ORIGINAL ARTICLE

Comparison of Species Distribution, Antifungal Susceptibility and Virulence Factors in Pregnant and Non-pregnant Women with Vulvo-vaginal Candidiasis

Rafat Siddiqui[1]

[1]*Department of Microbiology, Mahaveer Institute of Medical Sciences and Research, Bhopal-462036 (Madhya Pradesh) India*

Abstract:

Background: Vulvovaginal candidiasis is one of the common causes of morbidity afflicting women of reproductive age and is a significant cause of illness in the pregnant population. Studying the trends in spectrum and antimicrobial susceptibility of Candida species causing vulvovaginitis represents a vital area of research. *Aim and Objectives*: To understand the differences in Candida species causing vulvovaginal candidiasis in pregnant and non-pregnant women and compare their antifungal susceptibility pattern. *Material and Methods:* Cross-sectional study on 55 women of reproductive age who tested positive for vulvovaginal candidiasis. Growth of Candida was confirmed by colony morphology, Gram's stain and speciation done by its characteristics on cornmeal agar with Tween 80, sugar assimilation and fermentation tests. The virulence factors studied were biofilm formation and production of phospholipase and proteinase. Statistical analysis was done using Statistical Package for Social Sciences (SPSS) version 23. Categorical variables were presented in frequency and percentage. Chi square test was used to measure the association between pregnant status and different variables of interest. *Result:* Of the 55 women, 37 were non-pregnant and 18 were pregnant at the time of sample collection. *Candida albicans* was the predominant species in pregnant as well as non-pregnant women. There were observable differences in distribution of *Candida albicans* between the two groups which were not statistically significant. Voriconazole sensitivity was found twice in non-pregnant as compared to pregnant women by disc-diffusion as well as Minimum Inhibitory Concentration (MIC) determination by Vitek 2. Candida affecting pregnant women was twice as likely to produce biofilm as compared to non-pregnant women (OR= 2.115,95% CI=0.508-8.805). *Conclusion*: There are differences in Candida behaviour between pregnant and non-pregnant women that may affect treatment options. Further research with a larger sample size is recommended.

Keywords: *Candida albicans*, Non-albicans, Anti-Fungal, Susceptibility, Virulence

Introduction:

Vulvovaginal Candidiasis (VVC) is one of the common causes of morbidity afflicting women of reproductive age and is a significant cause of illness in the pregnant population [1]. Among the Candida species causing disease, *C. albicans, C. glabrata, C. parapsilosis, and C. tropicalis* represent 80 to 90% of infections experienced around the world [2]. Clinically, VVC is characterised by a lack of specific signs and symptoms. Without the supporting evidence of laboratory tests, its diagnosis is often impeded and is generally based only on history and physical examination [3]. VVC may complicate an otherwise normal pregnancy by increasing the risk of candidemia in preterm children. Hence early identification and suitable treatment are imperative for the health of the mother and the baby. On the other hand other causes of vaginitis may often be

misdiagnosed as VVC [3-4]. Such misdiagnosis often results in treatment attempts with antifungal agents. Such indiscriminate use coupled with availability of over-the-counter antifungals that are often used by women with recurrent vaginitis will undoubtedly increase the antifungal resistance of Candida species. Thus, the increased uses of antifungal drugs warrant for monitoring of the species distribution and the frequency of resistant Candida isolates [5-6].

Studying the trends in spectrum and antimicrobial susceptibility of Candida species causing vulvo-vaginitis hence represent a vital area of research that is largely ignored. Antifungal susceptibility testing plays a central role in the development of regional antibiogram to aid empiric selection of antifungals. This study was conducted in a tertiary care centre in India, with the aim to understand the differences in Candida species causing VVC in pregnant and non-pregnant women and compare their antifungal susceptibility pattern.

Material and Methods:

This study included 55 women who tested positive for VVC between January 01, 2016 and June 30, 2017, presenting at Obstetrics and Gynecology (Ob/Gy) Department of a tertiary care hospital of central India. Out of these, 37 women were non-pregnant and 18 were pregnant at the time of sample collection. The study was approved by institutional ethics committee. A detailed clinical examination was done and documented. Two vaginal swabs were subjected to Potassium Hydroxide (KOH) mount microscopy and Gram's stain for presence of fungal elements including budding yeast and pseudohyphae. Subsequently, third swab was inoculated on Sabouraud's Dextrose Agar (SDA) for fungal isolation. Growth of Candida was confirmed by colony morphology, Gram's stain and speciation done by its characteristics on cornmeal agar with Tween 80, sugar assimilation and fermentation tests. All tests for identification and speciation were followed according to standards [7]. The virulence factors studied were biofilm formation, and production of phospholipase, and proteinase.

