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Original Article

Quantification of Periodontopathic Bacteria in Saliva Using the Invader Assay

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SUMMARY: When quantifying periodontopathic bacteria, it is important to use a convenient method that does not produce false negative results. The Invader assay is a convenient method because it does not involve gene amplification. The purpose of this study was to evaluate the validity of the Invader assay to quantify periodontopathic bacteria. The Invader technology was applied in quantifying five periodontopathic bacteria (*Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella intermedia*, and *Treponema denticola*). The Invader assay produced a linear quantitative detection range over concentrations spanning seven exponential values, with a detection limit of $10^{3.7}$ copies/tube and intra-day and inter-day variance of 0.1% to 4.7% and 0.1% to 3.4%, respectively, in quantifying five periodontopathic bacteria. We compared the results of the Invader assay with those of real-time polymerase chain reaction (PCR) performed for quantifying five periodontopathic bacteria in 22 patients with periodontitis. Among the Invader-detectable bacterial strains of each species, significant correlations were observed in the counts of concerned bacterial species between these two methods, with correlation coefficients ranging from 0.757 to 0.996. This study validated repeatability and reproducibility of the Invader assay in quantifying periodontopathic bacteria and demonstrated consistent agreement between the Invader assay and real-time PCR in quantifying periodontopathic bacteria.

INTRODUCTION

Periodontal diseases have been reported to be caused by different periodontopathic bacteria. *Porphyromonas gingivalis* and *Tannerella forsythia* have been strongly implicated as major pathogens in the etiology of this disease (1–3). *Treponema denticola* has also been implicated as a major pathogen causing periodontitis (4). *Aggregatibacter actinomycetemcomitans* is the causative agent of localized aggressive periodontitis that occurs in adolescents (3,5). The level of *Prevotella intermedia* has been shown to increase in acute necrotizing ulcerative gingivitis (6).

Antibiotic therapy is now being used in addition to scaling and curettage (mechanical treatment), when mechanical treatment alone is not effective (7,8). The identification and quantification of periodontopathic bacteria are useful for confirming the effects of eliminating periodontopathic bacteria by antibiotic therapy (9). Furthermore, quantification of periodontopathic bacteria is a powerful tool for the epidemiological study of periodontal disease (10). Recent advances in molecular biology techniques have led to the development of methods for quantifying gene. Quantification of several periodontopathic bacteria has been achieved by performing real-time polymerase chain reaction (PCR), which detects the PCR amplicons (11). However, the use of real-time PCR in clinical practice is impractical for the quantification of periodontopathic bacteria in spite of its high sensitivity and specificity (11), because real-time PCR involves gene amplification and requires expensive equipments such as thermal cyclers. Furthermore, because the oral cavity's complex aerobic and anaerobic flora contain more than 300 different bacterial species (12), bacterial counts may be underestimated due to false positive results (13,14). When bacteria that have sequences similar to those of the periodontopathic bacteria are contaminated, they could be amplified and detected even at low concentrations (10^2–10^4/tube). These two drawbacks make real-time PCR unsuitable for clinical applications.

Invader technology provides an effective tool for analyzing single-nucleotide polymorphisms (15,16). The Invader assay utilizes the thermostable flap endonuclease cleavase XI, which cleaves invasive structures formed from single-base overlaps between the Invader oligo and the signal probe when hybridized to a complementary target DNA (15). This assay can determine the copy number of target genes easily and cheaply because it uses a common fluorescence resonance energy transfer (FRET) dye-labeled cassette and does not re-

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quire gene amplification. It also has the advantage of providing accurate values over a larger copy number range. Wong et al. reported the first application of the Invader assay to the quantification of pathogenic microorganisms for hepatitis B virus in patients with hepatocellular carcinoma (17). Invader technology has since been applied to quantify various genes.

In the present study, we used quantitative Invader assays for quantification of the major periodontopathic bacteria, *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia*, *P. intermedia*, and *T. denticola*. We validated the use of Invader assay using real-time PCR as the diagnostic reference standard. We also aimed to establish a system that assesses levels of periodontopathic bacteria in the general population and in regular dental examinations.

MATERIALS AND METHODS

Subjects: Invader assays and real-time PCR were performed at a clinical practice in Ayase city, Kanagawa Prefecture, on clinical samples from 22 patients (5 men and 17 women; mean age, 45.2 ± 12.3 years), with various rates of periodontal pockets of >3 mm, ranging from 3.1% to 96.4%. The patients consented to participate in this study. Exclusion criteria were as follows: patients who wore dentures or those with systemic diseases (diabetes mellitus, heart disease, renal disease, cancer, and so on). Out of the 22 patients, 5 patients (3 men and 2 women aged 41–64 years) and 3 employees from BML Inc. (1 man and 2 women aged 54–58 years), who did not fit the exclusion criteria participated in intra-day and inter-day experiments of Invader assay.

Informed consent was obtained from all the subjects. This trial was approved by the Ethics Committees of BML, Inc., Kawagoe, Japan and Tsurumi University, Yokohama, Japan.

