UNIVERSITY OF IOWA FINAL REPORT
Contract Number 17855700 (BR01) with Dentist Select
Approved: 1/4/12
Expiration: 3/10/12

Objective:

Test a prototype mouth rinse for antibacterial activity. The species to be tested are: *Streptococcus mutans* (UA159), *Lactobacillus acidophilus* (ATCC 4356), *Porphyromonas gingivalis* (ATCC 33277), *Aggregatibacter actinomycetemcomitans* (ATCC 43718), *Tannerella forsythia* (ATCC 43037), *Treponema denticola* (ATCC 35405), *Treponema socranskii* (ATCC 35535), *Prevotella intermedia* (ATCC 49046), and *Candida albicans* (ATCC 64124).

Materials:

Dentist Select supplied three versions of the rinse. The first version, hereafter designated Rinse 1, was hand-delivered by Dentist Select in early December, 2011. This was prior to the finalization of the contract. Rinse 1 was used only in some preliminary experiments. Dentist Select subsequently sent a fresh preparation of the rinse (late December, 2011) which we designated Rinse 2 (or just "Rinse" in e-mail correspondence for relaying interim results). Rinse 2 was used for the experiments that fulfilled our contractual obligations. In mid-February, 2012, Dentist Select sent a third preparation of the rinse, designated Rinse 3, for which they requested testing on a subset of the bacterial test species. The data provided in this report will be labeled as having been derived with Rinse 2 or Rinse 3 as appropriate. On February 5, 2012, Dentist Select requested that *Pseudomonas aeruginosa* and *Proteus mirabilis* be added to the list of test species.

Experimental Design:

Protocol 1

Our initial method of choice was to measure zones of inhibition in a lawn of the test species on agar plates. The zone of inhibition may reflect temporary inhibition of bacterial growth, or bacterial killing. Chlorhexidine (0.12%) was used as a positive control, and pH4.5 buffer was used as a control for the pH of the rinse. Different types of agar plates were necessary to promote growth of the different species. For *A. actinomycetemcomitans, S. mutans, P. aeruginosa* (ATCC 47085), and *P. mirabilis* (ATCC 14153) we used Brain Heart Infusion (BHI) agar plates. For *L. acidophilus* we used Lactobacilli MRS agar (Difco), for *C. albicans* YM agar (Difco) was used, and anaerobic blood agar plates (Difco) were used for *P. gingivalis, T. forsythia,* and *P. intermedia*. For the two *Treponema* species we used ATCC 1494 Modified NOS medium.

To create a lawn of the test bacterial species, 100µl containing an estimated 4×10^6 bacteria was spread over the surface of the plate. The estimation of bacterial numbers was based on the formula: Colony Forming Units (CFU) per ml = Optical Density $(OD)_{620} \times 2.5 \times 10^8$. Next, holes were punched and suctioned out using a sterile punch and house vacuum. A volume of 10µl of the rinse, or control solutions (chlorhexidine or buffer) was added to the appropriate wells. Each agar plate had six wells, allowing duplicate testing of the rinse and control solutions. The plates were incubated at 37°C for sufficient time for the lawn to grow (this differed by species). Anaerobic species were incubated in an anaerobic chamber (85% N_2, 10% CO_2, 5% H_2). Zones of inhibition were measured as diameters, including the diameter of the well (3mm).

Protocol 2

To test bacterial killing or inhibition in broth (minimum inhibitory concentration; MIC), we used the same types of media as in protocol 1 (minus the agar). The concentration of the media was doubled and mixed with an equal volume of the rinse or control solutions so that the media components were at 1x concentration. Initial experiments were done with a bacterial concentration of 4×10^6 CFU/ml. However, the protocol was then modified to use 4×10^5 CFU/ml; all results reported below used the latter bacterial concentration. The bacteria were diluted to the desired concentration using the double strength media and 50µl added to wells of a 96-well tissue culture plate. The two components of the rinse were mixed, and within one minute appropriate amounts of rinse and water – proportions of each based on creating a series of increasing dilution – always totaling 50µl, were added to the wells containing the bacteria. A similar protocol was used for control wells containing chlorhexidine. The range of dilutions tested was from 1:2 to 1:256. For clarity, the 1:2 dilution means that one part test rinse or control was mixed with one part bacteria in medium; a 1:4 dilution indicates that one part test rinse or control was mixed with three parts bacteria in medium.

