

# In vitro evaluation of the antibacterial activity of cured dentin/enamel adhesive incorporating the antimicrobial agent MDPB

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## SUMMARY

The aim of this study was to investigate in vitro the antibacterial effect of a dentin/enamel adhesive containing MDPB (Clearfil Protect Bond) after curing and to compare it with an adhesive not containing MDPB (Clearfil SE Bond). The antibacterial effect of the cured adhesives was tested on *Enterococcus faecalis* by the evaluation of growth inhibition in a 48-h microplate direct contact test (DCT). The presence of antibacterial activity due to soluble components was also investigated by an agar diffusion test (ADT) performed with the adhesives cured on the surface of dentin disks. In the DCT, both the tested materials showed complete inhibition of bacterial growth until the 7-h reading, regardless of whether direct contact with the bacterial inoculum was continued or limited to 1 h. Stationary growth phase turbidity levels were observed at 24 or 48-h incubation. The ADT produced no inhibition areas around the dentin disks. Both adhesives tested in vitro under conditions simulating those encountered in the restorative treatment of dental caries were equally able to delay bacterial growth demonstrating a similar bacteriostatic effect. As suggested by both the DCT and the ADT, the antibacterial effect of the two adhesives depends on direct contact and does not seem to be related to the diffusion of soluble components.

**KEY WORDS:** Dentin/enamel adhesives, MDPB, MDP, Antibacterial activity, Direct contact test, Agar diffusion test

Received January 30, 2009

Accepted May 05, 2009

## INTRODUCTION

The complete removal of carious dentin during cavity preparation is a primary goal in the treatment of dental caries. However, dentin can still show a low level of infection after conventional caries removal (Kidd *et al.*, 1996; Kidd and Banerjee, 2001).

Moreover, when restorative materials are placed on the cavity wall, complete sealing at the bonded surface is not always achieved. Residual bacteria harboured on the excavated lesions and leakage of bacteria through microgaps after restora-

tions are known to be the main cause of secondary caries and damage to the pulp (Bergenholtz *et al.*, 1982; Grieve *et al.*, 1991; Ratledge *et al.*, 2001). Therefore, the use of restorative materials, including adhesive systems, that maintain antibacterial activity even after being placed in a cavity may provide a supplementary treatment contributing to the suppression of residual infection and increasing the survival of the restored tooth. In the past decade, Imazato *et al.* reported that the incorporation of the antibacterial monomer 12-methacryloyloxydodecylpyridinium bromide (MDPB) is an effective method of providing dentin primer with antibacterial activity within the primer solution (Imazato *et al.*, 1997; Imazato *et al.*, 1998a).

The primer incorporating MDPB was shown to be promising for inactivating residual bacteria in cavities and, based on these experimental results, a self-etching/priming system with cavity disinfecting effects, *Clearfil Protect Bond*, was devel-

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oped (Imazato *et al.*, 1998a; Imazato *et al.*, 2003; Imazato *et al.*, 2004). Incorporation of MDPB with the phosphoric monomer (MDP) into resin-based materials was suggested to improve the bonding ability of the materials to tooth substrate, but also proved to enhance the bactericidal activity of the immobilized agent, possibly due to the influence of covalent bonding (Imazato *et al.*, 1998b).

In the past, the antimicrobial activity of root canal sealers was assessed using the agar diffusion test (ADT). Unfortunately, this technique is not suitable for testing water-insoluble materials, but it would be useful to test the eventual release of antibacterial substances and might detect unpolymerized residuals.

Recently, agar diffusion through dentin disks has been used to assess the behavior of dentin bonding agents under conditions encountered in the oral environment (Schmalz *et al.*, 2004). In 1996, Weiss *et al.* (Weiss *et al.*, 1996) described a direct contact test (DCT) assay designed to evaluate water insoluble antibacterial materials.

The DCT has been used to evaluate the *in vitro* antibacterial activities of quaternary ammonium nanoparticles immobilized in resin-based materials and of many endodontic sealers, such as the zinc-oxide-eugenol (ZOE)- and epoxy resin-based sealers, those ones containing calcium hydroxide, and the conventional glass-ionomer-based and compomers endodontic sealing cements (Beyth *et al.*, 2006; Gomes *et al.*, 2006; Matalon *et al.*, 2006; Pizzo *et al.*, 2006; Davidovich *et al.*, 2007).

The aim of this study was to determine the antibacterial effect of the dentin/enamel adhesive, *Clearfil Protect Bond*, containing both MDPB and MDP, compared to an adhesive with the same composition but not containing MDPB, *Clearfil SE Bond*, after curing under conditions simulating those encountered in the restorative treatment of caries.

