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Evaluation of antibacterial activity of an experimental dental adhesive containing synthesized quaternary ammonium compound: in vitro study

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Abstract

Background Dental adhesives with immobilized antibacterial agents are formulated to combat bacterial invasion along the tooth-restoration interface. This study aims to evaluate the antibacterial effect of synthesized quaternary ammonium compound (QAC) incorporated into commercial dental adhesive.

Methods QAC was synthesized from 2-(Dimethylamino) ethyl methacrylate and 1-Bromobutane and characterized using CHN (Carbon, Hydrogen, Nitrogen), FTIR (Fourier transform infrared) and H^+NMR (Proton nuclear magnetic resonance) analyses. The synthesized QAC was assessed for its cytotoxicity and its antibacterial activity against *S. mutans* using disc diffusion method, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), time-kill kinetics test, and TEM imaging. The QAC was added to the primer of a commercially available adhesive (OptiBond XTR) at two concentrations; 20 and 40 mg mL^{-1} representing the MIC and MBC, respectively. The antibacterial properties of the experimental adhesives, commercial antibacterial adhesive Clearfil SE Protect containing 12-methacryloyloxydodecylpyridinium bromide (MDPB), and commercial vehicle (OptiBond XTR) were compared using time-kill kinetics test. Statistical analysis by ANOVA followed by tukey post-hoc test ($P < 0.05$).

Results Disc diffusion and time-kill kinetics tests showed potent antibacterial action of QAC, both in the unpolymerized and the cured forms. MIC and MBC were 20 and 40 mg mL^{-1} respectively. There was no statistically significant difference between experimental adhesives and Clearfil Protect with more than 99% reduction in bacterial count, while OptiBond XTR showed no bacterial killing up for up to 10 h.

Conclusions The synthesized QAC added to a commercially available adhesive imparted antibacterial properties, thus providing an affordable adhesive system to the local market.

Article Highlights

- Quaternary ammonium compounds impart antibacterial action to a commercially available adhesive (OptiBond XTR).
- The experimental adhesive shows comparable antibacterial activity to Clearfil SE Protect adhesive.
- It is feasible to formulate an antibacterial adhesive with simple procedures and reduced cost.

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Keywords *Streptococcus mutans* · Quaternary ammonium compounds · Dental adhesives · Minimum inhibitory concentration · Minimum bactericidal concentration · Time-kill kinetics

Abbreviations

BisGMA	Bisphenol A-glycidyl methacrylate
CFU mL^{-1}	Colony-forming unit per milliliter
CHN	Carbon, Hydrogen, Nitrogen
CLSI	Clinical and Laboratory Standards Institute
CP	Clearfil SE Protect
DMAEMA	2-(Dimethylamino) ethyl methacrylate
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FTIR	Fourier transform infrared
^{1}H NMR	Proton nuclear magnetic resonance
HSF	Human Skin Fibroblast
LC_{50}	Median lethal concentration
MBC	Minimum bactericidal concentration
MDPB	12-Methacryloyloxydodecylpyridinium bromide
MIC	Minimum inhibitory concentration
QAC	Quaternary ammonium compound
SRB	Sulforhodamine B
TEM	Transmission electron microscope
TSY	Trypticase soy broth/agar medium supplemented with 3 g L^{-1} yeast extract

1 Introduction

Adhesive restorative systems have made a breakthrough in dentistry especially in the field of conservative dentistry. Continuous development of adhesive dental materials enabled the use of highly esthetic restorations and the application of minimally invasive techniques since they provide immediate bond strength. However, considering the long-term success of the restoration, it is important to study the strength and the durability of the tooth-restoration adhesive junction [1]. The adhesive junction comprises some challenges, such as polymerization shrinkage stresses, bacterial invasion, and degradation at the tooth-restoration interface [2]. As most systematic survival studies of resin composites and dental adhesives indicate, secondary caries is the foremost reason for resin-based restoration failure and life span reduction. Secondary caries between dental adhesive resins and the tooth structure is considered the primary reason for the failure of polymer-based bonded restorations [3]. Hence, there are trials to formulate dental adhesives with antimicrobial abilities to fight bacterial invasion and growth at the interface [4, 5].

To improve the long-term service of dental restorations, various antibacterial agents were added to experimental and commercial dental bonding agents [2]. An antibacterial agent interferes with the growth of bacteria, to fight both the residual bacteria in the tooth cavity and the new invading bacteria at the margins, thus, minimizing the risk of secondary caries [6].

Many trials were made to create adhesives with antibacterial properties, including the incorporation of chlorhexidine, glutaraldehyde, and nano-sized metallic particles such as Silver, Titanium and Copper [7]. However, the low-molecular-weight antimicrobial agents have the limitation of the possible toxicity, short-term effects, and the difficulty of controlling their rate of diffusion [8]. Also, the release of antimicrobial agents has a side effect on the mechanical properties of the adhesive layer [9].

These leachable agents were replaced by polymerizable antibacterial agents, which are immobilized in the resin matrix after curing, ensuring a more prolonged and durable antimicrobial action while maintaining the mechanical properties of the dental bonding agents [10, 11].

Such antibacterial monomers include cationic quaternary ammonium compounds (QAC), which show antimicrobial activity against both Gram-positive and Gram-negative bacteria, fungi, parasites, and enveloped viruses [12]. It was reported that QACs were used in 1970 in mouth rinses to inhibit oral biofilm formation [13, 14]. QACs were also

incorporated in dental composite materials in 1994 by Imazato et al. to help inhibit plaque accumulation and secondary caries [9]

One such QAC is 12-methacryloyloxydodecylpyridinium bromide (MDPB), which was synthesized by combining a polymerizable methacrylate group with an antibacterial quaternary ammonium group (dodecylpyridinium bromide) resulting in a compound which is rather hydrophobic [15]. It was initially synthesized in 1993 and introduced into a commercially available dental adhesive system—in 2003—known as Clearfil SE Protect Bond primer bottle (5% MDPB), and it has been used successfully in clinical practice. One study proved its bacteriostatic activity and antiadhesion property against oral streptococci especially *S. mutans* [16–18]. In contrast, there was no statistically significant difference between Clearfil Protect Bond and a dental adhesive without antibacterial function (All-Bond SE) either in enamel demineralization or in dental biofilm formation, which suggests that Clearfil Protect Bond was unable to inhibit secondary caries in situ [19].

