



MICROBIOLOGY FINAL REPORT  
ANTIMICROBIAL EFFICACY OF HIGH-BIOAVAILABLE CETYLPYRIDINIUM CHLORIDE  
RINSE

Technical Analyst: John Barnes  
Microbiology Study Director: Nivedita Ramji  
Study Sponsors: Jamie Fitzgerald and Nivedita Ramji  
Study Statistician: Roger Gibb  
Study Number: OC 0009 2002  
Lab Notebooks: HCS3400, page 1 and HCL7970, page 1  
Test Facility: Product Development Microbiology Facility  
The Procter & Gamble Company  
Health Care Research Center  
8700 Mason-Montgomery Road  
Mason, Ohio 45040  
Test Products: 0.05% High-Bioavailable Cetylpyridinium Chloride (HBA-CPC) Rinse  
Water  
Testing Date: August 17, 2002 – September 13, 2002

**MICRO OBJECTIVE**

The effectiveness of CPC as a broad spectrum antimicrobial has long been recognized. A number of studies conducted over the years have confirmed not only the ability of High-Bioavailable CPC (HBA-CPC) to kill a broad spectrum of microorganisms, both *in vitro* and *in vivo*, but also demonstrated significant clinical outcomes in team tests associated with this cidal activity, including reduction and control of intrinsic oral malodor, reduction in total number of anaerobes on tongue and reduction in total number of aerobes and gram negative anaerobes in supra-gingival plaque (refer to study #s 2000048 and LLBP24).

In the current study, two products, HBA-CPC and water, were evaluated for germ kill efficacy. The objective was to demonstrate that HBA-CPC is effective in killing bacteria in saliva and maintaining its efficacy for a period of two hours post product usage. The efficacy of cumulative rinses with HBA-CPC was tested against water rinses and the saliva samples collected during this study were quantified for total aerobes and total facultative anaerobes (TFA).

**SUMMARY**

This was a randomized, two-period, two-treatment crossover design team test involving sixteen panelists.

During the first period, one group rinsed their mouths with HBA-CPC rinse a total of 13 times over a five-day period. The other group rinsed with water a total of 13 times over the same five-day period. Saliva samples were collected prior to the first rinse (baseline sampling), immediately prior to the 13<sup>th</sup> rinse (pre-rinse sampling), and at several time points from two minutes to two hours after the 13<sup>th</sup> rinse. Each saliva sample was assayed to determine the concentration of total aerobic bacteria and TFA bacteria.

John Barnes 10/4/02 JBA 10/4/02

After a two-week wash out period this procedure was repeated as the second period. The group that used HBA-CPC during the first period used water during the second, while the group that used water during the first period now used HBA-CPC. Saliva samples from the second period were collected and assayed as in the first period.

One of the 16 panelists followed an improper rinsing schedule during the first period of the test. This panelist's results, for both periods of the test, were excluded from the statistical analysis, leaving N = 15. A lab error resulted in unusable data for one panelist at the 60 minute post-HBA-CPC rinse, total aerobes plated on TSA, leaving N = 14 for this one data point (see Table 1).

A statistical analysis of the data showed that rinsing with HBA-CPC resulted in significant reductions of both total aerobic and TFA bacteria in saliva versus rinsing with water. This reduction in bacteria was evident for every post-baseline sampling time, including the samples collected immediately prior to the 13<sup>th</sup> rinse and the two-hour sampling after the 13<sup>th</sup> rinse.

### MATERIALS

#### **Media:**

Diluent for serial dilutions of saliva:

D/E Neutralizing Broth (D/E Broth)-Difco, Detroit, Michigan

Plating media for aerobic bacteria:

Tryptic Soy Agar (TSA), made in-house

Plating media for total facultative anaerobic bacteria:

Enriched Tryptic Soy Agar (ETSA), Cat. No. AS-546, Anaerobe Systems, Morgan Hills, CA

#### **Apparatus:**

Spiral plater:

Autoplate 4000, Spiral Biotech, Norwood, MA

Walk-in warm room, DV1-407

Anaerobic chamber:

Model AALC, Coy Laboratory Products, Ann Arbor, Michigan, 10% CO<sub>2</sub>, 5% H<sub>2</sub> and 85% N<sub>2</sub>

Colony counter:

Q Count Imaging System, Model 510, Spiral Biotech, Norwood, MA

### SUBJECT INCLUSION / EXCLUSION CRITERIA

The panelists in this study were asked to confirm that they not:

- Have a history of any medical or dental condition that may interfere with the study, or anticipate any dental procedures like oral surgery, visits to dentist for cleaning, root canals, teeth extraction, fillings etc. to be conducted during the course of this team test.
- Be on antibiotics or prescription mouthrinses (e.g. Peridex).
- Be a panelist / participant in any other team test during this period.
- Be a panelist / participant in any other team test at least one week prior to this study.
- Wear full or partial dentures.

