Comparison of Three Dispersion Procedures for Quantitative Recovery of Cultivable Species of Subgingival Spirochetes

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Spirochetes are usually the predominant organisms observed microscopically in subgingival plaques removed from tooth sites associated with periodontitis, but these organisms are rarely isolated by cultural means, presumably because the media do not support their growth and/or because these fragile organisms are disrupted by the various procedures used to disperse plaque samples. In the present investigation, three dispersal procedures, sonification, mechanical mixing, and homogenization, were compared for their ability to permit the isolation of *Treponema denticola*, *Treponema vincentii*, *Treponema socranskii*, and *Treponema pectinovorum* from plaque samples on media that support the growth of these species. Plaque samples in which the spirochetes averaged 50% of the microscopic count were chosen. The highest viable recoveries of spirochetes were observed when the plaques were dispersed with a Tekmar homogenizer, and the lowest occurred with sonification. The highest recoveries averaged only about 1% of the total cultivable counts, indicating either that the sought-after species were minor members of the flora or that the dispersal procedures were still too harsh. A total of 91% of the isolates were *T. denticola*, 5% were *T. socranskii*, and 4% were a heretofore-undescribed species.

Spirochetes are the predominant organisms observed microscopically in subgingival plaques removed from sites associated with periodontitis (6, 7, 9). However, they are rarely isolated on most artificial media that are used to culture the plaque. Either these media do not provide the nutrients essential for their growth or the spirochetes are disrupted by the procedures used to disperse the plaque so as to optimize the recovery of cultivable species. Thus, the inability to quantitatively isolate spirochetes from the plaque could reflect technical problems related to dispersion as well as nutritional inadequacies of the media.

Certain species of spirochetes have been isolated from nondispersed plaques by methods that exploit the ability of the spirochetes to migrate through agar (11) or membrane filters (8) or to grow in the presence of rifampin (1, 5). These isolation tactics yield strains of pure spirochetes but do not provide quantitative information as to the relative proportions of the isolated species in the plaque flora. Four of the cultivable species, *Treponema denticola*, *Treponema vincentii*, *Treponema socranskii*, and *Treponema pectinovorum*, were grown in a modification of Laugon medium (4) which has the following composition per liter: tryptone (Difco Laboratories, Detroit, Mich.), 10.0 g; heart infusion broth, 5.0 g; yeast extract, 10.0 g; gelatin, 10.0 g; $(\text{NH}_4)_2\text{SO}_4$, 0.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g; K_2HPO_4 , 1.13 g; KH_2PO_4 , 0.9 g; NaCl, 1.0 g; and Noble agar (Difco), 10 g. The pH was adjusted to 7.2 with 4 N KOH prior to autoclaving of the medium. The following ingredients were mixed, neutralized with KOH, sterilized by filtration, and added aseptically to the autoclaved medium (final concentrations per liter): glucose, 1.0 g; cysteine hydrochloride, 1.0 g; thiamine PP_i , 0.0125 g; sodium pyruvate, 0.25 g; acetic acid, 0.27 ml; propionic acid, 0.10 ml; *n*-butyric acid, 0.064 ml; *n*-valeric acid, 0.016 ml; isobutyric acid, 0.016 ml; isovaleric acid, 0.016 ml; DL-methylbutyric acid, 0.016 ml; and heat-inactivated rabbit serum, 10% (vol/vol). Pectin (2.5

g/liter) was added so as to support the growth of *T. pectinovorum* (13).

In the present study this modified medium, from here on referred to as MTYGVS, was used for the quantitative recovery of these species from subgingival plaques after different dispersal procedures were used. It was postulated that because the nutritional needs of these species were met, the effect of the dispersal procedures on their quantitative recoveries from the plaques could be evaluated.

Prior studies showed that 20 s of sonification with a Kontes sonifier (Kontes Co., Vineland, N.J.) yielded a maximal recovery of bacteria from subgingival plaques without affecting the recovery of the fragile species *Fusobacterium nucleatum* (S. A. Syed and W. J. Loesche, J. Dent. Res. 57, Special Issue abstr. no. 982, 1978). A preliminary study had shown that dispersal with a Tekmar tissue homogenizer (Tissumizer; Tekmar Co., Cincinnati, Ohio) was less disruptive to *T. denticola* in vitro than was sonification with the Kontes sonifier (7). Accordingly, a protocol was developed to compare whether the dispersal of plaques with the Tekmar homogenizer would yield higher quantitative recoveries of the aforementioned species of cultivable spirochetes than would sonification. A third dispersal procedure, mechanical mixing with a mixer from VWR Co., Bohemia, N.Y., was also included, as microscopic examination of plaques has shown that mechanical mixing does not lyse the spirochetes (9, 10).

