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Colonization pattern of periodontal bacteria in Japanese children and their mothers

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Background and Objective: The purpose of this study was to determine the time of infection by anaerobic gram-negative rods associated with periodontal disease, and to clarify their transmission from mother to child.

Material and Methods: Seventy-eight Japanese children (including 10 siblings), aged from 3 to 9 years, and 68 mothers, were enrolled in this study. Colonization by 11 periodontal bacterial species was determined using polymerase chain reaction amplification of samples of subgingival plaque obtained from the children and their mothers.

Results: The detection rates of *Porphyromonas gingivalis*, *Tannerella forsythensis* and *Treponema denticola* increased in children after the age of 6 years. We found a high consistency in colonization by *P. gingivalis*, *T. denticola*, *Prevotella intermedia* and *Prevotella nigrescens* in 9 of the 10 siblings. The average number of bacterial species in plaque samples harboring *Fusobacterium nucleatum* and/or *Fusobacterium periodonticum* was significantly greater than in those without, in both children and mothers. Kappa statistical analysis revealed that the detection of *Capnocytophaga gingivalis*, *Capnocytophaga ochracea*, *Campylobacter rectus* and *T. denticola* in children was consistent with that in the mother.

Conclusion: Periodontal bacterial colonization in Japanese children increased with age and was associated with *F. nucleatum* and/or *periodonticum*, and the bacterial flora in children was similar to that in their mothers.

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More than 500 taxa of microorganisms have been identified in the human oral cavity biofilm, among which an increasing number of anaerobic gram-negative rods and spirochetes have been demonstrated to be closely associated with various types of periodontal disease (1,2). The predominant form of periodontal disease in children is gingivitis, initially a reversible inflammatory reaction in marginal gingival tissue. Several reports have suggested the involvement of specific anaerobic bacteria in the etiology of gingivitis in children (3,4). Anaerobic bacteria constitute a significant portion of the bacterial community in periodontal lesions (5). Their ability to adhere and survive by evading host defenses in the rapidly changing environment of early childhood is a fundamental factor in the organization of periodontopathic biofilm (6,7). Many research groups have demonstrated that colonization by periodontal bacteria is a key step in the development of periodontal disease (8–10). Although many research groups have revealed a relationship between specific pathogens in lesions in children and gingivitis (11–14), relatively little is known about when such colonization takes place or the

succession of such anaerobic bacterial species. Therefore, the detection of a specific bacterium in association with periodontal lesions may be an important tool in the diagnosis and treatment of periodontal disease (15–17).

A number of studies have suggested intrafamilial infection with periodontipathic bacteria (18–20). It is possible that periodontopathic bacteria are transmitted from mother to child as the first step in colonization. Umeda *et al*. (21) detected *Tannerella forsythensis*, *Prevotella intermedia* and *Prevotella nigrescens* more frequently in the oral cavities of children whose parents already harbored those bacteria, leading them to suggest intrafamilial transmission. Tanner *et al*. (22) found a similarity between the oral microbiota of preschool children and that of their caregivers. These reports suggested vertical transmission of periodontopathic bacteria. Further analysis is required, however, to determine the route and period of infection. Children with dental plaque harboring bacteria showed no manifest signs of gingivitis (4). Clarification of these points may offer a strong tool in the prognosis of periodontal conditions. The goal of the present study was to investigate the colonization by 11 species of periodontal bacteria, including *Fusobacterium nucleatum* and/or *Fusobacterium periodonticum*, in children by the polymerase chain reaction (PCR), to determine whether there is a relationship between the presence of such bacteria in children and their presence in the mothers, and to clarify the infection route and period.

Material and methods

Subjects

A total of 78 patients, including 10 siblings aged from 3 to 9 years, who visited the Department of Pediatric Dentistry, Tokyo Dental College, Chiba, Japan, and 68 mothers, were enrolled in this study. None had either moderate or severe gingivitis. No patient had received antibiotics for a period of 6 mo before the experiment. Informed consent was obtained from many of the children and from all of the mothers. This study was performed with the permission of the Ethical Committee of Tokyo Dental College.

