

## **The antibacterial activity of three endodontic sealers against *Enterococcus faecalis* in vitro**

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### **Abstract**

Conventional root canal therapy consists of chemo-mechanical treatment of the root canal to eliminate the microbial infection. The root canal is obturated with a root filling, which normally consists of a core and an endodontic sealer. The purpose of the sealer is to obturate the root canal, and should in addition have an antibacterial activity against bacteria that are left in the root canal after instrumentation and cleaning. The aim of this study was to investigate the antibacterial activity of three endodontic sealers with different chemical composition, AH Plus, RealSeal SE and TotalFill against *Enterococcus faecalis* in vitro. **Methods:** Agar diffusion test (ADT), Direct contact test (DCT) and Modified direct contact test (MDCT) were used to evaluate the antibacterial activity of the three endodontic sealers. **Results:** For the ADT a small clear zone of inhibition could be identified around AH Plus, which were absent for Real Seal SE and TotalFill. For the DCT, no bacterial growth was evident for all three sealers AH Plus, RealSeal SE and TotalFill compared to the control inoculum. This was in accordance with the MDCT, which showed that all three sealers eliminated all bacterial after 1 hour contact time with the sealers. **Conclusions:** Freshly prepared samples of AH Plus, Real Seal SE and TotalFill showed bactericidal activity against *E. faecalis*.

### **Introduction**

Root canal therapy is the main method for treatment of an infected pulp in modern dentistry. The etiology of pulpitis and apical periodontitis are microbes and their byproducts. Conventional root canal therapy consists of chemo-mechanical treatment of the root canal to eliminate the microbial infection. After cleaning and shaping, the root canal is obturated with a root filling, which normally consists of a core and an endodontic sealer. The principal functions of a root filling material is to prevent bacteria from invading the root canal space and infect the root canal system after completed endodontic treatment. This is accomplished by the formation of a tight, permanent seal with the surrounding tooth structure, leaving no space for invasion or colonization by bacteria. The materials can also have a direct antibacterial effect and kill the bacteria when in contact with them.

Root filling material such as endodontic sealers should have the following attributes; biocompatibility, ability to penetrate the dentin tubules, and it should be bactericidal or bacteriostatic. However, there is a positive correlation between antibacterial effects of sealers and their cytotoxic effect.<sup>1</sup>

*Enterococcus faecalis*, the most frequently recovered bacteria from refractory periapical periodontitis, has been used in numerous studies to assess the antibacterial properties of disinfecting agents because of its resistance to some medicaments and its ability to survive conventional root canal therapy.<sup>2</sup> According to Sundqvist et al. in 1998<sup>3</sup>, as much as 38% of root canal treatment failures contained *E. faecalis*.

Several studies have been done in the recent years to assess the antimicrobial activity of different endodontic sealers.<sup>4 5 6</sup> The aim of this study was to investigate the antibacterial

efficiency of three endodontic materials, an epoxy-amine resin based sealer AH Plus (De Trey Division Dentsply, Weybridge, England), a methacrylate based resin sealer, RealSeal SE (SybronEndo, USA), and a bioceramic based sealer TotalFill (Peter Brasseler Holdings, LLC, FKG Dentaire SA, Switzerland). The antibacterial effects of the three endodontic sealers were evaluated using the agar Diffusion Test (ADT), Direct Contact Test (DCT) and Modified Direct Contact Test (MDCT).

## **Material and methods**

### ***Endodontic sealers***

The three endodontic sealers, AH Plus, RealSeal SE and TotalFill, were prepared following the manufacturers recommendations. AH Plus were mixed using its own AH Plus Jet, Mixing Syringe and Mixing Tips with Intra Oral Tip. RealSeal SE was mixed using mixing syringe included in the package. TotalFill was applied directly with its tip included in the package. Freshly mixed materials of AH Plus and Real Seal SE were used after 20 minutes of setting time. TotalFill were covered with 30  $\mu$ L water for setting for 1 hour according to Zhang et al (2009).<sup>2</sup>

### ***Bacteria***

*Enterococcus faecalis* ATCC 19433 (American Type Cell Culture Collection) were grown aerobically from frozen stock cultures and were stored at -80°C in TSB (Oxoid) supplemented with 15% glycerol. For the antibacterial assays the bacteria were grown overnight (ON: 18 hours) in Tryptone Soya Broth (TSB, Oxoid) at 37°C, 5% CO<sub>2</sub> supplemented atmosphere. For the DCT and the MDCT, the ON culture was centrifuged at 5000 g x 5 min at room temperature before resuspended in PBS (Phosphor Buffered Sialine) to an Optical Density at 600 nm (OD<sub>600</sub>) of 1.0. From this solution 1 ml were centrifuged 5000 g x 5 min at room temperature and resuspended in 500  $\mu$ L corresponding approximately to  $2 \times 10^8$  CFU mL<sup>-1</sup>.

