Comparison of Dark-Field Microscopy and a Flagella Stain for Monitoring the Effect of a Water Pik® on Bacterial Motility*

Connie L. Drisko,† Connie L. White,‡ William J. Killoy§ and William E. Mayberry‖

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THE PURPOSE OF THIS EXAMINER-BLIND study was threefold: (1) to compare the microbial counts obtained by two different techniques for assessing bacterial motility, (2) to assess the inter-rater reliability of these two techniques and (3) to evaluate the effect of a water irrigating device (Water Pik®) on bacterial motility at 3- and 6-mm probing depths. Subgingival plaque samples were taken from 10 healthy patients having at least two sites that probed >6 mm, (one control, one experimental). Half of the patients were sampled at 3 mm, the other half at 6 mm, both at baseline (Day 0) and at Day 21. Two slides were prepared from each plaque sample, one for dark-field evaluation and one stained with a simplified silver-plating technique for flagella. All slides were read simultaneously by 3 observers, and the per cent motility calculated for spirochetes, motiles and all others. Strong positive inter-rater reliability correlations ranging from $r = 0.95$ to $r = 0.99$ were found for both the dark-field and flagella staining techniques. Spirochete counts obtained by both techniques were highly correlated ($r = 0.91$), whereas counts for motiles resulted in negative correlations between the techniques. Dark-field counts were consistently higher than the flagella stain counts for motile rods. Spirochetes were reduced, but not significantly, after irrigation of both 3-mm and 6-mm sites. Bacterial motility can be evaluated by both dark-field and flagella-staining techniques with a high degree of inter-rater reliability.

The most efficient and cost effective way to prevent periodontal disease is meticulous daily plaque removal. Healthy patients can usually accomplish this task by using traditional methods such as brushing and flossing along with other oral hygiene aids designed to assist in mechanically removing subgingival plaque. However, once deep periodontal pockets have developed, complete removal of plaque, particularly subgingival plaque, may be impossible, even with frequent professional intervention.

Periodontists have recognized the need for better plaque control methods that their patients can use on a daily basis and have continued to explore a variety of home care products that might fulfill this need.

Recently, there has been a renewed interest in the Water Pik® as an oral hygiene aid although conflicting reports exist regarding the effect of this irrigating device on the accumulation and maturation of dental plaque. Derdivanis et al.[1] found no significant differences between the amount of plaque accumulation when the oral irrigator was used with and without a commercial mouthwash, compared with no water irrigation. Lobene[2] and Fine and Baumhammers[3] found slight, but not statistically significant, reduction in plaque accumulation when water irrigation was used in addition to toothbrushing.

Others have reported reductions in both plaque and debris[4] and gingival inflammation.[2,4,5] Hugoson[6] reported a highly significant reduction of plaque and gingivitis in a group using the Water Pik as their only means of plaque control compared with a group who performed no plaque control measures at all. They failed to demonstrate any further reduction of plaque or gingivitis when the irrigator was introduced as an additional measure to brushing.

The effect of the Water Pik on bacterial motility was not shown until Aday[7] reported that the irrigator significantly reduced all morphologic bacterial types ex-

*Funded by the Roy J. Rinehart Memorial Foundation at University of Missouri-Kansas City School of Dentistry.

†Assistant Professor, Department of Periodontics, University of Missouri-Kansas City, School of Dentistry, Kansas City, MO 64108.

‡Assistant Professor, Diagnosis Department, University of Missouri-Kansas City.

§Professor and Chairman, Department of Periodontics, University of Missouri-Kansas City.

‖Associate Dean for Academic Affairs, University of Missouri-Kansas City.

cept cocci, in 3-mm pockets. West[8] later showed that water irrigation of 6-mm pockets also reduced the total bacterial counts and significantly reduced spirochetes but not other morphologic types. Both authors commented that the irrigator may be more effective in removing unattached plaque than attached plaque, thus making it more effective in reducing subgingival organisms.

