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Original Research Article

Isolation and Identification of some Oral Microorganisms from Healthy Sudanese Smokers and Oral Cancer Patients

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Abstract: This study was conducted to identify any possible association between the different microorganisms within oral cavity of smokers and oral cancer patients. Isolates were collected from 30 volunteers; smokers and oral cancer patients during August and October 2013 in Khartoum Dental Hospital, Sudan. Swabs were obtained from both patients and smokers who were not diagnosed as diabetics or immunosuppressed. Administration of antibiotic, steroid or other treatment were taken in concern as well as age, gender beside smoking history. Each specimen was aseptically cultured on nonselective media under both aerobic and anaerobic conditions, then the conventional methods for identification were done. A total number of 45 bacterial isolates were represented by 6 genera of bacteria; (37.7%) *Staphylococcus* spp, (26.6%) *Bacillus* spp , (22.2%) *Streptococcus* spp , (8.8%) *Peptococcus* spp., *Aerococcus* spp and *Micrococcus* spp (2.2%), whereas 25 yeast isolates were represented by *Candida albicans.* In the smokers group; *Peptococcus* spp was detected only in subjects who is being smoking. Moreover, the study showed significant($r = 0.563$) correlation between oral cancer and smoking. In patients group; *Streptococcus* spp and *C. albicans* were detected only in individuals without treatment. Counts of *C. albicans* were elevated in smokers group than patients, while, *Micrococcus* spp and *Aerococcus* spp were detected in patients but not in smokers. Study concludes that both of *Micrococcus* spp and *Aerococcus* spp may be considered as diagnostic indicators of oral cancer.

Keywords: *Staphylococcus* spp, oral cancer, smokers.

INTRODUCTION

Smoking and alcohol intake are the most important risk factors for oral and pharyngeal cancer. Evidence concerning a connection between smoking and cancer has been extensively documented. Many of the compounds in tobacco smoke are hazardous to health and some are undoubtedly carcinogenic [1]. A number of bacterial species have been associated with different cancers following either epidemiological or laboratory-based studies.

For instant, *Chlamydia trachomatis* infection has been associated with an increased risk for the development of invasive cervical carcinoma, Bacteremia and endocarditis due to *Streptococcus bovis* have likewise been linked with malignancies in the colon, and *Helicobacter pylori* infection has long since been considered a causative agent of both gastric adenocarcinoma and mucosa associated lymphoid tissue lymphomas. Moreover, several mechanisms by which different bacteria may play a role in cancer development have been proposed such as through the induction of chronic inflammation, by interference, either directly or indirectly, with eukaryotic cell cycle and signaling pathways, or via the metabolism of potentially carcinogenic substances.

The latter mechanism is of relevance in the oral cavity, where the local micro flora may promote carcinogenesis by converting ethanol into its carcinogenic derivative, acetaldehyde. Following the ingestion of alcohol, salivary bacteria have been shown to produce levels of acetaldehyde that can induce DNA damage, mutagenesis, and secondary hyper-proliferation of the epithelium.

Interestingly, microbial acetaldehyde production is increased in heavy drinkers and smokers, offering a possible explanation for these risk factors [2].

On the other hand, Fungal diseases have been recognized with clinical importance in the second half of the last century and the incidence of *candidal* infections has increased dramatically over the past few decades. Among *Candida* species *Candida albicans* is the most frequently associated normal commensal in 50% of healthy individuals [3]. However, if the balance of the normal flora is disrupted or the immune defenses are compromi

sed, *Candida* species often become pathogenic. The etiologic process very likely involves several factors. Changes in the oral environment due to smoking, tobacco consumption, lifestyle has been found to be few of the etiological factors in development of multiple precancerous lesions and few of them has been noted to have the ability to change in oral cancer, hence termed as 'Precancerouslesions'[2]. Various carcinogens play a vital role in altering cellular metabolism, damage to chromosome or damaging DNA directly in cells and chemical carcinogens are one of them [5]. For many years *Candida* spp have been implicated in various epithelial cancers as it is found to produce chemical carcinogens, *candidal* acetaldehyde and endogenousnitrosamine [6]. Candidosis is the most common fungal infection of the oral cavity and is caused by an overgrowth of commensals of the *Candida* spp [7]. A change from the harmless commensal existence of *Candida* to a pathogenic state can occur following alteration of the oral environment to one that favors the growth of *Candida*. The causes of such changes are the so called predisposing factors for *Candida* infection(Candidosis) and most often these relate to a weakening of host immune defenses [8].

