J. Med. Microbiol. — Vol. 39 (1993), 416–421

Comparison of three molecular typing methods in studies of transmission of *Porphyromonas gingivalis*

T. J. M. VAN STEENBERGEN, C. MENARD*, C. J. TIJHOF, C. MOUTON* and J. DE GRAAFF

*Department of Oral Microbiology, Academic Centre for Dentistry Amsterdam, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands and *Groupe de Recherche en Ecologie Buccale, Faculté de Medecine Dentaire, Université Laval, Quebec G1K7P4, Canada*

Summary. *Porphyromonas gingivalis* is associated strongly with severe periodontitis, but little information is available on possible transmission routes of this species. This study evaluated three DNA-based molecular typing methods for use in epidemiological surveys of *P. gingivalis*. In total, 32 isolates from eight married couples were investigated by: (i) restriction endonuclease analysis (REA) of whole chromosomal DNA; (ii) hybridisation of DNA fragments with ribosomal DNA (ribotyping); and (iii) amplification of DNA by the polymerase chain reaction with arbitrary primers (AP-PCR). The data obtained with the three methods were in broad agreement: in six of the eight couples, the isolates from husband and wife were indistinguishable, but isolates from unrelated individuals showed distinct types with all three methods. For some isolates, minor differences in REA pattern were obtained which could not be correlated with differences in ribotype or AP-PCR type. Ribotyping showed differences between isolates from one individual, which were indistinguishable with the other two methods. The patterns obtained with ribotyping or AP-PCR were simple in comparison to the relatively complex REA patterns. Although all three methods were concordant, AP-PCR was found to be the least time-consuming method. The data support the suggestion that *P. gingivalis* can be transmitted between spouses.

Introduction

Porphyromonas gingivalis is associated strongly with severe periodontitis, but despite much data on the prevalence, biology and virulence of this suspected pathogen,[1–4] little information is available on its possible routes of transmission. We have recently provided evidence for the transmission of *P. gingivalis* between spouses.[5] Similarly, although *P. gingivalis* is isolated rarely from children, Petit *et al.*[6] have shown that when this species is found in children, one of the parents is often colonised with the same type, indicating the possibility of transmission between parents and their children.

Several typing methods have been described for epidemiological studies of *P. gingivalis*, but classical typing methods, such as biotyping and serotyping, discriminate few distinct types and are, therefore, not suitable for studying routes of transmission. Modern typing methods based on molecular biology techniques have also been described for *P. gingivalis*. These include restriction endonuclease analysis (REA) of whole chromosomal DNA,[7–9] ribotyping, involving hybridisation of DNA fragments with ribosomal DNA,[10,11] and amplification of DNA fragments by the polymerase chain reaction with arbitrary primers (AP-PCR).[12] The aim of the present study was to compare these three DNA-based methods for typing *P. gingivalis*.

Received 5 April 1993; accepted 14 May 1993.
Correspondence to Dr. T. J. M. van Steenbergen.

Materials and methods

Bacterial isolates

The *P. gingivalis* isolates were obtained from the subgingival plaque, saliva and oral mucous membranes of eight patients with severe adult periodontitis, and their spouses, all colonised with *P. gingivalis*. The clinical and microbiological characteristics of these married couples have been described previously.[5] Between one and seven isolates were evaluated from each individual patient or spouse.

Restriction endonuclease analysis (REA)

Chromosomal DNA was isolated by phenol: chloroform extraction and ethanol precipitation after lysis of the bacterial cells with lysozyme, proteinase K and SDS, as described previously.[5] Samples (2 μg) of DNA were digested to completion (2 h) with *Pst*I or *Bam*HI (Boehringer Mannheim) according to the manufacturer's recommendations. DNA fragments

Table. Comparison of results obtained by restriction endonuclease analysis (REA), polymerase chain reaction with arbitrary primer (AP-PCR), and ribotyping for *P. gingivalis* isolates from periodontitis patients and their spouses

