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**Original Article**

## **Antiseptic Effect of Slightly Acidic Electrolyzed Water on Dental Unit Water Systems**

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### **Abstract**

Biofilm formation in dental unit water systems (DUWSs) can contaminate water from three-in-one syringes, air rotors, and low-speed handpieces. This may serve as a potential source of infection for dentists, dental staff, and patients, so these systems must be sterilized. Because slightly acidic electrolyzed water (SAEW) is often used as a disinfectant for food, the aim of this study was to investigate the possibility of using SAEW as a DUWS disinfectant. Slightly acidic electrolyzed water was injected into a dental unit and its effects evaluated. Chemical properties such as chlorine ion and potential hydrogen in the SAEW were measured. Detection of both ordinary and heterotrophic bacteria from the DUWS was performed by culture, and biofilm formation of the bacteria in the DUWS evaluated. Polymerase chain reaction (PCR) was used to detect contamination by nosocomial pathogens. Almost all the chlorine ions in the SAEW were exhausted during the two-day trials, and the pH value of the SAEW fell from 5 to 4. No viable cells were detected in the SAEW collected. Biofilm formation in the water from the DUWS with SAEW was almost at a baseline level, whereas that without SAEW was 4 times higher. The PCR analysis showed that no nosocomial infecting pathogens were detected in the SAEW. The present study demonstrated the antiseptic effect of SAEW in DUWS.

**Key words:** Dental unit water system (DUWS) —  
Slightly acidic electrolyzed water (SAEW) — Nosocomial infection

## Introduction

Water obtained from dental units *via* three-in-one syringes, air rotors, and low-speed handpieces may be heavily contaminated with microorganisms, and thus constitute a potential source of infection for dentists, dental staff, and patients<sup>6,46</sup>. Several studies have indicated that rates of respiratory infections are higher in dentists and dental staff than in the general public<sup>8,9</sup>, and contaminated handpieces are believed to be at least partially responsible for this phenomenon<sup>26</sup>. Elderly people, as compromised hosts, are particularly susceptible to infection with respiratory pathogens such as *Candida albicans*, MRSA, and *Pseudomonas aeruginosa* *via* the oral cavity<sup>1</sup>. In addition to contamination *via* a dental unit water system (DUWS), other instruments commonly used in dental practice such as air rotors and ultrasonic scalers can also lead to the creation of aerosols involving microorganisms. The range of microorganisms isolated from DUWSs includes both harmless environmental organisms and nosocomial pathogens such as *P. aeruginosa*, *Legionella pneumophila*, and *Staphylococcus* species<sup>2,5,7,15,48</sup>.

Electrolyzed oxidizing water (EOW) has recently been developed as a new type of disinfectant agent. One strong version of this type of agent is produced through the electrolysis of water containing a low concentration of sodium chloride in an electrolysis chamber in which anode and cathode electrodes are separated by a diaphragm, which imparts strong bactericidal and virucidal properties to the water collected from the anode<sup>43</sup>. Strongly acidic water obtained *via* this method has been used to disinfect dental material, dental instruments and clinical specimens<sup>16,29,30,47</sup>. There is a slightly or weakly acidic type, which is produced by electrolysis of water containing a low concentration of hydrochloride in an electrolysis chamber where the anode and cathode electrodes are not separated, which imparts strong bactericidal properties to the water<sup>28</sup>. Both types of EOW are permitted as food additives by the Japanese Ministry of Health, Labour and

Welfare<sup>41</sup>. Among these different types of EOW, the strongly acidic type has one disadvantage in that metallic material can be easily corroded, with Co-Cr alloys, in particular, showing a greater degree of corrosion with the strong type than with the slightly acidic type<sup>12</sup>. This suggests that the slightly acidic type of EOW would be more suitable for preventing bacterial contamination of dental instruments and dental units.

In the present study, we evaluated the ability of slightly acidic electrolyzed water (SAEW) to prevent microbial contaminations in DUWSs.

