequency of isolation was seemed to be higher among β-thalassemia major patients (20%) than healthy controls (7%) (Table 1). On the other hand, the same data indicated that the highest carriage rate (65%) among thalassemic patients was observed within the age range of 11-20 years, whereas the highest carriage rate (57%) among healthy subjects was recorded within 1-10 years. However, the statistical analysis using Chi square test revealed a significant correlation between the frequency of detection of *C. albicans* and Age within both groups (χ^2 *p*-value > 0.05). Inequality was demonstrated regarding the distribution of *C. albicans* in both sexes among healthy subjects in which the carrier rate was higher among females (71%) than males (29%), whereas *C. albicans* colonization rate was equal among male and female subjects within thalassemic group (Figure 1). The result of susceptibility testing of *C. albicans* isolates to a 25 µg/ml fluconazole disc was decided according to the work of Cronvall and Carlsson (2001). Briefly, isolates showed the diameter of zone of inhibition < 18 mm were regarded as resistant to fluconazole, whereas those showed zone diameters ≥ 18 mm were recorded as sensitive (Table 2). Out of 27 *C. albicans* isolates, 23 were resistant to fluconazole. High rates of fluconazole resistance among the tested isolates were recorded in both study groups (86% and 85% for the healthy subjects and thalassemic patients, respectively). The results of disc diffusion method to test the efficiency of fluconazole against susceptible and resistant *C. albicans* isolates are presented in Table 3. Generally, Great heterogeneity in the susceptibility pattern of the oral isolates was observed. The recorded inhibitory concentration for all the susceptible isolates from the control group and half of the susceptible isolates from thalassemias was higher than 16 µg/ml and the overall range was 4 - >16 µg/ml. On the other hand, all the resistant *C. albicans* isolates from both study groups were highly resistant to fluconazole (>128 µg/ml).

Table 1. Rate of oral *C. albicans* colonization in thalassemic patients and healthy controls according to age

Age (year)	Healthy controls (%)	Thalassemic patients (%)
1-10	4 (57)	5 (25)
11-20	1 (14)	13 (65)
21-30	2 (29)	2 (10)
Total	7/100	20/100

Table 2. Susceptibility pattern of oral *C. albicans* isolates sensitive to a 25 µg/ml fluconazole disc

MIC (µg/ml)	Healthy controls %	Thalassemic patients %	*P*-value
4	0.0	25	
8	0.0	25	
>16	100	50	
Total %	100	100	

Table 3. Susceptibility pattern of oral *C. albicans* isolates resistant to a 25 µg/ml fluconazole disc

MIC (µg/ml)	Healthy controls %	Thalassemic patients %	*P*-value
32	0.0	0.0	
64	0.0	0.0	
>128	100	100	
Total	100	100	

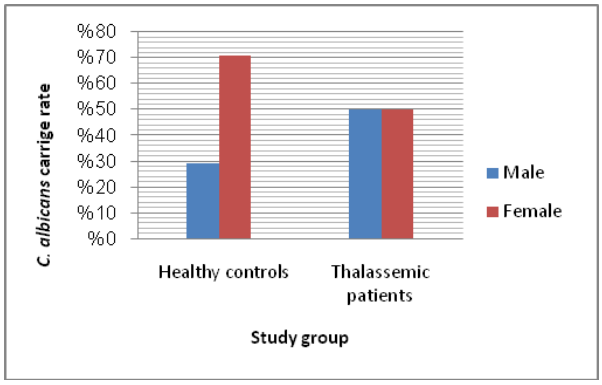

Figure 1. Rate of oral *C. albicans* colonization in thalassemic patients and healthy controls according to gender

4. Discussion

There is a global interest in conducting studies regarding the role of *Candida* in oral health and disease. In the present study, *C. albicans* was the only isolated species in both the healthy subjects and thalassemic patients in different frequencies (Table 1). A 100% recovery of *C. albicans* was also reported by Xu and Mitchell [17] in healthy subjects from eastern North America. Predominance of *C. albicans* over other *Candida* species was common demonstrated by Nneka and Ebele [18] and Martins *et al* [20]. The frequency of occurrence and isolation rate of *Candida* species are greatly influenced by different factors such as genetic factors, lifestyle, diet, regular dental care, age, general state of health, socioeconomic and cultural levels and sampling method [25,26,27]. Our results also showed that about 7% of the healthy subjects harbor *C. albicans* in their oral cavity. This detection rate is in agreement with that reported in a review study on oral *Candida* carriage by Scully *et al* [28] in which the total carrier rate among healthy individuals usually ranges from 2 to 69.1%. On the other hand, a significantly higher detection frequency (20%) was observed among thalassemic patients. Much higher carriage rate (74%) was recorded by Hazza'a *et al* [22] in a group of Jordanian patients with β-thalassemia major in comparison to healthy individuals (56%). Despite