A growth control well, without test rinse or chlorhexidine, was included. The bacteria were incubated at 37°C for as long as necessary to see growth in the growth control well. Anaerobic species were incubated in an anaerobic chamber. The highest dilution at which no bacterial growth was detected was designated the Minimum Inhibitory Concentration. Since growth of *T. denticola* was below the threshold of visible turbidity, medium from each well was tested for growth on an agar plate to determine the MIC.

Results:

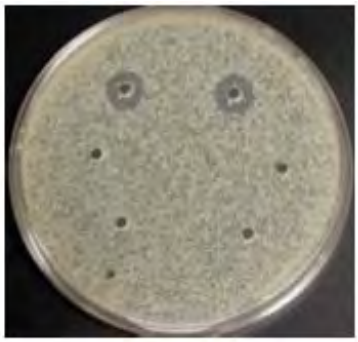
After testing multiple species using protocol 1, and not finding any zones of inhibition associated with the test rinse, we suspected that the rinse or its active ingredients did not readily absorb into the agar (examples are shown in Figure 1). Preliminary tests with the same test species using protocol 2 revealed anti-bacterial activity associated with the rinse that was not detected with protocol 1. Therefore, all subsequent testing utilized protocol 2.

Fig. 1

A. actinomycetemcomitans

S. mutans

C. albicans



Top wells: 0.12% chlorhexidine
Middle wells: Rinse 2
Bottom wells: pH 4.5 buffer

P. gingivalis

P. intermedia

Left side wells: 0.12% chlorhexidine
Middle wells: Rinse 2
Right side wells: pH 4.5 buffer

This figure shows representative plates from testing Rinse 2 for activity against various bacterial species. The zones of inhibition associated with chlorhexidine ranged from 10mm (C. albicans) to 20mm for the other species. No zones of inhibition were observed for the Rinse against A. actinomycetemcomitans, S. mutans, and C. albicans. Small (about 4mm), partial zones of inhibition (incomplete inhibition of bacterial growth) were evident for the Rinse against P. gingivalis and P. intermedia if the plates were viewed at an angle.

Table 1 lists the results of determining MICs using protocol 2. Rinse 2 was tested on the entire panel of bacterial species, whereas Rinse 3 was tested only on a subset of bacterial species. The MICs are expressed asfractions. The smaller the fraction, the higher the dilution and, in general, the more potent the test solution (or the more susceptible the bacterial species tested).

Table 1

	Minimal Inhibitory Concentration (Highest dilution of test solution that inhibits growth of the bacteria test species)			
Bacterial Test Strain	Rinse 2	Rinse 3	CHX	CHX normalized
A. a	1/32	1/256	1/256	1/8
L. acidophilus	1/2	1/16	1/128	1/4
P. aeruginosa	1/4	1/16	1/128	1/4
P. mirabilis	1/2	1/32	1/64	1/2
S. mutans	1/2	1/16	1/128	1/4
C. albicans	1/4	1/16	1/128	1/4
P. intermedia	1/2	1/8	< 1/256	< 1/8
P. gingivalis	1/4	Not Done	< 1/256	< 1/8
T. denticola	1/4	1/16	1/256	1/8
T. forsythia	1/2	Not Done	< 1/256	< 1/8
T. socranskii	1/2	Not Done	< 1/256	< 1/8

The dilutions shown are based on diluting the full strength Rinses following mixing of the two parts, or dilution of the 0.12% chlorhexidine (CHX) solution. The designation "< 1/256" (< 1/8 in the CHX normalized column) indicates that the bacteria grew at the highest dilution tested and so the MIC is actually a smaller fraction (higher dilution) than 1 to 256 (or 1 to 8 for the normalized CHX). The last column (right-most column) is a normalization based on differences in the parts per million (ppm) of the active ingredients between Rinse 2 and chlorhexidine. The active ingredients in the full strength Rinse 2 are at 37ppm whereas the ppm of chlorhexidine is 1200, or about 32-fold higher than Rinse 2. Referring to Table 2, it can be seen, for example, that a 1/128 dilution of CHX has approximately the same ppm of active ingredients as a 1/4 dilution of Rinse 2. Comparing the MICs in the Rinse 2 and CHX normalized columns gives a perspective on the relative activities of the two solutions when adjusted for equivalent ppm levels of the active ingredients.

