Prevalence And Pathogenicity of Non-Albicans Candida Species Among Diabetic Patients with Post Infection of Covid-19 in Basrah

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Abstract
Aims The aim of the current study is to investigate the prevalence of non albicans Candida species in diabetic patients with post Covid 19 infections with evaluation the virulence factors of yeast isolates. Materials and Methods A total of 75 oral and nasal swabs were collected from post Covid 19 infection associated with diabetic patients from both Al-Moani and Al-Faihaa hospitals in Basrah province/Iraq from September to November 2021. The age groups of the patients were ranged between 10 to 90 years old. For data collection, a questionnaire form including name, age, gender, underlying disease, type of Covid 19 vaccine, type of antibiotics, corticosteroids, or immunosuppressive therapy besides the date of hospital admission were collected for each patient.Results Depending on the sings and symptoms of yeast infections and examination of specimens the prevalence of fungal infections appeared in 46(61.3%), oral infections 35(46.6%) and nasal infections in 40(53.3%), while the age groups (51-70) years appeared as the highest incidence of fungal infection and female appeared in 33 (71.7%) cases. In spite of non vaccinated patients were showed high accidence of yeast infections 17(80.95%) with comparison of vaccinated patients4(19.04%) .The identification of pathogenic yeast isolates revealed that classification of eight species due to four genera that involving Candida albicans ,C. dubliniensis, C. glabrata, C. parapsilosis, C. tropicalis, Magnusiomyces capitatus,Pichia kudriavzevii, Magnusiomyces ,Rhodotorula and Trichosporon ashii After excluded C. abicans, the incidence of non abicans Candida species showed that C. dubliniensi appeared in high percentage 8/21(38.09%) followed by C. tropicalis (19.04%). The enzymatic activity of yeast isolates (proteinase, phospholipase, esterase and hemolysin) appeared that the majority of the yeast isolates produce esterase activity, with 18(85.71%), while proteinase production was demonstrated minority 3(14.28%), as well as, the biofilm formation of yeast isolates showed that ability of most isolates 17(80.95%) to produce slim layer
Keywords: Diabetic patients, non albicans Candida infections, Covid19.

1. Introduction

Invasive fungal infections (IFIs) have generally turned out to be one of the major pathogens that have threatened human life worldwide. The dramatic enhancement in the prevalence of invasive fungal infections is directly associated with a tremendous increment in the number of immunocompromised people that are generally linked to some diseases like HIV, diabetes mellitus, Covid 19 infections, and other diseases that consequently trigger immunosuppression (Riley, 2021).
Diabetes mellitus (DM) is a common endocrine metabolic disease (Kałużna,et al, 2019) so that, the level of hyperglycemia in diabetes has a vital function in changing the balance of the oral microbiota because it provides an ideal environment for the development of secondary infections including oral candidiasis and superficial or systemic fungal infections (Babatzia et al., 2020).
Alarmingly, the globe has suffered recently from the spread of Covid 19, a novel generation of SARS-CoV-2, that was broke out for the first time in Wohan, China at the end of 2019. According to WHO (2022) it is estimated that over 628 million confirmed infections with Covid 19 world widely till 2 nd of November 2022.
Because Covid 19 alters both the immune and metabolic response through dysfunction of T lymphocytes, patients with Covid 19 were highly susceptible to various coinfections including fungal infections, therefore, it is reported that fungal infections have emerged as one of the leading coinfections with about 3.7% in Covid 19 patients (Nazari et al, 2022). With respect to C. albicans that is considerably the leading opportunistic coinfections in immunocompromised patients including Covid 19 patients (Peckham et al, 2020) there is a dramatic enhancement in the incidence of non albicans Candida in Covid 19 patients (Roudbary et al, 2021).