The transition from commensalism to disease may be associated with the virulence characteristics of *Candida* such as adherence, germ tube formation,dimorphism, phenotypic switching, toxins, and hydrolytic enzymes [7].

Cernea *et al* were the first to recognize *Candida* infection in oral leukoplakias and introduced the term 'candidal leukoplakia'. [9] found that six out of ten tissue biopsies initially diagnosed as chronic hyper plastic candidiasis (CHC) progressed to oral squamous cell carcinoma (OSCC) while [10] reported that two of three CHC cases underwent malignant transformation. In recent years researchers have focused on the presence of pathogenic microorganisms, such as *Candida albicans* inpatients with potentially malignant lesions such as leukoplakia and oral lichen planus.

Walker and Arendorf (1980) have shown that *C. albicans* was isolated more frequently from the mouths of smokers than from non-smokers [12]. Production of carcinogens and Initiation of carcinogenesis *Candida* might induce OSCC by directly producing carcinogenic compounds e.g. nitrosamines and acetaldehyde (ACH) [11]. Such a carcinogen will bind with DNA to form adducts with bases, phosphate residues, and or hydrogen bonding sites that could cause miscoding or irregularities with DNA replication. Point mutations thus induced may activate specific oncogenes and initiate the development of oral cancer [6].

When oral cancer act as the sixth most common malignancy worldwide and is particularly prevalent in developing country and when a tobacco smoke is a human carcinogen and play important role in development of oral cancer without a shade of doubt therefore this study will focus on the identification of the microorganisms that can be associated with oral cancer and smoking in some Sudanese patients.

MATERIALS AND METHODS

Description of the samples

In the present study 30 random subjects were used, 50% of them were oral cancer patients whereas 50 % were smokers. The study was carried out to investigate the relationship between smoking habits, existence of microorganisms and appearance of the disease. The minimum age of the selected subjects was 20 year whereas the maximum age was 90 year with median of 53.9±16.6 year. About 23 (76.7%) males and 7 (23.3%) females were included in the study .The studied habits included both smoking cigarette (as a main habit), tobacco chewing and alcohol as well as number and duration of smoking cigarette . Moreover the cessation and duration of it from smoking habit was also investigated among the individual sample .

Sampling methodology

A total of 30 study subjects (15 smokers and 15 oral cancer patients) were investigated. The patients underwent biopsy, according to medical report (between August and October 2013 in Khartoum Dental Hospital, Sudan). All subjects under study were not diagnosed as diabetics or immunosuppressed (except one patient) and the administration of antibiotics, steroid or other treatment (chemo/radiotherapy for patients) were taken in concern . This study was ethically approved by the Research Department in the hospital. Samples were collected from lesions (patients) and oral cavity (smokers) with sterile swabs and stored at -4^{o}C.

Culture media

The following media and chemicals, were used to detect different types, of microorganisms.

1. Solid media

1.1Nutrient Agar

This was a general-purpose culture medium for bacteria. It was obtained in a dehydrated form. The constituents of the medium were beef extract, yeast extract, peptone, sodium chloride and agar. It was prepared according to the manufacture’s instructions by suspending 28g in one liter distilled water. The medium was allowed to boil until it was completely dissolved. The pH of medium was adjusted to pH 7.4±0.2 and then the medium was sterilized in an autoclave at 121^{o}C for 20 minutes [13].

1.2 Sulfide Indole motility(SIM)

The medium was used for the determination of motility. The medium was composed of tryptone, meat extract, disodium thiosulphate, cystein hydrochloride, sodium chloride and agar. It was prepared according to the manufacturer's instructions by suspending 43.7g in o

ne liter distilled water. The ingredients were dissolved i n water by heating. The medium was dispensed into test tubes and sterilized by autoclaving at 121^{o}C for15 minu tes [14].