Saliva sample preparation: In 2006 and 2007, saliva samples were obtained from subjects 2 h after they had brushed their teeth, during which time they were not allowed to eat or drink. The flow of whole saliva was stimulated by chewing paraffin gum, and the subjects spat into a cup. A sterile cotton stick was immersed in the saliva for 10 s and then placed in phosphate

Table 1. Probe and oligo for the Invader assay and primer for real-time PCR

(A) Invader assay

Primary probe or Invader oligo	Sequence (5′→3′)
Aa-p	CGCGCCGAGG**C**TAAACCCCAATCCCCA
Aa-io	GTCGATTTATCACGTTAGCTTCGGGCACCAGGGT
Pg-p	CGCGCCGAGG**C**GCAGTATGGCAAGC
Pg-io	CCCACGCCTTCGTGCTTCAGTGTCAGTT
Tf-p	CGCGCCGAGG**A**CTATATCGCAAACTCCTAG
Tf-io	CCCAGGTGGATTACTTAACGCTTTCGCTGTAGAGCTTACT
Pi-p	CGCGCCGAGG**C**GATGAATCTTTGGTCCA
Pi-io	CAACAAGCTAATCAGACGCATCCCCATCCTCCACA
Td-p	CGCGCCGAGG**A**TTCTTCATCTGCAAAAGAATT
Td-io	CATGACTACCGTCATCAAAGAAGCATTCCCTCTTCTTCTTT

(B) Real-time PCR

TaqMan probe or PCR primer	Sequence (5′→3′)
Aa-P	TGCGAGCGTTAATCGGAATAACTGGG
Aa-F	ATAGCATGCCAACTTGACGTTAAAT
Aa-R	GATTTCACACCTCACTTAAAGGTCC
Pg-P	CGTTGAAACTGCCGGGCTTGACTTC
Pg-F	GCGCTCAACGTTCAGCCT
Pg-R	CACGAATTCCGCCTGCC
Tf-P	AGATGAAGTAGGCGGAATGCGTGTA
Tf-F	TGAAAGTTTGTCGCTTAACGATAAAA
Tf-R	TCGTGCTTCAGTGTCAGTTATACCT
Pi-P	TGTGGACAACATCGGGTATTAGGCCG
Pi-F	CGTATCCAACCTTCCCTCCA
Pi-R	CGATGAATCTTTGGTCCACGT
Td-P	TGTGAATGAAGAAGGCCGAAAGGTTGTAAA
Td-F	CTTCCGCAATGGACGAAAGT
Td-R	CAAAGAAGCATTCCCTCTTCTTCTTA

Aa, *Aggregatibacter actinomycetemcomitans*; Pg, *Porphyromonas gingivalis*; Tf, *Tannerella forsythia*; Pi, *Prevotella intermedia*; Td, *Treponema denticola*.
(Invader) p, primary probe; (FAM) io, Invader oligo. Underlined sequence represents the 5′ flap of probe. boldface, cleavage site of primary probes.
(Real-time PCR) P, TaqMan probe. All probes were labeled at the 5′ end with FAM and at the 3′ end with TAMRA. F, forward PCR primer; R, reverse PCR primer.

buffered saline. All the saliva samples were stored at −20°C until use. Bacterial nucleic acids were extracted from 200 μl of saliva using the Magnesil Blood Genomic Max Yield System (Promega, Madison, Wis., USA) (18).

Bacterial strains: The following species of periodontopathic bacteria were obtained from the ATCC biological resource center: *A. actinomycetemcomitans* ATCC 43718, *P. gingivalis* ATCC 33277, *T. forsythia* ATCC 43037D, *P. intermedia* ATCC 49046, *T. denticola* ATCC 35404, and *Fusobacterium nucleatum* ATCC 25586. *Neisseria* spp. included clinical isolates of *Neisseria subflava* and *Neisseria sicca*. *F. nucleatum* and *Neisseria* spp. were added as oral commensal bacteria because they have genes that are not homologous to periodontopathic bacteria.

Preparation of standards: Nucleic acids were extracted from ATCC strains of each of the periodontopathic bacteria, and the full-length of the 16S-rRNA gene (rDNA) region was amplified using PCR. The PCR products were subcloned into a pCR Blunt vector using a Zero Blunt PCR Cloning Kit (Invitrogen, Carlsbad, Calif., USA), and the resulting genetic element was used to transform competent *Escherichia coli*. Transformed *E. coli* were cultured in lysogeny broth and the plasmid was purified from cultured *E. coli*. The copy number of purified plasmid was measured using the Picogreen dsDNA Quantitation Kit (Molecular Probes, Eugene, Oreg., USA). Standards for the Invader assay were prepared at $10^{3.7}$, $10^{4.7}$, $10^{5.7}$, and $10^{6.7}$ copies/tube. Standards for real-time PCR were prepared at 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, and 10^7 copies/tube. All the standards were stored at −80°C until use.

