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Comparison of restriction enzyme analysis and amplified fragment length polymorphism typing of *Porphyromonas gingivalis* isolated from spouses

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Introduction: In the past, theories on the transmission of *Porphyromonas gingivalis* between individuals have been based on, among other techniques, restriction enzyme analysis (REA) of bacterial DNA. Currently, amplified fragment length polymorphism (AFLP) may be a more sophisticated alternative. The possibility of automatic pattern analysis and digital storage of the typing data enables the comparison of patterns from a large number of strains in a broad time frame. The aim of this study was to compare REA profiles with AFLP patterns of *P. gingivalis* strains isolated from periodontitis patients and their spouses.
Methods: Forty-two *P. gingivalis* strains were isolated from different sites in the mouth from six adult patients with periodontitis and their spouses. DNA of the bacterial isolates was subjected to REA and AFLP analysis.
Results: One single type of *P. gingivalis* was found in each individual with both methods, regardless of the site of isolation. Indistinguishable types were found in four of the six couples with both techniques. Different types were found in two couples with both the REA and the AFLP method.
Conclusions: The AFLP typing technique confirms earlier observations on the transmission of *P. gingivalis* between spouses. This new technique can replace REA typing.

Key words: amplified fragment length polymorphism; periodontitis; *Porphyromonas gingivalis;* restriction enzyme analysis; spouses; typing; transmission

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Typing of oral bacterial strains is important for various reasons such as identification of specific virulent clones within a species and the study of epidemiological spread of bacterial clones in a population. In periodontal infections, transmission of specific pathogens has been evaluated mainly in family units (14, 15, 21) and most studies have been focused on the transmission of *Porphyromonas gingivalis* (19).

Biotyping methods, such as sugar fermentation, or phage typing are not available for *P. gingivalis* (20). Serotyping and antibiogram typing have been described for *P. gingivalis* but these methods recognize insufficient heterogeneity between strains to be used when studying transmission routes (14, 17). Within the *P. gingivalis* species, at least six serotypes have been described (5, 18) and this serotyping has been used in an epidemiological study (20).

DNA-based methods have been developed such as restriction enzyme analysis (REA) (7, 13, 14), random amplified polymorphic DNA/arbitrary primed-polymerase chain reaction (RAPD/AP-PCR) (3, 9, 13), multilocus enzyme electrophoresis (6), repetitive extragenic palindromic sequence PCR (13), ribotyping (1, 13), and ribosomal intergenic spacer region analysis (11) to genotype *P. gingivalis* isolates. A comparison of REA, ribotyping and

Table 1. Restriction enzyme sites used for REA and AFLP

REA		AFLP	
*Pst*I	CTGCA′G	*Pst*I	CTGCA′G
*Bam*HI	G′GATCC	*Mse*I	T′TAA

Table 2. Adapter and primer sequences of oligonucleotide probes used for the AFLP genotyping

Adapter	Nucleotide sequence
MSE-AD1	5′-**g**AC-GAT-GAG-TCC-TGA-3′
MSE-AD2	5′-CTA-CTC-AGG-ACT-**c**AT-3′
PST-AD1	5′-CTC-GTA-GAC-TGC-GTA-C**a**T-GCA-3′
PST-AD2	5′-**t**GT-ACG-CAG-TCT-AC-3′
Primer	
MSE-C	5′-GAT-GAG-TCC-TGA-GTA-A***C***-3′
PST-0-FAM	5′-GAC-TGC-GTA-CAT-GCA-G-3′

In the adapter the bold nucleotides in lowercase letters are modified to preserve the adapter in the restriction reaction. In the primer the bold italic nucleotide is introduced for selective amplification of the restriction fragments.

AP-PCR by van Steenbergen et al. (14) showed that the REA method is highly sensitive and reveals considerable heterogeneity between strains. However, the very complex banding patterns and sometimes minor differences between patterns made comparison and differentiation very difficult. Teanpaisan and Douglas (13) also concluded that ribotyping and AP-PCR result in a limited number of bands, making interpretation more easy.

