RESEARCH ARTICLE

The Study of Prevalence of Candida Species in Oral Cavity between Habitual and Non-Habitual of Gutka Chewers in Vitro Condition

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Candida is the most common yeast existing as normal flora of oral cavity. Sometime _Candida yeast_ can cause diseases in the oral cavity. Immune system is weakened due to widespread use of antibiotic, immunosuppressive drugs, age, genetic, malnutrition and hormone disorder as well as HIV infection. Immunocompromised person are more sensitive Aim of the study was to evaluate the prevalence of _Candid yeast_ in those persons that are habitual of chewing gutka and non-habitual. For this study, 450 oral rinse samples were collected aseptically in sterile test tube. These persons visited in dental OPD, Kalka dental college, Meerut. Groups were divided on the bases of habitual and non-habitual of gutka chewer and further clinical history of patients was recorded such as age, gender and socioeconomic status. Each sample were diluted and inoculated on Sabouraud's Dextrose Agar medium by spread culture technique. The samples were further processed for colony characterization and biochemical test to identify _Candida species_. As well as measurement of pH level of the oral rinse sample were recorded with pH strip method. 414 isolates were collected from 450 samples, eight _Candida_ species were isolated. _Candida albicans_ were observed as dominant yeast, showed creamy colored, smooth colonies on Sabouraud's dextrose agar medium. And observed germ tube test, psuedohyphae chlamydospore and various sugars fermentation as well as some other _Candida_ yeasts were also isolated.

Introduction

Candida albicans are the most common opportunistic pathogen and *Candida* yeast has drawn attention of the researcher due to the increased incidence of severe oral *Candidiasis*. The study reveals that more than 90% person visited in dental OPD were affected by *Candida*. Usually this is considered as normal oral flora but it may act as pathogen in case of weak immunity and this incidence has increased with wide spread use of corticosteroids, antibiotics and immunosuppressive drugs, these type of fungi called opportunistic pathogenic yeast. (Samarnayake L, P., 1990) *Candida albicans* is the dominant species to cause disease in oral cavity. Although some other *Candida* species are also involved such as *C.glabrata, Candida dulbigenesis, Candida tropicalis, Candida parapsilosis, Candida krusii, Candida dubliniensis, Candida famata* and *Candida guillerrmondii*. Among these, *Candida dubliniensis* is a new, recently seen species which was initially described from severe oral *Candidiasis* of subjects infected with human immunodeficiency virus (HIV) and it was therefore suspected to increase virulence (Hannula, 2000).

Candida causes endogenous infection known as *Candidiasis or oral thrush or lesions*. These lesions are soft white and slightly elevated plaque frequently occurring on the buccal mucosa of tongue but may also be seen on another part of oral cavity such as on the gingival palate and floor of the mouth. In acute condition the entire oral cavity may be affected. Lesions or thrush is very common in weak immunity due to *HIV* infection and the patients being treated by chemotherapy or radiotherapy and it may also be seen in children due to improper developed immune system. Chronic oral *Candidiasis* may be denture induced stomatitis or chronic hypertrophic condition. Denture induced is due to tight fitted denture. This may prevent saliva having antimicrobial activities. In chronic hypertrophic Candidiasis, plaques remain tightly sticked usually on tongues, cheeks and lips. (Arendroff., *et. al.*, 1979).

Candida dubliniensis, which is morphologically similar to *Candida albicans* (show germ tubes formation and chlamydospore among other similar traits), it has now been identified (Sullivan, *et. al.*, 1995) and is among the list of pathogens. Several medical microbiologists have

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suggested (Abi-Said *et. al.*, 1997; Costa *et. al.*, 2000;) that the dominant pathogen among species of *Candida* of human is *Candida albicans*. The study of research was found that *Candida albicans* more dominate and observed much higher in processed samples during research as comparatively other species of Candida. Next among the *Candida* pathogens is *C.tropicalis* (Knoke *et. al.*, 1997; Nucci *et. al.*, 1998. Coasta *et. al.*, 2000), *C.parapsilosi* (Nucci *et. al.*, 1998; Kao *et. al.*, 1999, Costa *et. al.*, 2000*), C globrata* These three species alternate as the second most common pathogen, it depends on sources of infection. Since long time several research paper and review has been written about morphology and physiology of *Candida* species. The colony characterization of *Candida* are creamy white coloured to yellowish, smooth, pasty colonies appear and rapid growth matured in 48h. Most species form psuedohyphae but chlamydospore are formed by some *Candida* species.

