이학석사 학위논문

Mucosal and salivary microbiota associated with recurrent aphthous stomatitis

재발성 아프타성 구내염과 연관된 구강 점막과 타액의 세균

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위 원 장 오 석 배 (인)

부 위 원 장 최 영 님 (인)

위 원 최 봉 규 (인)

ABSTRACT

Mucosal and salivary microbiota associated with recurrent aphthous stomatitis

Yunji Kim

Department of Dental medicine and life Sciences

Major of Immunology and Molecular Microbiology in Dentistry

Graduate School, Seoul National University

(Directed by professor Youngnim Choi, D.D.S., Ph.D.)

Background

Recurrent aphthous stomatitis (RAS) is common oral ulcerative disease to all ages. RAS is characterized by recurrent occurrence of ulceration which is extremely painful and heal slower than traumatic ulcers. Many factors, including stress, hormonal imbalance, oxidative stress, genetic predisposition,

viral and bacterial infection, food allergies, vitamin and microelement deficiencies, have been suggested to contribute to the occurrence of RAS. However, the etiopathogenesis of RAS is unclear.

Methods

The bacterial communities of the oral mucosa and saliva from RAS patients with active lesions (RAS, n = 18 for mucosa and n = 8 for saliva) and control subjects (n = 18 for mucosa and n = 7 for saliva) were analyzed by pyrosequencing of the 16S rRNA genes. The species richness and diversity index were calculated using the Ribosomal RNA database project's pyrosequencing pipeline (http://pyro.cme.msu.edu). The overall phylogenetic distance between communities was estimated using the weighted Fast UniFrac and was visualized using PCoA.

Immortalized human oral keratinocyte HOK-16B cells originated from the retromolar gingival tissue were maintained in keratinocyte growth-culture medium (Clonetics, San Diego, CA, USA) containing supplementary growth factors. HOK-16B cells plated into 48-well plates at $4x10^4$ cells/well in triplicate were cultured for 24 h and then infected with bacteria at the multiplicity of infection (MOI) 0, 100, 500, and 1000. The viability and total number of live cells in each well were determined by trypan blue exclusion under a microscope.

Results

There were no significant differences in the richness and diversity of the mucosal and salivary microbiota between the controls and the RAS. However, the mucosal microbiota of the RAS patients showed increased inter-subject variability. A comparison of the relative abundance of each taxon revealed decreases in the members of healthy core microbiota but increases of rare species in the mucosal and salivary microbiota of RAS patients. Particularly, decreased *Streptococcus salivarius* and increased *Acinetobacter johnsonii* in the mucosa were associated with RAS risk. A dysbiosis index, which was developed using the relative abundance of *A. johnsonii* and *S. salivarius* and the regression coefficients, correctly predicted 83 % of the total cases for the absence or presence of RAS. Interestingly, *A. johnsonii* substantially inhibited the proliferation of gingival epithelial cells and showed greater cytotoxicity against the gingival epithelial cells than *S. salivarius.*

Conclusion

Pyrosequencing analysis successfully characterized the oral microbiota of RAS patients compared with healthy controls up to the species level. The mucosal microbiota of RAS lesions are characterized as decreases in the members of healthy core microbiota but increases of rare species, and a decrease in *S. salivarius* and an increase in *A. johnsonii* are associated with RAS risk. These findings may provide a diagnostic tool and new targets for the therapeutic.

management of RAS.

Keywords: Recurrent aphthous stomatitis, Dysbiosis of oral bacteria, Pyrosequencing

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CONTENTS

국문초록

1. Introduction

Recurrent aphthous stomatitis (RAS) is a common oral ulcerative disease to all ages. This oral mucosal disorder occurs in 10 to 20 % of the general populations[1]. RAS is characterized by recurrent occurrence of ulceration which is well-circumscribed and extremely painful and heal more slowly than traumatic ulcers. The size of ulcer can vary, and single or multiple ulcers can occur[2]. Many factors, including stress, hormonal imbalance, oxidative stress, genetic predisposition, viral and bacterial infection, food allergies, vitamin and microelement deficiencies, have been suggested to contribute to the occurrence of RAS[3]. However, the etiopathogenesis of RAS is unclear. Therefore, the current therapy is limited to symptomatic treatment.

Bacterial infection has been suggested as the cause of RAS. For example, S*treptococcus* strain which isolated from the RAS lesions in associated with occurrence of cross-reactive antibody[4]. However, *Streptococcus oralis* turned out less association with RAS lesions than in healthy controls[5]. Also some reports said, *Helicobacter pylori* infection is associated with RAS, but the presence of the *H. pylori* affecting the occurrence of RAS is not clear[6,7]. To identify the difference in between RAS and healthy controls, Marchihi et al.[8] analyzed the mucosal and salivary bacteria using a culture-independent method. In that study, 57 and 38 phylotypes were detected in the RAS lesions and control mucosa, respectively. Recently, some studies characterized oral microbiota using high-throughput sequencing of the 16S

rRNA gene in RAS patients[9,10]. Also other studies actively Terminal-Restriction Fragment Length Polymorphism analysis of bacterial 16S rRNA genes, the human oral microbe identification microarrays, and matrix-assisted laser desorption/ionization time-of-flight analysis have been applied to study the oral microbiota of RAS patients[11,12]. Many studies suggested changing of microbiota in the RAS patients compare with those of controls, but specific species were not identified from those studies.