In another attempt, 2-(Dimethylamino) ethyl methacrylate (DMAEMA) was used as a precursor to synthesize two different quaternary monomers which are compatible with existing dental dimethacrylate-based monomers [10, 20], as DMAEMA is often used as a co-initiator in dental adhesives [21]. It was also used for the synthesis of several cationic antibacterial monomers which were investigated to assess their antimicrobial property against oral bacteria [22–26]. Moreover, four different QACs including dimethylaminobutyl methacrylate (DMABM), showed promising results against *S. aureus* and *E. coli* bacteria [27]. It should be noted that the incorporation of QACs into the adhesives had no adverse effect on their micro tensile bond strength [28–30]

To our knowledge, there are limited commercially available adhesive systems with antibacterial activity for clinical use in dental applications, such as Clearfil SE Protect (containing MDPB) and GLUMA 2Bond (containing 5% glutaraldehyde) [31]. However, the biocompatibility of GLUMA 2Bond is questionable due to the cytotoxicity of the aldehyde moiety [31]. Clearfil SE Protect is the only commercially available adhesive to date with antibacterial QAC (MDPB), which is rather expensive and not available in all markets. Moreover, it is important to formulate a QAC that is compatible with the adhesive composition, has low cytotoxicity, and needs a simple procedure for synthesis. Therefore, our aim was to formulate an affordable adhesive with comparable antibacterial characteristics. This was done through the synthesis and characterization of an antibacterial QAC, its incorporation into a commercially available adhesive system (Optibond), and assessment of its antibacterial properties, compared to the commercially available (MDPB-containing) antibacterial adhesive. Incorporation of QACs into Optibond has been performed in a previous study using Benzalkonium Chloride QAC, but the study did not evaluate the antibacterial activity of the resultant adhesive [32]. In the current study, the null hypothesis tested that incorporation of QAC into dental adhesive Optibond has no antibacterial effect.

2 Methods

The chemicals used for the synthesis of the QAC were 2-(Dimethylamino) ethyl methacrylate (Alfa Aesar, ThermoFisher, Kandel, Germany), 1-Bromobutane (Fisher Scientific, Loughborough, Leics, UK) and absolute ethyl alcohol as a solvent (Sphinx, Egypt). OptiBond XTR Universal Adhesive (Kerr Corporation, California, USA) was used as a vehicle for the experimental adhesive to which QAC was added. It is a two-step, self-etch adhesive system supplied in two 5 mL bottles (fig. S1). Clearfil SE Protect (Kuraray Medical Inc, Okayama, Japan) (fig. S2) is a two-step, self-etch antimicrobial adhesive. The compositions of both adhesive systems are shown in table S1. Clearfil SE Protect was used as the positive control (as a comparator against the experimental adhesive regarding the antibacterial activity), while Optibond without the QAC was considered the negative control in the antibacterial test.

2.1 Synthesis and characterization of the quaternary ammonium monomer

The QAC was obtained through the Menschutkin reaction [10]. In a tared beaker, 2-(N,N-dimethylamino) ethyl methacrylate DMAEMA (1.57 g, 10 mmol) and 1-Bromobutane (butyl bromide) (1.37 g, 10 mmol) were mixed, and 3 g ethanol were added as a solvent. Using a magnetic stir bar, the beaker was stirred for 24 h at 60 °C. Afterward, the solvent and residual reagents were removed by evaporation [14]. The resultant monomer was a clear, colorless, viscous fluid that turned white and showed increased viscosity and tackiness with time (fig. S3). The following characterization tests for the synthesized QAC were performed using one sample each.

2.1.1 CHN elemental analysis

Elemental analysis of the synthesized (QAC) was done using Automatic Analyzer CHNS (Vario EL III – elementar Analysensysteme GmbH – Germany) to determine the percentage of Carbon, Hydrogen, and Nitrogen found in the sample, compared to the calculated values.

2.1.2 Analysis using Fourier transform infrared spectroscopy (FTIR)

The functional groups of the starting materials and the quaternary ammonium salt were identified by FTIR spectroscopy in a KBr pellet (NICOLET 380 FT-IR, Thermo-scientific, China). FTIR spectra of the starting materials and the synthesized monomer were collected in the 4000 cm^{-1} to 400 cm^{-1} region with a wavenumber expanded uncertainty of 0.5 cm^{-1} [10].

2.1.3 Analysis using proton nuclear magnetic resonance (^{1}H NMR)

Proton NMR analysis was performed to identify the molecular structure of the synthesized QAC. High-resolution 400 MHz ^{1}H NMR spectra were acquired on Bruker NMR spectrometer (Bruker, Germany). Deuterated water (D_2O) was used as a solvent.

2.1.4 Cytotoxicity testing

Cell viability against the synthesized QAC was assessed by sulforhodamine B (SRB) assay according to the method described by [33, 34]. The Human Skin Fibroblast (HSF) cell line was used (Nawah Scientific Inc., El Mokattam, Cairo, Egypt). It is a normal cell line, Passage no. 4. The test was performed in triplicate.

The QAC was examined at different concentrations in complete media (10, 20, 40, 60, and 100 µg mL^{-1}). A solution of SRB at 0.4% was used for staining the proteins of viable cells [35]. The percentage of the viable cells exposed to the QAC was calculated with respect to the negative control, and the lethal concentration 50 (LC_{50}) was determined [36, 37].

2.2 Antimicrobial activity of the synthesized QAC

2.2.1 Test microorganism and growth conditions

A standard strain of *Streptococcus mutans* ATCC 25175 was used, grown in Trypticase soy broth/agar medium supplemented with 3 g L^{-1} yeast extract (TSY) for 24 h at 37 °C under anaerobic conditions in a CO_2 incubator [38].