Sampling of subgingival plaque

Collection of subgingival plaque was performed according to the method of Nakagawa *et al*. (12), with minor modification. Briefly, in the children, subgingival plaque was collected from the maxillary first deciduous molar and maxillary second deciduous molar with sterile toothpicks after removal of supragingival dental plaque. In the mothers, samples were collected from the maxillary first molar and maxillary second molar by the same method. The plaque samples collected from each subject were suspended and mixed in 100 μL of phosphate-buffered saline (pH 7.2), and the microorganisms were precipitated by centrifugation (18,870 *g*, 4°C, 10 min). The pellets were then stored at −20°C until used for detection of periodontal bacteria.

Detection of periodontal bacteria by the PCR

Collected samples were suspended in 100 μL of buffer consisting of 20 mM Tris-HCl, pH 8.0, 2 mM EDTA and 1% Triton X-100, and boiled at 100°C for 10 min. Genomic DNA was isolated by phenol extraction and ethanol precipitation. The presence of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *P. intermedia*, *P. nigrescens*, *Treponema denticola*, *T. forsythensis*, *Capnocytophaga sputi-*

Table 1. Species-specific and ubiquitous polymerase chain reaction primers for 11 periodontal bacteria

Primer pairs (5′–3′)	Amplicon length (bp)
Actinobacillus actinomycetemcomitans	
AAA CCC ATC TCT GAG TTC TTC TTC	557
ATG CCA ACT TGA CGT TAA AT	
Porphyromonas gingivalis	
AGG CGA CTT GCC ATA CTG CG	404
ACT GTT AGC AAC TAC CGA TGT	
Prevotella intermedia	
TTT GTT GGG GAG TAA AGC GGG	575
TCA ACA TCT CTG TGG GCT GCG T	
Prevotella nigrescens	
ATG AAA CAA AGG TTT TCC GGT AAG	804
CCC ACG TCT CTG TGG GCT GCG A	
Treponema denticola	
TAA TAC CGA AGC TCA TTT ACA T TCA AAG TCT CTG	316
TGG GCT GCG A	
Tannerella forsythensis	
GCG TAT GTA ACC TGC CCG CA	641
TGC TTC AGT GTG AGT TAT ACC T	
Capnocytophaga sputigena	
AGA GTT TGA TCC TGG CTC AG	185
GAT GCC GCT CCT ATA TAC CAT TAG G	
Capnocytophaga ochracea	
AGA GTT TGA TCC TGG CTC AG	185
GAT GCC GCT CCT ATA TAC TAT GGG G	
Capnocytophaga gingivalis	
AGA GTT TGA TCC TGG CTC AG	185
GGA CGC ATG CCC ATC TTT CAC CAC CGC	
Campylobacter rectus	
TTT CGG AGC GTA AAC TCC TTT TC	227
TTT CTG CAA GCA GAC ACT CTT	
Fusobacterium nucleatum/periodonticum	
CTG AAC ATT GGA AAC TAT ATA GTA GAA CAA ACA AG	142
GTC CTT CAT CGG CTC TTA CTA CCT AGG C	

gena, *Capnocytophaga ochracea*, *Capnocytophaga gingivalis*, *Campylobacter rectus* and *F. nucleatum/periodonticum* was determined by PCR. The specific primers for the PCR are listed in Table 1. All primers, except those for *F. nucleatum/periodonticum*, were designed by Hayashi *et al.* (14) and Ashimoto *et al.* (23). The primers for *F. nucleatum/periodonticum* were designed based on the 16S and 23S rRNA sequences of *F. nucleatum* in GenBank at the National Center of Biotechnology Information (Bethesda, MD, USA). The specificity of the primers was confirmed against 39 oral bacterial species. A 2-μL sample was added to 48 μL of buffer (Takara Bio Inc., Shiga, Japan) containing 0.2 mM dNTPs, 1 μM each specific primer pair and 0.25 U of *Taq* DNA polymerase (Takara Bio Inc.). All PCR runs, apart from those for *F. nucleatum/periodonticum*, were performed using a thermal cycler (Gene Amp PCR system 9700; Applied Biosystems, Foster City, CA, USA) according to the method of Ashimoto *et al.* (23) and Conrads *et al.* (24). PCR for *F. nucleatum/periodonticum* was performed as follows: 94°C for 5 min, 30 cycles of 98°C for 15 s, 65°C for 30 s and 74°C for 30 s. The PCR products were electrophoresed through a 2% agarose gel and then examined under ultraviolet light after staining with the SYBR Safe DNA stain (Molecular Probes, Eugene, OR, USA).