We previously characterized the murine oral microbiota to the species level by pyrosequencing[13]. Therefore, this study aimed to characterize the microbiota of the oral mucosa and saliva of RAS patients compared with those of control subjects at the species level. Pyrosequencing analysis successfully characterized the oral microbiota of RAS patients and identified two species associated with RAS risk.

2. Materials and Methods

2.1 Study population and sample collection

Twenty RAS patients, visiting the Oral Medicine Clinic at the Seoul national university Dental Hospital from February 2013 to January 2014 and 20 healthy control had other oral mucosal disorder were included. Subjects who had received steroid or antibiotics within the last month, and patients with xerostomia were excluded (unstimulated whole salivary flow rate < 0.1 ml/min).

Also subject with other oral mucosal disease or systemic that involve oral ulcer were excluded. All sampled subjects were requested to not eat and not mouthwash with antiseptic for two hours before sampling. Mucosa sample, which take to a sterilized 20 mm X 20 mm polyvinylidene difluorid membrane (Millipore, Billerica, MA, USA) on the ulcerated area for 30 seconds. A minimum of 2ml unstimulated whole saliva samples were collected by a split method.

2.2. DNA extraction, 16S rRNA gene amplification, DNA pyrosequencing

Genomic DNA was taken from the membrane and the pallets of centrifugated saliva using the PowerSoil DNA Isolation Kit (MO BIO Labratories, Carlsland, CA, USA). Forty mucosal (n= 20 and n=20 for healthy controls and RAS patients respectively) and 20 salivary (n=10 and n= 10 for healthy controls and RAS patients, respectively) were requested to pyrosequencing analysis. The genomic DNA was amplified using primers targeting the V1 to V3 hypervariable region of bacterial 16S rRNA gene, and the PCR products were sequenced according to the previously described method[13] using 454 GS FLX Titanium Sequencing System (Roche, Branford, CT, USA). Both the 16S rRNA gene amplification and sequencing were performed at ChunLab Inc. (Seoul, Korea). Out of the 60 samples analyzed, we successfully obtained data sets for 39 mucosal (n= 19 and n=20 for the Healthy controls and RAS patients,

respectively) and 17 salivary (n=8 and n=9 for healthy controls and RAS patients, respectively) microbiota communities. Four samples failed in pyrosequecing due to fail to amplification of 16s rRNA gene. After all sample exclusions, the final data in this study includes 36 mucosal (n=18 and n=18 for healthy controls and RAS patients, respectively) and 15 salivary (n=7 and n=8 for healthy controls and RAS patients, respectively) microbiota communities. The pyrosequencing data are available in the SRP database under the accession number SRP049562. The entire process from the enrollment of subjects to the acquisition of final data sets is illustrated as a flow chart (Figure. 1).

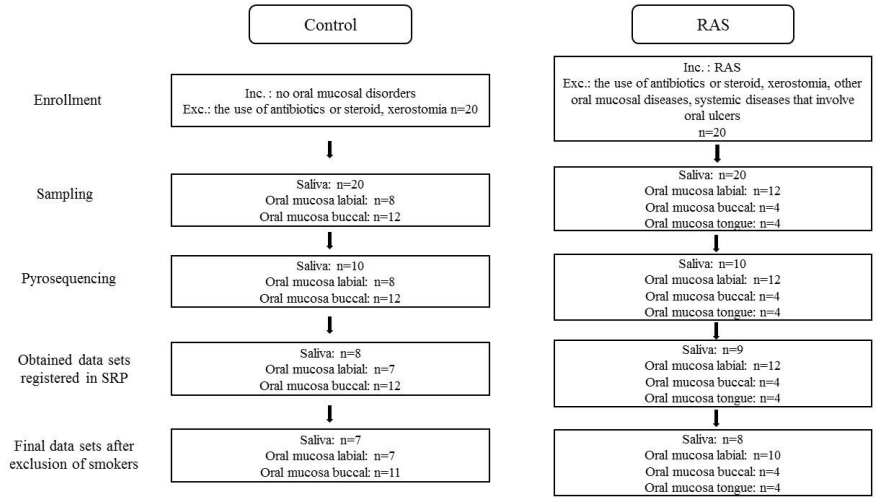

Figure 1. A flow chart from the enrollment of subjects to the acquisition of final data sets.

2.3. Pyrosequencing data analysis

The basic analysis were followed according to previously published descriptions[13]. After removing PCR primers, any reads shorter than 300bp, ambiguous nucleotides and chimera sequence were removed. The taxonomic classification of each read was assigned against the EzTaxon database-e (http://www.ezbiocloud.net/eztaxon)[14]. The species richness and diversity index were calculated using the Ribosomal RNA database project's pyrosequencing pipeline (http://pyro.cme.msu.edu). The cutoff value for assigning a sequence to the same phylotype was ≥ 97 % similarity. Random subsampling was conducted to equalize variation in the read counts among the samples. The overall phylogenetic distance between communities was estimated using the weighted Fast UniFrac[15] and was visualized using PCoA. In addition, the pyrosequencing data of buccal mucosa and saliva published by The Human Microbiome Project (HMP) Consortium[16] were also analyzed to compare with the healthy controls of this study.

2.4. Bacterial and cell culture

A. johnsonii KCTC 12405 (Korean Collection for type culture, Deajeon, Korea) was cultured in brain heart infusion medium at 30 °C and aerobic condition. *S. salivarius* KCTC 5512 (KCTC) and *Porphyromonas gingivalis* ATCC 33277 (American Type Culture Collection, Manassas, VA, USA) were cultured in ATCC medium 188 and BHI medium supplemented with 5 μg/ml hemin and

10 μg/ml vitamin K, respectively, at 37 °C under an anaerobic condition. Bacteria were harvested in log phage growth and bacterial concentration were measured by flow cytometry[17]. Immortalized human oral keratinocyte HOK-16B cells originated from the retromolar gingival tissue[18] were maintained in keratinocyte growth-culture medium (Clonetics, San Diego, CA, USA) containing supplementary growth factors.

