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# Examination of maternal gingival crevicular fluid for the presence of selected periodontopathogens implicated in the pre-term delivery of low birthweight infants

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**Key words:** prevalence, periodontopathogens, maternal gingival crevicular fluid, Rwanda

Background: Reports show that more than 20 million infants world-wide are born prematurely with 95% of all pre-term births occurring in developing countries. Oral colonization of Gram-negative anaerobes has been implicated as a risk factor for preterm delivery of low birth weight infants.

Results: Association of bacterial species with the risk of periodontal disease and thus the risk of preterm delivery was only observed when they occurred in pairs or groups of three or more. *Aa* appeared to be a necessary co-factor for significant associations of bacterial groups with the variables recorded.

Materials and Methods: This study comprised 200 women admitted to the department of obstetrics and gynecology of the teaching hospital of Butare in Rwanda. Gingival crevicular fluid was collected from each quadrant of the mother's mouth (using paper points) within 24 hours of delivery. A dichotomous score of presence or absence of gingival inflammation was recorded for each patient along with demographic data such as age, marital status etc. Samples were examined by PCR for the presence of *Aggregatibacter actinomycetemcomitans* and selected members of the red and orange complexes described by Socransky et al. (1998), and their presence associated with age, gingival inflammation and pregnancy outcomes.

## Introduction

Reports show that more than 20 million infants in the world are born prematurely (before 37 weeks of gestation) with low birth weight (weight <2,500 grams).[1-3] Ninety-five percent of all preterm low birthweight infants (PLBW) are born in developing countries with 15% occurring in sub-Saharan Africa. Reports indicate that the rate in developing countries is more than double that in developed countries (16.5% and 7% respectively).[4-6] Risk factors for PLBW include, amongst others, maternal age at delivery, socio-economic status, ethnicity, tobacco and alcohol use, genitourinary infections, maternal nutritional status.[7-10]

Recent studies proposed a link between the periodontal health status of pregnant women and adverse pregnancy outcomes. The risk of periodontal disease (infection of the tissues surrounding and supporting the teeth) in pregnant women is considered to be exacerbated by the increase of estrogen and progesterone concentrations.[11-21]

It is postulated that lipopolysaccharides from the anaerobic Gram-negative periodontopathogens, stimulate the release of inflammatory cytokines which may trigger pre-term delivery.[22] Among the bacteria implicated, are members of the red and orange complexes described by Socransky et al.[23] Using statistical analysis, Socransky et al. used colour coding to cluster frequently occurring bacterial species into complexes relating to the clinical parameters used in the diagnosis of periodontal disease.

Studies abroad have detected selected members of Socransky's red and orange complexes such as *Tannerella forsythia, Porphyromonas gingivalis, Treponema denticola, Prevotella intermedia, Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans* (previously known as *Actinobacillus actinomycetemcomitans* and hereafter referred to as *Aa)* in 18% of full-term and 100% of preterm deliveries.[1,18,22,24-26] To our knowledge, an association of these species with adverse pregnancy outcomes in an African population has not been reported. The objective of this study was therefore to examine a group of mothers from Rwanda (where infant mortality due to PLBW is high) for the presence of these anaerobic Gram-negative bacteria in order to determine whether they could effectively be used as microbial biomarkers for the risk of preterm delivery.

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**Table 1.** Nucleotide sequences of selected and modified 16S rDNA primer pairs

| Target | PCR primer pairs (5'–3') | Source |
|---|---|---|
| *Porphyromonas gingivalis*: Forward<br>Reverse | AGG CAG CTT GCC ATA CTG CG<br>ACT GTT AGC AAC TAC CGA TGT | Rocas et al. (2001) |
| *Tannerella forsythia*: Forward<br>Reverse | GCG TAT GTA ACC TGC CCG CA<br>TGC TTC AGT GTC AGT TAT ACC T | Rocas et al. (2001) |
| *Treponema denticola*: Forward<br>Reverse | TAA TAC CGA ATG TGC TCA TTT ACA T<br>TCA AAG AAG CAT TCC CTC TTC TTC TTA | Rocas et al. (2001) |
| *Prevotella intermedia:* Forward<br>Reverse | CAA AGA TTC ATC GGT GGA<br>GCC GGT CCT TAT TCG AAG | Kook et al. (2005) |
| *Fusobacterium nucleatum*: Forward<br>Reverse | ATT GTG GCT AAA AAT TAT AGT T<br>ACC CTC ACT TTG AGG ATT ATA G | Mayanagi et al. (2004) |
| *Aggregatibacter actinomycetemcomitans*: Forward<br>Reverse | GCT AAT ACC GCG TAG AGT CGG<br>ATT TCA CAC CTC ACT TAA AGG T | Avila-campos and Julio (2003) |
| Ubiquitous primers: Forward<br>Reverse | GAT TAG ATA CCC TGG TAG TCC AC<br>CCC GGG AAC GTA TTC ACC G | Rocas et al. (2001) |