However, ribotyping provides only information about specific regions within the genome, while other regions may vary which would be left undetected by the probe used.

PCR-based methods offer the convenience of speed, relative cheapness and the requirement of only small amounts of DNA.

Amplified fragment length polymorphism (AFLP) operates on selective restriction fragment amplification techniques after ligation of double-stranded oligonucleotide adaptors to genomic restriction fragments, followed by specific PCR amplification with adapter-specific primers. AFLP was first described by Vos et al. (22) and has been modified in recent years (4, 10, 12). Only a small amount of purified genomic DNA is required, which is digested with two restriction enzymes, a frequent cutter and a moderate cutter. The double-stranded oligonucleotide adapters are designed in such a way that the restriction site is not restored after ligation, enabling simultaneous restriction and ligation. The adapter-specific primers, one with a fluorescent label, can have at their 3′ ends an extension of one to two nucleotides running into the unknown chromosomal restriction fragment. For micro-organisms a pattern of proximally 50 bands is optimal for detection in an automatic sequence apparatus. Normalization of the complex AFLP patterns is easier through co-electrophoresis of sample and marker fragments of known length as internal standards (12).

The advantages of AFLP are the small amount of DNA needed, which is digested completely in a short time, the possibility of optimizing the number of fragments by using a different selective nucleotide in the amplification primer, the possibility of standardizing on a 1-base-pair discrimination level and ana-