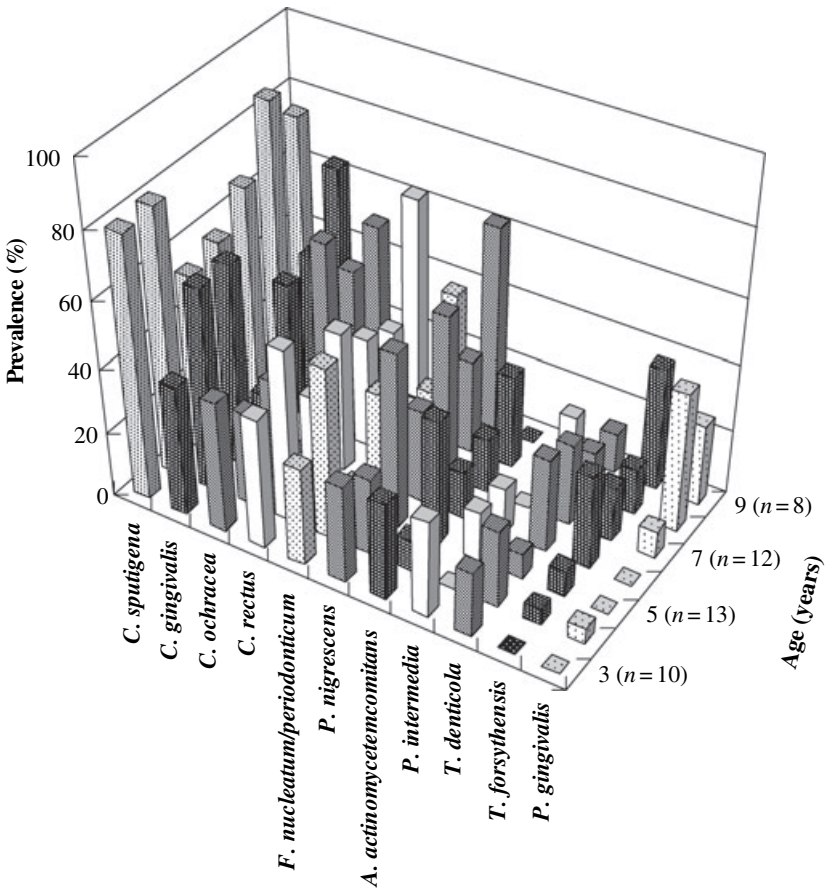

Fig. 1. Detection rates of 11 periodontal bacteria in subgingival plaque samples from 78 Japanese children. *n* = number of children examined. *A. actinomycetemcomitans*, *Actinobacillus actinomycetemcomitans*; *C. gingivalis*, *Capnocytophaga gingivalis*; *C. ochracea*, *Capnocytophaga ochracea*; *C. rectus*, *Campylobacter rectus*; *C. sputigena*, *Capnocytophaga sputigena*; *F. nucleatum/periodonticum*, *Fusobacterium nucleatum/periodonticum*; *P. gingivalis*, *Porphyromonas gingivalis*; *P. intermedia*, *Prevotella intermedia*; *P. nigrescens*, *Prevotella nigrescens*; *T. denticola*, *Treponema denticola*; *T. forsythensis*, *Tannerella forsythensis*.

Statistical analysis

To investigate the relationship between age and detection, Fisher's exact test was performed. To determine the degree of agreement, the Kappa statistic was used according to the method of Cohen (25). To clarify the relationship between colonization by *F. nucleatum/periodonticum* and detection number of other bacterial species, the Student's *t*-test was used.

Results

Specificity of primers for *F. nucleatum/periodonticum*

To confirm the specificity of the primers for *F. nucleatum/periodonticum*, 39 species of microorganisms were subjected to PCR. Only *F. nucleatum/periodonticum* were amplified.

