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著者名(英)	KAMAGUCHI, Arihide/NAKAMURA, Reiko/OKAMOTO, Akimasa/WATANABE, Toshihiro/OHYAMA, Tohru/BABA, Hisae
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Quantification of *Porphyromonas gingivalis*
by real time PCR: new
primers targeting the *rgpA* and *rgpB* gene encoding RGP

Arihide KAMAGUCHI*, Reiko NAKAMURA*, Akimasa OKAMOTO**,
Toshihiro WATANABE***, Tohru OHYAMA*** and Hisae BABA*

*Department of Oral Microbiology, School of Dentistry,
Health Sciences University of Hokkaido.

**Department of Oral Bacteriology, School of Dental
Medicine, Turumi University.

***Department of Food Sciences and Technology, Faculty of Bioindustry,
Tokyo University of Agriculture.

(Chief : Prof. Hisae BABA)

Abstract

We designed new primers for the quantification of *Porphyromonas gingivalis* by real time PCR. The new primer set targeted the *rgpA* and *rgpB* genes that encode arginine specific cysteine proteinase (Arg-gingipain or Rgp), one of the putative pathogenic factors of *P. gingivalis*. The PCR product obtained using our primers showed no by-products by melting curve analysis. The PCR product sequence showed no significant matches to other sequences by BLAST searching of genetic databases except for matches to *P. gingivalis* *rgpA* and *rgpB* sequence, and could not be amplified from template derived from other oral bacteria apart from *P. gingivalis*. Therefore, we concluded that our primers were specific for *P. gingivalis* *rgpA* and *rgpB*, and could be used to quantify from 10^3 to 10^7 *P. gingivalis* cells when applied to real time PCR.

Key words : *Porphyromonas gingivalis*, Real time PCR, Quantification.

Introduction

Porphyromonas gingivalis has been implicated as a major etiology of adult periodontitis and acute periodontitis¹⁾. As *P. gingivalis* numbers in adult periodontitis are thought to be related to disease prognosis, it is important to be able to quantify *P. gingivalis* cell numbers at the periodontal site. Until now, the measurement of specific bacterial numbers has involved the

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inoculation of sample material into selective media followed by culture the counting of colony numbers. However, one of the drawbacks of this approach is the relatively long time required for *P. gingivalis* colonies to appear. Recently, PCR has been applied not only to bacterial species identification, but also to bacterial cell quantification. A system of *P. gingivalis* identification based on PCR amplification of 16S rDNA has been reported²⁻¹¹, along with quantification by light cyclers or TaqMan systems using primers for 16S rDNA or ribosomal intergenic spacer region (ISR) sequences^{12,13}. For the PCR identification of bacteria, often two primer sets are used, one targeting 16S rDNA sequence, and the other targeting a pathogenic factor. Examples of pathogenic factors used include the Toxin A gene for *Clostridium difficile*, Vero toxin gene for *Escherichia coli*, streptococcal erythrogenic genes for *Streptococcus pyogenes*, and the cholera toxin gene for *Vibrio cholerae*¹⁴⁻¹⁷. While several primer sets have been reported for the detection of *P. gingivalis* 16S rDNA sequence³⁻¹¹, few primers have targeted pathogenic factors, with only primers for the *fimA* and *trpC* genes reported^{18,19}. It is well known that capsule, LPS, fimbriae, collagenase, Rgp and Lysine specific cysteine proteinase (Lys-gingipain or Kgp) are pathogenic factors for *P. gingivitis*. Rgp, a major pathogenic factor of *P. gingivalis*²⁰, is encoded by the *rgpA* and *rgpB*²⁰⁻²³. Nucleotide and amino acid sequence analysis have indicated that *rgpA* consists of a signal peptide, an N-terminal propeptide, a protease domain, and C-terminal adhesin domains. *RgpB* is completely identical to *rgpA* except for a defective C-terminal adhesin domain²⁰⁻²³. Rgp can degrade human collagen type I and fibronectin^{24,25}, as well as human immunoglobulin (IgG and IgA)^{24,25}, and cytokines²⁶. Rgp can also interfere with neutrophil^{24,25}, prevent the activation of the kallikrein and kinin cascades²⁷, the destruction of complement²⁸, prevent the resolution of fibrinogen and fibrin^{27,29}, and the activation of the X-factor involved in blood coagulation³⁰. Thus, *rgpA* is an important gene in the pathogenesis of *P. gingivalis*. It was thought that detection of trypsin-like enzyme activity was sufficient to determine the presence of Rgp. However, Fletcher et al reported a mutant of the *prtH* (*rgpA*) gene in *P. gingivalis* isolated from a periodontitis patient³¹, suggesting that it is important to determine the presence of the *rgpA* gene rather than Rgp activity. In this study, we present a set of primers for the quantification of *P. gingivalis* based on the genes encoding Rgp.

