©Operative Dentistry, 2007, **32-4**, 388-393

In Vitro Inhibition of Bacterial Growth Using Different Dental Adhesive Systems

R Walter • WR Duarte • PNR Pereira
HO Heymann • EJ Swift Jr • RR Arnold

Clinical Relevance

All materials tested, especially iBond, have a potential long-term antibacterial effect against the oral bacteria tested.

SUMMARY

Objectives: This study evaluated the antibacterial potential of four different adhesive systems.

Methods & Materials: Gluma Comfort Bond + Desensitizer, Gluma Comfort Bond, iBond and One-Up Bond F were tested against *Streptococcus mutans*, *Streptococcus sobrinus*, *Lactobacillus acidophilus* and *Actinomyces viscosus*. The inhibition of growth by calibrated preparations was quantified by the measurement of zones of inhibition on bacterial lawns. Bactericidal activity was determined as reductions in recoverable colony-forming units in bacterial suspensions exposed to test preparations. Results: All the preparations exhibited detectable zones of inhibition for all target bacteria through six months. When the bactericidal action was evaluated, all the materials were able to kill all the tested bacteria when tested immediately after polymerization. After one week of aging, iBond was the only material that continued to kill all of the test strains.

*Ricardo Walter, DDS, MS, assistant professor, Department of Operative Dentistry, The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Wagner R Duarte, DDS, PhD, assistant professor, Department of Periodontology, Dental Research Center, The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Patricia NR Pereira, DDS, PhD, assistant professor, Department of Operative Dentistry, Dental Research Center, The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Harald O Heymann, DDS, MEd, professor, Department of Operative Dentistry, The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Edward J Swift Jr, DDS, MS, professor, Department of Operative Dentistry, The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Roland R Arnold, PhD, professor, Department of Diagnostic Sciences & General Dentistry, Dental Research Center, The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

*Reprint request: Manning Drive & Columbia Street, CB #7450, Chapel Hill, NC 27599-7450, USA; e-mail: rick_walter@dentistry.unc.edu

DOI: 10.2341/06-115

INTRODUCTION

Bacteria present in the oral cavity might lead to undesired outcomes, such as caries development.[1] Once a carious lesion is established, caries removal and an adequate cavity preparation are required as part of the restorative treatment. To overcome residual caries and

reduce the possibility of cariogenic bacteria from being left in the cavity preparation, caries disclosing dyes have become available to guide the clinician in the removal of involved tissue. It has been suggested, however, that this method is not precise and might result in either under or over preparation of the tooth structure.[2-5]

In addition to residual caries, the prevention of secondary caries development remains a challenge for dentists. It is estimated that approximately half of the restorations placed by dentists are replacements, with secondary caries being the most frequent cause of restoration failure.[6-8] Secondary caries has been described as a lesion located at the margin of an existing restoration, while the relationship between marginal integrity and secondary caries is not entirely clear.[9] Likewise, little is known about the microbial etiology of this disease.[10] It seems that bacteria similar to that found in primary caries might also be responsible for secondary caries development.[9]

Considering that no consistent method to eliminate residual bacteria and prevent secondary caries formation is currently available, the antibacterial potential of current adhesive systems is of great importance. Until the development of self-etching adhesive systems, the phosphoric acid treatment prior to adhesive application was responsible for eliminating the remaining bacteria in the cavity preparation.[11] However, recent adhesive systems do not require a total-etch step with phosphoric acid, so that cavity disinfection is dependent on the constituent components of the self-etch adhesives themselves. A recent review has assessed the antibacterial properties of bonding agents, with glutaraldehyde and 12-methacryloyloxydodecylpyridinium bromide (MDPB) being cited as possible antibacterial components contained in the current systems.[12]

Glutaraldehyde was introduced in 1984 as a possible dentin adhesion promoter for bonding systems.[13] It has since been used as part of the composition of different adhesive systems. In addition to playing a possible role in the bonding of restorative materials to tooth structure, glutaraldehyde-containing adhesive systems are likely to be antibacterial. Several studies have demonstrated an antimicrobial effect of glurataldehyde-containing bonding agents against oral bacteria.[11,14-17] However, these studies have only investigated the effects of glutaraldehyde-containing adhesive systems shortly after polymerization.

