Detection of Two Anaerobic Periodontopathogens in Children by Means of the BANA and ELISA Assays

M.-R. WATSON[1], D.E. LOPATIN, W.A. BRETZ, I.J. ERTEL, and W.J. LOESCHE[2]

Department of Biologic and Materials Sciences, The University of Michigan School of Dentistry, 1011 North University, Ann Arbor, MI 48109

The mouths of young children become colonized by a variety of bacteria, but there have been only a few studies that have sought the presence of periodontopathic species in this population. Almost all of these studies used culturing techniques rather than the newer detection methodologies for various periodontopathogens.

Studies in adults have shown that *Treponema denticola* and *Porphyromonas (Bacteroides) gingivalis* can be detected in dental plaque by use of the BANA and ELISA diagnostic tests. In the present study, plaque samples from four subgingival sites in each of 157 children (aged from two to 18 years) were tested for BANA hydrolysis with a BANA reagent card, and for *T. denticola* and *P. gingivalis* with an ELISA assay. Anaerobic periodontopathogens hydrolyzing the BANA substrate were found to be present in at least one of four plaque samples in 88 children (56%). *T. denticola* and/or *P. gingivalis* were detected by ELISA in at least one plaque sample in each of 135 children (86%). This study shows that children are widely colonized by these micro-organisms. A higher proportion of Black children than Caucasian children was colonized by these BANA-positive organisms. Also, children having a parent with a documented history of periodontal disease were more likely to be BANA-positive than were children of parents with unknown periodontal status.

J Dent Res 70(7):1052–1056, July, 1991

Introduction.

The gingival crevice of young children becomes colonized by a variety of bacteria (de Araujo and MacDonald, 1964; Frisken *et al.*, 1987), including spirochetes (Mikx *et al.*, 1986), but there have been few studies of the bacterial composition of the subgingival plaque in this population. Furthermore, the age at which periodontopathic organisms associated with periodontitis first colonize the subgingival environment is not known (Wojcicki *et al.*, 1987).

In recent years, considerable data have become available that suggest that periodontal disease may be related either to the presence or to the overgrowth of one or more bacterial types in subgingival plaque (Loesche, 1987). Several studies have been designed with the purpose of correlating the profiles of healthy and diseased periodontal sites with subgingival microbes. From these studies, there is evidence that spirochetes, *Porphyromonas (Bacteroides) gingivalis, Bacteroides forsythus, Prevotella (Bacteroides) intermedius*, and *Actinobacillus actinomycetemcomitans* are strongly associated with gingivitis and/or periodontitis (Loesche *et al.*, 1985; Slots *et al.*, 1985; Moore, 1987; Dzink *et al.*, 1988).

An enzyme that mimics the activity of trypsin in its ability to hydrolyze the synthetic substrate N-benzoyl-DL-arginine-2-naphthylamide (BANA) is possessed by *Treponema denticola*, *P. gingivalis*, and *B. forsythus*, and not by 51 other bacterial species that can be isolated from dental plaque (Loesche *et al.*, 1990a). This finding indicates that the BANA test, since it measures enzyme activity presumably possessed only by these organisms, may be useful for the detection of anaerobic infections associated with one or more of these organisms (Loesche, 1986). The BANA assay has been tested with subgingival plaque of adult individuals, and it has been found to be a suitable test for the detection of *T. denticola* and *P. gingivalis* (Bretz *et al.*, 1990; Loesche *et al.*, 1990a,b).

Antibodies to *T. denticola* and *P. gingivalis* can detect these organisms by an enzyme-linked immunosorbent assay (ELISA) (Loesche *et al.*, 1990a) and could be useful for studies of the correlation between the frequency and the levels of both *T. denticola* and *P. gingivalis* in subgingival plaque samples and different clinical signs (Bretz *et al.*, 1990).

The aim of this study was to determine, by means of the ELISA assay, whether the periodontopathic organisms *T. denticola* and *P. gingivalis* could be found in the subgingival plaque of children, and to correlate the presence of these organisms with other parameters—such as BANA hydrolysis by the plaque, ethnic origin, gender, age, papillary bleeding scores, and plaque index. In particular, we wanted to determine whether BANA hydrolysis by the plaque would provide comparable information on the presence of, or the levels of, the BANA-positive species *T. denticola* and *P. gingivalis*, as could be attained by the immunological assay that used antibodies highly specific for *T. denticola* and *P. gingivalis* (Bretz *et al.*, 1990).