## Materials and Methods

### 1. Properties of SAEW in dental unit

The outline of the tested system is shown in Fig. 1. The SAEW was obtained by the electrolysis of municipal tap water after the addition of 2% HCl solution (Purestar Mate3, Morinaga Engineering, Tokyo, Japan) in a commercial EOW generator (Purestar Mp240-B, Morinaga Engineering). For testing, the Exceed Flora Over Arm type dental unit was

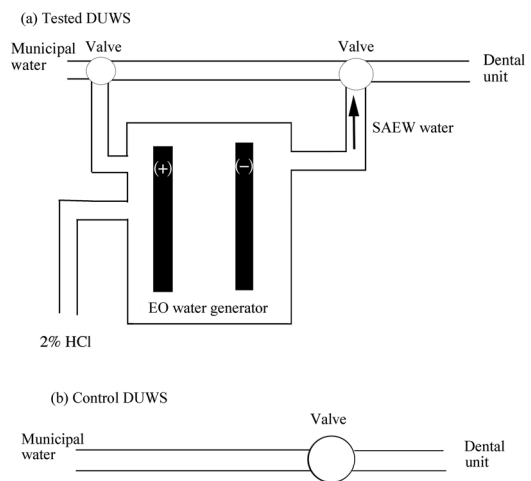


Fig. 1 Tested DUWS incorporating electrolysis chamber for SAEW

Circles indicate valves. Valves were opened after office hours and shut before office hours.

used (Yoshida Dental MFG, Tokyo, Japan). The generator was connected to the DUWS by tubes. All tested SAEW was obtained *via* three-in-one syringes. A control DUWS was connected to the dental unit and standard municipal tap. Water samples from both types of dental unit were collected after 64 hrs rest from dental use.

Levels of free and total chlorine ions were measured by diethyl-p-phenylenediamine (DPD) colorimetric and potassium iodide (KI) colorimetric method using a commercial kit (ATK100DA, ATD100DA; Advantec Toyo Kaisha, Tokyo, Japan). The pH value and temperature were measured with the pH tester 30 (Eutech Instrument, Ayer Rajah Crescent, Singapore).

## 2. Microbiological assessment by culture method

Heterotrophic bacteria and ordinary bacteria were detected by culture from both SAEW and municipal water obtained *via* the dental units. Total numbers of contaminated cells were determined according to the America Method 9215A, as set forth in the Standard Methods for the Examination of Water and Wastewater<sup>3)</sup>. Water samples collected from the DUWSs *via* a three-way syringe after 64 hrs rest from dental use were employed. An aliquot of 100  $\mu$ l water sample was immediately transferred into 900  $\mu$ l soybean-casein broth containing lecithin and polysorbate 80 (SCDLP; Nihon Seiyaku, Tokyo, Japan) and diluted in a 10-fold series down to  $10^{-3}$ . The SCDLP used as the dilution solution was prepared by the addition of 0.8 mm glass beads. Each 100  $\mu$ l SCDLP sample was plated onto an appropriate plate, spread evenly with a Conrage stick, and cultured at 37°C for 48 hrs. This procedure was carried out in 3 separate assays performed in triplicate. After culture, the number of colony forming units (CFUs) was counted. Bacteria were grown on R2A agar (Merck, Darmstadt, Germany) for heterotrophic bacteria, or standard agar (Nissui Pharmaceutical, Tokyo, Japan) for ordinary bacteria. Also, evaluation of Enterobacteriaceae contamination, which indicates

pollution by waste water, was also evaluated in the same way as described above, but using *Endo* agar (Eiken Chemical, Tokyo, Japan).

## 3. Biofilm formation

Two hundred-microliter aliquots of water from the dental units were inoculated into a 96-well tissue culture plate (Greiner Bio-One, Frickenhausen, Germany) at 37°C. Then, 100  $\mu$ l was added to the same samples every 3 days for 2 weeks to supplement loss by transpiration. Semi-quantification of biofilm formation using toluidine blue was performed by the method of Tanaka with modifications<sup>39)</sup>. The biofilm in the wells of the tissue cultures was incubated with 200  $\mu$ l of 0.1% toluidine blue for staining at room temperature. The dye was decanted 30 min later and excess stain removed by washing twice with distilled water. Next, 100  $\mu$ l ethanol was added and left for 30 min to extract the stain. The optical density of each sample was determined at 590 nm.

## 4. DNA extraction from water samples

A total of 500 ml each water sample was filtered through a polycarbonate filter (0.45- $\mu$ m pore size; HVLP; Millipore, Billerica, MA, USA) under sterile conditions. The filters were cut into eighths and each filter piece placed in 10 ml phosphate buffered saline (pH 7.2, PBS).