Design of bacteria-specific probes for Invader and

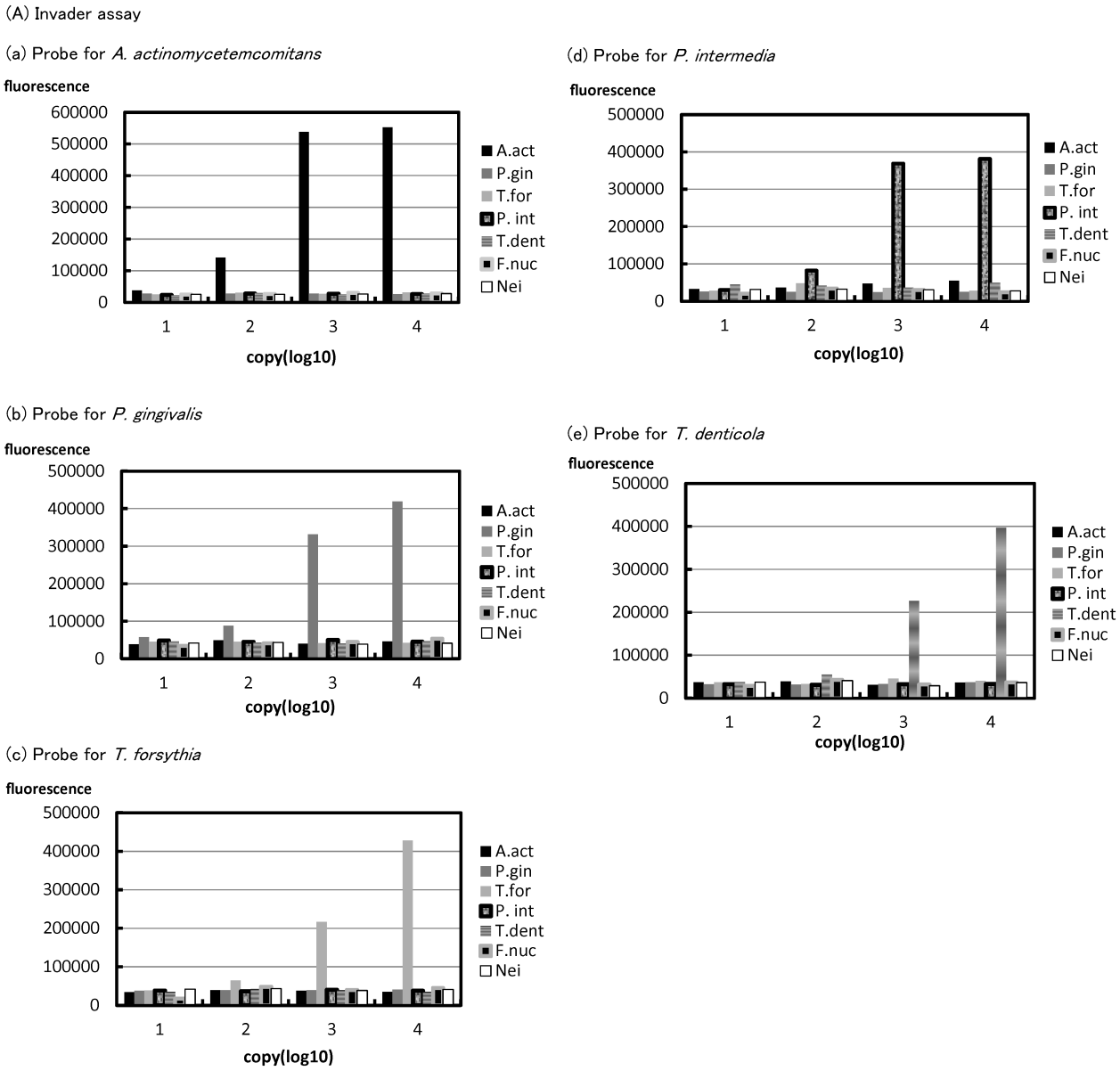

Fig. 1. Validation of probes for the Invader assay and primers for real-time PCR. (A) Invader assay. Each bacterial DNAs (10^1, 10^2, 10^3, and 10^4 pg) were applied in the Invader assay with each probe. (B) Real-time PCR. Each bacterial DNAs (10^1, 10^2, 10^3, and 10^4 pg) were applied in real-time PCR with each primer. A. act, *Aggregatibacter actinomycetemcomitans*; P. gin, *Porphyromonas gingivalis*; T. for, *Tannerella forsythia*; P. int, *Prevotella intermedia*; T. dent, *Treponema denticola*; F. nuc, *Fusobacterium nucleatum*; Nei, *Neisseria*.

primers for real-time PCR: Designs of primary probes and Invader oligos for the Invader assay and of primers for real-time PCR for each bacterial species were based on a region of the 16S rRNA gene as previously reported (19) (Table 1). Probes and primers were ascertained not to have considerable homology with known genes by conducting a BLAST search. Cleavage points for the Invader reaction to distinguish each periodontopathic bacterial species were selected based on a 16S rDNA hypervariable region, including species-specific sites. The primary probe and invasive oligonucleotides for the quantitative assay of periodontopathic bacteria were designed using the Invader Creator software (Third Wave Technology Inc., Madison, Wis., USA) to have theoretical annealing temperatures of 63°C and 77°C, respectively, using a nearest-neighbor algorithm on the basis of final probe and target concentrations (Table 1).

