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Relationship between the quantity of oral Candida and immunological vigor

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Abstract

OBJECTIVE: Oral Candida asymptomatically colonizes approximately 35 to 80% of individuals in a population at any given time. It is speculated that increases in oral Candida may be closely related to host immunity. Therefore, the aim of the present study was to determine whether a relationship exists between the number of oral Candida colonies and immunological status of the host.

MATERIALS AND METHODS:

We analyzed 32 subjects and their immunity was assessed and scored using the “Scoring of Immunological Vigor (SIV)”. Amount of oral candida was detected by culture of concentrated oral rinse solutions. The relationship between SIV and the count of oral Candida was investigated.

RESULTS: Oral Candida was detected in cultures of samples from all subjects. The median number of Candida colonies was 6.5 CFU (IQR: 28.5 CFU, range: 1 - 3480 CFU). There was a significant correlation between the count of oral Candida and the grade of SIV. The amount of oral candida was higher in the lower immunological status of the host. Significant correlations were also found between the amount of Candida and the number of T cells, naïve T cells, and Natural Killer (NK) cells.

CONCLUSIONS: The results of this study suggested that the detection of oral Candida may be a possible marker for determining immunological vigor of the host.

Key words: Oral Candida, Immunological Vigor, Candida infection

Running title: Oral Candida and immunological vigor

Introduction

Candida habitually resides in the oral cavity and the reported rate of oral Candida among healthy individuals varies between 35% and 80% depending on the population studied and detection methods used [1-2]. Candida species asymptomatically colonize individuals in a population at any given time, and they may cause mucosal and systemic infections in the condition where oral candida increased and/or host defenses are weakened.

It is reported that the amount of oral Candida increased in the local condition including denture wearing, hypoptyalism, low saliva pH, and the presence of dental carious lesions [3-8]. It is also suggested that increase of oral Candida may be closely related to host immunity. Previous studies reported that oral Candida increased in immunocompromised individuals, including HIV-positive and AIDS patients, organ transplant recipients, and patients with some factors that primarily act by inducing immunosuppression (e.g., corticosteroids, chemotherapy, malnutrition, malignancy, and neutropenia)[9-15]. These results strongly speculated that increase of oral Candida may be closely related to host immunity; however, this relationship has not yet been examined. Therefore, the aim of the present study was to determine whether a relationship exists between the number of oral Candida colonies and immunological status of the host by examining the number of Candida colonies in the oral cavity and immunological vigor of the host, as assessed by immunological indices.

Materials and Methods

The Committee for Ethics at Shinshu University Hospital (#2795) and Aizawa Hospital (#2012-091) approved this study protocol.

1. Subjects

Samples were obtained from 38 subjects who agreed to participate in the present study. Of these, 24 were recruited from consecutive patients who visited the department of dentistry and oral surgery at Aizawa Hospital or Shinshu University Hospital

complaining of dental and oral diseases (between February 2013 and November 2014). The other 14 subjects were healthy volunteers. Informed consent was obtained from all subjects.

Table 1. Relationship between an amount of oral Candida and subjects' sex, age, oral symptoms/sings, and systemic diseases

Characteristics	(n)	Amount of Candida (CFU) median (IQR)		
Sex	Women (19)	6	(1.5 – 160)	p = 0.81*
	Men (13)	10	(4 – 15)	
Age	20-59 (19)	4	(1 – 6)	p < 0.01* p < 0.001**
	60 < (13)	285	(20 – 2160)	(r = 0.64)
Oral symptoms/signs	Absence (19)	4	(1.25 – 6)	
	BMS (6)	10	(1 – 162)	p = 0.19* (vs. absence)
	Oral candidiasis (7)	2320	(160 – 2900)	p < 0.001* (vs. absence)
Denture wearing	(–) (19)	4	(1 – 6.5)	p < 0.001*
	(+) (13)	285	(15 – 2610)	
Systemic disease	Absence (14)	4	(1.3 – 5.8)	p < 0.01*
	Presence (18)	24.5	(10.3 – 1813.8)	