Eakle et al.[9] tested the depth of penetration of an oral irrigating device (Water Pik) into periodontal pockets ranging from 0 to >7 mm. Mean penetration ranged from 44 to 71%. Their results showed that the oral irrigator will deliver an aqueous solution to approximately half the depth of the pocket. These results further supported the findings of Aday and West, that water irrigation is capable of reaching 3 to 6 mm subgingivally.

Home use of oral irrigators to deliver local chemotherapeutic and antimicrobial agents has also been shown to successfully suppress plaque formation and proliferation.[10,11] Boyd et al.[12] compared the effect of daily use of the Water Pik by the patients at home, with and without 0.02% SnF_2. Their results showed that there were significant improvements in the periodontal health of the SnF_2 group during the first 10 weeks of their cross-over study. Plaque accumulation was less, but not statistically different, in the water irrigation only group than the control group which received no water irrigation. They reported significant decreases in per cent spirochetes for both their control and experimental groups. The SnF_2 group showed greater reductions in the per cent spirochetes, but these differences were not statistically significant. The effect of the irrigator on the microbial flora remains unclear in this study, however, since even the untreated controls had significant reductions in per cent spirochetes.

Studies that tested the effect of oral irrigators on bacterial motility monitored changes in the microbial contents of the pockets by dark-field or phase contrast microscopy.[7,8,12] With these techniques, viable organisms from subgingival plaque were viewed under the microscope, various morphologic bacterial types counted, and their percentages calculated.

Leggott et al.[13] pointed out the difficulty encountered with viable organisms moving in and out of the field using phase-contrast microscopy to assess bacterial motility. Boyd et al.[12] reported significant differences ($P < 0.05$) in counts of motile rods obtained by three calibrated observers using phase-contrast microscopy. Since the differential dark-field technique also counts viable organisms, this raises some question regarding the accuracy of these live assessment methods in research.

A simplified silver-plating flagella stain is available as an alternative to live methods for assessing bacterial motility.[14] With this technique, bacterial plaque smears are fixed, then stained before viewing with a light microscope. All motile, as well as nonmotile, bacteria can then be counted in a similar fashion as described for dark-field, except motile rods are identified by their flagella rather than by their motility. Mycoplasmas, and gliders, such as the *Capnocytophaga* species are not identifiable with this stain since they have no flagella.

There have been no reports comparing the reliability, particularly the inter-rater reliability, of flagella stains with dark-field for assessing bacterial motility. There have also been relatively few reports of the effect of oral irrigating devices on bacterial motility.

The purpose of this examiner-blind study was threefold: (1) to compare the microbial counts obtained by two different techniques for assessing bacterial motility, (2) to assess the inter-rater reliability of these two techniques and (3) to evaluate the effect of an oral irrigating device (Water Pik) on bacterial motility at 3- and 6-mm probing depths.

MATERIALS AND METHODS

Ten healthy adults from the patient population of the University of Missouri-Kansas City School of Dentistry participated in this study. Patients were selected who had at least two >6-mm pockets in the anterior region and a negative history of (1) rheumatic heart disease or valvular prostheses, (2) diabetes, (3) antibiotics taken within 6 months, (4) prophylaxis within 3 months and (5) current use of an oral irrigation device.

A preclinical study was conducted to train and calibrate three observers in the use of the dark-field and flagella-staining techniques for assessing bacterial motility from subgingival plaque samples. An American Optical 110 microscope equipped with three binocular eye-pieces was used so all three observers could read the slides simultaneously. Counts of spirochetes, motile rods and other morphologic types of bacteria found in subgingival plaque samples were performed. At the end of this portion of the study, inter-rater reliability correlations for the three observers were obtained that ranged from $r = 0.83$ to 0.99 for dark-field specimens, and required 3.5 hours of microscope time. Only 2.2 hours were needed to attain inter-rater correlations of $r = 0.95$ to 0.98 when reading the flagella-stained smears.

