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〔ORIGINAL〕

Quantification of *Porphyromonas gingivalis* by real time PCR: new primers targeting the *rgpA* and *rgpB* gene encoding RGP

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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 quantity 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 periodontitis[1]. 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 reported[2-11], along with quantification by light cycler 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 defficile*, 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 sequence[3-11], 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, fimbrie, collagenase, Rgp and Lysine specific cysteine proteinase (Lys-gingipaine 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 *repA* except for a defective C-terminal adhesin domain[20-23]. Rgp can degrade human collagen type 1 and fibronectin[24,25], as well as human immunoglobulin (IgG and IgA)[24,25], and cytokines[26]. Rgp can also interfere with neutrophil[24,25], prevent the activation of the kallikrein and quinine cascades[27], the destroy complement[28], prevent the resolution of fibrinogen and fibrin[27,29], and the activation of the X-factor involved in blood coagulation[30]. Thus, *rgpA* is an important gene in the pathogenesis of *P. gingivalis*. It was thought that detection of trypsin - like enzyme activity was sufficient determine the presence of Rgp. However, Fletcher et al reported mutant of the *prtH (rgpA)* gene in *P. gingivalis* isolated from a periodontitis patient[31], suggesting that 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 loesheii* 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 al[8]. 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 μl template in, 4 μl 10 x buffer, 2.5 μl each primer, 15 μl H_2O, and 0.2 μl 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 μl LC-DNA Master SYBR Green I 14 μl, 16.8 μl $MgCl_2$, 6.5 μl each primer (10 pmol/μl), and 82.2 μl H_2O was prepared and 18 μl aliquoted into cooled LC capillaries containing 2 μl 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 preimers 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 3bands 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.

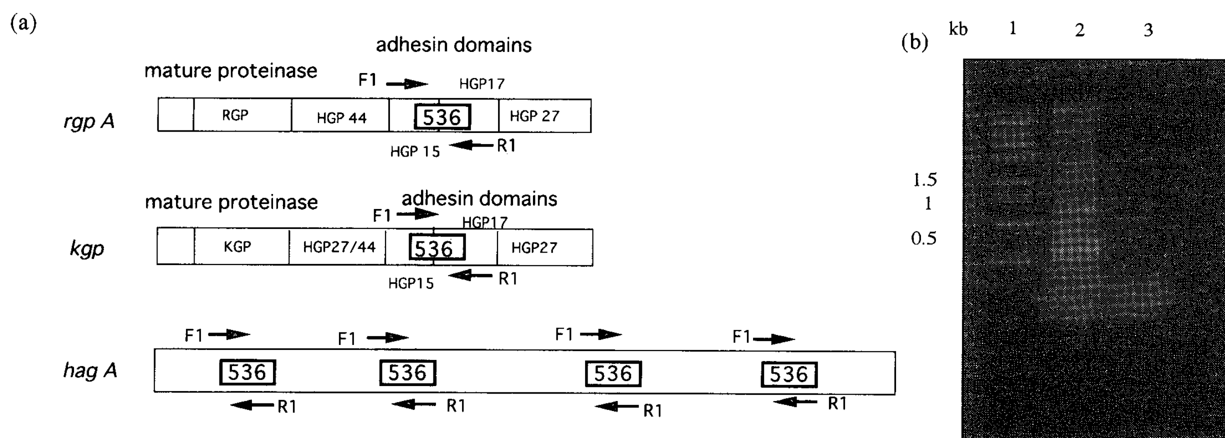

Fig. 1. PCR primer pair and agarose gel electrophoresis pattern of PCR products from *P. gingvalis* ATCC 33277 genomic DNA. (a) Putative location of the F1-R1 primer pair against *rgpA*, *kgp*, and *hagA*. (b) Agarose gel electrophoresis pattern of PCR product from *P. gingvalis* ATCC 33277 template. Lane 1, Mw marker; lane 2,PCR product; lane 3, H_2O 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).

