Original Article

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In vitro antibacterial activity of oligomer-based and calcium silicate-based root canal sealers

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Objective: To evaluate antibacterial effects of root canal sealers, oligomer-based (OB) and calcium silicate (BC) in comparison to epoxy-resin (AH Plus) and zinc oxide-eugenol (ZOE), against *Enterococcus faecalis*.

Materials and Methods: Antibacterial effects of the root canal sealers were evaluated by the modified direct contact method using 96-well plates. Each sealer was filled on the bottom of wells at 1-mm thickness, 20 wells of each sealer. The wells of each group was further divided into five subgroups depending on testing periods after sealer setting, i.e.- 20 min, 24 h, 3, 7, and 14 days. Next, 200-µl aliquot of *E. faecalis* (5×10^5 CFU) was placed in the well containing the set sealer and kept at 37°C for 24 h. The wells containing the bacterial suspension (without any sealer) and sterile culture media were used as positive and negative controls. Survival of bacteria were determined by 10-fold serial dilution in Brain-Heart Infusion (BHI) broth and cultured on BHI agar. In addition, elution test was carried out by incubating the bacterial suspension to culture media that exposed to the set sealers for 20 min, 24 h, and 3 days. Statistical analysis was conducted using Kruskal-Wallis non-parametric test (α=.05).

Results: From the direct contact test, at 20 min and 24h, BC sealer and AH Plus showed strong bactericidal effects while OB sealer did not display any antibacterial effect. At 3 days, antimicrobial effect of BC sealer was significantly reduced while AH Plus did not show the antibacterial effect. At 7 and 14 days, all sealers did not possess any antibacterial activity, except ZOE sealer that had exhibited the potent bactericidal effect until 14 days. For the elution test, eluted substances from the test sealers at all setting periods did not cause any significant reduction of the bacteria.

Conclusion: The root canal sealers showed different antimicrobial activity against *E. faecalis* after setting. OB sealer showed no antibacterial effects at all periods. ZOE sealer was the most effective sealer with antibacterial activity until 14 days. Antimicrobial effects of AH Plus and BC sealers gradually decreased within 24 h and 3 days after setting, respectively.

Key words: antibacterial, calcium silicate, direct contact, *Enterococcus faecalis*, oligomer, root canal sealer

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Introductions

Root canal obturation with the obturation cone(s) and sealer plays an important role to create apical sealing and entomb remaining bacteria within the root canal. It might be beneficial if survival microorganisms that still remain after the chemo-mechanical disinfection can be further eliminated by antimicrobial effect of root canal sealer [1-3]. This antibacterial effect of sealer could also delay bacterial penetration from coronal leakage overtime if occurs. Sealing ability and antibacterial activity of various root canal sealers have been focused in the literatures [4,5].

Several types of root canal sealers are currently available and proposed to have antibacterial activity [6-11]. Zinc-oxide eugenol sealer exhibits the strong antibacterial

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effect due to the eugenol component [1,12]. Other endodontic sealers that consisting of polymer materials contain the antimicrobial effect due to cytotoxicity of the component(s). For example, antimicrobial activity of epoxy resin-based sealer (AH plus) is related to the release of bisphenol-A diglycidyl [6] or formaldehyde [13,14]. For calcium hydroxide-based and calcium silicate-based sealers, the bactericidal action is provided from the alkalinity due to the release of hydroxyl ions during setting [7,15].

Bioceramic (BC) sealer Totalfill® BC Sealer™ (*FKG Dentaire SA, La Chaux-de-Fonds, Switzerland*) is also known as iRoot SP™ *(Innovative BioCeramix, Inc., Vancouver, Canada*) or EndoSequence BC sealer™ (*Brasseler, Savannah, GA, USA*). BC sealer is a biocompatible, calcium silicate-based material that might provide antibacterial activity from slow releasing of calcium hydroxide during and after setting. Setting process of this sealer produces high alkaline pH that is toxic to bacteria [1]. The pH and Ca^{2+} release of BC sealer are significantly higher during setting duration than those of AH Plus [16]. *In vitro* studies showed that high alkaline pH of BC sealer promotes elimination of *E. faecalis* [17,18]. In contrast, its antibacterial effect greatly decreased after one week even high pH value still remained [1].