Age of children and detection rate of periodontopathic bacteria

The detection rates for the 11 species of gram-negative bacteria targeted in the plaque samples from the 78 children are shown in Fig. 1. The detection rates of the three Capnocytophaga species were higher relative to those of *P. gingivalis*, *T. forsythensis* and *T. denticola*. Apart from the three Capnocytophaga species, the detection rates of most of the other species increased with age of the child. The detection rates of *P. gingivalis*, *T. forsythensis* and *T. denticola* increased from age 6–7 years onwards. Fisher's exact test revealed a significant difference between the group of children in whom *P. gingivalis* was detected by the age of 3–5 years and the 6–9-year-old group ($p < 0.05$).

Relationship between colonization by *F. nucleatum/periodonticum* and number of bacterial species detected

The average numbers of bacterial species detected in relation to *F. nucleatum/periodonticum* in children and their mothers are summarized in Table 2. We found that the number of bacterial species detected in children harboring *F. nucleatum/periodonticum* was significantly higher than that in children who did not harbor *F. nucleatum/*

Table 2. The average number of bacterial species detected in children and mothers harboring *Fusobacterium nucleatum/periodonticum* was significantly higher than that in children and mothers not harboring F. nucleatum/periodonticum

Fusobacterium nucleatum/ periodonticum	Harboring	Nonharboring
Children	4.8 ± 1.8*	2.7 ± 1.8
Mothers	5.5 ± 1.8*	3.9 ± 1.9

*, $p < 0.001$.

periodonticum ($p < 0.001$). The average number of bacterial species in mothers in whom *F. nucleatum/periodonticum* was detected was significantly higher than that in mothers who did not harbor *F. nucleatum/periodonticum* ($p < 0.001$).

Detection rate between mother and child

The detection rates in the children and their mothers, and in the children and mothers simultaneously, for all species targeted are shown in Fig. 2. The detection rates for *C. sputigena*, *C. gingivalis*, *C. ochracea*, *C. rectus*, *F. nucleatum/periodonticum*, *P. nigrescens*, *A. actinomycetencomitans*, *P. intermedia*, *T. denticola*, *T. forsythensis* and *P. gingivalis* in children were 71.8%, 50.0%, 35.9%, 42.3%, 29.4%, 38.5%, 9.0%, 19.2%, 19.2%, 10.2% and 9.0%, respectively; and those in the mothers were 80.9%, 63.2%, 39.7%, 51.5%, 39.7%, 57.6%, 23.5%, 26.5%, 39.7%, 14.7% and 17.6%, respectively. The rates of *C. sputigena*, *C. gingivalis*, *C. ochracea*, *C. rectus*, *F. nucleatum/F. periodonticum*, *P. nigrescens*, *A. actinomycetencomitans*, *P. intermedia*, *T. denticola*, *T. forsythensis* and *P. gingivalis* detected in children and their mothers simultaneously were 55.1%, 43.6%, 26.9%, 33.3%, 16.7%, 26.9%, 3.8%, 9.0%, 15.4%, 2.6% and 2.6%, respectively.

Consistency of detection in families

Kappa statistic analysis revealed that the detection of *C. gingivalis* ($\kappa = 0.43$), *C. ochracea* ($\kappa = 0.51$), *C. rectus* ($\kappa = 0.50$) and *T. denticola* ($\kappa = 0.46$) in children was highly consistent with that in their mothers (Table 3). It also showed an extremely high consistency of detection, or nondetection, for *P. gingivalis* ($\kappa = 1$), *T. denticola* ($\kappa = 1$), *P. intermedia* ($\kappa = 0.88$) and *P. nigrescens* ($\kappa = 1$) in siblings (Table 4).