Detection of Virulence Factors:

Phospholipase Detection:

All isolates were screened for their extracellular phospholipase activity by the following procedure: Egg yolk medium consisting of 13 g SDA, 11.7g NaCl, 0.111 g $CaCl_2$, and 10% sterile egg yolk was used. The egg yolk was centrifuged at 5000g for 30 min at room temperature, and 20 ml of the supernatant was added to the sterilized medium. Extracellular phospholipase activity was detected by inoculating 10µl aliquots of the yeast suspension (approximately 108 yeast cells /ml) into the wells punched onto the surface of the egg yolk medium. The diameter of the precipitation zone around the well was measured after incubation at 37°C for 48 h. Phospholipase index (Pz) was defined as the ratio of the diameter of the growth to the total diameter of the growth plus the precipitation zone. Pz $\geqslant$ 1 indicated no phospholipase activity. Pz<1 indicated positive phospholipase activity. *C. albicans* ATCC 10231 was used as positive control [7].

Proteinase Detection:

Candida proteinase was detected by modified Staib method. Proteinase activity was measured in terms of Bovine Serum Albumin (BSA) degradation. Bovine serum albumin medium (dextrose 2%, KH_2PO_4 0.1%, $MgSO_4$ 0.05%, agar 2% was mixed after

cooling to 50°C with 1% bovine serum albumin solution) was used. Proteinase activity was detected by inoculating 10µl aliquots of the yeast suspension (approximately 108 yeast cells/ml) into the wells punched onto the surface of the medium. The plates were incubated at 37°C for 2 days. After incubation, the plates were fixed with 20% trichloroacetic acid and stained with 1.25% amidoblack. Decolourisation was performed with 15% acetic acid. Opaqueness of the agar, corresponding to a zone of proteolysis around the wells that were not stained with amidoblack indicated degradation of the protein. The diameter of unstained zones around the well was considered as a measure of proteinase production. Pz was measured in terms of the ratio of the diameter of the growth of the unstained zone. Pz $\geqslant$ 1. No proteinase activity detected in the strain while Pz<1. Positive for proteinase production. C. albicans ATCC 10231 was used as positive control [7].

***In-vitro* Biofilm Formation/Slime Production:**

Biofilm production was determined by visual methods. Colonies from the surface of SDA plate were inoculated into a polystyrene tube (Falcon conical tube with screw cap) containing 10 ml of Sabouraud-Dextrose Broth (SDB) supplemented with glucose (final concentration 8%). After incubation at 35°C for 48 h, the broth in the tubes was gently aspirated. The tubes were washed with distilled water twice and then stained with 2% safranin for 10 min. They were then examined for the presence of an adherent layer. Biofilm production was scored as negative (.), weak (+), moderate (++) or strong (+++). The biofilm producer *Staphylococcus epidermidis* ATCC 35984 was used as a positive control [8].

Antifungal Susceptibility Testing:

Minimum Inhibitory Concentration (MIC) determination by VITEK 2:

MIC is the lowest drug concentration that prevents visible microorganism growth after overnight incubation. AST-YS01 card in Vitek 2 system was used for susceptibility testing by automation. Discs of antifungal Amphotericin B (AmB) 20µg (Himedia), Fluconazole (FLC) 25µg (Himedia) and Voriconazole (VRC) 01µg (Himedia) were used for susceptibility testing by disc diffusion method. MIC of AmB, FLC and VRC was determined as described in the company's manual [9]. Statistical analysis was done using SPSS version 23. Categorical variables were presented in frequency and percentage. Chi-square test was used to measure the association between pregnant status and different variables of interest.

