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# Effects of Extended Storage of Chlorhexidine Gluconate and Benzalkonium Chloride Solutions on the Viability of *Burkholderia cenocepacia*

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Chlorhexidine gluconate (CHX) and benzalkonium chloride (BZK) formulations are frequently used as antiseptics in healthcare and consumer products. *Burkholderia cepacia* complex (BCC) contamination of pharmaceutical products could be due to the use of contaminated water in the manufacturing process, over-diluted antiseptic solutions in the product, and the use of outdated products, which in turn reduces the antimicrobial activity of CHX and BZK. To establish a "safe use" period following opening containers of CHX and BZK, we measured the antimicrobial effects of CHX (2−10 µg/ml) and BZK (10−50 µg/ml) at sublethal concentrations on six strains of *Burkholderia cenocepacia* using chemical and microbiological assays. CHX (2, 4, and 10 µg/ml) and BZK (10, 20, and 50 µg/ml) stored for 42 days at 23°C showed almost the same concentration and toxicity compared with freshly prepared CHX and BZK on *B. cenocepacia* strains. When 5 µg/ml CHX and 20 µg/ml BZK were spiked to six *B. cenocepacia* strains with different inoculum sizes ($10^0$−$10^5$ CFU/ml), their toxic effects were not changed for 28 days. *B. cenocepacia* strains in diluted CHX and BZK were detectable at concentrations up to $10^2$ CFU/ml after incubation for 28 days at 23°C. Although abiotic and biotic changes in the toxicity of both antiseptics were not observed, our results indicate that *B. cenocepacia* strains could remain viable in CHX and BZK for 28 days, which in turn, indicates the importance of control measures to monitor BCC contamination in pharmaceutical products.

**Keywords:** Bactericidal effects, antiseptic, chlorhexidine gluconate, benzalkonium chloride, *Burkholderia cenocepacia*

## Introduction

Topical antiseptics, which are regulated by the FDA, are chemical germicides applied to living tissue to inhibit or destroy microorganisms. Chlorhexidine gluconate (CHX) and benzalkonium chloride (BZK) are among those antiseptics that are most commonly used worldwide [1-3]. BZK solutions for hospital use tend to be neutral to alkaline, non-corrosive on metal surfaces, non-staining, and safe to use on all washable surfaces [1−5]. BZK is also added to drug products as a preservative for multiple dose containers [6]. CHX is commonly used for hand hygiene or mouth rinses in the USA [1−3, 7]. BZK affects bacterial membrane permeability by the physical disruption and partial solubilization of the membrane and cell wall, whereas CHX enters the bacterial cell by destabilizing the association of divalent cations with the cell membrane and disrupting lipopolysaccharide [1, 8−10]. Based on their levels of antimicrobial effectiveness, CHX and BZK are classified as low-level antiseptics able to inactivate vegetative bacteria, some fungi, and viruses [1]. Notwithstanding these facts, these antiseptics have broad-spectrum activity against many organisms, including *Burkholderia cepacia* complex (BCC).

BCC species are increasingly recognized as human pathogens [11]. These bacteria are generally opportunistic, affecting people with impaired immunity or conditions such as cystic fibrosis (CF) or chronic granulomatous disease [11−13]. More than 40 outbreaks due to BCC-contaminated antiseptic solutions and equipment have been reported [14−26]. Recently, the Centers for Disease Control and Prevention investigated an outbreak of bloodstream infections caused by BCC [27]. As a result, contaminated prefilled saline flush syringes were identified as the source of the bacteria. Moreover, the FDA recently announced a voluntary nationwide recall of oral liquid docusate sodium products owing to a potential risk of product contamination with BCC [28].