Isolate	Couple no.	Subject	Site	REA type	AP-PCR type	Ribotype	
						*Bgl*I	*Bam*HI
HG 1293	1	Patient	Pocket	A	a	1	I
HG 1296	1	Spouse	Pocket	B	b	2	II
HG 1301	2	Patient	Pocket	C	c	3	III
HG 1306	2	Spouse	Pocket	C	c	3	III
HG 1312	3	Patient	Pocket	D′	d	4	IV
HG 1310	3	Patient	Saliva	D	d	4	IV
HG 1316	3	Spouse	Pocket	D′	d	4	IV
HG 1319	3	Spouse	Pocket	D	d	4	IV
HG 1321	4	Patient	Pocket	F	f	5	V
HG 1326	4	Spouse	Pocket	F	f	5	V
HG 1331	5	Patient	Pocket	G	g	6	VI
HG 1332	5	Patient	Pocket	G	g	6	VI
HG 1333	5	Patient	Tongue	G	g	6	VI
HG 1334	5	Patient	Buccal	G	g	6	VI
HG 1335	5	Patient	Tonsils	G	g	6	VI
HG 1336	5	Patient	Tonsils	G	g	6	VI
HG 1337	5	Patient	Saliva	G	g	6	VI
HG 1338	5	Spouse	Pocket	G	g	6	VI
HG 1343	6	Patient	Pocket	H	h	7	VII
HG 1347	6	Spouse	Pocket	J	j	8	VIII
HG 1352	7	Patient	Pocket	K	k	9	X
HG 1353	7	Patient	Saliva	K	k	10	X
HG 1354	7	Patient	Tongue	K	k	10	IX
HG 1355	7	Patient	Buccal	K	k	10	X
HG 1356	7	Spouse	Tongue	K	k	10	X
HG 1357	7	Spouse	Buccal	K	k	10	X
HG 1358	7	Spouse	Tonsils	K	k	10	X
HG 1359	7	Spouse	Saliva	K	k	10	X
HG 1493	8	Patient	Pocket	L′	l	11	XI
HG 1494	8	Patient	Buccal	L	l	11	XI
HG 1495	8	Spouse	Pocket	L″	l	11	XI
HG 1496	8	Spouse	Saliva	L″	l	11	XI

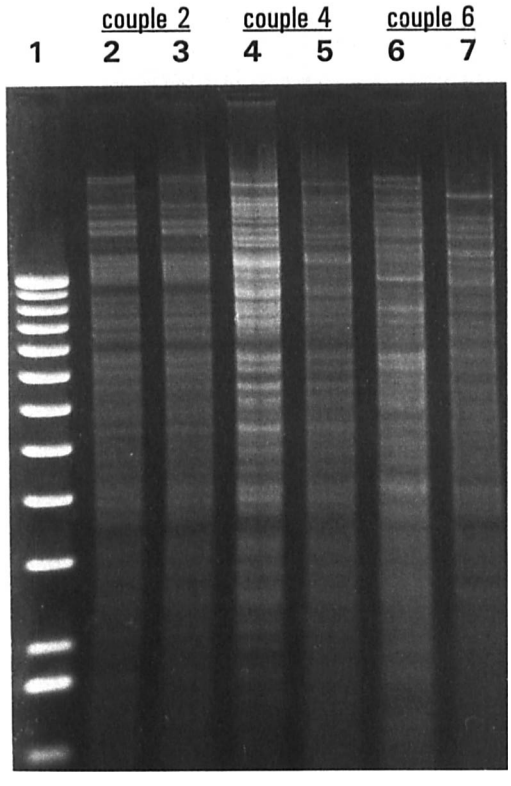

Fig. 1. *Bam*HI restriction digests of DNA from six *P. gingivalis* isolates from three married couples. Lane **1**, 1-kb DNA size marker ladder (Gibco, Gaithersburg, MD, USA); **2** and **3**, couple 2, patient and spouse; **4** and **5**, couple 4, patient and spouse; **6** and **7**, couple 6, patient and spouse.

