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Java project on periodontal diseases: a study on transmission of *Porphyromonas gingivalis* in a remote Indonesian population

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Abstract

Aim: To study transmission of *Porphyromonas gingivalis* in a population living in a remote area in Southern Java, Indonesia.

Material and Methods: Subgingival plaque samples from 167 subjects with varying degrees of periodontal breakdown were obtained and cultured for the presence of *P. gingivalis*. After extraction and purification of bacterial DNA, amplified fragment length polymorphism technique was applied to genotype the bacterial isolates. Computer-assisted analysis of the bacterial DNA profiles was used to study distribution of *P. gingivalis* genotypes within family units.

Results: One hundred and five of the 167 (63%) subjects were culture positive for *P. gingivalis*. In total, 371 *P. gingivalis* isolates were obtained from the 105 subjects. Of the 105 subjects, 30 were siblings representing 13 families. In six of the 13 families (46%), identical *P. gingivalis* genotypes were found among siblings. In the study group of 105 subjects, 13 married couples were identified of which both spouses were culture positive for *P. gingivalis*. None of the 13 couples shared an identical *P. gingivalis* genotype. Twenty *P. gingivalis*-positive subjects had spouses that were culture negative for *P. gingivalis*.

Conclusions: In this study population, vertical transmission of *P. gingivalis* has occurred within family units, most likely from parents to children. Transmission of *P. gingivalis* between spouses could not be established.

Key words: periodontitis; *P. gingivalis*; siblings; transmission

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Porphyromonas gingivalis is a Gram negative, strict anaerobic, black-pigmented coccobacillus and is considered a major pathogen in destructive periodontal diseases. Although the subgingival microflora in periodontitis is complex and comprises many different bacterial species, *P. gingivalis* has shown to be a strong marker of the disease in adult subjects (Griffen et al. 1998, van Winkelhoff et al. 2002, Boutaga et al. 2003). The natural habitat of the species is the human oral cavity where it can be isolated from subgingival plaque and oral mucosal surfaces (van Winkelhoff et al. 1988). There is evidence to show that *P. gingivalis* is isolated infrequently from healthy children by culture methods or immunoassays (Asikainen et al. 1996) suggesting that colonization occurs at later age. Most studies show that the majority of individuals that are colonized by *P. gingivalis* harbour one clonal type of the species (for a review see Asikainen & Chen 1999). Studies on transmission of *P. gingivalis* have been produced mainly in Western study populations and have revealed that the species is transmitted between spouses in 30–75% of the couples (Petit et al. 1993, Saarela et al. 1993a, b, for a review see van Winkelhoff & Boutaga 2005). The likelihood of periodontitis to develop after colonization of *P. gingivalis* has not been determined but an early observation suggests that spouses of patients

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with severe periodontitis suffer from more periodontal breakdown compared with spouses of periodontally healthy subjects and that simultaneous isolation of *P. gingivalis* occurs more often from couples of which one spouse suffers from periodontitis (von Troil-Linden et al. 1995). In our ''Java project on Periodontal Diseases'' we have been able to clinically and microbiologically monitor a group of subjects since 1987. These subjects, aged 15–25 years at the start of the study, live in a village in a remote area in Southern Java. In 1994, 160 subjects could be retrieved in which microbiological cultures were performed to study presence and levels of *P. gingivalis* in subgingival plaque samples. As the study population comprises subjects that live rather isolated, it was hypothesized that transmission of periodontal pathogens between family members and spouses could influence the periodontal condition. This hypothesis could not be confirmed in an analyses based on longitudinal clinical and microbiological data (van der Velden et al. 1993, 1996).

To further investigate the possible transmission of *P. gingivalis*, we used serotyping to study transmission among spouses as well as siblings (van Winkelhoff et al. 1999). Results showed that none of the couples with both spouses culture-positive for *P. gingivalis* shared the same serotype. However, in three out of 29 sib ships with more than one member positive for *P. gingivalis*, the same *P. gingivalis* serotype was found, suggesting that transmission of the species in family units had occured.

Only seven *P. gingivalis* serotypes are available and therefore a more accurate typing method is necessary to further study the distribution of different clonal types of *P. gingivalis* and to study the possible spread of this species in this particular population. For this purpose, we have used the amplified fragment length polymorphism (AFLP) technique to type all *P. gingivalis* isolates. The purpose of this investigation was (1) to study the distribution of different AFLP types of *P. gingivalis* in the population, and (2) to study possible transmission in family units.