Materials and methods.

Subjects.—The 157 child subjects (from two to 18 years old) were seen at the University of Michigan Pediatric Dentistry Clinic (n = 57); at the University Hospital Pediatric Continuity Clinic (n = 55); or at the Thurston Elementary School, Willow Run Community School District, Michigan (n = 45). All children examined were in good general health.

Clinical and demographic data.—For each child, the accompanying parent or guardian was asked to supply information on his/her child, such as birth date, gender, race, and child's status regarding the usage of antibiotics (frequency and current consumption in order to withdraw those who had received antibiotic therapy during the last six months). The parents were also asked to give their full names and were questioned about their own dental conditions in order to determine: (1) whether they were regular patients of the University Dental Clinic and/or the Department of Periodontics (in order to check their records); (2) whether they had ever before been diagnosed (by means of periodontal probing) as having periodontal disease or 'gum' disease; and (3) whether they had had periodontal surgery in the past.

The child was given a dental examination in which the dental

Received for publication November 21, 1989
Accepted for publication March 21, 1991

[1]Based on a thesis submitted as partial fulfillment of the requirements for a Master's of Science in Pediatric Dentistry, The University of Michigan School of Dentistry

[2]To whom correspondence and reprint requests should be addressed

This study was partially funded by Oral-B Laboratories, Redwood City, CA.

development status (primary, mixed, or permanent dentition) and the def/DMF scores were determined. Additional measurements were taken in the following order: Plaque Index (PI), collection of subgingival plaque samples, and Papillary Bleeding Scores (PBS). The PI (Silness and Löe, 1964) was used to ascertain the plaque score at the sites to be sampled. The PBS (Loesche, 1979) was used for diagnosis of gingivitis and is based on the bleeding of papillary sites when a Stimudent™ is inserted interproximally. The PBS expands the GI = 2 score (Silness and Löe, 1964) into three easily recognized clinical conditions, thereby increasing the sensitivity of the clinical observation concerned with gingival bleeding (Loesche, 1979).

Collection of plaque samples.—Dental floss was used to obtain the subgingival plaque samples. First, the supragingival plaque was removed from the site with dental floss. Following this, new dental floss was introduced sub-gingivally between the first and second primary molars of each quadrant (for the deciduous dentitions); between the second primary molar and the first permanent molar (for the mixed dentitions); and between the first permanent molar and the second premolar (for the late-mixed or permanent dentitions).

BANA assay.—The BANA reagent card (PerioScan™, Oral-B Laboratories, Redwood City, CA) was chosen because results obtained with this chair-side test were similar to those obtained with the liquid BANA test (Loesche *et al.*, 1990a). The card was easy to use in the field and gave test results in 15 min, as compared with the overnight incubation that was used with the liquid BANA test. The portion of dental floss with the plaque was placed onto the strip impregnated with BANA located at the bottom of the reagent card (PerioScan™). The reaction was immediately activated by application of water to the upper strip containing the fast black dye, and then both strips were brought together when the testing card was folded. A metallic clip was positioned to ensure proximity of both strips, and the card was then placed in an incubator at 55°C for 15 min. Positive results appeared as blue spots that indicated weak or strong BANA reactions, whereas negative results did not show any color change (Loesche *et al.*, 1990b).

ELISA assay.—In this method, the lower strip of the BANA reagent cards, which had been inoculated with the plaque samples, was cut horizontally into two pieces, so that one portion could be assayed for *T. denticola* and the other for *P. gingivalis*, with highly specific polyclonal antisera that can detect approximately 10^4 to 10^5 colony-forming units (cfu) of either *T. denticola* or *P. gingivalis* (Bretz *et al.*, 1990).

The cut lower strips were then blocked in 5% blotto (nonfat powdered milk) in 100 mL Tris-buffered saline (TBS) [0.05 molar Tris base + 0.2 molar sodium chloride] for 30 to 60 min and rinsed in TBS-Tween (TBS-T) [TBS + 0.05% Tween-20 detergent]. Cards were incubated with the appropriate specific hyperimmune rabbit antibody at 1:1000 dilution in 2% blotto in 100 mL of TBS-T for 60 min and then rinsed with TBS-T. Antibodies that bound to the plaque were stained for 60 min with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin-G diluted 1:000 in TBS-T containing 2% blotto. Cards were then rinsed with TBS-T followed by a final rinse with TBS. A developer solution containing Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyo phosphate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was added to TBS in a 1:1:10 ratio (100 mL), and development progressed until the background color became evident. The development was stopped by pouring off the developer solution and rinsing the cards three to four times with distilled water. Positive results appeared dark blue, whereas negative results did not show any color change.