2.2.2 Susceptibility testing using the disc diffusion method

The susceptibility testing was performed according to the Clinical and Laboratory Standards Institute (CLSI) recommendation [39]. A suspension of *S. mutans* of 1.5×10^8 colony-forming unit per milliliter (CFU mL^{-1}) was adjusted spectrophotometrically (T80 UV/VIS double beam Spectrophotometer, PG instruments Ltd, LEICESTERSHIRE, UK) to get a culture density equivalent to a 0.5 McFarland standard [40]. Then the culture was diluted to 10^6 CFU mL^{-1} and was surface inoculated onto the TSY agar plates using a sterile swap. The synthesized QAC was assessed at two concentrations (20 and 10 mg mL^{-1}). Clindamycin antibiotic disc (15μg) served as the positive control. TSY agar plates were incubated in a CO_2 incubator at 37 °C for 24 h. The inhibition zone was measured around each disc. The test was performed in triplicate.

2.2.3 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) by broth microdilution method

MIC and MBC of the synthesized QAC were determined using broth microdilution assay according to the CLSI and European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards [41]. Serial dilutions of the QAC starting from 40 mg mL^{-1} to 78 µg mL^{-1} were prepared in the wells of the 96-well microplate. A suspension of *S. mutans* in TSY broth at 10^6 CFU mL^{-1} was added to the wells. Positive control (bacteria and plain TSY medium) and negative control (plain TSY medium, to confirm the sterility of the medium) were prepared. The MIC was defined as the lowest concentration inhibiting the visible growth after incubation anaerobically at 37 °C for 24 h.

For the determination of MBCs, 20µl aliquots from each well showing no visible turbidity were diluted and transferred onto TSY agar plates. The plates were incubated overnight at 37 °C anaerobically in a CO_2 incubator. Viable colony count on agar medium was performed. MBC was defined as the concentration causing > 99.9% (> 3-log cycle) reduction of the initially inoculated colony counts [42]. MIC and MBC determinations were performed in triplicate.

2.2.4 Kill kinetics assay (Time-kill test) of QAC

Time-kill kinetics of the QAC against *S. mutans* was performed using concentrations equal to 2 × MIC, 3 × MIC, and 4 × MIC of the monomer [43, 44]. An inoculum of 10^6 CFU mL^{-1} was added to the wells containing the mentioned QAC concentrations, as well as positive and negative controls, and incubated at 37 °C for 12 h. Aliquots of 20µl were withdrawn at time intervals of one hour for up to 12 h. The aliquoted samples were diluted appropriately and inoculated onto TSY agar plates, then incubation for 24 h anaerobically at 37 °C was performed and the number of recovered viable cells (CFU mL^{-1}) was determined (fig. S4). The test was performed in triplicate [24, 45, 46].

2.2.5 Bacterial cell imaging by the transmission electron microscope (TEM)

TEM was used to monitor the structural changes in bacterial cells exposed to the antibacterial QAC. An overnight culture of *S. mutans* in TSY broth was prepared, mixed with the synthesized QAC (20 mg mL^{-1}) and incubated anaerobically in a CO_2 incubator at 37 °C for 12 h. Another culture was prepared without QAC to serve as positive control. A drop (2–5 µL) was pipetted from each suspension (the positive control and the QAC-treated bacteria suspension) and spread on a sheet of parafilm. The electron microscope grid (carbon-coated 400-mesh copper grids) was made directly on the specimen and stained with 2% Phosphotungstic acid (PTA) stain for 30 s. The prepared grids are taken for imaging by TEM (JEM-1400Flash Electron Microscope, JEOL Ltd., Tokyo, Japan).

2.3 Preparation of the experimental adhesive system.

The synthesized QAC was added to the primer bottle of the OptiBond XTR adhesive with concentrations 20 and 40 mg mL^{-1} (0.57% and 1.1% by weight), representing the MIC and MBC, respectively. Each bottle was sonicated using an ultrasonic processor (Hielscher UP50H, Hielscher Ultrasonics Germany) to ensure homogenous mixing.

2.4 Sample size calculation

In a previous study by Ma et al. in 2012 [25] the reduction in the number of viable bacteria (%) within group 1 (adhesive with antibacterial quaternary ammonium compound) and group 2 (MDPB-containing dental adhesive) was normally distributed with mean and standard deviation of 99.45 (0.59) and 32.80 (13.12) respectively. Therefore, we will need to study 3 subjects per group to be able to reject the null hypothesis that the means of the experimental and control groups are equal with probability (power) 0.8. The Type I error probability associated with this test of this null hypothesis is 0.05. The sample size was calculated using G*Power version 3.1.9.2 for windows using independent t test.

2.5 Kill kinetics assay of the experimental and commercially available adhesives

The rate of bacterial killing of experimental adhesive systems (containing either 20 or 40 mg mL^{-1} of the QAC) was compared against the commercially available antibacterial adhesive system, Clearfil SE Protect Bond (containing 5% MDPB monomer) as the positive control, while the commercially available vehicle OptiBond XTR was considered the negative control, to demonstrate that the primer itself is not antimicrobial. The adhesives were applied and cured according to the manufacturer's instructions into the wells of a 48-well plate (fig. S5).

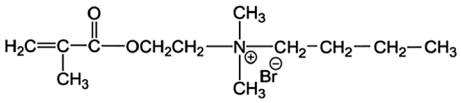
An amount of 500µL of *S. mutans* suspension in TSY broth with 10^6 CFU mL^{-1} inoculum size was added to the wells containing the polymerized adhesives and incubated at 37 °C for 12 h in a CO_2 incubator. Aliquots of 20µl samples were withdrawn every hour for up to 12 h. The cell count of surviving bacteria was determined by the plate count method as mentioned before. The test was performed in triplicate (n = 3) [24, 45, 46].

To express the change (reduction or increase) in the microbial population compared to a starting inoculum, the percentage decrease was determined for each time point as follows [47]:

Table 1 CHN analysis of the synthesized QAC

Element	Carbon	Hydrogen	Nitrogen
Found (wt%)	44.61%	8.18%	4.78%
Calculated (wt%)	43.63%	8.16%	4.24%

Fig. 1 The estimated structure of the synthesized QAC



$$\% \text{ Reduction} = \frac{\text{Initial count} - \text{Count at} \times \text{interval}}{\text{Initial count}} \times 100$$

And the Log reduction was calculated as follows [47]:

$$\text{Log10 (initial count)} - \text{Log10} \times \text{time interval} = \text{Log10 reduction}$$

2.6 Statistical analysis

Data for kill kinetics assay were expressed as mean and standard deviation of three replicates and were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey post-hoc test. Differences were considered statistically significant at $P < 0.05$. The statistical variables were evaluated using the Medcalc software, version 19 for windows (MedCalc Software Ltd, Ostend, Belgium).