Material and Methods

The present microbiological samples were obtained from subjects participating in a longitudinal, prospective study and living in a village with approximately 2000 inhabitants at the Malabar/Poerbasari tea estate on Western Java, Indonesia. This specific population was selected because it had not received regular dental care and had not been exposed to preventive dental-care programmes. Emergency dental treatment, consisting of extraction of teeth, was provided by a general physician. Therefore, this population was suitable for study of the natural development and progression of periodontitis. The population consisted mostly of tea labourers, receiving basic medical care, employed by a government-owned tea estate, PTP XIII.

The study populations of this ongoing investigation have been described in detail by Timmerman et al. (2001) and by van der Velden et al. (2006). In 1994, 158 (69 males, 89 females) subjects of the original population of 255 subjects were available for microbiological evaluation. In these subjects, the deepest bleeding site with the greatest amount of attachment loss in each quadrant of the dentition was selected and sampled with sterile paper points. Pooled samples were analysed for the presence and levels of *P. gingivalis*.

Microbiological procedures

The pooled subgingival plaque samples were vortexed for 30 s and 10-fold serial dilutions were prepared in sterile saline. Aliquots of 100 μl were plated on 5% sheep blood agar plates (Oxoid no.2, Basingstoke, UK), supplemented with haemin (5 mg/l) and menadione (1 mg/l) and anaerobically incubated (BBL Gaspak Anaerobic System, Beckton, Dickinson and Company, Sparks, MD, USA) at 37°C for up to 14 days. The total colony-forming units was determined and dark-pigmented colonies were streaked to purity and identified using standard techniques (van Winkelhoff et al. 1985). One to four *P. gingivalis* isolates per subject with a total of 371 isolates were kept on beads at -80°C until use.

AFLP typing

Bacterial strains were anaerobically grown as described above. Cells were suspended to a density of 0.5–1.0 McFarland in 2.5 ml TE 1-buffer [1 M Tris, 0.1 M EDTA (pH 8.0)]. 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, Almere, the Netherlands). The protocol included 1 h of pre-treatment 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 until use (2).

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

Five microlitres of the diluted restriction–ligation mixture was added to 5 μl of the AFLP amplification mixture [1 × GeneAmp PCR buffer (Applied Biosystems, Nieuwerkerk ad Ijssel, the Netherlands], 2 mM dNTP's (Promega, Leiden, the Netherlands), 15 mM $MgCl_2$, (Applied Biosystems) 20 ng *PST*-0-FAM primer (Eurogentec), 60 ng *MSE*-C primer (Eurogentec), 1 U AmpliTaq DNA polymerase (Applied Biosystems). The mixture was amplified in a GeneAmp PCR System 9700 machine (Applied Biosystems). The amplification conditions were 2 min. at 72°C followed by 12 cycles consisting of 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 deionised formamide and 0.5 μl of ROX-labelled GeneScan-500 as an internal standard. The samples were run on an ABI 3100 Genetic Analyser (Applied Biosystems). AFLP patterns were analysed with BioNumerics software version 3.0 (Applied Maths, Sint-Martens-Latum, Belgium).

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

Table 1. Number of subjects and number of *Porphyromonas gingivalis* isolates retrieved per subject

Number of subjects	Number of isolates/subject
8 (7.6%)	1
6 (5.7%)	2
13 (12.4%)	3
78 (74.3%)	4
Total 105	371

Reproducibility

The reproducibility of the AFLP typing method was established by triple testing of five *P. gingivalis* strains. On the basis of the calculated standard deviation, a cut off level of 85% similarity was used to designate isolates as one clonal type (data not shown).

Results

One hundred and five of the 158 subjects (67%) were culture positive for *P. gingivalis* and the mean percentage amounted to 23% of the subgingival microflora in culture-positive subjects. The number of subjects from which a given number of *P. gingivalis* isolates were obtained is summarised in Table 1.

Table 2. Number of sib ship with identical AFLP *Porphyromonas gingivalis* (Pg) profile in 30 subjects representing 13 different families

Number of Pg-positive sib ships in the study population	Number of sib ships with identical Pg genotype
Ten families with two siblings	Three sib ships
Two families with three siblings	One sib ship all three subjects
	One sib ship with two subjects
One family with four siblings	Two siblings
Total number of subjects = 30	Total number of subjects = 13

AFLP, amplified fragment length polymorphism.