Although all BCC species have been associated with infections in humans, *Burkholderia cenocepacia* is the most problematic species infecting CF patients [29−31]. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of CHX or BZK for most *B. cenocepacia* strains is relatively high [32, 33]. Rose *et al*. [33] reported MICs of 100 mg/l (CHX) and >400 mg/l (BZK) for *B. cenocepacia*, respectively. *B. cenocepacia* MU1 and LMG 18832 showed a CHX MBC of 1,000 mg/l, indicating that these strains may remain viable in commercial biocide formulations [33]. *B. cenocepacia* HI2718, isolated from a CF patient, could grow in the highest concentrations (500 mg/l) of CHX and BZK among several BCC strains tested [32]. CHX and BZK are used in a variety of commercial products in concentrations ranging from 0.02% to 5% (200–50,000 µg/ml) [32]. Although most BCC outbreaks were associated with antiseptic solutions containing less than 2% chlorhexidine, an outbreak has been reported involving an antiseptic solution of 2% to 4% chlorhexidine [34]. Most outbreaks were attributed to the use of contaminated water in the manufacturing process, over-diluted antiseptic solutions, and the use of outdated products, which in turn, reduces the bactericidal activity of CHX and BZK [3]. However, drug products containing preservatives and antiseptics may become contaminated with pathogens such as BCC when opened and are stored for extended periods of time. To the best of our knowledge, this is the first study that examined the antibacterial efficacy of CHX and BZK that had been diluted and stored in opened containers for prolonged periods of time. The objective of the present study was to provide fundamental information that could help in establishing a “safe use” period by determining the effect of extended storage of opened or diluted solutions of CHX and BZK on their antimicrobial effect on six strains of *B. cenocepacia*. In this study, we determined the abiotic and biotic changes in the toxicity, survival, and bacterial recovery of *B. cenocepacia* in CHX and BZK solutions.

## Materials and Methods

### Bacterial Strains

A collection of six strains of *B. cenocepacia* (AU1054, J2315, AU0222, AU19236, HI2976, and HI2485) was obtained from the *Burkholderia cepacia* Research Laboratory and Repository at the University of Michigan [32, 35]. This collection included isolates from clinical and environmental habitats. To use the same number of cells for each experiment, these organisms were freshly grown on tryptic soy agar (TSA) at 30°C for 48 h and transferred into sterile distilled water as previously described [35].

### Assessment of Bacteriostatic Activity of Opened or Diluted but Non-Contaminated CHX and BZK after Extended Storage

*B. cenocepacia* strains persisted in distilled water for 40 days [35] and the survival of BCC in antiseptics was evaluated after 20 min, and 24, 48, 168, and 336 h (14 days) [32]. The concentrations of CHX or BZK opened and exposed for 42 days at 23°C were compared with the initial (day 0) concentrations of these antiseptics prepared in sterile distilled water for chemical and microbiological analysis.

**Analytical chemistry methods.** The concentration of antiseptics opened over a long period was quantitatively assessed by measuring the abiotic transformation of CHX (Spectrum Chemical Mfg. Corp., USA) and BZK (Acros Organics, USA). Dilutions of CHX (10 µg/ml) and BZK (50 µg/ml) were prepared in 10 ml of distilled water and remained open for 42 days at 23°C. After 42 days, the concentrations of CHX and BZK were measured and calculated. CHX samples were filtered through a 0.22 µm pore size filter (Millipore Corp., USA) and analyzed by HPLC (1200 series; Agilent, USA) with a C-18 Gemini NX column (4.6 × 150 mm; 5 µm particle size; Phenomenex, USA) at 258 nm according to the manufacturer’s instructions. The mobile phase composition was 2 g/l SDS and 6 ml/l acetic acid in water:acetonitrile:tetrahydrofuran (4:4:2 (v/v/v)) at a flow rate of 0.6 ml/min. The temperature of the analytical column was maintained at 40°C.

For the BZK assay, 2 ml samples were extracted with 4 ml of acetonitrile and ethyl acetate (1:1 (v/v)) for 4 h. The acetonitrile:ethyl acetate extracts were pooled, dried, and reconstituted with 100 µl of acetonitrile prior to HPLC analysis. The BZK analysis was performed by a modified method as described previously [36, 37]. Briefly, samples were analyzed by HPLC (Agilent 1200 series) with a C-18 Luna SCX column (4.6 × 150 mm; 5 µm particle size; Phenomenex) with UV detection at 265 nm. The initial mobile phase composition was 70% mobile phase A (20 mM sodium perchlorate in water) at a flow rate of 0.5 ml/min. Solvent B (20 mM sodium perchlorate in acetonitrile) was increased from

30% to 100% over 50 min [36].