Materials and Methods

Bacterial strains

P. gingivalis ATCC 33277, JCM 8525, and clinical isolates, 1, 2, 3, 5, 6, 15, 32, 83, usui, *Prevotella intermedia* ATCC 25611, and clinical isolates, 21, 32, 83, *Prevotella nigrescens* ATCC 25261, ATCC 33563, IN 19, *Prevotella oris* JCM 8540, *Prevotella loeschei* JCM 8530, and *Prevotella melaninogenica* JCM 6321 were grown anaerobically in tryptic soy broth supplemented with yeast extract, hemin and menadione. *Actinomyces naeslundii* ATCC 12014, *Actinobacillus actinomycetemcomitans* ATCC 29523, *Streptococcus mutans* Ingbritt, *Streptococcus gordonii*

ATCC 10558, and *Streptococcus salivarius* 9222 were grown anaerobically in tryptic soy broth supplemented with yeast extract. Bacteria counted using a bacterial cell counting chamber.

Preparation of bacterial genomic DNA

P. gingivalis chromosomal DNA was extracted according to the method of Smith et al⁸⁾. Briefly, chromosomal DNA was obtained after treatment with SDS, proteinaseK, phenol/chloroform/isoamyl alcohol extraction, and ethanol precipitation, followed by RNase treatment, phenol/chloroform/isoamyl alcohol extraction, and ethanol precipitation.

Procedure of conventional PCR and real time PCR

Conventional PCR was performed using 2 μ l template in, 4 μ l 10 x buffer, 2.5 μ l each primer, 15 μ l H₂O, and 0.2 μ l Taq polymerase, PCR thermocycling of preheating at 94 °C for 5 min, followed by 35 cycles of 92 °C for 1 min, annealing at 57 °C for 1 min, and extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min. PCR samples were then applied to agarose gel electrophoresis and gels stained with ethidium bromide. Real time PCR was performed using a Light Cycler (Roche Diagnostics). Preparation of the reaction mixtures was done according to manufacture's instructions. Briefly, a mastermix consisting of 14 μ l LC-DNA Master SYBR Green I 14 μ l, 16.8 μ l MgCl₂, 6.5 μ l each primer (10 pmol/ μ l), and 82.2 μ l H₂O was prepared and 18 μ l aliquoted into cooled LC capillaries containing 2 μ l template or water. Capillaries were centrifuged and then placed into the Light Cycler. PCR conditions were an initial denaturation at 95 °C for 2 min (Temp transition rate 20 °C/sec), followed by 50 cycles of 95 °C for 0 sec (temp transition rate 20 °C/min), annealing at 57 °C for (temperature transition rate 20 °C/sec), and elongation at 72 °C for 10 sec (temperature transition rate 2 °C/sec). For melting curve analysis, conditions were cycle 1 of 95 °C for 0 sec (temp transition rate 20 °C/sec), 62 °C for 10 sec (temperature transition rate 20 °C/sec), and 95 °C for 0 sec (temperature transition rate 0.2 °C/sec).

