

# The identification of bacteria associated with periodontal disease and dental caries by enzymatic methods

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This brief review describes the potential usefulness of enzymatic reagents for the diagnosis of dental caries and periodontal disease. The cultural tests for salivary *S. mutans* levels should soon become commercially available in the U.S. and their success may preclude the immediate development of enzymatic diagnostic reagents for this group of organisms. However, in regards to periodontal disease, these enzyme reagents offer great promise, particularly those which measure the trypsin-like activity of plaque. The BANA hydrolytic activity of plaque, while not due exclusively to the spirochetes, nevertheless seems to reflect the spirochetal load of the plaque. Of even greater interest is the possibility that this and other enzymes can also be measured in the saliva. This would lend itself to routine screening for these enzymes in the saliva.

## Introduction

The cost efficiency of any antibacterial treatment in dentistry, be it preventive or therapeutic, increases to the extent that it can be focused upon those populations or individuals who are at actual risk of decay or periodontal disease. In the past, and in many places in the present, all children were considered at risk of decay, and all adults were considered at risk of periodontal disease, so that the need to diagnose an infection was moot. Now with the demonstration of *Streptococcus mutans* and lactobacilli as the prime etiologic agents in human dental decay (15), and spirochetes (13, 17, 19), *B. gingivalis* (19, 26), *H. actinomycetemcomitans* (26, 36) and others (21, 31, 33) as prime etiologic agents in periodontal disease, it is possible to identify those individuals with an odontopathic infection, and treat them either prospectively so as to prevent dental diseases, or therapeutically so as to cure these infections (14).

Approximately 200 to 300 bacterial species colonize human dental plaques

(22, 28), but as noted above only a finite number have been associated with either dental caries or periodontal disease. The diagnostic problem then is the identification and quantification of the odontopathogens against the background of a very complex and highly variable plaque flora. How can one reliably and routinely document that *H. actinomycetemcomitans* or *B. gingivalis* is present in a single plaque sample when it comprises less than 0.1% of the cultivable flora? What is the significance of 10% spirochetes vs 25% spirochetes, or 5% *S. mutans* vs 15% *S. mutans*?

Cultural studies have been essential for the implication of certain species as odontopathogens, but even with the very best transport and selective media, cultural methods may not be reliable or inexpensive enough to become the routine means by which odontopathic infections are diagnosed. This is certainly the current situation with periodontal infections, but may be otherwise with the culturing of saliva for *S. mutans* and lactobacilli (9, 15).

Microscopic examination of plaque

for the presence and levels of motile organisms and spirochetes is a reliable and simple procedure (13), that has been widely publicized (7) but apparently minimally accepted by the clinician (35). Additional information may be obtained if highly specific fluorescent antibodies are used in conjunction with microscopic procedures (27, 38), but this requires a battery of antibodies to recognize each serotype of each putative odontopathogen, and assumes that the sought after antigen(s) in the plaque is (are) biologically exposed to the antibody. An even more specific and sensitive diagnostic agent would be the use of a DNA probe for each odontopathogen. This would require that the DNA of odontopathic species can be made accessible in a stable form; i.e. is not hydrolyzed by the DNAses found in plaque (3).

An alternate diagnostic possibility is the monitoring of the presence and levels of certain bacterial or host enzymes directly in the plaque (30), gingival crevicular fluid (3) or in the saliva (23). This possibility has evolved from

the current usage of enzyme diagnostic reagents for the rapid identification of pure bacterial colonies that have been isolated from plaque (6, 11, 12, 25, 32). In particular the ability of the putative periodontal pathogens *B. gingivalis* (11, 25), *T. denticola* (11) and *B. forsythus* (32) to hydrolyze various synthetic substrates of trypsin led to studies which suggest that this trypsin-like enzyme(s) may be a diagnostic marker for both clinical disease and the magnitude of the spirochetal infection (17a).

In this review the use of enzyme-based markers as a means of diagnosing either a cariogenic *S. mutans* infection or a proteolytic anaerobic infection presumably due to spirochetes and/or bacteroides will be described.