2. Materials and Methods

Collection of specimens a total of 75 oral and nasal swabs, oral 35(46.6%) and nasal 40 (53.3%) were collected from diabetic patients between September 2021 till November 2021 from both Al-Moani and Al-Faihaa general hospitals in Al-Basrah province/ Iraq. The age group of patients were ranged from 10 to 90 years, 55 (73.33%) females and 20(26.7%) males.

doi.org/10.31838/hiv23.03.94 Received: 27.10.22, Revised: 21.11.22, Accepted: 03.12.22.

The total number of vaccinated patients was only 16 patients, and according to the type of vaccine the majority of patients were vaccinated with Pfizer vaccine 6(37.5%), so that, the percentage prevalence of fungal infections from DM vaccinated patients 4(19 %) , and non-vaccinated patients 17 (81%).

Diagnosis of oral and nasal Candidiasis was basically determined based on the signs and symptoms of patients in addition to examine of specimens microscopically by using the 10% KOH and Gram stain technique to detect fungal elements, yeast cells, pseudo hyphae, true hyphae and clusters of conidia. On the other hand, the swabs were cultured on Sabouraud Dextrose Agar (SDA) plus chloramphenicol and incubated at 37 °C under aerobic conditions for 24-72 h. The pure culture of all fungal isolates was performed by subculturing on SDA slant and then kept at 4º C for further studies.

Identification study

The preliminary identification of the isolates were carried out through culture pure colonies on chrome Candida agar (Hi media) and then incubated at 37° C for 24-48 h. to differentiate Candida spp. based on both the colors and morphology of the isolates . The germ tube assay (Matare, et al,2017) were performed to differentiated to Candida albicans ,C. dubliniesis from other species of Candida .

After cultivation of a single colony on SDA for 48 h at 37ºC, the genotypic identification of the isolates was examined through extraction of genomic DNA using Fungi/yeast genomic DNA extraction kit (Gained, Korea), then PCR amplification was carried out to amplify intergenic spacer region (ITS) using both ITS1 F-5'- TCC GTA GGT GAA CCT GCG G- 3'and ITS4 R-5'- TCC TCC GCT TAT TGA TAT GC-3'. The PCR mixture was prepared in a total volume of (25µl) containing master mix (10 µl)(Bioneer, Korea) , forward primer (2µl) ,reverse primer (2µl), extracted DNA (2 µl), RNAase free water (9µl) .The initial denaturation was carried out at 95ºC for 5 min, thereafter, Thirty cycles of amplification were accomplished for denaturation step at 94°C for 30 sec, an annealing step at 56°C for 30 sec, an extension step at 72°C for 1 min were conducted, followed by a final extension step at 72°C for 7 min, after that, the PCR products were electrophoresed in 2% agarose by adding 5µl of each reaction to 1µl of loading dye.

PCR products for all yeast isolates (n=43) were sent out for sequencing to Macrogen company (Korea), then the treated sequences were identified in "BLAST" provided by the NCBI software.

Detection of virulence factors

Enzymatic activity

The proteinase production was determined according to Ramos *et al*,(2015) using bovine serum albumin agar (BSA). Phospholipase activity by measuring the size of precipitation zone after growth on egg yolk agar according to Ellepola *et al* (2016). Hemolysin activity was evaluated with a blood plate assay according to beed *et al* (2022).Esterase production of yeast was achieved using Tween 80 opacity test (Fatahinia *et al*, 2015).

Biofilm formation assay

The ability of biofilm formation by current isolates were investigated using Microtiter plate method, the assay was performed on a sterile 96-well microtiter plate as described in the following steps (Marak and Dhanashree, 2018).

3. Results and Discussion

Population and demographic study

In the current study, a total of 75 nasal and oral swabs were collected from DM patients with Post Covid-19 infections, 20 (26.7%) male, and 55 (73.3%) female.

Table (1) reveals the age groups of our study population that were ranged from 10-90 years old. The majority in the number of patients were in (51-70) years. In contrast, the minority of patients was (10-30) years.

Table (1): Distribution of DM patients post Covid 19 according to age groups and gender.