Evaluation of parents' dental records.—Records of parents were searched for at the University of Michigan Dental Clinics, and when available, probing depths, periodontal diagnosis, as well as any history of periodontal surgery were recorded. The available records indicated that 20 of the parents had been treated in the Periodontal Clinic for moderate to advanced periodontitis. These 20 parents were entered into a group categorized as having a documented history of periodontal disease. The remaining parents were entered into a group categorized as having an unknown periodontal status.

Statistical analysis.—Site-specific analysis was performed for determination of the within-patient dependency of sites (Hujoel *et al.*, 1990). A beta binomial ANOVA model (SAS, SAS Institute, Inc., NC) was used to correlate the within-patient frequency of positive BANA or positive ELISA reactions with the frequency of periodontal disease in the 20 parents found to have a history of periodontal disease (generalized linear interacting model: GLIM). The results of the BANA test with the presence of *T. denticola* and *P. gingivalis*, as detected by the ELISA assay, were correlated by Cochran-Mantel-Haenzel Chi-square Statistics.

Results.

Fig. 1 shows the frequency distribution of the children by age and gender. The largest group of children was in the age range of four to six years (n = 83; *i.e.*, 53%). There was a similar number of males and females in the sample. Gender had no effect on the detection of the BANA-positive organisms. In the subsequent analysis, males and females were treated as a single population of children.

BANA results.—Fifty-six percent of the 157 children tested positive and/or weak-positive for the presence of BANA-positive organisms in one or more of their four plaque samples, while 44% of the children tested negative for all four plaque samples examined. Only 13% of the children had at least one sample with a strong BANA reaction. Twenty-one percent of the 620 plaque samples exhibited a BANA-positive or weak-positive reaction (Table).

Caucasians, Blacks, Asians, Indians/Arabs, and Hispanics accounted for 66, 20, 6, 5, and 3% of the population, respectively. Fifty-five percent of the Blacks were BANA-positive, compared with 37% of the Caucasian population. The higher numbers of BANA-positive reactions in the Blacks, compared with the Caucasian population, were statistically significant (Chi-square = 11.37; p value ≤ 0.02). The number of Blacks

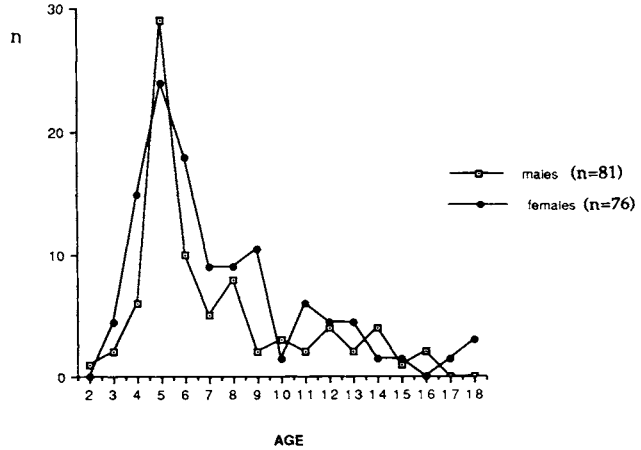

Fig.—Frequency distribution of children by age and gender (n = 157).

TABLE
FREQUENCY DISTRIBUTION OF POSITIVE REACTIONS TO THE BANA AND ELISA ASSAYS

By Subjects (n = 157)	BANA	ELISA
Children presenting positive reactions in one or more plaque samples[a]	88 (56%)[b]	135 (86%)[b]
Strong reactions to BANA	21 (13%)	NA[c]
ELISA reactions to *P. gingivalis* (alone)	NA	126 (80%)
ELISA reactions to *T. denticola* (alone)	NA	124 (79%)
ELISA reactions to *P. gingivalis* and *T. denticola*	NA	115 (73%)
By Plaque Samples (n = 620)		
Plaque presenting positive reactions	130 (21%)[d]	465 (75%)

[a]Including weak-positive and strong-positive reactions to BANA.
[b]Percentage of children showing at least one positive reaction.
[c]Not applicable.
[d]Percentage of plaque.

in this study was less than 1/3 of the Caucasian population sampled, which makes the fact that significance was observed even more impressive. There was no correlation between BANA scores and caries status, PI, or PBS of the subjects. Neither was there a statistical correlation between the presence of dental plaque and papillary bleeding when analyzed by sites.