Plaques were obtained from patients who had periodontal sites exhibiting active periodontitis, as judged by bleeding upon probing and high proportions of spirochetes in the subgingival plaque. The supragingival plaque was removed and discarded, and a second curette was used to remove the subgingival plaque and place it into 0.5 ml of reduced transport fluid (9). The plaque suspension was placed in an anaerobic glove box (Coy Laboratory Products, Ann Arbor, Mich.), where it was dispersed for 10 s by mechanical mixing. This mixing was done so as to yield as homogeneous a suspension as possible without destroying the morpholog-

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TABLE 1. Effects of dispersal procedures on the recovery of spirochetes and other bacteria from 18 plaque samples^a

Dispersal procedure	Counts (10 ⁴ CFU) of:					% of:			
	Total (<i>P</i> = 0.03) ^b	BPB	<i>A. odontolyticus</i>	Spirochetes on:		BPB	<i>A. odontolyticus</i>	Spirochetes on:	
				ETSA-R (<i>P</i> = 0.02) ^b	MTYGVS-R (<i>P</i> = 0.08) ^b			ETSA-R (<i>P</i> = 0.03)	MTYGVS-R (<i>P</i> = 0.06)
Kontes sonification	204.6	37.3	5.3	0.004	0.013	12.7	2.4	0.004	0.02
Mechanical mixing	91.6	17.8	1.8	0.023	0.114	11.1	1.8	0.08	0.38
Tekmar homogenization	138.8	27.7	3.5	0.067	0.148	11.3	2.9	0.18	0.38

^a Values in boldface type are significantly different from each other or from the other value(s) in the column at *P* < 0.05 (Scheffe test).

^b Values in columns are significantly different (Kruskal-Wallis test).

ical integrity of the spirochetes. Prior studies with American Type Culture Collection strains of *T. denticola*, *T. vincentii*, and *T. socranskii* indicated that this degree of mixing had no effect or a minimal effect on the quantitative recovery of these strains on MTYGVS agar medium. A 10-μl sample of the plaque suspension was removed for microscopic counting, and the remaining suspension was diluted to 9 ml and separated into three 3-ml portions. One portion was mixed with the mechanical mixer for 20 s, the second portion was dispersed with the Kontes sonifier for 20 s, and the third portion was dispersed with the Tekmar homogenizer for 20 s. The sequence of the dispersal procedure was rotated among the plaque suspensions.

The resultant plaque suspensions were serially diluted in reduced transport fluid and plated semiautomatically by means of a plating device (Spiral Systems, Gaithersburg, Md.) on enriched tryptic soy agar (ETSA) (15), on ETSA medium supplemented with 5 μg of rifampin per ml (5) (ETSA-R), and on MTYGVS agar medium supplemented with 2.5 mg of pectin and 5 μg of rifampin per ml (MTYGVS-R). ETSA medium was used for the enumeration of total cultivable CFU; for differential counts of black-pigmented bacteroides (BPB), which were used as a monitor of the recovery of easily disrupted gram-negative anaerobes; and for counts of *Actinomyces odontolyticus*, which was used as a monitor of the recovery of hard-to-disrupt gram-positive organisms. The recovery of these species was used as an indicator of the relative disruptive capabilities of the various dispersal procedures. ETSA-R and MTYGVS-R media were used to isolate spirochetes. The various agar media were incubated within the anaerobic chamber for 7 days (ETSA plates) or 14 days (ETSA-R and MTYGVS-R plates) at 35°C under an atmosphere of 85% N₂-10% CO₂-5% H₂. The spirochete, BPB, and *A. odontolyticus* counts were normalized as a percentage of the total CFU which grew on the corresponding ETSA medium.

The plates were examined with a dissecting microscope, and the total counts and distinctive pigmented colonies of BPB and *A. odontolyticus* were enumerated on ETSA medium. The MTYGVS-R and ETSA-R plates were examined for colonies which exhibited growth into the underlying agar. These and all other colonies on the rifampin media were examined by dark-field microscopy, and if spirochetes were present, they were restreaked to assure purity and characterized by conventional biochemical tests (3) and by their enzyme profiles in the Micro-Ident system (S. A. Syed, S. L. Salvador, W. J. Loesche, and M. A. Flynn, J. Dent. Res. **65**, Special Issue abstr. no. 305, 1986).