3 Results

3.1 Synthesis and characterization of the QAC

3.1.1 CHN elemental analysis:

The elemental analysis data of QAC by CHN analyzer is shown in Table 1. These experimental values (found) given by the analyzer were consistent with the theoretical value (calculated) according to the estimated structure of the synthesized QAC (Fig. 1).

3.1.2 Analysis using FTIR spectroscopy

FTIR spectra of the synthesized QAC and its starting reagents (DMAEMA and Bromobutane) showed the disappearance of the C–Br absorption bands from Bromobutane (667 cm^{-1}, 570 cm^{-1}) as well as the $N(CH_3)_2$ bands (2952 cm^{-1}, 2771 cm^{-1}) from DMAEMA. The prepared QAC displayed absorbance bands corresponding to the aliphatic methyl groups in the 3000 cm^{-1} to 2800 cm^{-1} region (2964 cm^{-1}, 2877 cm^{-1}). Also, the carbonyl ester group was evident in the 1750 cm^{-1} to 1725 cm^{-1} region (1720 cm^{-1}) (Fig. 2).

3.1.3 Analysis using ^{1}H NMR

The peak assignments δ (ppm) ^{1}H NMR were as follows (Fig. 3A):

^{1}H NMR (400 MHz, D_2O) δ 0.86 (t, $J = 7.6$ Hz, 3H, CH_3), 0.91–0.95 (m, 2H, CH_2), 1.06–1.10 (m, 4H, CH_2), 1.84 (s, 3H, CH_2=C–CH_3), 3.08 (s, 6H, $N(CH_3)_2$), 3.67–3.69 (m, 2H, OCH_2CH_2), 4.52–4.54 (m, 2H, OCH_2), 5.68 (s, 1H, C=CH_2), 6.06 (s, 1H, C=CH_2). This ^{1}H-NMR analysis confirmed the structure of the synthesized QAC (Fig. 3B).

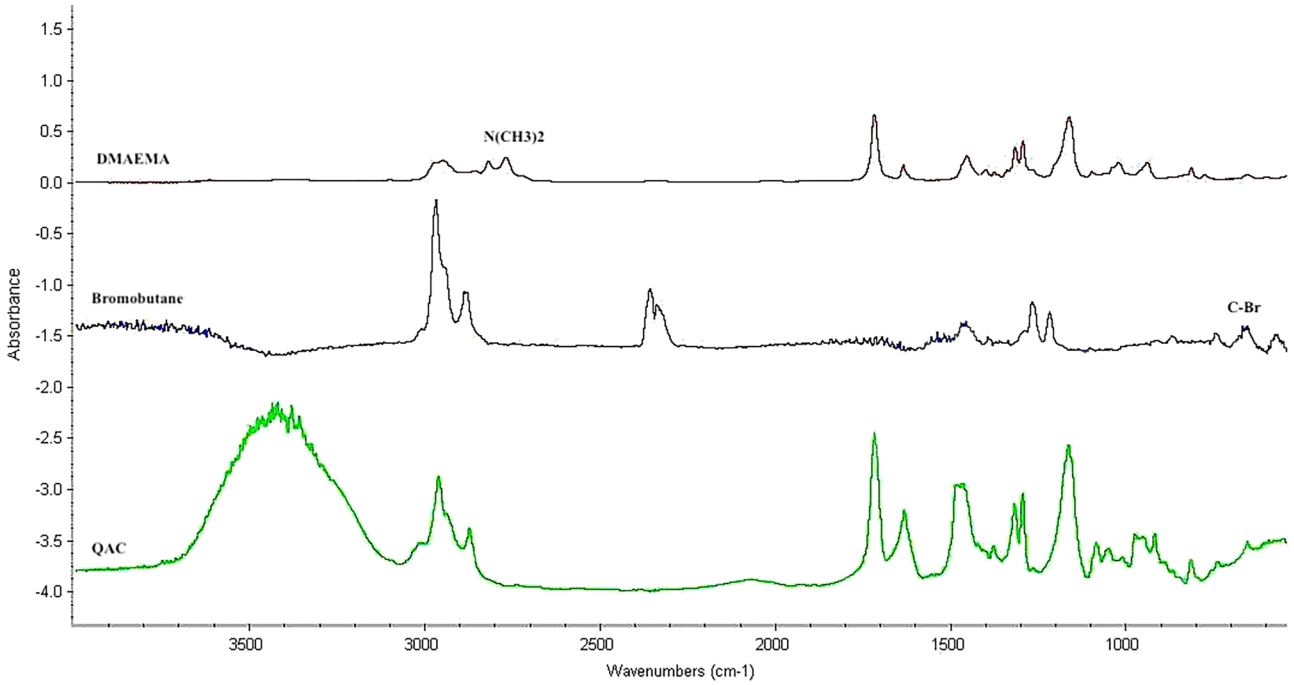


Fig. 2 FTIR spectra of the reactants (DMAEMA and Bromobutane) and the synthesized QAC

3.1.4 Cytotoxicity testing

The results are expressed as the viability % of HSF subjected to different concentrations of QAC, compared to the viability of the negative control (Fig. 4). Cell viability showed a mild decrease with increasing monomer concentration to reach HSF viability of approximately 85% at 100 µg mL^{-1}. The median lethal concentration LC_{50} for the QAC was 51.79 µg mL^{-1}.

3.2 Antimicrobial activity of the synthesized QAC

3.2.1 Susceptibility test using the disc diffusion method

The synthesized QAC showed antimicrobial activity against the tested strain of *S. mutans* ATCC 25175, which was more potent with a concentration 20 mg mL^{-1} than 10 mg mL^{-1} as evidenced by the diameter of the inhibition zone, 11 and 7.5 mm respectively (Fig. 5), However, clindamycin showed the highest level of inhibition resulting in an inhibition zone of 32.5 mm (table S2).