* Mann-Whitney's U test

** Spearman's rank correction test

BMS: burning mouth syndrome

2. Detection and count of oral Candida

An oral rinse solution was collected by rinsing the mouth with 5 mL sterile saline, which was held in the mouth for 30 seconds before collection in a sterile container. Concentrated oral rinse solution was prepared by centrifuging the rinse solution at 3,200 x g for 20 minutes. After the supernatant was removed, the precipitate was resuspended with 250 µL saline and 50 µL of the sample was inoculated onto the Chromagar Candida agar (Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo, Japan). Candida colonies on culture agars were counted after an incubation at 37 °C for 48 hours.

3. Assessment of host immunity

Immunity was assessed and scored using the "Scoring of Immunological Vigor (SIV)" reported by Hirokawa et al. [16-19]. Two milliliters of venous blood was collected in a tube containing ethylenediaminetetraacetic acid (EDTA-2K) at the same time as oral Candida sampling. Peripheral blood samples were sent to the Institute for Health and Life Sciences (HLS Tokyo, Japan) and SIV was measured using the patents of Tokyo Medical and Dental University (No.4608704, No.5030109). SIV evaluates comprehensive immunity strength by scoring various immune indexes including the total number of T

cells, number of CD8+ CD28+ T cells, the ratio of CD4+ T cells to CD8+ T cells, the number of naive T cells and ratio of naive to memory T cells, number of B cells, and number of Natural Killer (NK) cells. Each index was measured by the method described below. Consequently, the measurement results of each index were reported in three ranks (scores 1 to 3). Score 1 means "needs improvement", score 2 means "needs observation", and score 3 means "safe". The criteria for the ranking were not shown because they were protected by the patent. Each score of seven indexes was summed to obtain the total score, with the resulting score ranging between 7 and 21. Immunological vigor was classified into 5 grades: grade 5 (total score = 21, highest immunity), grade 4 (total score = 20-18), grade 3 (total score = 17-14), grade 2 (total score = 13-10), and grade 1 (total score = 9-7, lowest immunity). Serological C-reactive protein (CRP) levels were measured in order to assess the presence of local and systemic inflammation, which may influence the assessment of immunity.

The methods used to measure each index were as follows:

WBC count: A hematological analysis performed with a PENTRA80 analyzer (Horiba, Kyoto, Japan).

Mononuclear cell count: Mononuclear cells were stained with a combination of 5 monoclonal antibodies (mAbs) conjugated with 5 chromophores. A flow cytometer (Navios: Beckman Coulter, Miami, FL, U.S.A) was used to count the number of mononuclear cells.

Counts of the subtypes of lymphocytes using monoclonal antibodies (mAbs): The following mAbs (Beckman Coulter, Miami, FL, U.S.A) were used: fluorescein isothiocyanate (FITC)-conjugated anti-CD8 and anti-CD20; phycoerythrin (RD1)-conjugated anti-CD3; phycoerythrin-Texas Red (ECD)-conjugated anti-CD45RA; phycoerythrin-cyanin 5.1 (PC5)-conjugated anti-CD28 and CD16; phycoerythrin-cyanin 7 (PC7)-conjugated anti-CD45; allophycocyanin (APC)-conjugated anti-CD4CD, and anti-CD56. The following combinations of mAbs were used: CD3-RD1/CD20-FITC/CD16-PC5/CD45-PC7/CD56-APC and CD4-APC/CD8-FITC/CD45RA-ECD/CD28-PC5/CD45-PC7. These mAbs enabled us to identify B cells (CD20+ cells), NK cells (CD56+ CD16+ cells), T cells (CD3+), and the following subpopulations of T cells: CD4+ T cells and CD8+ T cells.

4. Statistical assessment

Statistical assessments were carried out using PC running software (GraphPad Software Prism 6 for Windows). Spearman's rank correction test or Mann-Whitney's U-test was utilized to test the relationship between the variables. P-values less than 0.05 were used to indicate significance.