**Microbiological methods.** To determine the antimicrobial effects of antiseptics on each of the six *B. cenocepacia* strains, a change in the start of growth time ($\Delta SGT$) method [38] was carried out using CHX (2 and 4 μg/ml) and BZK (10 and 20 μg/ml). First, CHX and BZK were diluted with 10 ml of distilled water to achieve final concentrations of CHX (20 and 40 μg/ml) and BZK (100 and 200 μg/ml) and then reserved for 42 days at 23°C. Freshly diluted CHX and BZK served as negative controls for abiotic transformation. To prepare the inocula, each of the six *B. cenocepacia* strains were grown on 1/10× TSA at 30°C for 48 h, washed with sterilized distilled water, and transferred into 20 ml of sterilized distilled water to achieve approximately $1.1 \times 10^7$ CFU/ml (optical density, $OD_{600}$ = 0.08−0.1). Then, 1 ml each of the above suspensions was transferred into 9 ml of sterilized distilled water to achieve approximately $1.1 \times 10^6$ CFU/ml. Finally, 20 μl each of antiseptic stock solutions and suspended cells was added into a 96-well plate containing 160 μl of tryptic soy broth (TSB) medium. Thus, the final inoculum of bacterial culture was approximately $1.1 \times 10^5$ CFU/ml, and the final concentrations were 2 and 4 μg/ml of CHX and 10 and 20 μg/ml of BZK, respectively. The 96-well plates were incubated at 23°C for 12 h and growth was measured by $OD_{600}$ with a Synergy MX spectrophotometer (BioTek Instruments, USA) [32, 33]. After 12 h incubation at 23°C, the number of wells in which growth had occurred (the negative control was $OD_{600} < 0.045$) was recorded. Wells with 0.12 $OD_{600}$ were recorded as the start point of the lag phase. The growth curves from the wells containing CHX (2 and 4 μg/ml) and BZK (10 and 20 μg/ml) were standardized to compare their bacteriostatic effects and were expressed as a change in the start of growth time ($\Delta SGT$). The $\Delta SGT$ measured between treated ($SGT_{\text{Treated}}$) and untreated ($SGT_{\text{Untreated control}}$) is defined as $\Delta SGT = SGT_{\text{Treated}} - SGT_{\text{Untreated control}}$, where treated ($SGT_{\text{Treated}}$) is derived from wells containing antiseptics, and untreated ($SGT_{\text{Untreated control}}$) is from wells without antiseptics. All other recorded conditions were measured as described previously [32, 35].

## Assessment of Bacteriostatic Activity of BCC-Contaminated CHX and BZK

**Analytical chemistry methods.** The influence of bacterial contamination on the concentration of antiseptics was quantitatively evaluated by analytical chemistry methods using six *B. cenocepacia* strains. First, CHX and BZK were diluted with 20 ml of distilled water to achieve final concentrations of 150 μg/ml (CHX) and 500 μg/ml (BZK). Each of the freshly grown six strains of *B. cenocepacia* was prepared on 1/10× TSA, transferred to distilled water, and diluted to approximately $10^6$, $10^5$, $10^4$, $10^3$, and $10^2$ CFU/ml. Then, 2 ml each of antiseptic stock solutions and suspended cells were added to a test tube containing 16 ml of distilled water, so that the final concentrations of CHX and BZK were 15 and 50 μg/ml, respectively. The final inocula were approximately $10^5$, $10^4$, $10^3$, $10^2$, and 10 CFU/ml. Samples were periodically withdrawn over the incubation period and the remaining CHX and BZK were measured using HPLC as described above. Experiments were done in triplicate.

**Microbiological methods.** To determine the effect of intrinsic contamination of antiseptics on the bacteriostatic activity, *B. cenocepacia* HI2976 (approximately $10^5$ CFU/ml) was inoculated into CHX (5 μg/ml) and BZK (20 μg/ml) and reserved for 28 days at 23°C. After 28 days of incubation, the *B. cenocepacia* HI2976 was removed aseptically by filtration (pore size rating 0.22 μm). Dilutions of uncontaminated CHX (5 μg/ml) and BZK (20 μg/ml) served as negative controls and were prepared in distilled water and reserved for 28 days at 23°C. The antimicrobial activity of the above filtered antiseptic samples was evaluated after the inoculation of *B. cenocepacia* J2315 by comparing the growth in the filtered antiseptics with that in the negative control samples. To prepare the inocula, *B. cenocepacia* J2315 was grown on 1/10× TSA at 30°C for 48 h and then transferred into 10 ml of sterilized distilled water (approximately $1.1 \times 10^8$ CFU/ml). Then, 1 ml of the suspension was diluted into 9 ml of sterilized distilled water (approximately $1.1 \times 10^7$ CFU/ml). Finally, 20 μl each of 10× TSB medium and suspended *B. cenocepacia* J2315 (approximately $1.1 \times 10^6$ CFU/ml) were added to a 96-well plate containing 160 μl of the filtered and control antiseptic solutions. The 96-well plate was incubated at 23°C for 40 h and the bacterial growth was measured as described above.