**Table 2.** Prevalence of the six bacterial species as detected by PCR and their distribution according to age

| Bacterial species | Total positive n (%) | ≤20 years n (%) | 21–25 years n (%) | 26–30 years n (%) | 31–35 years n (%) | ≥36 years n (%) | p value |
|---|---|---|---|---|---|---|---|
| ***Fn*** | 163 (86.2) | 12 (80.0) | 49 (86.0) | 50 (87.7) | 28 (80.0) | 21 (95.5) | 0.513 |
| ***Pi*** | 139 (73.5) | 13 (86.7) | 46 (80.7) | 37 (64.9) | 24 (68.6) | 17 (77.3) | 0.229 |
| ***Tf*** | 91 (47.6) | 10 (66.7) | 29 (50.9) | 27 (47.4) | 10 (28.6) | 11 (50.0) | 0.110 |
| ***Aa*** | 85 (45) | 10 (66.7) | 30 (52.6) | 19 (33.3) | 14 (40.0) | 11 (50.0) | 0.096 |
| ***Pg*** | 54 (28.4) | 3 (21.4) | 13 (22.8) | 18 (31.6) | 15 (42.9) | 5 (22.7) | 0.258 |
| ***Td*** | 46 (24.3) | 3 (20.0) | 14 (24.6) | 17 (29.8) | 6 (17.1) | 4 (18.2) | 0.640 |
| ***Tf/Aa*** | | 8 (53.3) | 16 (28.1) | 9 (15.8) | 4 (11.4) | 7 (31.8) | 0.009 |
| ***Tf/Pi*** | | 9 (60.0) | 23 (40.4) | 17 (29.8) | 5 (14.5) | 9 (40.9) | 0.014 |
| ***Pi/Aa*** | | 9 (60.0) | 24 (42.1) | 12 (21.1) | 10 (28.6) | 8 (36.4) | 0.027 |

*Fn*, *F. nucleatum*; *Pi*, *P. intermedia*; *Tf*, *T. forsythia*; *Aa*, *A. actinomycetemcomitans*; *Pg*, *P. gingivalis*; *Td*, *T. denticola*.

**Table 3.** Association of bacterial species with frequency of tooth brushing

| | Frequency of brushing | | | |
|---|---|---|---|---|
| Bacterial species | 1 x day n (%) | 2 x day n (%) | 3 x day n (%) | p value |
| ***Fn*** | 74 (85.1) | 45 (81.8) | 5 (100.0) | 0.541 |
| ***Pi*** | 70 (80.5) | 37 (67.3) | 2 (40.0) | 0.045 |
| ***Tf*** | 41 (46.1) | 26 (47.3) | 2 (40.0) | 0.950 |
| ***Aa*** | 34 (39.1) | 25 (45.5) | 1 (20.0) | 0.176 |
| ***Pg*** | 26 (29.5) | 14 (25.5) | 2 (40.0) | 0.733 |
| ***Td*** | 21 (24.1) | 15 (27.3) | 1 (20.0) | 0.883 |
| ***Pg/Tf/Aa*** | 5 (5.7) | 6 (10.9) | 2 (40.0) | 0.025 |
| ***Pg/Tf/Pi/Aa*** | 5 (5.7) | 4 (7.3) | 2 (40.0) | 0.018 |
| ***Pg/Tf/Aa/Fn*** | 4 (4.6) | 6 (10.9) | 2 (40.0) | 0.012 |

## Results

Gingival crevicular fluid samples could only be collected from 189 of the 200 patients due to the availability of the dentist. The frequency of each bacterial species is therefore expressed as a percentage of 189 samples. In **Table 1**, the distribution of bacterial species within each age group is expressed as a percentage of the number in each age category to facilitate comparison with bacterial combinations of 2 or more. *F. nucleatum* (*Fn*) and *P. intermedia* (*Pi*) were the species most frequently detected, followed by *T. forsythia* (*Tf*), *A. actinomycetemcomitans* (*Aa*), *P. gingivalis* (*Pg*) and lastly *T. denticola* (*Td*). Their prevalence increased between the ages of 21–30 years but not significantly so (**Table 2**). Although no singular bacterial species exhibited a significant association with age, significant associations were detected with different combinations of *Tf/Aa/Pi* (**Table 2**).