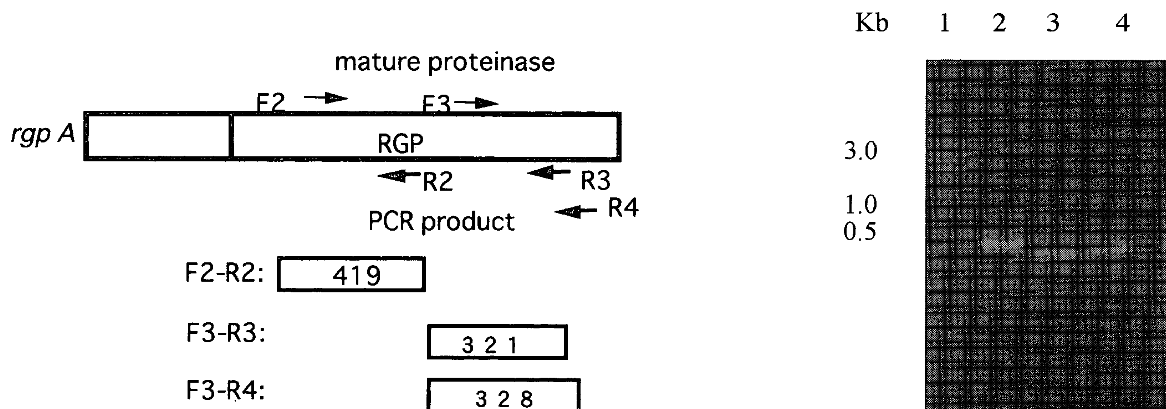

Fig. 2. PCR primer pair and agarose gel electrophoresis pattern of PCR products from *P. gingivalis* ATCC 33277 genomic DNA. (a) Putative locations of F2-R2, F3-R3, and 3-R4 primer pairs against rgpA. (b) Agarose gel electrophoresis pattern of PCR products from *P. gingivalis* ATCC 33277. Lane 1, Mw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

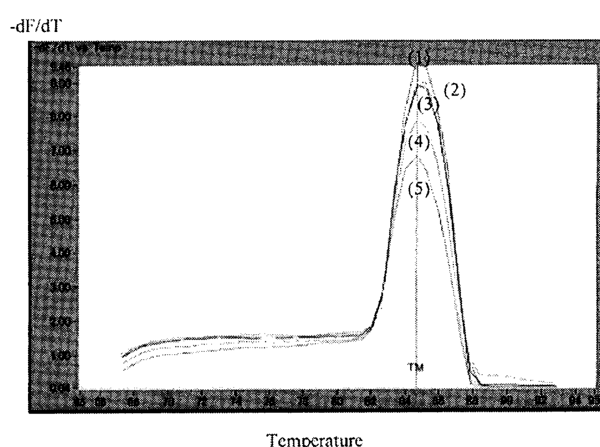

Fig. 3. Specificity of the amplified PCR products assayed by melting curve analysis using Light Cycler For the F3-R3 primer pair. Lines indicate (1) DNA form 10^7 *P. gingivalis* cells, (2) DNA from 10^6 *P. gingvalis* cells, (3) DNA from 10^5 P. gingivalis cells, (4) DNA form 10^4 *P. gingvalis* cells,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