All clinical data were gathered and treatment performed by a fourth investigator, thus maintaining the examiner-blind format of the study.

Subgingival plaque samples were taken at two >6-mm sites per patient. Half of the patients were sampled at 3 mm and the other half at 6 mm, on Day 0 and Day 21. No treatment was performed until Day 21.

Baseline Data Collection. On Day 0, one site in each patient was randomly assigned to the control group, the other to the experimental group. Prior to subgingival sampling, supragingival plaque was removed with a sterile curet and the area dried and isolated. Plaque samples were obtained by placing a sterile barbed

broach, wrapped with calcium alginate fibers, to the predetermined depth of 3 mm or 6 mm (Fig. 1). The broach was turned 5 times in a clockwise direction, then placed immediately in a sterile, capped tube containing 0.25 ml phosphate-buffered saline (PBS = 0.01 M Sodium phosphate, 0.145 M Sodium chloride, pH 7.4).

Sample Preparation and Microscopic Counts. Dark-field specimens were prepared immediately after collection by removing the sampling broach from each tube containing the plaque suspension before placing it in a Bransonic 220® ultrasonic bath for 40 seconds. Following sonication, a 1-mm tuberculin syringe with a 27-gauge needle was used to aspirate and expel the suspension 4 times. One drop of the plaque suspension was placed on a clean glass slide, and cover glasses were then sealed onto the slides with petroleum jelly. Three observers read the slides simultaneously within 30 to 40 minutes of plaque collection. An American Optical 110 microscope equipped with 2 additional binocular bodies with 10× widefield eyepieces and 10 mm/100 squares micrometer discs was used to read the slides at × 1000 magnification. At least three representative fields and a minimum of 200 bacteria were counted. The bacteria were grouped into three types; (1) spirochetes, (2) motiles and (3) all others. Motiles and spirochetes were counted first in each field.

A second slide from each plaque suspension sample was then prepared for silver-plating flagella staining. One running drop of the suspension was placed on an alcohol-cleaned slide, air dried, then fixed with a mordant and stained with a silver plating flagella stain.[14] Bacterial groups were identified and counted as (1) spirochetes (spiral), (2) motiles (flagellated) and all others (nonflagellated). As in the dark-field counts, the same three calibrated observers performed these counts simultaneously and were unaware of the sample origin or the treatment condition.

Day 21. At Day 21, samples from the control sites were obtained in the same manner as described for the baseline collection. Prior to sampling the experimental sites, the supragingival plaque was removed and the sites irrigated for 8 seconds with a water irrigating device (Water Pik) filled with tap water. A standardized pressure of 70 pounds per square inch (psi) and a fixed distance of 3 mm was used (Fig. 2). The area was dried and isolated and the plaque sample taken immediately after irrigation of the experimental sites. Dark-field and flagella-stained specimens were prepared and read as previously described.

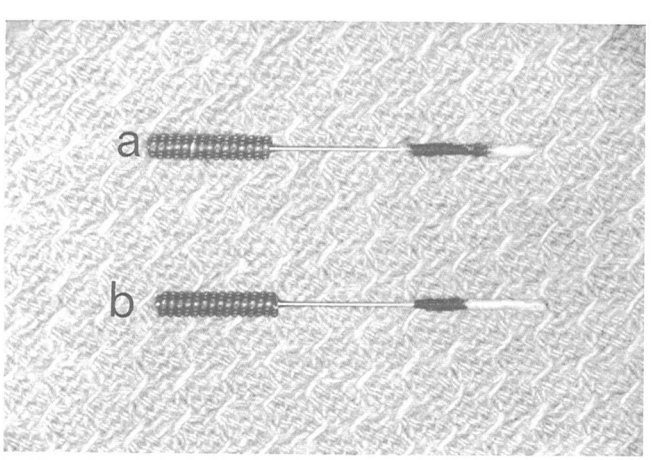

Figure 1. *Extra fine barbed broaches wrapped with calcium alginate fibers used to obtain plaque samples at* (a) *3 mm and* (b) *6 mm.*