the lower carriage frequency recorded in our study, there is a general agreement on that the total carriage rate is higher in compromised patients than healthy individuals [5,29,30]. The possible explanation for the increased yeast carriage rate in thalassemias than healthy subjects is that in the absence or inadequate chelation therapy, there is abnormal accumulation of plasma non-transferring binding iron, free iron, due to repeated blood transfusion, ineffective erythropoiesis, increased gastrointestinal absorption of iron and lack of a physiologic mechanism for excreting excess iron [31,32]. The free plasma iron, then, can infiltrate different body cells resulting in oxidative damage [33]. Skoutelis and colleagues [34] demonstrated a defective neutrophil and macrophage chemotaxis, impaired phagocytosis and B-lymphocyte differentiation in those with plasma iron overload, resulting in weakening of the systemic immune status of the patient. In addition, Vaiopoulos and co-workers [35] found a decreased salivary secretion due to iron accumulation in salivary gland cells in β-thalassemia major patients. It is well-known that saliva is the main fluid in the oral cavity that has antimicrobial properties through its flushing action that removes non-adherent cells away from oral surfaces, and perhaps due to increased quantities of anti-*Candida* molecules including histatins, lysozyme, peroxidase, lactoferrin and salivary immunoglobulin A (sIgA) [36,37,38,39]. Since, the optimal secretion rate of saliva is important for maintaining oral health; decreased quantities might result in increased recovery of yeasts. As expected, children (1-10 years) showed the highest carrier rate (57%) in comparison to other age groups. The predominanace of yeasts in the oral cavity of children is in agreement with the finding of Martins *et al* [20] and Ugun-Can *et al* [40]. Immature immune system together with motor and cognitive immaturity in children, that makes it hard for the children to realize and apply hygienic measures, are the main precipitating factors to disturb the normal balance of the oral cavity. Improvements are usually seen among older children, adolescents and adults resulting in the decrease in the frequency of yeast carriage [41]. On the hand, thalassemic patients aged 11-20 years, were the frequent carriers (65%) for *C. albicans.* This high carriage rate among adolescent subjects could be attributed to the growth retardation and endocrine dysfunction usually seen in β-thalassemia major patients [42]. In this study, the frequency of carriage rate was higher among female healthy subjects (71%) than males (29%). However, similar carriage rates were observed among male and female thalassemic patients (Figure 2). Our result is completely in consistence with those observed by Hazza'a *et al* [22] regarding both study groups, but differed from the findings of Nneka and Ebele [18] and Martins *et al* [20] in which in healthy individuals both sexes are similar in their oral yeast carriage. *Candida albicans* isolates showed a wide range of sensitivity to fluconazole (4 to >128 µg/ml). Interestingly, the high rate of resistance among *C. albicans* isolates recorded in our study exceeds most of the previously recorded data. Fothergill (2012) [43] recorded a lower range of the MIC of fluconazole (≤0.125-64 µg/ml) against clinical *C. albicans* isolates from 2000-2009. However, a relatively similar observation was recorded by Sun and his colleagues [44] in which approximately 70% of *C. albicans* starins isolated from the oral cavity of cancer patients under chemotherapy were resistant to fluconazole and the MIC of all the resistant isolates was >128 µg/ml. He has attributed this great variation in fluconazole susceptibility to the genotypic heterogeneity among *C. albicans* isolates.

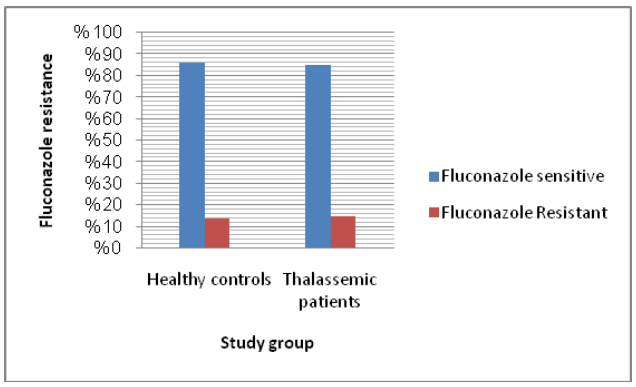

Figure 2. Sensitivity of oral *C. albicans* isolates to a 25 µg fluconazole disc in the study groups

In a conclusion, *C. albicans* constitutes the main isolated species from the oral cavity of thalassemic patients and healthy subjects. Higher oral carriage was observed among thalassemias than healthy controls. Most of the isolated strains were resistant to fluconazole. Further studies are required to assess the sensitivity of oral *Candida* isolates to other antifungal drugs.

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