3.2.2 MIC and MBC by broth microdilution method

The synthesized QAC showed average MIC and MBC values of 20 and 40 mg mL^{-1}, respectively, against S. mutans. The MIC (20 mg mL^{-1}) resulted in a one-log cycle reduction of the bacterial count, equivalent to 90% inhibition of the growth of the initially inoculated count.

3.2.3 Kill kinetics assay (Time-kill test)

The time-kill kinetics profile of the synthesized antibacterial monomer against *S. mutans* at concentrations equivalent to 2 × MIC, 3 × MIC, and 4 × MIC showed a reduction in number of viable cells after 2 h at all tested concentrations. While the bacteria in the positive control group showed continuous viability and growth. Moreover, a complete bacterial death was observed after 10 h at QAC concentrations equivalent to 3 × MIC, and 4 × MIC (60 and 80 mg mL^{-1}, respectively). Bacteria treated with 2 × MIC (40 mg mL^{-1}) of the QAC showed complete death at 12 h (Fig. 6).

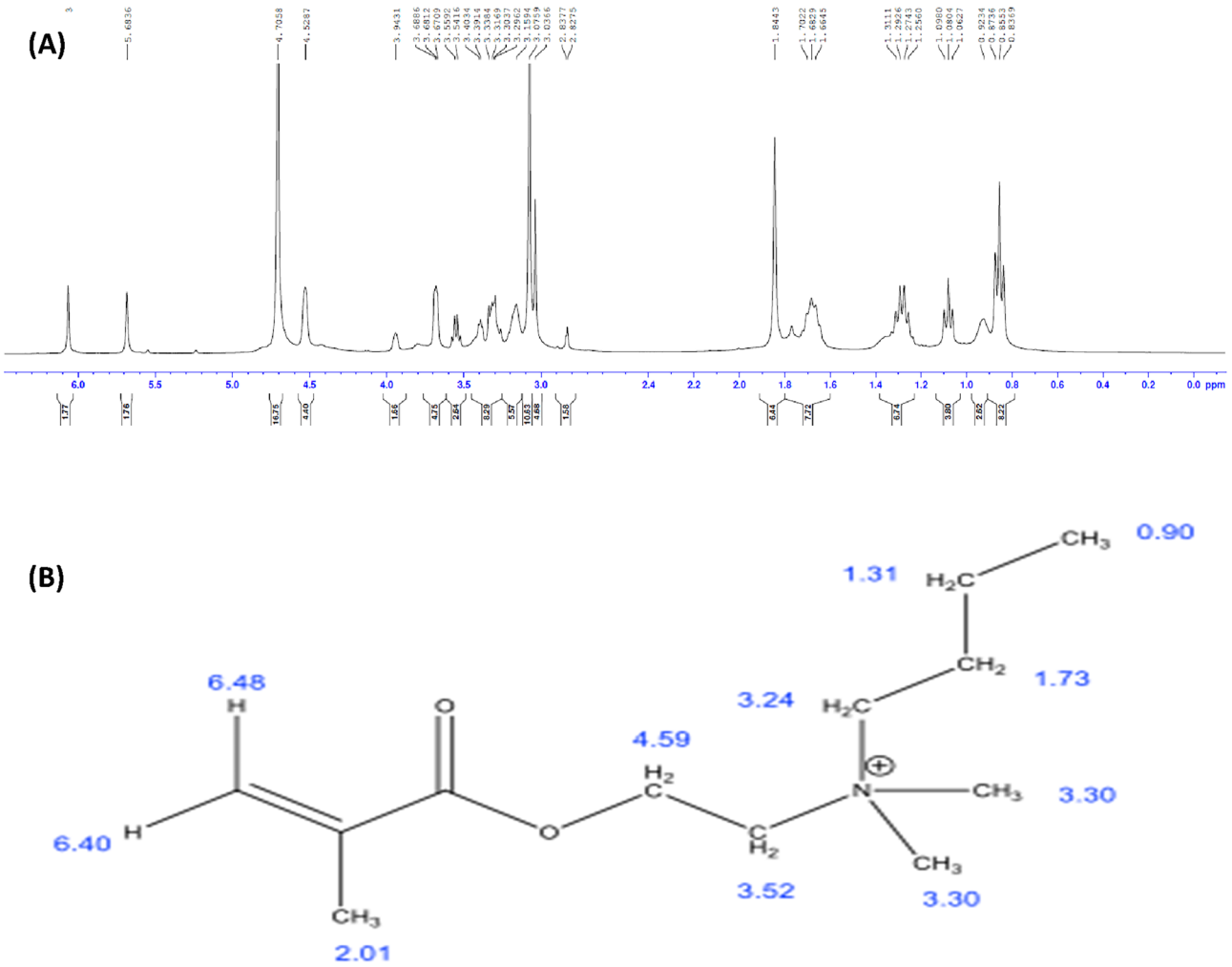


Fig. 3 **A** ^{1}H NMR spectrum of the synthesized QAC. **B** The estimated structure of the synthesized QAC

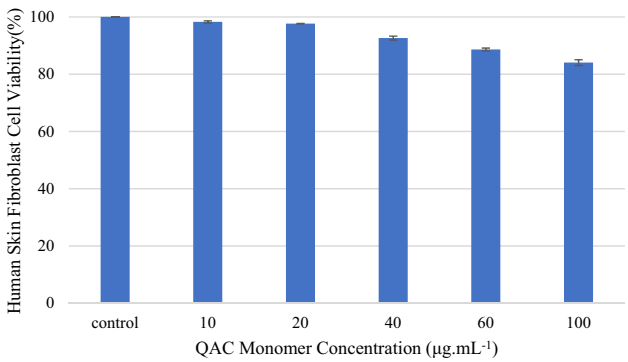


Fig. 4 Mean and standard deviation of cell viability results of different concentrations of the QAC against human skin fibroblasts

3.2.4 Bacterial cell imaging by TEM

For the untreated bacteria, the TEM micrograph showed actively growing bacteria having several initial membrane invaginations for septum formation and well-defined cell wall (Fig. 7A). While the bacteria treated with QAC appeared longer than usual with no sign of membrane invagination, and the dividing septum was not evident at all, moreover, a distorted cell wall appeared (Fig. 7B).