A 10-ml portion of the water sample was centrifuged at 20,000  $\times$ g for 30 min. The sediment was then transferred to a sterile 1.5-ml tube and centrifuged at 20,000  $\times$ g for 10 min, and the resulting segment re-suspended in 300  $\mu$ l PBS. The suspension was then divided into 3 tubes. After another centrifugation step at 20,000  $\times$ g for 10 min, the resulting pellet was used for DNA extraction, which was carried out with a commercially available kit (QiAamp DNA Mini Kit; Qiagen, Hilden, Germany) according to the manufacturer's instructions. First, bacterial cells were lysed by enzymatic treatment. For Gram-negative rods, a pellet was resuspended in ATL buffer and treated with 10  $\mu$ g/ml lysozyme (Sigma-Aldrich, St. Louis, MO, USA)<sup>31)</sup>. For *Staphylococcus*

*aureus*, a pellet was resuspended in TEN buffer and treated with 20 µg/ml lysostaphin (Sigma-Aldrich)<sup>18</sup>. For *C. albicans*, a pellet was resuspended in Zymolyase buffer and treated with 10 µg/ml lyticase (Sigma-Aldrich)<sup>25</sup>. All samples that were treated with each enzyme were incubated at 37°C for 60 min. Then, 200 µl kit buffer AL and 20 µl proteinase K were added and incubated at 56°C for 10 min, after which 200 µl ethanol was added to each tube. After brief centrifugation, the supernatant was transferred to the column and washed twice with AW1 and AW2 by spin down. Isolated DNA was then eluted in 50 µl elution buffer AE (supplied in the kit) and stored at -20°C until analysis by PCR.

### 5. Detection of nosocomial pathogens by PCR

PCR was used to detect *P. aeruginosa*<sup>10</sup>, *L. pneumophila*<sup>45</sup>, *S. aureus*<sup>32</sup>, MRSA<sup>32</sup>, and *C. albicans*<sup>17</sup>. The oligonucleotides used as primers for PCR are shown in Table 1. Oligonucleotide primers and probes were synthesized at GE Healthcare, UK Ltd. (Amersham Place, England, UK). The PCR reagent used was from the Ready-to-go PCR kit (GE Healthcare, UK). PCR amplification was performed in the DNA thermal cycler Dice (TaKaRa Bio, Shiga, Japan). The PCR temperature profile for *L. pneumophila*, *S. aureus*, MRSA and *C. albicans* included an initial denaturation step at 95°C for 3 min, followed by 40 cycles

of a denaturation step at 95°C for 1 min, a primer annealing step at 55°C for 1 min, an extension step at 72°C, and a final step at 72°C for 5 min. The PCR temperature profile for *P. aeruginosa* included an initial denaturation step at 95°C for 3 min, followed by 40 cycles of a denaturation step at 95°C for 1 min, a primer annealing step at 60°C for 1 min, an extension step at 72°C, and a final step at 72°C for 5 min. A negative control (*i.e.*, sterile water without bacteria) was included in each batch of samples for DNA preparation and PCR performed to exclude contamination of the buffer solutions.

Five microliters of each of the amplified products was analyzed by electrophoresis in 3% agarose gel (NuSieve 3:1 Agarose; Cambrex Bio Science, ME, USA) in 1×TBE buffer (90 mM Tris-borate, 2 mM EDTA; pH 8.3) at 100V for 40 min using the Mupid system (Advance, Tokyo, Japan). A low-DNA mass ladder (TaKaRa Bio) was used as the molecular size standard. The gel was stained with ethidium bromide (0.5 µg/liter) and photographed under ultraviolet illumination with Polaroid film (Polaroid, St. Albans, England, UK) and Tri-X film (Eastman Kodak, Rochester, NY, USA).

### 6. Statistical analysis

Viable cell numbers in biofilm formation were analyzed using a one-factor ANOVA (StatView Program, SAS Institute Inc., San

Table 1 Primers for PCR detection of nosocomial pathogens in waters

Strain	Direction	Sequence (5'-3')	Product	Annealing temperature
<i>Pseudomonas aeruginosa</i>	F	TTC CCT CGC AGA GAA AAC ATC	520 bp	60°C
	R	CCT GGT TGA TCA GGT CGA TCT		
<i>Legionella pneumophila</i>	F	AGG GTT GAT AGG TTA AGA GC	386 bp	55°C
	R	CAA ACA GCT AGT TGA CAT CG		
<i>Staphylococcus aureus</i>	F	AAT CTT TGT CGG TAC ACG ATA TTC TTC ACG	422 bp	55°C
	R	CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA		
MRSA	F	AAA ATC GAT GGT AAA GGT TGG C	533 bp	55°C
	R	AGT TCT GCA GTA CCG GAT TTG C		
<i>Candida albicans</i>	F	CCT GAA CCA CAA GAT GGA ACC ATT A	490 bp	55°C
	R	CGC AGT TTT CTA CTA CCA CCA TCG		

Francisco, CA, USA), and Fisher's PLSD as a post hoc test at a 5% level of significance.