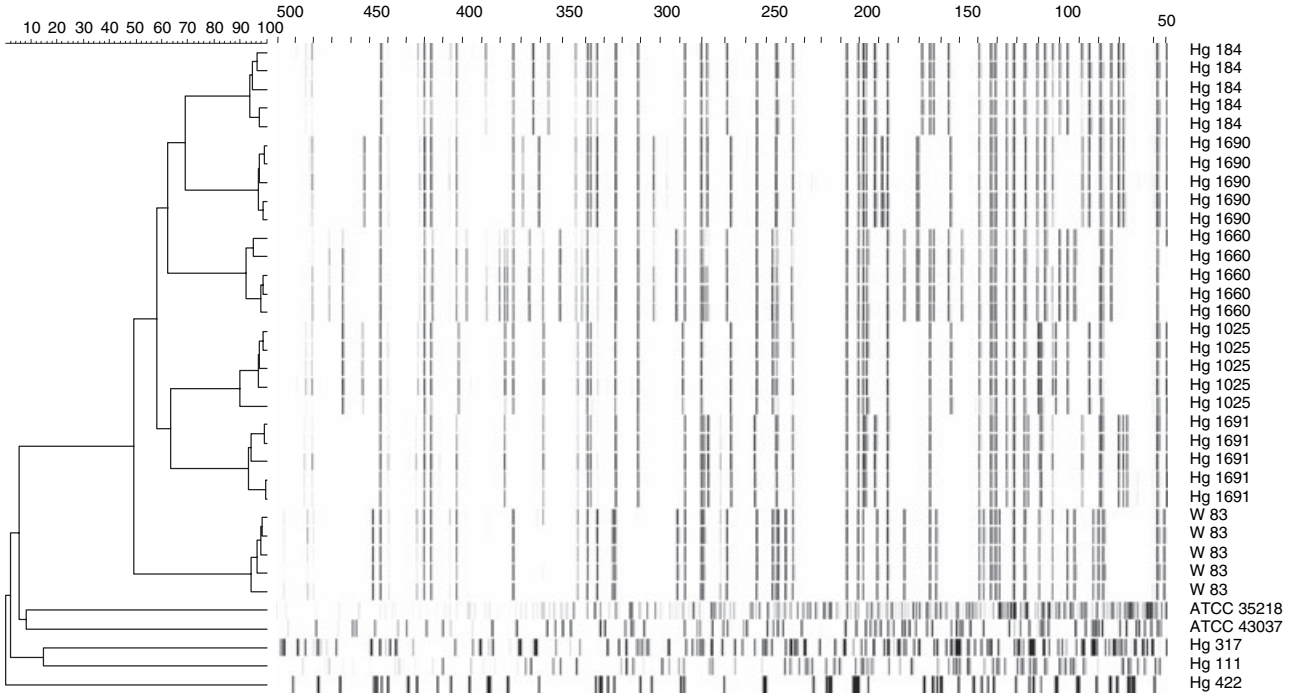

Fig. 1. AFLP patterns of the reproducibility of *Porphyromonas gingivalis* strains within a run and between different runs. Related strains *Porphyromonas asaccharolytica* HG 111, *Porphyromonas macacae* HG 317 and *Porphyromonas endodontalis* HG 422 and non-related strains *Tannerella forsythensis* ATCC 43037 and *Escherichia coli* ATCC 35218 were also tested. Each *P. gingivalis* strain was tested five times. Numbers on the horizontal axes indicate percentage similarity of the normalized AFLP patterns (range 50–500 base pairs) and calculated with the Pearson correlation. The dendrograms were obtained by the unweighted pair group method using arithmetic averages (UPGMA). Multiple testing of different *P. gingivalis* strains showed that a similarity ≥85% indicates that the strains are in the same clonal type; similarity between 42% and 72% indicates the same species but a different clonal type; and similarity <20% indicates a different species.

lysis in an automatic sequencer; these all make this method suitable for digital database construction. The aim of this study was to compare REA and AFLP techniques for typing *P. gingivalis* strains isolated from family units.

Materials and methods

Patient selection and the clinical and microbiological examination and REA typing of the *P. gingivalis* strains have been described previously (15). In short, patients suffered from severe periodontitis and had not received periodontal treatment in the past. Couples were married for at least 10 years. *P. gingivalis* strains from six couples were used in this study. Both spouses were subgingivally colonized with *P. gingivalis* as well as on extra-crevicular sites including the dorsum of the tongue, the buccal mucosa, tonsils, and saliva. In this study we tested 42 isolates from six patients and their spouses using AFLP and REA.

REA

The DNA isolation and purification was carried out as described by van Steenbergen et al. (15, 16). In short, bacterial cells were grown for 6 days in an anaerobic environment at 37°C on blood agar plates (Oxoid no. 2, supplemented with 5% horse blood, 5°mg/l hemin and 1 mg/l menadione). Cells were washed in TES buffer (10 mmol/l Tris–HCl, 5 mmol/l EDTA, 50 mmol/l NaCl, pH 8.0), and lyzed at 37°C for 1 h with lysozyme (1 mg/ml) and then for 30 min at 60°C with sodium dodecyl sulfate (1%) and proteinase K (0.1 mg/ml). DNA was purified by phenol–chloroform extractions, followed by a chloroform–isoamylalcohol extraction and ethanol precipitation. The DNA was washed in ethanol and suspended in TE buffer (10 mmol/l Tris–HCl, 1 mmol/l EDTA, pH 7.6) and treated with RNAse (0.1 mg/ml). The concentration of the DNA was estimated by comparison with known quantities of phage λ DNA after electrophoresis. For the treatment with enzymes, 2 µg DNA was digested to completion in a volume of 20 µl with the restriction endonucleases *Pst*I or *Bam*HI, 5 U/µg DNA (Boehringer, Mannheim, Germany) (see Table 1) for 2 h according to the manufacturer's recommendations. DNA fragments were separated for 20 h at 25 V in a horizontal gel containing 0.6% agarose in TAE buffer (40 mmol/l Tris–acetate, 1 mmol/l EDTA, pH 8.0). DNA was stained for 1 h in 1 mg/l ethidium bromide and photographed with a Polaroid 35 camera.