2.5. Trypan blue assay

HOK-16B cells plated into 48-well plates at 4 x 10^4 cells/well in triplicate were cultured for 24 h and then infected with bacteria at the multiplicity of infection (MOI) 0, 100, 500, and 1000 as previously described[19]. Atmosphere of 95 % air and 5 % CO_2, cells were harvested, including the dead cells in the supernatant. The viability and total number of live cells in each well were determined by trypan blue exclusion under a microscope.

2.6. Statistical analysis

The data are presented as the mean ± the standard errors of means, unless described otherwise. The differences in relative abundance and in UniFrac distances between the two groups were determined with the Mann-Whitney U test and t-test, respectively. The association of bacterial species with RAS risk was determined with a logistic regression analysis. Differences in the viability and proliferation between control and infected cells were analyzed by t-test. All

statistics were performed using the SPSS Statistics19 software (SPSS Inc., Chicago, IL, USA). Significance was set at $P < 0.05$.

3. Results

3.1. Subjects

The demographic information of healthy control subjects and RAS patients are summarized in Table 1. Eighteen patients included and distribution of age was 19 to 81 years. Sampling site of RAS patients included the lip labial mucosa, the buccal mucosa and tongue tips. Eighteen healthy control included and distribution of age was 21 to 71 years. Sampling site of healthy control subject were the lip labial mucosa and buccal mucosa.

Table 1. The demographic data of the control subjects and RAS patients

	Control subjects (n = 18)	RAS patients (n = 18)
Gender	8 males, 10 females	9 males, 9 females
Age	43.6 ± 3.7	43.8 ± 3.9
Ulcer numbers	-	Single: 12 (66.7%) Multiple: 6 (33.3%)
Sampling sites	Lip labial mucosa: 7 (38.9%), Buccal mucosa: 11 (61.1%)	Lip labial mucosa: 10 (55.6%), Buccal mucosa: 4 (22.2%) Tongue tip: 4 (22.2%)
Unstimulated salivary flow rates	0.48 ± 0.09 ml/minute	0.67 ± 0.08 ml/minute

3.2. The alpha and beta diversities of the oral microbiota

In the 51 communities, total 484,501 filtered reads were obtained and each sample showed in greater than 99% Good's coverage. First, we analyzed the alpha diversity of healthy controls and RAS patients. Chao 1, which represents the species richness was not significantly different between RAS patients and healthy controls both in the mucosa (314 ± 19 and 292 ± 22) or in the saliva (377 ± 14 and 447 ± 0.06). The diversity of the microbiota communities as determined by the Shannon index presented similar patterns. The diversities of RAS mucosal microbiota were slightly lower (3.57 ± 0.11 and 3.56 ± 0.06) but in the RAS saliva microbiota showed slightly higher (4.02 ± 0.09 and 4.12 ± 0.1) without significance (Figure. 2a).

UniFrac-based principal coordinate analysis (PCoA) to determine variation among the samples revealed that the microbiota profile was differentiated better by the anatomical sites, i.e., mucosal surfaces vs. saliva, than by disease. The different locations in the mucosa, i.e., tongue tip, labial, or buccal, did not show distinct clustering (Fig. 2b). Although PCoA clustering did not reveal clear separation between the control and RAS communities, the intergroup UniFrac distance (0.061 ± 0.001) was higher than the intragroup distance of controls (0.057 ± 0.001), suggesting a significant difference in the bacterial profile between control and RAS samples. In addition, a higher intragroup UniFrac distance in the RAS (0.067 ± 0.001) compared to the control

group suggested the increased inter-subject variability for RAS lesions (Fig. 2c). In the salivary communities that reflect not only the diseased sites but also the healthy sites of patients, no significant differences were observed in intra- or intergroup UniFrac distances.