Age Groups	Female	Male	Total
10-30	5 (6.7%)	1(1.3%)	6(8%)
31-50	12(16%)	5(6.7%)	17(22.7%)
51-70	28(37.3%)	9(12%)	37(49.3%)
71-90	10(13.3%)	5(6.7%)	15(20%)
Total	55 (73.3%)	20(26.7%)	75(100%)

Based on the source of specimens, the percentage of nasal swabs was 40 (53.3%), and oral swabs were 35 (46.6%), while in terms of Covid 19 vaccination , among the 75 diabetic patients, the total number of vaccinated patients was only 16 (21.33%) patients, as well as, depending on the type of vaccine, the most of patients were vaccinated with Pfizer vaccine 6(37.5%), followed by sinopharm vaccine where 5 (31.25%) patients and only 1 (6.25%) patient was vaccinated with Asterzenca vaccine ,and 4 (25%) patients did not know the type of vaccine.

Detection of fungal infections the investigation of pseudomembranous candidiasis was performed based on the appearance of clinical signs such as whitish to milky plaques with erythematous zone in the oral cavity of the patients, in addition to burning and itching sensation so, this step proves the presence of fungal infections

Furthermore, microscopic examination of nasal and oral swabs showed that 46 patients (61.3%) showed positive results of fungal infections that appeared as true hyphae, pseudo hyphae, and clusters of conidia under the microscope.

Prevalence of fungal infections

The current findings showed that the incidence of fungal infection was in 46 (61.3%), where female appeared in 33 (71.7%) cases and 13(28.3%) male. In terms of age groups (51-70) years old revealed as the highest incidence of fungal infection, (table 2). Moreover, there were three nasal swabs were failed to grow on SDA when compared with the results of microscopic examination and clinical signs, this is due to many reasons it may be a small amount of the sample, or have antibiotics that inhibit the growth of fungi on the culture media or experimental conditions, so, this investigation agreed with Yang and co-authors (2021) who reported that out of 459 clinically diagnosed isolates, only 127 isolates appeared positive for laboratory culture .

Table (2): Prevalence of fungal infections according to gender and age groups of DM patients.

Gender Age groups	10-30	31-50	51-70	71-90	Total	Chi-square (p-value)
Female	1(3%)	8(24.24%)	16(48.48%)	8(24.24%)	33(71.73)	22.34 (0.01)
Male	1(7.69%)	4(30.76%)	4(30.76%)	4(30.76%)	13(28.26%)	3.78 (0.134)
Chi-square	1.11 (0.35)	2.29 (0.19)	20.43 (0.001)	2.29 (0.091)	18.89 (0.001)	

The results of table (2) appeared that fungal infections of mucous membranes associated with the inflammatory host response are very common and can severely affect the quality of life for a female more than males, due to the complex interplay of sex hormones, genetic variability, and the environment against a background of intrinsic effects of sex chromosome differences. Indeed, each adult human somatic cell exhibits sex-specific differences in gene expression and epigenetic profile to varying degrees (Enfert et al,2021;Kraševec, 2022). This investigation agreed with Kraševec (2022), Benedict et al (2020) and Strati et al (2016) who reported that women were more than men to recognize fungal infections.

The current investigation found that the rate of fungal oral infection was higher than those of nasal infection where they were 30(85.7%)in comparison with colonization rate in nasal (40%) as shown in figure (1). The majority of oral fungal infections are resultant of opportunistic conditions, host resistance impairment allows for the initiation and progression of pathogenic conditions through local colonization in the oral cavity. The frequency of oral infection has remarkably enlarge globally with the increased use of immunosuppressive drugs and immunodeficiency viral infections (Santosh ,2021). The present study agreed with Santosh *et al* (2021) and Vila *et al* (2020) who reported that the oral candidiasis is most often times observed in patients with immune dysregulation or immunosuppressive medications.