Design of primer pairs

Primer sequences were analyzed using OLIGO 4.0 to minimize the formation duplex or hairpin loop structures between forward and reverse primer. A region including *rgpA*, *kgp*, and *hagA* were amplified using the primers 5'-TGGACTGTTTCGGCGACCCC-3'(forward, F1) and 5'-TCGGTGCATCCCACTTGAGCG-3' (reverse, R1). Sequences containing *rgpA* and *rgpB* were amplified using primers 5'-GGCTCTTGTATTGCTTCGGC-3'(forward, F2), 5'-AGACTGGTTGATCGTAGACGC-3'(reverse, R2), 5'-CTCGTTCGTACACTTGTCCCG-3' (forward, F3), 5'-TTTCTGACCCTGCGTTGTGC-3'(reverse, R3), and 5'-GCGTTACTTTCTGTCCCTGCG-3'(reverse, R4).

DNA sequencing

DNA was sequenced using the ABI PRISM BigDye terminator cycle sequencing Kit (Applied Biosystems) and the ABI PRISM 310 DNA Sequencer (Applied Biosystems). Sequence homology similarity searches were carried out using BLAST and sequence alignments carried out using DNAsis.

Results

PCR of 6 regions of *P. gingivalis* genomic DNA

As *rgpA*, *kgp* and *hagA* contain 6 identical copies of a 536 bp sequence, it was thought that the repeated sequence would make a good target for PCR when only few bacteria. Conventional PCR was performed using a primer pair specific for the 536bp region, but 3 bands were detected by agarose gel electrophoresis (Fig. 1). From these results it was concluded that the primers target the 536 bp sequences were unsuitable for PCR-based quantification.

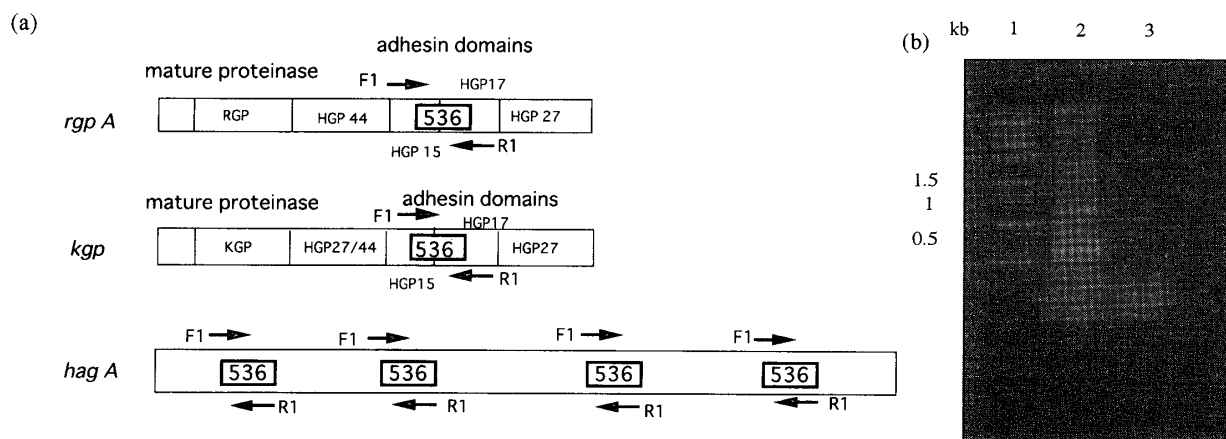


Fig. 1. PCR primer pair and agarose gel electrophoresis pattern of PCR products from *P. gingivalis* ATCC 33277 genomic DNA. (a) Putative location of the F1-R1 primer pair against *rgpA*, *kgp*, and *hagA*. (b) Agarose gel electrophoresis pattern of PCR product from *P. gingivalis* ATCC 33277 template. Lane 1, Mw marker; lane 2, PCR product; lane 3, H₂O only control.

PCR of *rgpA* and *rgpB*

We then generated 3 primer pairs that each amplified less than 500 bp regions of the *rgpA* gene. Expected bands sizes of the PCR products were 419, 321, and 328bp. Conventional PCR was used to confirm that each primer pair product a single PCR product (Fig. 2).