The clinical entity of oral thrush has been known for several centuries and described by Hippocrates in 400 B.C. as oral ulcer (Odds. 1998). Although these all organisms typically colonize on mucocutanous surface and the later entered into the tissue when immunsystem is weakened (Rogers and Balish., 1980).

Odds (1998) studied a large number of papers on oral carriage from the literature. In the children, it was on peak between 1 week and 18 months. C.albicans I the dominant species followed by C.tropicalis, C.glabrata, C. parapsilosis and C.krusei. Other Candida specie existing in oral cavity interacts with oral environment and diseased caused by enzyme that released from growing point of the cells of *Candida* where enzymes are synthesized. The phospholipases are concentrated at the tips of fungal hyphae and localized in vicinity of host cellular compartments where the invasion is occurring. (Stenderup, P.A., 1990). Although some factors that influence carriage include salivary factor, temporal variation chewing tobacco, immune status.

Materials and methods

For this study, total four hundred fifty, clinical samples were collected from patients, who visited in dental OPD at Kalka dental college, Meerut Uttar Pradesh during the period of July 2016 to December2017. The patients were divided in two groups habitual and nonhabitual of gutka chewer. Clinical oral rinse samples were collected from oral cavity. The samples were collected in sterile test tube aseptically. Oral rinse technique was followed for the collection of samples. For this, the patients were asked to 10 mL of sterile phosphate buffer saline water (PBS, pH 7.2) to rinse properly oral cavity for one minute. Oral rinse samples were collected in sterile test tube and immediately transported to the laboratory. The samples were centrifuged (Sigma 2k 15) at 3000 rpm for 10 minutes. The supernatant of samples was discarded and sediments were resuspended to prepare uniform suspension in 1 mL of sterile phosphate buffer saline (pH 7.2) by vortex for 1 minute and 100 µL of suspension was inoculated onto Sabouraud's dextrose agar medium (Hi-Media, Mumbai) containing antibiotics (chlramphenicol 20 µL/mL, streptomycin 25 µg/mL) aseptically. It provides selective nature for fungi and was spread evenly with sterile L-spreader. The inoculated SDA plates were incubated at 37° C for 48 h. After incubation, the colonies of *Candid*a yeast grown on SDA plates were counted to determine CFU/mL of rinse sample under digital colony counter and these values (total colonies) were multiplied by dilution factor to yield the CFU/mL of original oral rinse sample (Samarnayake *et. al.*, 1986; Ripon, 1988). Some other factors were also considered such as measurement of pH of saliva and salivary flow rate were measured. Saliva was collected using spitting method described by Satish, (2006). For this, patients were asked to swallow any saliva in the mouth and asked to sit normally for 5 min. After that asked to patients to expectorate the saliva in sterile container during this collection period, the patients were requested not to swallow the saliva. And second method was mechanical stimulation method, for this paraffin wax or rubber was provided to the patients and ask for chewing it for one min and the expectorated saliva were collected in sterile container. The pH measurement of saliva was carried out using pH strip from Hi- Media (Mumbai). The saliva was added on pH strip and colour change compared with chart provided by manufacturer and pH was recorded of the saliva samples.

Whole salivary flow retain normal condition is 0.2-0.4 mL/min. and it is 2-5 mL/min. on stimulation while unstimulated whole saliva flow rate of <1.0 mL Without stimulation is suggested abnormally low and indicate salivary hypofunction. (Satish, 2006)

Further isolated yeast colonies (pure culture) cultured on Sabouraud's Dextrose Agar media were processed to identify species of *Candida*. The methods were followed such as Gram's staining, it was performed for identification of yeast morphologically. For this, thin smear of the growth was prepared on properly clean glass. The smear was heat fixed by passing slide 3-4 times through flame of sprit lamp. Crystal violet was added on smear for one minute. The slide's smear was washed properly using wash bottle with distilled water. Excess water was removed from the slide. Gram's iodine was added on smear for 60 seconds. The slide was washed again using distilled water. The smear was decolorized with decolorizing solution (75% ethyl alcohol + 25% acetone) drop by drop for 20-30 seconds. The smear was washed with distilled water properly. Excess water was removed. The smear was counter stained with safranin (red in colour) for 30 seconds. The smear was washed with double distilled water. The slide was air dried. One drop of oil immersion was added on smear and then smear was examined under oil immersion lens (100X) of the microscope for confirmation of the culture as Gram's positive *Candida yeasts* morphologically.