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

The ADT was performed essentially as described by Weiss et al. (1996).<sup>7</sup> Briefly, ON cultures of *E. faecalis* were diluted 1:10 in pre-warmed TSB. From this solution, 200  $\mu$ L were spread on TSB agar plates and left to dry before dots of approximately 5 mm diameters of each sealer were applied onto the agar plates. The agar plates were incubated at 37°C, 5% CO<sub>2</sub> supplemented atmosphere for 24 h. The day after, the agar plates were examined for bacterial growth inhibition.

### ***Direct contact test - (DCT)***

The DCT was also performed as described by Weiss et al. (1996) and was used for AH Plus and Real Seal SE. It is performed by determination of bacterial growth in 96-well microtiter plates. The bacterial outgrowth is measured by using Optical density at 600 nm at 37°C and recorded by Synergy™ H1, Hybrid Reader Bio-Tek, every hour for 18 hours. The microtiter plate with the tested materials and bacteria were automixed before each recording to ensure a homogeneous bacterial cell suspension.

Group A	Material and control (TSB) + bacteria $1 \times 10^6$ CFU in 10 $\mu$ L add 300 $\mu$ L after 1 hour incubation (without transfer)
Group B	Transfer 40 $\mu$ L from wells marked green into wells with 220 $\mu$ L TSB = Total volume 260 $\mu$ L (with transfer)
Group C	Material and control alone without bacteria with 260 $\mu$ L TSB

**Table 1: DCT test in microtiter wells.** Shows the module of wells and the sealers application in it. 3 sets of wells were used for both AH Plus, Real Seal SE and the control medium had 2 wells.

A 96-well microtiter plate with flat bottom was held vertically. One of the side walls of 3 wells was coated with freshly mixed materials of AH Plus and Real Seal SE. Even and thin coating was achieved by using a small size round ended dental instrument. Special care was taken to avoid the material's flow to the bottom of the well, which would interfere with the light path through the microtiterplate well and result in false readings. Approximately 20 min later, which corresponded to the recommended working time of the sealers, a 10  $\mu$ L of the PBS solution containing  $2 \times 10^8$  CFU  $\text{mL}^{-1}$  was placed on the mixed material.<sup>7</sup> While the plate remained in vertical position, wells were inspected for evaporation of the suspension's liquid, which occurred within 1 h at 37°C. This ensured direct contact between bacteria and tested material. TSB (300  $\mu$ L) was added to each of the Group A 1 wells and gently mixed for 2 min; 40  $\mu$ L were then transferred from Group A wells respectively to an adjacent set of 3 wells containing fresh medium (220  $\mu$ L) designated as Group B. This resulted in two sets of 3 wells for each tested material containing an equal volume of liquid medium, so that bacterial growth could be monitored both in the presence and in the absence of the tested material. Two sets of wells in the same microtiter plate served as control, Group C. The plate was placed for incubation at 37°C in the Synergy™ H1 microplate reader and the optical density in each well at 600 nm was followed automatically for 18 h. Growth after 18 hours incubation were also evaluated by visual inspection.