For this purpose, a DCT was performed with the polymerized material applied to the wells of a microplate. Furthermore, an ADT was performed from the materials cured on the surface of sterile dentin disks. *Enterococcus faecalis* was used for the susceptibility tests because of its implication in endodontic failures and in primary root canal infections (Siqueira *et al.*, 2002; Rocas *et al.*, 2004; Siqueira and Rocas, 2004; Pirani *et al.*, 2008).

## MATERIALS AND METHODS

### *Materials tested*

Two self-etching dentin/enamel adhesives were tested: *Clearfil Protect Bond* (CPB; Kuraray Medical, Tokyo, Japan), containing MDPB and fluoride in its primer and *Clearfil SE Bond* (CSEB; Kuraray Medical) with the same composition with the exception of MDPB. Both adhesives include two components, primer and bonding, and were applied and photopolymerized following the manufacturer's instructions. A LED curing unit (Elipar Freelight 2, ESPE, Grafenau, Germany) was used for light-polymerization with an exposition of 1000 mW/cm<sup>2</sup> for 20 seconds.

### *Test microorganism and growth conditions*

A collection strain of *Enterococcus faecalis* (ATCC 29212; American Type Culture Collection, Rockville, MD) was used to evaluate the antibacterial activity of the two adhesives. Bacteria from frozen stock cultures were grown aerobically to late logarithmic or early stationary phase in brain heart infusion (BHI) broth (Oxoid Ltd, Basingstoke, UK) at 37°C. Cells were harvested by centrifugation and resuspended in fresh medium. Inocula were prepared by adjusting the cell suspension to predetermined optical densities (ODs) corresponding to 10<sup>8</sup> CFU/ml.

### *Direct contact test (DCT)*

The DCT was performed in 96-well microtiter plates following the method of Weiss *et al.* (Weiss *et al.*, 1996), with minor modifications. Both the materials tested were applied with a microbrush to the side wall of four wells of the microtiter plate and light-polymerized. Ten microliters of the bacterial inoculum (approximately 10<sup>6</sup> bacteria) were placed on the polymerized materials and direct contact between bacteria and the tested materials was allowed for 1 h in a humid atmosphere at 37°C (group A wells). BHI broth (250 µl) was then added to each well and gently mixed for 2 min. A 50-µl inoculum was transferred from group A wells to an adjacent set of four wells containing 200 µl of fresh medium each (group B wells). This resulted in two sets of four wells for each resin, so that the bacterial growth could be monitored both in the presence and absence of the tested material. Two sets of four uncoated wells served as positive control and were inocu-

lated with 10 µl of bacterial suspension (group A growth control) and 50 µl of 1:25 diluted inoculum (group B growth control), respectively. The negative control consisted of two sets of wells coated with the tested materials and containing uninoculated fresh medium (250 µl). Plates were incubated at 37°C in a humid chamber. The bacterial growth was monitored by densitometric measurement in a microplate reader (Multiskan MCC/340, LabSystems, Helsinki, Finland) recording the OD at 600 nm every 30 min for 7 h, and after 24 and 48 h incubation. At each recording time, the mean OD value was calculated from the negative control wells and it was subtracted from the value obtained from group A wells. All experiments were carried out under aseptic conditions and repeated twice to ensure reproducibility. The data obtained at 24 h and at the end of 48 h incubation were subjected to one-way ANOVA and post hoc comparisons were done using the Fisher's protected least significant difference (PLSD) test.

#### *Agar diffusion test (ADT)*

Dentin disks with a 6-mm diameter and 2-mm thickness were cut from non-carious human molars and sterilized by autoclaving (121°C for 25 min). These sterile dentin disks were used for the ADT. Two disks were obtained from each tooth: one was coated with CPB and the other with CSEB. Following light-polymerization, the coated disks were applied face down to the surface of BHI agar plates inoculated with 100 µl of *E. faecalis* suspension, and incubated at 37°C for 48 h. Plates were inspected at 24 and 48 h for the presence of inhibition zones around the disks. The test was performed in triplicate and uncoated dentin disks were used as controls.

## RESULTS

#### *Direct Contact Test*

The results of the DCT are shown in Figure 1. Each point on the growth curve is the average of the OD measurements in eight wells at every time interval, four each in two separate experiences. The negative control wells containing uninoculated adhesives showed high baseline turbidity levels (ranging from 500 to 600 OD units) that were subtracted each time from the values cal-