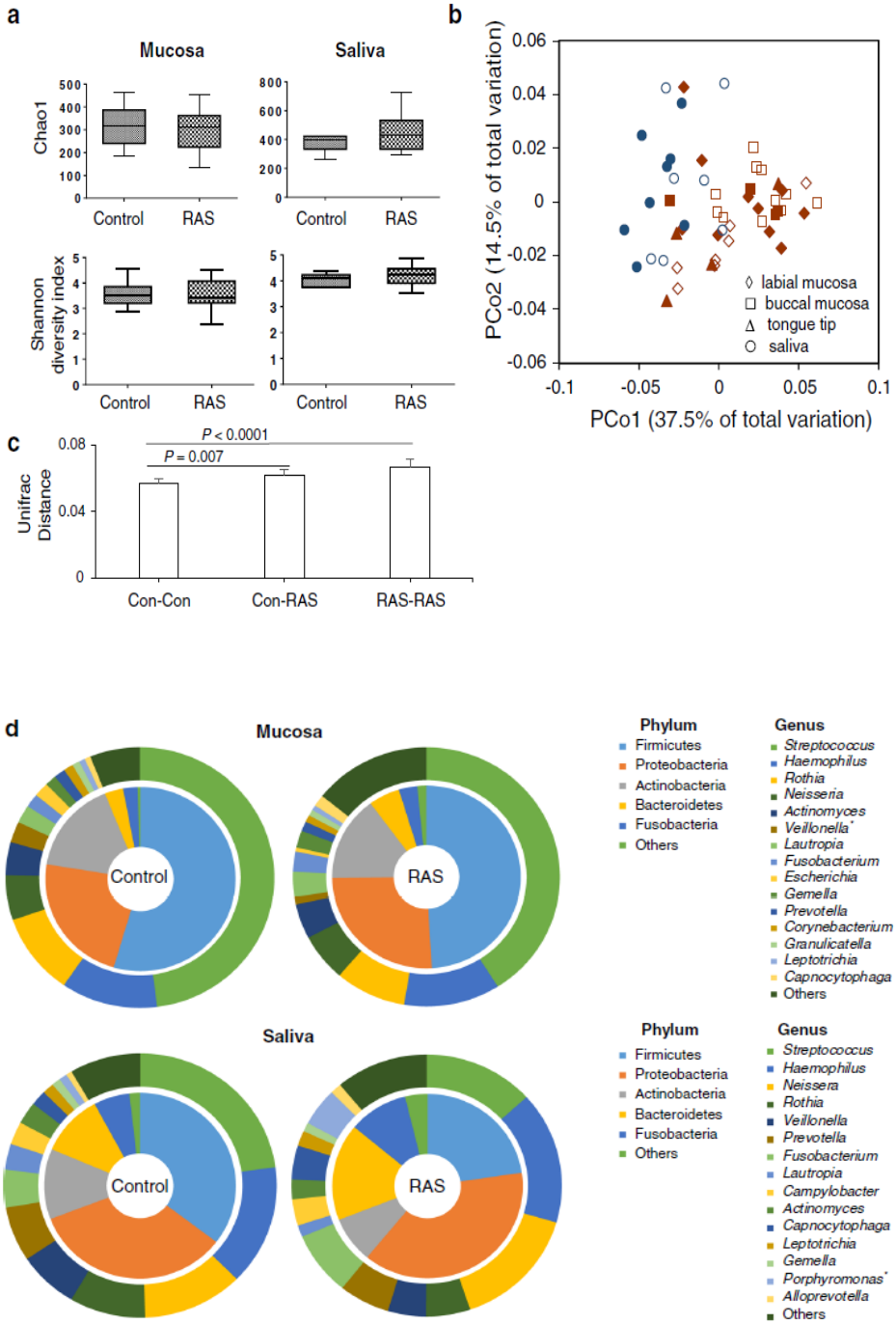
a
Mucosa
Saliva
Chao1
Shannon diversity index
Control
RAS
b
PCo2 (14.5% of total variation)
PCo1 (37.5% of total variation)
labial mucosa
buccal mucosa
tongue tip
saliva
c
Unifrac Distance
P < 0.0001
P = 0.007
Con-Con
Con-RAS
RAS-RAS
d
Mucosa
Saliva
Control
RAS
Phylum
Firmicutes
Proteobacteria
Actinobacteria
Bacteroidetes
Fusobacteria
Others
Genus
Streptococcus
Haemophilus
Rothia
Neisseria
Actinomyces
Veillonella
Lautropia
Fusobacterium
Escherichia
Gemella
Prevotella
Corynebacterium
Granulicatella
Leptotrichia
Capnocytophaga
Others
Phylum
Firmicutes
Proteobacteria
Actinobacteria
Bacteroidetes
Fusobacteria
Others
Genus
Streptococcus
Haemophilus
Neissera
Rothia
Veillonella
Prevotella
Fusobacterium
Lautropia
Campylobacter
Actinomyces
Capnocytophaga
Leptotrichia
Gemella
Porphyromonas
Alloprevotella
Others

Figure 2. Comparison of mucosal and salivary microbiota between control and RAS. (a) The species richness estimated by Chao1 and Shannon diversity index are expressed using box and whisker plots. (b) PCoA plot generated using weighted Unifrac metric. The two components explained 52% of variance. (unfilled symbols: control samples, filled symbols: RAS samples). (c) The intra- and intergroup Unifrac distances of mucosal communities were obtained using weighted metric. (d) Double pie charts present the mean relative abundance of dominant phyla (top 5) and genera (top 15). * denotes significant difference by Mann-Whitney U test ($P < 0.01$)

3.3. Comparison of oral microbiota composition between the healthy subjects and RAS patients.

A total of 26 different phyla were identified by comparison of relative abundance of each taxon from the control and RAS mucosal samples. Four phyla, *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Fusobacteria* account for the majority of the sequences (>99% in controls and 97% in RAS). The relative abundance of the major phyla showed in the controls was not different from those of the RAS patients. But a minor phylum, *Streptophyta* was significantly increased in the RAS patients (P=0.03). In the genus level, *Streptococcus* encompassed almost half of the mucosal microbiota in the control group. Other major genera were consist of *Haemophilus*, *Rothia*, *Neisseria*, *Actinomyces*, and *Veillonella*, which significantly reduced abundance in the RAS patients (Figure. 2d). Also fourteen other genera showed differences in the relative abundance. At the species level, the abundance of several *Streptococcus*, incorporating *S. salivarius*, *V. dispar*, *R. dentocariosa*, *A. odontolyticus*, and *Prevotella histicola* was decreased in the RAS patients. Instead, the abundance of six species, *Acinetobacter oryzae*, *A. johnsonii*, *Capnocytophaga sputigena*, *N. oralis*, *Myxococcus xanthus*, *Ruminococcus gnavus*, and *Treponema denticola* were increased (Table 2). In the RAS salivary microbiota, an increase in *Firmicutes* and a decrease in *Proteobateria* were observed among the major phyla, but the differences were not significant. Only, SR1 showed significant difference (0.01 ± 0.004 and 0.6 ± 0.5, P= 0.02). At the

genus level, four genera, including *Porphyromonas*, *GU410548_g (SR1)*, *Abiotrophia*, and *Streptococcaceae_uc*, were significantly increased in the RAS salivary microbiota (Table 3). In the RAS samples, the amount of *S. salivarius* was decreased at the species level. Instead, the abundance of eight species was decreased (Table 3).

