EFFECT OF SMOKING ON THE PERI-IMPLANT BACTERIAL ECOLOGY

Miss Maneerat Kuptanon

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Esthetic Restorative and Implant Dentistry

Faculty of Dentistry Chulalongkorn University

Academic Year 2012

ผลของการสูบบุหรี่ต่อระบบนิเวศของแบคทีเรียรอบรากเทียม

นางสาวมณีรัตน์ คุปตานนท์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาทันตกรรมบูรณะเพื่อความสวยงามและรากเทียม
คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2555

Thesis Title	Effect of Smoking on the Peri-implant Bacterial Ecology
By	Miss Maneerat Kuptanon
Field of Study	Esthetic Restorative and Implant Dentistry
Thesis Advisor	Anjalee Vacharaksa, Ph.D.

Accepted by the Faculty of Dentistry, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

…….. Dean of the Faculty of Dentistry
(Assistant Professor Suchit Poolthong, Ph.D.)

THESIS COMMITTEE

…….. Chairman
(Associate Professor Chalermpol Leevailoj)

…….. Thesis Advisor
(Anjalee Vacharaksa, Ph.D.)

…….. External Examiner
(Somying Patntirapong, Ph.D.)

มณีรัตน์ คุปตานนท์: ผลของการสูบบุหรี่ต่อระบบนิเวศของแบคทีเรียรอบรากเทียม. (EFFECT OF SMOKING ON THE PERI-IMPLANT BACTERIAL ECOLOGY) อ.ที่ปรึกษาวิทยานิพนธ์: อ.ทพญ.ดร.อัญชลี วัชรักษะ, 50 หน้า.

วัตถุประสงค์ เพื่อศึกษาแบคทีเรียก่อโรคปริทันต์คือ *พอร์ไฟโลโมนัส จินจิวาลิส ทรีโพนีมา เด็นติโคลา แทนเนอเรลลา ฟอร์ไซเทีย และพรีโวเทลลา อินเตอร์มีเดีย* ในร่องปริทันต์ปกติรอบรากเทียมของคนสูบบุหรี่และไม่สูบบุหรี่

วิธีทดลอง ผู้ป่วยรากเทียมจะถูกคัดเลือกแบบสุ่มและแบ่งเป็นกลุ่มคนสูบบุหรี่จำนวน 7 คน และไม่สูบบุหรี่จำนวน 7 คน เชื้อโรครอบรากเทียมจะถูกซับจากร่องเหงือกด้วยกระดาษปลายแหลมนำมาสกัดดีเอ็นเอ และทดสอบปฏิกิริยาลูกโซ่พอลิเมอเรสด้วยไพรเมอร์ที่มีความจำเพาะต่อแบคทีเรียทั้ง 4 ชนิด

ผลการทดลอง เชื้อแบคทีเรียก่อโรคปริทันต์ตรวจพบได้ในกลุ่มคนสูบบุหรี่เป็นสัดส่วนมากกว่าคนไม่สูบบุหรี่ โดยตรวจพบเชื้อ*ทรีโพนีมา เด็นติโคลา* ในกลุ่มคนสูบบุหรี่มากกว่าคนไม่สูบบุหรี่อย่างมีนัยสำคัญทางสถิติด้วยการทดสอบฟิชเชอร์เอ็กแซคท์ที่ระดับนัยสำคัญ 0.029 และสัดส่วนปริมาณเชื้อ*ทรีโพนีมา เด็นติโคลา*ต่อปริมาณเชื้อแบคทีเรียทั้งหมดในกลุ่มคนสูบบุหรี่ที่วัดได้มีค่าสูงกว่ากลุ่มคนไม่สูบบุหรี่อย่างมีนัยสำคัญทางสถิติด้วยการทดสอบแมนวิทนีย์ ยูที่ระดับนัยสำคัญ 0.026

สรุปผลการทดลอง ผลการทดลองบ่งชี้ว่าการสูบบุหรี่เป็นปัจจัยที่มีอิทธิผลต่อระบบนิเวศของแบคทีเรียรอบรากเทียมและอัตราผลสำเร็จของการบูรณะด้วยรากเทียม

สาขาวิชา ทันตกรรมบูรณะเพื่อความสวยงาม ลายมือชื่อนิสิต.....

และทันตกรรมรากเทียม ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา 2555

5276156432: MAJOR ESTHETIC RESTORATIVE AND IMPLANT DENTISTRY
KEYWORDS: smoking, periimplantitis, Oral Microbiota, *Treponema denticola*, the red complex bacteria

MANEERAT KUPTANON: EFFECT OF SMOKING ON THE PERI-IMPLANT BACTERIAL ECOLOGY. ADVISOR: ANJALEE VACHARAKSA, DDS, Ph.D., 50pp.

Objective To investigate periodontal pathogenic bacteria, *Porphyromonas gingivalis, Treponema denticola, Tannerella forsythia,* and *Prevotella intermedia* in the healthy peri-implant sulci of smokers or nonsmokers using 16S rRNA-based PCR.

Materials and Methods Patients who received implant-supported fixed partial prostheses were randomly selected and categorized in to smokers (n=7) and nonsmokers (n=7).Sterile paper points were used to collect submucosal samples from healthy peri-implant sulci, and then DNA was extracted. Endpoint and quantitative PCR were performed to identify *Porphyromonas gingivalis, Treponema denticola, Tannerella forsythia, Prevotella intermedia* and total bacteria.

Results The prevalence of periodontal pathogenic bacteria was higher in smoker with statistical significant different in *Treponema denticola* by Fisher's exact test (p=0.029). Quantitative PCR showed the ratio of *Treponema denticola* to total bacteria were significantly higher in smokers when compared to nonsmokers by Mann-Whitney U Test (p=0.026).

Conclusions These results suggested that smoking was the environmental factor that strongly influenced the peri-implant microbiota and success rate of the dental implant.

Field of Study: Esthetic Restorative and Implant Dentistry Student's Signature.....
Academic Year: 2012 Advisor's Signature.....

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to the thesis committees, Associated Professor Chalermpol Leevailoj, Assistant Professor Pravej Serichetapongse, Dr. Anjalee Vacharaksa and Dr.Somying Patntirapong who gave important suggestions that enhanced this thesis edition.

I would like to thank my family, Dad, Mom, None, Ao, Tieng, Bank, who always stay beside me throughout my life.

I am indebted to all agents at Esthetic and implant dentistry clinic, Police Hospital and Department of microbiology for all the facilitation.

This thesis is dedicated to Esthetic restorative and implant dentistry program where gave me the best education and provided all supplement for sample collection.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Background and Rationale

The oral cavity is a habitat of the complex microbial community where they interact with their host and environment. Oral bacteria forms a biofilm, or the so-called dental plaque, by which may cause bacterial infection and inflammatory response of periodontal and peri-implant mucosal tissue. Periodontitis and periimplantitis show similar histological and clinical pathogenesis [1], and smoking is also one the risk factors contributing to the progression of both periodontitis and periimplantitis [2, 3]. However, it remains less clear how smoking involves in the bacterial-induced inflammation in the supporting tissue of teeth or dental implants. Several studies have shown that smoking affects on the composition of oral microbiota [4-6]. The composition shift of the bacterial biofilm around dental implants may promote the growth of putative pathogens as described in the ecology plaque hypothesis [7]. The putative periodontal pathogens, *Porphyromonas gingivalis (P. gingivalis), Treponema denticola (T. denticola),* and *Tannerella forsythia (T. forsythia),* known as the red complex, and *Prevotella intermedia (P. intermedia)* shown in the orange complex [8], might play a role in the inflammatory destruction of tooth and dental implant

supporting tissue [1]. In this study, we investigated whether smoking affected on the peri-implant microbiota, and caused the increase of putative pathogens. This finding may suggest the environmental-related mechanism that involved in the destructive inflammatory responses in periimplantitis.