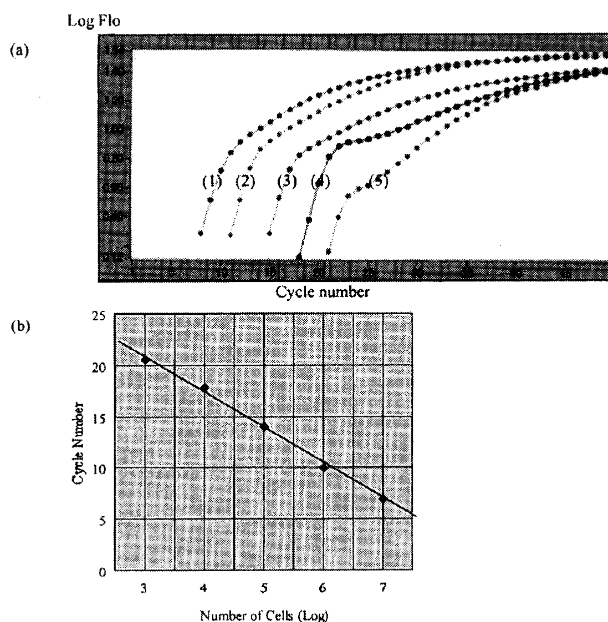

Fig. 4. Runs profiles and the standard curve. (a) Run profiles of PCR product using F3-R3 primer pair. Lines indicate (1) DNA form 10^7 *P. gingivalis* cells, (2) DNA from 10^6 *P. gingivalis* cells, (3) DNA from 10^5 *P. gingivalis* cells, (4) DNA form 10^4 *P. gingivalis* cells, and (5) DNA from 10^3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

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. naeslundii*, *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

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.

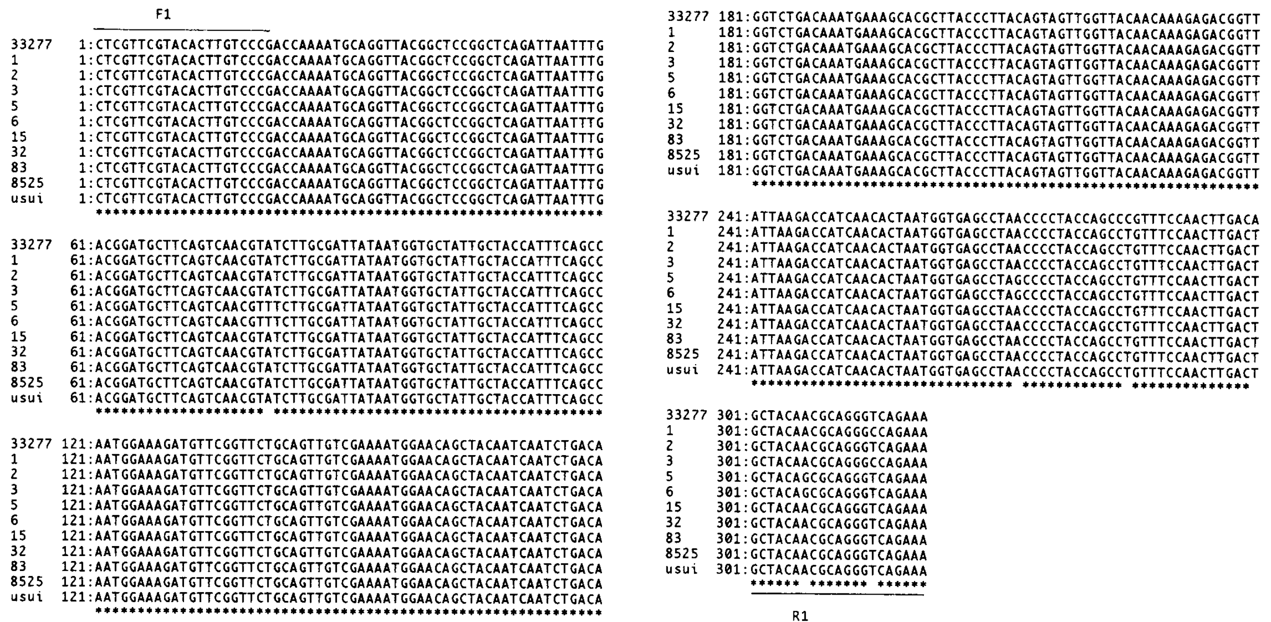

Fig. 5. Alignment of F3-R3 primer PCR product necleotide sequence from various *P. gingivalis* strains.

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 cells[4], 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 DNA[3], 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 polymorphisms[32], while Amano et al[33] reported that type II *fimA* strains were predominant in peridontal 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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