Germ tube formation test is a screenings test described by Tashdjian *et. al.,* (1960). The method of germ tube formation test with some modification was followed in this study. For this, just touched the colony of yeast with help of sterile inoculation loop and then the small mass of the test organism was inoculated into sterile test tube containing 0.5 mL of human serum + 0.5% glucose. The inoculated serum was then incubated at 37°C for 2-3 hours. After 2.5 hours, at every 10 min. intervals a drop of inoculated yeast-serum suspension was placed on clean glass slid (thickness 1± 0.1 mm), kept a cover slip on serum suspension. The incubated serum was examined under the microscope using 10X, 40X, and 100X objectives. Low power objective was used just to locate objectives and high-power objectives for confirmation germ tube formation. The appearance of short hyphal (filaments like structure) arising from the cell surface confirmed the formation of germ tube. The germ tube formation test is useful in rapid identification for *Candida albicans*. And is the main characteristics feature of 95% of the clinical isolate of C. albicans.

Dalmau plate culture technique was followed for study psuedhyphae and chlamydospore suggested by Khan *et. al.,* (2005). Isolated Candida were cultured on corn meal agar medium (CMA), Hi-Media, Mumbai for chlamydospore and Tobacco agar medium for psuedohyphae and chlamydospore formation were studied.

Chlamydospore and psuedhyphae are also main characteristics helped for identification of *Candida* species. For this Dalmau plate culture technique was followed. As suggested by Khan *et. al.,* (2005). Corn Meal Agar (CMA), Hi-media Laboratory, Mumbai was used. 17-gram corn meal agar medium was dissolved in 1000 mL of double distilled water. Heated the medium to dissolve with frequent agitation and boiled for one minute. Then 10 mL of tween 80 was added. The culture medium was sterilized in autoclave at 15 Pa for 15 min after that medium was allowed to cool down approximately to 45-50°C and poured into polystyrene disposable sterile Petridis (90 mm diameter) Although corn meal have sufficient nutrition for growth of *Candida* but 1% tween 80 (polysorbate) made it more reliable for formation of chlamydospore.

Tobacco tween 80 agar medium was preferred to differentiate *C.albicans and C.dubliniensis.* The method to prepare tobacco agar medium was the same as described by Tendolkar (2003); khan *et. al.,* (2004); Girish and Menon (2005). Tobacco was commercially available in the local market in different brand (Newla and Lummi), used in preparation of media. 10 gram tobacco was added in 1000 mL distilled water. This mixture was boiled for 10 min and then boiled mixture was filtered with several layers of muslin cloth. 15 g agar was added and boiled until liquefied as well as 10 mL Tween 80 was added and autoclaved at 15 Pa for 15 minutes. Then it was allowed to cool down to 45-50°C. The medium was gently mixed and poured into sterile Petriplate (90 mm diameter). A small amount of test organism was streaked on tobacco agar medium after that. Inoculated streak area was covered with sterile coverslip (thickness 1± o,1 mm). The inoculated plate were incubated at 28°C for 72 h and examined under microscope (Olympus) using low objectives lance for location of object and high objective lance for confirmation of colony characteristics, such as surface for colony morphology (smooth or rough), formation of hyphal fringes at the periphery of colonies and psuedohyphae formation. Formation of chlamydospore and their position on psuedohyphae were also studied.

Sugars fermentation test were carried out in YPD broth with 0.5% concentration of different sugars such as glucose, sucrose maltose, lactose, galactose and trehalose along with pH, indicator, bromothymol blue detection for acid production and durhum tube was kept in inverted position in test tube individually for detection gas formation during growth of Candida in culture medium. Test culture (2.5 x 10^3 CFU/mL) were inoculated in each test tube and incubated at 37°C for 24 hours yellow colour indicated acid production while red colour no sugar fermentation and accumulation of gas in durhum tube indicated gas production. And urea hydrolysis test was done for identification of Candida krusei.