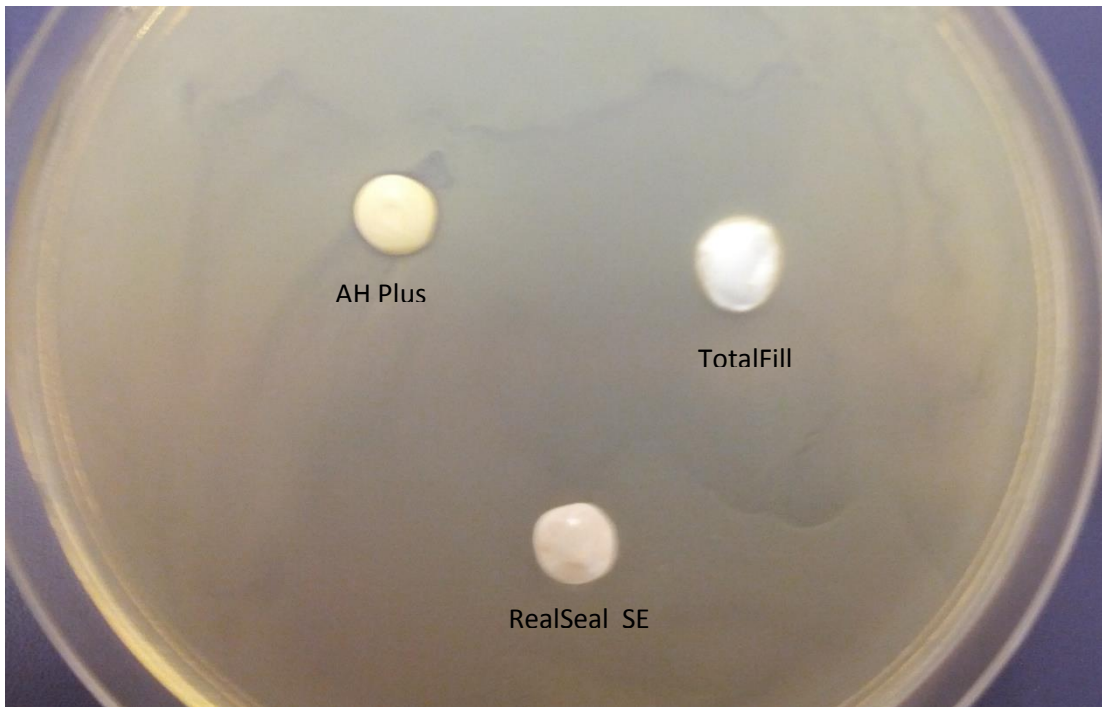
#### ***Modified direct contact test – (MDCT)***

The MDCT method is described by Zhang et al. (2009)<sup>2</sup> and was essentially performed like the DCT described above for AH Plus and RealSeal SE. However, TotalFill needs moisture to initiate its setting reaction<sup>6</sup>, therefore 30  $\mu$ L sterile distilled water (SDW), were applied to both TotalFill and AH Plus as control, after mixing and application of sealers in the microtiter wells. The hardening process took one hour in incubation at 37°C. There was added 300  $\mu$ L PBS in the bacterial suspension, and thereafter 100  $\mu$ L of the bacteria suspension with PBS was plated directly on TSB agar plates and incubated ON at 37°C, 5% CO<sub>2</sub> supplemented atmosphere. For wells with bacteria and TSB serial dilution was performed before plating. After incubation ON, the colonies on the plates were counted.

## Results

### *Agar diffusion test*

The first test to evaluate the antibacterial activity of the sealers was done by performing the ADT. The antibacterial effect is measured when a halo is formed, called inhibition zone, around the tested material on the agar plate. The size of this zone reflects the antibacterial effect of the sealer. The ADT showed that freshly mixed AH Plus made an inhibition zone. There were no inhibition zones observed for RealSeal SE and TotalFill (Fig.1).