1.3 Sabouraud Dextrose Agar (SDA)

This was a suitable culture medium for cultivat ion and differentiation of Fungi. It was obtained in a de hydrated form. The constituents of the medium were pe ptone, dextrose and agar. It was prepared according to t he manufacturer's instructions by suspending 65g in one liter distilled water. The medium was allowed to boil un til it was completely dissolved. The pH of medium was adjusted to pH 5.6 and then the medium was sterilized i n an autoclave at 121^{o}C for 20 minute. Then 0.1 g cholr maphenicol was added to one liter of medium after auto claving to inhibit bacterial growth (Oxoid).

2. Semi-solid media

2.1 Hugh and Leifson's medium

This was used for differentiating oxidative and fermentative metabolism of carbohydrates. The medium consisted of tryptone, yeast extract, D-glucose, bromocr esol purple and agar. The ingredients were added to one liter distilled water and dissolved by steaming. The pH was adjusted to pH 7.0 and then the medium was sterili zed by autoclaving at 115^{o} C for 20 minutes and sterile glucose (sterilized by tendallization) was aseptically ad ded to the previously sterilized basal medium to give a f inal concentration of 1%. The medium was steamed for 10-15 minutes before use to expel excess oxygen [13].

3. Liquid media

3.1 Peptone water

This medium was used for glucose (acid) test. The medium consisted of peptic digest of animal tissue and sodium chloride (BIOMARK). The ingredients wer e dissolved in distilled water. Then the pH was adjuste d to pH 7.2±0.2 andthe medium was sterilized in an aut oclave at 121^{o}C for 15 minutes [13].

Purification and identification of isolates

Predominant microorganisms from morphologi cally different colony types were selected from plate ag ar. Sterile nutrient agar and sabouroud dextrose agar for bacterial and yeast growth, respectively. Sub-culturing purified these isolates; typical colony was streaked onto sterile nutrient agar plates. The plates were incubated at 37^{o}C for 24 hours. The representative colonies of variou s microorganisms were sub-cultured onto the same med ia (slope) and then the cultures were kept in the refriger ator at 4^{o}C until used for further test. The identification of purified isolates was carried out according to [15].

Gram stain test

A distinct colony was picked carefully with st erile wire loop. The colony was emulsified in a drop of physiological saline (0.85%NaCl), placed on a clean sli de and spread evenly to make a thin film. The slide was allowed to dry. The smear was fixed by using a flame. Then the smear was stained as described by [13].

Endospore stain test

This demonstrates the presence of endospores, which were highly resistant to high temperature, lack of moisture and toxic chemicals. The smear was prepared i n the usual way, then the smear was fixed and stained as described by [13].

Motility test

A tube of motility medium (SIM medium with concentration of 0.4% of nutrient agar) was inoculated with a 24-48 hours culture. This was done aseptically us ing a straight wire to half depth of the tube. During gro wth, motile bacteria will migrate from the line of inocul ation to form turbidity in the surrounding medium. Non -motile bacteria will grow only along the line of inocula tion [14].

Catalase test

This demonstrates the presence of catalase, an enzyme that catalyzes the release of oxygen from hydro gen peroxide.

One drop of 3% hydrogen peroxide solution w as placed on a clean slide. A loop full from 24 hours cul ture was added. The release of bubbles of oxygen indica ted the presence of catalase in the culture under test [13] .

Oxidase test

A piece of filter paper was impregnated with o xidase test solution (HIMEDIA). Then a loop full from a 24 hour culture was streaked onto the filter paper. A p ositive reaction was indicated by purple color after 10-1 5 seconds, any later reaction being recorded as negative [13].

Glucose (acid) test

After preparing the peptone water medium the glucose (0.5-1.0%) was added. Andrade's (1%) was add ed as indicator and the pH was adjusted to pH 7.4, then the medium was distributed in test tubes with inverted Durham tubes. Some bacteria ferment certain sugars wit h the production of acid and gas; others produce neither acid nor gas. The positive result is the change in color a cid (pink) and production of gas in the Durham tubes [1 3].

Oxidation-fermentation (O/F) test

Fresh culture (18-24 hours) was tested for O/F test by stab inoculation onto freshly steamed Hugh and Leifson's medium, contained in test tubes. One of the tu bes was sealed with sterile paraffin oil and the other left unsealed. Inoculation was carried out at 37^{o}C for 2-7 da ys. Acid production is shown by change in the color of t he medium from blue to yellow but fermentative organi

sms produce acid in both tubes, and oxidative organisms produce acid in the open tube only [13].