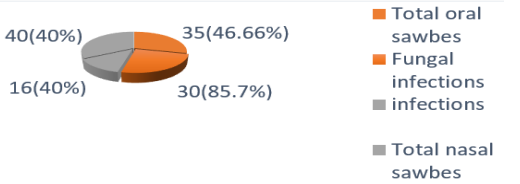

Figure (1) : Prevalence of fungal infection based on the source of specimens there is a significant difference (P ≤ 0.05).

Vaccines save millions of lives each year, the development of safe and effective Covid-19 vaccines are a crucial step in present, however corona virus vaccine preventing serious complications or hospitalization, thus reducing infection to many opportunistic microorganisms such as bacteria and fungi, so that, our findings revealed that the percentage prevalence of fungal infections from vaccinated patients 4(19%), and non-vaccinated patients in 17 (81%) (fig 2)

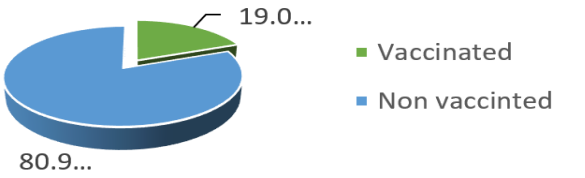

Figure (2): Percentage of fungal infections in vaccinated and non-vaccinated patients a there is a significant difference (P≤0.05)

Identification study

All fungal isolates were identified by different methods that included phenotypic characterizations involving morphological features and microscopic properties that assisted to identify yeast isolates genus Candida, Trichsporon and Geotrichum.

Germ tube has a crucial role in adherence which indeed played a vital role for colonization and initiation of pathogenesis. The morphological transitions between yeast and filamentous forms are considered as one of the most important virulent factors in C. albicans or C. dubliniensis. The results of this assay showed that 30 isolates (76.92%) produced germ tube out of 39 isolates of Candida spp (except Trichsporon, Geotrichum and Rhodotorula sp) and consequently these isolates presumed to be either C. albicans or C. dubliniensis . The current finding was similar with the study of Modrzewska and Kurnatowski ,(2015).

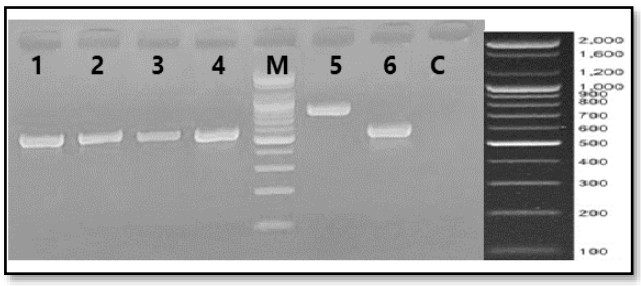

Figure (3): 2 % agarose gel electrophoresis analysis for PCR products using FITS1 and RITS4 primers

Candida species were identified presumptively by using Candida chrome agar ,where ,the results showed that Candida albicans / C.dubliniesis was appeared in 30(76.92%) followed by C.tropicalis

4(10.25%). This findings agreed with many studies who reported that Chrome agar was routinely exploited to differentiate between Candida species (Devi and Maheshwari, 2014; Obeed and Alrubayae, 2022).The identification of all yeast isolates were confirmed using molecular approaches based on the PCR amplifications of the ITS regions (figure 3)

Sequencing

The total of amplified sequencing of 43 yeast isolates showed that identification of eight yeast species that described in table (3).

After excluded the yeast isolates that identified as C. albicans 22(51.16%) C. dubliniensis revealed with high occurrence among non Candida albicans isolates 8(38.09%) followed by C. tropicalis 4(19.04%) appeared in table (3). On the other hand there is a significant difference between the yeasts isolates.