#### Lessons learned from *S. mutans*

*S. mutans* was initially thought to be a single species but extensive serological (1) and genetic (4) studies led to the recognition of 9 serotypes and at least 6 genetically distinct species (4, 15). This complexity along with the observation that most if not all individuals harbored one of the mutans streptococci frustrated the development of highly specific fluorescent antibody reagents. As a result a greater reliance was placed upon cultural studies for the obtaining of reliable quantitative data. In this process the relationship between salivary and plaque levels of *S. mutans* was established (29, 34), and in turn the relationship between salivary levels of *S. mutans* and the degree of present and prospective caries activity was empirically determined (8, 9, 39, 40).

This serological and genetic complexity led Shklair and Keene (24) to develop a phenotype characterization of the various mutans streptococci that resulted in a simple biotyping scheme (Table 1), that relies upon characteristics that reflect enzyme activity. Thus the mutans streptococci ferment mannitol and form adherent glucans via a variety of glucosyltransferases (GTF) (2).

The various species react uniquely to the utilization of raffinose, melibiose, the liberation of  $\text{NH}_3$  from arginine and in their resistance to bacitracin, thereby forming 4 distinct groups which corresponded to the genetic groups. Note that only *S. mutans* and *S. sobrinus* are human organisms (15), so that a diagnostic test in most instances need only identify these two species.

Tests that sought to reveal all the mutans streptococci in a dichotomous fashion by virtue of their ability to ferment mannitol were not specific and were unable to distinguish between high and low levels of the mutans streptococci (16). A breakthrough was the description of a selective agar medium (MSB agar) which relied upon 20% sucrose and 0.2  $\mu\text{g/ml}$  bacitracin to provide quantitative data on the numbers of the mutans streptococci in plaque and saliva (5). The widespread usage of this MSB medium allowed investigators to determine what salivary levels of mutans streptococci were associated with decay and which levels were associated with the caries free condition. Thus, individuals with more than  $10^6$  mutans streptococci per ml saliva are highly infected and should be aggressively treated (9). This treatment should be repeated if the mutans streptococci do not drop below  $2.5 \times 10^5$  CFU/ml (8, 39).

These empirically determined guidelines have put the delivery of dental care on a scientific basis (9, 15), but require that a professionally-staffed bacteriology laboratory perform the actual tests. Efforts have been made to package the MSB medium in a form that allows it to be used in the dental office. One such kit is a modification of the dip-culture initially described for salivary lactobacilli counts (10), whereas another kit is simply a small tube incubated at an angle that takes into account the ability of the mutans streptococci to form adherent polysaccharides (20). The latter test has been shown to reflect salivary mutans streptococci

levels, but no data have apparently been published showing its ability to reflect the caries status of the individual. There are no published data on the dip-culture kit. We have compared both kits using the same saliva sample and have related the findings to the caries morbidity (the DMFS score) and activity (the D score) of the subjects (Table 2). Both tests identified individuals with high levels of morbidity and activity, but the dip-culture exhibited the greater specificity as it recognized those mutans streptococci negative and caries negative subjects.

This brief review of diagnostic tests for the mutans streptococci shows how the combination of cultural and enzymatic methodologies has led to the development of a simple, inexpensive and apparently reliable diagnostic tests that can be used in the dental office.

#### The use of enzyme methodologies in periodontal disease

The periodontal microbiology is more complex than that associated with dental decay and it is in this area that enzyme technology offers promise in the area of diagnosis. Commercially available enzyme diagnostic kits have been particularly helpful in the identification of pure cultures of plaque isolates. Moreover, as we accumulate information regarding the enzyme profiles of plaque isolates, one or more patterns are emerging that suggest certain enzymes may be characteristic of the periodontopathogens, and it appears that these enzymes can be reliably measured directly in the plaque or saliva. If this can be validated then the possibility exists for direct enzyme readings on the

Table 1. Biochemical scheme used to speciate the mutans streptococci<sup>a</sup>

Species	Serotypes	Raffinose	Melibiose	$\text{NH}_3$ from Arginine	Resistance to Bacitracin
<i>S. mutans</i>	c, e, f	+	+	-	+
<i>S. sobrinus</i>	d, g, h	-	-	-	+
<i>S. rattus</i>	b	+	+	+	+
<i>S. cricetus</i>	a	+	+	-	-

a) All strains ferment mannitol and form extracellular glucans adapted from Shklair and Keene (24).