Results:

This study included 18 pregnant and 37 non-pregnant women with confirmed vulvo-vaginal candidiasis. Table 1 presents the information regarding the species distribution of Candida among the study group according to their pregnancy status. A total of 18 pregnant and 37 non-pregnant women with VVC were compared for species distribution of Candida. It is noted that *Candida albicans* is the predominant species in pregnant as well as the non-pregnant women. However, the differences in distribution of *C. albicans* between the two groups are not significant. The non-albicans species isolated were *C. glabrata, C. tropicalis, C. dubliniensis, C. lusitaniae, C. parapsilosis, C. krusei* and *C. guillermondi*. Among these, *C. tropicalis* was the most common in pregnant and *C. glabrata* in the

non-pregnant females. Within the non-albicans group, there were no statistical differences in the distribution of various species when compared by pregnancy status.

The two groups of women were compared for antifungal susceptibility of infecting species of Candida and the results are presented in Table 2. Three drugs, namely Amphotericin B, Fluconazole and Voriconazole were compared on two methods, Disc-diffusion and MIC determination by Vitek 2. In disc-diffusion method, Voriconazole sensitivity was found twice in non-pregnant as compared to pregnant women by disc-diffusion method; however, this difference was not statistically significant. Also, in MIC determination by Vitek2,Voriconazole sensitivity was twice in non-pregnant as compared to pregnant women; however this difference was not statistically significant.

Table 3 presents the data of virulence factors produced by Candida. Biofilm production, proteinase and phospholipase synthesis were compared between pregnant and non-pregnant women and the result suggests that Candida affecting pregnant women is twice as likely to produce biofilm as compared to non-pregnant women (OR= 2.115,95 % CI=0.508-8.805). While on comparing proteinase by pregnancy status, an odds ratio of less than 1 was obtained, indicating that pregnancy had a protective effect on proteinase synthesis. Similar result was obtained for phospholipase. None of the differences were statistically significant.

Table 1: Comparison of Candida Species by Pregnancy Status (n=55)

Candida Species	Non-Pregnant	Pregnant	Total	χ^2	OR	95%CI	*p*
C. albicans	12(32.43)	7(38.88)	19(100)	0.223	0.754	0.23-2.43	0.428
C. non albicans	25(67.57)	11(61.12)	36(100)	2.019	-	-	0.959
C. glabrata	9	3	12				
C. tropicalis	8	5	13				
C. dubliniensis	3	1	4				
C. lusitaniae	2	1	3				
C. parapsilosis	1	1	2				
C. krusei	1	0	1				
C. guillermondi	1	0	1				

Table 2: Comparison of Drug Sensitivity of Candida Species by Pregnancy Status

Sensitivity test	Drug	Test result	Pregnant		Chi value	OR (95%CI)	*p*
			No	Yes			
Disc Diffusion	Amphotericin B	Resistant	2(66.7)	1(33.3)	0.405	–	0.817
		Intermediate	4(80)	1(20)			
		Sensitive	31(66)	16(34)			
	Fluconazole	Resistant	6(75)	2(25)	0.255	–	0.880
		Intermediate	2(66.7)	1(33.3)			
		Sensitive	29(65.9)	15(34.1)			
	Voriconazole	Resistant	4(80)	1(20)	0.405	2.02 0.21-19.90	0.467
		Sensitive	33(66)	17(34)			
MIC By (VITEK 2)	Amphotericin B	Resistant	2(66.7)	1(33.3)	0.001	–	0.999
		Intermediate	2(66.7)	1(33.3)			
		Sensitive	33(67.3)	16(33.7)			
	Fluconazole	Resistant	7(77.8)	2(22.2)	0.730	–	0.694
		Intermediate	3(75)	1(25)			
		Sensitive	27(64.3)	15(35.7)			
	Voriconazole	Resistant	5(83.3)	1(16.7)	0.789	2.65 0.28-24.60	0.351
		Sensitive	32(65.3)	17(34.7)			

OR=Odds Ratio

Table 3: Comparison of Virulence Factors by Pregnancy Status

Virulence factor	Reaction	Pregnant	Non-pregnant	OR	CI	Chi square	*p*
Biofilm	Negative	3(16.7)	11(29.7)	2.11	0.508-8.805	1.08	0.24
	Positive	15(83.3)	26(70.3)				
Proteinase	Negative	6(33.3)	10(27)	0.74	0.219-2.507	0.23	0.42
	Positive	12(66.7)	27(73)				
Phospholipase	Negative	5(16.4)	4(10.8)	0.31	0.073-1.361	2.54	0.11
	Positive	13(83.6)	33(89.2)				

Discussion:

Pregnancy is a well-known risk factor for Candida and many studies have reported frequent colonization of the vagina of pregnant women with *Candida spp.* compared to non-pregnant women [10]. In the current study, pregnant and non-pregnant women with VVC were compared for species distribution of Candida, the antifungal susceptibility differences and virulence factor differences.