The current study investigated retention of the antibacterial activities of different adhesive systems against standard oral bacteria, following polymerization over time. The null hypothesis tested was that these adhesive systems do not have long-term antibacterial potential.

METHODS AND MATERIALS

Four dental adhesives were used in this study: Gluma Comfort Bond + Desensitizer (GL+) and Gluma Comfort Bond (GL), both of which are adhesives used with a total-etch technique; and iBond (iB) and One-Up Bond F (OUB), both of which are self-etching adhesive agents (Table 1). GL and GL+ differ due to glutaraldehyde being found in the latter. The same component is found in the composition of iB, while OUB contains fluoride.

Each bonding system was applied to 6.5 mm paper disks (Becton Dickinson and Company, Sparks, MD, USA) as follows: a drop of the material (26–33 µL, depending on the adhesive dropper) was placed on a disk using the respective dropper. The solvents and excess material were then removed by blowing air, using an air-syringe at a distance of about 5 mm for approximately 10 seconds. The adhesives on all the disks were light-cured for 20 seconds on each side at a distance of approximately 2 mm using an Optilux 501 unit (Kerr Corp, Orange, CA, USA) with an output of 700mW/cm^2. Blank disks (no material applied) were used as controls.

Inhibition Zones Assay

Four species of bacteria were used: *Streptococcus mutans* strain ATCC 10449 (serotype c), *Streptococcus sobrinus* strain NCTC 6715 (serotype d/g), *Lactobacillus acidophilus* strain ATCC 4356 and *Actinomyces viscosus* strain ATCC 43146. The stock cultures were stored in aliquots in skim milk at –80°C. Inocula from those stock cultures were cultivated in

Table 1: *Materials Compositions, Manufacturers and Batch Numbers*

Material	Manufacturer	Composition	Batch #
Gluma Comfort Bond+ Desensitizer (GL+)	Heraeus Kulzer Inc, Hanau, Germany	4-META, HEMA, UDMA, maleic acid, polycarboxylic acid ester, glutaraldehyde, ethanol, water, CQ	020047
Gluma Comfort Bond (GL)	Heraeus Kulzer Inc, Hanau, Germany	4-META, HEMA, UDMA, maleic acid, polycarboxylic acid ester, ethanol, water, CQ	010048
iBond (iB)	Heraeus Kulzer Inc, Hanau, Germany	4-META, UDMA, glutaraldehyde, acetone, water, CQ	010047
One-Up Bond F (OUB)	Tokuyama Corp, Tokyo, Japan	Methacrylate, water, fluoroaluminosilicate glass filler	042 (liquid A), 5391(liquid B)

Wilkins-Chalgren Anaerobic (W-C) broth (Oxoid Ltd, Basingstoke, Hampshire, UK) in an atmosphere of 5% CO_2/10% H_2/85% N_2 (Coy anaerobic chamber, Coy Laboratory Products Inc, Ann Arbor, MI, USA) after being screened by Gram-staining to confirm purity. The bacteria also were cultivated on sheep blood agar plates for the analysis of colony morphology and confirmation of purity. Loopful inoculations of *S mutans*, *S sobrinus*, *L acidophilus* and *A viscosus* were transferred to 10 ml of appropriate broth and incubated at 37°C under anaerobic conditions. Bacterial suspensions were prepared to 0.5 MacFarland standard.[18] Equal aliquots of the W-C broth were then added to each bacterial inoculation and 200 µl was taken and spread-plated using a "hockey stick" and turntable to ensure confluent bacterial distribution on the plates.