Table 2. Relationship between salivary *S. mutans* tests and active decay or caries morbidity (DMFS scores)

	Adherence Test		Dip-culture	
	+ <sup>a</sup>	-	+ <sup>a</sup>	-
Activity				
D=0	5	3	3	5
D=1 or 2	4	3	6	2
D ≥ 3	14	2	12	4
Morbidity				
DMFS=0	1	1	0	2
2	1	2	1	2
3-5	4	1	3	2
>5	17	4	17	5

a) Adherence: + = score ≥ 3. Dip-culture: + = score ≥ 3.

plaque using procedures suitable for the dental office.

#### A. Enzymatic identification of plaque isolates

Commercially available enzyme kits such as API ZYM (API System, La Balmes les Grottes, Montalieu-Vercieu, France) and An-Ident (Analytab Products, Plainview, N.Y.), have been used by several groups to characterize plaque isolates. These enzyme assays have permitted the rapid and reliable speciation of the black pigmented *Bacteroides* (BPB) (11). While it had long been known that multiple species exist among these organisms, it has only been in recent years that one of these species, *B. gingivalis* has emerged as a true periodontal pathogen (19, 26). Thus, in the diagnosis of a periodontal infection it is appropriate to demonstrate that it is *B. gingivalis* that is present, and if one were

to initiate interceptive treatment, then it is imperative to show that *B. gingivalis* is involved. This can be easily done by showing that the isolated BPB possess a trypsin-like activity, as only *B. gingivalis* of the human BPB gives a positive trypsin-like test (Table 3) (11, 25). This identification is confirmed by negative enzyme assays for  $\alpha$ -glucosidase and N-acetyl- $\beta$ -glucosaminidase. In fact, with these 3 enzyme assays the majority of the oral BPB can be adequately specified (Table 3).

Enzyme assays can be used to distinguish *H. actinomycetemcomitans* from the closely related *H. aphrophilus* and from several *Actinobacillus* species (Table 4) (25). Of particular interest was the finding that *Bacteroides forsythus*, a slow growing, anaerobe isolated from sites of active periodontal destruction (31) yielded strong positive reactions for  $\alpha$ -fucosidase, N-acetyl- $\beta$ -glucosami-

nidase,  $\alpha$ -glucosidase,  $\beta$ -glucuronidase and N-acetyl glucosaminidase, all reactions which suggest that this fastidious organism uses glycoproteins as energy substrates. By selection of a few enzyme markers this organism could be easily differentiated from several other fastidious gram negative anaerobes as well as other fusiform organisms such as the *Capnocytophaga* and *Fusobacterium* species (Table 5) (32).

**B. Plaque levels of trypsin-like enzymes**  
*B. forsythus* also possesses a trypsin-like enzyme, thereby sharing this marker with *B. gingivalis*, the small spirochete *T. denticola* and an unspecified *Capnocytophaga* species (12). This enzyme activity is usually measured as the hydrolysis of the colorless substrate N-benzoyl-DL-arginine-2-naphthylamide (BANA), thereby releasing the chromophore ( $\beta$ -naphthylamide) which turns a bright orange-red when a drop of fast garnet is added to the solution (11). The BANA activity of various subgingival gram negative species is listed in Table 6.