When questioned about their oral health care, 135 of the patients reported that they never visited a dentist (data not shown), 87 reported brushing their teeth once a day, 55 reported brushing twice a day and 5 reported brushing after every meal. With the exception of *Pi*, frequency of brushing did not significantly correlate with the detection of any other single bacterial species (**Table 3**). However, significant correlations were observed when three or more species were detected simultaneously, particularly when these groups included *Aa*, *Tf* and *Pg* (**Table 3**). Similar results were found for associations with bleeding when brushing (**Table 4**). Although a strong but not significant association was found with *Td* (p = 0.082), combinations of two or more species

yielded significant associations, particularly when *Td*, *Tf*, *Pi* and *Aa* were present.

Gingival inflammation appeared to be strongly associated with *Aa* but only significantly so with *Pi* (**Table 5**). However, when we analysed the combinations, the only significant combinations observed were *Pg*/*Pi* and *Fn*/*Pi*, with no *Aa* detected. As summarized in **Table 6**, this study failed to demonstrate an association between proposed risk factors for periodontal disease and PLBW with the exception of gingival inflammation which demonstrated a significant difference between the NT and PLBW groups.

## Discussion

Although it has been suggested that oral infection can act as the site of origin for dissemination of periodontopathogens to distant body sites thus linking them with PLBW, results have been inconclusive. Some studies show a significant correlation, while others do not.[18,22,26-29] These reports need not necessarily contradict each other if other factors such as race, geographical location and socio-economical standards are adequately adjusted for. Thus studies done in USA and Europe differ from those in Latin America or Africa, largely because of socio-economic factors such as easy access to adequate and affordable health care.

The objective of this study was to examine an African population for the presence of anaerobic bacteria frequently associated with periodontal disease and implicated in adverse pregnancy outcomes such as PLBW. It is known that PLBW infants are exposed to serious health problems, including, neurodevelopment disturbunces, ear infections, respiratory infections, asthma and death.[30] Ten% of neonatal mortality world-wide is caused by prematurity.[31] Infant mortality due PLBW is relatively high in Rwanda and for that reason we selected this group of mothers for this study. We elected to use gingival crevicular fluid instead of dental plaque for this study because it enabled us to use the same sample to examine for bacterial species (using PCR) as well as for inflammatory cytokines (work in progress) which may serve as biomarkers for preterm delivery.

We recruited 200 patients (100 cases and 100 controls) but data analysis and interpretation was sometimes complicated by the lack of response to questions asked of the patients and unavailability of the dentist for sample collection. Thus data analysis was largely confined to 189 patients instead of 200 for variables relating to the oral health of the patient. One sample was collected from each quadrant of the month and the four samples pooled to yield a single sample for each patient. PCR was used to detect six suspected periodontopathogens and the PCR results matched with selected risk factors (age, oral health care) and indicators (bleeding when brushing, gingival inflammation) for periodontal disease. In the absence of adequate diagnosis of periodontal disease, we concede that the associations may be inconclusive and for that reason, we present these results as an association between proposed risk factors for periodontal disease and PLBW rather than the presence of periodontal disease and PLBW. The six anaerobic bacteria examined for in this study, have been implicated in PLBW but no overt association exists.[1,18,22,24-26]

**Table 4.** Association of bacterial species with reported bleeding when brushing

| Bacterial species | Yes n (%) | No n (%) | p value |
|---|---|---|---|
| ***Fn*** | 81 (83.5) | 49 (86.0) | 0.436 |
| ***Pi*** | 75 (77.3) | 38 (66.7) | 0.105 |
| ***Tf*** | 49 (50.5) | 25 (42.4) | 0.206 |
| ***Aa*** | 47 (48.5) | 20 (35.1) | 0.073 |
| ***Pg*** | 30 (30.9) | 15 (25.9) | 0.314 |
| ***Td*** | 28 (28.9) | 10 (17.5) | 0.082 |
| ***Aa/Tf*** | 28 (28.9) | 8 (14.1) | 0.026 |
| ***Aa/Pg*** | 18 (18.6) | 4 (7.1) | 0.041 |
| ***Aa/Td*** | 16 (16.5) | 3 (5.3) | 0.032 |
| ***Aa/Pg/Pi*** | 17 (17.5) | 3 (5.3) | 0.022 |
| ***Aa/Tf/Td*** | 11 (11.3) | 1 (1.8) | 0.027 |
| ***Aa/Td/Pi*** | 16 (16.5) | 3 (5.3) | 0.032 |
| ***Aa/Pg/Tf/Pi*** | 10 (10.3) | 1 (1.8) | 0.041 |
| ***Aa/Tf/Td/Pi*** | 11 (11.3) | 1 (1.8) | 0.027 |
| ***Aa/Tf/Td/Fn*** | 11 (11.3) | 1 (1.8) | 0.027 |
| ***Aa/Td/Pi/Fn*** | 15 (15.5) | 3 (5.3) | 0.046 |