Germ tube test (GTT)

This test was used to rapid identification and specific for *Candida albicans*. A standard GTT was performed by inoculating 0.5 ml of serum with a loop full of the test strain, followed by incubation at 37°C for 3 hours [16]

RESULTS

A total number of 45 bacterial isolates were successfully identified, in addition to 25 yeast isolates from both patients and smokers. The identified bacteria belong to 6 genera; 17 (37.7%) *Staphylococcus* spp, 12 (26.6%) *Bacillus* spp, 10 (22.2) *Streptococcus* spp, 4 (8.8%) *Peptococcus* spp ,1 (2.2%) *Aerococcus* spp and 1(2.2%)*Micrococcus* spp .Whereas, the 25 yeast isolates were represented by *Candida albicans*.

Fifteen patients were found free from both diabetes and immunosuppression for all age classes. The age class 51-65 had the highest number of cancer patients than other age classes followed by age classes 36-50, 66-80 and 81-95 respectively whereas the age class 20-35 showed no cancer patients. On the other hand, the age class 51-65 also reported to be infected by cancer and immunosuppression.

About 76.7% (23) of the microorganisms existence was associated with males, corresponding to only 23.3% (7) females. The higher existence of microorganisms in males observed for one genus(Staphylococcus or Streptococcus or Bacillus) and *C. albicans* (11) followed by more than one genus of bacteria and *C. albicans* (8), *Staphylococcus spp* (2), *Streptococcus spp* (1) and finally *Bacillus spp*(1). For females the higher existence of microorganisms was in both one genus and *C. albicans* and more than one genus and *C. albicans* .

Both Aerococcus and Micrococcus were appeared only in females but there were no genera related to males alone however both Staphylococcus, Streptococcus, *Peptococcus* and Bacillus were found in both gender .

The study illustrated that great number of smokers (46.7%) were free from cancer , whereas non-smokers subjects (33.3%) were cancer positive. On the other hand, tobacco chewers (3.3%), alcoholics (3.3%), tobacco chewers and alcoholics (3.3%) and smokers, tobacco chewers and alcoholics (3.3%) were also cancer positive .Whereas smoker and tobacco chewers represents 6.7% were 50% normal and 50% with immunosuppression and cancer.

Individuals who smoke 1-5 (6.7%) were either associated with one genus of bacteria and *C. albicans* 3.3% or more than one genus of bacteria and *C. albicans* infested, while smokers who smoke 6-10 (23.3%) were also one bacterial genus and C. albicans (42.9%) and more than one bacterial genus and *C. albicans* (28.6%) . The smokers who smoke more than ten were found to have higher infection either by one genus of bacteria and *C. albicans* (62.5%) or more than one genus of bacteria and *C. albicans* (37.5%).

In all smokers who smoked 1-5 cig/day, Staphylococcus or Streptococcus appeared only with *C. albicans* and the same type of bacteria was observed in smokers who smoke 6-10 cig/day ,but to a lesser percent when compared to Bacillus and Staphylococcus or Bacillus and Streptococcus alone with *C. albicans*. On other words, the smokers who smoke more than 10 cig/day were highly infected either by (Staphylococcus or Bacillus) or by more than one genera represented by *Staphylococcus* and *Streptococcus* or Staphylococcus and *Peptococcus* or in combination with Bacillus and constant appearance of *C. albicans*.

The highest percentage of occurance of the microorganisms was reported for smokers who smoke for more than 5 years by existence of Staphylococcus or *Bacillus* as single genera with Candida as well as both *Peptococcus*, *Streptococcus*, *Bacillus* and *Staphylococcus* together with Candida followed by smoking for less than 5 years.

The highest existence of microorganisms (53.4%) mainly (one genus of bacteria and *C.albicans*) and (more than one genus of bacteria and *C. albicans*) was reported for those who did not stop smoking as compared to those stopped smoking 3.3%.

The highest existence of microorganisms mainly either *Staphylococcus*, *Streptococcus*, *Peptococcus* or *Bacillus* as well as both *Peptococcus*, *Staphylococcus* and *Bacillus* with constant existence of *C. albicans* with all.