Regarding the distribution of Candida species by pregnancy status, it was noted that *Candida albicans* is the predominant species in pregnant as well as the non-pregnant women. Within the non albicans group, *C. tropicalis* was the most common in pregnant and *C. glabrata* in the non-pregnant females. While these differences were not statistically significant, they are in concordance with most of the studies that indicate a higher proportion of *Candida albicans* infection in pregnant females [3, 11]. Similar findings were reported in a study from Bosnia where the most commonly detected species for both groups was *C. albicans.* The most commonly detected non-albicans species for the test group (pregnant) were *C. glabrata and C. krusei* and for the control group (non-pregnant) were *C. glabrata* and *C. parapsilosis* [12]. Another study from Ghana reported the predominant species as *Candida albicans* followed by *Candida krusei Candida glabrata* and *Candida tropicalis* [13]. The differences in infection rate between pregnant and non-pregnant is explained by the physiological changes in pregnancy like the high concentration of estrogen during pregnancy which provides a favourable environment for the growth of *Candida spp*. [14]. Further research is needed to elaborate the differences in species distribution which has to be guided by the changes in epidemiological pattern of Candida. This is particularly important, given that the recent trend indicates a shift towards rising non-albicans infections [15].

Poorly treated or untreated VVC in pregnancy can lead to many complications. It can cause chorio-amnionitis with subsequent abortion, prematurity and congenital infection of the neonate [16]. Studies have also reported the association of *C.*

albicans with gestational diabetes and *C. krusei* or *C. glabrata* with other gestational complications [3]. Thus, it is imperative to treat antenatal VVC in time with the correct antifungal medications. The antifungal susceptibility and resistance patterns of our study sample has been reported in a previous paper where we reported high antifungal sensitivity towards Amphotericin B, Voriconazole and Ketoconazole [17].

In this paper we compared the three drugs, Amphotericin B, Fluconazole and Voriconazole based on the pregnancy status of the cases. There were observable differences using different methods. The most sensitivity is towards Voriconazole in the non-pregnant women as compared to pregnant women. By disc-diffusion method, Voriconazole sensitivity found was twice in non-pregnant as compared to pregnant women; however this difference was not statistically significant. Also, in MIC determination by Vitek 2, Voriconazole sensitivity was twice in non-pregnant as compared to pregnant women; however this difference was not statistically significant. This is an interesting finding and fuels the question whether pregnancy affects the response to antifungal medications and needs further exploration. In a recent report, all *C. albicans* and *C. famata* isolates were susceptible to fluconazole while *C.glabrata* isolates were dose dependent susceptibility [18]. The emergence of fluconazole resistance has been reported among *C. albicans, C. tropicalis and C. parapsilosis* isolates from candidemia patients [19]. In a Ghanaian study, except *Candida krusei* all isolates were 100% susceptible to Amphotericin-B while resistance rate was high against Itraconazole and Ketoconazole [13]. Recent literature highlights drug resistance in non albicans strains than in any other [20-21]. Thus, rapid identification of these strains provides valuable information concerning treatment regimens in clinical settings [22-23]. In the current study, species-wise differences in antifungal susceptibility in relation to the pregnancy status was not analysed due to small sample and it is a limitation of this study. Safety considerations in pregnancy and worries about antifungal resistance warrant further research on other treatment modalities for Candida [24].

Differences in virulence factors were studied between the two groups and also revealed interesting findings. Three virulence factors, namely, biofilm production, proteinase and phospholipase synthesis was compared between pregnant and non-pregnant women. The result suggests that Candida affecting pregnant women is twice as likely to strongly impact biofilm production. Proteinase test on the other hand indicates that pregnancy had a protective effect on its synthesis. Similar result was obtained for phospholipase. Although, none of the differences were statistically significant, however differences suggest a need to further study these mechanisms as they have an important role in the diagnosis and management of Candida. Previous studies have reported similar finding that Candida isolates derived from pregnant patients with VVC produced significantly higher amounts of phospholipase and proteinase compared with the controls [25]. The determination of virulence factors may lead to the effective and prompt treatment of VVC, particularly in pregnant women. Although it is established that different strains of *C. albicans* and other Candida species differ in their capacities to form biofilms and virulence factors [26-27], the study of these differences by pregnancy status need further focus.