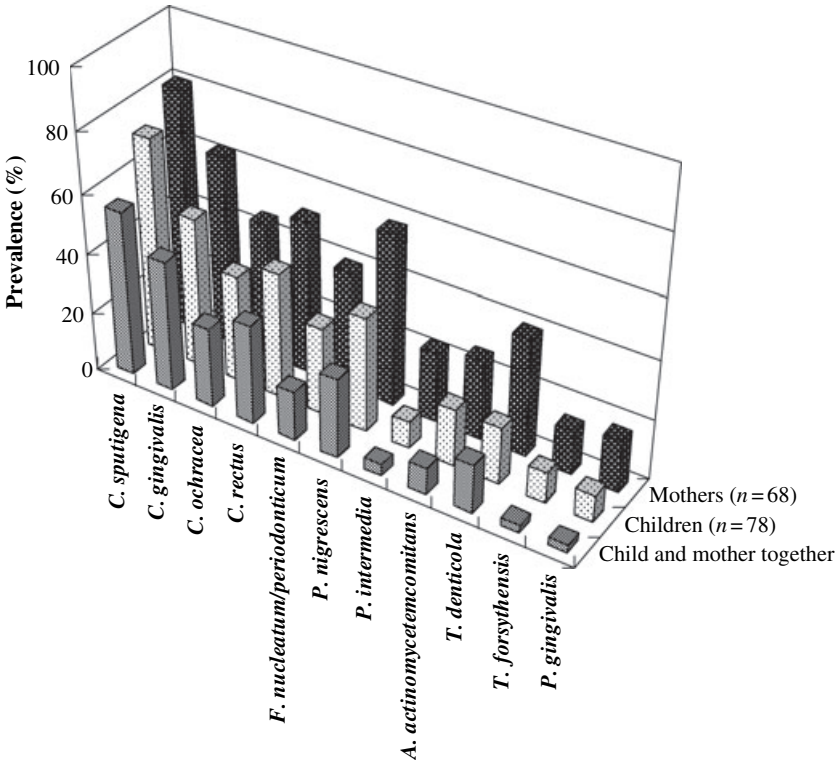

Fig. 2. Detection rates of 11 periodontal bacteria in subgingival plaque samples from 78 children, 68 mothers and child and mother together. n = number of children and mothers examined. *A. actinomycetemcomitans*, *Actinobacillus actinomycetemcomitans*; *C. gingivalis*, *Capnocytophaga gingivalis*; *C. ochracea*, *Capnocytophaga ochracea*; *C. rectus*, *Campylobacter rectus*; *C. sputigena*, *Capnocytophaga sputigena*; *F. nucleatum/periodonticum*, *Fusobacterium nucleatum/periodonticum*; *P. gingivalis*, *Porphyromonas gingivalis*; *P. intermedia*, *Prevotella intermedia*; *P. nigrescens*, *Prevotella nigrescens*; *T. denticola*, *Treponema denticola*; *T. forsythensis*, *Tannerella forsythensis*.

Table 3. Kappa statistic of detection of 11 periodontal bacterial species analyzed in 78 children and 68 mothers

Bacterial species	Kappa value
Capnocytophaga sputigena	0.003
Capnocytophaga gingivalis	0.43
Capnocytophaga ochracea	0.51
Campylobacter rectus	0.50
F. nucleatum/periodonticum	0.23
Prevotella nigrescens	0.18
Prevotella intermedia	0.14
Actinobacillus actinomycetemcomitans	0.27
Treponema denticola	0.46
Tannerella forsythensis	0.17
Porphyromonas gingivalis	0.13

Discussion

Among the 11 periodontal bacteria targeted, the detection rates of *C. sputigena*, *C. gingivalis* and *P. nigrescens* were higher than those of the red complex in the dental plaque samples from the children. *C. sputigena*, *C. ochracea* and *C. gingivalis* have been reported to be highly prevalent in Japanese children (11,14). In the present study, *C. rectus* was detected in 42.3% of Japanese children. Hayashi *et al.* (26) reported that of 10 children aged 4–6 years with complete primary dentition, all were positive for *C. rectus*, and that the rate of positive sites was 17.6 ± 2.4%. The high detection

Table 4. Kappa statistic of detection of 11 periodontal bacterial species analyzed in 10 siblings

Bacterial species	Kappa value
Capnocytophaga sputigena	0.36
Capnocytophaga gingivalis	0.20
Capnocytophaga ochracea	0.40
Campylobacter rectus	0.20
F. nucleatum/periodonticum	0.35
Prevotella nigrescens	1
Prevotella intermedia	0.88
Actinobacillus actinomycetemcomitans	0.35
Treponema denticola	1
Tannerella forsythensis	0.11
Porphyromonas gingivalis	1

rate of *C. rectus* in the present study agrees with the result of Hayashi *et al.* (26). In the present study, *P. nigrescens* was detected in 38.5% of the children. Umeda *et al.* (21) reported a similar detection rate (42.9%). Taken together, these results suggest that the three Capnocytophaga species, *C. rectus* and *P. nigrescens* became established at an early age in Japanese children.

