Analysis of microbiota associated with peri-implantitis using 16S rRNA gene clone library

Tatsuro Koyanagi[1,2]*, Mitsuo Sakamoto[2], Yasuo Takeuchi[1], Moriya Ohkuma[2] and Yuichi Izumi[1,3]

[1]Section of Periodontology, Department of Hard Tissue Engineering, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan; [2]Microbe Division/Japan Collection of Microorganisms, RIKEN BioResource Center, Wako, Saitama, Japan; [3]Global Center of Excellence (GCOE) Program, International Research Center for Molecular Science in Tooth and Bone Diseases, Tokyo Medical and Dental University, Tokyo, Japan

Background: Peri-implantitis (PI) is an inflammatory disease which leads to the destruction of soft and hard tissues around osseointegrated implants. The subgingival microbiota appears to be responsible for peri-implant lesions and although the complexity of the microbiota has been reported in PI, the microbiota responsible for PI has not been identified.

Objective: The purpose of this study was to identify the microbiota in subjects who have PI, clinically healthy implants, and periodontitis-affected teeth using 16S rRNA gene clone library analysis to clarify the microbial differences.

Design: Three subjects participated in this study. The conditions around the teeth and implants were evaluated based on clinical and radiographic examinations and diseased implants, clinically healthy implants, and periodontally diseased teeth were selected. Subgingival plaque samples were taken from the deepest pockets using sterile paper points. Prevalence and identity of bacteria was analyzed using a 16S rRNA gene clone library technique.

Results: A total of 112 different species were identified from 335 clones sequenced. Among the 112 species, 51 (46%) were uncultivated phylotypes, of which 22 were novel phylotypes. The numbers of bacterial species identified at the sites of PI, periodontitis, and periodontally healthy implants were 77, 57, and 12, respectively. Microbiota in PI mainly included Gram-negative species and the composition was more diverse when compared to that of the healthy implant and periodontitis. The phyla *Chloroflexi*, *Tenericutes*, and *Synergistetes* were only detected at PI sites, as were *Parvimonas micra*, *Peptostreptococcus stomatis*, *Pseudoramibacter alactolyticus*, and *Solobacterium moorei*. Low levels of periodontopathic bacteria, such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, were seen in peri-implant lesions.

Conclusions: The biofilm in PI showed a more complex microbiota when compared to periodontitis and periodontally healthy teeth, and it was mainly composed of Gram-negative anaerobic bacteria. Common periodontopathic bacteria showed low prevalence, and several bacteria were identified as candidate pathogens in PI.

Keywords: *dental implants; peri-implant infection; periodontal disease; peri-implant microbiota; biofilm; microbiology*

Osseointegrated titanium implants have become an important alternative to conventional prostheses for the replacement of missing teeth (1, 2). On the other hand, with the increasing demand for dental implants, dental implant failure is also being reported more frequently (3–8). Peri-implantitis (PI) is an inflammatory disease affecting soft and hard tissues around the osseointegrated implants, which can cause an early implant failure (9–11). Several factors, such as bacterial infection and/or excessive occlusal stress, are associated with the occurrence of the disease and the microbiological factors of PI have been of particular interest (11–13). After the insertion of titanium implants, rapid colonization of bacteria has been observed at the peri-implant sulcus (14). Some microbiological studies have shown that implants affected by PI tend to harbor microbiota

encompassing periodontal pathogen species, including *Porphyromonas gingivalis*, *Tannerella forsythia*, *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, and *Fusobacterium* species (15–20). Leonhardt et al. (17) also reported that less common oral species, such as staphylococci, enteric species, and yeasts, were recovered from failing implants. These findings indicate the complexity of the microbiota in PI and the species responsible for PI remain unclear. It is also possible that unknown bacteria are involved in the lesions. As pockets around the remaining teeth may act as a bacterial reservoir, the composition of the peri-implant microbiota is likely to be similar to that around teeth. However, few studies have evaluated the differences in bacterial composition between dental implants and remaining teeth in the same subjects.

In a recent study, molecular techniques such as oligonucleotide probes, polymerase chain reaction (PCR), and checkerboard DNA–DNA hybridization have been applied to identify the bacteria in PI (20–25). However, these approaches only detect specific target bacteria and are not practical for identifying the true diversity of potential pathogens in the pockets of PI. In contrast, PCR amplification of conserved regions of the 16S ribosomal RNA (rRNA) gene followed by clone library construction has been used to comprehensively identify various microbiotas. This approach allows the detection of almost every species in a given sample and is able to indicate the presence of previously uncultivated and unknown bacteria (26).