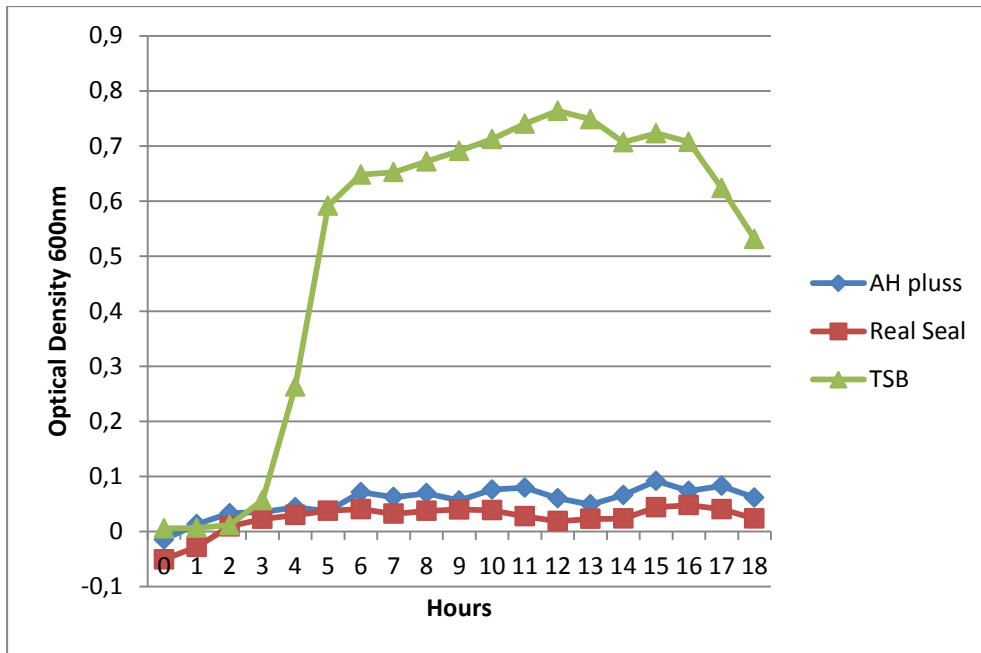


**Fig.1: ADT for AH Plus (yellow), RealSeal SE (pink) and TotalFill (white).**

The ADT showed that freshly mixed AH Plus had a small clear zone of inhibition (halo). There were no inhibition zones observed for RealSeal SE and TotalFill.

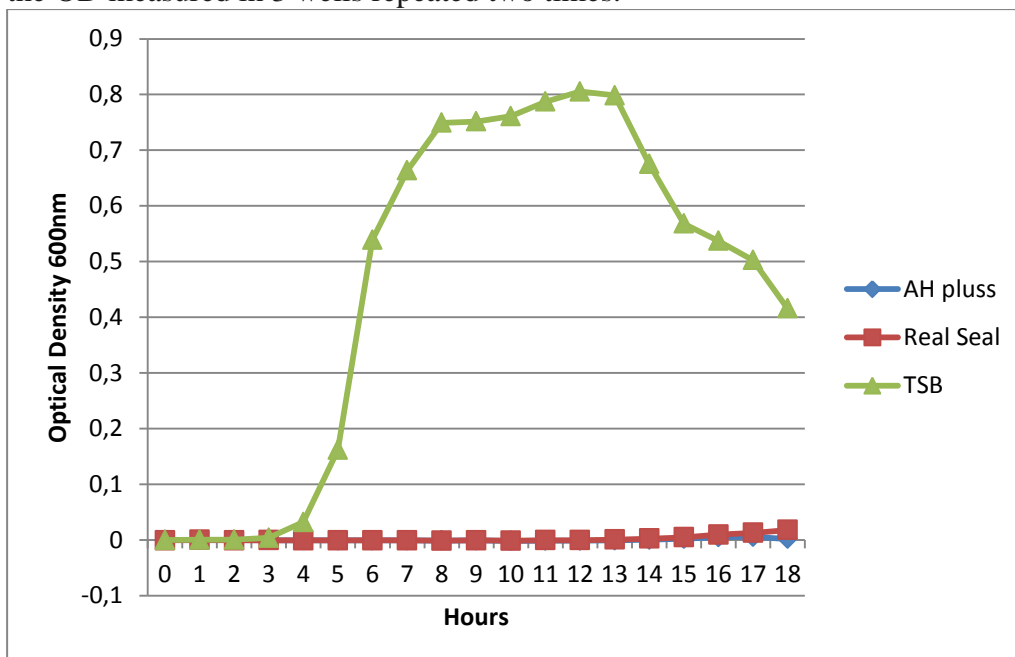
### *Direct contact test*

DCT was used to evaluate the bacterostatic and bactericidal effect of the sealers by measuring the growth rate of the bacteria by calculating the average of OD over time (18 hours). Both AH Plus and RealSeal SE showed bactericidal effect against *E. faecalis*. No growth was observed in either Group A wells (Fig.2) and Group B wells (Fig.3).