## Results

### 1. Properties of SAEW from dental unit

The amount of chlorine ions, pH value, and temperature are shown in Table 2. Almost all chlorine ions in the SAEW were exhausted during the 64 hrs trials, and the pH value of the SAEW fell from 5 to 4. These results indicate that the SAEW was resolved to 2% HCl solution. The amount of chlorine ions and the pH value of the municipal water showed no change over the 64 hrs period.

### 2. Microbiological assessment by culture method

No viable cells were detected in the SAEW collected from the dental units after 64 hrs (Fig. 2). In municipal water, bacteria grown on standard agar showed a significant increase to approximately 300 CFU/ml (Fig. 2A) after 64 hrs; heterotrophic bacteria grown on R2A agar, however, showed no significant increase after 64 hrs (Fig. 2B). This indicated a large number of heterotrophic bacteria in the municipal water prior to testing. No enterobacteriaceae were detected in either of the two dental unit systems (data not shown).

### 3. Biofilm formation

Figure 3 shows the effect of SAEW on bio-

film formation in the micro-wells under static conditions. The results of staining revealed biofilm formation was very low, almost at baseline level with SAEW. Biofilm formation with municipal water, on the other hand, was approximately 4 times greater than that with SAEW at one week, indicating that SAEW inhibited growth more strongly than the control (Fig. 3).

### 4. PCR detection

No nosocomial pathogens were detected in SAEW (Fig. 4), whereas *S. aureus* and *P. aeruginosa* were detected in municipal water. *P. aeruginosa* was detected only in the morning, whereas *S. aureus* was detected both in the evening and the morning. Both bands of *S. aureus* and *P. aeruginosa* were faint. This finding, that template DNAs obtained from municipal water *via* dental units were present in quite small amounts, indicates that minute amounts of *P. aeruginosa* or *S. aureus* had contaminated the municipal water.

## Discussion

In 2004 the American Dental Association (ADA) issued a statement on DUWSs recommending that water delivered during non-surgical dental procedures consistently contain no more than 200 CFU/ml aerobic mesophilic heterotrophic bacteria in an unfiltered dental unit output<sup>4)</sup>. This level is equivalent to

Table 2 Properties of tested waters *via* dental unit

	Slightly acidic electrolyzed water		Municipal water	
	Start	After 64 hrs	Start	After 64 hrs
Free chlorine ion (mg/liter)	3.26 ± 1.80	0.09 ± 0.04	0.05 ± 0.04	0.03 ± 0.01
Total chlorine ion (mg/liter)	3.14 ± 1.69	0.20 ± 0.08	0.06 ± 0.03	0.04 ± 0.03
pH	4.99 ± 0.32	3.96 ± 0.18	7.32 ± 0.11	7.37 ± 0.31
Oxidation Reduction Potential (ORP, mV)	857 ± 110	503 ± 71	555 ± 105	391 ± 76
Temperature (°C)	23.1 ± 1.9	21.2 ± 1.9	23.0 ± 0.9	21.5 ± 1.7

Results are expressed as mean ± standard deviation from 5 separate assays. n = 15.

the assurance standard for dialysis fluids. On the other hand, the American Public Health Association, the American Water Works Association, and the Environmental Protection Agency have all set the standard for drinking water at less than 500 CFU/ml of aerobic mesophilic heterotrophic bacteria. Water supplied from dental units should also abide by this standard. The Centers for Disease Control and Prevention consider that the

number of bacteria in water supplied through a dental unit should be the same as for drinking water, which should contain less than 500 CFU/ml of aerobic mesophilic heterotrophic bacteria<sup>19)</sup>. Palenik *et al.* reported that only one waterline specimen contained more than 500 CFU/ml among 10 tested specimens<sup>35)</sup>. In Europe, while the guidelines for potable water standards have recommended a bacterial contamination level of <100 CFU/ml,