On the basis of the above criteria, sixteen panelists were enrolled in this team test.

Sharon Barner 10/4/02

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## OBSERVATIONS/MEASUREMENTS

Measurements were made in colony forming units per milliliter (CFU/ml) for total aerobic and TFA populations of bacteria in saliva. CFU/ml were converted to log<sub>10</sub> CFU/ml for statistical analysis.

## STUDY DESIGN

### **Prior to Start:**

On Friday prior to the start of the study, panelists were instructed to follow a particular brush and rinse regime on Saturday and Sunday for acclimatization.

Each panelist received a "test kit" and the instructions on product usage for the first period of the study. Each test kit contained the following:

- One timer
- Two water rinse measuring containers
- Two treatment rinse measuring containers
- Two saliva collection containers
- Four Styrofoam cups with lids to transport saliva samples on ice
- One tube of Crest Cavity Protection
- Two soft toothbrushes (three were included in the test kit for the second period)
- One 470 ml bottle of HBA-CPC rinse for panelists on the HBA-CPC treatment, or no HBA-CPC for panelists on the water treatment
- Prepackaged paper towels
- Instruction sheet

The instructions for Saturday and Sunday included brushing with a brush head full of Crest Cavity Protection for one minute in the morning followed by expectoration of the dentifrice slurry and two subsequent rinses each with 20 ml water for 15 seconds. In the afternoon and at bedtime (bedtime was defined as before 11:00 PM) the panelist brushed with water for one minute followed by the same rinsing procedure as mentioned above.

### **Day 1 of Study:**

On Monday morning each panelist collected 5 ml of stimulated saliva into a sterile centrifuge tube before eating, drinking, brushing, or rinsing their mouth. The panelists then brushed their teeth with a brush head full of Crest Cavity Protection for one minute, expectorated the dentifrice slurry, and rinsed twice with 20 ml water for 15 seconds. This was followed by a 30 ml 30 second rinse with the test product (water or HBA-CPC). The saliva sample was held on ice in a Styrofoam cup provided in the kit and transported to the microbiology lab for processing. This gave the baseline reading for each panelist. The panelists refrained from eating or drinking for at least 30 minutes after the product usage. In the afternoon and at bedtime (bedtime was defined as before 11:00 PM) the panelists brushed with water for one minute followed by expectoration and two subsequent rinses each with 20 ml water for 15 seconds, followed by a 30 ml 30 second rinse with the test product (water or HBA-CPC). The panelists refrained from eating or drinking for at least 30 minutes after the product usage. This accounts for three product exposures for 30 seconds each time.

### **Days 2, 3 and 4 of Study:**

Panelists followed the same schedule as for Day 1 from Tuesday through Thursday (for a total of 12 rinses) except that no saliva was collected on Tuesday, Wednesday or Thursday.

### **Day 5 of Study:**

On Friday morning each panelist reported to the Micro Lab prior to eating, drinking, or oral hygiene. A 5 ml sample of stimulated saliva was collected from each panelist into a sterile centrifuge tube at the Micro Lab. Immediately thereafter each panelist rinsed with 30 ml of test product (water or HBA-CPC). This was the 13<sup>th</sup> exposure to the product. After the 13<sup>th</sup> rinse, saliva was collected 2, 15, 30, 60 and 120 minutes post-rinsing and held on ice until plated.

John Barnes 10/4/02

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**Wash Out Period:**

A two-week wash out period was allowed between the two treatments periods.

**MICROBIOLOGICAL ANALYSES**

**Saliva Samples:**

Saliva samples were diluted in sterile chilled D/E broth and spiral plated at  $10^{-1}$ ,  $10^{-3}$  and  $10^{-5}$  dilutions onto TSA for total aerobes and onto ETSA for TFAs. The TSA plates were incubated aerobically at  $33^{\circ}\text{C}$  while the ETSA plates were incubated anaerobically at  $35^{\circ}\text{C}$ . The TSA plates were read after 48 hours of incubation while the ETSA plates were read after 72 hours of incubation. Readings were conducted using a Spiral Biotech Q Count.