Forty percent of the children from the 20 parents having a documented history of periodontal disease (as determined by evaluation of their dental records) had one or more BANA-positive plaques. Only 16% of the children of parents whose periodontal status was not documented had one or more BANA-positive plaques. This difference was statistically significant when a beta binomial model was used (Chi-square = 15.30; p value $\leq$ 0.001).

ELISA results.—About 75% of the 620 plaque samples contained *P. gingivalis* and/or *T. denticola*, as detected by the ELISA assay. *T. denticola* was present in 124 children (79%) and *P. gingivalis* was present in 126 children (80%). *T. denticola* and *P. gingivalis* together were found in 115 children (73%), whereas 86% of the children had either *T. denticola* or *P. gingivalis* (Table). When the ELISA results for *T. denticola* and/or *P. gingivalis* in the children were correlated with the history of documented periodontal disease in their parents, by means of a beta binomial model, no significant association was observed (Chi-square = 2.49; p value = 0.14).

BANA and ELISA results.—The correlation between the BANA reaction and the ELISA reaction for *T. denticola* was highly significant (Cochran-Mantel-Haenzel Chi-square Statistics = 19.30; p value $\leq$ 0.01). The same degree of correlation was also obtained for *P. gingivalis* (Cochran-Mantel-Haenzel Chi-square Statistics = 19.90; p value $\leq$ 0.01) and when *T. denticola* and *P. gingivalis* were combined (Cochran-Mantel-Haenzel Chi-square Statistics = 27.60; p value $\leq$ 0.01).

When the ELISA test was used as the reference standard, then the specificity of the BANA test relative to the ELISA test was high (92%), *i.e.*, the BANA reaction was negative when *T. denticola* and/or *P. gingivalis* were not detected by the immunological reagents. However, the sensitivity of the BANA test relative to the ELISA test was low (25%), *i.e.*, when the ELISA was detecting *T. denticola* and/or *P. gingivalis*, the BANA reagent card was often negative. The same was true when *T. denticola* and *P. gingivalis* were analyzed independently.

Discussion.

Initially, it was suspected that BANA reactions in the children's plaque samples would be mainly negative, since periodontopathogens such as *P. gingivalis, T. denticola*, and *B. forsythus* have rarely been reported to be present in children's mouths (Van Oosten *et al.*, 1988). Most of the microbiological studies in children prior to 1980 mentioned the presence of black-pigmented bacteroides (BPB) species that were classified as *Bacteroides melaninogenicus* (de Araujo and MacDonald, 1964; Bailit *et al.*, 1964; Mackler and Crawford, 1973). Black-pigmented anaerobic rods now include two new genera—*Porphyromonas* and *Prevotella* (Shah and Collins, 1988, 1990)—that can be isolated from humans; therefore, it is difficult to know which of these species were studied by previous investigators (Mayrand and Holt, 1988).

Subsequent studies have reported the presence of *P. intermedius* and *P. melaninogenicus* in plaque removed from young individuals (Delaney *et al.*, 1986; Yanover and Ellen, 1986; Sweeney *et al.*, 1987; Wojcicki *et al.*, 1987; Van Oosten *et al.*, 1988). Several researchers reported *P. gingivalis* among their findings (Frisken *et al.*, 1987; Sweeney *et al.*, 1987; Robert and Mouton, 1989). Spirochetes are very difficult to isolate by culturing methods (Cheng and Chan, 1983; Salvador *et al.*, 1987), but microscopic examination of plaque has shown them to be present in children's mouths (de Araujo and MacDonald, 1964; Mackler and Crawford, 1973; Moore *et al.*, 1984; Delaney *et al.*, 1986; Mikx *et al.*, 1986; Ashley *et al.*, 1988; Loesche, 1988; Van Oosten *et al.*, 1988). Other putative periodontopathogens found in young children are fusobacteria (Sweeney *et al.*, 1987) and *A. actinomycetemcomitans* (Delaney *et al.*, 1986; Sweeney *et al.*, 1987).

The data of this study indicate that 56% of the children had at least one BANA-positive plaque, indicating the presence of one or more of the BANA-positive species, *P. gingivalis*, *T. denticola*, and *B. forsythus*. The ELISA results indicated that 86% of the children were positive for either *P. gingivalis* or *T. denticola*. This is a much higher frequency of occurrence than previously reported by the cultural or microscopic studies cited above.