Table 2. Relative abundance[a] of taxa differently distributed between the controls and RAS in the mucosal microbiota

		Controls (n=18)	RAS(n=18)	*P* value
Genus	*Veillonella*	1.86 (0.49–10.44)	0.82 (0–3.33)	0.003
	Acinetobacter	0 (0–2.54)	1.04 (0–7.69)	0.001
	DQ241813_g (Flavobacteriaceae) [b]	0.05 (0–1.72)	0.14 (0–11.22)	0.04
	Lachnoanaerobaculum	0.08 (0.02–0.87)	0.04 (0–0.47)	0.017
	Blautia	0 (0–0.24)	0.02 (0–3.73)	0.01
	Myxococcus	0 (0–0.14)	0.04 (0–3.73)	0.031
	Alloprevotella	0.04 (0–0.22)	0.09 (0–0.49)	0.031
	Pseudomonas	0 (0–0.33)	0.02 (0–2.28)	0.027
	Atopobium	0.04 (0–0.44)	0 (0–0.42)	0.037
	Ruminococcus_g6	0 (0–0.55)	0.02 (0–0.86)	0.031
	Faecalibacterium	0 (0–0.09)	0.02 (0–0.58)	0.014
	Staphylococcus	0 (0–0.15)	0.02 (0–0.28)	0.009
	Streptococcaceae_uc	0.02 (0–0.06)	0 (0–0.04)	<0.0001
	Flavobacterium	0 (0–0)	0 (0–0.38)	0.047
	Clostridium_g6	0 (0–0)	0 (0–0.14)	0.047
Species	*Streptococcus salivarius*	4.84 (0.08–18.08)	0.61 (0–10.20)	0.001
	Veillonella dispar	1.63 (0.29–5.30)	0.60 (0–2.16)	0.003
	Streptococcus parasanguinis	1.12 (0–11.45)	0.06 (0–0.98)	0.001
	Rothia dentocariosa	0.53 (0.01–9.91)	0.10 (0–3.71)	0.034
	Acinetobacter oryzae	0 (0–2)	0.75 (0–5.88)	0.01
	Actinomyces odontolyticus	0.43 (0–1.73)	0.11 (0–1.45)	0.02
	Capnocytophaga sputigena	0.01 (0–0.53)	0.08 (0–3.26)	0.047
	Acinetobacter johnsonii	0 (0–0.54)	0.21 (0–1.86)	0.001
	Streptococcus_uc	0.14 (0.03–1.35)	0.05 (0–0.16) 0	0.001
	FM997095_s (Streptococcus) [b]	0.17 (0.01–0.91)	0.03 (0–1.25)	0.005
	Neisseria oralis	0 (0–0.16)	0.03 (0–1.83)	0.017
	HQ757980_s (Streptococcus) [b]	0.07 (0–1.15)	0 (0–2.32)	0.017
	4P003152_s (Streptococcus) [b]	0.06 (0–1.27)	0 (0–0.09)	0.006
	Campylobacter concisus	0.09 (0–0.52)	0.02 (0–0.14)	0.002
	Prevotella histicola	0.01 (0–2.29)	0 (0–0.29)	0.027
	Myxococcus xanthus	0 (0–0.14)	0.04 (0–3.73)	0.031
	Streptococcus vestibularis	0.02 (0–0.33)	0 (0–1.25)	0.001
	Streptococcus lactarius	0.02 (0–0.87)	0 (0–0.10)	0.031
	4P002810_s (Prevotella) [b]	0.05 (0–0.38)	0 (0–0.34)	0.024
	Ruminococcus gnavus	0 (0–0.53)	0.02 (0–0.86)	0.034
	BABG01000051_s (Faecalibacterium	0 (0–0.07)	0.01 (0–0.52)	0.016
	Treponema denticola	0 (0–0.10)	0.02 (0–0.14)	0.031
	Streptococcaceae_uc_s	0.02 (0–0.06)	0 (0–0.04)	<0.0001
	FJ976422_s (Alloprevotella) [b]	0 (0–0.17)	0.01 (0–0.14)	0.047

[a]Relative abundance expressed as the median and range

[b]The lowest taxonomic rank classified to which the unclassified genus or species belongs

Table 3. Relative abundance[a] of taxa differently distributed between the controls and RAS in the salivary microbiota