The aim of this study was to determine the microbiota in subjects with PI, clinical healthy implants, and periodontal teeth using 16S rRNA gene clone library analysis, and to clarify the microbial differences.

Materials and methods

Subjects and clinical examination

Three subjects with PI, a clinically healthy implant, and a periodontally diseased tooth were selected. Subjects were non-smokers and in good general health. They had not received systemic antibiotics, anti-inflammatory drugs, or oral anti-microbial agents within the last 3 months. The investigation was approved by the Ethics Committee of the Tokyo Medical and Dental University, and a written informed consent was obtained from all subjects.

Clinical examinations were performed for the selected teeth and implants. The following clinical parameters were assessed at six sites per tooth and at six sites per implant (mesiobuccal, buccal, distobuccal, mesiolingual, lingual, and distolingual): (1) probing depth (PD), (2) bleeding on probing (BOP), (3) suppuration (SUP), and (4) Gingival Index (GI) (27). Intra-oral periapical radiographs (Insight dental films, Eastman Kodak Company, SP, Japan) were obtained using the parallel technique.

Radiographs were analyzed for peri-implant bone loss by the same examiner using the smooth components and threads of the implants as reference points. Based on clinical and radiographic data, a diseased implant, a clinically healthy implant, and a periodontally diseased tooth were selected for plaque sampling in each subject. Diseased implants (implants with PI) showed PD ≥ 5 mm with BOP and/or SUP and concomitant radiographic bone loss (bone loss more than three threads up to half of the implant length). Healthy implants (H) showed PD < 4 mm without BOP and SUP, and radiographic bone loss. All implants for sampling were treated as single stand prostheses. Periodontally diseased teeth (P) showed PD ≥ 4 mm with BOP.

Sample collection and bacterial DNA isolation

Subgingival plaque samples were obtained from the deepest pockets at the implants with/without PI. In addition, samples from the deepest pockets of the periodontally diseased tooth, not adjacent to the implant were collected. Two weeks before sampling, we performed periodontal examination for all of the residual teeth and implants. PD, BOP, and SUP were measured at six points per tooth as pre-examination and together with radiographic evaluation, sampling sites were decided. Sampling sites were isolated with sterile cotton rolls. Supragingival plaque was removed with sterile cotton pellets. Three paper points were inserted into a pocket until resistance was felt. After 30 s, all paper points were removed and placed in a sterile tube with 1 ml of sterile distilled water.

Samples were mixed for 1 min using a vortex mixer. After removing the paper point, each sample was collected by centrifugation at 12,000g for 5 min. The resulting pellet was resuspended in 150 μl of lysis buffer from a bacterial DNA extraction kit (Mora-extract, AMR Inc., Tokyo, Japan). Samples were then incubated for 10 min at 90°C and total bacterial genomic DNA was isolated using the Mora-extract kit. Total bacterial DNA was eluted with 200 μl of TE buffer (AMR Inc.) and was stored at −20°C.

16S ribosomal RNA (rRNA) gene clone library analysis

16S rRNA gene clone library analysis was performed as described previously (28, 29). Briefly, the primers used for PCR amplification of the 16S rRNA gene were 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). PCR reaction mixture (100 μl) containing 10 μl of extracted DNA, 2.5 U of TaKaRa Ex taq® (TAKARA BIO Inc., Otsu, Japan), 10 μl of 10 × Ex Taq buffer, 8 μl of dNTP mixture (0.2 mM each), and 50 pmol of each primer. PCR amplification was performed using a Veriti 200 PCR Thermal Cycler (Applied Biosystems, Foster City, CA,

USA) with the following program; 95°C for 3 min, followed by 15 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 1.5 min and a final extension period of 72°C for 10 min. PCR products were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA).

Purified amplicons were ligated into plasmid vector pCR®2.1 and then transformed into One Shot® INVaF′ competent cells using the Original TA Cloning kit (Invitrogen, San Diego, CA, USA). Plasmid DNAs were prepared using the TempliPhi DNA Amplification Kit (GE Healthcare, Buckinghamshire, UK) from randomly selected recombinants and used as templates for sequencing. Sequencing was conducted using the 27F and 520R primers, a BigDye Terminator Cycle Sequencing kit (Applied Biosystems), and a 3130*xl* Genetic Analyzer (Applied Biosystems).