**Table 5.** Association of bacterial species with gingival inflammation

| Bacterial species | Presence n (%) | Absence n (%) | p value |
|---|---|---|---|
| ***Fn*** | 89 (86.4) | 71 (85.5) | 0.515 |
| ***Pi*** | 83 (80.6) | 54 (65.1) | 0.013 |
| ***Tf*** | 53 (51.0) | 35 (41.7) | 0.131 |
| ***Aa*** | 40 (38.8) | 42 (50.6) | 0.072 |
| ***Pg*** | 35 (33.7) | 18 (21.7) | 0.050 |
| ***Td*** | 28 (27.2) | 17 (20.5) | 0.187 |
| ***Pg/Pi*** | 30 (29.1) | 14 (17.1) | 0.040 |
| ***Pi/Fn*** | 72 (69.9) | 47 (56.6) | 0.043 |

With the exception of *Pi* which showed a significant association with the frequency of tooth brushing ($p = 0.045$), none of the other singly detected species could be significantly associated with age, frequency of brushing, bleeding when brushing or gingival inflammation. Bacterial combinations however, yielded significant correlations especially when Aa, *Tf* and *Pi* were present. Gingival bleeding was the only variable with which *Td* showed any association, particularly when detected along with other members of the red complex viz. *Pg* and *Tf*. Because anaerobic spirochaetes have been consistently associated with periodontal disease,[32] one may speculate that these patients who reported bleeding when brushing their teeth either had or were at risk for having periodontal disease.

The association with gingival inflammation in our study supports the positive correlation reported by other researchers between poor periodontal health and PLBW.[18,22,26,27,33] However, our dichotomous recording of "presence" or "absence" of gingival inflammation as opposed to the use of periodontal indices as a measurement of periodontal health or disease, provides a rather crude assessment of the periodontal health of the mother and thus caution should be exercised in interpretation of this finding.

**Table 6.** Association between proposed risk factors for periodontal disease and pregnancy outcomes

| | NT | PLBW | p value |
|---|---|---|---|
| **Bacterial species** | **n (%)** | **n (%)** | |
| ***Fn*** | 82 (83.7) | 81 (89.0) | 0.197 |
| ***Pi*** | 69 (70.4) | 70 (76.9) | 0.198 |
| ***Tf*** | 43 (43.0) | 48 (52.7) | 0.115 |
| ***Aa*** | 48 (49.0) | 37 (40.7) | 0.158 |
| ***Pg*** | 28 (28.3) | 26 (28.6) | 0.546 |
| ***Td*** | 22 (22.4) | 24 (26.4) | 0.323 |
| **Age** | | | 0.557 |
| **<20 yrs** | 8 (8.2) | 9 (9.3) | |
| **21–25 yrs** | 28 (28.6) | 32 (33.0) | |
| **26–30 yrs** | 31 (31.6) | 27 (27.8) | |
| **31–35 yrs** | 22 (22.4) | 15 (15.5) | |
| **≥36 yrs** | 9 (9.2) | 14 (14.4) | |
| **Frequency of tooth brushing** | | | 0.522 |
| **1 x day** | 44 (58.7) | 48 (60.0) | |
| **2 x day** | 27 (36.0) | 31 (38.8) | |
| **3 x day** | 4 (5.3) | 1 (1.2) | |
| **Bleeding when brushing** | | | 0.522 |
| **Yes** | 48 (61.5) | 51 (60.7) | |
| **No** | 30 (38.5) | 33 (39.3) | |
| **Gingival Inflammation** | | | 0.004 |
| **Present** | 45 (45.5) | 59 (65.6) | |
| **Absent** | 54 (54.5) | 31 (34.4) | |

In studies where differences in the presence of the six bacteria were reported when comparing NT and PLBW, the results yielded no statistical significance between the groups when bacterial load was taken into account[28,29,34] and therefore, as in our study, failed to establish a direct association between these species and PLBW.