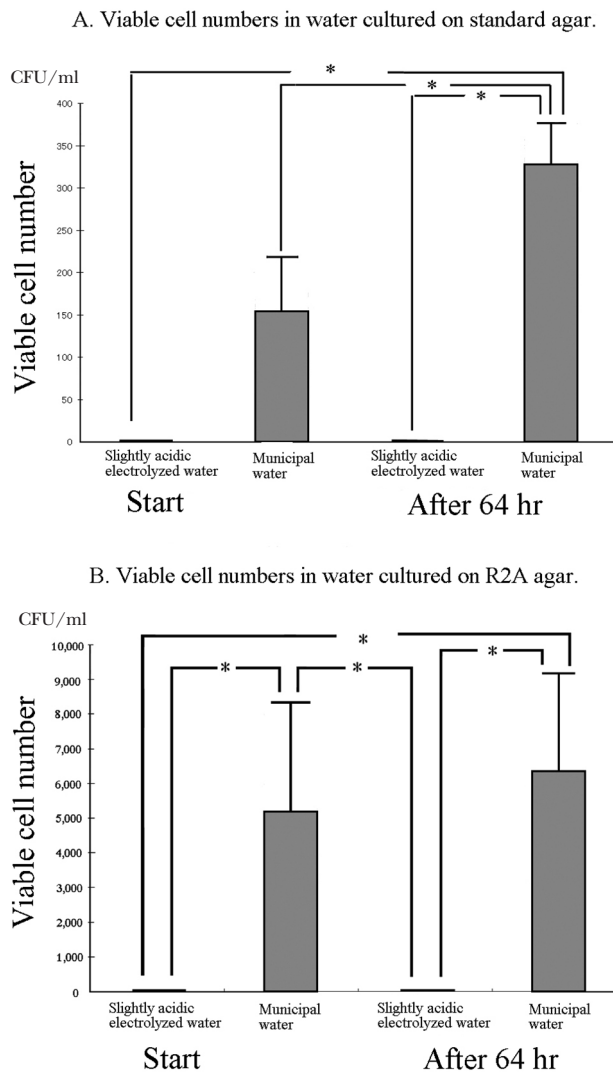


Fig. 2 Microbiological assessment by culture method  
 Bar indicates standard deviation from 3 separate assays. n=9.  
 \*Significantly different ( $p<0.05$ ) by one-way ANOVA.

there are as yet no guidelines in the European Union for the control of microbial contamination in DUWS<sup>40</sup>. Several investigators reported that most units tested were unsatisfactory according to the recommendations set by the ADA<sup>44</sup>. In Japan, the Ministry of

Health, Labour and Welfare requires that municipal water have less than 100 CFU/ml<sup>21</sup>, yet there are presently no guidelines in Japan on the control of microbial contamination in DUWSs. Some investigators have reported that DUWSs in Japan also failed to meet the recommendations set by the ADA, and sometimes over 1,000 viable bacterial cells were obtained from 1 ml of dental unit system water<sup>3,20</sup>. In the present study, over 200 CFU/ml planktonic bacteria were found in the control DUWS. In the DUWS employing SAEW, however, no viable heterotrophic or ordinary bacteria were detected. The use of SAEW allowed the DUWS to meet the water quality standards set for prevention of bacterial contamination in the USA, EU, and Japan.

Chlorine ions produced by the electrochemical hydrolysis are believed to be the main agent responsible for disinfection in EOW<sup>23</sup>. The antimicrobial effects of electrolyzed water are similar to those of hypochlorite solution. Diluted sodium hypochlorite is effective in improving the quality of effluent

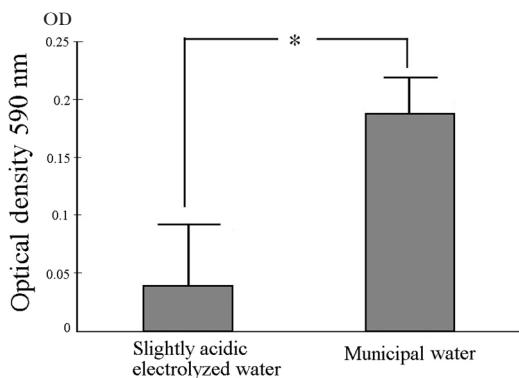


Fig. 3 Biofilm formation by microorganisms in water from DUWS

Bar indicates standard deviation from 3 separate assays. n=9. \*Significantly different ( $p < 0.05$ ) by one-way ANOVA.