Observation

During the study, physical examinations of patients were carried out, showed normally white tongue, oral thrush or lesions, depapllation. Colony characterizations were observed as white to cream colour, smooth, glabrous. Gram's staining showed oval shape and purple colour called as Gram positive yeasts. On corn meal agar fringed colony with chlamydospore. And on tobacco agar medium showed chlamydospore and their position psuedohyphae was also studied. *C.albicans* and C.dublniensis produced chlamydospore and psuedohyphae showed by C.*albicans C.tropicalis C.parapsilosis C.krusei pseudo C.dublniensis* and *C.guillermondii*. Growth of *Candida albicans, Ctropicalis, C.dublniensis and C.guillermondii* were observed on cyclohexamide containing medium. Germ tube formation test showed as outgrowth from the cell as short hyphal or filamentous structure. In sugar fermentation test observed the acid, alkali or gas formation resulted in visible changes in the experiments showed yellow colour for acid production and accumulation of gas in inverted durhum tube.

Fig. 1. 48 h old culture of Candida on Sabouraud's dextrose agar plate.

Result

In this study total 450 oral rinse sample were collected aseptically during the period of July 2016 to December 2018. In this present study eight *Candida species* were isolated in 414 isolates from 450 oral rinse samples. *Candida albicans* revealed creamy white coloured, smooth, glabrous colonies on Sabouraud's dextrose agar medium. (**Fig. 1**) Gram's staining showed oval shaped Gram's positive yeasts (**Fig. 2**). It produced germ tube as a short hyphal (filamentous) outgrowth extending from yeasts cells in human blood serum after 2 h of incubation observed under low power just to locate the objective and high-power objectives to confirm the presence of formation of germ tube. It is a very helpful to identify *Candida species such as Candida albicans*. (**Fig. 3**). On corn meal agar and tobacco agar medium showed Psuedohyphae and chlamydospore sugar fermentation test were observed positive for glucose and maltose and variable for trehalose and galactose. *Candida globrata* showed cream coloured smooth on Sabouraud's dextrose agar medium. Psuedohyphae was not observed which help in diagnosis. Sugar fermentation was observed positive for glucose and trehaose. *Candida tropicalis* showed cream coloured, smooth, raised colonies on Sabouraud's dextrose agar medium while on tobacco agar medium, showed abundant long wavy, multibranched psuedohyphae, chlamydospore was not produced which help in differentiation from C.albicans .And sugar fermentation was seen positive for glucose, maltose and sucrose .Although galactose and trehalose shoed variable result. This sugar fermentation test was helpful to differentiate C.tropicalis other than C.albicans. *Candida dubliniensis* also showed white to cream growth on Sabouraud's dextrose agar medium. On tobacco agar medium It produces psuedohyphae and chlamydospore at 28°C. Candida dubliniensis produce chlamydospore in pair and in cluster. (Khan *et. al.*, (2004) has used tobacco agar medium to distinguish C.dublniensis and C.albicans on the bases of pair of chlamydospore. It was the additional differentiating feature in C.dublniensis. *Candida parapsilosis* produced cream coloured, smooth growth on Sabouraud's dextrose agar medium. Highly branched hyphae were observed but No chlamydospore was formed, Sugar fermentation test showed positive for glucose only and variable result for galactose. *Candida krusei* also showed growth cream coloured, flat, dry growth on Sabouraud's dextrose agar medium. On corn meal agar, long wavy branched psuedohyphae. No chlamydospore was formed C.krusei showed urease positive. Sugar fermentation was positive only for glucose (**Table 1**). *Candida famata* produced white to cream coloure smooth growth on Sabouraud's dextrose agar medium.On tobacco medium, produced spherical, budding yeast. And sugar fermentation test showed variable for glucose, maltose sucrose and trehalose. (**Table 1**). *Candida guillerrmondii* showed creamy color, smooth and glabrous growth on Sabouraud's dextrose agar medium. And corn meal agar medium produced branched psuedohyphae. Sugar fermentation test showed for glucose and sucrose, but it was variable in result for galactose.

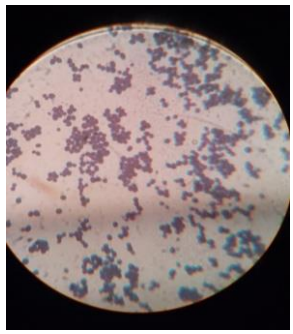

Fig. 2. Gram's-stained slide of Candida yeast.