The specimens were immediately placed on freshly inoculated agar plates to provide initial values, or they were stored in the dark, submersed in distilled water (approximately 7 ml) at 37°C and placed on freshly inoculated spread plates after 24 hours, one week, one month, three months and six months of storage. Each plate contained five disks: one disk of each adhesive group and a blank disk. The plates were anaerobically incubated for 48 hours at 37°C. This assay was performed twice in triplicate, totaling six specimens per subgroup. The diameter of the zone of inhibition of bacterial growth around the disks was measured using a caliper. This approach was designed to emulate the standard Kirby-Bauer test used to test antimicrobial susceptibility,[18] a standard method currently recommended by the National Committee for Clinical Laboratory Standards.[19] If a zone of inhibition was not evident around the disk, it was removed to determine whether there was growth under the disk. If inhibition was evident under the disk, this outcome was recorded as a qualitative observation, indicating retention of antibacterial activity on the surface, however, it was not meant to be interpreted as a 6.5 mm diffusion zone.

Bactericidal Activity

Cultures of each bacteria were adjusted to 1.0 x 10^8 colony forming units (CFU) ml^{-1} (as determined by A_{660nm} compared to a standard curve) in sterile 20 mmol potassium phosphate buffered saline (PBS, pH 6.8), and 100 µl of this suspension was placed on the surface of the cured adhesive specimen or a blank disk. After incubation at 37°C for one hour, the suspension was diluted by adding 100 µl of PBS. The bacteria were recovered by vigorously shaking the inoculum using a vortex mixer. The recovered bacteria suspension was serially (10-fold) diluted with PBS and spread plated (100 µl) onto W-C agar plates. The plates were incubated for 48 hours at 37°C. The number of recovered viable bacteria was then determined by counting the colonies of countable dilutions (dilutions yielding 30 to 300 CFU per plate) and the recoverable CFU/ml of the original suspension was calculated. A 100-fold reduction in recoverable CFU/ml, when compared to that with blank disks, was interpreted as significant killing. Besides one-hour specimens, polymerized samples were stored for one day and one week prior to testing. This experiment was performed in triplicate.

Each of the four test materials was treated in the same manner and compared with that from the blank disks for recoverable CFU as described above.

RESULTS

Figures 1 through 4 show the measurements of the inhibition zones for the adhesive systems tested according to the different bacteria. Most of the diffusible activities of GL and GL+ were lost one day after curing.

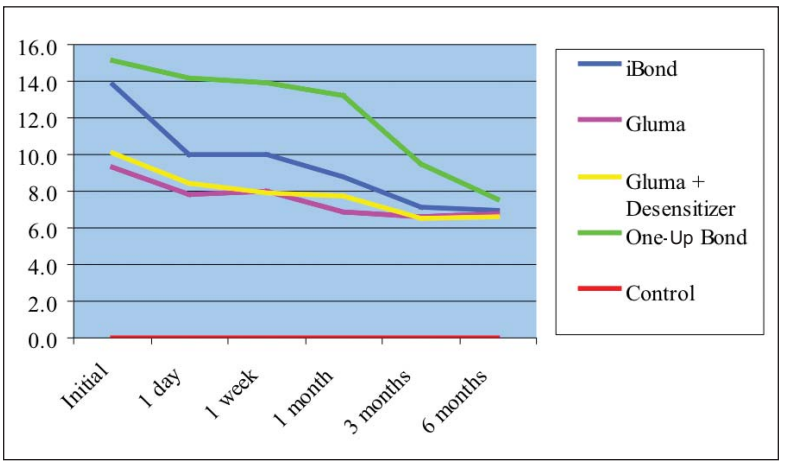

Figure 1: *Inhibition zones measurements of iB, GL, GL+ and OUB against* S mutans.