Invader assay: 16S rDNA sequences of the bacteria of interest were researched in the NCBI database and aligned using the GENETYX program (Cosmo Bio Inc., Tokyo, Japan). The reaction was performed in 96-well plates using a Cleavase XI Invader core reagent kit (Third Wave Technology). Each reaction mixture contained 5 μL of no-target control, and standard or bacterial genomic DNA denatured by incubating at 95°C for 5 min. After 15 μL of mineral oil (Sigma, St. Louis, Mo., USA) was overlaid on all the reaction wells, the plate was incubated isothermally (63°C for *P. gingivalis*, *T. forsythia*, *P. intermedia*, and *T. denticola*; 65°C for *A. actinomycetemcomitans*) for 4 h, and fluorescence intensity was measured every 2 min using a fluorescence multi-well plate reader (FluoDia T70; Otsuka Electronics, Osaka, Japan) for carboxyfluorescein dyes (wavelength/bandwidth: excitation, 485/20 nm; emission, 530/25 nm). Bacterial copy number was calculated according to a previously reported method (17).

Real-time PCR assay: Real-time PCR was performed using the ABI PRISM 7900 Sequence Detection System (SDS) (Applied Biosystems, Tokyo, Japan). Each reaction well contained 15 μL of reaction mixture, which contained 2× TaqMan® Universal PCR Master Mix (Applied Biosystems), 3 μL of DNA extracted from a sample, 3 pM of TaqMan® probe, and 0.75 μM of