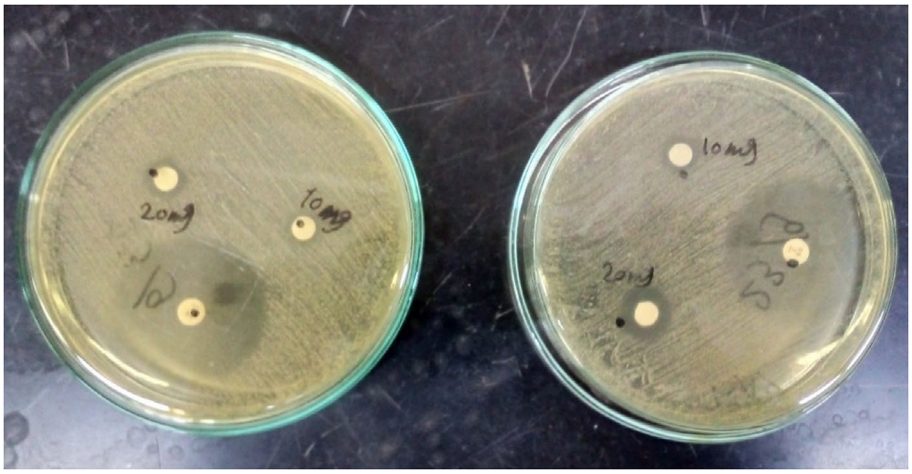


Fig. 5 Antimicrobial susceptibility test using disc diffusion method

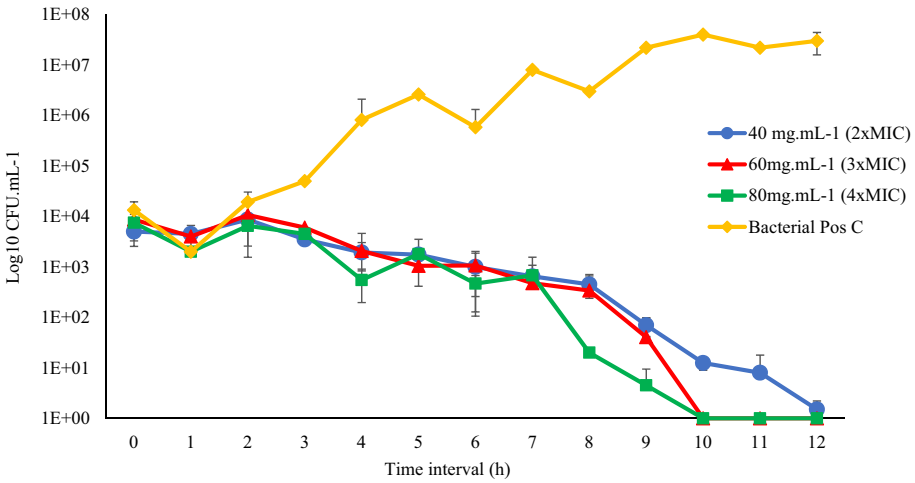


Fig. 6 Kill kinetics of *S. mutans* treated with QAC at concentrations equivalent to 2 × MIC (40 mg mL^{-1}) (Blue line), 3 × MIC (60 mg mL^{-1}) (Red line), 4 × MIC (80 mg mL^{-1}) (Green line) and positive bacterial control (Orange line). Data is presented as Mean and standard deviation of Log bacterial count

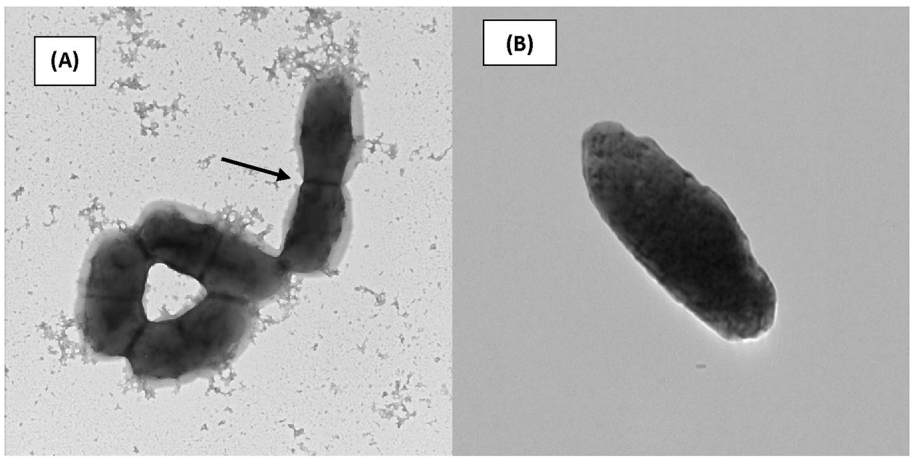


Fig. 7 **A** TEM micrograph of the untreated bacterial cells (× 30,000) with the arrow indicating the dividing septum. **B** The bacteria treated with the synthesized QAC (× 40,000)

3.3 Kill kinetics assay of the experimental and commercially available adhesives

The tested groups were the experimental adhesives containing 20 and 40 mg mL^{-1} QAC, denoted OB20 and OB40 respectively, MDPB-containing adhesive Clearfil Protect, OptiBond adhesive without an antibacterial agent, and positive control bacteria group. The time kill kinetics profile for the tested adhesives is shown in comparison to the