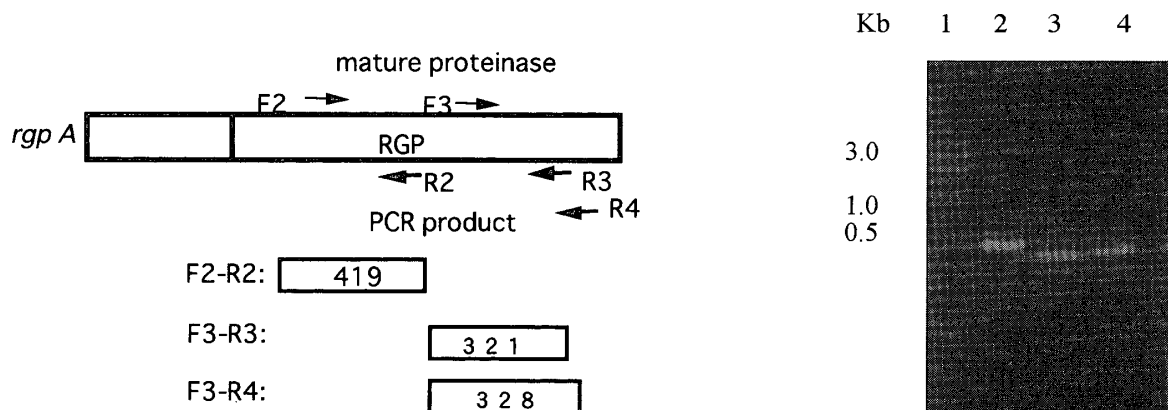


Fig. 2. PCR primer pair and agarose gel electrophoresis pattern of PCR products from *P. gingivalis* ATCC 33277 genomic DNA. (a) Putative locations of F2-R2, F3-R3, and F3-R4 primer pairs against *rgpA*. (b) Agarose gel electrophoresis pattern of PCR products from *P. gingivalis* ATCC 33277. Lane 1, Mw marker; lane 2, PCR product using the F2-R2 primer pair; lane 3, PCR product using the F3-R3 primer pair; lane 4, PCR product using the F3-R3 primer pair.

Quantification with real time PCR

The primer pairs were then applied to bacterial quantification by real time PCR using Light Cycler. Attempts at quantification using the F2 and R2 primer pair showed 2 major peaks by melting curve analysis of the PCR product (data not shown). This indicated that these primers produced by-products and were therefore unsuitable for PCR-based quantification. Likewise the F3 and R4 primer pair showed a large and small peak by melting curve analysis (data not shown), and was also considered unsuitable. However, real time PCR using the F3 and R3 primer pair showed only one peak by melting curve analysis (Fig. 3) indicating that a single PCR product was produced with no by-products. The F3 and R3 primers then used to quantify DNA concentration from 10^3 to 10^7 *P. gingivalis* cells (Fig. 4.). Gel electrophoresis of the resultant PCR products showed a clear band for template DNA derived from 10^3 cells (data not shown).

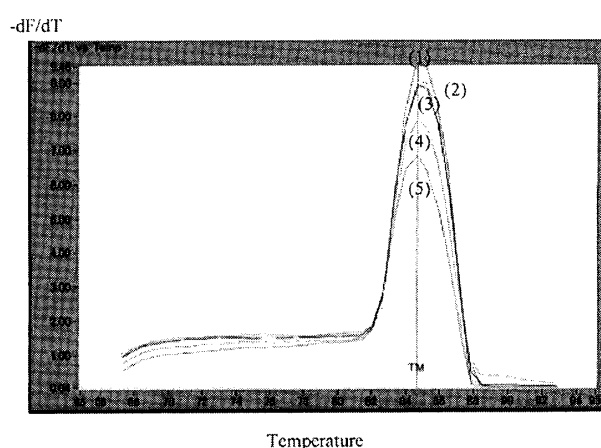


Fig. 3. Specificity of the amplified PCR products assayed by melting curve analysis using Light Cycler For the F3-R3 primer pair. Lines indicate (1) DNA from 10^7 *P. gingivalis* cells, (2) DNA from 10^6 *P. gingivalis* cells, (3) DNA from 10^5 *P. gingivalis* cells, (4) DNA from 10^4 *P. gingivalis* cells, and (5) DNA from 10^3 *P. gingivalis* cells.