**Fig.2: DCT for Group A wells for AH Plus, RealSeal SE and TSB.**

Antibacterial effect of endodontic sealer AH Plus (blue curve) and RealSeal SE (red curve) using DCT in a timeframe of 18 hours (x-axis) at an Optical Density of 600 nm (y-axis) and the control inoculum, TSB (green curve). Each point on the growth curves is the average of the OD measured in 3 wells repeated two times.

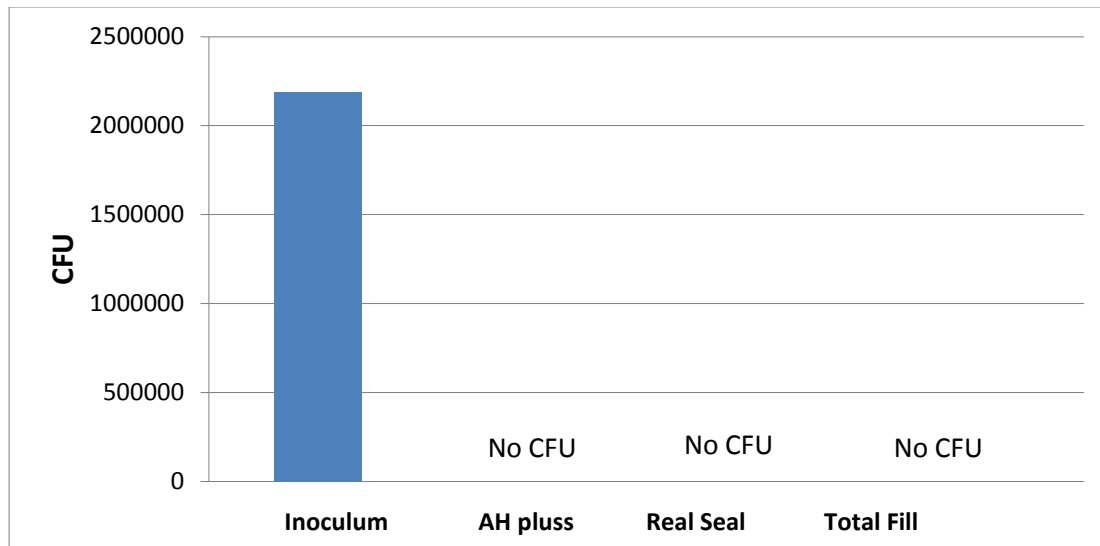


**Fig.3: DCT for Group B wells for AH Plus, RealSeal SE and TSB.**

Antibacterial effect of endodontic sealer AH Plus (blue curve) and RealSeal SE (red curve) using DCT in a timeframe of 18 hours (x-axis) at an Optical Density of 600 nm (y-axis) and the control inoculum, TSB (green curve). Each point on the growth curves is the average of the OD measured in 3 wells repeated two times.

### Modified direct contact test

The MDCT is done to measure the bactericidal effect instead of bacteriostatic effect of the materials. We can directly calculate the exact numbers of surviving bacteria after each contact time by counting colony-forming units. We report that all three sealers AH Plus, RealSeal SE and TotalFill were bactericidal against *E. faecalis* and killed all bacteria after 1 hour contact time with the material (Fig.4).



**Fig.4: MDCT for AH Plus, RealSeal SE, TotalFill and Inoculum**

MDCT shows the bactericidal effect of the sealers by counting colony-forming units (CFU y-axis) of the tested after 1 hour incubation. AH Plus, RealSeal SE and TotalFill killed all of after 1 hour contact time. The experiment was performed three times with three parallels for AH Plus and Real Seal SE and one time with three parallels for TotalFill.

### Discussion

Persistence of bacteria after root canal treatment may lead to persistent infection and treatment failure. *E. faecalis* is associated with persistent apical periodontitis, and have therefore been chosen as the test microorganism for this study.<sup>5</sup> The endodontic sealers are used in root canal therapy to eliminate the microorganisms after the chemomechanical preparation and to prevent recolonization of the root canal system. A sealer should be biocompatible and dimensionally stable, as well as having a long-lasting antibacterial effect.<sup>8</sup> The antibacterial effects of endodontic sealers have been investigated several times by using ADT, DCT and MDCT.<sup>7 2</sup>