AFLP

Bacterial strains were grown anaerobically as described above. A final concentration of approximately 5 ng/µl DNA in 100 µl elution buffer (Magna Pure LC DNA I Isolation kit III, bacteria and fungi, Almere, the Netherlands) was obtained from a suspension of the bacterial strain in 2.5 ml TE 1-buffer (1 M Tris–HCl, 0.1 M EDTA, pH 8.0) with a strength of 0.5–1.0 McFarland. A volume of 100 µl of this bacterial suspension was used for automated DNA extraction and purification with the MagNA Pure DNA Isolation kit III (Bacteria, Fungi; Roche Molecular Diagnostics). The protocol included 1 h of pretreatment with proteinase K (20 mg/ml) and lysis buffer (Bacteria, Fungi; Roche Molecular Diagnostics) at 56°C. After isolation, the DNA was eluted in 100 µl elution buffer (Bacteria, Fungi; Roche Molecular Diagnostics) and stored at –20°C till use (2).

AFLP typing is based on the procedure as described elsewhere (4, 19, 21). Five microliters of DNA solution was added to 5 µl restriction–ligation reaction mixture containing 1 × T4 DNA ligase buffer (New England Biolabs, Beverly, MA), 0.05 M NaCl, 1 mg/ml bovine serum albumin (New England Biolabs), 5 pmol *Pst*I adapter (Eurogentec, Seraing, Belgium), 20 pmol *Mse*I adapter (Eurogentec), 80 U T_4 DNA ligase (New England Biolabs), 0.5 U *Pst*I (New England Biolabs), 2 U *Mse*I (New England Biolabs) (see Table 1). This mixture was incubated for 3 h at 37°C. After incubation, the restriction–ligation reaction was diluted 1 : 20 with 0.1 TE-buffer pH 8.0.

Five microliters of the diluted restriction–ligation mixture was added to 5 µl of the AFLP amplification mixture [1X GeneAmp PCR buffer (Applied Biosystems, Nieuwerkerkaanden Ijssel, the Netherlands), 2 mM dNTPs (Promega b.v., Leiden, the Netherlands), 15 mM $MgCl_2$ (Applied Biosystems), 20 ng *PST*-0-FAM primer (Eurogentec) (Table 2), 60 ng *MSE*-C primer (Eurogentec) (Table 2), 1 U AmpliTaq DNA polymerase (Applied Biosystems)]. The mixture was amplified in a GeneAmp PCR System 9700 machine (Perkin Elmer). The amplification conditions were 2 min at 72°C followed by 12 cycles comprising 30 s at 94°C, 30 s at 65°C (with this temperature decreasing 0.7°C with each succeeding cycle) and 60 s at 72°C. This sequence was followed by 23 cycles consisting of 30 s at 94°C, 30 s at 56°C and 60 s at 72°C and a final incubation of 10 min at 72°C (4). The product was stored at 4°C.

The samples were prepared for capillary electrophoresis by adding 2.5 µl of the PCR product to 22 µl deionized formamide and 0.5 µl ROX-labeled GeneScan-500 as an internal standard. The samples were run on an ABI 3100 Genetic Analyzer. AFLP patterns were analyzed with BIONUMERICS software version 3.0 (Applied Maths, Sint-Martens-Latum,

Table 3. REA and AFLP genotypes of *Porphyromonas gingivalis* isolates from various oral sites of six periodontitis patients and their spouses