Specificity of the primer pair for *P. gingivalis*

Conventional PCR was performed using the F3 and R3 primer pair on a variety of other oral bacteria including *P. intermedia*, *P. nigrescens*, *P. oris*, *P. loeshii*, *P. melaninogenica*, *A. naeshundii*, *A. actinomycetemcomitans*, *S. mutans*, *S. gordonii*, *S. salivarius*. However, no PCR bands were detected. (data not shown). These results suggested that the F3 and R3 primers were specific for *P. gingivalis*. PCR products from 11 *P. gingivalis* strains amplified using the

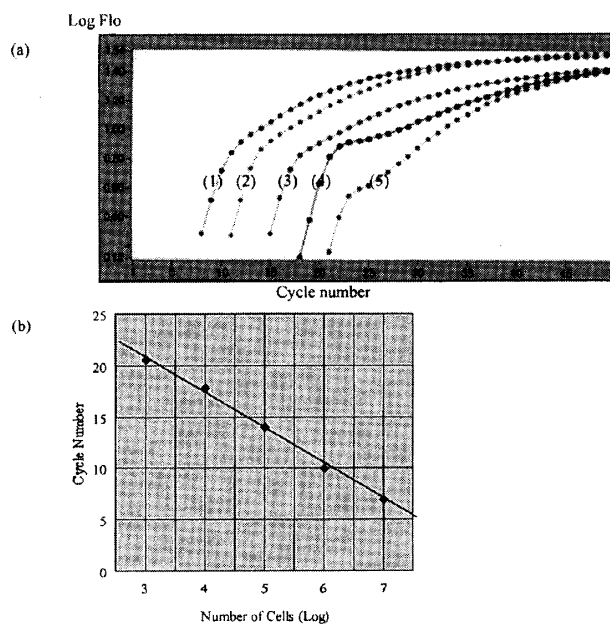


Fig. 4. Run profiles and the standard curve. (a) Run profiles of PCR product using F3-R3 primer pair. Lines indicate (1) DNA from 10^7 *P. gingivalis* cells, (2) DNA from 10^6 *P. gingivalis* cells, (3) DNA from 10^5 *P. gingivalis* cells, (4) DNA from 10^4 *P. gingivalis* cells, and (5) DNA from 10^3 *P. gingivalis* cells. (b) A standard curve generated from the run profiles in panel (a).

F3 and R3 primer pair were subjected to nucleotide sequencing (Fig. 5). While a low level of sequence variation was noted between strains, these differences were not great enough to cause problems in terms of primer recognition.