Recent studies correlating the results of DNA probe tests for *P. gingivalis* with culture data for *P. gingivalis* (Savitt *et al.*, 1988; Maiden *et al.*, 1989) show sensitivities of about 30 to 40%. This means that the DNA probes are detecting the presence of *P. gingivalis* in plaque more frequently than is the culturing approach and suggests that culturing may be greatly underestimating the true presence of *P. gingivalis* in plaque. The same conclusion has been reached from immunological approaches (Robert and Mouton, 1989; Loesche *et al.*, 1990b). Thus, the higher prevalence of *P. gingivalis* in these periodontally healthy children as shown by our immunological reagents, *i.e.*, 86%, may be more accurate than the collective data shown by the cultural studies. The fact that spirochetes are very difficult to cultivate—or, if present in low numbers, will not be detected by microscopic examinations—may account for the inability of previous investigations to detect them

as routinely as we did. In this study, *T. denticola* was detected by the ELISA assay in 79% of these periodontally healthy children.

It is not established when periodontopathic bacteria colonize the human mouth, and what or who were the sources of these bacteria (Wojcicki *et al.*, 1987). This study indicated that children who presented with a parent with documented evidence of periodontal disease had significantly more positive BANA reactions than children whose parents' periodontal status was not known. Blacks were colonized significantly more frequently than were Caucasians. This is consistent with the report of Mikx *et al.* (1986), but they were comparing African children with Dutch children, whereas we were comparing children living in the same community.

The discrepancy between the detection of *T. denticola* and/or *P. gingivalis* by the ELISA antibodies, and the detection of the organisms as BANA-positive species in the PerioScan™ card, may be explained by the different detection limits of these modalities (Loesche and Hujoel, 1991). The ELISA will detect about 10^4 cells of *P. gingivalis* and *T. denticola*, whereas the PerioScan™ card will detect, on the average, about 2×10^5 cells of *P. gingivalis* (ATCC strain 33277) and 8×10^5 cells of *T. denticola* (ATCC strain 35405) (Loesche *et al.*, 1990a). A plaque sample containing either *P. gingivalis* or *T. denticola* in numbers less than 10^5 cells will be positive by the ELISA reaction, but negative by the BANA. There is also the possibility that the immunological reagents are interacting with other species and are giving rise to false-positive reactions. In this regard, the antibodies used did not cross-react with related species of BPB and spirochetes (Bretz *et al.*, 1990).

In the present study, 21% of the plaque samples were BANA-positive, whereas 75% were ELISA-positive. This would suggest that these children were almost universally colonized by *P. gingivalis* and/or *T. denticola*, but that fewer children presented with the higher levels of these organisms that could be detected by the BANA reaction. The question of which is more important—the fact of being colonized (as detected by ELISA), or that a certain threshold of bacteria be present (such as indicated by the BANA reaction)—needs to be answered. The ability of BANA to detect certain anaerobic bacteria if they are at higher concentrations in the plaque sample may be more useful than sensitive tests that detect low levels of the organisms in the plaque. This may explain the fact that, in this study, BANA reactions were significantly correlated with a documented history of periodontal disease in the children's parents, whereas the ELISA reactions were not. These results, which suggest that parents with periodontal disease may serve as a reservoir of periodontopathic organisms for their children, offer a subject that deserves further investigation.

The BANA and ELISA assays utilized in this investigation may offer a suitable means for screening for the presence of putative periodontal pathogens *T. denticola* and *P. gingivalis* in dental plaque in a manner that can be used in epidemiological surveys. Furthermore, the ability of BANA to detect a particular threshold of anaerobic periodontopathic bacteria may be a valuable tool for the screening of populations to detect individuals at risk for anaerobic infection. However, it should be noted that the population sampled was a "convenient sample", and the results obtained may not apply to studies in which random sampling is used to obtain a representative sample.

Acknowledgments.

Our sincere gratitude to Philippe Hujoel for the extensive statistical help, to Neal Van Poperin for his assistance when processing the immunological analysis, and to Carol Gerlach for the preparation of the manuscript.

REFERENCES

ASHLEY, F.P.; GALLAGHER, J.; and WILSON, R.F. (1988): The Occurrence of *Bacteroides gingivalis, Bacteroides intermedius*, and Spirochetes in the Subgingival Flora of Adolescents and Their Relationship with the Amount of Supragingival Plaque and Gingivitis, *Oral Microbiol Immunol* 3:77–82.