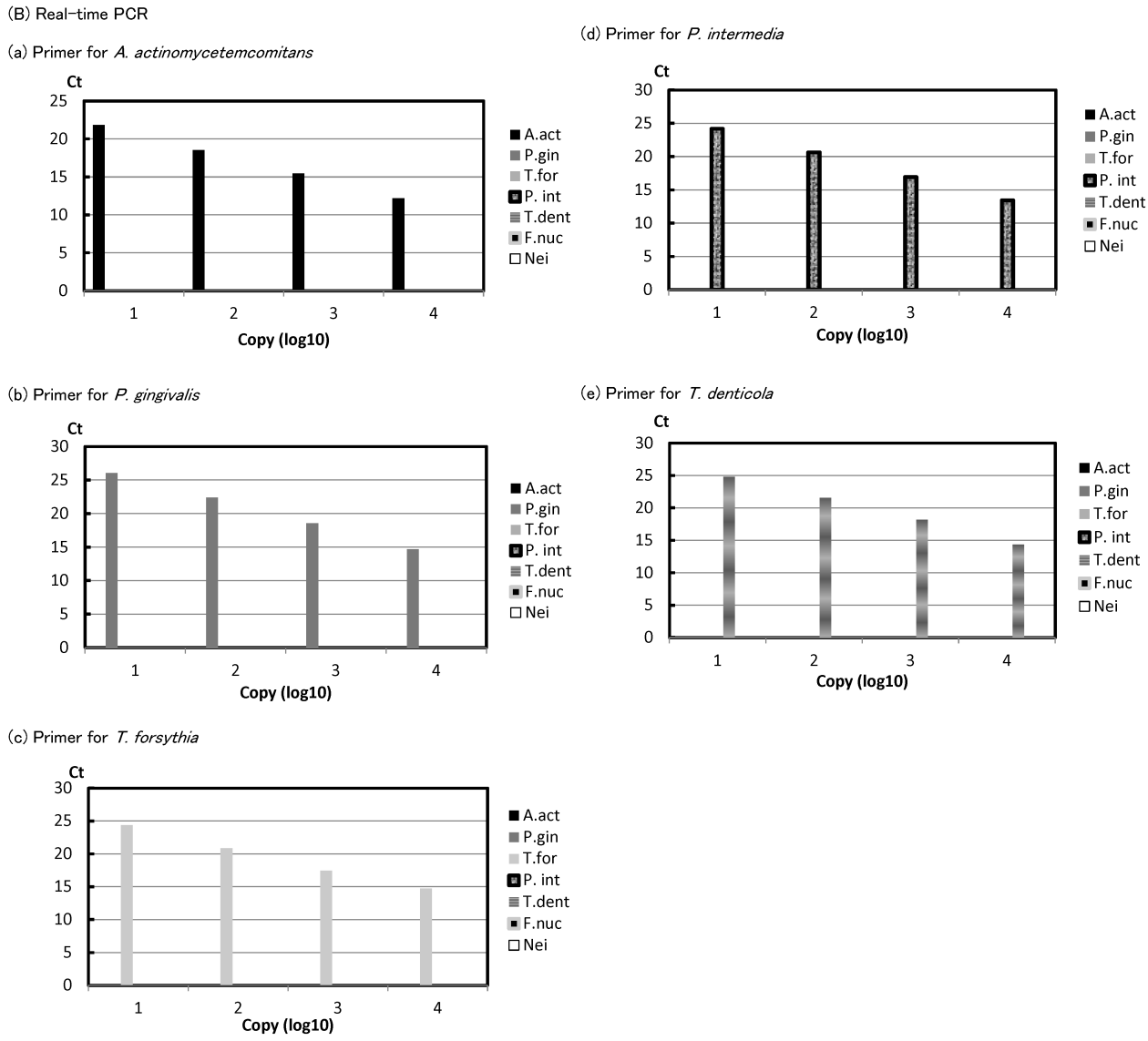

Fig. 1. Continued.

each primer. The thermal cycling conditions were as follows: 2 min at 50°C (uracil *N*-glycosylase; this treatment prevents carry-over cross-contamination by digesting uracil-containing PCR fragments generated in the prior PCR assays): 10 min at 95°C (activation of AmpliTaqGold) and 40 cycles of 15 s at 95°C (denaturation) and 1 min at 59°C (annealing and extension). Raw data were analyzed using the SDS software, version 2.3.

Validation of probes for the Invader assay and primers for real-time PCR: The probes for the Invader assay and the primers for real-time PCR used in this study were validated by comparing their reactivity with DNAs from the seven bacterial species (Fig. 1). DNA from bacterial species at a concentration of 10^{1-4} pg/tube was used in this validation. As shown in Fig. 1, the Invader assay demonstrated that each probe exhibited a higher intensity of fluorescence with DNA from corresponding bacteria than with DNA from other bacterial species at a concentration of 10^{2-4} pg/tube of DNA. The intensity of fluorescence of 10^4 pg/tube DNAs derived from other bacterial species was equivalent to that of 10 pg/tube of corresponding bacterial DNA. In real-time PCR, each primer pair reacted only with DNAs from corresponding bacterial species. Each probe or primer showed no reactivity with saliva samples in which none of the five periodontopathic bacteria were detected by the culture methods.

Intra-day and inter-day experiments: In the intra-day experiments, DNA preparations were evaluated 5 times in 1 day. In the inter-day experiments, DNA prepara-