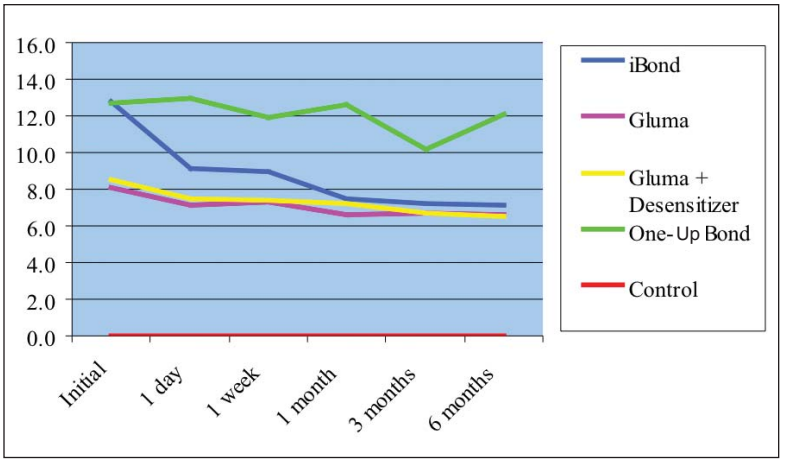

Figure 2: *Inhibition zones measurements of iB, GL, GL+ and OUB against* S Sobrinus.

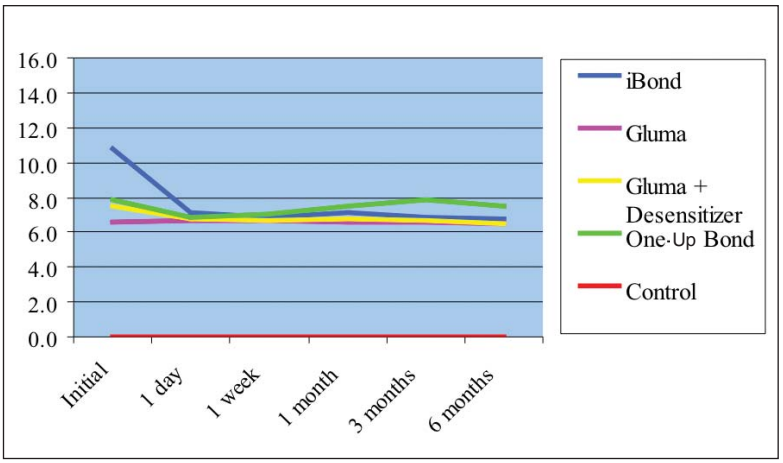

Figure 3: *Inhibition zones measurements of iB, GL, GL+ and OUB against* L acidophilus.

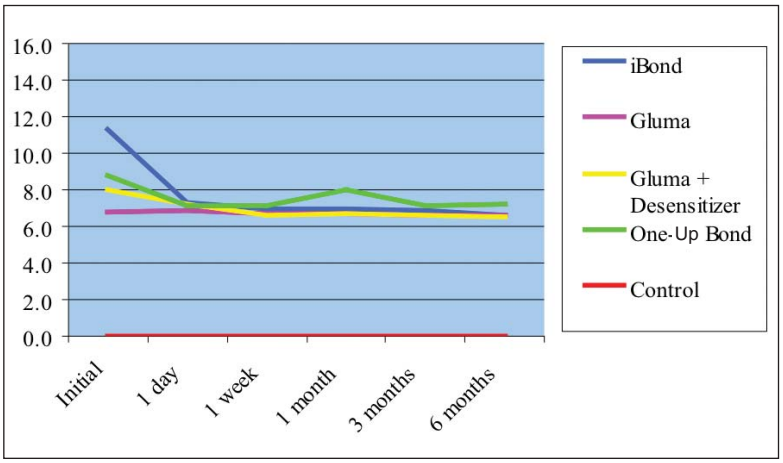

Figure 4: *Inhibition zones measurements of iB, GL, GL+ and OUB against* A Viscosus.