were separated by electrophoresis on agarose 0·6% w/v gels and stained with ethidium bromide.[5]

Polymerase chain reaction with arbitrary primer (AP-PCR)

Amplification of DNA fragments in the polymerase chain reaction with arbitrary primers was as described by Menard *et al.*[12] Briefly, one colony was harvested into 100 μl of H_2O with added activated charcoal, heated for 10 min at 100°C, and centrifuged briefly in a microfuge; 5-μl samples were used as template for the PCR. Two primers were used: no. 970-11 (5′-GTAAGGCCG), that can detect heterogeneity in *P. gingivalis*,[12] and no. 910-05 (5′-CCGGCGGCG), that has also been useful for discriminating between *P. gingivalis* strains (C. Menard and C. Mouton, unpublished results). Amplification with *Taq* polymerase (Pharmacia) was as described previously,[12] with 30 amplification cycles. Amplification products were evaluated by electrophoresis on agarose 1·6% w/v gels and staining with ethidium bromide.

Ribotyping

Ribotyping was as described by Saarela *et al.*[10] Chromosomal DNA was isolated as described for REA. The DNA was digested to completion with *Bgl*I or *Bam*HI (Boehringer Mannheim), used according to

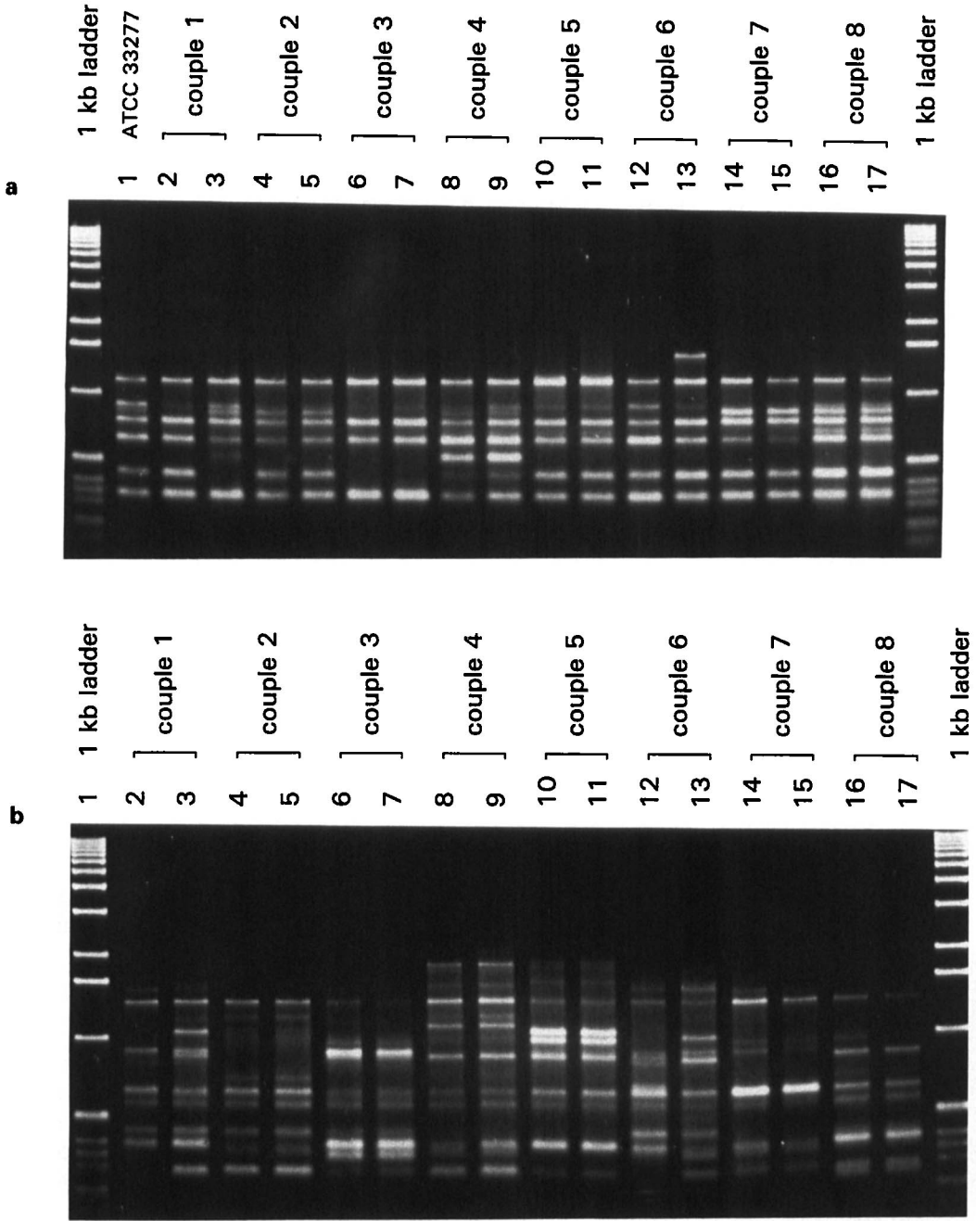

Fig. 2. Fingerprints obtained after PCR with **a**, primer 970-11; **b**, primer 910-05 and DNA from 16 *P. gingivalis* isolates from eight married couples.