culated from group A wells. When compared to the positive control wells, bacterial growth was delayed in both test groups with both of the tested materials. Both CPB and CSEB showed complete inhibition of bacterial growth until the 7-h reading regardless of whether the direct contact with the bacterial inoculum was continued (group A wells) or limited to 1 h (group B wells). Due to the high baseline turbidity levels, turbidity in group A wells was considered to be indicative of the progression towards or the achievement of a stationary bacterial growth phase only when the OD reading exceeded the mean absorbance of the uninoculated wells by at least 500 OD units. On the contrary, bacterial growth in group B wells was considered to have taken place if turbidity was at least equal to the value of the mean of ODs in positive controls minus two standard deviations. According to these criteria, bacterial growth was determined after ≤7-h, 24-h and 48-h incubation as shown in Table 1. At the end of the test, turbidity levels suggesting stationary growth phase were observed in four group A and four group B wells of the CPB test, and in five group A and three group B wells of the CSEB test. Statistical analysis of turbidity level measurements indicated that both materials significantly reduced bacterial growth when compared to controls at 24-h ( $P < 0.0001$ , Fisher's PLSD) and 48-h incubation ( $P = 0.0006$  for CPB and  $P = 0.0016$  for CSEB) in the continuous contact test (group A) and at 24-h incubation in the 1-h contact test (Group B;  $P = 0.0218$  for CPB and  $P = 0.0373$  for CSEB). There was no significant difference ( $P > 0.2$ ) between the two materials when compared to each other regardless of the test protocol used. Plating of the content of the wells after 48-h incubation confirmed that the increased OD in high turbidity wells was due to *E. faecalis* and that viable bacteria were still present in low turbidity wells (data not shown).

#### *Agar Diffusion Test*

No inhibition areas were observed around the coated dentin disks used for the test after 24 and 48 h incubation, indicating no diffusion of antibacterial substances. Only one of the three CPB treated disks showed a small inhibition spot, corresponding to a limited portion of its perimeter, whose width was 1 mm after 24-h and 2.5 mm after 48-h incubation.

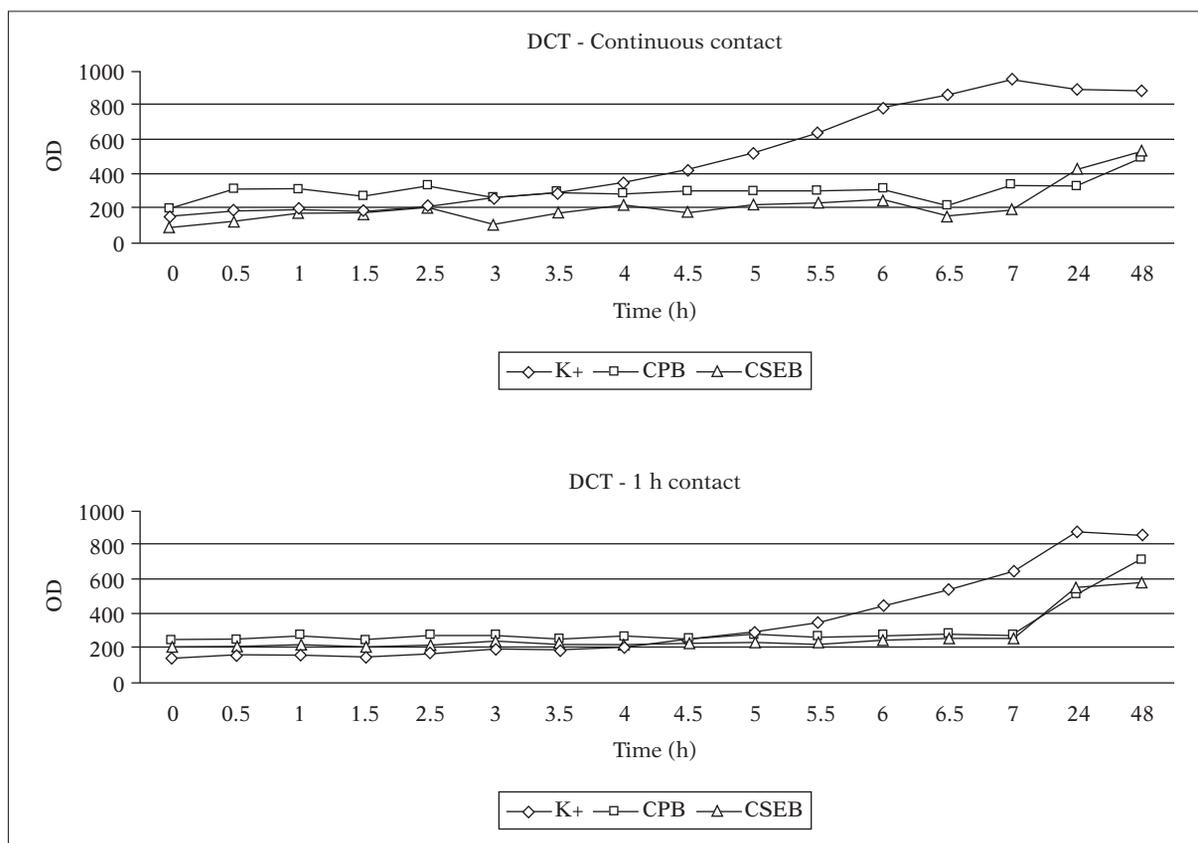


FIGURE 1 - Direct contact test (DCT) of dentin adhesives. Turbidometric readings were recorded for 48 h in the continuous presence (top) and in the absence after 1 h contact (bottom) of the tested materials. Each point on the growth curve is the mean of the OD measurements in two separate sets of four wells. K+ = growth control.