Research Questions

Whether smoking increases the prevalence of *P. gingivalis, T. denticola, T. forsythia and P. intermedia* in the submucosal peri-implant microbiota

Research Objectives

To detect the prevalence of *P. gingivalis, T. denticola, T. forsythia and P. intermedia* in the peri-implant sulci of smokers and nonsmokers using 16S rRNA-based PCR

Hypothesis

Null hypothesis

- There is no significant difference in the prevalence of *P. gingivalis, T. denticola, T. forsythia and P. intermedia* in smokers and nonsmokers.

Alternative hypothesis

- There is a significant difference in the prevalence of *P. gingivalis, T. denticola, T. forsythia and P. intermedia* in smokers and nonsmokers.

Keywords

smoking, periimplantitis, Oral Microbiota, *Treponema denticola*, the red complex bacteria

Research design

Cross sectional analytical study

Population: Smokers and nonsmokers who received implant-supported fixed partial prostheses at Faculty of Dentistry, Chulalongkorn University or Police Hospital, Bangkok, Thailand from year 1998 to 2011.

Intervention: None

Outcome measurement: The prevalence of *P. gingivalis, T. denticola, T. forsythia* and *P. intermedia* in smokers and nonsmokers.

CHAPTER II

LITERATURE REVIEW

The microbiota was the living microorganisms that resided symbiotically within the human body including the oral cavity. The microbiota played an important role to maintain microbial balance at the mucosal tissue for the health of its host [9]. Although the microbiota included bacteria, fungi, viruses, and archeae, bacteria were the majority in the oral microbial ecology [10, 11]. Disturbance of the bacterial ecology may influence some oral health problems [11]. Non-pathogenic bacteria could provide a natural competitive barrier against pathogen colonization and invasion at the mucosal surfaces using specific mechanisms such as disrupting the lipid membrane of pathogenic microbes [12], or reducing the permeability of the epithelium [13]. The commensal bacteria were shown to promote barrier integrity and reverse adverse effects of pathogens at the mucosal surfaces of the gastrointestinal tract [13]. In addition, the presence of non-pathogenic bacteria might stimulate and maintain the host innate and adaptive immune responses at readily state for preventing pathogenic microbes [9]. Host and oral bacteria maintained mutual relationship at the oral mucosa. Host immune system balanced a number of commensals and pathogens at the healthy level. Hundreds bacterial species predominately with gram-

positive bacteria such as *Streptococcus*, *Actinomyces* and others were associated with healthy periodontal sites [14, 15]. Internal or external influences, for example hormone changes, contacts to tobacco, or regular use of antibiotics, might alter healthy oral bacterial composition and disturbed the balance of host and microorganisms ecosystem [16].

The plaque-induced pathological conditions involved inflammatory responses to the persistent bacterial infections of periodontal tissue [17]. Periodontal disease onset and progression were thought to result from the accumulating of dental plaque as this might be referred to 'the non-specific plaque hypothesis' [18]. However, several evidences had shown that considerable amount of dental plaque or calculus did not necessarily cause destructive periodontal disease in many individuals. Localized destructive periodontitis had developed in the proximity with healthy adjacent periodontal tissue. These observations led to another hypothesis called 'the specific plaque hypothesis' which recently became more popular. The specific plaque hypothesis suggested that neither all bacteria in dental plaque were equally pathogenic nor share similar responsibility to develop periodontal diseases. Therefore, certain bacterial species implicated as pathogens in periodontal infections [19, 20].

Socransky et al. [8] classified subgingival bacteria into five major complexes. The periodontitis-associating complexes were the red complex consisting *T. forsythia,*

P. gingivalis and *T. denticola* and the orange complex consisting *F. nucleatum, F. periodonticum, P. intermedia, P. nigrescens* and *P. micros (*Figure 1). Interestingly, colonization of these pathogens could be observed within healthy oral sites.

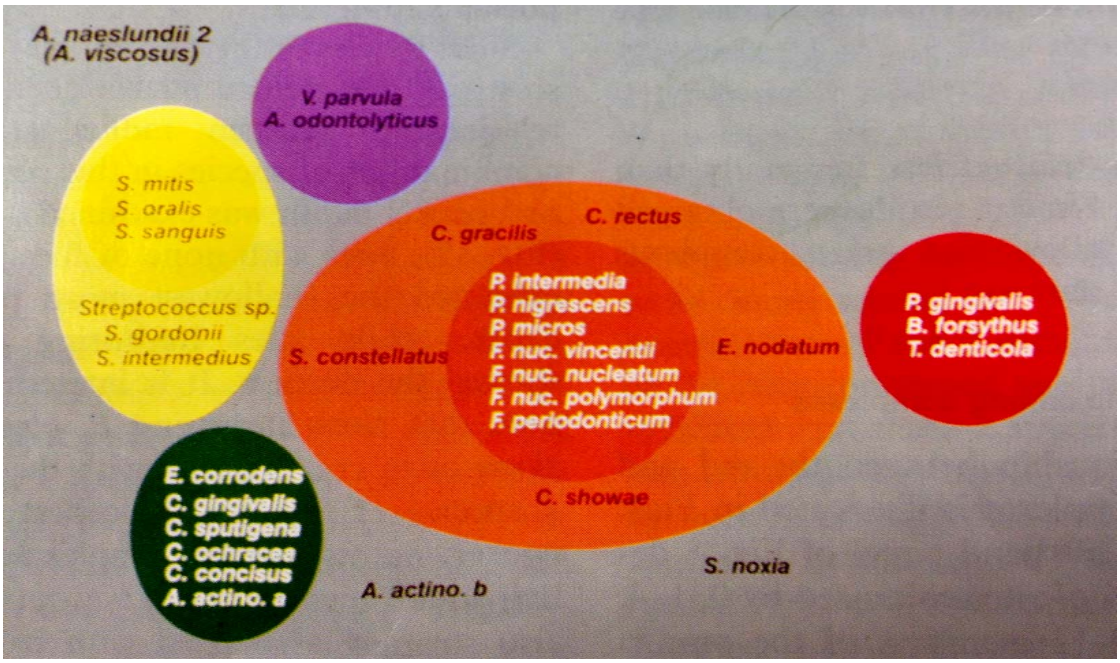


Figure 1. Major microbial complexes in subgingival plaque [8]

Thus, the questions were raised how the pathogens increased and dominated the bacterial ecology in the periodontal diseases. In the attempt to answer these questions, the ecological plaque hypothesis was proposed that the commensals might shift to pathogenic relationship with hosts due to factors that trigger a shift in the proportions of oral microbiota [7]. Despite the fact that pathogen infections caused periodontal diseases, the host inflammatory responses were also responsible for the destructive consequences of the periodontium [21]. Therefore, variety bacterial composition could differentially influence host

factors that lead to altered environmental conditions. The ecological plaque hypothesis additionally accommodated contributions from bacteria, host, and altered ecology in developing pathological process.

The success of dental implant is depend on integration between implant and oral hard and soft tissue [22]. There are many etiologic factors that can compromise implant-tissue integration and cause tissue inflammation or bone loss such as surgical trauma, microgap, occlusal overload, and periimplantitis [22, 23]. Periimplantitis is one of the leading problems during maintenance phase in implant dentistry. The definitions of peri-implant diseases, revised in the 6th European Workshop on Periodontology in 2008, were that “peri-implant mucositis is the presence of inflammation in the mucosa at an implant with no signs of loss of supporting bone; and periimplantitis, in addition to inflammation in the mucosa, is characterized by the loss of supporting bone” [24]. The recent study showed that facultative gram-positive cocci and gram-positive rods were dominating in healthy peri-implant sites with small fraction of anaerobic gram-negative rods. In contrast, anaerobic gram-negative bacteria were found increasing in the periimplantitis. The red complex and the orange complex species were also present in the infection of peri-implant tissue [1]. Therefore, similar group of pathogens may be the cause of periodontitis and periimplantitis.