All sequences were checked for possible chimeric artifacts by the Chimera Check program of the Ribosomal Database Project-II (RDP-II) and compared to similar sequences of the reference organisms by BLAST search (30). A 16S rRNA gene sequence similarity of 98% was used as the cut-off for positive identification of taxa (operational taxonomic unit – OTU). Less than 98% identity in the 16S rRNA gene sequence was the criterion used to identify bacteria at the species level. The sequences were aligned with the Clustal X 2.0.12 program (31) and corrected by manual inspection. Nucleotide substitution rates (K_{nuc} values) were calculated (32) after gaps and unknown bases were eliminated. The phylogenetic tree was constructed by the neighbor-joining method (33). Bootstrap resampling analysis (34) was performed to estimate the confidence of tree topologies. Sequences for novel phylotypes were deposited in the DDBJ database under accession numbers AB538407 to AB538428.

Libraries were analyzed using the Mothur program v.1.7.2 (35). Distance matrices were calculated using the Dnadist program within the PHYLIP software package version 3.69. The Shannon index was used to measure community diversity. The Chao1 index was applied to measure community richness.

Table 1. Clinical data of the subjects

Subject	A	B	C
Age	60	60	60
Gender	Female	Female	Female
Smoking habit	Non	Non	Non
Mean of all teeth PD (mm)[a]	2.7±0.76	2.2±0.42	2.5±1.27
Residual teeth	21	20	13
Residual implants	2	6	8

[a]Mean±SD.

Table 2. Clinical information of sampling sites

	Subject A		Subject B		Subject C	
Condition	PI	P	PI	P	PI	P
Site	37	44	16	13	11	24
PD (mm)	5	5	9	4	5	4
BOP or SUP	+	+	+	+	+	+
GI	2	2	2	2	2	2
Time of implant load (year)	10		7		3	
Implant surface	Rough		Machine		Rough	

Results

Clinical data of subjects and sites selected for bacterial sampling are summarized in Tables 1 and 2, respectively. A total of nine sites (three PI, three periodontitis, and three healthy implants) were selected and collected for subgingival plaque samples. One sample (periodontally healthy implant) was missed in the process of sample preparation, so eight samples were analyzed. A total of 335 sequences from eight samples were subjected to sequence analysis, which revealed 112 species; 51 (46%) were uncultivated phylotypes, of which 22 were novel. The total numbers of bacterial species identified at the sites of PI, periodontitis, and periodontally healthy implants were 77, 57, and 12, respectively. Each clone