TABLE 1 - Bacterial growth in the DCT determined after  $\leq 7$  h, 24-h and 48 h incubation performed in the presence (group A wells) and in the absence (group B wells) of the tested material.

Tested material	Group wells	Wells showing bacterial growth after		
		$\leq 7$ h	24 h	48 h
CPB	A*	0/8	1/8	4/8
	B <sup>§</sup>	0/8	3/8	4/8
CSEB	A*	0/8	4/8	5/8
	B <sup>§</sup>	0/8	3/8	3/8

\*Turbidity in group A wells was considered to be indicative of bacterial growth only when the OD reading exceeded by at least 500 OD units the average absorbance of the uninoculated wells.

§Bacterial growth in group B wells was considered to have taken place if turbidity was at least equal to the value of the mean of the ODs of positive controls minus two standard deviations.

## DISCUSSION

Incorporation of MDPB and MDP has been shown to be an effective method of providing resin-based restorative materials with antibacterial activity (Imazato *et al.*, 1997; Imazato *et al.*, 1998a; Imazato *et al.*, 2004; Kuramoto *et al.*, 2005; Lobo *et al.*, 2005; Imazato *et al.*, 2006; Yoshikawa *et al.*, 2007). The primer incorporating unpolymerized MDPB has been demonstrated to possess bactericidal effect before being cured (Imazato *et al.*, 1999). Cured adhesive resin incorporating MDPB is also able to inhibit bacterial growth on its surface and this bacteriostatic effect was ascribed to non-released immobilized bactericide (Imazato *et al.*, 1998b; Imazato *et al.*, 2003).

To date, the DCT is considered to be the most valuable in vitro assay to study the antimicrobial properties of solid dental materials (Weiss *et al.*,

1996; Cobankara *et al.*, 2004). The DCT provides a quantitative measure of the effect of direct and close contact between microorganisms and the tested material on microbial outgrowth, and allows a distinction to be made between bactericidal or just bacteriostatic effects. Another advantage of the DCT is its ability to follow bacterial growth in both the presence and absence of the tested material, allowing not only the direct contact effect, but also the effect of those components which are capable of diffusing into the liquid medium to be measured. Recently, Eldeniz *et al.* tested the antibacterial effect of the CPB and CSEB adhesives using DCT, and conclude that the two materials did not show any significant difference against *E. faecalis* in fresh samples, although the MDPB-containing bonding inhibited *E. faecalis* more effectively than CSEB in 3-day set samples (Eldeniz *et al.*, 2006). However, these Authors did not follow the bacterial growth in the presence of the tested materials, which is a better simulation of the condition encountered in vivo.

The results of the DCT performed in the presence (group A wells) and absence (group B wells) of the tested materials showed that the CPB resin containing MDPB delayed bacterial growth in both of the test conditions. However, based on our findings, the antibacterial effect does not seem to be entirely linked to the presence of the immobilized antimicrobial MDPB, since the resin not incorporating MDPB also exhibited comparable bacteriostatic activity. Both materials tested delayed bacterial growth equally in the continuous and in the 1-h contact condition (group A and B wells, respectively). This finding would be consistent with the absence of diffusible antibacterial components. Accordingly, the absence of inhibition areas in the ADT suggests that if antibacterial components were released by the adhesives, they were not diffusible in agar. Finally, the local inhibition spot occasionally produced by the CPB resin in the ADT might be related to the antibacterial effect of unpolymerized residuals.

Antibacterial properties of dentin/enamel adhesives have also been suggested to depend on components that were originally incorporated to promote adhesion (Atac *et al.*, 2001). The intrinsic antibacterial activity of the unpolymerized dentin primer of the CPB system has been demonstrat-

ed to be superior to that of a primer containing only MDP using an agar disc-diffusion test (Imazato *et al.*, 2006). The results of our study, performed after curing under conditions simulating those encountered in the restorative treatment of caries, indicate that the antimicrobial effectiveness of the dentin/enamel adhesive containing both MDPB and MDP is equivalent to that of the one not containing MDPB. As already suggested by surface growth tests (Imazato *et al.*, 1998b; Imazato *et al.*, 2003), the antibacterial effect of both tested materials was bacteriostatic and was shown to depend on direct contact and not to be related to the diffusion of soluble components. Further research is needed to investigate the exact mechanism of action of the different components of the adhesives tested and to assess the role of unpolymerized residuals.

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