Table 2: *CFU ml^{-1} of* S mutans *Counting After Direct Bacterial Killing Assay*

	Initial	1 Day	1 Week
Control	$8x10^4$	$4x10^6$	$4x10^6$
GL	0	$8x10^5$	$2.4x10^6$
GL+	0	$2x10^5$	$4x10^5$
iB	0	0	0
OUB	0	0	$1.2x10^5$

Table 3: *CFU ml^{-1} of* S sobrinus *Counting After Direct Bacterial Killing Assay*

	Initial	1 Day	1 Week
Control	$8x10^5$	$1.2x10^6$	$2x10^5$
GL	0	$4x10^4$	$4x10^5$
GL+	0	$6x10^4$	$4x10^4$
iB	0	0	0
OUB	0	0	$1.2x10^5$

OUB showed diffusible inhibition of growth of all target strains at each of the test times through six months. The inhibition of bacterial growth noted with iB, especially against mutans streptococci, tended to be greater than the inhibition recorded for GL and GL+. When no diffusible inhibition was detected, the disks were lifted and the absence of lawn growth was noted. All materials tested inhibited development of the bacterial lawn immediately under the disk even when aged up to six months prior to testing. These observations are consistent with the surface retention of antimicrobial activities but should not be interpreted as other than qualitative evidence of activity. No inhibition of bacterial growth was present under the control blank disks with any of the bacterial lawns.

Results of the bactericidal assay showed that all the tested materials were capable of killing the test strains at the initial time. OUB killed mutans streptococci up to one day of aging. iB was the only adhesive system tested that was able to kill all the bacteria through at least one week of aging. Considering the variation in bacterial concentration presented among the controls and, therefore, the sensitivity of the test, no difference in activity could be determined among GL, GL+ and OUB. A minimal difference of 100-fold was set *a priori* as a significant biological difference in activity when compared to the controls, which was not presented at any time (Tables 2-5).

DISCUSSION

The assays revealed differences among the adhesives in terms of diffusability of their growth inhibiting properties and their bactericidal effects against the different target bacteria, especially after storage.

The two Gluma products lost bactericidal activity and diffusible activity after one day of aging, and their activities were equivalent despite their differences in glutaraldehyde content. To date, no study reported in the literature has used two products with the same composition, except for the presence of glutaraldehyde to test its effect as part of the material composition. The lack of difference in antibacterial activity between those two products suggests that glutaraldehyde, which is a known disinfecting agent, might exhibit a transient antimicrobial effect, or its effect might later be suppressed by other components of GL+ after polymerization and aging. On the other hand, iB, which also contains glutaraldehyde but in a different formulation, showed a tendency to inhibit

Table 4: *CFU ml^{-1} of* L acidophilus *Counting After Direct Bacterial Killing Assay*

	Initial	1 Day	1 Week
Control	$4x10^6$	$8x10^6$	$4x10^6$
GL	$4x10^2$	$3.2x10^7$	$4x10^7$
GL+	0	$1.2x10^6$	$2.4x10^6$
iB	0	0	0
OUB	0	$2.8x10^5$	$8x10^6$

Table 5: *CFU ml^{-1} of* A viscosus *Counting After Direct Bacterial Killing Assay*

	Initial	1 Day	1 Week
Control	$4x10^6$	$2.4x10^6$	$1.2x10^7$
GL	0	$1.2x10^7$	$4x10^7$
GL+	0	$8x10^5$	$4x10^6$
iB	0	0	0
OUB	0	$2.8x10^5$	$8x10^6$

mutans streptococci better than GL+ and retained its bactericidal activity against all the tested strains through at least one week of aging. Differences between the compositions of iB and GL+ might have influenced the effect glutaraldehyde had against the tested bacteria.