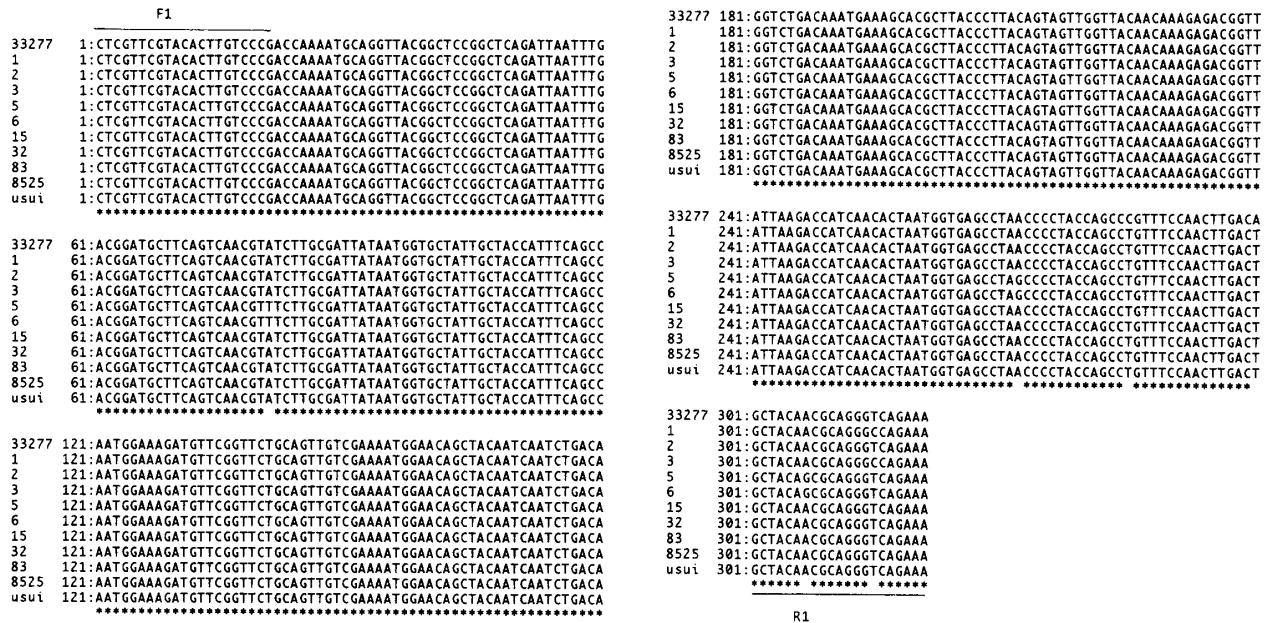


Fig. 5. Alignment of F3-R3 primer PCR product nucleotide sequence from various *P. gingivalis* strains.

Discussion

We investigated the use of PCR primers that targeted the *rgpA* gene, encoding a pathogenic factor, for the quantification of *P. gingivalis* by real time PCR. While methods for the identification of *P. gingivalis* by PCR using primers for 16S rDNA or ISR from clinical material have already been reported, it was thought that the accurate determination *P. gingivalis* numbers by PCR required the design of further specific primers. The *rgpA* gene product is relatively large, and is processed to become mature RGP. *RgpA* contains 4 adhesion domain protein, HGP44, HGP15, HGP17, HGP27, as well as a copy of a 536 bp DNA sequence corresponding to part of HGP15 and HGP17. A further copy of this sequence is present in the *kgp* gene, while the *hagA* gene has 4 copies. While the 6 copies of the 536 bp sequence would appear to make it comparable to 16s rDNA as a PCR target, PCR amplification of the 536 bp sequence was unsuitable for quantification. Further investigation showed that one of three primer pairs designed to amplify part of the *rgpA* encoding RGP was suitable for quantification. Target DNA sequence for this primer pair exists as 2 copies between the *rgpA* and *rgpB* gene. We conducted a gene database search using the BLAST tool for DNA sequences similar to the primer target region and detected no significant similarities except to *rgpA* and *rgpB* sequences. Also, using the primer pair no PCR products were generated using template DNA from many other species of oral bacteria. Therefore, it was considered that the F3/R3 primer

set was specific for *P. gingivalis*.

Analysis of the amount of *P. gingivalis* PCR product produced against the amount of template added showed that amplification was linear from 10^3 to 10^7 *P. gingivalis* cells. While Lyons et al reported that their system could quantify between 10^2 and 10^8 cells⁴⁾, their method was based nested PCR. Sakamoto et al reported that quantification was possible from 10^3 to 10^8 cells by using a primer pair for 16Sr DNA³⁾, which is similar to our present findings. Using our primer set, *rgpA* sequence was detected in all 11 *P. gingivalis* strains including 9 clinical strains. While sequence variation between the PCR products from the different strains, the sequence differences did not interfere in the amplification of the target region.

It has been reported that some clinical strains are negative for the *prtH* (*rgpA*) or *prtC* genes^{31,19)}. Therefore, we decided it was important to quantify *P. gingivalis* cell numbers using primers for the *rgpA* and *rgpB*. Recently, Griffen et al reported differences in virulence among *P. gingivalis* strains with regard to periodontitis after assaying for ISR polymorphisms³²⁾, while Amano et al³³⁾ reported that type II *fimA* strains were predominant in periodontal patients. Thus, it appears that virulence in periodontitis can vary between *P. gingivalis* strains, and it may be important to quantify cell numbers according to the presence of a gene encoding a major pathogenic factor.