Results

1. Subjects

Of the 38 subjects who participated in the study, six were compromised by inflammation (CRP level higher than 0.30 mg/dL) and were excluded from the study. Therefore, 18 patients (7 males and 11 females with a mean age of 70.6 years old ranging between 48 and 88 years old) and 14 volunteers (6 males and 8 females with a mean age of 31.3 years old, ranging between 26 and 42 years old) were available for the assessments. Seven patients had oral candidiasis, 6 had burning mouth syndrome (BMS), and other 5 had chronic periodontal diseases. Systemically, three patients had malignant tumors, 6 had hypertension, 2 had hyperlipidaemia and hyperuricemia, and other 2 had insomnia, 1 had diabetes mellitus and rheumatoid arthritis, and another one had multiple myeloma, diabetes mellitus, schizophrenia, pneumonia, respectively. Oral candidiasis was diagnosed based on clinical findings, such as creamy-white plaques, redness, and atrophic mucosa, and the microscopic observation, such as the identification of the candida hyphae and yeast with the periodic acid-Shiff (PAS) method, of Candida in oral lesion samples. BMS was defined as burning pain in the tongue or oral mucosa membranes without accompanying clinical and laboratory findings [20, 21].

Table 2. Relationship between the distribution of grade of immunological vigor and subjects' sex, age, oral symptoms/sings, and systemic diseases

		Number of subjects in each grade of immunological vigor						
		Grade 1	Grade 2	Grade 3	Grade 4	Grade 5		
Sex	Women	0	2	9	8	0	p =0.81*	
	Men	0	3	6	3	1		
Age	20-59	0	1	8	9	1	p = 0.01*	p < 0.01**
	60 <	0	4	7	2	0		(r = –0.51)
Oral symptoms/signs	Absence	0	0	8	10	1		
	BMS	0	0	4	2	0	p < 0.05* (vs. absence)	
	Oral candidiasis	0	4	3	0	0	p < 0.001* (vs. absence)	
Denture wearing	(–)	0	0	9	9	1	p < 0.01*	
	(+)	0	5	6	2	0		
Systemic disease	Absence	0	0	4	9	1	p < 0.01*	
	Presence	0	5	9	4	0		