The fluoride-containing product (OUB) showed potent diffusible antimicrobial activity against the mutans streptococci through at least six months of aging and lost its bactericidal activity after one week of aging. A recent study demonstrated the short-term antibacterial effects of OUB against oral bacteria.[20] It was suggested that this effect of OUB against bacteria was probably related to the low pH of the material. The data of the current investigation suggest that factors, such as fluoride content in OUB formulation, other than low pH, make up the differential. Among the selected bacteria, *L acidophilus* is the most resistant bacterium to acidic environments and *A viscosus* is the least resistant. Since OUB had a similar effect against both bacteria, no direct correlation between pH and the presented antibacterial activity should be made. On the other hand, fluoride contained in the composition of OUB might have been the component responsible for this bactericidal effect up to one day, especially for its well-known effect against mutans streptococci. Regardless of the component responsible for this material activity, its ability to kill bacteria was lost sometime between one day and one week. From that point, the material was still able to inhibit bacterial growth according to the first assay. Moreover, the agar plate is a buffering environment that should minimize the effect of a low pH.

It is important to emphasize that the inhibition of the bacterial growth assay was complemented by the bactericidal experiment that showed the mechanism of action of these products. While the first experiment showed that all materials were able to inhibit bacterial growth in contact and, in some cases, away from the disks, the second experiment showed that the materials were also able to kill the bacteria.

Based on the overall data evaluated from the inhibition zones assay, it can be speculated that the materials tested might be able to keep the tested bacteria and, consequently, their end products away from clinically placed restorations. Bacterial binding to the tooth/restoration interface or leakage through a marginal gap might not occur for a specific period of time when using these materials.

Equally important is the effect that the tested materials have on residual bacteria left in the preparation prior to the restorative procedure *per se*. In a clinical scenario, the materials are freshly applied to a cavity preparation that might contain some residual bacteria, then light-cured before the restoration is placed. To test the effect that the materials have on bacteria in contact with and away from the surface, a direct bacterial killing assay was performed. This assay was designed to determine how much killing of bacteria would occur within one hour. It is important to emphasize that this assay was done to determine the mechanism of action of the materials and, as in the previous assay, it was not checked for any effect of the materials on the metabolism of the bacteria.

Lastly, there are a few issues that have to be noted as limitations of the assay. First, the interactions that the adhesive components have with dentin, which might affect their possible antibacterial effect, are not well understood. When adhesive materials are tested after being applied to dentin, an increase or a decrease in antibacterial effect has been shown.[21-22] Second, the inhibitory properties of solid materials placed on agar plates are dependent upon the hydrophilicity of the material. The diffusion coefficient in agar gel and the ability of the material to wet the agar surface might not correspond to what occurs clinically.[23] Finally, different amounts of the adhesive systems remained in the disks after removing the excess material (data not shown). Twice as much material was found in the OUB disks than in their counterparts, all of which have similar droppers. These differences should be considered if comparisons among materials are made.

CONCLUSIONS

These assays revealed potentially important differences in the antimicrobial properties of distinct formulations of dental adhesives. It was demonstrated that the tested materials have a potential antibacterial

effect against representative strains of oral plaque bacteria with cariogenic potential. This effect was demonstrated to be long-lasting in an *in vitro* simulation. Further studies need to be performed to clarify the action that glutaraldehyde has on the metabolism of cariogenic oral bacteria.

Acknowledgement

The authors thank Eric Simmons and Dr Hong-Hong Wei for their help with the laboratory experiments. This study was supported by grant #5-58834/0-110-4375 from Heraeus Kulzer Inc.

(Received 21 August 2006)