Table (3): The prevalence of yeast infections of non *abicans Candida* spp from Post Covid 19 in DM patients	
Yeast isolates	Percentage of infections (%)
C. dubliniensis	8(38.095%)
C. glabrata	3(14.285%)
C. parapsilosis	1(4.761%)
C. tropicalis	4(19.047%)
Magnusiomyces capitatus	1(4.761%)
Pichia kudriavzevii	1(4.761%)
Rhodotorula mucilaginosa	2(9.523%)
Trichosporon ashii	1(4.671%)
Total	21(100%)
p-value	0.01

Moreover, the majority of our isolates were registered in the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast) and the accession numbers of the registered isolates were shown in table (4).

Table (4): The accession number of yeast isolates that registered in NCBI .		
Number	Yeast isolates	Accession number
1	*C. dubliniesis*	OP382361
2	*C. dubliniesis*	OP442935
3	*C. dubliniesis*	OP404197
4	*C. dubliniesis*	OP404198
5	*C. dubliniesis*	OP410923
6	*C. dubliniesis*	OP412355
7	*C. dubliniesis*	OP412357
8	*C. dubliniesis*	OP412361
9	*C. glabrata*	OP382964
10	*C. glabrata*	OP422499
11	*C. glabrata*	OP410930
12	*C. parapsilosis*	OP442933
13	*C. tropicalis*	OP404361
14	*C. tropicalis*	OP412360
15	*Magnusiomyces capitatus*	OP415526
16	*Pichia kudriavzevii*	OP404365
17	*Rhodotorula mucilaginosa*	OP412345
18	*Rhodotorula mucilaginosa*	OP412302
19	*Trichosporon asahii*	OP443873

The present study agreed with Katz,(2021) and Tumer et al, (2021) they showed that high prevalence of candidiasis in Covid 19 patients , because these patients have exposed to many drugs like corticosteroids that promote functional impairments of several immune cells, including neutrophils ,monocytes, macrophages, and also T cells (Hoenigl et al.,2022). Moreover, Salehi et al, (2020) reported that corticosteroids and cytokine blockers despite their relevance to Covid-19 management, these drugs hamper the activation of innate and adaptive antimicrobial responses and therefore represent important predisposing factors to secondary fungal infections, whereas, ICU patients, central venous catheters, corticosteroids and antibiotics which presented common risk factors in Coivd -19.

Phylogenetic construction

the results showed the close relationship between our isolates with reference strains that confirmed the identification of current study isolates based on molecular approaches.

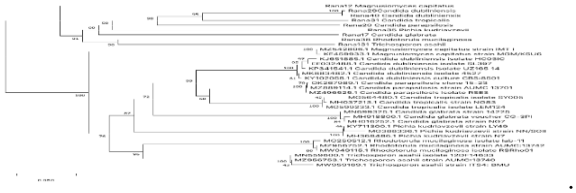

Figure (7) Phylogenetic construction of 9 Non albicans

Candida isolates and reference strains using Neighbor-joining

Investigation of virulence factors

The results of enzymatic activity revealed that the most of isolates of non albicans Candida, appeared esterase activity that exhibited by 18 (85.71%) isolates, while the proteinase production was demonstrated minority 3(14.28%), as were as, the phospholipase activity of the total isolates 16(76.2%) failed to produce phospholipase while, 5 (23.8%) isolates appeared phospholipase activity, in addition to hemolysis activity revealed that 10(47.61%) isolates showed success to produce hemolysin activity. This investigation disagreed with previous studies that showed the majority of yeast isolates have the ability to produce proteinase enzyme (deMelo et al, 2019; Al-Rubayae et al, 2020). The high negative ratio of the production of the two enzymes phospholipase and proteinase may be due to the inability of the isolate to secrete these two enzymes in vitro and the possibility of their secretion in the host in vivo. This present study corresponds to Wasnik et al (2022) and Karishma et al ,(2022) who reported that among non candida albicans including by C. glabrata and one of C. parapsilosis produce hemolysin and that C. glabrata and C. parapsilosis did not appear to produce phospholipase.