(A) Invader assay

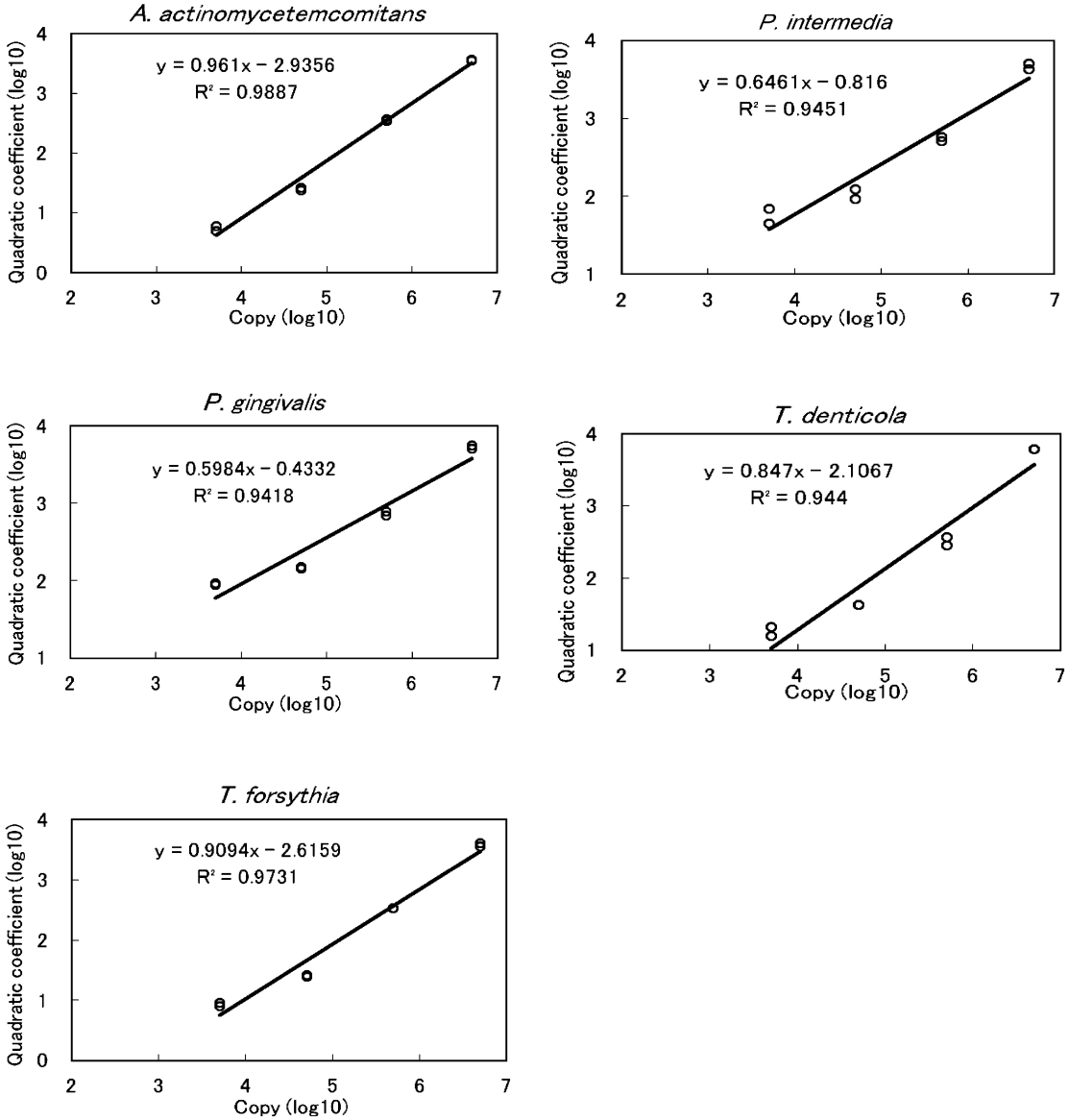

Fig. 2. Standard curves for the Invader assay and real-time PCR. (A) Invader assay. The graphs are standard curves generated from quadratic coefficients, calculated with a $10^{-4.7}$ dilution of the control plasmid, of a 10-fold dilution series of periodontopathic bacteria with non-target DNA (5 ng of human DNA and 5 ng of extracted DNA from periodontopathic bacteria). (B) Real-time PCR. The graphs are standard curves generated from the threshold cycle numbers of a 10-fold dilution series of periodontopathic bacteria with non-target DNA (5 ng of human DNA and 5 ng of extracted DNA from periodontopathic bacteria).

tions were evaluated every 3 consetutive days.

Data analysis: Signal generation of the Invader assay follows quadratic kinetics, and there is a linear relationship between the target copy level and the quadratic coefficient (20). For each reaction, the quadratic coefficient of the generation of the fluorescent signal was determined. Results for both methods were compared with a standard curve obtained using a known number of DNA copies. Bacterial counts were log transformed. To compare quantitative results between the two methods, Spearman's correlation coefficient was used. Statistical analyses were performed using the SPSS 10 software.

RESULTS

Figure 2 shows standard curves for quantification of DNA from five periodontopathic bacteria for the Invader assay and real-time PCR. Standard curves for the DNA of these bacterial species were similar in shape. Both methods presented high correlation efficiency in each range of plasmid concentration examined (correlation factors R^2 Invader assay, 0.9418–0.9887; real-time PCR, 0.9955–0.9993).

Table 2 shows the results of the intra-day and inter-day experiments for the Invader assay for quantification of the five periodontopathic bacterial species. Two subjects were examined for each bacterial species. We performed three independent measurements, and the values were averaged for each intra-day and inter-day experiment, resulting in a coefficient of variation (CV) of less than 5% for all the subjects.

The distribution of subjects by each bacterial count is shown in Table 3. The threshold for detecting bacterial DNA in the Invader assay is $10^{3.7}$ copies/tube. In all the bacterial species except *P. gingivalis*, the number of subjects with $>10^4$ copies/tube was almost identical in the Invader assay and real-time PCR (Table 3A). Of the subjects in whom each bacterial species could not detected with the Invader assay (*A. actinomycetemcomi-*