* Mann-Whitney's U test

** Spearman's rank correction test

BMS: burning mouth syndrome

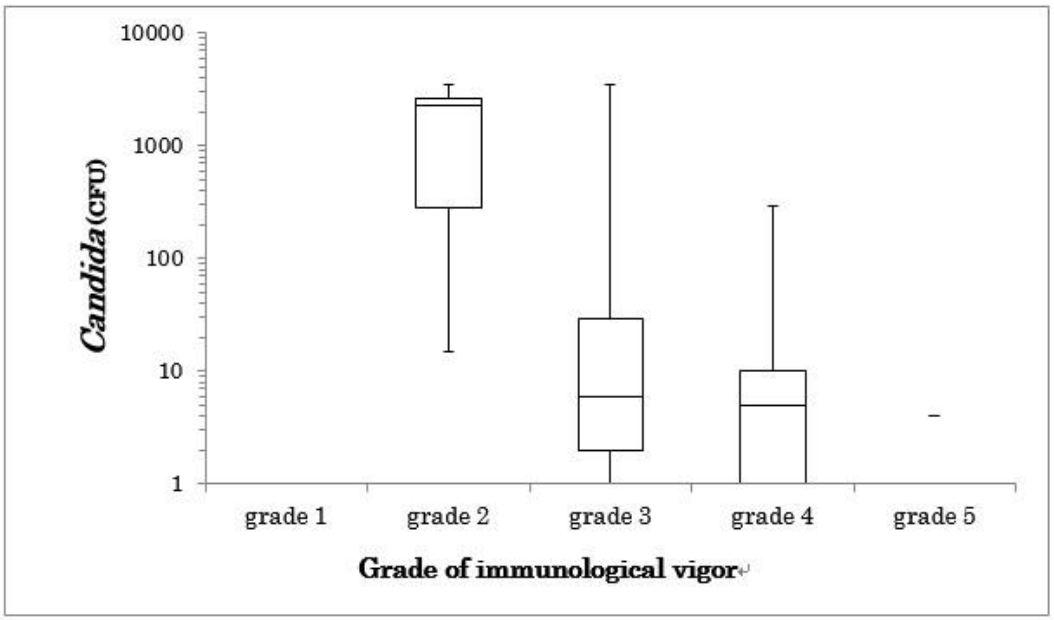
2. Detection and count of oral Candida

Oral Candida was detected in cultures of samples from all subjects. The median number of Candida colonies was 6.5 CFU (interquartile range (IQR): 28.5 CFU, range: 1 - 3480 CFU). The relationships between the count of oral Candida and subject's age, gender, oral candidasis/BMS, and systemic diseases were summarized in Table 1. A significant correlation was observed between the count of oral Candida and age (Spearman's rank correction test, $r = 0.64$, $p < 0.001$). The count of oral Candida increased with advancing age. In addition, the presence of oral candidiasis was related to a significantly higher count of oral Candida (Mann-Whitney's U test, $p < 0.001$). Based on results of this study, the cut-off point of the number of candida colonies for the patients diagnosed as oral Candidiasis was 160 CFU. This cut-off point reveals 100 % of sensitivity and 96 % of specificity, and may divide into the patients with asymptomatic candida carrier and with candidiasis. The status of denture wearing and the presence of systemic disease were also significantly related to a higher count of oral Candida (denture wearing: Mann-Whitney's U test, $p < 0.001$, systemic disease: Mann-Whitney's U test, $p < 0.01$). On the other hand, a significant difference was not recognized for gender ($p = 0.81$) and BMS ($p = 0.19$).

3. Assessment of immunological vigor

SIV ranged between 10 and 21, with a median score of 17 (IQR 2.25). No subject was classified as grade 1, 5 were grade 2, 15 were grade 3, 11 were grade 4, and 1 was grade 5. The relationships between the grade of immunological vigor and subject's gender, age, oral candidiasis/BMS, and systemic disease were summerized in Table 2. A significant correlation was observed between the grade of immunological vigor and age (Spearman's rank correction test, $r = -0.51$, $p < 0.01$). The grade of immunological vigor decreased with advancing age. In addition, the presence of BMS and oral candidiasis was related with a significantly lower grade of immunological vigor (Mann-Whitney's U test, BMS; $p < 0.05$, oral candidiasis: $p < 0.001$). The status of denture wearing and the presence of systemic disease were also significantly related to lower grade of immunological vigor (denture wearing: Mann-Whitney's U test, $p < 0.01$, systemic disease: Mann-Whitney's U test, $p < 0.01$). On the other hand, a significant difference was not noted for gender ($p = 0.81$).

Figure 1. The correlation between grads of immunological vigor and count of *Candida*



A correlation was observed between these factors ($p<0.05$) (the coefficient of correlation, $r = -0.520$, $p = 0.002$; Spearman's rank correction test).

4. Relationship between the count of oral Candida and grade of immunological vigor

A statistically significant correlation was found between the count of oral Candida and grade of immunological vigor (the coefficient of correlation, $r = -0.520$, $p = 0.002$; Spearman's rank correction test, Fig. 1). The count of oral Candida was higher in the subjects with the lower grade of immunological vigor. It is well known that there was a strong association between denture wearers and oral candidiasis [2]. Therefore, the relationship between oral Candida and SIV was additionally analyzed in the subgroup with or without wearing denture. The results of sub-analyses revealed that no significant correlation was observed between the amount of oral Candida and grade of immunological vigor in both sub groups (Mann-Whitney test, grade 3 with denture vs grade 3 without denture : P=0.09, grade 4 with denture vs grade 4 without denture : P=0.25, Table 3).