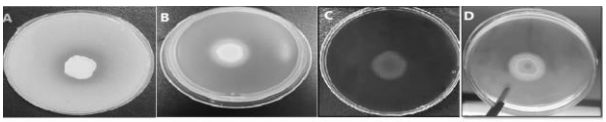

Figure (8): Enzymatic activity (a) proteinase on BSA (B) phospholipase activity on egg yolk agar (C)hemolytic activity on glucose and blood enriched Sabouraud dextrose agar (D) esterase activity on tween 80 opacity medium.

Table (5): Distribution of enzymatic activity of non albicans Candida isolates in oral and nasal swabs.

Yeast isolates (n=21)	Proteinase	Phospholipase	Hemolysin	Esterase
C. dubliniensis (8)	-	2(25 %)	3(37.5%)	7(87.5%)
C.glabrata (3)	-	-	1(33.3%)	1(33.3%)
C.parapsilosis (1)	1(100%)	-	1(100%)	1(100%)
C.tropicalis (4)	2(50%)	2(50%)	3(75%)	4(100%)
Magnusiomyces capitatus (1)	-	1(100%)	-	1(100%)
Pichia kudriavzevii (1)	-	-	1(100%)	1(100%)
Rhodotorula mucilaginosa(2)	-	-	-	2(100%)
Trichosporon ashi(1)	-	-	1(100%)	1(100%)
Total	3(14.28%)	5(23.8%)	10(47.61%)	18(85.71%)

Biofilm formation

The majority of isolates showed positive of biofilm production 17(80.95%) isolates (table 6). Nouraei et al(2021) believed that increased hemolysin activity among Candida spp ,Magnusiomyces capitatus, Rhodotorula mucilaginosa and Trichosporon ashii isolates in DM patients because of increasing blood glucose concentration, and also, an increased salivary glucose concentration in DM patients may contribute directly or indirectly to rising hemolysin production in Candida spp.in contrast , the statically analysis appeared there is a significant difference in biofilm formation among non Candida isolates.

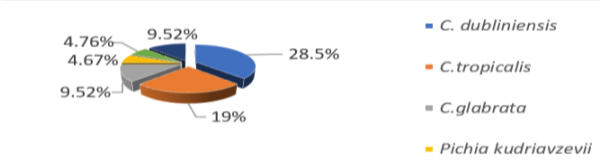

Figure (10): Biofilm formation assay of yeast isolates

4. Conclusions

The current study shows that the DM patients they infected previously with Covid-19 are highly susceptible to fungal colonization. As well as, the non vaccinated patients has more susceptible to fungal infection. The present finding reveals that the cases of oral infections more than nasal infections of DM patients. The results showed that the most important virulence factors is biofilm which assisted the adherence of pathogens and increased the pathogenicity and drug resistance.

References

Obeed, F. K., and Alrubayae, I. M. (2022). Evaluation of virulence factors of clinical yeast isolates from nosocomial fungal infections with the determination of their antifungal susceptibility profile. Iranian Journal of Ichthyology, 9, 61-68

Alrubayae, I. M., Al-laaeiby, A., Minati, M. H., and ALibraheem, S. A. (2020). Determination of genetic relationships and pathogenicity of oral candidiasis etiological agents in pediatric malignant patients in Basrah province, Iraq. Sys. Rev. Pharm, 11.

Babatzia, A., Papaioannou, W., Stavropoulou, A., Pandis, N., Kanaka-Gantenbein, C., Papagiannoulis, L., and Gizani, S. (2020). Clinical and microbial oral health status in children and adolescents with type 1 diabetes mellitus. International dental journal, 70(2), 136-144.

Devi, L. S., and Maheshwari, M. (2014). Speciation of Candida species isolated from clinical specimens by using Chrom agar and conventional methods. International Journal of Scientific and Research Publications, 4(3), 1-5.

Enfert, C., et al (2021). The impact of the Fungus-Host-Microbiota interplay upon Candida albicans infections: current knowledge and new perspectives. FEMS microbiology reviews, 45(3), fuaa060.