## Effects of Inoculum Size on the Survival of *B. cenocepacia* in Antiseptic Solutions

To determine the effect of cell numbers on the survival of *B. cenocepacia* in sub-MICs of CHX or BZK, each of the six *B. cenocepacia* strains was cultured on 1/10× TSA and transferred into 10 ml of sterilized distilled water. Various numbers of cells (approximately $10^5$, $10^4$, $10^3$, $10^2$, and 10 CFU/ml) were introduced into freshly prepared CHX (5 μg/ml) and BZK (20 μg/ml) as described above. The survival of *B. cenocepacia* in the antiseptics was evaluated after 20 min, and 1, 2, 3, 4, 7, 14, 21, and 28 days at 23°C. At each time point, 10 μl of serial dilutions of antiseptics with *B. cenocepacia* strains was inoculated onto 1/10× TSA and incubated at 23°C for 48 h [39]. Negative controls were prepared in distilled water and stored for 28 days at 23°C. All counts were performed in triplicate, and bacterial colonies were counted as CFU per 1 ml sample after incubation [32].

## Effects of Preincubation in Distilled Water on the Survival of *B. cenocepacia* in Antiseptic Solutions

To evaluate the effect of preincubation in distilled water on bacteriostatic activity, *B. cenocepacia* held in sterilized distilled water after 14 and 28 days was diluted to approximately $1.5 \times 10^8$ CFU/ml. Freshly diluted *B. cenocepacia* in sterilized distilled water served as a day 0 control. The survival of *B. cenocepacia* preincubated in distilled water was evaluated in freshly prepared CHX (5 μg/ml) and BZK (20 μg/ml) solutions. After 14 days of incubation, serial dilutions of the antiseptic solutions containing *B cenocepacia* were used to inoculate 1/10× TSA and incubated for

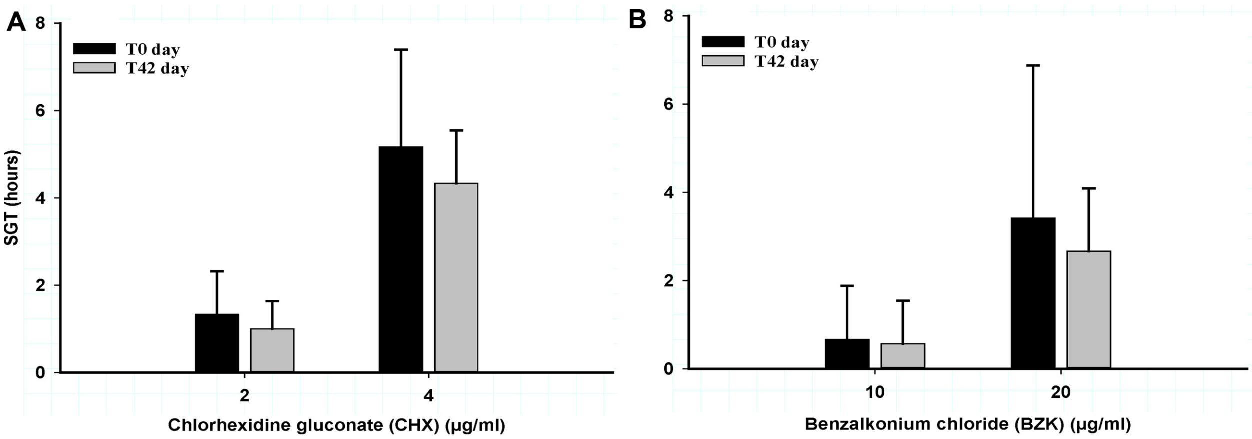


**Fig. 1.** Change in the start of growth time (Δ*SGT*) by chlorhexidine gluconate (CHX) and benzalkonium chloride (BZK) against six *Burkholderia cenocepacia* strains after opening containers at initial (0) day and 42 days.