Couple no.	Patient/ spouse	Location	REA	AFLP
1	P	Pocket	A	1
		Saliva	A	1
	S	Pocket	B	2
		Buccal mucosa	NT	2
		Tonsils	B	2
		Saliva	B	2
2	P	Pocket	C	3
		Buccal mucosa	C	3
		Tonsils	C	3
		Saliva	C	NT
	S	Pocket	C	3
		Buccal mucosa	C	3
3	P	Pocket	D	4
		Buccal mucosa	D	4
		Tonsils	D	4
	S	Pocket	D	4
		Tongue	D	NT
		Buccal mucosa	D	4
		Tonsils	D	4
		Saliva	D	4
4	P	Pocket	E	5
		Tongue	E	5
		Buccal mucosa	E	5
		Tonsils	E	5
		Saliva	E	5
	S	Pocket	E	5
		Tongue	E	5
		Buccal mucosa	E	5
		Tonsils	E	5
5	P	Pocket	E	6
		Buccal mucosa	E	NT
		Tonsils	E	6
		Saliva	E	6
	S	Pocket	F	7
		Tongue	F	7
		Buccal mucosa	F	7
		Tonsils	F	7
		Saliva	F	7
6	P	Pocket	G	8
		Tongue	G	8
		Buccal mucosa	G	8
		Saliva	G	NT
	S	Tongue	G	8
		Buccal mucosa	G	8
		Tonsils	G	8
		Saliva	G	8

P, for patient; S, spouse; NT, not tested.

Belgium). The similarities between normalized AFLP patterns (range 50–500 base pairs) were calculated with the Pearson correlation and dendrograms were obtained by the unweight pair group method using arithmetic averages (UPGMA) clustering.

The reproducibility of the AFLP was validated by multiple testing in separate test runs ($n = 5$) using different *P. gingivalis* reference strains including W 83, HG 184, HG 1660, HG 1690, HG 1691, and HG 1025. Related species *Porphyromonas asaccharolytica* HG 111, *Porphyromonas macacae* HG 317, and *Porphyromonas endodontalis* HG 422 and the unrelated strains *Tannerella forsythensis* ATCC 43037 and *Escherichia coli* ATCC 35218 were also tested.

Results

Multiple testing by AFLP of the reference strains W 83, HG 184, HG 1025, HG 1660, HG 1690, and HG 1691 resulted in a similarity ≥85% for the same clonal type. Similarity between 42% and 72% indicated the same species but a different clonal type. Similarity lower than 20% discriminated between *P. gingivalis* and related species such as *P. asaccharolytica*, *P. macacae* and *P. endodontalis* and unrelated species strains like *T. forsythensis* and *E. coli* (Fig. 1). One isolate was typed from each *P. gingivalis*-positive site in the patient and the spouse by REA and AFLP (Table 3). Indistinguishable patterns were obtained for *P. gingivalis* isolates from each individual, whereas isolates from unrelated individuals were clearly different. Based on the isolates tested, both typing methods showed that individuals carry one predominant clonal type. In couples 1 and 5, spouses had different *P. gingivalis* REA types, whereas in the four other couples, indistinguishable *P. gingivalis* strains were found for both husband and wife (Fig. 2). The same results were obtained by AFLP typing of the *P. gingivalis* isolates and no differences in results were observed between the techniques.

Discussion

In this study we compared REA and AFLP genotyping of *P. gingivalis* isolates from patients with destructive periodontal disease and their spouses. In the past, transmission studies have often been performed and conclusions on transmission have been drawn based on the REA typing technique (16, 18). This and the other techniques have shown transmission between spouses.

In this study we found indistinguishable REA patterns (data not shown) and AFLP patterns within patients and spouses. Based on AFLP, all the *P. gingivalis* isolates from an individual showed a similarity of >85%. This suggests that various oral sites, including the subgingival area, harbor one clonal type of *P. gingivalis*. This observation is in agreement with earlier observations (8). One strain from each oral site was tested because previous results have shown that each oral site harbors one clonal type. All strains isolated from couples 2, 3, 4, and 6 showed a similarity of > 85%, indicating possible transmission between spouses. With both techniques transmission was indicated in four of six couples. The present results of the AFLP genotyping confirm the results obtained with REA typing. With AFLP, a similarity between 42% and 72% indicates the same species but a different clonal type (11). In couples 1 and 5 the similarity was between 44% and 65%, indicating a different *P. gingivalis* clonal type. The present observations show that AFLP genotyping leads to the same conclusions as REA genotyping. The earlier observations on the clonal distribution of *P. gingivalis* based on REA typing are still valid using the more sophisticated AFLP genotyping technique. AFLP has