Periodontitis and periimplantitis also shared the similar risk factors including poor oral hygiene, genetic traits, diabetes mellitus, and smoking [1, 2]. Among those factors, smoking seemed to be a profound environmental factor associating with the diseases. During smoking, oral temperature was apparently increased and smoke provided direct contact of some cigarette ingredients to the oral cavity. Due to smoking-induced environmental change, oral microbial ecology and host immunity might be modulated. The composition of oral microbiota could shift to a specific profile that maintained high abundance in disease-associating microbiota. The signature bacterial ecology may associated to current smoking habit including smoking, or never-smoking [4, 25, 26], or smoking cessation [5]. The oral bacteria, including *Parvimonas, Fusobacterium, Bacteroides, Porphyromonas, Campylobacter,* and *Treponema* increased [4, 27, 28], while *Veillonella, Neisseria,* and *Streptococcus* decreased in smokers [4].

Earlier studies used culture-dependent methods for bacterial identification. However, culturing conditions selectively favored certain microorganisms therefore the majority of oral bacteria from specific sites was apparently uncultivated, and that several uncultivated bacteria may play an important role in disease etiology and pathogenesis [29]. Some molecular approaches, for example checkerboard DNA-DNA hybridization or species-specific PCR were limited to the examination of only previously known genomic

sequences. The recent investigation using 16S rRNA gene cloning and sequencing [30] provided unique bacterial signature that allow for accurate bacterial identification. While sequencing a large number of clones from each sample provides quantitative information on the relative abundance of each organism within a community [4]. Since the conventional method is labor-extensive and relatively difficult to do the analysis, the 16S rRNA gene cloning and sequencing method would be an alternative way for qualitative and quantitative evaluation the oral bacterial profile.

CHAPTER III

METHODOLOGY

Materials and Methods

1. Participant selection

Patients who received implant-supported fixed partial prostheses from Esthetic restorative and implant dentistry clinics, Faculty of Dentistry, Chulalongkorn University, or dental clinic in Police Hospital, Bangkok, Thailand from January 1998 to December 2011 were examined. Fourteen participants were randomly included in this study based on the following criteria (Table 1). All participants were thoroughly explained about the steps of microbial collection, benefits and possible risks of the study approved by ethics committee of Faculty of Dentistry, Chulalongkorn University (No. 081/2011) and Police Hospital (No. จว.65/2554). The consent forms were signed before attending the study and participants were free to withdraw from the study at any time.

Participants were categorized according to smoking habits into smoking group and nonsmoking group. Demographic data including name, date of birth, age, sex, medical status, medication, oral health care, and detailed information on dental implant prostheses were assessed through an interview.

Table 1. Criteria for participant selection.

Inclusion Criteria	Exclusion Criteria
- Patient with smoking habit (exposure to more than 5 pack year [31] or never smoking) - Having 1-3 implant supported fix partial dentures - Not underwent previous periodontal treatment for at least 3 months - Obtained consent from patient	- Presented with uncontrolled diabetes mellitus - Having Immunosuppressant medications or antibiotics within the past 3 months - Pregnancy - Visible peri-implant mucosal infection and/or inflammation

Periodontal parameters such as bleeding on probing and probing depth were evaluated on the mesiobuccal, buccal, distobuccal, mesiolingual, lingual, and distolingual aspects of each implant by plastic periodontal probe (Periowise, Premier Dental, Playmouth Meeting, PA,) (Figure 2).

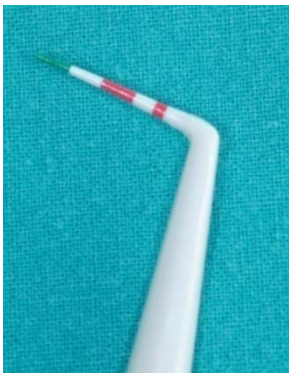


Figure 2. Plastic periodontal probe

Intraoral radiographs were taken on implant with periapical technique to measure the distance from the mesial and distal margins of fixture-abutment interface to the most coronal point where the bone appears to be in contact with the implant.

2. **Microbial sample collection**

For microbial sample collection, one implant per participant was randomly selected according to the criteria (Table 1). The site was isolated with sterile gauze and the supragingival plaque or calculus was removed with plastic sickle and curette (IMPLACARE™ handle #6 with tips, Hu-Friedy, Chicago, IL, USA) (Figure 3 and 4) in order

Figure 3. Plastic sickle

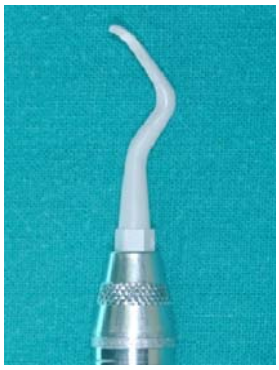


Figure 4. Plastic curette

to avoid contamination when the submucosal plaque was collected. Four paper points were inserted under light pressure in the peri-implant sulcus that presented the largest probing depth until resistance was felt and was kept there for 10 seconds to obtain peri-implant crevicular fluid [32] (Figure 5). These paper points were cut in half with sterile scissors and forceps and kept in 1.5 ml. plastic collection tube (Figure 6). The samples were stored at -20 ºC until processed.

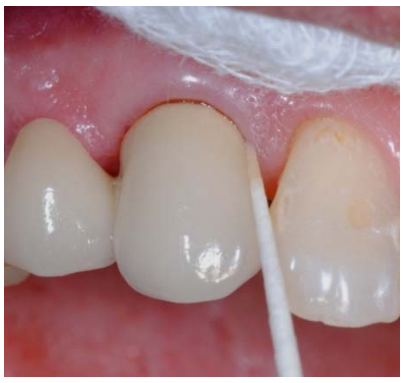


Figure 5. Peri-implant subgingival fluid collection

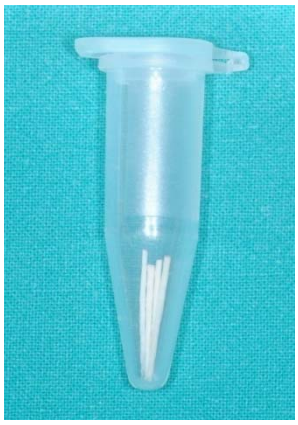


Figure 6. Sample Collection tube

3. **DNA extraction**

Bacterial DNA was extracted using PowerBiofilm™ DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instruction. Paper points in each plastic collection tube were washed with 350 µl BF1 buffer (BF1 needed to be warm at 55ºc for 10 minutes before used) then transferred to a bead tube. BF2 buffer (100 µl) was added and mixed using a vortex mixer. The bead tubes were incubated at 65ºc for 5 minutes. After the incubation, all samples were homogenized using Vortex Adapter at maximum speed for 10 minutes followed by centrifugation at 13,000 rcf for 1 minute at room temperature. Then, the supernatant was transferred to 2 ml collection tube. BF3 buffer (100 µl) was added and mixed using a vortex mixer. All samples were incubated at 4ºc on ice for 5 minutes follow by centrifugation at 13,000 rcf for 1 minute at room temperature. Thereafter, the supernatant was transferred to 2 ml collection tube avoiding

pellet. BF4 buffer (900 µl) was added and mixed using a vortex mixer. The supernatant (650 µl) was loaded onto spin filter and centrifuged at 13,000 rcf for 1 minute at room temperature. Then, discarding the flow through and repeating this step until the entire supernatant was loaded. After this step, the spin filter basket was placed into 2 ml collection tube. BF5 buffer (650 µl) was added followed by centrifugation at 13,000 rcf for 1 minute at room temperature and discarding the flow through. BF6 buffer (650 µl) was added followed by centrifugation at 13,000 rcf for 1 minute at room temperature and discarding the flow through. The centrifugation was repeated at 13,000 rcf for 2 minute at room temperature to remove the residual wash. Afterward, the spin filter basket was placed into the new 2 ml collection tube. BF7 buffer (50 µl) was added to the center of the white filter membrane and all samples were stored for 5 minutes at room temperature followed by centrifugation at 13,000 rcf for 1 minute at room temperature, and then the spin filter basket was discarded. NanoDrop2000 Spectrophotometer (Thermo SCIENTIFIC, Wilmington, DE, USA) (Figure 7) was used to quantify DNA.