Table 3. Bacterial phyla and genera detected in this study

| Actinobacteria	Proteobacteria	TM7
Actinobaculum	*Campylobacter*	TM7
Actinomyces	*Cardiobacterium*	
Atopobium	*Desulfobulbus*	Tenericutes
Propionibacterium	*Eikenella*	*Mycoplasma*
Rothia	*Hemophilus*	
	Lautropia	Synergistetes
Firmicutes	*Neisseria*	*Synergistes*
Catonella	*Terrahaemophilus*	
Dialister		
Eubacterium	Bacteroidetes	
Gemella	*Bacteroidetes*	
Granulicatella	*Capnocytophaga*	
Lachnospiraceae	*Porphyromonas*	
Lactobacillus	*Prevotella*	
Mogibacterium		
Parvimonas	Chloroflexi	
Peptostreptococcs	*Chloroflexi*	
Pseudoramibacter		
Selenomonas	Fusobacteria	
Solobacterium	*Fusobacterium*	
Streptococcus	*Leptotrichia*	
Veillonella		
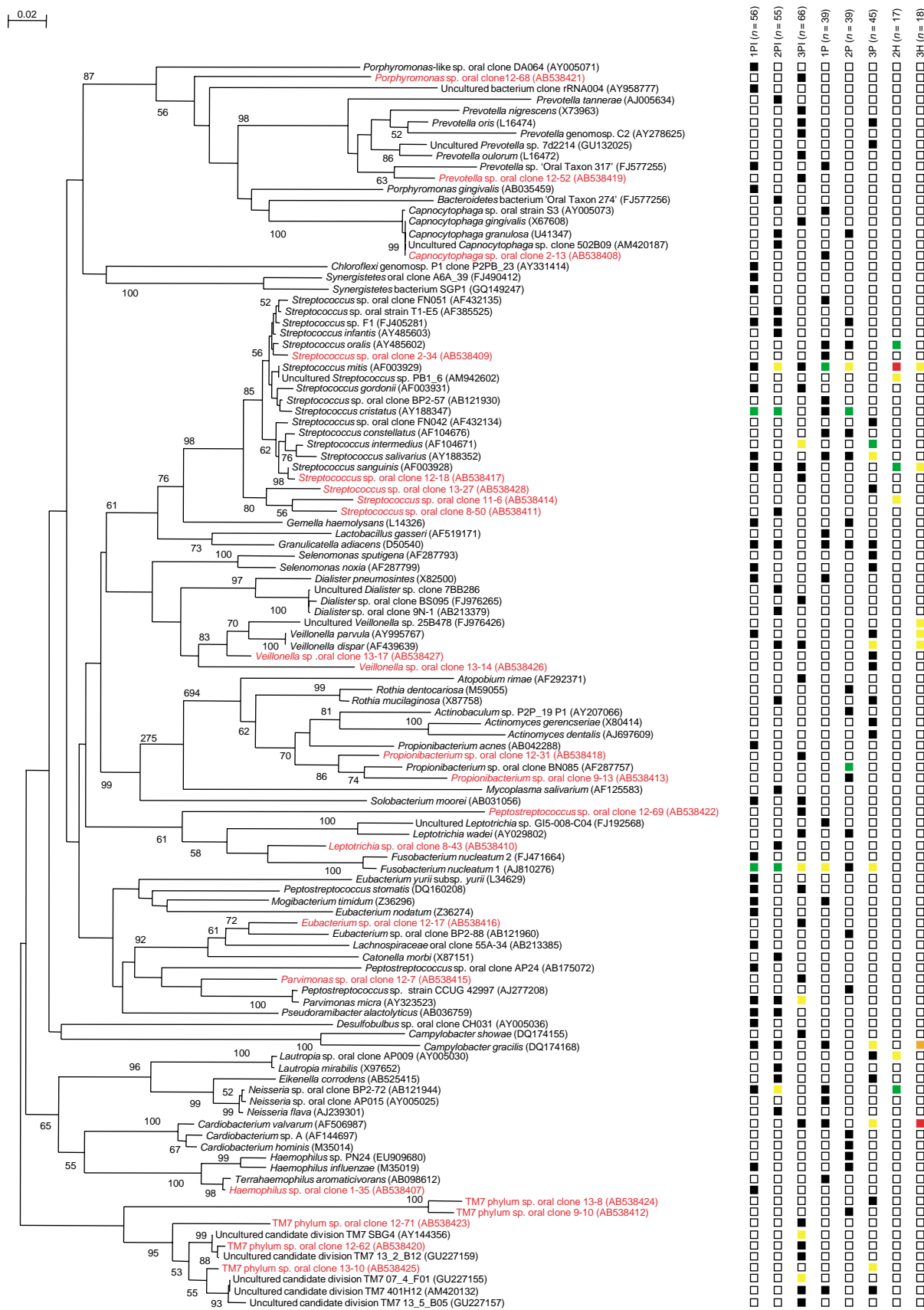
Fig. 1 (*Continued*)

Table 4. Comparison of diversity and richness of sequenced clones between peri-implantitis and periodontitis[a]

Sample source	No. of sequences	No. of OTUs[b]	Shannon index	Richness[c]	Coverage (%)[d]
Peri-implantitis	177	77	3.8 (3.7–4.0)	161 (110–278)	75.7
Periodontitis	123	57	3.7 (3.5–3.8)	78 (61–124)	79.7

[a]Shannon index and richness are estimated based on 2% differences in nucleic acid sequence alignments. Values given in parentheses are 95% confidence intervals, as calculated by the Mothur program.
[b]OTU – operational taxonomic unit.
[c]Chao1 values, a non-parametric estimate of species richness.
[d]Coverage values for a distance of 0.02, as calculated by the Mothur program.

was classified into several clusters corresponding to phylum-level classification (Table 3, Fig. 1). Microbiota of PI primarily included Gram-negative species and the composition was more diverse than that for healthy implants or periodontitis. *Chloroflexi*, *Tenericutes*, and *Synergistetes* phyla were only detected at PI sites. Also *Parvimonas micra*, *Peptostreptococcus stomatis*, *Pseudoramibacter alactolyticus*, and *Solobacterium moorei* were only observed at PI sites. *Fusobacterium nucleatum* was identified at all of the PI sites and *Granulicatella adiacens* was identified at two thirds of PI sites; these two species were also detected at periodontitis sites but not at healthy implants. Most of the bacterial species found in the healthy implants were also detected in the PI and periodontitis sites.