the manufacturer's instructions. DNA fragments were separated on agarose 0·6% w/v gels by electrophoresis, stained with ethidium bromide, and photographed under UV illumination. Thereafter, DNA was transferred to a positively charged nylon filter (Boehringer Mannheim), fixed on to the membrane by UV illumination for 3 min, and hybridised for 16 h at 68°C with a probe consisting of digoxigenin-labelled pKK3535 DNA. This plasmid contains the rDNA operon from *Escherichia coli*,[13] and was obtained from M. Saarela, Helsinki, Finland. The plasmid was isolated from a strain of *E. coli* by the Magic Miniprep procedure (Promega, Madison, WI, USA), and was then labelled with digoxigenin (Boehringer Mannheim) according to the manufacturer's instructions. Hybridised fragments were detected by incubation with anti-digoxigenin antibodies labelled with alkaline phosphatase, followed by incubation with nitro-blue-tetrazolium, or with the luminescent substrate Lumigen-PPD (Boehringer Mannheim). As detection with Lumigen-PPD gave the clearest bands, this procedure was used in most experiments.

Results

Three different methods (REA typing, ribotyping and AP-PCR) for typing *P. gingivalis* were compared for a total of 32 isolates obtained from 16 individuals (table). These isolates were chosen as being representative of the different REA types that were found