(B) Real-time PCR

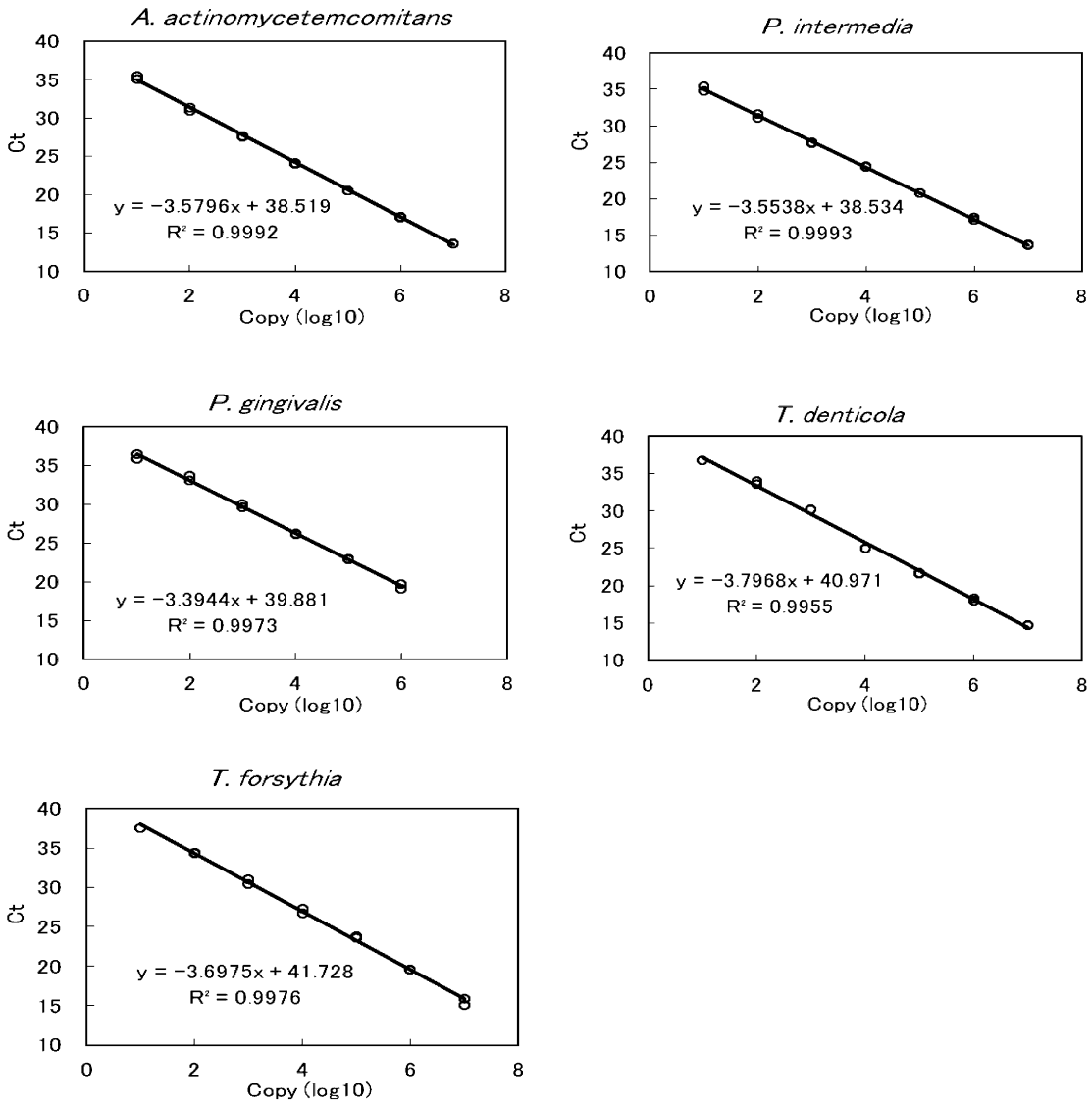

Fig. 2. Continued.

Table 2. Intra-day and inter-day experiments for measurement of periodontopathic bacteria

Intra-day experiments

	Log copy number of DNA/tube			
	Subject	Average	S.D.	CV (%)
A. actinomycetemcomitans	A	5.6	0.1	2.1
	B	4.4	0.1	2.4
P. gingivalis	B	5.5	0.0	0.4
	C	4.8	0.2	4.7
T. forsythia	D	5.4	0.0	0.6
	E	5.2	0.0	0.2
P. intermedia	F	5.2	0.0	0.4
	G	4.1	0.0	0.3
T. denticola	E	5.3	0.0	0.1
	H	5.1	0.0	0.3

Inter-day experiments

	Log copy number of DNA/tube			
	Subject	Average	S.D.	CV (%)
A. actinomycetemcomitans	A	5.7	0.0	0.5
	B	4.4	0.0	0.6
P. gingivalis	B	5.6	0.0	0.8
	C	5.0	0.0	0.1
T. forsythia	D	5.5	0.1	1.9
	E	5.2	0.1	2.2
P. intermedia	F	5.2	0.0	0.9
	G	4.1	0.1	1.3
T. denticola	E	5.3	0.2	3.1
	H	5.1	0.2	3.4

Two subjects were examined for each bacterial species. Two out of the 5 patients were examined for two bacterial species.
Subjects A, F, and G were volunteers.
Subjects B, C, D, E, and H were patients.
CV, coefficient of variation.

tans 19, *P. gingivalis* 15, *T. forsythia* 3, *P. intermedia* 13, and *T. denticola* 11), some showed the presence of corresponding bacteria at detectable levels in real-time PCR (*A. actinomycetemcomitans* 1, *P. gingivalis* 9, *T. forsythia* 3, *P. intermedia* 5, and *T. denticola* 6) (Table 3B). No strain undetected by the Invader assay scored higher than the critical bacterial level of 10^5 copies/tube in the real-time PCR.

We compared counts of bacterial strains obtained by the two methods. In all the bacterial species examined, there were statistically significant correlations, and the correlation coefficients ranged from 0.757 to 0.996 (Table 4).

DISCUSSION

In the present study, we investigated the validity and reliability of Invader technology in the quantification of periodontopathic bacteria. Since Invader technology does not use gene amplification, is a low cost technique, and has less risk of generating false positives than real-time PCR has, it is considered to be a valuable application for quantifying periodontopathic bacteria.