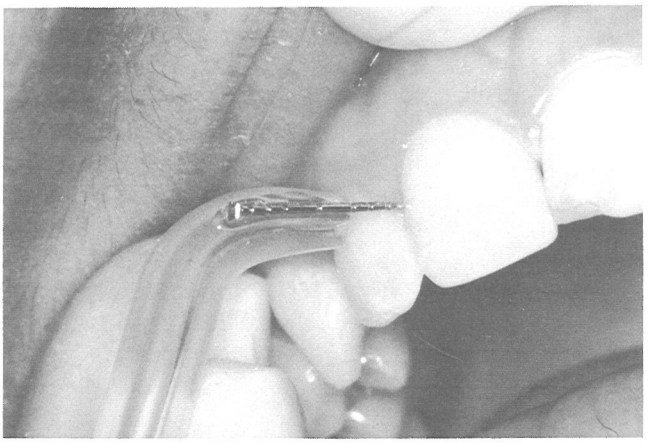

Figure 2. *Water Pik tip modified to standardize 3-mm distance from site to be irrigated at 90 degree angle to the tooth and 70 psi.*

Data Analysis. All data were converted and presented as percentages of the pocket population, e.g., per cent spirochetes (spiral), motiles (flagellated) and all others. Inter-rater reliability was determined by Cronbach's coefficient alpha.[15] One factor within and two factors between analyses of variance were performed to determine the effect of treatment on the per cent motility.

RESULTS

The preclinical trials to calibrate three observers using two techniques to measure bacterial motility resulted in inter-rater reliability coefficients of $r = 0.83$ for motiles and $r = 0.99$ for spirochetes using the dark-field technique. Flagella-stained smears yielded inter-rater reliabilities of $r = 0.95$ for motiles and $r = 0.98$ for spirochetes.

Similar correlation coefficients were later found between the three observers reading the clinical plaque specimens by both techniques. The mean values of three observers reading 20 plaque samples simultaneously can be seen in Table 1. The degree of agreement between observers ranged from $r = 0.95$ (others) to $r = 0.98$ (spirochetes) for the dark-field technique, and $r = 0.97$ (motiles) to $r = 0.99$ (spirochetes) for the flagella-staining technique. These correlations were only slightly higher than those in the preclinical trial, and essentially replicated our earlier findings.

A strong positive correlation ($r = 0.91$) was found when comparisons were made between counts obtained

Table 1
Inter-Rater Reliability Coefficients for Three Observers

Organisms	Dark-Field ($n = 20$)	Flagella ($n = 20$)
Motiles	$r = 0.97$	$r = 0.97$
Spirochetes	$r = 0.98$	$r = 0.99$
Others	$r = 0.95$	$r = 0.99$

Table 2
Mean Per Cent Spirochetes and Motiles for Pretest and Post-test Observations

Organisms	Mean	SD	Minimum	Maximum
Spirochetes ($n = 10$)				
PRE-CD*	6.4	13.9	.00	45.5
PST-CD	6.4	7.9	.00	23.0
PRE-CF	9.9	13.08	.00	44.60
PST-CF	8.69	9.54	.00	23.0
PRE-XD	3.26	2.59	.00	8.7
PST-XD	1.8	2.83	.00	7.5
PRE-XF	5.03	5.37	0.50	17.7
PST-XF	2.26	2.50	.00	7.5
Motiles ($n = 10$)				
PRE-CD	11.82	12.1	0.50	34.1
PST-CD	20.95	14.54	.00	43.5
PRE-CF	1.07	1.17	.00	3.10
PST-CF	0.80	0.66	.00	1.90
PRE-XD	19.16	13.59	0.50	45.0
PST-XD	15.94	15.69	.00	50.40
PRE-XF	0.53	1.04	.00	3.4
PST-XF	0.33	0.41	.00	1.3