When the diversity and richness of the resident bacterial species were compared between PI and periodontitis, higher values for the Shannon index and richness were observed at PI sites (Table 4), thus suggesting that the bacterial community at PI sites were more diverse when compared to periodontitis.

Discussion

In the present study, we identified the bacteria that compose biofilm at sites with PI. It is believed that the source of infecting bacteria on implants is mainly plaque from residual teeth or saliva, and that microbiota around the implants tend to be similar to that of residual teeth (36–38). The periodontal status of remaining teeth would thus determine the bacterial composition at PI sites (37, 38). Our results show that some *Streptococcus* spp. and *F. nucleatum* are common to sites with periodontitis and PI. Sites with PI tend to show a more complex microbiota when compared to periodontitis/healthy implant sites and Gram-negative anaerobic bacteria are particularly common at such sites.

The presence of periodontopathic bacteria is generally considered to be a risk factor for PI, and indeed, many studies have reported the high prevalence of bacteria, such as *P. gingivalis* and *A. actinomycetemcomitans* in PI lesions (15–20). In contrast, several researchers have argued that PI and/or implant failure does not always harbor periodontopathic bacteria (39–41). *F. nucleatum* is reported to be a pathogen involved in periodontitis and was found at all PI sites in the present study. However, we were unable to confirm the high prevalence of other periodontopathic bacteria in peri-implant/periodontal lesions. It should be emphasized that the sample size was limited and our results do not rule out an association between 'established' periodontopathic bacteria and PI. Although numerous species reside in peri-implant lesions when compared to periodontitis sites, potentially important bacteria may have been overlooked as disease pathogens. To our knowledge, this is the first study using the 16S rRNA gene clone library technique to analyze the microbiota in PI, to confirm that the biofilm of PI is composed of a greater variety of bacterial species when compared to periodontitis. Bacteria isolated only in PI, such as *P. micra*, *P. stomatis*, and *P. alactolyticus*, have been reported to be present in periodontal and/or endodontal lesions (42, 43). Because most of these bacteria are difficult to grow in culture, they have not yet been characterized by their bacterial properties. Also the *Chloroflexi*, *Tenericutes*, and *Synergistetes* phyla were only detected at PI sites. This is in contrast to Vartoukian et al. (44) who reported a high prevalence of the phylum 'Synergistetes' in both periodontitis and healthy subjects. We considered that the discrepancy of the results was mainly derived from the bacterial sampling method; they used pooled plaque samples taken by curette from four periodontal pockets. Since the number of subjects attended in the studies was small (Vartoukian; 10, ours;

Fig. 1. Phylogenetic tree of bacterial species and phylotypes detected in peri-implantitis (PI), periodontitis (P), and healthy implants (H). Novel phylotypes identified in this study are indicated in red letters. The scale bar represents 0.02 substitutions per nucleotide. Accession numbers for 16S rRNA gene sequences are given for each strain. Right columns 1PI, 2PI, 3PI, 1P, 2P, 3P, 2H, and 3H represent subject, sample, and the numbers of bacterial species identified at each site (see text). Boxes used to indicate abundance levels, based on total number of clones assayed: not detected (blank box), 1–5% (black), 6–10% (yellow), 11–20% (green), 21–40% (orange), and ≥40% (red).

3), further studies are necessary for clarifying the role of the phylum *Synergistetes* in peri-implant diseases, moreover for other bacteria.

Differences in bacterial diversity between PI and periodontitis lesions may be explained by the characteristics of surfaces to which the bacteria adhere. Surface roughness and free energy (wettability) are thought to have a significant impact on biofilm formation (45) and the higher levels of free energy on the implant surfaces are likely to affect biofilm components.

In conclusion, PI biofilms showed a more complex microbiota when compared to periodontitis and periodontally healthy implants, and were mainly composed of Gram-negative anaerobic bacteria. Previously established periodontopathic bacteria showed low prevalence and several bacteria were identified as candidate of pathogens in PI, although it is unclear whether the importance of these species is higher when compared to established periodontopathic bacteria.

Acknowledgements

Conflict of interest and funding

There is no conflict of interest in the present study for any of the authors.

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*Tatsuro Koyanagi
Section of Periodontology
Department of Hard Tissue Engineering
Graduate School, Tokyo Medical and Dental University
Bunkyo-ku, 1-5-45 Yushima, Tokyo 113-8549, Japan
Email: koyanagi.peri@tmd.ac.jp