Conclusion:

This study raises some important questions regarding the differences in species distribution, antifungal susceptibility, drug resistance and virulence patterns of Candida in pregnant and non-pregnant women diagnosed with VVC. The variances point towards probable underlying mechanisms that need to be investigated, all the more considering the alarming changes in the species distribution and antifungal resistance patterns observed of late.

Strengths and Limitations:

This study is first to explore the differences in pregnant and non-pregnant women diagnosed with VVC with regard to species distribution, antifungal susceptibility, drug resistance and virulence patterns. The study was limited by a small sample size. It is recommended to conduct in-depth studies to examine the differences in maternal and foetal outcomes related with Candida infection in pregnancy.

Acknowledgement:

This work would not be possible without the support of Professor Dr. D.K. Mendiratta and Dr. Atul Rukadikar, Department of Microbiology and Dr. Sandhya Gadre, Department of Obstetrics and Gynaecology, Chirayu Medical College and Hospital, Bhopal.

References

1. Willems HME, Ahmed SS, Liu J, Xu Z, Peters BM. Vulvovaginal candidiasis: a current understanding and burning questions. *J Fungi (Basel)* 2020;6(1):27.
2. Turner SA, Butler G. The Candida pathogenic species complex. *Cold Spring Harb Perspect Med* 2014; 4(9):a019778.
3. Sustr V, Foessleitner P, Kiss H, Farr A. Vulvovaginal candidosis: current concepts, challenges and perspectives. *J Fungi (Basel)* 2020;6(4):267.
4. Zeng X, Zhang Y, Zhang T, Xue Y, Xu H, An R. Risk factors of vulvovaginal candidiasis among women of reproductive age in Xi'an: A cross-sectional study. *Biomed Res Int* 2018; 2018:9703754.
5. Marchaim D, Lemanek L, Bheemreddy S, Kaye KS, Sobel JD. Fluconazole-resistant Candida albicans vulvovaginitis. *Obstet Gynecol* 2012; 120(6):1407-1414.
6. Ghaddar N, Anastasiadis E, Halimeh R, Ghaddar A, Dhar R, Al Fouzan W, *et al*. Prevalence and antifungal susceptibility of Candida albicans causing vaginal discharge among pregnant women in Lebanon. *BMC Infect Dis* 2020;20(1):32.
7. Chander J. Candidiasis., Textbook of Medical Mycology. 2011; 3rd edition 275:507-530.
8. Yigit M, Aktas E, Dagistan S, Ayyildiz A. Investigating biofilm production, coagulase and hemolytic activity in candida species isolated from denture stomatitis patients. *Eurasian J Med* 2011; 43(1): 27-32.
9. Pfaller MA, Diekema DJ, Procop GW, Rinaldi MG. Multicenter comparison of the VITEK 2 antifungal susceptibility test with the CLSI broth microdilution reference method for testing amphotericin B, flucytosine, and voriconazole against Candida spp. *J Clin Microbiol* 2007; 45(11): 3522-3528.
10. Leli C, Mencacci A, Meucci M, Bietolini C, Vitali M, Farinelli S, *et al*. Association of pregnancy and Candida vaginal colonization in women with or without symptoms of vulvovaginitis. *Minerva Ginecol* 2013; 65(3):303-309.
11. Waikhom SD, Afeke I, Kwawu GS, Mbroh HK, Osei GY, Louis B, *et al*. Prevalence of vulvovaginal candidiasis among pregnant women in the Ho municipality, Ghana: species identification and antifungal susceptibility of Candida isolates. *BMC Pregnancy Childbirth* 2020; 20(1): 266.
12. Babić M, Hukić M. Candida albicans and non-albicans species as etiological agent of vaginitis in pregnant and non-pregnant women. *Bosn J Basic Med Sci.* 2010;10(1):89-97.
13. Tsega A, Mekonnen F. Prevalence, risk factors and antifungal susceptibility pattern of Candida species among pregnant women at Debre Markos Referral Hospital, Northwest Ethiopia. *BMC Pregnancy Childbirth* 2019;19(1):527.