Figure 7. Nanodrop2000

4. **Microbial identification**

Periodontal pathogens were detected and quantified by endpoint PCR and quantitative PCR using bacterial-specific primers. The sequences of *P. gingivalis-, P. intermedia-, T. denticola-, T. forsythia-* and *total bacteria-* specific primers were shown (Table 2).

For endpoint PCR reaction, 1 µl of DNA (50 ng) was mixed with 12.5 µl of TopTaq Master Mix (TopTaq DNA Polymerase, dNTPs, and the innovative TopTaq PCR Buffer; Qiagen, Valencia, CA, USA), 0.4 µM of forward and reverse primers, and the PCR water was added to total volume of 25 µl. The end point PCR mixtures were processed with DNA Engine® Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA) (Figure 8). The program consisted of an initial step at 94°C for 3 min, and amplifications was performed for 35 cycles, with denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and elongation at

72°C for 60 s, followed by extension at 72°C for 10 min. Reaction products were electrophoresed in 1.5% agarose gels (Figure 9), stained with ethidium bromide and photographed under ultraviolet light. A 100 base pair DNA Ladder (Invitrogen™, Carlsbad, CA, USA) was used as a marker. The presence of a sharp band of the expected molecular size was scored as positive for the species.

For quantitative PCR, 1 µl of DNA (20 ng) was mixed with 10 µl of EXPRESS SYBR® GreenER™ qPCR Super Mixes and Universal (Invitrogen™, Carlsbad, CA, USA), 0.1 µM of forward and reverse primer, and the PCR water was added to total volume of 20 µl. The quantitative PCR mixtures were analyzed using MiniOpticon™ RealTime PCR System (Bio-Rad, Hercules, CA, USA) (Figure 10). The program consisted of an incubation step at 50°C for 2 min, an initial step at 95°C for 2 min, Then amplifications was performed for 39 cycles, at 95°C for 15 s and 58°C for 1 min. The melting curve analysis was performed at 65°C for 5 s, and 95°C for 50 s.

Table 2. Sequences of bacterial-specific primers

Primer	5' → 3' Sequences
Porphyromonas gingivalis (Forward)	AGGCAGCTTGCCATACTGCG
Porphyromonas gingivalis (Reverse)	ACTGTTAGCAACTACCGATGT
Prevotella intermedia (Forward)	TTTGTTGGGGAGTAAAGCGGG

Prevotella intermedia (Reverse)	TCAACATCTCTGTATCCTGCGT
Treponema denticola (Forward)	TAATACCGAATGTGCTCATTTACAT
Treponema denticola (Reverse)	TCAAAGAAGCATTCCCTCTTCTTCTTA
Tannerella forsythus (Forward)	GCGTATGTAACCTGCCCGCA
Tannerella forsythus (Reverse)	TGCTTCAGTGTCAGTTATACCT
Universal primer RW01 (Forward)	AACTGGAGGAAGGTGGGGAT
Universal primer DG74 (Reverse)	AGGAGGTGATCCAACCGCA

Figure 8. DNA Engine® Peltier Thermal Cycler

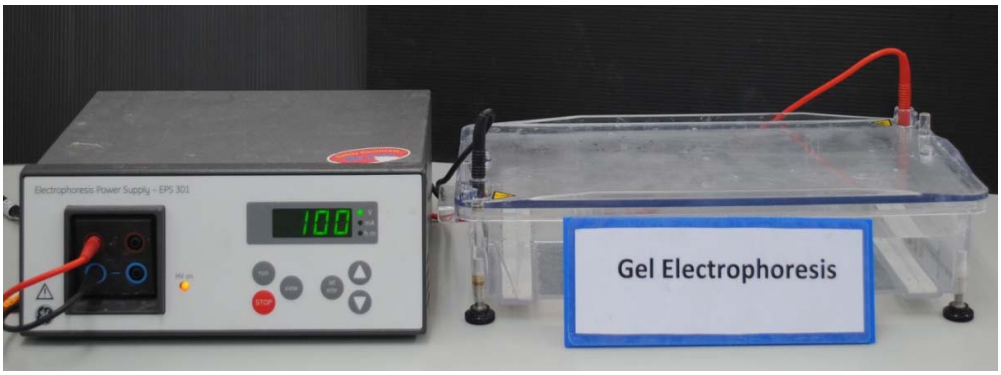


Figure 9. Gel electrophoresis

Figure 10. MiniOpticon™ Real–Time PCR System

5. Statistical analysis

SPSS program (SPSS version 16.0, SPSS Inc., Chicago, IL) was used to analyze all data. The prevalence of periodontal pathogenic bacteria was evaluated by Fisher's exact test. Mann-Whitney U Test was used to analyze the relative expression of *P. gingivalis, P. intermedia, T. denticola, T. forsythia* to total bacteria.

CHAPTER IV

RESULTS

Fourteen participants were included in this study. Participants, smokers (n=7) and nonsmokers (n=7), were male. The mean age (±SD) of smokers and nonsmokers was 47.5±11.12 and 55.86±11.36 years, respectively. The mean age of nonsmokers was higher, however, the average year of implant in function of nonsmokers were slightly lower. The average pack year of smoking was 11.56±4.34. The implant sites appeared healthy with approximately 3 to 4 mm. probing depth (Table 3).

Table 3. Summary of the demographic data of the participants

	Smokers	Nonsmokers
Age* (years)	47.5 ± 11.12	55.86 ± 11.36
Gender	Male	Male
Year implant loaded* (years)	6.61±4.08	5.29±3.65
Pack year*	11.56±4.34	0±0.00
Probing depth* (millimeter)	3.29±0.49	3.5±0.58