In conclusion, we have designed new primer set specific for the *rgpA* and *rgpB* genes. Our results indicated that this primer set can be used to quantify *P. gingivalis* by real time PCR.

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References

1. Slots J : Host-parasite interactions in periodontal diseases, *American Society for Microbiology*, 1982, 27-45.
2. Ashimoto A, Chen C, Bakker I et al. : Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions., *Oral Microbiol Immunol*, **11** : 266-273, 1996.
3. McClellan DL, Griffen AL and Leys EJ : Age and prevalence of *Porphyromonas gingivalis* in children., *J Clin Microbiol*, **34** : 2017-2019, 1996.
4. Slots J, Ashimoto A, Flynn MJ et al. : Detection of putative periodontal pathogens in subgingival specimens by 16S ribosomal DNA amplification with the polymerase chain reaction., *Clin Infect Dis*, **20** : S304-S307, 1995.
5. Tran SD and Rudney JD : Multiplex PCR using conserved and species-specific 16S rRNA gene primers for simultaneous detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*., *J Clin Microbiol*, **34** : 2674-2678, 1996.
6. Wahlfors J, Meurman JH, Vaisanen P et al. : Simultaneous detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* by a rapid PCR method., *J Dent Res*, **74** : 1796-1801, 1995.
7. Matto J, Saarela M, Alaluusua S et al. : Detection of *Porphyromonas gingivalis* from saliva by PCR by using a sample-processing method., *J Clin Microbiol*, **36** : 157-160, 1998.
8. Choi BK, Park SH, Yoo YJ et al. : Detection of

- major putative periodontopathogens in Korean advanced adult periodontitis patients using a nucleic acid-based approach., *J Periodontol*, **71** : 1387-1397, 2000.
9. Siqueira JF Jr, Rjcas IN, Oliveira JC et al. : Detection of putative oral pathogens in acute periradicular abscesses by 16S rDNA-directed polymerase chain reaction., *J Endod*, **27** : 164-167, 2001.
 10. Siqueira JF Jr, Rjcas IN, Oliveira JC et al. : Molecular detection of black-pigmented bacteria in infections of endodontic origin., *J Endod*, **27** : 563-566, 2001.
 11. Tran SD and Rudney JD : Improved multiplex PCR using conserved and species-specific 16S rRNA gene primers for simultaneous detection of *Actinobacillus actinomycetemcomitans*, *Bacteroides forsythus*, and *Porphyromonas gingivalis*., *J Clin Microbiol*, **37** : 3504-3508, 1999.
 12. Sakamoto M, Takeuchi Y, Umeda M et al. : Rapid detection and quantification of five periodontopathic bacteria by real-time PCR., *Microbiol Immunol*, **45** : 39-44, 2001.
 13. Lyons SR, Griffen AL, and Leys EJ : Quantitative real-time PCR for *Porphyromonas gingivalis* and total bacteria., *J Clin Microbiol*, **38** : 2362-2365, 2000.
 14. Fluit AC, Wolfhagen MJ, Verdonk GP et al. : Nontoxic strains of *Clostridium difficile* lack the genes for both toxin A and toxin B., *J Clin Microbiol*, **29** : 2666-2667, 1991.
 15. Paton AW, Paton JC, Goldwater PN et al. : Direct detection of *Escherichia coli* Shiga-like toxin genes in primary fecal cultures by polymerase chain reaction., *J Clin Microbiol*, **31** : 3063-3067, 1993.
 16. Tyler SD, Johnson WM, Huang JC et al. : Streptococcal erythrogenic toxin genes : detection by polymerase chain reaction and association with disease in strains isolated in Canada from 1940 to 1991., *J Clin Microbiol*, **30** : 3127-3131, 1992.
 17. Shirai H, Nishibuchi M, Ramamurthy T et al. : Polymerase chain reaction for detection of the cholera enterotoxin of *Vibrio cholerae*., *J Clin Microbiol*, **29** : 2517-2521, 1991.
 18. Watanabe K and Frommel TO : Detection of *Porphyromonas gingivalis* in oral plaque samples by use of the polymerase chain reaction., *J Dent Res*, **72** : 1040-1044, 1993.
 19. Bodinka A, Schmidt H, Henkel B et al. : Polymerase chain reaction for the identification of *Porphyromonas gingivalis* collagenase genes., *Oral Microbiol Immunol*, **9** : 161-165, 1994.
 20. Okamoto K, Misumi Y, Kadowaki T et al. : Structural characterization of argingipain, a novel arginine-specific cysteine proteinase as a major periodontal pathogenic factor from *Porphyromonas gingivalis*., *Arch Biochem Biophys*, **316** : 917-925, 1995.
 21. Nakayama K, Kadowaki T, Okamoto K et al. : Construction and characterization of arginine-specific cysteine proteinase (Arg-gingipain)-deficient mutants of *Porphyromonas gingivalis* : evidence for significant contribution of Arg-gingipain to virulence., *J Biol Chem*, **270** : 23619-23626, 1995.
 22. Nakayama K : Domain-specific rearrangement between the two Arg-gingipain-encoding genes in *Porphyromonas gingivalis* : possible involvement of nonreciprocal recombination., *Microbiol Immunol*, **41** : 185-196, 1997.
 23. Pavloff N, Potempa J, Pike RN et al. : Molecular cloning and structural characterization of the Arg-gingipain proteinase of *Porphyromonas gingivalis*. Biosynthesis as a proteinase-adhesin polyprotein., *J Biol Chem*, **270** : 1007-1010, 1995.
 24. Kadowaki T, Yoneda M, Okamoto K et al. : Purification and characterization of a novel arginine-specific cysteine proteinase (argingipain) involved in the pathogenesis of periodontal disease from the culture supernatant of *Porphyromonas gingivalis*., *J Biol Chem*, **269** : 21371-21378, 1994.
 25. Abe N, Kadowaki T, Okamoto K et al. : Biochemical and functional properties of lysine-specific cysteine proteinase (Lys-gingipain) as a virulence factor of *Porphyromonas gingivalis* in periodontal disease., *J Biochem (tokyo)*, **123** : 305-312, 1998.
 26. Calkins CC, Platt K, Potempa J et al. : Inactivation of tumor necrosis factor-alpha by