Among the 371 *P. gingivalis* isolates, 142 distinguishable AFLP profiles were found. Of the 105 *P. gingivalis*-positive subjects, 30 subjects were siblings and represented 13 different families. Ten sib ships consisted of two *P. gingivalis*-positive siblings, two sib ships of three *P. gingivalis*-positive siblings and in one family was represented by four *P. gingivalis*-positive siblings (Table 2).

Results of the AFLP evaluation showed that in three out of the 10 sib ships with two siblings positive for *P. gingivalis*, identical *P. gingivalis* clones were found (Fig. 1). In the two families with three *P. gingivalis*-positive siblings each, two of the three siblings had identical *P. gingivalis* clones in one family, whereas in the second family, all three siblings had the same clonal type. The one family represented by four *P. gingivalis*-positive siblings, two siblings harboured the same clonal type of *P. gingivalis*. In total, in six of the 13 families (46%) identical clones were observed among siblings (Fig. 2).

In the group of 105 *P. gingivalis*-positive subjects, 23 married couples could be identified. The average time of marriage was 10 years (range 7–14 years). In 13 married couples, both

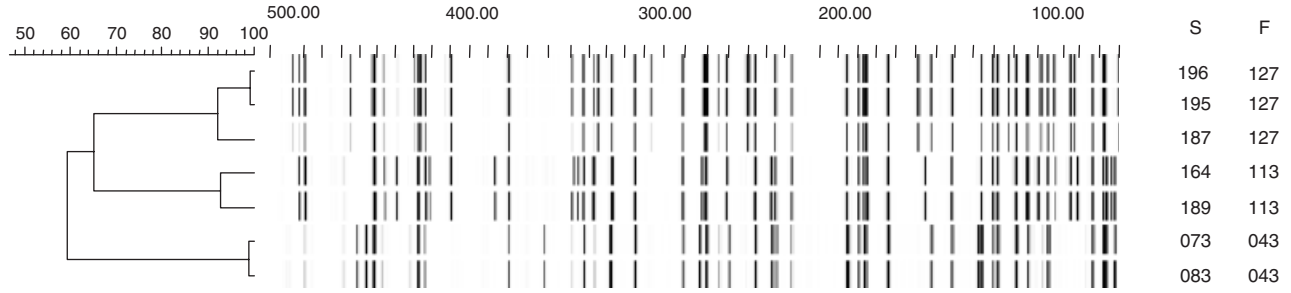

Fig. 1. Amplified fragment length polymorphism profiles of *Porphyromonas gingivalis* isolates from siblings. (S, subject identification number; F, family identification number). It is shown that subjects from the same family unit share similar *P. gingivalis* genotypes. The cut off value for identical clones was determined 85%.

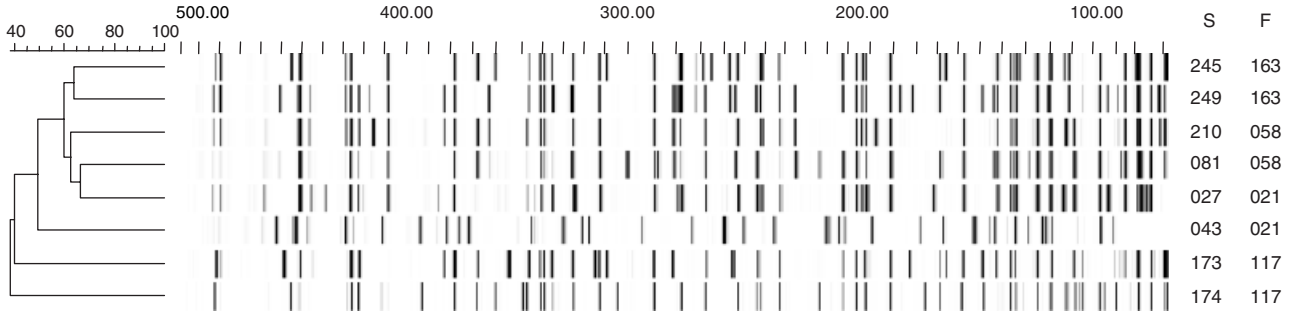

Fig. 2. Amplified fragment length polymorphism profiles of *Porphyromonas gingivalis* isolates from siblings. (S, subject identification number; F, Family identification number). It is shown that subjects from the same family unit have different *P. gingivalis* genotypes. The cut off value for identical clones was determined 85%.

spouses were culture positive for *P. gingivalis*. In no case was the same genotype found in spouses. Ten *P. gingivalis*-positive subjects had spouses that were culture negative for *P. gingivalis*.