Recently, a new oligomer-based root canal sealer (OB sealer) has been developed to use with a new thermoplastic elastomer-based (TPE) root canal obturation cone. Polyolefin-based TPE showed some promising properties to produce a root canal obturation cone such as chemically inert, highly flexible, light weight and non-toxic in nature [19]. For use with TPE cone, OB sealer is based on synthetic, bi-functional oligomer, mainly consisting of acrylate or methacrylate functional group with back-bone oligomer molecules. The bi-functional group might provide adhesion to TPE cone by cross-linking polymerization between the partial polymerized functional groups of the sealer to the TPE component. From the previous study, the methacrylate-based OB sealer exhibits high biocompatibility to the L929 cultured cells [20]. However, other information of this newly developed sealer, i.e. - antimicrobial activity, is limited.

Most freshly mixed sealers exhibit highest antimicrobial activity and cytotoxicity that gradually decrease during and after setting [21,22]. Prolong antibacterial effect of the sealer is desirable to prevent reinfection or recolonization of remaining bacteria in the treated root canals. Results from laboratory studies have been limited to antibacterial activity of the initial-set sealers [7,9,11]. It might be profitable if the persisted bacteria can be further eradicated by prolong antimicrobial activity of root canal sealers. Therefore, long-term evaluation of antimicrobial activity of sealers has been suggested [23,24].

Several methods have been used to evaluate antimicrobial effect of endodontic sealers, such as agar diffusion test (ADT), direct contact test (DCT) or elution test [25-27]. Antimicrobial results from the ADT method does not only depend on antibacterial effect of material, but also relate to diffusion and solubility of material into medium [28,29]. For the DCT, it can be used to evaluate antimicrobial effect of either water soluble or insoluble substances, which overcomes the disadvantage of the ADT [28-30]. Result from DCT is based on observing the growth of bacteria after contact with the testing materials. For root canal sealer, dissolution of the components released from incomplete set materials at the early stage after mixing is possible. The antimicrobial action from these components might inhibited the growth of those bacteria, which survived from the direct contact. Antimicrobial effect from eluted-components of sealers can be evaluated via an elution test.

Among bacteria found in the infected root canals, *E. faecalis* is the most common species detected from the failed endodontic treated teeth [31]. It participates in the persistent root canal infection and is difficult to eliminate from the infected root canal [32]. Moreover, *in vitro* studies have shown its ability to invade deep into dentinal tubules [33-36]. Thus, *E. faecalis* is frequently used as bacteria of choice for antimicrobial testing in endodontic research.

The purpose of this study was to evaluate antibacterial effects of four root canal sealers, i.e. - newly developed oligomer-based (OB sealer), bioceramic (BC

sealer), epoxy resin-based (AH Plus), and zinc oxide eugenol (ZOE sealer), against *E. faecalis* using modified direct contact method and elution test.

Materials and Methods

Four root canal sealers, i.e. - oligomer based sealer (OB sealer), bioceramic sealer (BC sealer), epoxy resin sealer (AH Plus), and zinc oxide eugenol (ZOE) sealers, were tested in this study. Manufacturers, preparations, compositions, setting times and methods of use are summarized in Table 1.

Direct contact test

Modified direct contact test was conducted to evaluate antimicrobial activity of the endodontic sealers using *Enterococcus faecalis* (ATCC 29212) as bacterial indicator. All sealers were mixed and prepared following the manufacturers' instructions. Using two 96-well microtiter plates *(Sarstedt Inc., Newton, NC, USA)*, the wells were divided into 4 experimental groups of the

Table 1: Details of four root canal sealers in this study.