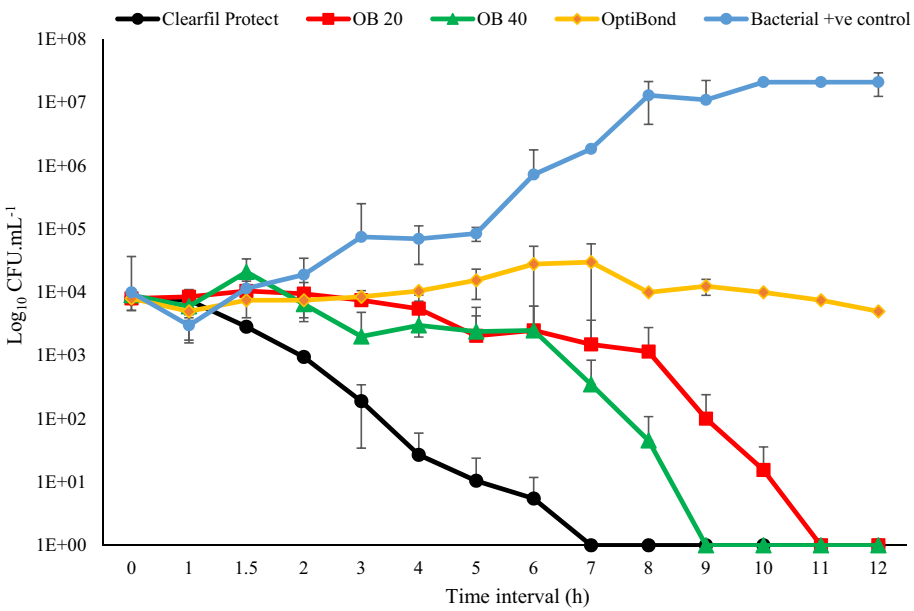


Fig. 8 Kill kinetics of experimental adhesives and commercial controls. "Black line" represents the effect of the commercial Clearfil Protect, "Green line" and "Red line" represent the kill effect of the experimental adhesive systems, supplemented with 40 and 20 mg mL^{-1} QAC respectively (OB40 and OB20), "Orange line" represents the kill effect of the commercial OptiBond XTR adhesive system without an antibacterial agent. "Blue line" represents the growth rate of the bacterial positive control. Data is presented as Mean and standard deviation of Log bacterial count

positive control bacteria group, which showed continuous viability and growth as represented by Log_{10} CFU mL^{-1} (Fig. 8).

The percentage bacterial count showed a significant decrease with time for OB20, OB40 and Clearfil Protect ($P=0.006$, $P=0.047$ and $P=0.026$) respectively after 10 h, while for Optibond the bacterial count increased with time ($P=0.661$). After 10 h there was no statistically significant difference between OB20, OB40 and Clearfil Protect with more than -99% reduction in the bacterial count, however, the 3 groups differed significantly from Opibond (Table 2).

4 Discussion

Insoluble antimicrobial agents incorporated into dental adhesives aim to target microorganisms along the tooth restoration interface [48]. A compound with a methacrylate functional group that copolymerizes with the comonomer mixture of the adhesive will improve antibacterial activity and prevent leakage and losing therapeutic effects over time [49]. These agents include QACs which have shown successful antibacterial action against oral pathogens [50].

The classical Menshutkin reaction provides a successful and simple approach to producing antibacterial QACs with minimal post-reaction purification [13, 14, 51].

The multifunctional monomer N,N-dimethylaminoethyl methacrylate (DMAEMA) has several reactive groups (ether, double bond and amino groups) and can copolymerize with other vinyl monomers with good biological compatibility [27, 52]. It has been used as the basis for QAC formulation which proved high antibacterial activity both in vitro and in vivo [20, 51]. It is well known that the alkyl chain length of a QAC is one factor that influences its antibacterial activity, where an increase in antibacterial efficiency was observed by increasing chain length from 3 to 16, but it decreased when reached 18 [51]. For the current study, the reaction of DMAEMA with butyl bromide resulted in QAC with four carbon atoms. This is considered a relatively short length of alkyl group, with more exposed positively charged groups enhancing the antibacterial effectiveness in the polymerized form compared to long chain compounds [53].

The results of CHN elemental analysis match the basic structure of the synthesized monomer, with the molecular formula ($C_{12}H_{24}NO_2Br$) (fig. S3). Spectra from FTIR spectroscopy showed the disappearance of the C–Br band from bromobutane, and the disappearance of $N(CH_3)_2$ bands from DMAEMA, which is characteristic of the quaternization reaction [10]. Results of ^1H NMR further confirmed the structure of the synthesized QAC and were in accordance with other studies [27].

The cytotoxicity test results have shown a favorable response of HSF cells to the QAC, where the cell viability was 85% using SRB assay. Previous studies used fibroblasts when testing the cytotoxicity of QACs [54, 55]. Moreover, the high value of LC_{50} (51.79 µg. mL^{-1}) indicated good biocompatibility, where bisphenol A-glycidyl methacrylate (BisGMA), one of the primary components in dental adhesives, showed LC_{50} values ranging between [56]. Studies also reported that

Table 2 The percentage change (decrease or increase) in bacterial count at different time points for tested materials, represented by means and standard deviation of three replicates

Time (hour)	Bacterial reduction (%)	Ob20	Ob40	CP	Ob	P value
1	Mean	5.555	− 28.750	8.335	− 36.665	$P=0.751$
	SD	7.856	58.3363	82.4981	4.7164	
1.5	Mean	30.95	125	− 62.500	− 8.335	$P=0.095$
	SD	3.3658	106.066	10.6066	11.7875	
2	Mean	− 15.075	− 20.000	− 87.500	− 8.335	$P=0.504$
	SD	41.5284	98.9949	3.5355	11.7875	
3	Mean	− 3.965	− 74.975	− 97.835	8.335	$P=0.022$
	SD	25.8165	35.32	1.1809	11.7875	
4	Mean	− 34.125	− 62.490	− 99.565	41.665	$P=0.111$
	SD	32.5481	53.0189	0.5586	58.9232	
5	Mean	− 71.505	− 69.995	− 99.830	125	$P=0.197$
	SD	32.435	42.4193	0.2263	176.7767	
6	Mean	− 64.280	− 68.745	− 99.910	333.335	$P=0.343$
	SD	50.5016	44.1871	0.1131	471.4069	
7	Mean	− 78.565	− 95.620	− 99.985	366.665	$P=0.331$
	SD	30.2995	6.1801	0.007071	518.5426	
8	Mean	− 83.565	− 99.435	− 99.985	33.335	$P=0.018$
	SD	23.2285	0.7849	0.007071	47.1428	
9	Mean	− 98.565	− 99.990	− 99.985	75	$P=0.068$
	SD	2.0153	0	0.007071	106.066	
10	Mean	− 99.780	− 99.990	− 99.985	33.335	$P=0.011$
	SD	0.297	0	0.007071	47.1428	
	P value	$P=0.006$	$P=0.047$	$P=0.026$	$P=0.661$	

DMAE-CB exhibited LC_{50} values between 2 and 5 µg. mL^{-1}. As the LC_{50} values of Bis-GMA, the most used monomer in dental materials, were reported to be µg. $mL.^{-1}$ [57]

Streptococcus mutans was chosen for the antimicrobial testing as it is one of the main pathogens in the development of primary and secondary dental caries [45, 58, 59]. Inhibition zone measurement was carried out to test the quaternary ammonium monomer's antibacterial abilities to estimate the potent concentrations against a certain bacterial strain [60].