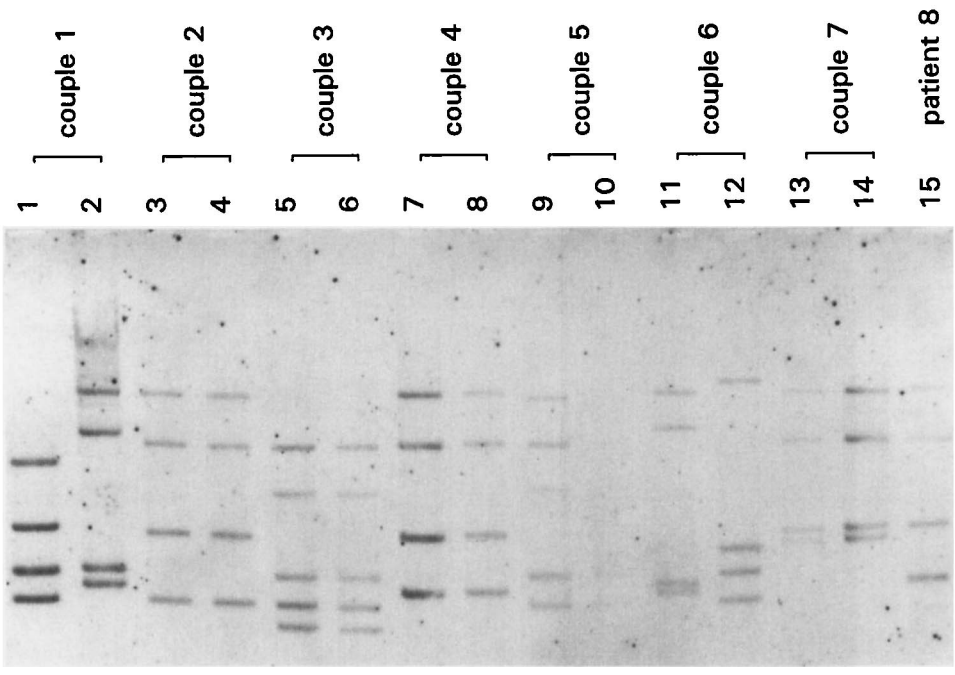

Fig. 3. Ribotypes visualised after *Bam*HI digestion of DNA from 15 *P. gingivalis* isolates from eight married couples.

previously in these individuals.[5] REA typing showed a total of 10 distinct types. Examples of the different REA types are shown in fig. 1 for six isolates from three married couples (*Bam*HI digest); lanes 2 and 3 (couple 2) were indistinguishable, as were lanes 4 and 5 (couple 4); the two isolates from couple 6 were distinct (lanes 6 and 7). The clearest differences in the banding patterns were found in the molecular size region above 5 kb. In two couples (3 and 8), minor differences indicative of subtypes were found between different isolates (results not shown). Isolates from unrelated individuals all had distinct DNA patterns, whereas the partners in six couples shared indistinguishable REA types. Isolates from different sites of a single individual gave indistinguishable patterns (table; couples 5 and 7). The data obtained by REA typing were in agreement with earlier results.[5]

Typing by AP-PCR was evaluated with the same set of isolates. Fig. 2 shows the AP-PCR patterns obtained with primers 970-11 and 910-05 for 16 isolates of *P. gingivalis*, one from each individual. The pattern obtained with primer 970-11 for ATCC strain 33277 was similar to that reported in an earlier study,[12] indicating good inter-assay reproducibility. Isolates from unrelated individuals gave distinct patterns, whereas those from six of the eight couples yielded indistinguishable DNA patterns, irrespective of the primer used. The banding patterns obtained with primer 910-05 were usually more complex than with primer 970-11, but allowed the same discrimination. The size of the PCR amplicons with both primers ranged from 0·3 to 2 kb. The AP-PCR and REA typing data were in agreement with respect to the main types found (table). The subtypes differentiated by REA (on the basis of minor differences in the high molecular size region of the DNA fingerprint) could not be distinguished by AP-PCR.

Ribotyping was performed after digestion of chromosomal DNA with restriction endonucleases *Bgl*I or *Bam*HI, followed by hybridisation of the digests with ribosomal DNA from *E. coli*. Fig. 3 shows examples of the ribotypes obtained for 15 isolates after digestion with *Bam*HI. The patterns obtained after digestion with *Bam*HI were more distinct than those obtained after digestion with *Bgl*I (not shown). The size of the DNA fragments identified by ribotyping ranged from 5 to 20 kb. Ribotyping with both enzymes identified 11 distinct patterns. The ribotyping results were largely in agreement with the results obtained by REA typing and AP-PCR. As with AP-PCR, ribotyping was not able to discriminate between the strains with minor differences observed by REA typing in couples 3 and 8 (table). These differences in the REA pattern were observed after digestion with *Bam*HI, electrophoresis and staining with ethidium bromide. After blotting of the same gel, no differences were found after hybridisation of the fragments with rDNA.