Details	BC sealer (TotalFill)	OB	AH Plus	ZOE sealer (MU sealer)
Manufacturers	FKG Dentaire SA, La Chaux-de-Fonds, Switzerland	M Dent, Bangkok, Thailand	Dentsply-Maillefer, Tulsa, OK, USA	M Dent, Bangkok, Thailand
Preparations	Pre-mixed syringe	Two pastes	Two pastes	Powder and liquid
Compositions	Tricalcium silicate, dicalcium silicate, calcium hydroxide, zirconium oxide, phosphate monobasic, filler and thickening agents	Bi-functional oligomer (di-acrylate), light curing initiator, self-cure initiator (peroxide), catalyst, radiopaque (barium sulfate), fillers and oil	Paste A: bisphenol-A epoxy resin, bisphenol-F epoxy resin, calcium tungstate, zirconium oxide, silica, iron oxide pigments Paste B: dibenzyldiamine, aminoadamantane, tricyclodecane-diamine, calcium tungstate, zirconium oxide, silica, silicone oil	Powder: zinc oxide, resin, bismuth subcarbonate, barium sulfate, sodium borate Liquid: clove oil
Setting types	Self-curing	Dual-curing	Self-curing	Self-curing
Setting times	4 h, (can be more than 10 h in very dry root canal)	Immediately after light curing, approximately 1-2 h self curing in anaerobic condition	8 h at 37°C	90 min
Mixing methods	No mixing is required	Mixing powder-liquid or equal volume of two pastes, on a glass slab or a mixing pad using a metal spatula, to a homogeneous consistency.		

sealers, 20 wells of each, and 2 control groups. Each sealer group was further divided into five subgroups depending on the testing periods after setting at 20 min, 24 h, 3 days, 7, days and 14 days (Fig. 1). Then, each well was filled with equal amount of freshly mixed OB sealer, BC sealer, AH Plus or ZOE sealer using a cavity liner applicator to obtain approximately 1-mm thickness of sealer. The suspension of 24-h *E. faecalis* in Brain Heart Infusion (BHI) broth was prepared at cell density of 5 x 10^5 CFU/ml. An aliquot of 200-µl bacterial suspension was placed on the surface of each sealer at different times after setting, i.e.- 20 min, 24 h, 3, 7, and 14 days. The positive controls were the wells with bacterial suspension but without sealer, and the negative controls were the wells with sterile culture media. The plates were incubated at 37 °C for 24 h. After exposure to the sealers for 24 h, the bacterial suspension from each well was gently mixed with a pipette tip, and 100 µl of suspension was transferred to another 96-well microtiter plates. The suspension was 10-fold serially diluted and cultured on BHI agar plates *(Difco Laboratories, Detroit, MI, USA)*. After incubation at 37°C for 24 h, bacterial colonies were observed and calculated into CFU/ml. The experiment was repeated twice to confirm the reliability of results (a total n = 8 of each testing period after setting/ sealer).

Elution test

To investigate whether release substances during setting of the sealers had any antibacterial effect, the elution test was performed. In brief, the 96-well microtiter plate were divided into the 4 experimental sealer groups, as previously mentioned. Each sealer was filled at the bottom of the well as previously described and then further divided into three subgroups of setting periods, i.e.- after 20 min, 24 h, and 3 days (n = 2). At each setting period, 200 µl of BHI broth *(Difco Laboratories, Detroit, MI, USA)* was added into each well containing the set sealer and then incubated at 37°C for 60 min. Next, 100 µl of the BHI broth from each well was transferred into another 96-well microtiter plate. The 100 µl of *E. faecalis* suspension (10^5 CFU) was added, gently mixed, and incubated at 37°C. The wells filled with the bacterial suspension and the culture media (without elute of sealer) were used as positive control. After 24 h, the suspension from each well was subjected to bacterial count as previously described. The experiment was repeated twice to confirm the reliability of results (a total n =4 of each testing period after setting/ sealer).