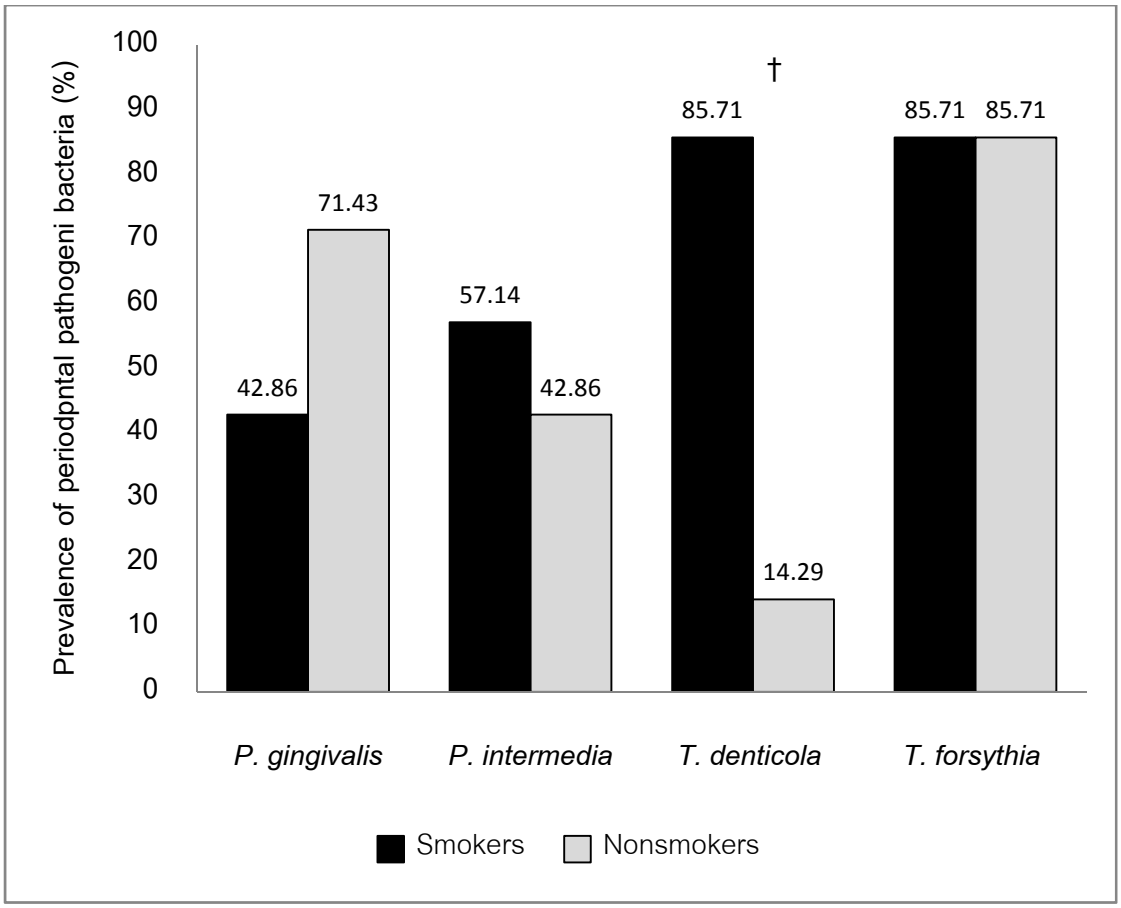
* Mean±SD were shown

The endpoint PCR revealed that the prevalence of periodontal pathogenic bacteria was higher in smokers. The number of participants positive for *P. gingivalis, P. intermedia, T. denticola, T. forsythia* were 42.86%, 57.14%, 85.71%, 85.71% from the total of seven smokers. While, *P. gingivalis, P. intermedia, T. denticola, T. forsythia* were respectively found in 71.43%, 42.86%, 14.29%, 85.71% from the total of seven nonsmokers. However, the statistically significant difference was observed only in *T. denticola* by Fisher's exact test ($p<0.05$) (Table 4 and Figure 11).

Table 4. Prevalence of putative pathogens detected by endpoint PCR

Bacteria	Smokers							Nonsmokers							p-value
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
P. gingivalis	-	+	-	+	+	-	-	-	+	+	+	+	-	+	0.582
P. intermedia	+	+	-	+	-	-	+	+	+	-	+	-	-	-	1
T. denticola	-	+	+	+	+	+	+	-	-	-	-	-	-	+	0.029†
T. forsythia	+	+	+	+	+	-	+	-	+	+	+	+	+	+	1

† Significant different with Fisher's exact test ($p=0.029$)

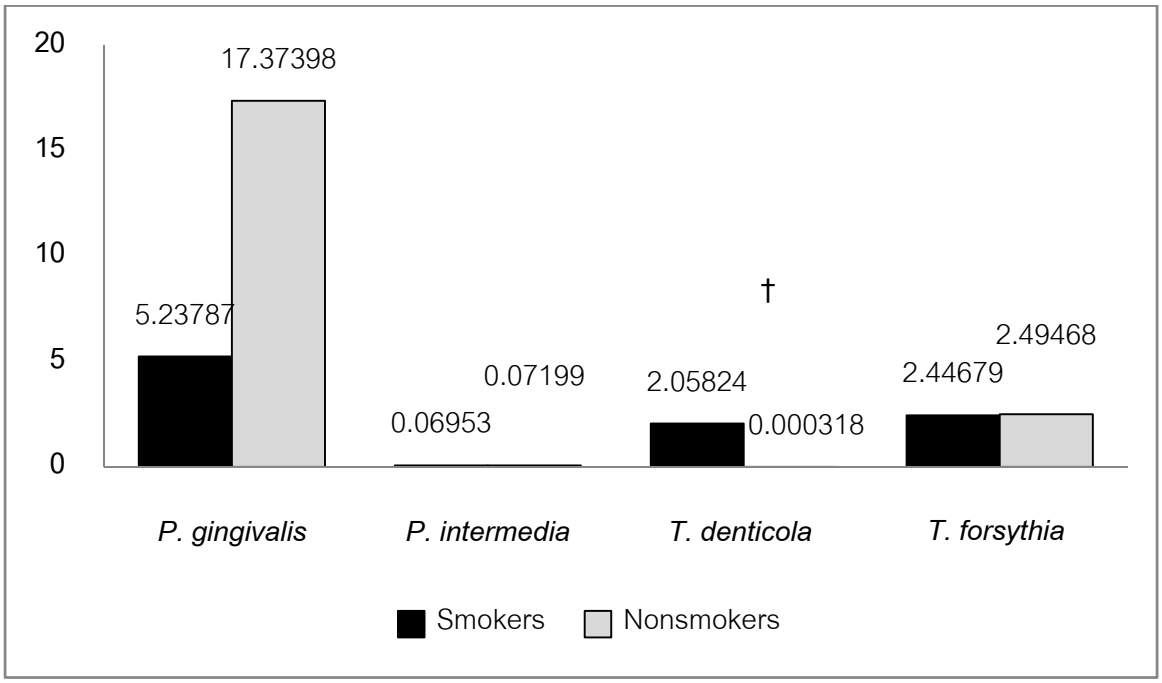


† Significant different with Fisher's exact test (p=0.029)

Figure11. Percentage of prevalence of periodontal pathogenic bacteria

To investigate the abundance of each red complex bacterium, quantitative PCR was performed and the expression of *P. gingivalis, P. intermedia, T. denticola*, and *T. forsythia* to total bacteria was estimated in smokers and nonsmokers (Figure 12). The results showed that the abundance of *P. gingivalis, P. intermedia,* and *T. forsythia* were not statistically different between smokers and nonsmokers. However, the relative expression of

T. denticola to total bacteria was significantly higher in smokers (p=0.026) by Mann-Whitney U test.



† Significant different with Mann-Whitney U test (p=0.026)

Figure12. Relative expression of periodontal pathogenic bacteria to total bacteria

CHAPTER V

DISCUSSION AND CONCLUSIONS

Discussion

Smoking was not an absolute contraindication for dental implants, but smoking might increase the risk of early implant failure [33] or bacterial-induced peri-implantitis [3, 34, 35]. The purpose of this study was to investigate whether smoking increased the putative pathogens around dental implants. Other factors that might affect the peri-implant bacterial ecology were controlled. All participants included in this study were male in order to eliminate the possibility of hormonal differences in female [16, 36]. All implants were restored with fixed partial prostheses since the magnitude and direction of occlusal forces were well-designed in comparison to those on removable prostheses [37]. The cut point of 5 pack year has been applied to the inclusion criteria to eliminate the very light or occasional smokers [31], and the average pack year (±SD) of smoking was 11.56±4.34. Nonetheless, this study was performed in the dental school of Chulalongkorn University and Police Hospital, therefore the number of participants with smoking habit was relatively small due to the limited number of cases.

Submucosal plaque accumulation is relatively low in healthy peri-implant sites, therefore, instead of using Gracey curettes [25], we used paper points for microbial DNA collection [32]. The use of paper points has gained most of planktonic bacteria in the crevicular fluid, and the total bacterial DNA was then revealed as shown by the amplification using total bacteria primers. This method was easy to handle, reproducible, and less painfulness for patients, however, it was also selective. We might fail to detect those species that could not be detached into the crevicular fluid. Further studies will be needed to investigate whether the collection method using paper points has omitted some important microorganisms.