The results showed that 96.7% (29 individuals) are continue to smoking and all of them were infected by microorganism. About 44.8% (13 individuals) out of them infected by one genus and *C. albicans*, 37.9% (11 individual) infested by > 1 genus and *C. albicans* and 6.9% infested by *Staphylococcus* spp, 6.9% infested by *Streptococcus spp* and 3.5% infested by *Bacillus spp*. On the other hand the rested sample (one individual) was stop from smoking for about 1-3 month and infested by one genus(Bacillus but this reported as cancer patient) and *C. albicans* .

It was also found that, 50% of the studied subjects were free from any of the studied diseases whereas the rest wasdivided between cancer 46.7% (14 patients) and cancer and immunosuppressive 3.3% (one patient). About 35.7% (5 patients) of the cancer patients were infected by one genus and *C. albicans* , 35.7% (5 patients) were infected by > one genus of bacteria and *C. allbicans*, 14.2% (2 patients) were infected by *Staphylococcus*

spp, 7.1% infected by *Streptococcus* spp and 7.1% infected by *Bacillus*s pp.

Moreover. about 20% (3 patients) of cancer had cancer treatment. The distribution of microorganisms for this ratio was one by *Staphylococcus*spp, one by Bacillus spp and one by > one genus of bacteria and *C. allbicans*. The rest of the cancer patients who represent 80% (12 patients) about 50% of them were infected by one genus and *C. albicans*, 41.7% infected by > one genus of bacteria and *C. allbicans* and 8.3% infected by *Streptococcus* spp.

Although, about 86.7% (26 individuals) of studied sample did not antibiotic administration and only 13.3% (4 individuals) had antibiotic. The existence of *Staphylococcus* spp, *Bacillus*spp, one genus and *C. albicans* and > one genus and *C. albicans* was equally distributed (25% for each) among the individuals who administrated antibiotics.

Medium negative significant correlation ($P < 0.01$) ,was found between the diagnosis of the diseases and age, whereas there was medium positive significant correlation($P=0.003$) with sex and habit. Moreover, there was strong positive and significant correlation($P= 0.000$) between diagnosis and treatment. In contrast, strong negative and significant correlation ($P= 0.000$) was showed with number of cigarette, duration of smoking and stopping of smoking. On the other hand, there was positive non-significant correlation($P > 0.05$) between diagnosis and duration of stopping and there was a weak positive significant correlation($p= 0.043$) between diagnosis and application of drugs .

DISCUSSION AND CONCLUSION

The results obtained from this descriptive study revealed a number of different associations in relation to oral cancer. The number of male cancer patients when compared to the females, showed higher incidence than females, this was similar to the global statistics which was done by Jemal *et al*., [17] and Siegel *et al*., [18] who reported that about 25,240 new cases of oral cavity and pharynx cancer were males and 10,480 were only females and about 29,620 new cases of the same cancer were males and 11,760 were females respectively. And when considering the age limit , a higher incidence of oral cancer was found to occurred among subjects in age class 51 – 65 a finding which goes in contrast with [19] who indicated that the incidence of oral cancer increase becomes more rapid after the age 50 or increased at younger ages (> 60 years).

The study record a number of microorganisms that occur in relation to the gender, mainly *Bacillus spp, Staphylococcus spp, Streptococcus spp, Peptococcus spp, Micrococcus spp, Aerococcus spp* and *C. albicans.* Moreover ,certain gender association was noticed for some species like *Micrococcus spp* and *Aerococcus spp* which were present only in females and absent in males. In the case of males ,they show some sort of single bacterial species dominance either *Bacillus*, *Staphylococcus* or *Streptococcus* to occur in a highest percent together with the fungus *C. albicans.*

For females, also the dominance of single bacterial species like *Micrococcus* or *Bacillus* was noticed to occur in a highest percent.Also a single association was noticed between both bacterial species *Micrococcus* and *Bacillus* with the fungus*C. albicans* alone without any appearance to other bacterial species.

The study observed that, the smoking habit can lead to cancer disease but when it coupled with other habit such as alcohol or tobacco chewing, this observation was also noticed by [20] who reported that on the basis of the two-stage hypothesis of cancer induction, alcoholic beverages may act as a co-carcinogen and as a solvent, enhancing the penetration of oral epithelium by organic carcinogens present in tobacco smoke.