The relationship between the amount of oral Candida and score of each index in SIV was summarized in Table 4. Correlations were observed between the amount of Candida and numbers of T cells, naive T cells, and NK cells (Spearman's rank correction test, $r = -0.353$, $r = -0.351$, $r = 0.361$, $p < 0.05$). The numbers of T cells and naive T cells decreased with increases in the amount of oral Candida.

Table 3. Relationship between grades of immunological vigor and count of *Candida with/without denture wearing*.

Grade of immunological vigor	Amount of *Candida* (CFU) median (IQR)	p value
Grade 2/denture (+)	2320 (285-2610)	-
Grade 3/denture (-)	4.5 (1.8-11.8)	NS*(p = 0.09)
Grade 3/denture (+)	15 (8-1605)	
Grade 4/denture (-)	4 (1-7)	NS*(p = 0.25)
Grade 4/denture (+)	148 (74.5-221.5)	
Grade 5/denture (-)	4 (4-4)	-

Mann – Whitney's U test

***Not significant.**

Table 4. Relationship between the amount of oral Candida and score of each index in SIV

		Amount of Candida (CFU) median (IQR)		
Number of total T-cells	Score 1	29	(8.5 - 1302.5)	p < 0.05*
	Score 2	12	(1.75 - 687.75)	r = -0.35
	Score 3	5.5	(2.5 - 10)	
Number of CD8+ CD28+ T-cells	Score 1	2	(2 - 2.5)	p < 0.01*
	Score 2	15	(4.5 - 1307.5)	r = -0.52
	Score 3	3	(1 - 5.5)	
Ratio of CD4+ T-cells to CD8+ T-cells	Score 1	5.5	(2.75 - 12.75)	p = 0.47*
	Score 2	27.5	(9.75 - 2755)	r = 0.13
	Score 3	4.5	(1.25 - 13.75)	
Number of naive T-cells	Score 1	2465	(1743.75 - 2827.5)	p < 0.05*
	Score 2	2.5	(2.5 - 33.5)	r = -0.35
	Score 3	5.5	(1.25 - 10)	
Ratio of naive to memory T-cells	Score 1	35	(9.5 - 2465)	p < 0.05*
	Score 2	6	(1 - 24.5)	r = -0.44
	Score 3	6	(4 - 9.75)	
Number of B-cells	Score 1	15	(15-15)	p = 0.36*
	Score 2	2320	(2320-2320)	r = -0.17
	Score 3	6	(2 - 26.75)	
Number of Natural Killer-cells	Score 1	3.5	(2.25 - 4.75)	p < 0.05*
	Score 2	6	(2 - 15)	r = 0.36
	Score 3	29	(4 - 295)	

*Spearman's rank correction test

Discussion

1. Count of oral Candida

Although oral Candida was detected using various sampling and measurement methods; an optimum method has not yet been established. The reported rate of oral Candida among healthy individuals varies between 35% and 80% [1,2] depending on the studied population and the detection method employed. In recent years, DNA-based methods have been developed to detect oral Candida. However, they are laborious and costly, and are associated with a risk of contamination. Kurita et al. described a method that used a Candida mannan antigen to detect oral Candida [22]. However, this method is costly and may not be able to detect the species of bacteria of the subtype that specifically react to the lasting mannan antigen of Candida albicans. The conventional detection method for oral Candida is a culture of oral samples. Although it is a relatively simple and easy detection method, many studies reported a low rate of detection [23-25]. We speculate that the method to collect Candida from the oral cavity may partly be responsible for the low rate of Candida detection by culture. Tooyama et al. recently demonstrated that a concentrated oral rinse culture (the use of a concentrated oral rinse solution) led to a higher detection rate for Candida than the conventional method (no concentrated oral rinse solution) [26]. Therefore, in the present study, we utilized the concentrated oral rinse culture and detected oral Candida in all subjects.