As the BANA positive species are putative periodontal pathogens, it was of interest to determine whether the presence of one or more of these species could be detected by the ability of the plaque to directly hydrolyze BANA. Subgingival plaque was removed from one pocket per quadrant in periodontal patients. This plaque was assayed for BANA hydrolysis, microscopic and cultural counts and these findings were related to the clinical parameters. In some instances the effect of scaling and root planing alone or in conjunction with metronidazole on these parameters was assessed.

Fifty-three percent of over 400 plaque samples were either positive (43%) or weakly positive (10%) for the BANA reaction. Of the known BANA positive species, *T. denticola* (measured indirectly as a small spirochete) was detected in 93% of the BANA positive plaques. *B. gingivalis* and the *Capnocytophaga* species were detected in only 27% and 6% of the BANA positive plaques. *B. forsythus* was not monitored. The proportions of small spirochetes which would include *T. denticola* exhibited a highly significant correlation with the BANA response of the plaque (Table 7). A similar highly significant relationship was also observed with the proportions of intermediate and large size spirochetes, indicating that spiro-

Table 3. Distinguishing enzyme patterns of black pigmented bacteroides<sup>a)</sup>

Species	Trypsin-like	$\alpha$ -glucosidase	N-acetyl- $\beta$ -glucosaminidase
<i>B. gingivalis</i>	+	-	-
<i>B. endodontalis</i>	-	-	-
<i>B. intermedius</i>	-	+	-
<i>B. melaninogenicus</i>	-	+	+
<i>B. asaccharolyticus</i>	-	-	±
non human strains <sup>b)</sup>	±	-	±

a) Data taken from Slots, 1981; Laughon et al. 1982.

b) *B. levii*, *B. macacae*, catalase + beagle dog isolates.

Table 4. Distinguishing enzyme patterns of *Actinobacillus* and *Hemophilus* species

Species	$\beta$ -galactosidase	$\alpha$ -glucosidase	$\beta$ -glucuronidase	esterase-lipase
<i>H. actinomycetemcomitans</i>	-	-	-	±
<i>H. aphrophilus</i>	+	+	-	-
<i>A. equuli</i>	+	-	-	+
<i>A. lignieresii</i>	+	-	-	-
<i>A. suis</i>	+	+	-	-
<i>A. seminis</i>	-	-	+	+

Adapted from Slots, 1981, J. Clin. Microbiol. 14: 288-294.

Table 5. Enzyme reactions of fastidious oral gram negative species

Species	Trypsin-like	$\alpha$ -glucosidase	$\alpha$ and $\beta$ galactosidase	proline amino peptidase	esterase lipase
<i>B. forsythus</i>	+	+	-	-	-
<i>B. gracilis</i>	-	-	-	-	weak
<i>B. ureolyticus</i>	-	-	-	-	weak
<i>B. buccae</i>	-	+	+	-	-
<i>Capnocytophaga</i> sp.	-	+	-	+	-
<i>Wolinella</i> sp.	-	-	-	-	weak
<i>Campylobacter concisus</i>	-	-	-	-	weak
<i>E. corrodens</i>	-	-	-	+	+
<i>Selenomonas sputigena</i>	-	+	+	-	-

Adapted from Tanner et al. 1985. J Clin. Microbiol. 22: 333-335.

chetes representative of all morphotypes contributed to the ability of the plaque to hydrolyze BANA. Thus, BANA hydrolysis could be considered as a measure of the presence of spirochetes in the plaque.

The ability of the plaque to hydrolyze BANA could also be associated with periodontal morbidity, using probing depths as the indicator of morbidity. Plaques obtained from sites with probing depths of 2 or 3 mm had either undetectable spirochetes or low proportions of spirochetes and exhibited only 10% positive BANA reactions. Plaques obtained from sites  $\geq 7$  mm deep had 34% to 46% spirochetes and exhibited from 80 to 92% positive BANA reactions. No relationship between percentage of *B. gingivalis* or *Capnocytophaga* and probing depth was evident (17a).