* PRE-CD = Pretest control Dark-field
PST-CD = Post-test control Dark-field
PRE-CF = Pretest control Flagella
PST-CF = Post-test control Flagella
PRE-XD = Pretest experimental Dark-field
PST-XD = Post-test experimental Dark-field
PRE-XF = Pretest experimental Flagella
PST-XF = Post-test experimental Flagella

for spirochetes by both the dark-field and flagella-staining techniques. A negative correlation ($r = -0.32$) was obtained when the two techniques were compared in counting motile rods.

Table 2 shows the mean per cent spirochetes and motiles at baseline and after the experimental sites had been irrigated. Standard deviations and ranges are also given. No statistically significant differences were found between the percentage of organisms in each category between experimental group pretest and post-test values. In general, dark-field counts were higher than the flagella-stained counts for motiles.

Table 3 gives the mean values obtained for per cent spirochetes in all treatment conditions. There were no statistically significant differences between the reduction of spirochetes found at depths of 3 and 6 mm after water irrigation by either the flagella or dark-field measure (Table 3, I). Neither the flagella stain nor dark-field measurement showed a significant reduction in per cent spirochetes when preexperimental and postexperimental sites were averaged across depths (Table 3, II). The control and experimental groups differed considerably in their mean per cent spirochetes. Comparison of the postcontrol with postexperimental group may not be valid; however, it is interesting to note that the flagella-stained samples showed that spirochetes were reduced to a level which approached significance ($P = 0.053$), when comparing postcontrol samples with postexperimental samples (Table 3, III). The dark-field measure also showed a reduction, but to a lesser degree.

DISCUSSION

The results of this study indicate that a high degree of agreement can be obtained when counting spirochetes in subgingival plaque specimens by both the

Table 3
Mean Values Obtained for Per Cent Spirochetes in All Treatment Conditions*

Depth	Control		Experimental		
	Day 0 Pretest	Day 21 Post-test	Day 0 Pretest	Day 21 Post-test	
Flagella stain					
3 mm	12.56	8.92	3.48	1.76	I
6 mm	7.32	8.46	6.58	2.76	I
Mean	9.94	8.69	5.03	2.26	
		III	II		
Dark-field					
3 mm	10.52	5.22	2.80	1.02	I
6 mm	2.32	7.60	3.72	2.58	I
Mean	6.42	6.41	3.26	1.80	
		III	II		

* One factor between two factors within repeated measures ANOVA:
I = No significant differences between per cent spirochetes at 3- or 6-mm depths in the experimental post-test values.
II = No significant differences between pre- and postexperimental test values for spirochetes averaging across depths.
III = No significant differences between per cent spirochetes in control groups postexperimental and test groups postexperimental ($P = 0.053$).

dark-field and flagella-staining techniques. The same was not true for counting motile organisms. The dark-field counts were consistently higher for motiles than the flagella-stained counts. This lack of agreement may have been due to overestimation of motiles by the dark-field technique. The genus *Capnocytophaga* consists of motile rods that are frequently found in periodontal pockets but do not have flagella. Since these organisms move by mechanisms other than flagella, they may have contributed to the higher counts for motiles by the dark-field technique.

It is also possible that the flagella-staining technique may destroy some flagella, thus reducing the number of flagellated (motile) organisms available for counting and therefore adding to the disagreement between counts by the two techniques. However, West et al.[14] have reported the flagella-staining technique to be 91% reliable when counting flagellated organisms in pure and mixed cultures.