14. Konadu DG, Owusu-Ofori A, Yidana Z, Boadu F, Iddrisu LF, Adu-Gyasi D, *et al*. Prevalence of vulvovaginal candidiasis, bacterial vaginosis and trichomoniasis in pregnant women attending antenatal clinic in the middle belt of Ghana. *BMC Pregnancy Childbirth* 2019;19(1):341.
15. Taei M, Chadeganipour M, Mohammadi R. An alarming rise of non-albicans Candida species and uncommon yeasts in the clinical samples; a combination of various molecular techniques for identification of etiologic agents. *BMC Res Notes* 2019; 12(1): 779.
16. Maki Y, Fujisaki M, Sato Y, Sameshima H. Candida chorioamnionitis leads to preterm birth and adverse fetal-neonatal outcome. *Infect Dis Obstet Gynecol* 2017; 2017:9060138.
17. Siddiqi R, Mendiratta D, Rukadikar A, Gadre S. Study of virulence markers and antifungal susceptibility by vitek-2 in various candida species isolated from cases of vulvovaginal candidiasis. *Int J CurrMicrobiol Appl Sci* 2017; 6(12): 3593-3605.
18. Masri SN, Noor SM, Nor LA, Osman M, Rahman MM. Candida isolates from pregnant women and their antifungal susceptibility in a Malaysian tertiary-care hospital. *Pak J Med Sci* 2015;31(3):658-661.
19. Amran F, Aziz MN, Ibrahim HM, Atiqah NH, Parameswari S, Hafiza MR, *et al*. In vitro antifungal susceptibilities of Candida isolates from patients with invasive candidiasis in Kuala Lumpur Hospital, Malaysia. *J Med Microbiol* 2011;60(9):1312–1316.
20. Yassin MT, Mostafa AA, Al-Askar AA, Bdeer R. In vitro antifungal resistance profile of Candida strains isolated from Saudi women suffering from vulvovaginitis. *Eur J Med Res* 2020; 25(1):1.
21. Kołaczkowska A, Kołaczkowski M, Drug resistance mechanisms and their regulation in non-albicans Candida species. *J Antimicrob Chemother* 2016; 71(6):1438-1450.
22. Shi Y, Zhu Y, Fan S.Liu X, Liang Y, Shan Y. Molecular identification and antifungal susceptibility profile of yeast from vulvovaginal candidiasis. *BMC Infect Dis* 2020; 20(1): 287.
23. Manzoor S, Aziz M, Sheikh AS. Identification and characterization of Candida on CHROMAgar™ in pregnant women of Multan, Pakistan. *J Women's Health Care* 2018(7):424.
24. Kumar A, Thakur S, Thakur VC, Kumar A, Patil S, Vohra MP. Antifungal activity of some natural essential oils against Candida species isolated from blood stream infection. *J Krishna Inst Med Sci Univ* 2012; 1(1): 61-66.
25. Kalkanci A, Güzel AB, Khalil II, Aydin M, Ilkit M, Kuştimur S. Yeast vaginitis during pregnancy: susceptibility testing of 13 antifungal drugs and boric acid and the detection of four virulence factors. *Med Mycol* 2012;50(6):585-93.
26. Talapko J, Juzbaši´c M, Matijevi´c T, Pustijanac E, Beki´c S, Kotris I, *et al*. *Candida albicans*-The virulence factors and clinical manifestations of infection. *J Fungi (Basel)* 2021; (7):79.
27. Rajmane VS, Mohite ST. Comparison of the VITEK 2 yeast antifungal susceptibility testing with CLSI brothmicrodilution reference method for testing four antifungal drugs against Candida species isolated from blood samples. *J Krishna Inst Med Sci Univ* 2018; 7(1): 39-46.

Author for Correspondence:

Dr. Rafat Siddiqui, Department of Microbiology, Mahaveer Institute of Medical Sciences and Research, Bhopal-462036, Madhya Pradesh, India

Email: drrafatsiddiqui@gmail.com Cell: 8602249433

How to cite this article:

Siddiqui R. Comparison of Species Distribution, Antifungal Susceptibility and Virulence Factors in Pregnant and Non-pregnant Women with Vulvo-vaginal Candidiasis. *J Krishna Inst Med Sci Univ* 2021; 10(4): 80-88.

Submitted: *09-May-2021* ***Accepted:*** *02-Aug-2021* ***Published:*** *01-Oct-2021*