Statistical analysis

Number of bacterial colonies in CFU/ml was calculated and expressed as $\log_{10}$ CFU/ml. Mean and standard deviation (SD) were calculated. Normal

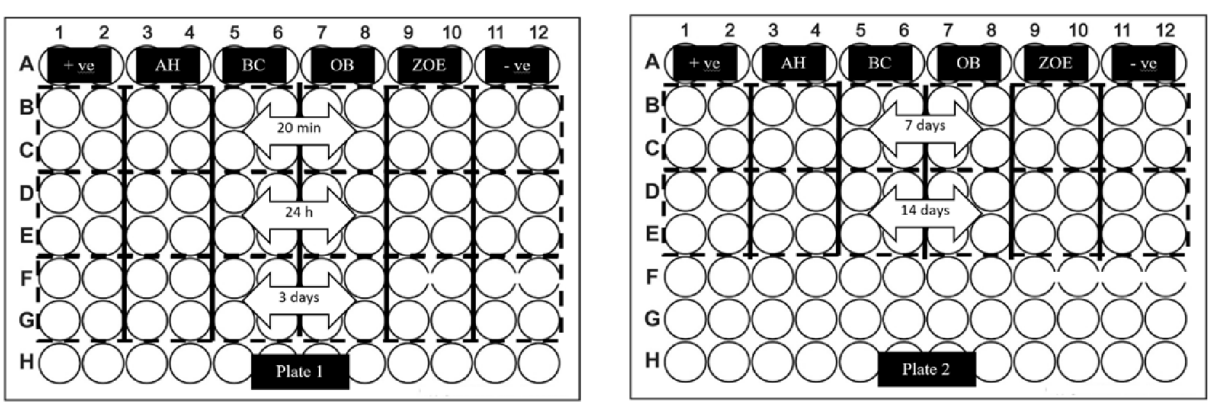


Figure 1. Illustrations present the experimental design in the modified direct contact test using 96-well microtiter plates.
Four experimental sealer groups (n = 20 each) divided into 5 subgroups of different testing periods (n = 4 each), and the positive (+ve) and negative (-ve) control groups were included; AH = AH Plus sealer, BC = BC sealer, OB = OB sealer, ZOE = ZOE sealer.

distribution of data was tested using Shapiro-Wilk test. Since the data were not normally distributed, non-parametric Kruskal-Wallis test was used to compare antibacterial effects among the tested sealers and the periods after setting at a significance level of .05.

Results

For antimicrobial activity by the modified direct contact test, the amounts of *E. faecalis* (CFU/ml) after 24-h exposure to the sealers are present in Table 2. Statistical analysis of antimicrobial effectiveness among the sealers at different periods is shown in Table 3. ZOE sealer was considered as a gold standard of antimicrobial activity since it completely eradicated the bacteria through the periods. In contrast, OB sealer did not cause any significant bacteria reduction when compared to the control at all periods ($p > .05$). Freshly mixed (at 20 min) and early set (at 24 h) AH Plus and BC sealers completely eradicated the test bacteria, which was significantly different from that of the control ($p < .05$). At 3 days, AH Plus entirely loss the antibacterial activity while BC sealer presented markedly decreased antibacterial activity (Table 3). At 7 and 14 days, the two sealers did not show any bacterial reduction that was not significantly different to that of the control ($p > .05$).

Comparing the results among the setting periods, OB sealer showed insignificant antimicrobial effect at all setting time. AH Plus had a strong antibacterial effects at 20 min and 24 h after setting that were significantly higher than those at 3, 7, and 14 days ($p < .05$). BC sealer had potent antibacterial effects after setting up to 3 days that were significantly higher than those at 7 and 14 days ($p < .05$).

Results of antibacterial activity of the sealers from the elution test are presented in Table 4 and 5. No antimicrobial effect of the eluted media 60-min exposed to the set sealer was observed. Eluted-medium from all root canal sealers did not cause significantly reduction of CFU-counts in comparison with the positive control. No statistically significant antibacterial effect was observed among tested sealers at all setting periods ($P > .05$)