In this study, no significant differences in reductions of spirochetes were found between the post-test samples taken at 3 and 6 mm after irrigation. This was contrary to predicted differences; e.g., that the water irrigation would have less effect at 6 mm than at 3 mm, based on the results from similar studies by Aday[7] and West.[8] Our results may have been in closer agreement with these studies if the patients had been matched as to per cent motility before assigning them to the 3- or 6-mm treatment groups. Further evaluation of the data revealed that patient Number 5 in the 3-mm category had 45.5% spirochetes, which was approximately 40% higher than any other single site at 3 or 6 mm. By eliminating that patient's data from the analysis, the mean spirochete reduction was more in the expected direction but still not statistically different at 3 and 6 mm.

Eakle et al.[9] tested the depth of penetration of the Water Pik into periodontal pockets by irrigating the sites with water and a red dye prior to extraction of the teeth. Mean penetration into pockets of 0 to 3 mm gave the highest score at 71%. Moderate pockets of 4 to 6 mm were penetrated to 44% of the depth, and deeper pockets (>7 mm) 67%. Their results showed that the oral irrigator will deliver an aqueous solution to approximately half the depth of the pocket when used at 70 psi at a 90 degree angle of application.

The experimental design of this study did not include evaluation of the depth of penetration of the oral irrigator; however, results here showed a reduction of spirochetes in 70% of the sites following irrigation. Based on the work of Eakle et al.,[9] it was assumed that the water irrigation penetrated to varying degrees at depths of 3 and 6 mm, thus the reduction of spirochetes occurred at both levels.

Mean values for per cent spirochetes were very similar in all categories throughout the study using both techniques for assessing motility. However, the variation within each individual site at each sampling period was high. For example, spirochete counts in the pretest controls as seen on Table 1 ranged from 0.0% to a maximum of 45.5%. At the second sampling period, these same sites ranged from 0.0% to 23%, a decrease of almost 50% even though no therapy had been rendered. Because of the high degree of site variability and the small sample size used in this study, care must be exercised in generalizing these results to a large population group.

Magnusson et al.[16] reported similar site variations when multiple samples were taken over a period of 32 days. Their results showed that the variation within the site was of about the same magnitude as when the samples were taken within a few minutes. They implied that the sampling procedure may be the primary cause of variation and cautioned that single site samples may not be as representative of the microbial contents as averaging multiple samples of that site over a period of time.

The results showed that water irrigation of 3- and 6-mm pockets reduced spirochetes from pretreatment levels and that an irrigant from a Water Pik can reach depths of up to 6 mm. If an antimicrobial can be found which safely inhibits subgingival microflora, the Water Pik may be a useful delivery system. The clinical importance of the spirochete reduction reported in this study cannot be determined at this time since the specific organisms that initiate adult periodontitis are yet to be identified. Whether spirochetes are the causative organisms or merely opportunistic may be academic, since their presence is strongly associated with deep pockets and bleeding upon probing.[17–21] Until more information is available regarding the role of spirochetes in periodontal disease activity, it would seem prudent to recommend oral hygiene methods to patients that can aide in the daily removal of these organisms in subgingival plaque.

CONCLUSIONS

1. Dark-field microscopic counts of subgingival plaque resulted in inter-rater reliability coefficients of $r = 0.98$ for spirochetes and $r = 0.95$ for motiles.

2. Flagella-stained smears of subgingival plaque resulted in inter-rater reliability coefficients of $r = 0.99$ for spirochetes and $r = 0.97$ for motiles.

3. Negative correlation coefficients ($r = -0.32$) were found between the dark-field and flagella-staining techniques when measuring per cent motile organisms. Dark-field counts were consistently higher than flagella-stained counts.

4. A high degree of agreement ($r = 0.91$) was achieved with both the dark-field and flagella-staining techniques when measuring per cent spirochetes in subgingival plaque samples.

5. Water irrigation reduced spirochetes, but not significantly. This reduction was equally effective at 3- and 6-mm depths.

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Send reprint requests to: Dr. Connie L. Drisko, University of Missouri-Kansas City, School of Dentistry, 650 East 25th St, Kansas City, MO 64108.