BAILIT, H.L.; BALDWIN, D.C.; and HUNT, E.E., Jr. (1964): The Increasing Prevalence of Gingival *Bacteroides melaninogenicus* with Age in Children, *Arch Oral Biol* 9:435–438.

BRETZ, W.A.; LOPATIN, D.E.; and LOESCHE, W.J. (1990): Benzoyl-Arginine Naphthylamide (BANA) Hydrolysis by *Treponema denticola* and/or *Bacteroides gingivalis* in Periodontal Plaques, *Oral Microbiol Immunol* 5:275–279.

CHENG, S.L. and CHAN, E.C.S. (1983): The Routine Isolation, Growth and Maintenance of the Intermediate-size Anaerobic Oral Spirochete from Periodontal Pockets, *J Periodont Res* 18:362–368.

DE ARAUJO, W.C. and MacDONALD, J.B. (1964): The Gingival Crevice Microbiota in Five Preschool Children, *Arch Oral Biol* 9:227–228.

DELANEY, J.E.; RATZAN, S.K.; and KORNMAN, K.S. (1986): Subgingival Microbiota Associated with Puberty; Studies of Pre-, Circum- and Postpubertal Human Females, *Pediatr Dent* 8:268–275.

DZINK, J.L.; SOCRANSKY, S.S.; and HAFFAJEE, A.D. (1988): The Predominant Cultivable Microbiota of Active and Inactive Lesions, *J Clin Periodontol* 15:316–323.

FRISKEN, K.W.; TAGG, J.R.; LAWS, A.J.; and ORR, M.B. (1987): Suspected Periodontopathic Microorganisms and Their Oral Habitats in Young Children, *Oral Microbiol Immunol* 2:60–64.

HUJOEL, P.P.; LOESCHE, W.J.; and MOULTON, L. (1990): Estimation of Sensitivity and Specificity of Site-specific Diagnostic Tests, *J Periodont Res* 25:193–196.

LOESCHE, W.J. (1979): Clinical and Microbiological Aspects of Chemotherapeutic Agents Used According to the Specific Plaque Hypothesis, *J Dent Res* 38:2404–2412.

LOESCHE, W.J. (1986): The Identification of Bacteria Associated with Periodontal Disease and Dental Caries by Enzymatic Methods, *Oral Microbiol Immunol* 1:65–70.

LOESCHE, W.J. (1987): The Bacterial Etiology of Periodontal Disease: The Specific Plaque Hypothesis. In: **Clinical Dentistry**, J. W. Clark, Ed., Hagerstown, MD: Harper & Row Publishers, Ch. 12, pp. 1–11.

LOESCHE, W.J. (1988): The Spirochetes. In: **Oral Microbiology and Immunology**, M.G. Newman, Ed., Philadelphia: The Saunders Co., pp. 263–271.

LOESCHE, W.J.; BRETZ, W.A.; KERSCHENSTEINER, D.; STOLL, J.; SOCRANSKY, S.S.; HUJOEL, P.P.; and LOPATIN, D.E. (1990a): Development of a Diagnostic Test for Anaerobic Periodontal Infections Based on Plaque Hydrolysis of Benzoyl-DL-Arginine-Naphthylamide, *J Clin Microbiol* 28:1551–1559.

LOESCHE, W.J.; BRETZ, W.A.; LOPATIN, D.E.; STOLL, J.; RAU, C.F.; HILLENBURG, K.C.; KILLOY, W.J.; DRISKO, C.L.; WILLIAMS, R.; WEBER, H.P.; CLARK, W.; MAGNUSSON, I.; WALKER, C.; and HUJOEL, P.P. (1990b): Multi-Center Clinical Evaluation of a Chairside Method for Detecting Certain Periodontopathic Bacteria in Periodontal Disease, *J Periodontol* 61:189–196.

LOESCHE, W.J. and HUJOEL, P.P. (1991): Microbiological-based Diagnostic Tests for Periodontitis. Considerations in Regard to Sensitivity, Specificity and Accuracy. In: **Markers of Disease Susceptibility and Activity for Periodontal Diseases**, Royal Society of Medicine Symposium, London: Cambridge University Press, pp. 417–440.