The ADT was long the most commonly used test method.<sup>9 10 11</sup> However, there are many limitations of this test that are well known nowadays. This method does not only depend on the materials toxicity to a given microorganism, but is also depend on diffusion and affinity of the material in bacteria suspension. Materials with easier diffusion abilities may produce larger bacterial inhibition zones.<sup>12</sup> The results obtained did not reflect the true antibacterial potential for the various sealers. Therefore, ADT is no longer the only recommended test to evaluate the antibacterial activity of endodontic sealers. There are also other difficulties experienced when comparing the results of various studies using ADT because there have been used different strains and growth media. The viability of the microorganism's ability to test and compare also lacks by using ADT. Another drawback is

inability to distinguish between the bacteriostatic (bacteria-inhibiting) and bactericidal (bacteria-killing) effect of the endodontic sealer. ADT has its limitations<sup>13</sup> and it was chosen to compare the findings with the DCT method.<sup>7</sup>

The results for ADT showed a small inhibition zone for AH Plus. The study done by Gomes et al. (2004)<sup>14</sup> showed no inhibition zone when tested for AH Plus against *E. faecalis*. The ADT test gave no results for Epiphany (RealSeal), which are also the findings of other studies conducted.<sup>15</sup> <sup>12</sup>According to Maekawa et al (2012)<sup>15</sup> there was no antibacterial activity, no inhibition zone or halo formed on the tested microorganisms, including *E. faecalis* for Epiphany, but AH Plus showed a inhibition halo compared to Epiphany. The same finding was done by Pinheiro (2009)<sup>12</sup>, and they compared their results in comparison with Bodrumlu (2006). Most authors that performed ADT tests with various microorganisms always concluded that *E. faecalis* showed a higher resistance to the evaluated sealers than any other bacteria tested. TotalFill gave no inhibition zone either and there are not performed any studies with ADT for this sealer to compare our results with.

The DCT is a quantitative method and provides information on growth rate of the bacteria. At the same time, it's a reproducible method that mimics the direct contact between and the endodontic sealers inside a root canal. In DCT, the optical density of the suspension allows detecting the growth and prevention of growth (bacteriostatic effect). An advantage of the way the DCT is setup, is the fact that it can follow the bacterial growth whether the sealer is present or not. By using DCT not only is the direct antimicrobial activity of the tested endodontic sealers assessed, but also components of endodontic sealers capable of diffusing into the medium may be assessed.<sup>16</sup>

The challenge facing us when we did the DCT test was that during the 18 hours where the 96-well microtiter plates were being scanned and optical density was being measured, some of the sealer would tear off the wells sidewalls and end up as loose particles at the bottom. This would result in less amount of light being transmitted through the wells, which would show up on the results as higher number of cells present in that well. This problem was the main reason why we had to remove other endodontic materials such as the ProRoot MTA cement from our study. MTA had a much shorter working time than the other sealers. It would easily get detached from the sidewall and float on top of the well as a flake shaped matter. This made it impossible to get a correct DCT read of ProRoot MTA cement. The other three sealers which were applied using their own syringe were much more user friendly.

The DCT was modified by plating the bacteria suspension from wells, and the inoculum from controls directly on TSB agar after 1 hour contact time. This may reduced the risk of carryover effect compared to standard DCT. It also provided us with the opportunity to directly evaluate the bactericidal effect of the sealers.

There have been a numerous studies of which antimicrobial activity of different endodontic sealers has been tested. Zhang et al. (2009)<sup>2</sup> concluded that fresh AH Plus killed *E. faecalis* effectively. Pizzao et al. (2006)<sup>17</sup> also used the DCT method to measure the antibacterial activity of AH Plus and it reported that only fresh AH Plus showed antibacterial activity. They also concluded that the samples of AH Plus that were a day or a week older did not show any antibacterial activity.

## **Conclusion**

We report that the three endodontic sealers tested, AH Plus, RealSeal SE and TotalFill, shows good antibacterial activity. From freshly prepared sealers all (100%) of the bacteria was killed within the 1 hour contact time for AH Plus, Real Seal SE and TotalFill. Future studies that would be interesting is to evaluate the antibacterial activity of these endodontic sealers within a timeframe from 2 minutes to 60 minutes. In addition, the sealers should be tested for their

antibacterial activity after 1, 3 or 7 days, and the pH in solution could be monitored.

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