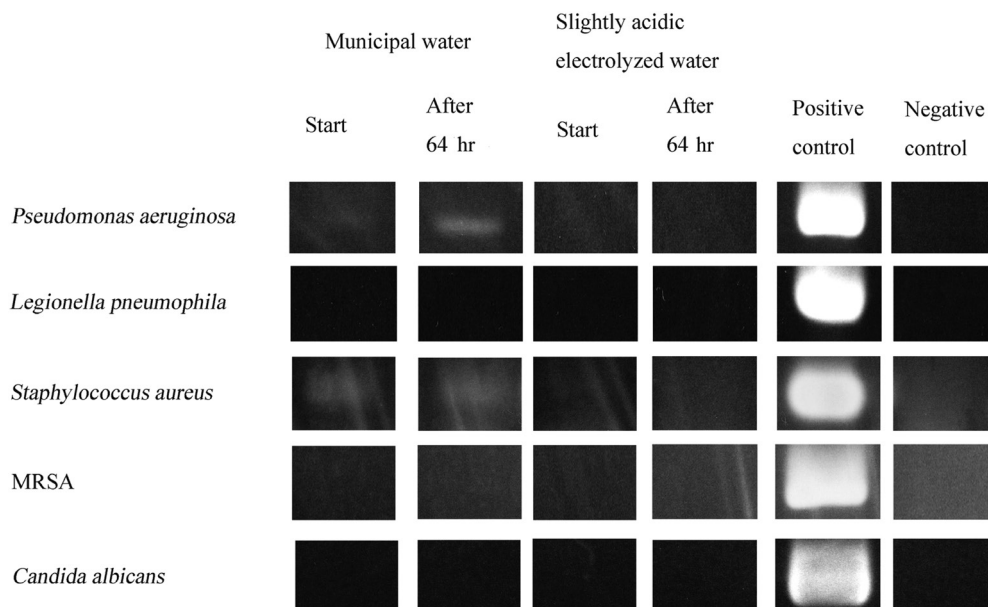


Fig. 4 Detected nosocomial pathogens by PCR  
Representative of 5 separate assays. n = 15.



water and reducing the amount of biofilm in DUWSs<sup>11</sup>. With EOW, it has been reported that approximately 40% of the chlorine was lost under closed dark storage conditions for 2 months<sup>22</sup>. Chlorine dioxide also inhibits the formation of natural biofilms<sup>37</sup>. Our results showed that SAEW which had passed through the DUWS inhibited biofilms formation to within almost baseline levels.

The type of chlorine ions often used in the decontamination of water, ordinary sodium hypochlorite, is usually referred to as bleach. Disinfection with chlorine has been the single most important measure in ensuring the microbiologic safety of potable water supplies. Since the institution of the routine chlorination of water supplies, waterborne outbreaks of infectious agents have been exceedingly rare. Most waterborne outbreaks are believed to result from the use of untreated water, systems receiving inadequate treatment, or contamination after treatment<sup>36</sup>. The Japanese Ministry of Health, Labour and Welfare recommends the removal of chlorate ions to below 0.6 mg/ml in the concentration of municipal water as stipulated by the tap water quality standards<sup>21</sup>. As a food additive, EOW is called hypochlorous acid water, and must be removed before the completion of the final product according to Japanese specifications and standards for food additives<sup>42</sup>. However, electrolyzed water is considered easy to remove, and is completely removed immediately after being replaced with municipal water<sup>11,36</sup>. Use of SAEW in a DUWS meets these criteria for hypochlorous water and chlorate.

Several disinfectant agents have been used for washing and disinfecting DUWSs. Chlorhexidine<sup>13,14,34</sup>, tetraacetyl enediamine<sup>38</sup>, hydrogen peroxide<sup>24,33</sup>, strong-type acidic electrolyzed water<sup>20</sup>, bleach, and other chemicals<sup>27</sup> have been used in dental unit waterline decontamination. In our study, we found MRSA and *P. aeruginosa* in the control DUWS by PCR. We believe that the PCR detection method used was highly sensitive. Even so, only a very small amount of these bacteria was detected. As shown in Table 1, two annealing

temperatures were used in the PCR, with PCR for each strain carried out separately. However, no significant difference was observed in the amplification efficiency of the positive control, which is shown in Fig. 4. No amount of pollution of a DUWS with nosocomial infection-inducing agents is permissible, so every effort must be made to eradicate them completely. In the present study, injection of SAEW into a DUWS for 64 hrs resulted in no detection of nosocomial pathogens, indicating that SAEW offers an effective means of disinfecting such units.

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