Discussion

None of the sealers was able to sustain its antimicrobial activity up to one week, except ZOE sealer that the effect lasted through the experimental period of 14 days. It may imply that these endodontic sealers could eliminate residual microorganisms that have survived in root canal. However, their effects were sustained for only a limited short period. Thus, these sealers might not prevent microbial re-contamination from coronal leakage if occurs. Initial antibacterial activity of epoxy resin-based sealer might relate to the release of bisphenol-A diglycidyl component, and minor release of formaldehyde [6,13,14]. Using DCT or modified-DCT, freshly-mixed AH Plus had potent antimicrobial effect. Freshly mixed sealers are highly toxic to cells, which is caused by highly initial release of cytotoxic components at early stage of setting [21,22]. However, several studies have shown that its antibacterial effect decreased over time to ineffective level [1,27,37]. In the present study, AH Plus had significant antimicrobial effect until 24 h after setting. Its antibacterial effect was abolished within few days, which is consistent with those of the previous studies [1, 27, 37].

It is believed that bioceramic sealer provides antibacterial effect from the release of calcium hydroxide by-product, causing a very high alkaline pH that is toxic to the bacteria [1,38]. The alkaline pH from BC sealer promotes elimination of bacteria such as *E. faecalis*, *in vitro* [17,18]. The present experiment showed that BC sealer exhibited immediate, potent antibacterial effect up to 24 h after setting. However, its antibacterial effect considerably decreased within 3 days and was completely diminished at 7 days. This is in agreement to the result of *in vitro* study that reported a short antibacterial action of BC sealer against *E. faecalis,* using a modified direct contact test [1]. Increase of pH level from the release of calcium hydroxide at early stage of setting creates an unsuitable environment for bacterial growth [39]. Later, calcium hydroxide release might be reduced, so the alkaline pH level decreased. This may explain the decreased antibacterial effect over time and the disappearance after long-term setting.

Table 2: Antimicrobial activity of the root canal sealers against *E. faecalis* at different periods after setting (means ± standard deviation; total n=8).

Setting periods	Types of sealer	Number of the bacteria (× 10^9 CFU/ml)			
		Mean	Range	Mean log_{10}	Range log_{10}
		Freshly mixed sealers			
20 min	+ ve control	3.26 ± 1.64	1.72 – 6.80	9.47 ± 0.19	9.24 – 9.83
	OB	1.16 ± 0.38	0.40 – 1.64	9.03 ± 0.19	8.60 – 9.21
	AH Plus	0	0	0	0
	BC	0	0	0	0
	ZOE	0	0	0	0
		Set sealers			
24 h	+ ve control	1.32 ± 0.62	0.60 – 2.24	9.08 ± 0.21	8.78 – 9.35
	OB	0.91 ± 0.78	0.24 – 2.40	8.82 ± 0.37	8.38 – 9.38
	AH Plus	0	0	0	0
	BC	0	0	0	0
	ZOE	0	0	0	0
3 days	+ ve control	1.52 ± 0.44	0.92 – 2.32	9.17 ± 0.13	8.96 – 9.37
	OB	0.88 ± 0.44	0.28 – 1.60	8.89 ± 0.24	8.45 – 9.20
	AH Plus	1.52 ± 0.56	0.40 – 2.00	9.14 ± 0.24	8.60 – 9.30
	BC	0.27 ± 0.34	0.001 – 1.00	7.86 ± 0.98	6.16 – 9.00
	ZOE	0	0	0	0
7 days	+ ve control	1.37 ± 0.30	0.92 – 1.68	9.12 ± 0.10	8.96 – 9.23
	OB	1.42 ± 0.45	0.88 – 2.24	9.13 ± 0.13	8.94 – 9.35
	AH Plus	0.91 ± 0.24	0.52 – 1.24	8.94 ± 0.13	8.72 – 9.09
	BC	1.00 ± 0.37	0.40 – 1.60	8.97 ± 0.18	8.60 – 9.20
	ZOE	0	0	0	0
14 days	+ ve control	1.23 ± 0.20	0.88 – 1.48	9.08 ± 0.08	8.94 – 9.17
	OB	0.98 ± 0.34	0.64 – 1.48	8.97 ± 0.15	8.80 – 9.17
	AH Plus	0.86 ± 0.26	0.32 – 1.16	8.90 ± 0.18	8.50 – 9.06
	BC	0.79 ± 0.22	0.40 – 1.16	8.88 ± 0.13	8.60 – 9.06
	ZOE	0	0	0	0

+ve control = positive control, OB = OB sealer, AH Plus = AH Plus sealer, BC = BC sealer, ZOE = ZOE sealer.