These findings suggested that the

ability of subgingival plaques to hydrolyze BANA could be used as a measure of periodontal morbidity in the patient. Seventy-one percent of the plaques removed from untreated patients were BANA positive, while only 8% of the plaques removed from treated and maintained patients seen at recall visits were BANA positive. Within each patient category the ability of the plaque to hydrolyze BANA identified those sites which had elevated levels and proportions of spirochetes. For example, among the recall patients the 8 BANA positive plaques averaged 20% spirochetes and 5 spirochetes per high power microscopic field (hpf), while the 90 BANA negative plaques averaged 6% spirochetes and one spirochete per hpf. This suggests that the BANA hydrolyzing activity of the plaque was able to identify those plaques with a high spirochetal load (infections) (17a).

We were interested in whether the BANA test could be used to monitor the plaque response to various forms of periodontal treatment. We evaluated in a double-blind half mouth study what effect mechanical debridement alone or in conjunction with one week of systemic metronidazole treatment would have upon the ability of the plaque to hydrolyze BANA. Prior to treatment about 85% of the monitored sites were BANA positive (Table 8). One week after treatment the proportions of plaques which exhibited a positive BANA reaction had decreased by 62% to 93% with the greatest reduction occurring in those sites which received both scaling and metronidazole. Concurrent measurements showed that the spirochetes decreased significantly, primarily in the patients who received metronidazole.

These findings suggest that BANA hydrolysis by subgingival plaque has the potential to be an objective indicator of periodontal disease activity and could be used in combination with clinical criteria both to initiate therapy and as a means to monitor the efficacy of treatment. However, before this is done additional information is needed concerning all sources of the BANA hydrolyzing activity of the plaque, as it is possible that when this is known, the BANA test can be made both more specific and sensitive for periodontal disease activity.

Table 6. BANA activity of bacterial species found in subgingival plaque

BANA Positive		BANA negative	
Treponema denticola	T. socranskii	B. gracilis	
Bacteroides gingivalis	T. vincentii	B. ureolyticus	
B. forsythus	T. pectinovorum	B. buccae	
unspesiated Capnocytophaga	B. intermedius	Capno. sputigena	
	B. melaninogenicus	C. gingivalis	
	B. loeschii	C. ochracea	
	B. endodontalis	F. nucleatum	
	Wolinella recta	Selenomonas sputigena	
	W. curva	Campylobacter concisus	
	W. succinogenes		

Data taken from Refs. 11, 12, 25, 32 and unpublished results.

Table 7. Relationship between BANA hydrolysis and proportions of certain subgingival bacteria

BANA Hydrolysis	% Small Spirochetes	% Intermediate Spirochetes	% Large Spirochetes	% <i>B. gingivalis</i>	% Capnocytophaga
Positive	26 $\pm$ 15	11 $\pm$ 8	6 $\pm$ 9	2 $\pm$ 8	0.04 $\pm$ 0.2
Weakly Positive	14 $\pm$ 15	7 $\pm$ 8	2 $\pm$ 7		
Negative	7 $\pm$ 12	1 $\pm$ 3	2 $\pm$ 1	0.1 $\pm$ 0.1	0.1 $\pm$ 0.2
Kruskal-Wallis	p < 0.0001	p < 0.0001	p < 0.0001	NS	NS

Table 8. Effect of mechanical treatment plus metronidazole or placebo on the ability of subgingival plaque to hydrolyze BANA

Treatment Group	Half-Mouth Design				
	% BANA Positive		% Change	Due to	
	Before	After		Scaling	Metro
Placebo	83 (24) <sup>a</sup>	70	16		
Placebo + Scaling	85 (23)	32	62	46	
Metronidazole	82 (22)	21	74		58
Metronidazole + Scaling	84 (25)	6	93	77 additive	

a) Number of samples.