Regarding the number of Candida colonies, although a significant difference was not recognized with gender, the count of oral Candida significantly increased with advancing age. Mun et al. examined oral Candida carriage in asymptomatic patients and found no significant difference in carriage with gender or age [27]. Previous studies showed that a large number of factors including cigarette smoking, denture wearing, xerostomia, low saliva pH, the presence of carious lesions, and immunosuppression are associated with higher Candida carriage rates [3-8]. The prevalence of some of these factors (including denture wearing and xerostomia) is known to increase with advancing age. Due to the characteristics of our study subjects, a close relationship was observed between the oral Candida load and age.

2. Assessment of immunological vigor

Difficulties are associated with objectively evaluating and expressing systemic immunity with a numerical value. There is currently no standard laboratory procedure to evaluate systemic immunity. Hirokawa and Utsuyama [16-19] developed an immunological scoring method to standardize various immunological parameters, combine them, and express the immune status of individuals as a simple numeral, termed “immunological vigor”. In the present study, the host immune status was evaluated using SIV reported

by Hirokawa and Utsuyama. We entrusted an analysis of SIV to their laboratory (Institute for Health and Life Science). The relationship between SIV and the onset of disease has not yet been investigated from an immunological aspect. However, Hirokawa et al. reported a decline in T cell-related immune functions in cancer patients and an attempt to restore them through the infusion of activated autologous T cells by the SIV method [18]. Age-related changes of the T cell-dependent immune system were characterized by a decrease in T cell number, changes in T cell subsets and in qualitative changes such as proliferation and cytokine production [17]. In the SIV method, T cells and their characteristics were selected as core indices for the assessment of immune status [17]. Therefore, we investigated the relationship between quantity of oral Candida and the immunological status using SIV.

The results of this study revealed a correlation between the grade of immunological vigor and age. Immunological vigor decreased with advancing age. The gradual deterioration of the immune system is caused by natural age advancement [28]. Immunological function has been reported to decline with age in humans and animal model experiments [16, 29, 30]. Our results were consistent with these findings. Furthermore, not only the presence of oral candidiasis, but also BMS was related to a significantly lower grade of immunological vigor. Although conditions that have been reported to be associated with BMS include mechanical irritation, chronic anxiety or depression, nutritional deficiency, diabetes, changes in salivary function, dentures, candida infection, parafunctional habits [31-35], and allergy, the cause of BMS remains still unclear. Regarding the correlation between low-SIV grades and BMS in the present study, one of the causes of BMS may be opportunistic infections by the oral bacteria flora with a decline in immune activity. The further investigation of the correlation of between low-SIV grades and BMS is needed on based on large number of cases.

3. Correlation between the count of oral Candida and SIV

We herein found a correlation between the count of oral Candida and SIV. Since the relationship between host immunity and the count of oral Candida has not yet been examined, this is the first study to show that the immune status correlates with the oral Candida load. Thanyasrisung et al. showed that, among HIV-infected patients, a CD4 count of 200 cells mm–3 was associated with a higher prevalence of the oral carriage of Candida species. They suggested that the host immune status may influence oral colonization by Candida species [36]. Our result was compatible with their results.

In the host defense against Candida, recognition of Candida by host cells lead to activation of a cytokine response profile [37]. Interleukin-8 recruits neutrophils to the epithelium, subsequently including neutrophil-dependent mucosal defense against