Hoenigl, M., Seidel, D., Sprute, R., Cunha, C., Oliverio, M., Goldman, G, et al (2022). COVID-19-

associated fungal infections. Nature microbiology, 7(8), 1127-1140.
Kałużna, M., Trzeciak, I., Ziemnicka, K., Machaczka, M., and Ruchała, M. (2019). Endocrine and metabolic disorders in patients with Gaucher disease type 1: a review. Orphanet Journal of Rare Diseases, 14(1), 1-14.
Katz, J. (2021). Prevalence of candidiasis and oral candidiasis in COVID-19 patients: A cross-sectional pilot study from the patients' registry in a large health center. Quintessence Int, 52(8), 714-718.
Kraševec, N. (2022). The Multifaceted Role of Mating Type of the Fungus and Sex of the Host in Studies of Fungal Infections in Humans. Journal of Fungi, 8(5), 461.
Matare, T., Nziramasanga, P., Gwanzura, L., and Robertson, V. (2017). Experimental germ tube induction in Candida albicans: An evaluation of the effect of sodium bicarbonate on morphogenesis and comparison with pooled human serum. BioMed research international, 2017.
Nazari, T., Sadeghi, F., Izadi, A., Sameni, S., and Mahmoudi, S. (2022). COVID-19-associated fungal infections in Iran: A systematic review. PLoS One, 17(7), e0271333.
Obeed, F. K., Al-Saad, L. A., and Alrubayae, I. M. N. (2022). The Prevalence of Hospital Acquired Fungal Infections in Some Hospitals of Basrah Province. Health Education and Health Promotion, 10(1), 1000-1012.
Peckham, H., de Gruijter, N. M., Raine, C., Radziszewska, A., Ciurtin, C., Wedderburn, L. R., et al (2020). Male sex identified by global COVID-19 meta-analysis as a risk factor for death and ITU admission. Nature communications, 11(1), 6317.
Riley, M. M. S. (2021). Invasive Fungal Infections Among Immunocompromised Patients in Critical Care Settings: Infection Prevention Risk Mitigation. Critical Care Nursing Clinics, 33(4), 395-405.
Salehi, M., Ahmadikia, K., Mahmoudi, S., Kalantari, S., Jamalimoghadamsiahkali, S., Izadi, A., et al (2020). Oropharyngeal candidiasis in hospitalised COVID-19 patients from Iran: Species identification and antifungal susceptibility pattern. Mycoses, 63(8), 771-778.
Santosh, A. B., Muddana, K., and Bakki, S. R. (2021). Fungal infections of oral cavity: diagnosis, management, and association with COVID-19. SN comprehensive clinical medicine, 3(6), 1373-1384.
Vila, T., Sultan, A. S., Montelongo-Jauregui, D., and Jabra-Rizk, M. A. (2020). Oral candidiasis: a disease of opportunity. Journal of Fungi, 6(1), 15.
Wang, Y., Duan, L., Li, M., Wang, J., Yang, J., Song, C., et al (2022). COVID-19 vaccine hesitancy and associated factors among diabetes patients: a cross-sectional survey in Changzhi, Shanxi, China. Vaccines, 10(1), 129.
WHO (2022). Diabetes key facts Available at:- https://www.who.int/news-room/fact-sheets/detail/diabetes, Accessed 3rd May 2020 Google Scholar.
Yang, Y; Lin, L; Li, Y; Jiang, Z; Li, C; Liu, M; and Lin, X. (2021). Etiology, microbiological isolates, and antibiotic susceptibilities in culture-proven pediatric endophthalmitis: a 9-year review. Graefe's Archive for Clinical and Experimental Ophthalmology, 259(1), 197-204.
Fatahinia, M., Poormohamadi, F., and Mahmoudabadi, A. Z. (2015). Comparative study of esterase and hemolytic activities in clinically important Candida species, isolated from oral cavity of diabetic and non-diabetic individuals. Jundishapur Journal of Microbiology, 8(3)