		Control (n=7)	RAS (n=8)	*P* value
Genus	*Porphyromonas*	0.40 (0.17–2.65)	4.51 (0.88–12.27)	0.006
	GU410548_g (SR1) [b]	0.01 (0–0.02)	0.06 (0–4.72)	0.021
	Abiotrophia	0 (0–0)	0.04 (0–0.28)	0.014
	Streptococcaceae_uc	0 (0–0.02)	0.03 (0–0.04)	0.029
Species	*Streptococcus salivarius*	2.18 (0.76–10.42)	0.74 (0.03–3.84)	0.021
	Neisseria flava	0.06 (0–1.16)	0.53 (0–6.99)	0.04
	Capnocytophaga gingivalis	0.21 (0.06–0.62)	0.68 (0.04–3.50)	0.029
	Aggregatibacter segnis	0.02 (0–0.19)	0.42 (0–2.65)	0.021
	Capnocytophaga sputigena	0.06 (0–0.20)	0.38 (0.06–1.62)	0.004
	FM995684_s (Porphyromonas) [b]	0 (0–0.27)	0.30 (0–3.91)	0.029
	4P003196_s (Actinomyces) [b]	0.03 (0.01–0.42)	0.14 (0.10–2.09)	0.04
	Neisseria sicca	0 (0–0.25)	0.17 (0.02–1.37)	0.006
	Porphyromonas_uc	0 (0–0.03)	0.06 (0–0.55)	0.04
	Abiotrophia defectiva	0 (0–0)	0.03 (0–0.28)	0.04
	4P004176_s (SR1) [b]	0 (0–0.01)	0.03 (0–0.23)	0.021
	treptococcaceae_uc_s	0 (0–0.02)	0.03 (0–0.04)	0.029

[a]Relative abundance expressed as the median and range

[b]The lowest taxonomic rank classified to which the unclassified genus or species belongs

3.4. Identification of bacterial species associated with RAS risk

To explore the bacteria, associated with RAS risk, we analyzed the top 100 species in the mucosal microbiota by logistic regression analysis. In forward method, the abundance of *S. salivarius* was associated with reduced RAS risk (OR 0.734 per 1% increase, CI 95% 0.565-0.954, *P*=0.02), and the abundance of *A. johnsonii* was associated with an increased RAS risk (OR 211 per 1% increase, CI 95% 1618-2.7E4, *P*=0.03). In the RAS salivary microbiota, none of the species showed significant association with RAS.

A dysbiosis index was defined as 5.35 × [*A. johnsonii*] - 0.309 × [*S. salivarius*] using the relative abundance of *A. johnsonii* and *S. salivarius* in the mucosa where 5.35 and -0.309 are the regression coefficients. The dysbiosis index was significantly associated with RAS risk (OR 2.76. CI 95 % 1.26-6.05, *P* = 0.01) and correctly predicted 83 % of the total cases for the absence or presence of RAS (94 % of control and 72 % of RAS, Fig. 3).

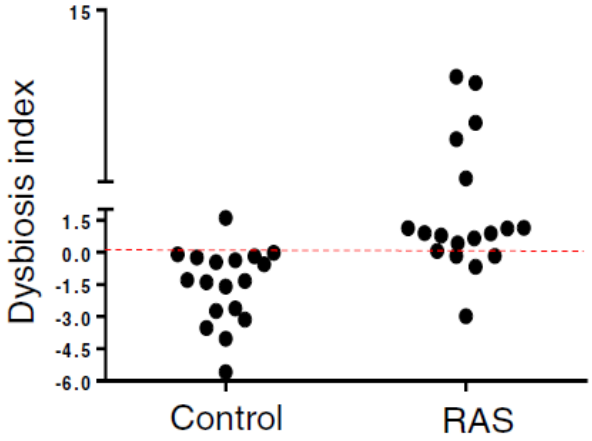

Fig 3. Dysbiosis index of RAS. A dysbiosis index was defined as 5.35 × [*A. johnsonii*] - 0.309 × [*S. salivarius*] using the relative abundance of *A. johnsonii* and *S. salivarius* in the mucosa where 5.35 and -0.309 are the regression coefficients. The dysbiosis index of 18 control samples and 18 RAS samples are shown. The dotted line indicates cutoff for RAS.

3.5. Role of RAS associated bacteria in the etiopathogenesis of RAS

Two RAS-associated bacteria were incubated with oral epithelial cells to understand the potential role of bacteria in the pathogenesis of RAS and examined the viability and proliferation ability of cells. Also other periodontal pathogen, *P. gingivalis* that has been reported to inhibit wound healing in an *in vitro* scratch assay, was used as a control. *A. johnsonii* increased cytotoxicity against HOK-16B cells at all MOIs, but *S. salivarius* showed low levels of cytotoxicity only at MOI 1000 (Figure. 3a). Also *A. johnsonii* showed inhibition of HOK-16B cell proliferation in a dose dependent manner (Figure. 4b).