One may interpret that the detection of all six species in cases and controls, with or without gingival inflammation may suggest a "carrier" state for all of these species. However, the lack of quantification of bacterial load in all of the samples, presents an obstacle in drawing a finite conclusion or comparing our results with other studies.

The consistent finding of *Aa* in all the bacterial combinations which showed a significant correlation with the variables described, deserves some discussion. *Aa* has been significantly associated with aggressive periodontitis.[35] It has also frequently been found in healthy individuals.[36] Studies of different population groups have revealed that a unique *Aa* clone (JP2) is responsible for the aggressive form of periodontitis observed in adolescents from north and west Africa[37,38] and from individuals of African origin living in Europe and USA. Being an Africa population, the prevalence of Aa in theses subjects is not surprising, particularly when observing that besides the combinations of *Pg*, *Pi* and *Fn* found in **Table 5** and the combination of *Tf* and *Pi* in **Table 2**, all the other combinations included *Aa*. One might argue that the presence of *Aa* is nothing more than an indicator of a "carrier" state, but the increased significance of the associations of the bacteria in combination with the variables described, would suggest otherwise. All of the combinations significantly associated with gingival bleeding when brushing include *Aa*. It has been suggested that *Aa* may occur in 2 forms, either as an opportunistic pathogen occurring world-wide with a diversity of *Aa* clones or as an exogenous pathogen with a particular clone (JP2) racially restricted to persons of African origin.[37] Further studies on this population group will inform us of the nature of the *Aa* detected in this population and the associated of risk of PLBW.

This study implies that specific groups of oral anaerobes may be risk factors for periodontal disease in these subjects and thus suggest a risk for PLBW. Although PCR has often been proposed as the new gold standard for detecting pathogens in clinical samples[39,40] detection of endogenous microbes (as in the oral cavity) can only yield meaningful results if bacterial load is quantified.[34,41] This study also demonstrates the limitation of studies which target a selected group of bacterial species. One should bear in mind that because of the complexity of the aetiology of periodontal disease, concentration on selected species alone may result in many potential pathogens going undetected. Until a fully standardized protocol is available to establish the exact role of periodontal pathogens in PLBW, comparison with other studies will always be hampered by differences in diagnosis, sampling, detection methods and data analysis. This, compounded by differences in race, socio-economic status and geographical position may lend itself to speculation and often affect interpretation of results in such a way as to favour a proposed hypothesis.

We are aware that if the pregnancy outcomes were adjusted for other risk factors such as alcohol and tobacco usage, level of education and general nutrition and well-being, amongst others, the presence of these anaerobic species might indeed prove to be a co-factor for the risk of PLBW. With so many contradicting reports regarding the presence and role of the oral anaerobes in PLBW, research in this area continues in the quest for conclusive evidence of their role in adverse pregnancy outcomes.

## Material and Methods

**Patient selection.** This case-control study requested the consent and participation of 200 pregnant women admitted to the department of obstetrics and gynecology of the teaching hospital of Butare in Rwanda. Patients were informed of the purpose of the study and required to complete consent forms if they agreed to participate in the study. Demographic data including age, marital status, educational level and socio-economic standards were recorded from the patient's medical records. Patients were also questioned about their oral health care habits in order to establish their risk for periodontal disease, and their responses linked to their gestation terms and time of delivery.

**Inclusion and exclusion criteria.** We elected to examine mothers from Rwanda since indicators from the Rwanda Ministry of Health (2005) reported the incidence of mortality due to PLBW

as 86/1,000 and maternal mortality as 1,071/1,00,000 live births. The mean age of the participants was 28 years ( 6.09). Preterm birth was defined as <37 weeks of gestation and low birth weight <2,500 grams. The stage of gestation was determined by recording the last date of menstruation of the patient or by ultrasound. No instruction for oral hygiene had preceded the collection of clinical samples. Patients on antibiotics or who had received antibiotics 2 weeks prior to examination were excluded from the study.