References

1. Bergenholtz G, Cox CF, Loesche WJ & Syed SA (1982) Bacterial leakage around dental restorations: Its effect on the dental pulp *Journal of Oral Pathology* **11(6)** 439-450.
2. Anderson MH, Loesche WJ & Charbeneau GT (1985) Bacteriologic study of a basic fuchsin caries-disclosing dye *The Journal of Prosthetic Dentistry* **54(1)** 51-55.
3. Boston DW & Graver HT (1989) Histological study of an acid red caries-disclosing dye *Operative Dentistry* **14(4)** 186-192.
4. Boston DW & Liao J (2004) Staining of non-carious human coronal dentin by caries dyes *Operative Dentistry* **29(3)** 280-286.
5. Banerjee A, Kidd EA & Watson TF (2003) *In vitro* validation of carious dentin removed using different excavation criteria *American Journal of Dentistry* **16(4)** 228-230.
6. Burke FJ, Cheung SW, Mjör IA & Wilson NH (1999) Reasons for the placement and replacement of restorations in vocational training practices *Primary Dental Care* **6(1)** 17-20.
7. Burke FJ, Cheung SW, Mjör IA & Wilson NH (1999) Restoration longevity and analysis of reasons for the placement and replacement of restorations provided by vocational dental practitioners and their trainers in the United Kingdom *Quintessence International* **30(4)** 234-242.
8. Burke FJ, Wilson NH, Cheung SW & Mjör IA (2001) Influence of patient factors on age of restorations at failure and reasons for their placement and replacement *Journal of Dentistry* **29(5)** 317-324.
9. Mjör IA & Toffenetti F (2000) Secondary caries: A literature review with case reports *Quintessence International* **31(3)** 165-179.
10. Fontana M & González-Cabezas C (2000) Secondary caries and restoration replacement: An unresolved problem *Compendium of Continuing Education in Dentistry* **21(1)** 15-30.
11. Emilson CG & Bergenholtz G (1993) Antibacterial activity of dentinal bonding agents *Quintessence International* **24(7)** 511-515.
12. Imazato S (2003) Antibacterial properties of resin composites and dentin bonding systems *Dental Materials* **19(6)** 449-457.
13. Munksgaard EC & Asmussen E (1984) Bond strength between dentin and restorative resins mediated by mixtures of HEMA and glutaraldehyde *Journal of Dental Research* **63(8)** 1087-1089.
14. Felton D, Bergenholtz G & Cox CF (1989) Inhibition of bacterial growth under composite restorations following GLUMA pretreatment *Journal of Dental Research* **68(3)** 491-495.
15. Fraga RC, Siqueira JF Jr & de Uzeda M (1996) *In vitro* evaluation of antibacterial effects of photo-cured glass ionomer liners and dentin bonding agents during setting *The Journal of Prosthetic Dentistry* **76(5)** 483-486.
16. Herrera M, Carrión P, Bravo M & Castillo A (2000) Antibacterial activity of four dentin bonding systems *International Journal of Antimicrobial Agents* **15(4)** 305-309.
17. Schmidlin PR, Zehnder M, Göhring TN & Waltimo TM (2004) Glutaraldehyde in bonding systems disinfects dentin *in vitro The Journal of Adhesive Dentistry* **6(1)** 61-64.
18. Bauer AW, Kirby WM, Sherris JC & Turck M (1966) Antibiotic susceptibility testing by a standardized single disk method *American Journal of Clinical Pathology* **45(4)** 493-496.
19. National Committee for Clinical Laboratory Standards (1979) *Performance Standards for Antimicrobial Disc Susceptibility Tests: Approved Standard ASM-2* 2nd ed NCCLS, Villanova, P aeruginosa.
20. Imazato S, Kuramoto A, Kaneko T, Ebisu S & Russell RR (2002) Comparison of antibacterial activity of simplified adhesive systems *American Journal of Dentistry* **15(6)** 356-360.
21. Schmalz G, Ergücü Z & Hiller KA (2004) Effect of dentin on the antibacterial activity of dentin bonding agents *Journal of Endodontics* **30(5)** 352-358.
22. Ergücü Z, Hiller KA & Schmalz G (2005) Influence of dentin on the effectiveness of antibacterial agents *Journal of Endodontics* **31(2)** 124-129.
23. Prati C, Fava F, Di Gioia D, Selighini M & Pashley DH (1993) Antibacterial effectiveness of dentin bonding systems *Dental Materials* **9(6)** 338-343.