The number of colony forming units, total aerobes and TFAs, present in the saliva at baseline and post-treatment with the test product were statistically analyzed on the base 10 logarithm scale using an Analysis of Covariance for Repeated Measures. Statistically significant differences were deemed to exist between treatment groups when the treatment comparative  $p$ -value was  $\leq 0.05$ .

**RESULTS**

Table 1 and Figure 1, provided by Roger Gibb, show the log CFU/ml adjusted mean concentrations in saliva of total facultative anaerobes (plated on ETSA) and total aerobes (plated on TSA) for each post-baseline sampling time.

- "Overall Baseline" is the mean concentration of organisms in saliva on Monday morning prior to the first use of product.
- "Pre-Rinse" is the mean concentration of organisms in saliva on Friday morning immediately prior to the 13<sup>th</sup> use of product (approximately 9 hours after the 12<sup>th</sup> use of product).
- "Minute 2", "Minute 15", etc. is the mean concentration of organisms in saliva on Friday morning 2 minutes, 15 minutes, etc. after the 13<sup>th</sup> rinse.

HBA-CPC showed a statistically significant reduction, relative to water, of mean total aerobic and TFA bacteria in stimulated saliva at all time points from immediately prior to the 13<sup>th</sup> use of the product (pre-rinse) through 2 hours after the 13<sup>th</sup> use of the product.

HBA-CPC's advantage over water (reduction of bacteria relative to water, see Table 1) was:

**TFAs**

- 0.42 log immediately prior to the 13<sup>th</sup> rinse
- 2.74 log 2 minutes after the 13<sup>th</sup> rinse
- 0.70 log 2 hours after the 13<sup>th</sup> rinse.

**Total Aerobes**

- 0.45 log immediately prior to the 13<sup>th</sup> rinse
- 3.18 log 2 minutes after the 13<sup>th</sup> rinse
- 0.82 log 2 hours after the 13<sup>th</sup> rinse

It is particularly interesting that HBA-CPC exhibited a significant reduction of organisms in saliva at the pre-rinse time point. By the time this sample of saliva was collected, immediately prior to the 13<sup>th</sup> rinse, approximately nine hours had passed since the 12<sup>th</sup> rinse. Therefore, the data indicate that HBA-CPC, used multiple times over several days, has the ability to suppress the bacterial concentration in saliva for several hours.