All three methods were reproducible. This was illustrated by the finding that all seven isolates from different sites of the patient in couple 5 were indistinguishable by all three methods. With typing by REA and AP-PCR, all isolates tested from couple 7 gave indistinguishable patterns; in contrast, ribotyping of these isolates showed heterogeneity: isolate HG 1352 was distinct from the other seven isolates after digestion with *Bgl*I, and isolate HG 1354 was distinct after digestion with *Bam*HI (table). Fig. 4a shows that the REA patterns from isolates HG 1354 and HG 1359 were indistinguishable, whereas hybridisation with rDNA of the fragments from the same

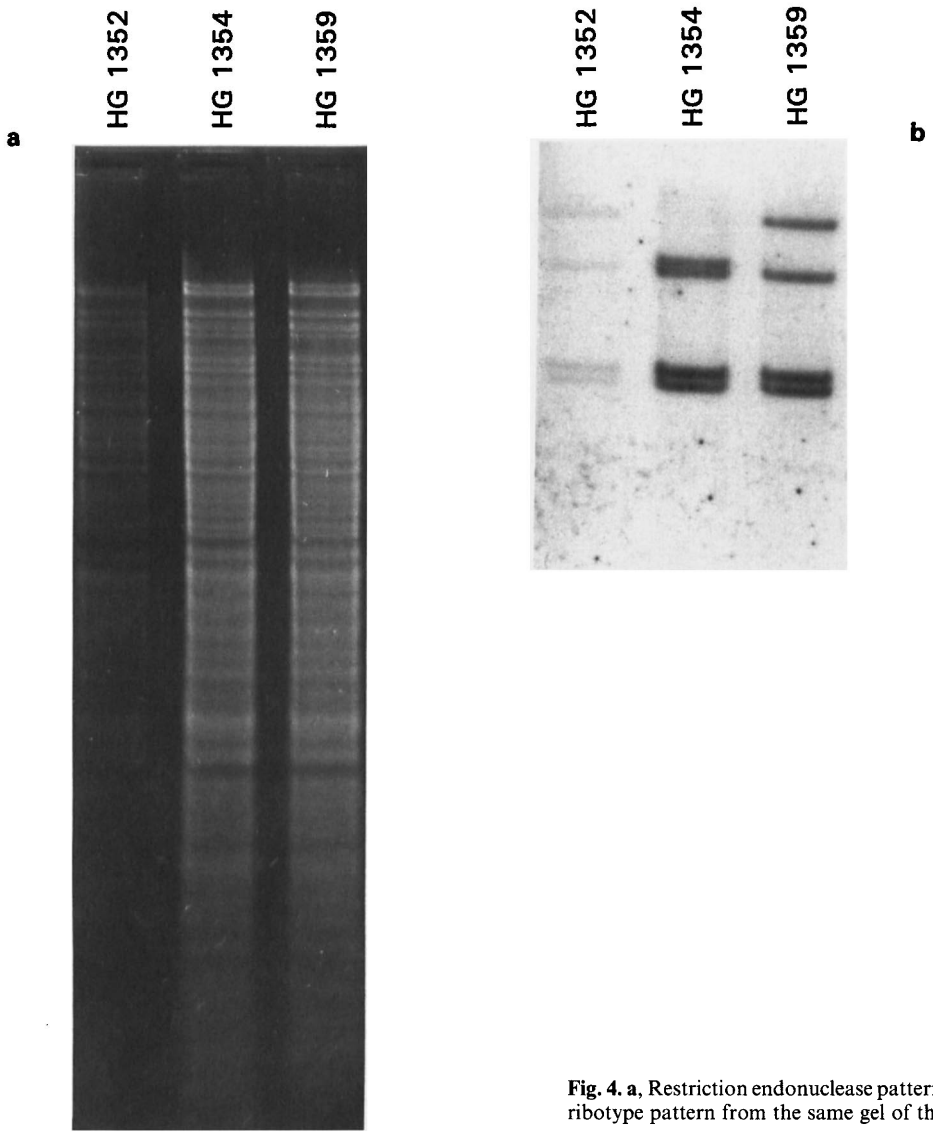

Fig. 4. a, Restriction endonuclease pattern after *Bam*HI digestion; **b**, ribotype pattern from the same gel of three isolates from couple 7.

gel showed a clearly distinct pattern for HG 1354 (fig. 4b). These results were found to be reproducible in three separate experiments.