LOESCHE, W.J.; SYED, S.A.; SCHMIDT, E.; and MORRISON, E.C. (1985): Bacterial Profiles of Subgingival Plaques in Periodontitis, *J Periodontol* 56:447–456.

MACKLER, S.B. and CRAWFORD, J.J. (1973): Plaque Develop-

ment and Gingivitis in the Primary Dentition, *J Periodontol* 44:18–24.

MAIDEN, M.; TANNER, A.; McARDLE, S.; NAJPAVER, K.; and GOODSON, J.M. (1989): Multicenter Evaluation of Tetracycline Fiber Therapy. IV. DNA Probe and Cultural Assay of Microorganisms, *J Dent Res* 68:241, Abst. No. 479.

MAYRAND, D. and HOLT, S.C. (1988): Biology of Asaccharolytic Black-pigmented Bacteroides Species, *Microbiol Rev* 52:134–152.

MIKX, F.H.M.; MATEE, M.I.; and SCHAEKEN, M.J.M. (1986): The Prevalence of Spirochetes in the Subgingival Microbiota of Tanzanian and Dutch Children, *J Clin Periodontol* 13:289–293.

MOORE, W.E.C. (1987): Microbiology of Periodontal Diseases, *J Periodont Res* 22:335–341.

MOORE, W.E.C.; HOLDEMAN, L.V.; SMIBERT, R.M.; CATO, E.P.; BURMEISTER, J.A.; PALCANIS, K.G.; and RANNEY, R.R. (1984): Bacteriology of Experimental Gingivitis in Children, *Infect Immun* 46:1–6.

ROBERT, J.C. and MOUTON, C. (1989): *Bacteroides gingivalis* and other Black-pigmented Bacteroides in Children, *J Dent Res* 68:987, Abst. No. 961.

SALVADOR, S.G.; SYED, S.A.; and LOESCHE, W.J. (1987): Comparison of Three Dispersion Procedures for Quantitative Recovery of Cultivable Species of Subgingival Spirochetes, *J Clin Microbiol* 25:2230–2232.

SAVITT, E.D.; STRZEMPO, M.N.; VACCARO, K.K.; PEROS, W.J.; and FRENCH, C.K. (1988): Comparison of Cultural Methods and DNA Probe Analyses for the Detection of *Actinobacillus actinomycetemcomitans, Bacteroides gingivalis*, and *Bacteroides intermedius* in Subgingival Plaque Samples, *J Periodontol* 59:431–438.

SHAH, H.N. and COLLINS, M.D. (1988): Proposal for Reclassification of *Bacteroides asaccharolyticus, Bacteroides gingivalis* and *Bacteroides endodontalis* in a New Genus, *Porphyromonas, Int J System Bacteriol* 38:128–131.

SHAH, H.N. and COLLINS, M.D. (1990): *Prevotella*, a New Genus to Include *Bacteroides melaninogenicus* and Related Species Formerly Classified in the Genus *Bacteroides, Int J System Bacteriol* 40:205–208.

SILNESS, J. and LÖE, H. (1964): Periodontal Disease in Pregnancy, II. Correlation Between Oral Hygiene and Periodontal Condition, *Acta Odontol Scand* 22:121–135.

SLOTS, J.; HAFSTROM, C.; ROSLING, B.; and DAHLÉN, G. (1985): Detection of *Actinobacillus actinomycetemcomitans* and *Bacteroides gingivalis* in Subgingival Smears by the Indirect Fluorescent-Antibody Technique, *J Periodont Res* 20:613–620.

SWEENEY, E.A.; ALCOFORADO, G.A.P.; NYMAN, S.; and SLOTS, J. (1987): Prevalence and Microbiology of Localized Prepubertal Periodontitis, *Oral Microbiol Immunol* 2:65–70.

VAN OOSTEN, M.A.C.; MOMBELLI, A.; GUSBERTI, F.A.; and LANG, N.P. (1988): Black-pigmented Bacteroides and Spirochetes in the Subgingival Microbiota of Prepubertal Schoolchildren, *J Periodont Res* 23:199–203.

WOJCICKI, C.J.; HARPER, D.S.; and ROBINSON, P.J. (1987): Differences in Periodontal Disease-associated Microorganisms of Subgingival Plaque in Prepubertal, Pubertal and Postpubertal Children, *J Periodontol* 4:219–223.

YANOVER, L. and ELLEN, R.P. (1986): A Clinical and Microbiologic Examination of Gingival Disease in Parapubescent Females, *J Periodontol* 57:562–567.