Discussion

The aim of the Java Project on Periodontal Diseases was to study risk factors for the onset and progression of naturally developing periodontitis. We hypothesized that transmission of *P. gingivalis* could be one of those factors. On the basis of the isolated nature of the study population, we speculated that transmission of certain clonal types of *P. gingivalis* would occur frequently. To study this hypothesis, we have isolated *P. gingivalis* from family members including spouses and siblings. In the majority of the subjects (74%) we were able to obtain four *P. gingivalis* isolates, in 14 subjects only two or one isolate. These one or two isolates were the only isolates that could be found on the plates, in most cases only after prolonged incubation. We cannot rule out the possibility that four isolates from all study subjects might have changed the results to some extent. In six (46%) of the 13 families, identical clones were found based on the AFLP typing methodology. This observation indicates that siblings were either infected by the same source, e.g. one of the parents, or they may have transferred the *P. gingivalis* to each other. Unfortunately, the study did not allow the search for a common source. Transmission of the species between spouses has been documented in several studies. Asikainen et al. (1996) found 20% of the couples to be infected with the same *P. gingivalis* genotype using AP-PCR. van Steenbergen et al. (1993) selected 18 adult periodontitis patients culture-positive for *P. gingivalis*, of which 10 spouses also appeared *P. gingivalis* positive. The isolates of eight of these couples were genotyped using restriction enzyme analysis. They found six of the eight couples (75%) to be infected with indistinguishable *P. gingivalis* REA types. Both studies also showed that every couple was infected with a unique *P. gingivalis* genotype. In this study we found no evidence of transmission of *P. gingivalis* between spouses, in no case the same genotype was found in spouses' *P. gingivalis* profile. Moreover, 20 subjects who were culture positive for *P. gingivalis* had spouses with no detectable *P. gingivalis*. The lack of transmission of the species between spouses in this population is difficult to explain. All spouses suffered from periodontitis to some degree and none of the subjects had received regular dental care. Saliva in periodontitis subjects usually has detectable levels of *P. gingivalis* (van Winkelhoff et al. 1988, van Steenbergen et al. 1993) and also in this population; salivary *P. gingivalis* was detected in 87% of the subjects (Timmerman et al. 1998). In addition, tongue kissing is a common habit in this population. The average time of marriage in this study was 10 years and ranged from 7 to 14. In the study of van Steenbergen et al. (1993), the time of cohabitation ranged from 10 to 27 years. This difference may also have influenced the transmission rate, although the complete absence of evidence for horizontal transmission in this study is still unexpected. One can speculate that the colonisation resistance of the existing oral microflora was obviously high enough to prevent transmission of *P. gingivalis* despite cohabitation of at least 7 years (mean 10 years). That transmission of *P. gingivalis* is not readily achieved is also exemplified by the observation that 10 *P. gingivalis*-negative subjects had spouses culture positive for *P. gingivalis*.

In summary, this study provides evidence for the vertical transmission of *P. gingivalis* in family units in this study population. We found no evidence of transmission between spouses in the experimental period of on average 10 years, which is in contrast to observations in Western populations. This may implicate that mechanisms of spread of this periodontal pathogen may be different among world populations. This may be explained by differences in colonization resistance as a consequence of life style and oral health level.

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Clinical Relevance

Scientific rationale for the study: To study the transmission of *P. gingivalis* in an isolated population of individuals using analyses of bacterial DNA and computer-assisted analysis of the DNA profiles.

Principle findings: We found evidence for transmission in six out of 13 (46%) sib ships in this study. No evidence was found for horizontal transmission of *P. gingivalis* between spouses in 13 married couples, despite cohabitation of on average 10 years.

Practical implications: Transmission patterns in this study indicate that *P. gingivalis* can be transmitted with families units, possibly from parents to children. This may contribute to the odds developing periodontal disease in the children. Prevention of transmission may therefore represent a preventive measure for periodontitis to develop.