Discussion

Several conventional typing methods, including biotyping, serotyping and antibiogram typing, have been described for *P. gingivalis*,[14,15] but these methods recognise insufficient heterogeneity between strains to study transmission routes of this suspected periodontal pathogen. New molecular typing methods for bacteria have recently been applied to *P. gingivalis*. REA typing of *P. gingivalis*[7-9] is highly sensitive and reveals considerable heterogeneity between strains, but produces large numbers of DNA fragments, resulting in patterns which can be difficult to interpret when large numbers of isolates have to be evaluated. In contrast, ribotyping[16] results usually in only four to eight bands. This method has been applied to *P. gingivalis* isolates from six[11] and nine[10] unrelated subjects, all of which were found to be distinct. Random amplification of DNA by PCR with an arbitrary primer (AP-PCR) can also be used for typing bacteria.[17,18] Random amplification of genomic DNA, directed by a single oligonucleotide of arbitrary sequence, produces a set of varying short DNA products. Menard *et al.*[12] applied this method to *P. gingivalis* and found that nine strains yielded nine distinct banding patterns. The present study compared these three typing methods with a set of 32 *P. gingivalis* isolates from eight periodontitis patients and their spouses.

All three methods demonstrated considerable heterogeneity in *P. gingivalis*, observed as DNA fragment length polymorphisms, with 10 main types and three additional subtypes by REA, 10 types by AP-PCR, and 11 distinct types by ribotyping. The epidemiological conclusions arrived at by the three methods largely agreed: in six couples, the *P. gingivalis* types found in husband and wife were indistinguishable; in two couples (1 and 6), isolates from husband and wife were clearly distinct; and isolates from unrelated individuals, i.e., from different couples, were always

distinct. Indistinguishable patterns obtained with isolates from different sites in the same individual were indicative of monoclonal colonisation. These data strengthen the conclusion, based on previous experiments with the same set of strains, that *P. gingivalis* can probably be transmitted between spouses.[5]

Relatively few differences were observed between the results obtained with the three methods. The minor differences between some strains found by REA (type D′, L′ and L″), observed as a variable banding pattern in the high molecular size region, were not reflected in differences by AP-PCR or ribotype. This suggests that such minor polymorphisms reflect mutations restricted to a small part of the genome of these isolates. Such mutations outside the primer-target pairing site are overlooked by AP-PCR. A further discrepancy between the three methods was found with some isolates from couple 7. One of eight isolates tested was distinct from the other seven isolates after ribotyping with *Bgl*I, and a second isolate was distinct after ribotyping with *Bam*HI. As this discrimination was not obtained by REA or AP-PCR, it can be speculated that these differences in ribotype represent relatively small polymorphisms in the genome of these isolates. This would imply that clear differences in ribotype among strains do not necessarily mean that the isolates are unrelated, and that minute differences can be detected even if their genomes have a high degree of similarity when measured by other techniques.

Since all three methods demonstrated considerable heterogeneity among independently obtained strains of *P. gingivalis*, it is questionable whether any of these methods is useful to study genetic relationships and to make correlations with virulence and pathogenicity. However, all three methods are suitable for studies of the routes of transmission, although AP-PCR may offer some advantages over the other two methods. REA typing provides a complex pattern with a large number of visible bands (usually > 30), which makes comparison of lanes difficult, whereas ribotyping and AP-PCR both result in a limited number of bands, rendering comparison easier. A disadvantage of ribotyping is that it is rather time-consuming, in contrast to AP-PCR which is relatively easy to perform, gives reproducible data in less than 24 h, and can be applied to a single colony. We conclude that AP-PCR may be of great value for epidemiological studies of *P. gingivalis*, and may also be useful for studies of other bacteria.

This work was supported in part by a grant from the Netherlands Organization for Scientific Research (NWO).