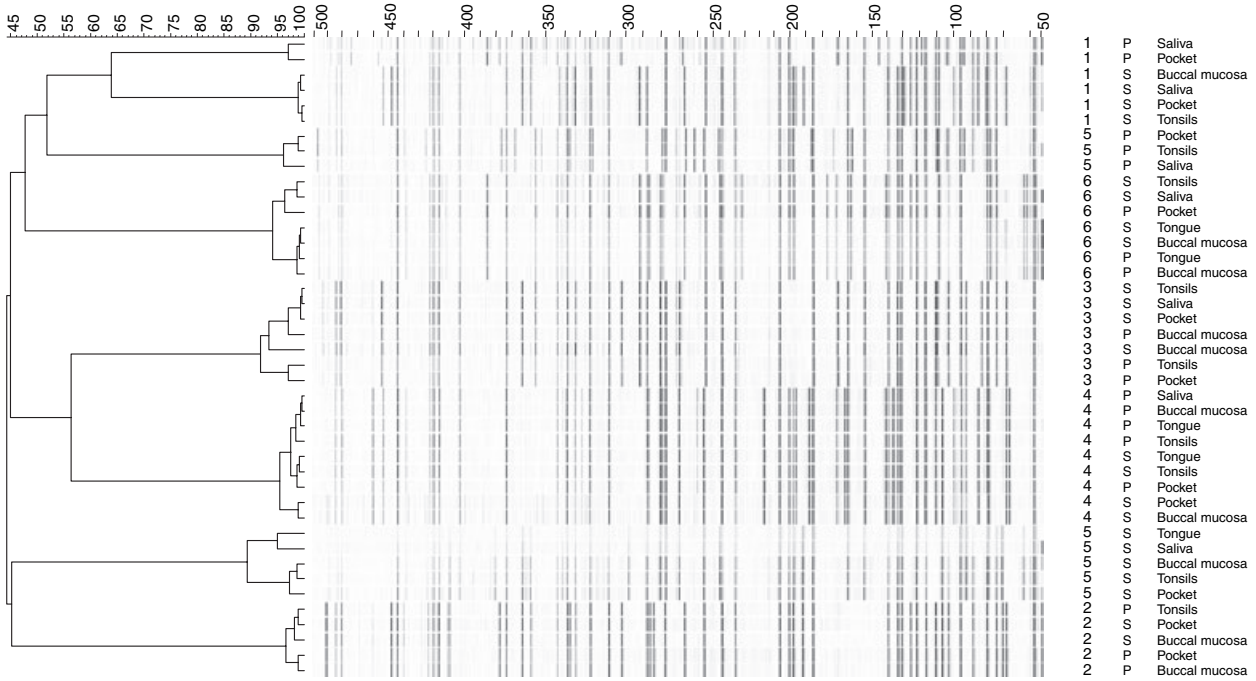

Fig. 2. AFLP patterns of *Porphyromonas gingivalis* isolates from periodontitis patients and their spouses. Numbers on the horizontal axes indicate percentage similarity of the normalized AFLP patterns (range 50–500 base pairs) and calculated with the Pearson correlation. The dendrograms were obtained by the unweighted pair group method using arithmetic averages (UPGMA). First vertical row indicates family number, in the second row P stands for patient, S stands for spouse, third row indicates the location. A similarity higher than 85% indicates the same clonal type; similarity between 42% and 72% indicates the same species but a different clonal type; and similarity lower than 20% indicates a different species.

several advantages over the REA technique, one of which is the computer-assisted data analysis and storage. Disadvantages may be that it is laborious and requires specific equipment.

Based on our observations we conclude that AFLP is an optimal technique for bacterial typing; it can be used to study clonal distribution of oral bacteria in a population and it can be used to study transmission of oral pathogens.

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