John Borges 10/4/02

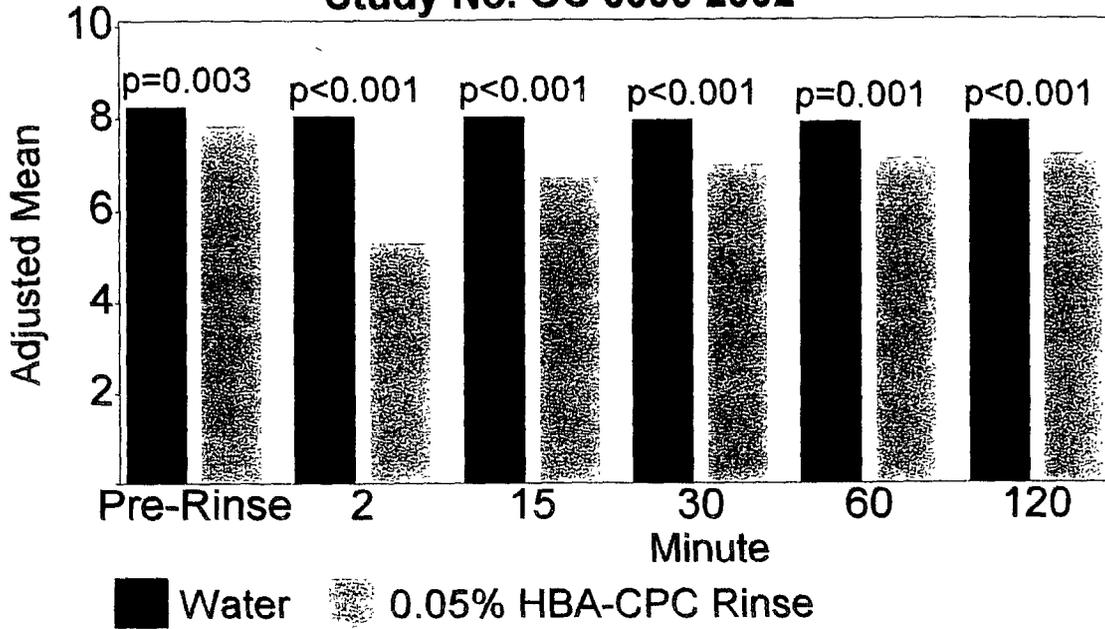
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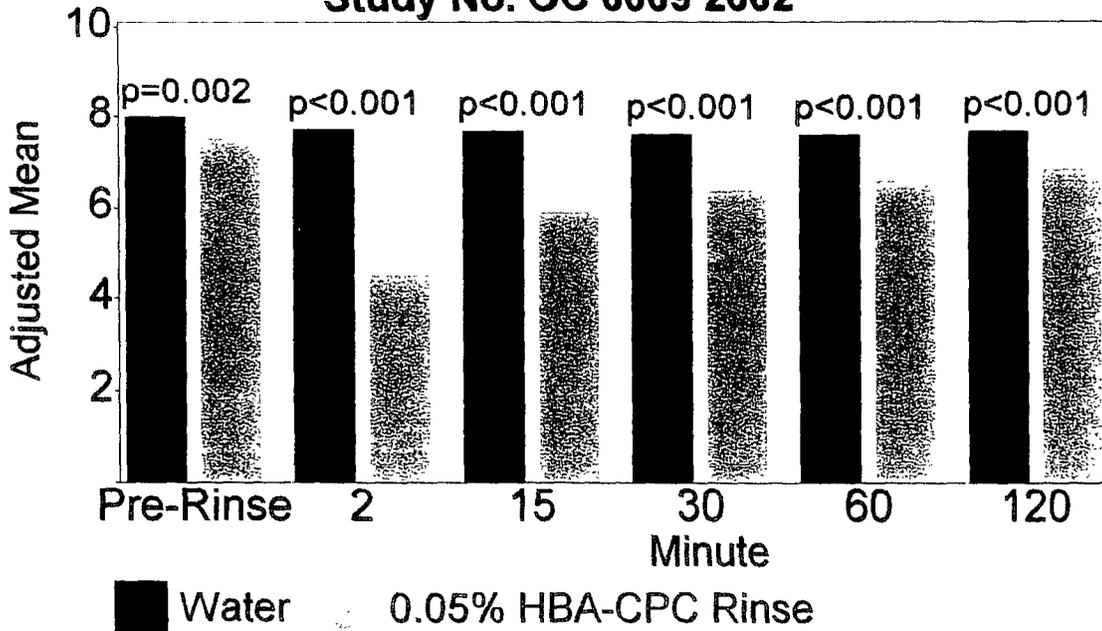
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Study No. OC 0009 2002									
TABLE 1 EFFICACY ANALYSIS – ANALYSIS OF COVARIANCE FOR REPEATED MEASURES LOG COLONY FORMING UNITS / ML									
MEDIA/ TIME OF SAMPLING	OVERALL BASELINE	WATER (PLACEBO)		0.05% HBA-CPC RINSE		ADJUSTED MEAN DIFFERENCE <sup>a</sup>	P-VALUE <sup>b</sup>	STATISTICAL MODEL VARIANCE ESTIMATES	
		N	ADJ. MEAN (SE)	N	ADJ. MEAN (SE)			SUBJECT	ERROR
<b>TOTAL FACULTATIVE ANAEROBES PLATED ON ETSA</b>									
Pre-Rinse	8.41	15	8.27 (0.083)	15	7.85 (0.083)	-0.42	0.0032	0.0069	0.0936
Minute 2	8.41	15	8.05 (0.212)	15	5.31 (0.212)	-2.74	<.0001	0	0.6633
Minute 15	8.41	15	8.03 (0.155)	15	6.73 (0.155)	-1.30	<.0001	0.0571	0.2960
Minute 30	8.41	15	7.95 (0.122)	15	6.99 (0.122)	-0.96	<.0001	0.0186	0.1986
Minute 60	8.41	15	7.89 (0.124)	15	7.12 (0.124)	-0.77	0.0009	0	0.2247
Minute 120	8.41	15	7.90 (0.094)	15	7.20 (0.094)	-0.70	<.0001	0.0378	0.0915
<b>TOTAL AEROBES PLATED ON TSA</b>									
Pre-Rinse	8.21	15	7.98 (0.093)	15	7.53 (0.093)	-0.45	0.0022	0.0283	0.1010
Minute 2	8.21	15	7.71 (0.189)	15	4.53 (0.189)	-3.18	<.0001	0	0.5310
Minute 15	8.21	15	7.68 (0.190)	15	5.93 (0.190)	-1.75	<.0001	0.0144	0.5221
Minute 30	8.21	15	7.61 (0.156)	15	6.39 (0.156)	-1.22	0.0001	0.0055	0.3578
Minute 60	8.19	15	7.60 (0.130)	14	6.61 (0.134)	-0.99	0.0003	0	0.2484
Minute 120	8.21	15	7.67 (0.092)	15	6.85 (0.092)	-0.82	<.0001	0.0108	0.1139
<sup>a</sup> Adjusted mean for the 0.05% HBA-CPC Rinse treatment minus the adjusted mean for the Water (Placebo) treatment.									
<sup>b</sup> All p-values are two-sided.									