### C. Multiple Enzyme Assays

A potential way of increasing the specificity and sensitivity of an enzymatic diagnostic test would be to increase the number of enzymes that are monitored. When subgingival plaques were removed from deep pockets in treated and untreated periodontal patients and examined for their ability to hydrolyze 8  $\beta$ -naphthylamide derivatives, the ability to hydrolyze L-phenylalanine, L-leucine and L-alanine was similar to that observed with BANA (Table 9) (30). However, there was no apparent added benefit derived from the usage of these additional substrates. Thus, 83 to 90% of the plaques from the untreated patients yielded a positive reaction, whereas 29 to 42% of the plaques from the treated patients gave a positive reaction.

The collection of plaque from a single site creates a sampling problem as the clinician may not know which tooth site should be sampled. This can be addressed by the collection of multiple samples or pooled samples from several teeth.



In either case this is not as satisfactory as having a single sample that reflects the entire mouth in the way a salivary culture for the mutans streptococci can be used to diagnose caries risk. In this regard many of these enzymes can be measured in the saliva either in the supernatant or sediment (23). Higher enzyme activities were found in the supernatants obtained from 10 periodontitis patients than were found in the supernatants obtained from 10 periodontally healthy patients for alkaline phosphatase, esterase, esterase-lipase, leucine-valine- and cystine aminopeptidases,  $\beta$ -glucuronidase and  $\alpha$ -glucosidase. No difference in trypsin-like activity was found. In addition the supernatants from the periodontitis patients showed significantly greater ability to hydrolyze 9 of over 50-naphthylamide derivatives that were evaluated (23).

These findings offer the possibility that enzyme tests of saliva may yield information concerning the periodontal

status of the individual. This was further suggested by studies in which the levels of the same large panel of enzymes were compared in salivary supernatant before and after periodontal treatment in 5 adults (37). The results show a significant reduction in the levels of the trypsin-like enzyme, and also in the levels of 10 other enzyme activities, 6 of which had previously been shown to be elevated in periodontitis patients (Table 10) (23). This suggests that perhaps the periodontitis patient can be recognized by a unique pattern of salivary enzymes that have a unique specificity for arginine, glycine or phenylalanine-containing compounds. It remains to be shown whether these results can be obtained with whole saliva, thereby avoiding the centrifugation step.

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Table 9. Enzymatic activities against  $\beta$ -naphthylamide derivative in plaques removed from pocket depths  $\geq 5$  mm

$\beta$ -naphthylamide derivatives	% of Sites with Positive Reaction		
	Untreated Periodontitis n=41	Treated Patients Seen at Recall n=24	Ratio Untreated/Treated
BANA	85	29	2.9
pyrrolidine	10	8	1.3
L-phenylalanine	88	38	2.4
L-leucine	90	42	2.1
L-valine	12	0	
L-serine	5	0	
L-arginine	24	8	3
L-alanine	83	33	2.5

Adapted from Syed et al. 1984. *J. Perio. Res.* **19**: 618-621.

Table 10. Effect of periodontal therapy<sup>a)</sup> on salivary enzymatic activity

Enzymes	Pre-treatment	2 wks. post-treatment <sup>a)</sup>
Trypsin-like	5 (3) <sup>b</sup> mM	1 (2)
Cystine aminopeptidase	10 (0)	4 (2)
$\beta$ -galactosidase	12 (4)	7 (4)
Hydrolysis of $\beta$ -naphthylamide derivations		
Arginine	23 (5)	11 (5)
$\beta$ -glutamine	3 (2)	0
Proline	23 (5)	11 (5)
Arginyl-arginine	37 (5)	13 (6)
Glycyl-arginine	17 (5)	8 (2)
Glycyl-phenylalanine	10 (0)	6 (2)
Lysyl-alanine	20 (0)	12 (4)
Phenylalanine-arginine	33 (5)	15 (9)
Salivary bacteria	$3.54 \times 10^{10}$	$6.0 \times 10^{10}$
Spirochetes	2%	0%
Motile rods	13%	8%

a) 5 adults received 2 or more sessions of scaling and root planing and 1 g/day of Tetracycline-HCL for 14 days. Adapted from Zambon et al. 1985. *J. Perio. Res.* **20**: 652-659.

b) Standard deviation in parentheses.

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