In this study, the MIC and MBC values of the synthesized QAC agree with previous studies where a similar QAC, dimethyl ammonium ethyl dimethacrylate) was tested [56]. The value of MIC calculated for the synthesized monomer against *S. mutans* was so close to a similar monomer (DMAEDM) studied before [56]. Other researchers studied the antibacterial properties of QAC and the MIC value was 15 mg mL^{-1} [24]. Moreover, the MIC (20 mg mL^{-1}) caused the death of 90% of the bacterial colonies, with suppression of growth to the remaining colonies, so even the MIC showed considerable antibacterial action against *S. mutans*.

As regards the bactericidal activity of the synthesized QAC in its unpolymerized form, it showed potent action at a concentration of 40 mg mL^{-1}, where more than 99.9% of the bacterial culture was dead, which is beneficial in the clinical application as the primer will initially get in contact with the cut dentin, causing disinfection of the cavity [38, 58]. The MBC values reported in previous studies against *S. mutans* range between 31mg mL^{-1} and 62 mg mL^{-1} [24].

It should be noted that it was not feasible to compare the synthesized QAC with MDPB present in the commercial adhesive Clearfil Protect due to the difficulty in isolating the monomer from the adhesive.

The TEM images of the untreated bacteria revealed its normal bead-like appearance previously described in many studies [23, 61]. While for the bacteria treated with the QAC, an altered morphology could be seen in accordance with a similar study [23] and only a few cells were observed, whose appearance was altered into elongated cells with distorted membranes, such description of the affected cells was observed by other researchers [62]. It is understood that the mechanism of action of antibacterial QAC is mainly through binding to bacterial membranes and causing bacterial lysis [13, 63]. That is because the positively charged quaternary amine (N^+) is adsorbed to the negatively charged bacterial cell wall and binds with the phosphate part of the cell wall through an ionic interaction. It then penetrates the cell wall and attaches to the cytoplasmic membrane leading to its disruption. This causes a disturbance in the electric balance

and denaturation of proteins resulting in the leakage of intracellular components and the death of bacteria, moreover, the bacterial cell could explode under its own osmotic pressure [64–66]. It should be noted that *S. mutans* belongs to Gram-positive bacteria, where there is a thick peptidoglycan cell wall covering the inner cytoplasmic layer. The QAC can penetrate such layer, due to their alkyl groups, which are lipophilic groups enhancing the bactericidal ability [67, 68].

The primer of the parent commercial adhesive, Optibond, was used to serve as a vehicle to incorporate the synthesized antimicrobial QAC, since the primer is applied directly to the exposed cavity wall [58].

Time-kill test monitors the progressive death of bacteria exposed to the antibacterial monomer in relation to time [39]. Upon observing the time-kill assay of the unpolymerized QAC and the experimental adhesive after polymerization, it was seen that the adhesive system with 40 mg mL^{-1} QAC showed the total killing of *S. mutans* bacteria after only 9 h, which is faster than unpolymerized QAC (12 h). It was stated by other researchers that the polymerized QACs possess stronger antibacterial activities than their monomer counterparts, due to the higher charge density of the polymer, which increases the binding sites of the antimicrobial active groups to the negatively charged bacterial surface, resulting in multiple points of disruption of the cell membrane [52, 53, 69].

Optibond was used as a negative control to exclude the effect of the solvent, it was found that the bacterial count increased after 10 h of exposure (Table 2). On the other hand, the experimental adhesives with both QAC concentrations (OB20 and OB40), as well as the commercially available MDPB-containing adhesive (Cearfil Protect), showed a statistically significant reduction in the bacterial count with time reaching almost 100% bacterial death after 10 h (Table 2), thus the null hypothesis was rejected. There was no statistically significant difference between the positive control antibacterial adhesive (Clearfil Protect) and the experimental adhesives even when the concentration of 20 mg mL^{-1} was used, which is equivalent to the MIC value.

When comparing the time-to-kill of the tested adhesives, the experimental adhesives with 20 and 40 mg mL^{-1} QAC and MDPB-containing adhesive showed 10, 9, and 7 h respectively (Fig. 8). The different performance between MDPB and the synthesized QAC is probably due two one of two reasons, both are related to the polymer structure. First, the quaternary ammonium group in MDPB is located at the terminal end of the alkyl chain, making it more accessible to interact with the bacterial cell wall [17], while the synthesized QAC has the quaternary ammonium group almost in the middle of the chain, so it is less likely to encounter the bacterial cell wall. The second possible reason is the presence of the pyridine ring in MDPB, where the conjugation tends to pull away the electrons from around the N^{+}, creating a negative inductive effect on the nitrogen atom, rendering it more positive [70].

5 Limitations and recommendations

It is recommended to investigate the long-term antibacterial action of the experimental adhesive containing the synthesized QAC. Further studies are necessary to determine whether the addition of QAC to the adhesive system has any effect on its degree of conversion, mechanical properties, or dentin bond strength. It is also recommended to evaluate the antibacterial activity of multiple QACs synthesized using reagents with variable chain length.

6 Conclusions

The experimental adhesive system used in this study showed promising antibacterial properties, through incorporation of QAC to the commercial adhesive resin, which provides an opportunity for the formulation of an affordable antibacterial dental adhesive system, comparable to the commercially available antibacterial adhesive Clearfil Protect.

Author contributions All authors contributed to the study conception and design. Synthesis and characterization of QAC, and preparation of the experimental adhesives were done by MEI-D and EI-RK. For the antibacterial testing, adhesive samples were prepared by MEI-D and NA. H, while the preparation of the bacterial cultures and the antibacterial testing were performed by MM. I. The first draft of the manuscript was written by MEL-D and revised by MM. I, EI-RK, and NA. H. All authors read and approved the final manuscript.

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Data availability The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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