**Figure 1**  
**ETSA Media Adjusted Mean Log CFU/mL**  
**By Treatment and Time of Sampling**  
**Study No. OC 0009 2002**



**Figure 2**  
**TSA Media Adjusted Mean Log CFU/mL**  
**By Treatment and Time of Sampling**  
**Study No. OC 0009 2002**



*John Barnes 10/8/02*

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KEY LEARNINGS

HBA-CPC, used multiple times over several days, exhibited a statistically significant reduction, compared to water, of total aerobic and total facultative anaerobic bacteria in saliva. This reduction in the concentration of salivary bacteria persisted for several hours.

DATA MANAGEMENT

This study is documented in laboratory notebooks HCS3400, page 1 and HCL7970, page 1.

ARCHIVE

This report will be filed in the Microbiology Archives.

APPROVAL SIGNATURES

Approved by: Nivedia Ranji / 9/27 Date: 10/4/02  
Microbiological Study Director

Approved by: Jamesina J. Fitzgerald Date: 10/4/02  
Microbiological Study Sponsor

Approved by: John Barnes Date: 10/4/02  
Microbiological Analyst

John Barnes 10/4/02

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J.B. 10/4/02

Statistical Analysis Report  
OC 0009 2002

**STUDY OBJECTIVE AND DESIGN**

A 0.05% high bioavailable CPC rinse treatment (0.05% HBA-CPC) was compared to a water (Placebo) rinse in a two-period cross-over design experiment. Two measurements, ETSA colony forming units/ml (ETSA CFU/ml) and TSA colony forming units/ml (TSA CFU/ml), were collected at seven times; Baseline, Pre-Rinse, and Minutes 2, 15, 30, 60, and 120 post-rinse. The washout between treatments was approximately 14 days.

**DATA ANALYZED**

Sixteen team members completed the study. The Period 2 TSA CFU/ml data for subject 9 was not available due to lab error. Subject 3 did not comply with the terms of the study and was not included in any statistical analyses. The Minute 2 data were marked too few to count (TFTC) for several subjects. These data were set to the lower limit of detection, 4000 CFU/ml, and included in the analyses. The subjects and the respective measurements that were marked TFTC for the first period Minute 2 sample were Subjects 1, 10, 12 and 15 for TSA CFU/ml and Subject 1 for ETSA CFU/ml. Similarly, the second period Minute 2 sample TSA CFU/ml data were marked TFTC for Subjects 4, 6, and 13.

**STATISTICAL METHODS**

Analysis of covariance for repeated measures methods were applied to model the mean level of each study endpoint following treatment. A separate model was fit for each endpoint using the respective baseline measurement as a covariate and including class variables for Period and Treatment. Subject was included as a random effect. Treatment carryover effect was assessed in each model. Both measurements were analyzed on the base 10 logarithm scale.

The false-positive error rate for each treatment comparison was set to 10%. All treatment comparisons were made two-sided. Due to the exploratory nature of this experiment, no attempt was made to control the experiment-wise false-positive error rate.

**RESULTS**

Study results are summarized in Table 1 and Figures 1-2. The results indicate that the 0.05% HBA-CPC treatment was statistically significantly more effective than the Placebo treatment with respect to both germ kill measures at all post-treatment time points. For both measures the largest absolute adjusted mean difference occurred at Minute 2 and decreased at each successive time point. The smallest treatment effect for both measures occurred at the Pre-Rinse measurement. ETSA log CFU/ml adjusted mean difference (Placebo minus 0.05% HBA-CPC) ranged from -2.74 to -0.42 and TSA log CFU/ml ranged from -3.18 to -0.45. There was not a significant carryover effect for either measurement.

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Study No. OC 0009 2002

TABLE 1  
EFFICACY ANALYSIS – ANALYSIS OF COVARIANCE FOR REPEATED MEASURES  
LOG COLONY FORMING UNITS / ML

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Johanna Barner 10/8/02

SB 10/8/02