Table 3: Statistical comparison of antimicrobial effectiveness against *E. faecalis* of the root canal sealers at different periods after setting (median value of $\log_{10}$ CFU/ml; total n=8).

Type of sealers	Freshly mixed sealers	Testing periods after sealer setting			
	20 min	24 h	3 days	7 days	14 days
+ ve control	9.44 [a, A]	9.07 [c, B]	9.19 [e, A]	9.16 [g, B]	9.08 [h, B]
OB	9.09 [a, C]	8.75 [c, C]	8.88 [e, f, C]	9.13 [g, C]	8.95 [h, C]
AH	0 [b, D]	0 [d, D]	9.24 [e, E]	8.98 [g, E]	8.95 [h, E]
BC	0 [b, F]	0 [d, F]	8.16 [f, F, G]	8.98 [g, G]	8.90 [h, G]

* Different lowercase letters (in column) indicate statistically significant difference ($p < 0.05$) among sealer types at each testing period.
** Different uppercase letters (in row) indicate statistically significant difference ($p < 0.05$) among testing periods of each sealer.
+ve control = positive control, OB = OB sealer, AH Plus = AH Plus sealer, BC = BC sealer.

Table 4: Antimicrobial activity of eluted-media from the root canal sealers against *E. faecalis* at different periods after setting (mean ± standard deviation; total n=4).

Setting periods	Types of sealer	Number of the bacteria (× 10^9 CFU/ml) Mean	Range	Mean $\log_{10}$	Range $\log_{10}$
		Freshly mixed sealers			
20 min	+ ve control	1.31 ± 0.40	1.00 – 1.88	9.10 ± 0.12	9.00 – 9.27
	OB	0.90 ± 0.23	0.76 – 1.24	8.95 ± 0.10	8.88 – 9.09
	AH Plus	1.27 ± 0.36	0.84 – 1.60	9.09 ± 0.13	8.92 – 9.20
	BC	1.51 ± 0.35	1.12 – 1.88	9.17 ± 0.10	9.05 – 9.27
	ZOE	0.65 ± 0.53	0.20 – 1.36	8.69 ± 0.38	8.30 – 9.13
		Set sealers			
24 h	+ ve control	1.58 ± 0.50	0.84 – 1.92	9.18 ± 0.17	8.92 – 9.28
	OB	1.53 ± 0.12	1.36 – 1.64	9.18 ± 0.04	9.13 – 9.21
	AH Plus	1.89 ± 0.34	1.52 – 2.32	9.27 ± 0.08	9.18 – 9.37
	BC	1.64 ± 0.26	1.36 – 1.88	9.21 ± 0.07	9.13 – 9.27
	ZOE	0.50 ± 0.26	0.28 – 0.88	8.66 ± 0.20	8.45 – 8.94
3 days	+ ve control	1.61 ± 0.12	1.44 – 1.72	9.20 ± 0.03	9.16 – 9.24
	OB	1.06 ± 0.37	0.80 – 1.60	9.00 ± 0.13	8.90 – 9.20
	AH Plus	1.71 ± 0.59	0.92 – 2.24	9.20 ± 0.18	8.96 – 9.35
	BC	1.60 ± 0.40	1.04 – 1.92	9.19 ± 0.12	9.02 – 9.28
	ZOE	0.40 ± 0.29	0.24 – 0.84	8.53 ± 0.26	8.38 – 8.92

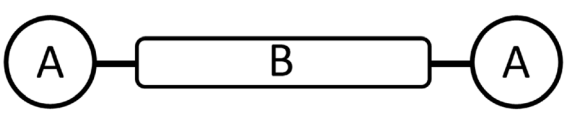
+ve control = positive control, OB = OB sealer, AH Plus = AH Plus sealer, BC = BC sealer, ZOE = ZOE sealer.