The 16S rRNA-based PCR was used to detect the putative pathogenic bacteria because of its benefit of accuracy and sensitivity. All collected samples were pooled from submucosal sites of a single implant. PCR-positive bacterium in the sample represented the prevalence of that bacterium of each participant. It appeared that the red complex bacteria and *P. intermedia* were present in some peri-implant sulci of both smokers and nonsmokers (Table 2). Consistent to the previous reports, that these putative pathogens were parts of the oral microbiota, and might be found in healthy sites [38, 39].

All smokers were positive for more than one putative pathogen, and two smokers were positive for all tested bacteria. In contrast, none of nonsmokers were positive for all

tested bacteria. Since the complexity of the peri-implant microbiota increased in disease sites [40], it reflected that the microbiota in smokers might be more shifted to disease state. Increase of the red complex bacteria also suggested that the peri-implant ecosystem of smokers could be more anaerobic than that of nonsmokers. Additionally, smoking could have detrimental effect on host immunity by reduction of leukocyte macrophage and PMN function[41, 42]. Moreover, previous study showed that the oxidation reduction potential of dental plaque have been shown to be decreased by smoking [43]. This ecology change resulted in the elevation of anaerobic species and the risk of pathogen invasion.

Although the prevalence of putative pathogens seemed to be higher in smokers than nonsmokers, the statistical significant difference was found only in *T. denticola* in our results. The previous study by *Ata-Ali et al* [44] using different technique also demonstrated greater amount of *T. denticola* in peri-implant microbiota of smokers. These results were consistent to the reports in natural teeth. The investigation of Umeda *et al* [39], on the prevalence of *P. gingivalis, T. denticola, T. forsythia, A. actinomycetemcomitans, P. intermedia* and *P. nigrescens*, showed that smokers had increased the risk of *T. denticola* colonization with an odd ratio of 4.61. In addition, Delima *et al* [5] showed that the level of *T. denticola* around natural teeth was significantly decreased after smoking cessation for 12 months. Finally, the study of Haffagee *et al* [25] showed significant

differences in the prevalence of *T. denticola,* and other species including, *T. forsythia*, *P. gingivalis* and *E.nodatum, P. micros, P. nigrescens*, *P. intermedia, F. nucleatum* in current smokers than nonsmokers.

In this study, we have estimated the abundance of putative pathogenic bacteria by using quantitative PCR. Because the data were not normally distributed, we then compared the median of smokers and nonsmokers. Our result revealed that the prevalence of *P. intermedia* was lower, but the median ratio of *P. intermedia* to total bacteria was higher in nonsmokers. Nonetheless, the abundance of bacteria was not different among smokers and nonsmokers, except *T. denticola.*

Smoking, therefore, affected both prevalence and abundance of *T. denticola* in peri-implant microbiota. The increase of putative pathogens, especially *T. denticola,* in smokers might result in the change of host immune responses. Since *T. denticola* is the motile anaerobic bacterium that can penetrate deep tissue and induce inflammatory responses [45], the increase of *T. denticola* may contribute to the initiation of bacterial-induced inflammatory bone loss in the regulation of host-microbial interactions [46]. Thus, the smoking-related bone resorption mechanism should be further investigated.

Conclusion

Our study demonstrated that smoking was the environmental factor that influenced the peri-implant microbiota. The prevalence and abundance of *T. denticola* in the peri-implant microbiota of smokers were significantly higher than that in nonsmokers.

REFERENCES

[1] Heitz-Mayfield LJ, Lang NP: Comparative biology of chronic and aggressive periodontitis vs. peri-implantitis. *Periodontol 2000* 53 (2010):167-181.

[2] Heitz-Mayfield LJ: Disease progression: identification of high-risk groups and individuals for periodontitis. *J Clin Periodontol* 32 Suppl 6 (2005):196-209.

[3] Haas R, Haimbock W, Mailath G, Watzek G: The relationship of smoking on peri-implant tissue: a retrospective study. *J Prosthet Dent* 76(6) (1996): 592- 596.

[4] Shchipkova AY, Nagaraja HN, Kumar PS: Subgingival microbial profiles of smokers with periodontitis. *J Dent Res* 89(11) (2010):1247-1253.

[5] Delima SL, McBride RK, Preshaw PM, Heasman PA, Kumar PS: Response of subgingival bacteria to smoking cessation. *J Clin Microbiol* 48(7) (2010): 2344-2349.

[6] Gomes SC, Piccinin FB, Oppermann RV, Susin C, Nonnenmacher CI, Mutters R, Marcantonio RA: Periodontal status in smokers and never-smokers: clinical findings and real-time polymerase chain reaction quantification of putative periodontal pathogens. *J Periodontol* 77(9) (2006):1483-1490.

[7] Marsh PD: Are dental diseases examples of ecological catastrophes? *Microbiology*

149(Pt 2) (2003):279-294.

[8] Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL, Jr.: Microbial complexes in subgingival plaque. *J Clin Periodontol* 25(2) (1998):134-144.

[9] Backhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI: Host-bacterial mutualism in the human intestine. *Science* 307(5717) (2005):1915-1920.

[10] Siqueira JF, Jr., Rocas IN: The oral microbiota: general overview, taxonomy, and nucleic acid techniques. *Methods Mol Biol* 666 (2010):55-69.

[11] Bik EM, Long CD, Armitage GC, Loomer P, Emerson J, Mongodin EF, Nelson KE, Gill SR, Fraser-Liggett CM, Relman DA: Bacterial diversity in the oral cavity of 10 healthy individuals. *ISME J* 4(8) (2010):962-974.

[12] Cederlund A, Gudmundsson GH, Agerberth B: Antimicrobial peptides important in innate immunity. *FEBS J* 278(20) (2011):3942-3951.

[13] Ulluwishewa D, Anderson RC, McNabb WC, Moughan PJ, Wells JM, Roy NC: Regulation of tight junction permeability by intestinal bacteria and dietary components. *J Nutr* 141(5) (2011):769-776.

[14] Loesche WJ, Syed SA: Bacteriology of human experimental gingivitis: effect of plaque and gingivitis score. *Infect Immun* 21(3) (1978):830-839.

[15] Colombo AP, Boches SK, Cotton SL, Goodson JM, Kent R, Haffajee AD, Socransky

SS, Hasturk H, Van Dyke TE, Dewhirst F *et al*: Comparisons of subgingival microbial profiles of refractory periodontitis, severe periodontitis, and periodontal health using the human oral microbe identification microarray. *J Periodontol* 80(9) (2009):1421-1432.

[16] Sanz M, van Winkelhoff AJ: Periodontal infections: understanding the complexity-consensus of the Seventh European Workshop on Periodontology. *J Clin Periodontol* 38 Suppl 11 (2011):3-6.

[17] Page RC: The role of inflammatory mediators in the pathogenesis of periodontal disease. *J Periodontal Res* 26(3 Pt 2) (1991):230-242.

[18] Theilade E: The non-specific theory in microbial etiology of inflammatory periodontal diseases. *J Clin Periodontol* 13(10) (1986):905-911.

[19] Socransky SS, Haffajee AD: The bacterial etiology of destructive periodontal disease: current concepts. *J Periodontol* 63(4 Suppl) (1992):322-331.

[20] Feres M, Cortelli SC, Figueiredo LC, Haffajee AD, Socransky SS: Microbiological basis for periodontal therapy. *J Appl Oral Sci* 12(4) (2004):256-266.

[21] Graves DT, Oates T, Garlet GP: Review of osteoimmunology and the host response in endodontic and periodontal lesions. *J Oral Microbiol* 3 (2011).

[22] Oh TJ, Yoon J, Misch CE, Wang HL: The causes of early implant bone loss: myth or

science? *J Periodontol* 73(3) (2002):322-333.

[23] Quirynen M, De Soete M, van Steenberghe D: Infectious risks for oral implants: a review of the literature. *Clin Oral Implants Res*, 13(1) (2002):1-19.