**Sample collection.** Samples were collected within 24 hours of delivery**.** Prior to sampling, the patients were examined for signs of gingival inflammation and/or bleeding which might be attributed to lack of oral health care. No attempt was made for an accurate diagnosis of gingivitis or periodontitis using clinical indices or radiographs and instead, gingival inflammation was recorded by the dichotomous score of "presence" or "absence". All clinical examinations and sample collections were done by the same clinician. Gingival crevicular fluid (GCF) was collected from four teeth (16, 26, 36, 46) by inserting filter paper strips (PropFlow, Inc., Amityville, NY) into the base of the pocket for one minute per tooth.[42] Care was taken to prevent saliva or blood contamination during collection. Each paper strip was placed into 50 l phosphate buffered saline sampling buffer in an Eppendorf tube with added 0.05% tween-20 (PBS-T) and stored at -80°C.[43] Samples were transported on dry ice to South Africa and stored at -80°C until analysis.

**Sample analysis.** Polymerase Chain Reaction (PCR) was used for the detection of the presence of the 6 selected target bacteria in this study. Samples from the freezer were thawed by incubation at 37°C for 10 min, then centrifuged (10,000x g) for 15 minutes at 4°C. The supernatents of the 4 tubes of each patient were pooled to yield a single sample representing all four quadrants of the mouth for each patient.[44] Samples were vortexed for 30 seconds and centrifuged at 2,500x g for 2 minutes. The supernatent was removed and the pellet resuspended in 100 l of distilled water. Another step of vortexing and centrifugation was done and the pellet was resuspended in 500 l of distilled water. The suspension was heated at 94°C for 10 min and the vials immediately chilled on ice for 5 min.

Reference DNA from *Tannerella forsythia* (ATCC 43037), *Porphyromonas gingivalis* (ATCC 33277), *Treponema denticola* (ATCC 335521), *Prevotella intermedia* (ATCC 25611), *Fusobacterium nucleatum* (NTCC 10562) and *Aggregatibacter actinomycetemcomitans* ( ATCC 33396) were used as positive controls. Chilled samples were centrifuged for 10 seconds at 9,000X and 5 l aliquots of the supernatents were used in the PCR assay. Twenty-five of the Dreamtaq™ Green PCR Master Mix(2X) (FE K1081, Inqaba biotec), 0.1–1.0 M of each primer and 18 l of water nuclease were added to the 5 l of template DNA. Species-specific primers (Inqaba biotec) were used to detect the presence of the six target organisms in this study. The expected product lengths were 641 bp for *T. forsythia*, 404 bp for *P. gingivalis*, 316 bp for *T. denticola*, 307 bp for *P. intermedia*, 500 bp for *A. actinomycetemcomitans* and 705 bp for *F. nucleatum*. Confirmation of PCR reaction was achieved by the use of a pair of ubiquitous primers product length (602 bp) which matches most bacterial 16S rRNA genes at the same position. Nucleotide sequences of selected and modified 16S rDNA primer pairs are listed in **Table 1**.

The negative control contained 5 l of distilled water in place of the sample and the positive control consisted of 49 l from the master mix and 1 l (100 ng) of the reference genomic DNA. A brief vortexing of samples was done. PCR amplifications were perfomed as follows:

*P. gingivalis*[45]: an initial denaturation step at 94°C for 2 minutes, followed by 36 cycles of a denaturation step at 94°C for 30 seconds, a primer annealing step at 60°C for 1 minute, an extention step at 72°C for 2 minutes and a final step at 72°C for 10 minutes.

*T. forsythia*, *T. denticola* and ubiquitous primers[45]: an initial denaturation step at 95°C for 2 minutes, followed by 36 cycles of a denaturation step at 95°C for 30 seconds, a primer annealing step at 60°C for 1 minute and extention step at 72°C for 1 minute and a final step at 72°C for 2 minutes.

*Prevotella intermedia*[46]: an initial denaturation step at 95°C for 2 minutes, followed by 36 cycles as one cycle at 94°C for 30 s (denaturation) followed by 55°C for 1 min (annealing) with an elongation of 72°C for 1 minute and a final step at 72°C for 10 minutes.

*A. actinomycetemcomitans* and *F. nucleatum*: Same conditions as described previously by Rocas et al.[45]

The PCR products were analyzed by electrophoresis in 1% agarose gels using Tris-Borate EDTA buffer at 90 V. A 100 bp size ladder (O'GeneRuler 100 bp DNA ladder, Fermentas) was used as the molecular weight marker. The DNA was stained with ethidium bromide and visualized under UV light.

**Data analysis.** Data were entered into Excel 2003 and then transferred to the Statistical Package for Social Sciences (SPSS 14.0) for analysis. Frequencies, means and standard deviations were calculated using descriptive statistics. The significance of associations was determined using chi-squared and Fisher's exact test. A p value of <0.05 was considered significant.



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