Table 5: Statistical comparison of antimicrobial effectiveness against *E. faecalis* of eluted-media from the root canal sealers at different periods after setting (median value of $\log_{10}$ CFU/ml; total n=4).

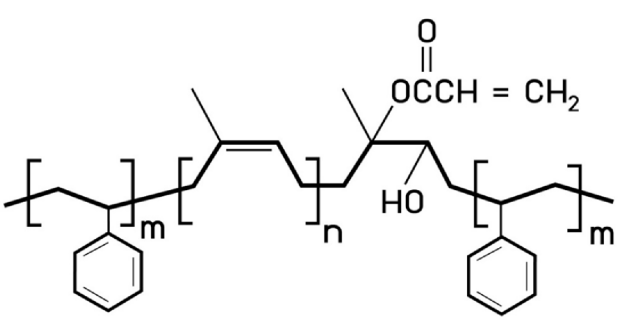
Types of sealer	Freshly mixed sealers	Testing periods after sealer setting	
	20 min	24 h	3 days
+ ve control	9.07	9.25	9.21
OB	8.90	9.19	8.96
AH	9.12	9.27	9.26
BC	9.18	9.22	9.23

* No statistically significant antibacterial effect among tested sealers at all setting periods.
+ve control = positive control, OB = OB sealer, AH Plus = AH Plus sealer, BC = BC sealer.

Effectiveness of OB sealer against the bacteria was considerably lower than those of the other sealers even at the initial period. This can be explained by the biocompatible characteristic of its structures and components. OB sealer mainly consists of acrylate/methacrylate functional group and back-bone oligomer molecule (Fig. 2). With the stable molecular structure of oligomer, instead of the monomer, it may be possible that the release of cytotoxic components is greatly reduced. The results from an *in vitro* cytotoxicity test showed the high biocompatibility of OB sealer to the cultured cells [20]. It seems that the synthetic, di-functional oligomer



A) The structure of synthetic bifunctional oligomer molecule; A = acrylate or methacrylate functional group, B = back-bone oligomer molecule.



B) Chemical structure of acrylate oligomer used in OB sealer

Figure 2. The structure of OB sealer.

structure makes the sealer become a highly biocompatible material. Nevertheless, OB sealer was light cured in this study, so a further study is required to investigate antibacterial effect in the self-curing mode. Releases of free oligomer and other components may be higher in the self-cured condition, which might affect the biocompatibility and antibacterial activity.

Antibacterial activity of ZOE-based sealer is based on the release of eugenol component, which possesses potent antibacterial effect against microorganisms [40-42]. The result of this study demonstrated that ZOE sealer had the strongest antibacterial effect against *E. faecalis* and exhibited prolonged antibacterial activity until two weeks. It is indicated that the amount of eugenol release is sustainable and high enough to inhibit bacterial growth. In accordance with the previous findings, this sealer had the greatest antimicrobial effect against microorganisms [5,26,40,43,44].

From the direct contact test, it reported the decrease of antimicrobial activity after setting of the sealers. Reduction of bacteria at the early stage might be related to direct contact between the surface of initial-set sealer and bacterial suspension. In addition, dissolution and releasing of effective components from the initial set materials could improve its antibacterial activity. Nevertheless, the results from the elution assay showed elutes of the sealers had insignificant effect on reduction of bacteria. Thus, it can be concluded that the antibacterial activity of these sealers was primarily due to the direct-contact effect between the sealer and bacteria, rather than elute of sealer.

In summary, none of test sealers was able to sustain the antimicrobial action against *E. faecalis* through 14 days after setting, except ZOE sealer. BC sealer and AH Plus provided a short antibacterial effect that were diminished within few days after setting. Light-cured OB sealer was not effective at all setting periods. Further investigation is required to verify antibacterial activity of OB sealer in the self-curing condition.

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Competing interests: None declared.

Ethical approval: Faculty of Dentistry/ Faculty of Pharmacy, Mahidol University Institutional Review Board COE. No. MU-DT/ PY-IRB 2016/010.1706.

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