[24] Lindhe J, Meyle J: Peri-implant diseases: Consensus Report of the Sixth European Workshop on Periodontology. *J Clin Periodontol* 35(8 Suppl) (2008):282-285.

[25] Haffajee AD, Socransky SS: Relationship of cigarette smoking to the subgingival microbiota. *Journal of Clinical Periodontology* 28(5) (2001):377-388.

[26] Mager DL, Haffajee AD, Socransky SS: Effects of periodontitis and smoking on the microbiota of oral mucous membranes and saliva in systemically healthy subjects. *Journal of Clinical Periodontology* 30(12) (2003):1031-1037.

[27] Zambon JJ, Grossi SG, Machtei EE, Ho AW, Dunford R, Genco RJ: Cigarette smoking increases the risk for subgingival infection with periodontal pathogens. *J Periodontol* 67(10 Suppl) (1996):1050-1054.

[28] van Winkelhoff AJ, Bosch-Tijhof CJ, Winkel EG, van der Reijden WA: Smoking affects the subgingival microflora in periodontitis. *J Periodontol* 72(5) (2001):666-671.

[29] Kumar PS, Leys EJ, Bryk JM, Martinez FJ, Moeschberger ML, Griffen AL:

Changes in periodontal health status are associated with bacterial community shifts as assessed by quantitative 16S cloning and sequencing. *J Clin Microbiol* 44(10) (2006):3665-3673.

[30] Bittar F, Richet H, Dubus JC, Reynaud-Gaubert M, Stremler N, Sarles J, Raoult D, Rolain JM: Molecular detection of multiple emerging pathogens in sputa from cystic fibrosis patients. *PLoS One* 3(8) (2008):e2908.

[31] Grossi SG, Zambon JJ, Ho AW, Koch G, Dunford RG, Machtei EE, Norderyd OM, Genco RJ: Assessment of risk for periodontal disease. I. Risk indicators for attachment loss. *J Periodontol* 65(3) (1994):260-267.

[32] Slots J, Ashimoto A, Flynn MJ, Li G, Chen C: Detection of putative periodontal pathogens in subgingival specimens by 16S ribosomal DNA amplification with the polymerase chain reaction. *Clin Infect Dis* 20 Suppl 2 (1995):S304-307.

[33] Silverstein P: Smoking and wound healing. *Am J Med* 93(1A) (1992):22S-24S.

[34] DeLuca S, Habsha E, Zarb GA: The effect of smoking on osseointegrated dental implants. Part I: implant survival. *Int J Prosthodont* 19(5) (2006):491-498.

[35] Bain CA, Moy PK: The association between the failure of dental implants and cigarette smoking. *Int J Oral Maxillofac Implants* 8(6) (1993):609-615.

[36] Holm-Pedersen P, Loe H: Flow of gingival exudate as related to menstruation and pregnancy. *J Periodontal Res* 2(1) (1967):13-20.

[37] Berglundh T, Persson L, Klinge B: A systematic review of the incidence of biological and technical complications in implant dentistry reported in prospective longitudinal studies of at least 5 years. *J Clin Periodontol* 29 Suppl 3:197-212 (2002); discussion 232-193.

[38] van Winkelhoff AJ, Goene RJ, Benschop C, Folmer T: Early colonization of dental implants by putative periodontal pathogens in partially edentulous patients. *Clin Oral Implants Res* 11(6) (2000):511-520.

[39] Umeda M, Chen C, Bakker I, Contreras A, Morrison JL, Slots J: Risk indicators for harboring periodontal pathogens. *J Periodontol* 69(10) (1998):1111-1118.

[40] Leonhardt A, Renvert S, Dahlen G: Microbial findings at failing implants. *Clin Oral Implants Res* 10(5) (1999):339-345.

[41] Kamma JJ, Nakou M, Baehni PC: Clinical and microbiological characteristics of smokers with early onset periodontitis. *J Periodontal Res* 34(1) (1999):25-33.

[42] Noble RC, Penny BB: Comparison of leukocyte count and function in smoking and nonsmoking young men. *Infect Immun* 12(3) (1975):550-555.

[43] Kenney EB, Saxe SR, Bowles RD: The effect of cigarette smoking on anaerobiosis

in the oral cavity. *J Periodontol* 46(2) (1975):82-85.

[44] Ata-Ali J, Flichy-Fernandez AJ, Alegre-Domingo T, Candel-Marti ME, Penarrocha D, Balaguer-Martinez JF, Penarrocha MA: Analysis of the peri-implant microbiota in 90 dental implants and its relationship to crevicular fluid volume. *Med Oral Patol Oral Cir Bucal* 16(7) (2011):e944-947.

[45] Sela MN: Role of Treponema denticola in periodontal diseases. *Crit Rev Oral Biol Med* 12(5) (2001):399-413.

[46] Fenno JC: Treponema denticola interactions with host proteins. *J Oral Microbiol* 4 (2012).

APPENDIX

Smokers

Subject	Area of implant	Brand	Implant design	Restoration
1	#12	ITI	Platform switching	PFM crown
2	#36	Centerpulse	Bone level	Splint crown
3	#21	Centerpulse	Bone level	Splint crown
4	#46	Centerpulse	Bone level	PFM crown
5	#36	Ankylos	Bone level	PFM crown
6	#46	Replace	Bone level	PFM crown
7	#46	ITI	Platform switching	Full metal crown

Nonsmokers

Subject	Area of implant	Brand	Implant design	Restoration
1	#21	ITI	Platform switching	PFM crown
2	#36	Zimmer	Bone level	PFM crown
3	#16	Astra	Platform switching	PFM crown
4	#36	Paragon	Bone level	PFM crown
5	#23	Frialit-2	Bone level	PFM crown
6	#36	Zimmer	Bone level	PFM crown
7	#26	Zimmer	Bone level	PFM crown

Crosstabs

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
Smoking habit * *Porphyromonas gingivalis*	14	100.0%	0	.0%	14	100.0%
Smoking habit * *Prevotella intermedia*	14	100.0%	0	.0%	14	100.0%
Smoking habit * *Treponema denticola*	14	100.0%	0	.0%	14	100.0%
Smoking habit * *Tannerellaforsythus*	14	100.0%	0	.0%	14	100.0%

Smoking habit * *Porphyromonas gingivalis*

Crosstab

Count

		Porphyromonas gingivalis		
		Negative	Positive	Total
Smoking habit	Smoker	4	3	7
	Nonsmoker	2	5	7
Total		8	6	8

Chi-Square Tests

	Value	Df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.167[a]	1	.280		
Continuity Correction[b]	.292	1	.589		
Likelihood Ratio	1.185	1	.276		
Fisher's Exact Test				.592	.296
Linear-by-Linear Association	1.083	1	.298		
N of Valid Cases[b]	14				

a. 4 cells (100.0%) have expected count less than 5. The minimum expected count is 3.00.

b. Computed only for a 2x2 table

Smoking habit * *Prevotella intermedia*

Crosstab

Count

		Prevotella intermedia		
		Negative	Positive	Total
Smoking habit	Smoker	3	4	7
	Nonsmoker	4	3	7
Total		9	7	7

Chi-Square Tests

	Value	Df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.286[a]	1	.593		
Continuity Correction[b]	.000	1	1.000		
Likelihood Ratio	.287	1	.592		
Fisher's Exact Test				1.000	.500
Linear-by-Linear Association	.265	1	.606		
N of Valid Cases[b]	14				

a. 4 cells (100.0%) have expected count less than 5. The minimum expected count is 3.50.

b. Computed only for a 2x2 table

Smoking habit * *Treponema denticola*

Crosstab

Count

		Treponema denticola		
		Negative	Positive	Total
Smoking habit	Smoker	1	6	7
	Nonsmoker	6	1	7
Total		7	7	14