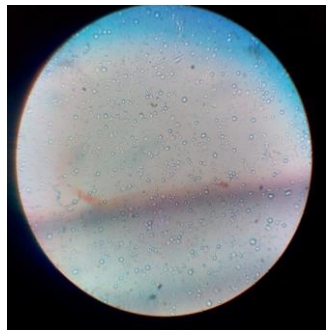

Fig. 3. Candida showing germ tube in 2h old serum.

After correlation of cultural characteristics, microscopic and biochemical study, eight *Candid*a species were obtained in 414 *Candida* isolates from 450 oral

Table 1. Identification of *Candida spp.*, Cultural and biochemical characteristics of 414 isolates from 450 clinical samples.

Isolated test yeast	Number of isolates	Colony morphology	Gram's Stain		Physiological test			Dalmau Culture		Urea hydrolysis	Sugar fermentation test					
					Growth at 37 °C	Growth On cyclohexa. Containing medium	Germ tube test	Chlamydospore	Psuedohyphae		Glucose	Galactose	Lactose	Maltose	Sucrose	Trehalose
C.alb.	281	C.S.	+		+	+	+	+	+	-	+	±	-	+	-	±
C.glab.	56	C.S.	+		+	-	-	-	-	-	+	-	-	-	-	+
C.trop.	29	C.S.	+		+	+	-	R	+	-	+	±	-	+	+	±
C.dubl.	14	C.S.	+		+	+	+	+	+	-	ND	ND	ND	ND	ND	ND
C.para.	11	C.S.	+		+	-	-	-	+	-	+	±	-	-	-	-
C.kru.	10	C.S.	+		+	-	-	-	+	-	+	-	-	-	-	-
C.fam.	8	C.S.	+		+	±	-	-	+	-	±	-	-	±	±	±
C.guil.	5	C.S.	+		+	+	-	-	+	-	+	±	-	-	+	-

C. = Candida, alb. = albicans, tro. = tropicalis, dub. = dubliniensis, para. = parapsilosis, kru. = krusei, fam. = famata, guill. = guillerrmondii, r = rare strain, nd = not detected, Gr. = Growth, (+) = positive, (-) = negative, C. S. = creamy smooth,

rinse samples. *Candida albicans* were 281 (67.87%), *C.glabrata* 56 (13.53%), *Candida tropicalis* 29 (7.00%), *Candida dubliniensis* 14(3.38%), *Candida parapsilosis* 11 (2.66%), Can*dida krusei* 10 (2.42%), *Candida famata* 8 (1.93%), *Candida guillerrmondii* 5 (1.21%) (**Table 2**) and (**Fig. 4**). The finding of the present study confirms predominance of *Candida albicans* (67.87%) and nonalbicans Candida (NAC) has been recorded 32.13% (**Fig. 5**). It may therefore conclude that Candida albicans is the most dominant opportunistic pathogenic species in oral cavity.

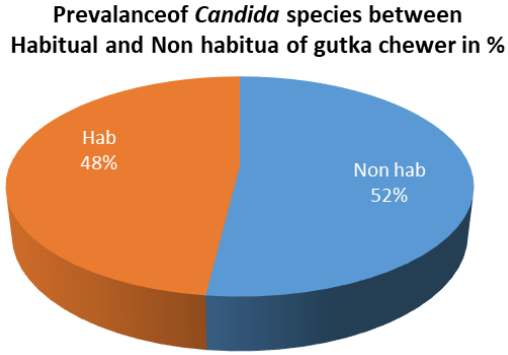

Fig. 4. Showing prevalence of *Candida* species in obtained isolates.

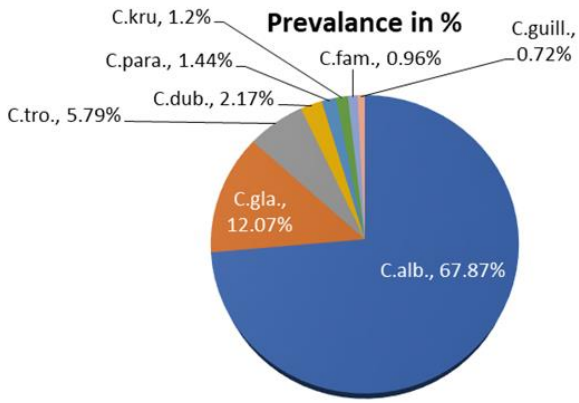

Fig. 5. Showing prevalence of *Candida* species between Habitual and Non habitual of gutka chewer.