We detected *P. gingivalis*, *T. forsythensis* and *T. denticola* in 9.0, 10.2 and 19.2%, respectively. These species are known as the red complex, and are believed to be intimately associated with chronic periodontitis (27). McClellan *et al.* (28) detected *P. gingivalis* in 37.0% of 198 0–18-year-old subjects in Ohio State, USA. Umeda *et al.* (21) reported the detection rate of the three species to be 8.9–48.2% in children. On the other hand, Kimura *et al.* (29) reported no detection of *P. gingivalis* or *T. denticola* in dental plaque samples from 144 Japanese children with negligible periodontal inflammation. The detection rates of these species in the present study were within a similar range to that found in previous studies.

The detection rate of *A. actinomycetemcomitans* was 0.9% in 78 children with no definite gingivitis in this study. Okada *et al.* (30) reported that the percentages of *A. actinomycetemcomitans* in healthy, gingivitis and periodontitis groups in 2–12-year-old Japanese children were 4.8, 6.8 and 20.0%, respectively. On the other hand, Kimura *et al.* (29) reported that *A. actinomycetemcomitans* was found in $\approx$50% of dental plaque samples collected with a Gracy currette from all age groups in 2–13-year-old children. *A. actinomycetemcomitans* has several pathogenic factors, such as fimbriae and leukotokin (31–33). These factors play an important role in colonization by these species. It is possible that the difference in detection rates between this and the study of Kimura *et al.* (29) are a result of genetic variation in these factors or differences in the method of dental plaque sampling.

We found that the detection rates of the red complex periodontal pathogens were higher after eruption of the permanent teeth, and that, among them, detection of *P. gingivalis* was significant ($p < 0.05$). We detected all three species of the red complex in the dental plaque sample from one child, and detected *T. forsythensis and T. denticola* in another 8-year-old child. The detection rates of these species, especially those of *P. gingivalis and T. forsythensis*, increased in children after the age of 7 years. The present results suggest that the eruption of a permanent tooth is involved in colonization by these species.

The detection rate of *F. mucleatum/periodonticum* was 32.1% in all 78 children, and the average number of periodontal bacteria species in subjects with these species was significantly higher than that in those without. Bradshaw *et al.* (34), Foster & Kolenblander (35) and Edwards *et al.* (36) showed that the presence of *F. nucleatum* in dental plaque played a significant role in biofilm formation by co-aggregation with other periodontal bacteria. Growth support of *P. gingivalis* by *F. nucleatum* under oxygenated and CO_2-depleted environments was reported (37). It is possible that colonization by *F. nucleatum/periodonticum* triggers periodontal bacterial colonization.

Detection rate profiles were similar between mother and child. Kappa statistical analysis revealed that the detection of two of the Capnocytophaga species and *T. denticola* in children was consistent with detection in the mother. Notably, the detection rate of *T. denticola* in both mother and child was only 16.4%. In addition, highly consistent detection of *P. gingivalis*, *T. denticola*, *P. intermedia* and *P. nigrescens* was found in the 10 sibling children, although the overall detection rate of these microorganisms in children was less then 19.2%. The similarity in detection rate profiles between mother and child, and the consistency in detection of the species with a low detection rate between family members, suggests intrafamilial transmission of periodontopathic bacteria. Many research groups, including our department, have demonstrated intrafamilial transmission of periodontal bacteria (18–20,38–40). Tanner *et al.* (22) reported that there were significant positive associations in species detection between caregiver and child. Lee *et al.* (41) investigated the transmission of red complex species by the benzoyl-arginine naphthylamide test and reported that, if the caregiver was benzoyl-arginine naphthylamide positive, the odds of the child also being benzoyl-arginine naphthylamide positive were 35 times higher than for a child with a benzoyl-arginine naphthylamide-negative caregiver, after adjustment for the child's age and papillary bleeding score. In an earlier study, *P. gingivalis* infection from the spouse was observed in 6 out of 16 couples (19). Taken together, our results and those of these earlier reports suggest that intrafamilial transmission of periodontopathic bacteria is an important factor in the organization of periodontopathic dental plaque biofilm. To clarify this process, further study on the intrafamilial transmission of these periodontal bacteria using pulsed field electrophoresis is required.

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