Chi-Square Tests

	Value	Df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	7.143[a]	1	.008		
Continuity Correction[b]	4.571	1	.033		
Likelihood Ratio	7.925	1	.005		
Fisher's Exact Test				.029	.015
Linear-by-Linear Association	6.633	1	.010		
N of Valid Cases[b]	14				

a. 4 cells (100.0%) have expected count less than 5. The minimum expected count is 3.50.

b. Computed only for a 2x2 table

Smoking Habit * *Tannerella forsythus*

Crosstab

Count

		Tannerella forsythus		
		Negative	Positive	Total
Smoking Habit	Smokers	1	6	7
	Nonsmokers	1	6	7
Total		2	12	14

Chi-Square Tests

	Value	Df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.000[a]	1	1.000		
Continuity Correction[b]	.000	1	1.000		
Likelihood Ratio	.000	1	1.000		
Fisher's Exact Test				1.000	.769
Linear-by-Linear Association	.000	1	1.000		
N of Valid Cases[b]	14				

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 1.00.

b. Computed only for a 2x2 table

Mann-Whitney Test

Ranks

	Habit	N	Mean Rank	Sum of Ranks
Relative Expression of Pg to Total Bacteria	Smokers	4	4.00	16.00
	Nonsmokers	5	5.80	29.00
	Total	9		

Test Statistics[b]

	Relative Expression of Pg to Total Bacteria
Mann-Whitney U	6.000
Wilcoxon W	16.000
Z	-.980
Asymp. Sig. (2-tailed)	.327
Exact Sig. [2*(1-tailed Sig.)]	.413[a]

a. Not corrected for ties.

b. Grouping Variable: Habit

Mann-Whitney Test

Ranks

	Habit	N	Mean Rank	Sum of Ranks
Relative Expression of Pi to Total Bacteria	Smokers	7	7.00	49.00
	Nonsmokers	7	8.00	56.00
	Total	14		

Test Statistics[b]

	Relative Expression of Pi to Total Bacteria
Mann-Whitney U	21.000
Wilcoxon W	49.000
Z	-.447
Asymp. Sig. (2-tailed)	.655
Exact Sig. [2*(1-tailed Sig.)]	.710[a]

a. Not corrected for ties.

b. Grouping Variable: Habit

Mann-Whitney Test

Ranks

	Habit	N	Mean Rank	Sum of Ranks
Relative Expression of Td to Total Bacteria	Smokers	7	10.00	70.00
	Nonsmokers	7	5.00	35.00
	Total	14		

Test Statistics[b]

	Relative Expression of Td to Total Bacteria
Mann-Whitney U	7.000
Wilcoxon W	35.000
Z	-2.239
Asymp. Sig. (2-tailed)	.025
Exact Sig. [2*(1-tailed Sig.)]	.026[a]

a. Not corrected for ties.

b. Grouping Variable: Habit

Mann-Whitney Test

Ranks

	Habit	N	Mean Rank	Sum of Ranks
Relative Expression of Tf to Total Bacteria	Smokers	4	4.75	19.00
	Nonsmokers	5	5.20	26.00
	Total	9		

Test Statistics[b]

	Relative Expression of Tf to Total Bacteria
Mann-Whitney U	9.000
Wilcoxon W	19.000
Z	-.245
Asymp. Sig. (2-tailed)	.806
Exact Sig. [2*(1-tailed Sig.)]	.905[a]

a. Not corrected for ties.

b. Grouping Variable: Habit

No. 081/ 2011

Study Protocol and Consent Form Approval

The Human Research Ethics Committee of the Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand has approved the following study to be carried out according to the protocol and patient/participant information sheet dated and/or amended as follows in compliance with the **ICH/GCP.**

Study Title	: Effect of Smoking on the Peri-implant Bacterial Ecology
Study Code	: HREC-DCU 2011-087
Study Center	: Chulalongkorn University
Principle Investigator	: Dr. Maneerat Kuptanon
Protocol Date	: December 2, 2011
Date of Approval	: December 9, 2011
Date of Expiration	: December 8, 2013

S. Amatyakul

(Associate Professor Dr. Supathra Amatyakul)
Chairman of Ethics Committee

(Assistant Professor Dr. Suchit Poolthong)
Associate Dean for Research and International Affairs

*A list of the Ethics Committee members (names and positions) present at the Ethics Committee meeting on the date of approval of this study has been attached (upon requested). This Study Protocol Approval Form will be forwarded to the Principal Investigator.

โรงพยาบาลตำรวจ
สำนักงานแพทย์ใหญ่
492/1 ถนนพระรามที่ 1 แขวงวังใหม่
เขตปทุมวัน กรุงเทพฯ 10330

เอกสารรับรองโครงการวิจัย

โดย

คณะกรรมการจริยธรรมและวิจัยในมนุษย์ โรงพยาบาลตำรวจ

เลขที่หนังสือรับรอง ๒๐. ๖๕ /๓๓๑๑

ชื่อโครงการ/ภาษาไทย	-ผลของการสูบบุหรี่ต่อระบบนิเวศของแบคทีเรียรอบรากเทียม
ชื่อหัวหน้าโครงการ / หน่วยงานที่สังกัด	น.ส.มณีรัตน์ คุปตานนท์ ,ทันตแพทย์หญิง นิสิตปริญญาโท วิทยาศาสตรมหาบัณฑิต หลักสูตรนานาชาติ จุฬาลงกรณ์มหาวิทยาลัย
รหัสโครงการ	-
สถานที่ทำการวิจัย	โรงพยาบาลตำรวจ
เอกสารรับรอง	1. โครงร่างวิทยานิพนธ์ ฉบับที่ 1.0 ลงวันที่ 9 ธันวาคม พ.ศ. 2554 (Version 1.0 Date 9 December 2011)(ฉบับอังกฤษ) 2. โครงร่างวิทยานิพนธ์ ฉบับที่ 1.0 ลงวันที่ 9 ธันวาคม พ.ศ. 2554 (Version 1.0 Date 9 December 2011)(ฉบับภาษาไทย) 3. แบบฟอร์มการให้ข้อมูลแก่ผู้เข้าร่วมวิจัย ฉบับที่ 1.0 ลงวันที่ 9 ธันวาคม พ.ศ. 2554 (Version 1.0 Date 9 December 2011) 4. เอกสารชี้แจงข้อมูลและเอกสารลงนามยินยอม ฉบับที่ 1.0 ลงวันที่ 9 ธันวาคม พ.ศ. 2554 (Version 1.0 Date 9 December 2011) 5. แบบสอบถาม ฉบับที่ 1.0 ลงวันที่ 9 ธันวาคม พ.ศ. 2554 (Version 1.0 Date 9 December 2011) 6. อัตตประวัติผู้วิจัย
รับรองโดย	คณะกรรมการจริยธรรมและวิจัยในมนุษย์ โรงพยาบาลตำรวจ
วันที่รับรอง	9 ธันวาคม 2554
วันหมดอายุ	8 ธันวาคม 2555

หนังสือรับรองฉบับนี้ออกโดยความเห็นชอบในการพิจารณาจากคณะกรรมการจริยธรรมและวิจัยของโรงพยาบาลตำรวจ ตามกฎเกณฑ์สากล

พลตำรวจตรี ทรงชัย สิมะโรจน์

(ทรงชัย สิมะโรจน์)

ประธานคณะกรรมการจริยธรรม และวิจัยในมนุษย์โรงพยาบาลตำรวจ

BIBILOGRAPHY

NAME	Miss Maneerat Kuptanon
DATE OF BIRTH	28 May1985
PLACE OF BIRTH	Bangkok
INSTITUTIONS ATTENDED	Chulalongkorn University, 2003-2009: Doctor of Dental Surgery, DDS.
RESEARCH GRANT	Supported by Chulalongkorn University Research Fund