Candida [38]. Neutrophils can induce epithelial cell-mediated protection against Candida infections through upregulation of Toll-like receptor 4 [38]. Neutrophils can also directly kill Candida cells through ingestion and killing, degranulation, or through the Neutrophils extracellular Traps [39,40]. A secreted chemokine (C-C motif) ligand 20 recruits the Th-17 cell subset [41]. The results of this study revealed correlations between the amount of Candida and the number of T cells, naive T cells, and NK cells. The amount of oral Candida was the higher in the subjects with the lower number of T cells and naive T cells. On the other hand, the amount of oral Candida was the lower in the subjects with the higher number of NK cells. Th-17 cells, which differentiate from T cells, were previously reported to participate in immunity to Candida [42]. The presentation of Candida-specific antigens by dendritic cells triggers the priming of T cells into a specific subset [43]. Furthermore, Dectin-2 is a specific receptor of α-mannan produced by Candida on dendritic cells and macrophages. Dectin-2 participates in the differentiation of Th-17 cells, and has been shown to play an important role in Candida infection defense [44]. The Th-17 cells responses mediate protection against mucosal candidiasis, especially since secretory IgA antibodies can inhibit the adherence of Candida to epithelial cells [45]. Additionally, Th-17 cell differentiation was strongly induced in naïve CD4+T cells cultured with C. albicans-stimulated bone marrow dendritic cells- conditioned medium [44]. These results seem to support our finding that the number of Th-17 and naïve T cells, which involved in the defense against Candida infection, were lower in the subject with increased oral Candida colonies. Although NK cells exhibit antifugal activity against fungi, the activity of NK cells is markedly weaker than that of phagocytes. In the review by Renshaw et al, the discrepancies at the levels of NK cell number and function in aged human [46]. There has been reported a significant decline in patients with oral candidiasis compared with healthy volunteers in NK activity, whereas no significant age-related decline in NK activity in healthy volunteers [47]. Although a cause of this decline was reported to be due to the ability of C. albicans to block NK activity [47], it remains unclear why there is a decline in NK activity in patients with oral candidiasis. In this study, the correlation between a number of NK cells and an amount of Candida may be supposed the possibility of C, albicans to block NK activity. Furthermore, the function of B cells in defense against Candida remains unclear. In this study, because there was no significant correlation between the number of B cell and the amount of Candida colonies, the further examination of immunomechanism of B cell in Candida infection is needed. The number of oral Candida colonies associated with T cells and their characteristics in the SIV method. These findings suggest the importance of T cells in the oral Candida load and infection.

The association between denture wearers and oral candidiasis has been reported [2]. The results of this study also showed that denture wearer had a significantly higher amount of oral Candida than non-wearers. Therefore, there is a possibility that a factor of denture wearing might have more influence on the amount of oral Candida than host immunity (a factor of denture wearing might be a cofounding factor). The comparison of amount of oral Candida between denture wearers and non-wearer was summarized in Table 3. There was no significant difference as to amount of oral Candida in each grade of SIV (Mann-Whitney test, grade 3 with denture vs grade 3 without denture : P=0.09, grade 4 with denture vs grade 4 without denture : P=0.25). In the results of this study, there was a strong correlation between denture wearing and host immunity. The status of denture wearing was correlated with not only the higher count of oral Candida but also the lower grade of SIV. These results suggested that there may be a multiplex collinearity characteristic between a factor of denture wearing and the grade of SIV, which influence the amount of oral Candida.

However, the results of this study showed that significant correlation was found between the account of oral Candida and immunological vigor whether in the subjects with or without denture wearer. Other local factors (hyposalivation, etc.) may have some influence on oral Candida colonies, however these conditions might also be related with immunological state of the host.

Conclusion

The relationship between the number of oral Candida colonies and immunological status of the host and that between the number of Candida colonies detected in the oral cavity and immunological vigor of the host were investigated in the present study. A relationship was observed between the number of oral Candida colonies and immunological status of the host. The detection of oral Candida may be a possible marker for determining immunological vigor of the host. However, it is necessary to consider the effect of local condition, such as denture wearing and xerostomia, on the amount of oral Candida colonies.

Author contributions

Hayashi K analyzed the data and drafted the manuscript.

Tooyama H and Kurashina K designed the study.

Tanaka H, Aizawa H, and Shimane T collected the data.

Yamada S drafted and edited the manuscript.

Kurita H designed the study and drafted and edited the manuscript.

Disclosure of conflicts of interest

The authors state that they have no conflicts of interest.

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