Table 2

Dilution	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
Rinse 2 ppm	18.5	9.3	4.6	2.3	1.2	0.6	0.3	0.1
0.12% CHX ppm	600	300	150	75	38	18.8	9.4	4.7

Discussion:

The protocols selected for testing the rinse solutions provide an efficient manner for documenting anti- bacterial activity. Since the test product remains in the assays throughout the test period, a lack of bacterial growth could indicate a bacteriostatic effect or a bactericidal effect. Bacteriostatic agents prevent bacterial growth when present but do not kill the bacteria. Bactericidal agents kill bacteria. Some agents can be bacteriostatic at one concentration and bactericidal at a higher concentration. It seems likely that the Rinse is acting in a bactericidal manner since the availability of the active ingredient, chlorine dioxide, dissipates relatively rapidly after mixing the two components (based on the time-course curve of chlorine dioxide availability in the Dentist Select Rinse provided by Dentist Select in a Jan. 31, 2012 e-mail).

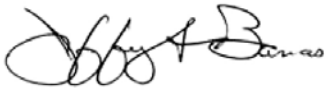
This may be an important point for another reason as well. Some scientists and clinicians question the relevancy of MIC testing for agents intended to be applied clinically for only a short time precisely because the MIC protocol keeps the agent in contact with bacteria for the duration of the assay. If the chlorine dioxide is, in fact, only available at significant concentrations for a short time during the assay, then the MIC assay is a de facto equivalent of an assay in which the effect of a brief exposure is tested. We suspect that the chlorine dioxide did not efficiently diffuse into the agar in protocol 1, or that the time necessary for the rinse to diffuse into the agar was too long relative to the availability of the chlorine dioxide. Consequently, the MIC assay was a better choice for detecting anti-bacterial activity in the Rinse.

Both protocols 1 and 2 involve a relative measure of potency. For protocol 1 it is the size of the zone of inhibition; for protocol 2 it is the MIC itself. However, the MIC or "relative potency" of an agent is only a guideline for its application. An agent with a lower MIC (higher dilution that is inhibitory) than another agent is not necessarily a ëbetterí agent. The data in Table 1 were expressed as MICs for Rinses 2 and 3 and the 0.12% chlorhexidine control. If the chlorhexidine MICs are normalized based on ppm of active ingredients, then they are sometimes higher, sometimes lower, and sometimes the same as the MICs for Rinses 2 and 3. This provides context for the potency of Rinses 2 and 3 relative to a known bactericidal agent, but again, it does not mean the Rinses are "better" or "worse" than chlorhexidine. Importantly, there was a concentration for both Rinses that inhibited the growth of every bacterial (and fungal) species tested. An appropriate conclusion to be drawn is that the MIC assays established the anti-bacterial potential of the test Rinses.

While higher or lower MICs do not of themselves establish agents as better or worse than each other, there may still be value to estimating potency. The MIC assay uses a fixed concentration of bacteria and varies the concentration of the test agent. It is probable that the more potent an agent, the higher the concentration of bacteria it will be able to kill or inhibit. This can have in vivo relevance given the high concentration of bacteria within the oral cavity, especially within dental plaque. So another assay that may provide perspective is one in which the concentration of the test agent is held constant and the concentration of bacteria is varied. Moving upwards in terms of sophistication, the Rinse could also be tested on bacteria within a biofilm rather than in a planktonic state since bacteria in vivo most often exist within a biofilm. Each of these in vitro tests represent a means of screening for in vivo potential. Agents showing potential are worthy of further study whereas agents that fail to exhibit in vitro activity will almost certainly lack in vivo activity. Thereafter, there are many other factors that will determine whether the potential shown by an agent is actually realized in vivo.

Overall Summary and Conclusion:

The experimental Rinse exhibited broad spectrum anti-microbial activity against Gram-positive and Gram-negative bacterial species common to the oral cavity, including cariogenic and periodontal pathogens, and against the opportunistic fungal pathogen *C. albicans*.



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