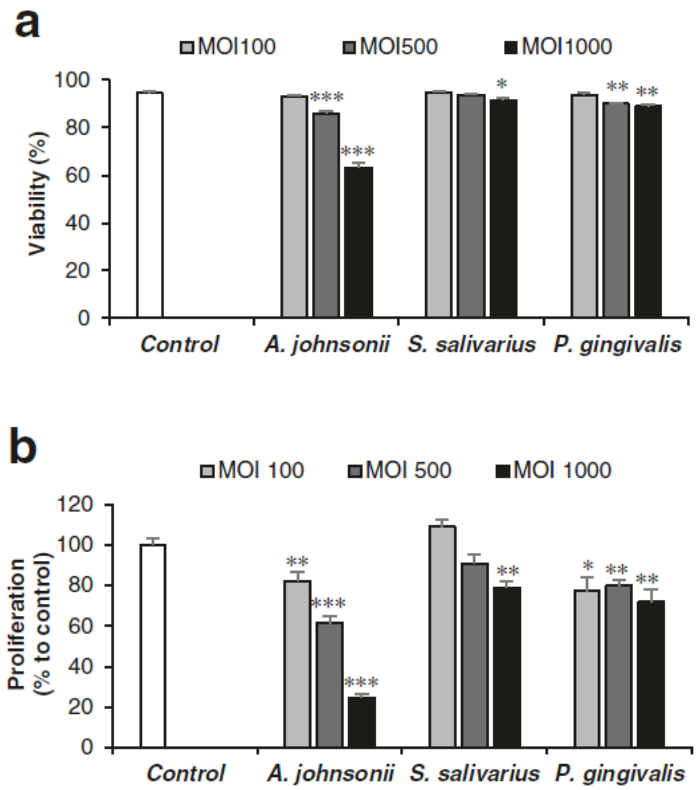

Fig 4. The effect of RAS-associated bacterial species on the viability and proliferation of human oral epithelial cells. HOK-16B cells were infected with *A. johnsonii*, *S. salivarius*, and *P. gingivalis* at MOIs of 100, 500, and 1000 for 24 hours. The viability (a) and the number of live HOK-16B cells (b) in six wells from two independent experiments were determined by trypan blue exclusion and compared with control cells without bacterial infection.*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ versus control.

IV. Discussion

In this study, imbalances between commensals and rare species in the oral mucosal and salivary microbiota were characterized in RAS patients. Periodontitis and inflammatory bowel disease are well known examples for the diseases the pathogenesis of which involves dysbiosis of microbiota. Whereas periodontitis is associated with an increase in the diversity of plaque microbiota, Crohn's disease is associated with the reduced bacterial diversity of colonic microbiota[20,21]. RAS was not associated with changes in the diversity of the mucosal or salivary microbiota, which agrees with previous studies[9,11]. However, increased inter-subject variability of the mucosal microbiota was observed in the RAS patients.

The PCoA plot revealed no separate clustering of RAS from the healthy control subjects, which means there was no big difference in microbial composition. However, difference in several taxonomic levels were observed. By regression analysis, two bacterial species, *S. salivarius* and *A. johnsonii* were identified to be associated with decreased and increased RAS risk respectively. A dysbiosis index that was calculated using the relative abundance and regression coefficients of *A. johnsonii* and *S. salivarius* correctly predicted RAS in 83% of total cases. We can use the index to diagnose RAS using and provide the more accurate treatment than present methods.

In the current study, the bacterial species that were increased in RAS compared to control group, did not belong to the normal oral mucosal and

salivary flora. *A. johnsonii*, a species associated with increased RAS risk, is known to be a member of skin flora[22] and has been isolated from the blood from the clinical samples in association with bacteremia[23]. *R. gnavus* which also increased in the RAS patients, is associated with Crohn's disease[24]. In addition, increased *Capnocytophaga sputigena* was showed in the salivary microbiota of RAS patients. *Capnocytophaga* is normally found in the oral cavity but considered as an opportunistic pathogen in the endodontic infections, emphysema and bacteremia[25-27].

Among the 15 species/phylotypes significantly decreased in the RAS mucosa compared to the controls, nine species including *S. salivarius, S. parasanguinis, S. peroris, S. vestibularis, S. lactarius, V. dispar, Rothia dentocariosa, Campylobacter concisus, Actinomyces odontolyticus, and P. histicola* belonged to those defined as the normal flora of the oral mucosa[1,22], and the six unclassified phylotypes also belonged to *Streptococcus* and *Prevotella*. It has been reported that the relative abundance of *Streptococcus* was negatively associated with the concentrations of IL-1β and IL-8 in saliva[11]. To sum up pyrosequencing data analysis, increased *A. johnsinii* and decreased *S. salivarius* may contribute to ulceration, delayed healing, and severe pain caused by inflammatory cytokines, all of which are associated with RAS.

The current study has several limitations. First, the mucosal specimens of the control subjects were sampled from the labial and buccal mucosa, while the sampling sites from the RAS patients also included the tip of the tongue.

Second, the limited number of total cases requires further study in larger cohorts and also in diverse populations, considering the differences in the relative abundance of major phyla comprising healthy microbiota between Koreans and the Human Microbiome Project subjects[17]. Third, the non-ulcer sites of RAS patients were not studied. According to the study by Bankvall et al., the mucosal microbiota at the non-ulcer sites of RAS patients was different from that of controls, and the differences were most profound in patients who had lesions during sampling [11].

For the therapeutic approaches, *S. salivarius* could use as probiotic application and antibiotics, which can kill harmful bacteria such as *A. johnsonii*. These findings may provide a diagnostic tool and new targets for the therapeutic management of RAS.

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국문초록

재발성 아프타성 구내염과 연관된 구강 점막과 타액의 세균

김 윤 지

서울대학교 대학원

치의생명과학과 면역 및 분자미생물치의학 전공

(지도교수: 최 영 님)

1. 목 적

재발성 아프타성 구내염은 구강 점막에서 흔하게 일어나는 질병 중의 하나로 유병률이 약 10-20%에 이른다고 보고되고 있다. 이 질병의 임상적인 특성으로는 궤양이 발생되며 재발된다. 아프타성 구내염의 궤양은 극심한 통증과 더불어 다른 구강 점막 궤양에 비해 치유되기까지 오랜 시간이 걸리는 것으로 알려져 있다. 이 질병의 원인으로 생각되는 요소들은 유전적인 영향, 면역학적인 장애, 바이러스나 세균의 감염, 음식물에 대한 알러지 반응 등이다. 하지만 이 질병의 원인 병인론은 정확하게 밝혀지지 않았기 때문에, 구강 세균이 병인에 관여할 가능성과 더 나아가 정확한 질병의 치료법을 연구하고자 하였다.