Tadokoro et al. showed that the Invader Plus method could quantify periodontal pathogens with good correlation with the results of real-time PCR (19). In this assay, DNA sequences were amplified and quantified using the Invader technology. The Invader Plus technique increases the risk of generating false positives through the reaction of amplified DNAs of periodontal pathogens with the Invader probe.

The Invader assay is different from the Invader Plus method in that it can detect DNA sequences of interest without gene amplification. It can make the quantification of DNA more convenient and reduce the incidence of false positives and operation costs by omitting gene amplification process. Omission of the gene amplification process opens the way for the development of kits that would enable clinicians to quantify periodontopathic bacteria in clinical practice and epidemiological studies. These merits suggest that the establishment of periodontopathic bacteria quantification by the Invader assay provides a necessary tool for the prevalence of quantification of these microorganisms. Therefore, the Invader assay claims to be routinely used in clinical practice when its quantification was demonstrated to have reliability and consistency.

In the present study, the Invader assay showed linearity over a broad range of quantities of periodontopathic bacteria (10^4–10^8 copies/tube) and validated the specificity of reactions with target bacterial DNA. We analyzed 22 patients for five bacterial species using the Invader assay technique. Among the Invader-detectable bacterial strains of each species, the significant correlations between bacterial counts obtained using the Invader assay and real-time PCR was observed for all the species. However, some of the periodontopathic pathogens detected by both the methods were found in small numbers; therefore, further examination in larger samples is required. A tendency that bacterial level of *P. gingivalis* was higher in those with greater rates of periodontal pockets >3 mm was observed in the Invader assay, similar to that observed in the real-time PCR (data not shown). Real-time PCR was established as the quantifying method for periodontopathic bacterial species (11). Our results indicate that the Invader assay could provide reliable data for analyzing the periodontopathic bacteria, without gene amplification.

One limitation of the Invader assay is that its threshold for detecting bacterial DNA ($10^{3.7}$ copies) makes it difficult to quantify small bacterial counts. In fact, all the strains which were not detected with the Invader assay had counts lower than 10^5 in real-time PCR. However, levels of periodontopathic bacterial species in subgingival plaque and saliva of patients with periodontitis ranged from 10^4–10^7 copies (11,21–23). These results suggest that the Invader assay is capable of detecting levels of periodontopathogens in patients with clinically problematic periodontal conditions.

In the present study, we report a novel quantifying system for periodontopathic bacteria. Our findings demonstrate that the Invader assay is superior to real-time PCR for evaluating periodontopathic bacteria because of its ability to acquire reliable values without gene amplification and expensive instruments. Pathogens of the two major dental diseases, periodontal dis-

Table 3. Distribution of subjects by bacterial count

(A) Number of subjects by bacterial count

(Copy number of DNA/tube)

		ND	1.E+01	1.E+02	1.E+03	1.E+04	1.E+05
A. actinomycetemcomitans	Inv	19	0	0	0	3	0
	PCR	18	0	1	1	2	0
P. gingivalis	Inv	15	0	0	2	3	2
	PCR	6	2	2	4	5	3
T. forsythia	Inv	3	0	0	1	7	11
	PCR	0	0	1	3	15	3
T. denticola	Inv	11	0	0	4	7	0
	PCR	5	1	4	6	6	0
P. intermedia	Inv	13	0	0	2	6	1
	PCR	8	1	2	4	7	0

(B) Number of subjects in real-time PCR in whom bacterial species were not detectable with the Invader assay

(Copy number of DNA/tube)

	ND	1.E+01	1.E+02	1.E+03	1.E+04	1.E+05
A. actinomycetemcomitans	18	0	1	0	0	0
P. gingivalis	6	2	2	3	2	0
T. forsythia	0	0	1	1	1	0
T. denticola	5	1	3	2	0	0
P. intermedia	8	1	2	2	0	0

Inv, Invader assay; PCR, real-time PCR; ND, not detected.

Table 4. Correlation coefficients between bacterial counts with Invader assay and real-time PCR

	Correlation coefficient	*P*
A. actinomycetemcomitans	0.996	<0.001
P. gingivalis	0.757	<0.001
T. forsythia	0.788	<0.001
P. intermedia	0.879	<0.001
T. denticola	0.845	<0.001

Sample with not detection of concerned bacteria was given a score of 0.

ease and dental caries inhabit the oral cavities of a major portion of the adult population. Increased levels of these pathogens increase the risk of occurrence of the diseases. Practicing routine bacterial examinations to monitor these pathogens is necessary for preventing these dental diseases. Implementation of bacterial examination for oral pathogens requires less cost and decrease in the number of false positive results. The merit of the Invade assay, generating less false positive results and its low cost for not requiring gene amplification is convenient to motivate individuals to acquire oral bacterial examinations routinely. The Invader assay would contribute to preventing diseases in the dental field. We believe that the Invader assay will become a widely used tool in clinical practice in the near future.

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Conflict of interest None to declare.

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