- proteinases (gingipaines) from the periodontal pathogen, *Porphyromonas gingivalis*. Implication of immune evasion., *J Biol Chem*, **273** : 6611-6614, 1998.
27. Imamura T, Potempa J, Pike RN et al. : Effect of free and vesicle-bound cysteine proteinases of *Porphyromonas gingivalis* on plasma clot formation : implications for bleeding tendency at periodontitis sites., *Infect Immun*, **63** : 4877-4882, 1995.
28. Wingrove JA, DiScipio RG, Chen Z et al. : Activation of complement components C3 and C5 by a cysteine proteinase (gingipain-1) from *Porphyromonas (Bacteroides) gingivalis*., *J Biol Chem*, **267** : 18902-18907, 1992.
29. Okamoto K, Nakayama K, Kadowaki T et al. : Involvement of a lysine-specific cysteine proteinase in hemoglobin absorption and heme accumulation by *Porphyromonas gingivalis*., *J Biol Chem*, **273** : 21225-21231, 1998.
30. Imamura T, Potempa J, Tanase S et al. : Activation of blood coagulation factor X by arginine-specific cysteine proteinases (gingipain-Rs) from *Porphyromonas gingivalis*., *J Biol Chem*, **272** : 16062-16067, 1997.
31. Fletcher HM, Schenkein HA, Morgan RM et al. : Virulence of a *Porphyromonas gingivalis* W83 mutant in the *prtH* gene., *Infect Immun*, **63** : 1521-1528, 1995.
32. Griffen AL, Lyons SR, Becker MR et al. : *Porphyromonas gingivalis* strain variability and periodontitis., *J Clin Microbiol*, **37** : 4028-4033, 1999.
33. Amano A, Nakagawa I, Kataoka K et al. : Distribution of *Porphyromonas gingivalis* strains with *fimA* genotypes in periodontitis patients., *J Clin Microbiol*, **37** : 1426-1430, 1999.