2. 방 법

20 명의 재발성 아프타성 구내염을 가지고 있는 환자와 20 명의 건강한 사람의 구강 점막과 타액에서 세균을 채취하였다. 구강 점막의 세균 채취는 20 mm X 20 mm 의 크기의 멸균된 polyvinylidene difluorid membrane 를 환부에 30 초간 접촉시켜 얻었으며, 타액은 비자극성 타액을 5 분간 채취하였다.

Power Soil Bacterial DNA Isolation Kit를 이용하여 membrane 과 타액에서 세균 DNA를 추출하였다. 샘플 중에서 점막에서 얻은 샘플 40개(건강한 사람 :20개, 재발성 아프타성 구내염 환자 :20개), 타액에서 얻은 샘플 20개(건강한 사람 : 10개, 재발성 아프타성 구내염 환자 :

10개)를 파이로시퀀싱 분석을 하였다. 의뢰한 총 60개의 샘플 중, 39개의 점막 샘플과 17개의 타액 샘플의 파이로시퀀싱 결과를 얻을 수 있었다. 건강한 사람과 재발성 아프타성 구내염 환자간의 구강 세균 조성 차이를 비교하기 위하여 파이로시퀀싱 결과를 이용하여 alpha 다양성 분석을 수행하였고, 각 그룹간의 계통발생학적인 차이를 밝히기 위하여 Unifrac 분석을 통하여 그 결과를 PCoA로 시각화 하였다. 회귀분석을 통하여 *Acinetobacter johnsonii*와 *Streptococcus salivarius*가 재발성 아프타성 구내염의 생성과 관련이 있는 것으로 확인이 되었다.

파이로시퀀싱의 결과로 얻은 재발성 아프타성 구내염에 관련된 두가지 균, *A. johnsonii* KCTC 12405와 *S. salivarius* KCTC 5512를 각각 30℃로 호기성 조건, 37℃로 혐기성 조건에서 배양하여 이용하였다. 이 두 세균이 구강 상피세포에 미치는 독성을 확인하기 위하여 세포를 48-well plate에 $4x10^4$으로 배양한 후, 두 세균을 24시간동안 감염시켜 살아있는 세포와 죽은 세포를 trypan blue로 염색하여 현미경 하에서 수를 세었다.

3. 결 과

건강한 사람 군과 재발성 아프타성 구내염 환자 군 사이의 alpha 다양성을 비교해 본 결과 점막과 타액 모두 종 풍부성의 지수, 종 다양성의 지수의 값에서 유의한 차이를 보이지 않았다. 각 샘플끼리의 비교하기 위하여 Unifrac-based principal coordinate 분석을 해 본 결

과 정확한 구분이 지어지지는 않았지만 건강한 사람들과 비교하였을 때 재발성 아프타성 구내염을 가진 환자들의 점막 샘플에서 세균의 다양성이 커진 것을 발견 할 수 있었다.

각 점막 샘플에서 세균 조성 비율을 분석해 본 결과, 유의한 차이를 볼 수 없었으며 소수로 존재하던 *Streptophyta*는 건강한 사람과 비교했을 때 재발성 아프타성 구내염 환자에서 그 값이 유의하게 증가하는 것을 볼 수 있었다. 속 수준에서 비교를 해 본 결과, 건강한 사람들의 세균 조성과 비교하여 재발성 아프타성 구내염 환자에서 *Veillonella* 속의 수치가 유의하게 감소한 것을 관찰 할 수 있었으며, 나머지 14개의 속에서도 차이를 볼 수 있었다. 종 수준의 비교에서는 *S. salivarius*, *Veillonella dispar*, *Rothia dentocariosa*, *Actinomyes odontolyticus*, *Prevotella histicola*가 건강한 사람에 비해 재발성 아프타성 구내염 환자에서 감소했으며, *Acinetobacter oryzae*, *A. johnsonii* 등 총 7개의 종이 재발성 아프타성 구내염 환자에서 증가하는 것을 관찰 할 수 있었다.

회귀분석을 통하여 점막에서 발견된 세균들 중에서 순위 100안에 드는 세균들을 분석해본 결과, *S. salivarius*의 감소와 *A. johnsonii*의 증가가 재발성 아프타성 구내염 발생과 유의한 관련이 있었다. 또한 세균 조성 비율의 값을 이용하여 dysbiosis index를 얻어 재발성 아프타성 구내염 생성여부가 83%의 값으로 예측됨을 알 수 있었다.

회귀분석의 결과로 확인한 두 세균이 구강 상피 세포에 미치는 영향

을 확인하기 위하여 구강 상피세포를 감염을 시킨 후 세포의 증식과 사멸을 관찰 해 보았다. 그 결과 *A. johnsonii*가 *S. salivarius*에 비하여 매우 큰 세포 독성을 가지는 것을 관찰 할 수 있었고 특히 *A. johnsonii*의 경우 구강 상피세포의 증식도 억제하였다.

4. 결 론

파이로시퀀싱 결과 분석을 통하여 아프타성 구내염 환자와 건강한 사람군 사이의 구강 점막, 타액에서의 세균의 차이를 종의 수준까지 밝혀내었다. 아프타성 구내염 환자의 점막에서는 일반적인 세균들의 감소와 적은 수로 존재하는 세균들의 증가를 관찰 할 수 있었다. 특히 *S. salivarius*의 감소와 *A. johnsonii*의 증가를 관찰 할 수 있었다. 본 연구의 결과는 더욱 효과적인 아프타성 구내염 치료법의 고안과 진단 기술 개발을 위한 초석이 되는 지식을 제공한다.

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