References

1. Mayrand D, Holt SC. Biology of asaccharolytic black-pigmented *Bacteroides* species. *Microbiol Rev* 1988; **52**: 134–152.
2. Slots J, Listgarten M. *Bacteroides gingivalis, Bacteroides intermedius* and *Actinobacillus actinomycetemcomitans* in human periodontal diseases. *J Clin Periodontol* 1988; **15**: 85–93.
3. van Steenbergen TJM, van Winkelhoff AJ, de Graaff J. Black-pigmented oral anaerobic rods: classification and role in periodontal disease. In: Hamada S, Holt HC, McGhee JR (eds) Periodontal disease: pathogens and host immune responses. Tokyo, Quintessence Publishing Co. 1991: 41–52.
4. van Winkelhoff AJ, van Steenbergen TJM, de Graaff J. The role of black-pigmented *Bacteroides* in human oral infections. *J Clin Periodontol* 1988; **15**: 145–155.
5. van Steenbergen TJM, Petit MDA, Scholte LHM, van der Velden U, de Graaff J. Transmission of *Porphyromonas gingivalis* between spouses. *J Clin Periodontol* 1993; **20**: 304–345.
6. Petit MDA, van Steenbergen TJM, Scholte LHM, van der Velden U, de Graaff J. Epidemiology and transmission of *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* among children and their family members. A report of 4 surveys. *J Clin Periodontol* 1993 (in press).
7. Loos BG, Mayrand D, Genco RJ, Dickenson DP. Genetic heterogeneity of *Porphyromonas* (*Bacteroides*) *gingivalis* by genomic DNA fingerprinting. *J Dent Res* 1990; **69**: 1488–1493.
8. van Steenbergen TJM, van Winkelhoff AJ, van der Velden U, de Graaff J. Taxonomy, virulence and epidemiology of black-pigmented *Bacteroides* species in relation to oral infections. *Infection* 1989; **17**: 194–196.
9. van Steenbergen TJM, van der Velden U, Abbas F, de Graaff J. Microflora and bacterial DNA restriction enzyme analysis in young adults with periodontitis. *J Periodontology* 1991; **62**: 235–241.
10. Saarela M, Stucki AM, von Troil-Linden B, Alalusua S, Jousimies-Somer H, Asikainen S. Intra- and inter-individual comparison of *Porphyromonas gingivalis* genotypes. *FEMS Immunol Microbiol* 1993; **6**: 99–102.
11. Socransky SS, Martin L. Ribotyping of *P. gingivalis* and *C. rectus*. *J Dent Res* 1992; **71**: 246 (abstract).
12. Ménard C, Brousseau R, Mouton C. Application of polymerase chain reaction with arbitrary primer (AP-PCR) to strain identification of *Porphyromonas* (*Bacteroides*) *gingivalis*. *FEMS Microbiol Lett* 1992; **95**: 163–168.
13. Brosius J, Ullrich A, Raker MA *et al.* Construction and fine mapping of recombinant plasmids containing the rrnB ribosomal RNA operon of *E. coli*. *Plasmid* 1981; **6**: 112–118.
14. Loos BG, Dyer DW, Genco RJ, Selander RK, Dickinson AP. Natural history and epidemiology of *Porphyromonas gingivalis*. In: Shah HN, Mayrand D, Genco RJ (eds) *Porphyromonas gingivalis*. Boca Raton, FL, CRC Press Inc. 1993 (in press).
15. van Steenbergen TJM, van Winkelhoff AJ, de Graaff J. Classification and typing methods of black-pigmented Gram-negative anaerobes. *FEMS Immunol Microbiol* 1993; **6**: 83–88.
16. Grimont F, Grimont PAD. Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. *Ann Inst Pasteur/Microbiol* 1986; **137B**: 165–175.
17. Welsh J, McClelland M. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res* 1990; **18**: 7213–7218.
18. Williams GK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res* 1990; **18**: 6531–6535.