Although numerous factors affecting the prevalence of growth of *Candida species* has been seen such as age, more than 30 year, recorded 238 (57.48%) for prevalence of oral *Candidiasis* and below 30 year of age 176 (42.18%) (**Fig. 6**). And in socio economic status revealed middle class 198 (47.82%), and poor 142(34.29%) and upper middle class. 17.87% (**Fig. 7**). And pH level 363(87.68%) was recorded acidic in 414 patients. These all were *Candida* positive. Salivary factor was studied that showed reduction in salivary rate of 314(75.84%) in 414 patients that promote oral Candidiasis. The study reveal higher saliva flow rate lower the growth of *Candida*. Salivary flow rate is very important in preventing oral Candidiasis because it removes loose or unattached *Candida species* of the oral cavity. Salivary flow rate, quality and quantity of saliva affect clearance of microbes from oral cavity. (Wyk and Steenkamp. 2011)

Table 2. Isolation of various *Candida spp.* from oral cavity of habitual (chewing gutka) and non-habitual patient.414 isolates of Candida species isolated from 450 clinical samples.

Isolates	Number of total isolates with prevalence percentage% (Total isolates 414) A+B		Number of isolates with prevalence % (Nonhabitual) (Total isolate-414) 'A'		Number of isolates with prevalence % (habitual Gutka chewing) (Total isolates 414) 'B'	
Candida. albicans	281	67.87%	142	34.30%	139	33.57
Candida globrata	56	13.53%	29	7.00%	27	6.52%
Candida tropicalis	29	7.00%	17	4.11%	12	2.90%
Candida dubliniensis	14	3.38%	8	1.93%	6	1,45%
Candida parapsilosis	11	2.66%	6	1.45%	5	1.21%
Candida. krusei	10	2.42%	5	1.21%	5	1.21%
Candida famata	8	1,93%	5	1.21%	3	0.72%
Candida guillerrmondii	5	1.21%	3	0.72%	2	0.48%

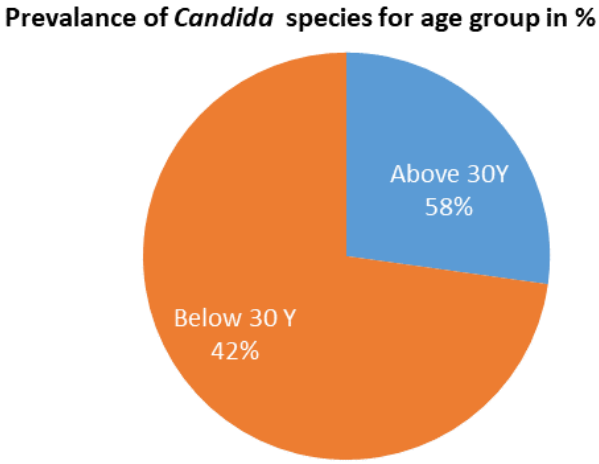

Fig. 6. Showing prevalence of Candida species for age group in %.

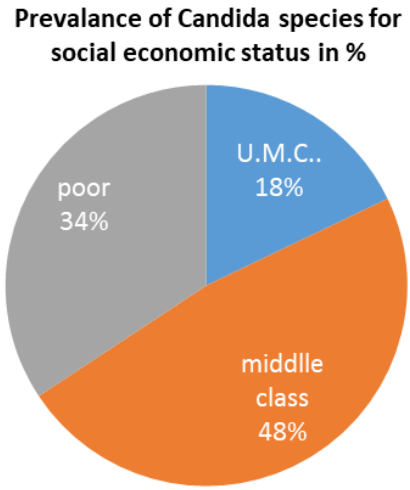

Fig. 7. Showing prevalence of Candida species for social economic status in %.
U.M.C.: Upper Middle Class

Conclusion

Candida yeasts are most common opportunistic pathogens of the oral cavity. The study reveal *Candida albicans* is the predominant yeasts. Factors studied during this period also suggested that saliva flow rate, age socioeconomic status and gutka chewer effect the environment of oral cavity. Low salivary flow rate increase Candidiasis because saliva removes yeasts, which are free in oral cavity or not attached with surface of oral cavity and increase pH support the growth of *Candida*. And gutka chewer showed slightly reduction in number of *Candida* because gutka stimulates saliva production that removes free *Candida* of the oral cavity.

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