With regard to the smoking habits, although the study found that, smokers who had 6-10 cig/day were cancer patients but those who had less than 6 or > 10 cig/day were not, this may not mean that the occurrence of cancer decrease when the smoking of < 6 or >10 cig/day but may be for statistical reasons.

Some correlation was also noticed between the observed microorganisms and the number of smoked cig/ day .The appearance of more than one bacterial genus with *C. albicans* increased linearly when range of having cig/day was increased, therefore the appearance of (*Bacillus* spp, *Staphylococcus* spp, *Streptococcus* spp, *Peptococcus* spp and *C. albicans*) were related to smokers who had > 10 cig/day as well as in smoker who had 6-10 cig/day with exception in the disappearance of *Peptococcus* spp ,but in smokers who had 1-5 cig/day both *Peptococcus* spp and *Streptococcus* spp were also absent.

Moreover, the existence of microorganisms increased with duration per year in that the appearance of the same microbial population in smokers who had > 10 cig/day were present in those who smoked > 5 years and also *Peptococcus*spp was observed in smokers who smoke 1-5 years and not detected in smokers > 5 years.

Furthermore, the study showed that the highest detection of microorganisms was associated with subjects who carried on to smoking when compared with stopped smokers that illustrated by the detection of *Bacillus* spp alone with C. albicans in subjects who stopped the smoking while, all other bacterial genera were associated with subjects who did not stop. This was also noticed by [20] who examined 200 buccal smears from alcoholics and cigarette smokers and found that the alcohol consumption and cigarette smoking are possible risk factors that can cause a typical cellular changes that lead to possibly oral infection, and the degree of these changes dep

ends on both the duration of alcohol consumption and ci garette smoking.

Studying the possible effect of cancer treatmen t application on the distribution of microorganisms reve aled that, the appearance of the bacterium *Streptococcus spp* alone with the fungus *C. albicans* was associated wi th untreated patients than in treated, a finding which wa s similar to [21] who reported that both the commonly e ncountered oral *Streptococci* and yeasts possess metabo lic pathways for the carcinogenic. Whereas in 3 patients with treatment , two of them infected by only one bacter ial genus specifically *Staphylococcus spp* or *Bacillus sp p* but the third one was infected by more than one bacte rial genus (*Staphylococcus spp*, *Streptococcusspp* and *P eptococcusspp*) and *C. albicans*, Nevertheless, Jukka, [2 1] reported that *Candida albicans* was found in one or more sites in 54% of the subjects who had received radi otherapy to the head and neck in comparison to 15% of the controls with other bacterial genera than those in the study.

The effect of antibiotic application on the exist ence of certain microorganisms was clearly noticed acc ording to the finding of higher existence of one bacteria l genus alone or with *C. albicans* associated with subjec ts who did not administrated with antibiotic than those who had it, this was also indicated by [22] who reported that antibiotic as well as impaired immune system and d iabetes promote *Candida* infection since an imbalance a ppears between bacteria and fungi and can cause a disea se even if it commensal ones.

Although, a huge number of studies were indic ates to presence of *C. albicans* in oral cavity of both sm okers [23] and patients with cancer [2], but this study ill ustrated that several bacterial genera detected in the pati ents were not detected in the smoker for instance, *Micro coccus spp* and *Aerococcusspp* were isolated only from tumorous specimens and not from non-tumorous ones a nd these were similar to other study by Hooper *et al*., [2] who indicated that several species detected in the non- tumorous control tissue were not detected in the tumor t issues, and *vice versa*. For instance *Staphylococcus aur eus*, and *Micrococcus* were isolated only from tumorous specimens and not at all from non-tumorous ones.

The present study indicated that: the incidence of oral cancer was higher in males than females and incr eased more rapidly after age 50: although, the number o f subjects was very little and only conventional detectio n methodologies were used, several bacterial genera and *C. albicans* were isolated from the two groups. Both *Mi crococcus* spp and *Aerococcus* spp were isolated from p atients and absent in smokers as well as present in femal es and absent in males. The appearance of *C. albicans* i n smokers was higher when compared with patients wit h cancer. *Peptococcus* spp was detected only in subjects who smoking for long time. All the detected microorga nisms on the smoker group disappeared completely in t he ones who stopped this habit except the *Bacillus* spp a nd *C. albicans*. The association of both *Streptococcus* s pp and *C. albicans* was detected in patients without trea tment than ones under therapy.

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