The highest recoveries of total bacterial counts as well as recoveries of BPB and *A. odontolyticus* were achieved when the plaques were dispersed by sonification (Table 1). However, the differences between dispersal procedures were only significant with regard to the total count after sonifica-

tion being significantly greater than the total count after mechanical mixing (*P* < 0.05, Mann-Whitney U test). Very few spirochetal colonies were observed on either spirochetal medium; those that grew on MTYGVS-R medium accounted for about 0.4% of the cultivable flora that grew on ETSA medium, and those that grew on ETSA-R medium amounted to 0.004 to 0.2% of the flora on ETSA medium. The highest spirochetal recoveries occurred when the plaques were dispersed with the Tekmar homogenizer, and the lowest were associated with dispersal by sonification.

We conducted a second experiment in which sonification was omitted and the spirochetal recoveries obtained with the mechanical mixer were directly compared with those obtained with the Tekmar homogenizer. Eighteen plaque samples were collected, dispersed, and plated as described previously. There was a tendency (but no statistical significance) for the spirochetal recoveries to be better when the plaques were dispersed with the Tekmar homogenizer and plated on MTYGVS-R medium.

The spirochetes averaged about 56% of the microscopic count but only 1% of the cultivable flora in the 18 Tekmar homogenizer-dispersed plaques (Table 2). This meant that more than half the total organisms present in these plaques were uncultivable spirochetes or possibly cultivable spirochetes that had been killed during the isolation procedure. As such, the cultivable spirochetes accounted for about 0.5% of the total microscopic count (Table 2). The cultivable spirochetes were all small, indicating that the intermediate and large spirochetes, which averaged 20 and 9%, respectively, of the total microscopic count, were apparently not cultivable. These data suggest that the overwhelming majority of the spirochetes observed microscopically in the plaque were not cultivable by the methods used.

Ninety-six strains of cultivable spirochetes were characterized. Eighty-seven were *T. denticola*, as judged by a positive enzyme reaction for α-N-benzoyl-DL-arginine-naphthylamide hydrolysis, α- and β-glucosidase activities, and no alkaline phosphatase activity (4). Representative strains of the *T. denticola* isolates were shown by electron

TABLE 2. Spirochetes as percentages of microscopic and viable counts

% of spirochetes that were:	% of spirochetes that were:		
	Small (diam, 0.1 to 0.25 μm)	Intermediate (diam, 0.2 to 0.5 μm)	Large (diam, >0.5 μm)
Detected by microscope	26.3	20.0	9.4
Cultivable ^a	1.0 ^b	— ^c	—

^a (No. of spirochetes on MTYGVS-R medium/total number of cultivable bacteria on ETSA medium) × 100.

^b Mainly *T. denticola*, which may appear as an intermediate spirochete.

^c —, None (no growth).

microscopic examination to have a 2-4-2 axial filament arrangement and were considered to be small spirochetes (12). Recently, oral spirochetes with a higher axial filament number were described as being intermediate in size (1) but as having a guanine-plus-cytosine content consistent with *T. denticola* (2). Thus, *T. denticola* may appear upon microscopic examination both as small or as intermediate spirochetes. The *T. denticola* isolates were larger than the five strains which were identified as *T. socranskii*, which fermented glucose and exhibited an enzyme pattern identical to that of the American Type Culture Collection strains of *T. socranskii* (14). Four strains did not conform to any described *Treponema* species and appear to be a heretofore-undescribed species (S. L. Salvador, M. Suzuki, S. A. Syed, K. K. Makinen, P.-L. Makinen, C. A. Edwards, and W. J. Loesche, J. Dent. Res. **66**, Special Issue abstr. no. 1604, 1987). An increased isolation frequency of *T. denticola* from diseased plaques has been reported previously with a semiquantitative broth tube dilution procedure (10).

This study shows that when the nutritional needs of cultivable species are met and when presumably gentle dispersal procedures are used, the quantitative recovery of spirochetes from subgingival plaques is discouragingly low. This result suggests that the cultivable species are either minor contributors to the spirochetal flora or that the dispersal procedures used are still too harsh to permit the survival of the majority of their members. Thus, in the plaques included in this investigation, it was not possible to cultivate or identify at the species level those organisms, namely, spirochetes, which comprised the majority of the flora. This would be true for most, if not all, other investigations which seek to culture the bacterial flora associated with periodontal disease.

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