(**A**) Δ*SGT* of 2 and 4 μg/ml CHX. (**B**) Δ*SGT* of 10 and 20 μg/ml BZK. Values represent the mean ± standard deviation of six *B. cenocepacia* strains in triplicate.

48 h at 23°C. All counts were performed in triplicate, and bacterial colonies were counted as described above.

## Results

### Bacteriostatic Effects of Non-Contaminated Antiseptic Solutions after 42 Days

The concentrations of CHX (10 μg/ml) or BZK (50 μg/ml) opened and exposed for 42 days at 23°C were compared with the initial (day 0) concentrations of these antiseptics prepared in sterile distilled water. After 42 days, the concentrations of CHX or BZK in the sterilized distilled water were approximately 9.8 ± 0.7 and 49.7 ± 1.4 μg/ml, respectively, which were close to the initial concentrations of the antiseptics. No abiotic transformation of CHX or BZK was observed in the antiseptic solutions (data not shown).

*B. cenocepacia* grew well in 1/10× TSB containing up to MIC of CHX (100–500 μg/ml) or BZK (200–500 μg/ml) (data not shown). Better growth was observed in the media spiked with low concentrations of CHX or BZK. The bacteriostatic effect was assessed by growth kinetic assays and defined as a change in start of growth time (Δ*SGT*) (Fig. 1). These data represent the average of Δ*SGT* of the six strains. The *B. cenocepacia* strains showed a higher value of *SGT* with 4 μg/ml of CHX or 20 μg/ml of BZK than with 2 μg/ml of CHX or 10 μg/ml of BZK. The high Δ*SGT* at the higher concentrations of CHX or BZK indicated a prolonged lag phase and thus increased bacteriostatic activity CHX or BZK against *B. cenocepacia* in distilled water. Means of Δ*SGT* at $T_0$ and $T_{42\text{days}}$ were 1.3 ± 0.9 and 1.0 ± 0.6 h, respectively, in 2 μg/ml of CHX and 5.2 ± 2.2 and 4.3 ± 1.2 h, respectively, in 4 μg/ml of CHX. For BZK, the means of Δ*SGT* at $T_0$ and $T_{42\text{days}}$ were 0.7 ± 1.2 and 0.6 ± 0.9 h, respectively, in 10 μg/ml and 3.4 ± 3.5 and 2.7 ± 1.4 h, respectively, in 20 μg/ml of BZK. The Δ*SGT* on the initial day showed slightly higher values in all concentrations than 42 days reserved antiseptics; however, there was no difference between the initial day and 42 days.

### Bacteriostatic Effects of Contaminated Antiseptic Solutions after 28 Days

Various concentrations of the six *B. cenocepacia* strains ($10^1$–$10^5$ CFU/ml) survived in sterilized distilled water containing CHX (15 μg/ml) or BZK (50 μg/ml) for 28 days at 23°C. Culture filtrates from each of the six *B. cenocepacia* strains incubated with CHX or acetonitrile:ethyl acetate extracts from BZK were analyzed by HPLC. Although suspended cells (approximately $10^5$, $10^4$, $10^3$, $10^2$, and 10 CFU/ml) were introduced into freshly prepared CHX or BZK, there was no net change in the concentrations of BZK or CHX (Fig. 2). These data represent the average of concentrations of BZK or CHX for the six strains. Evaluation of net change in the concentrations of antiseptics indicated that the *B. cenocepacia* strains did not degrade CHX (10 μg/ml) and BZK (50 μg/ml) in sterilized distilled water during the incubation for 28 days at 23°C. Furthermore, we could not find any metabolites of CHX and BZK (data not shown).

The six *B. cenocepacia* strains survived in 50–100 μg/ml of CHX or 100–500 μg/ml of BZK. Strain HI2976 was resistant at the higher concentrations of CHX (100 μg/ml) or BZK (200 μg/ml) than strain J2315 at 50 μg/ml of CHX or 50 μg/ml of BZK. *B. cenocepacia* HI2976 (approximately $10^5$ CFU/ml)

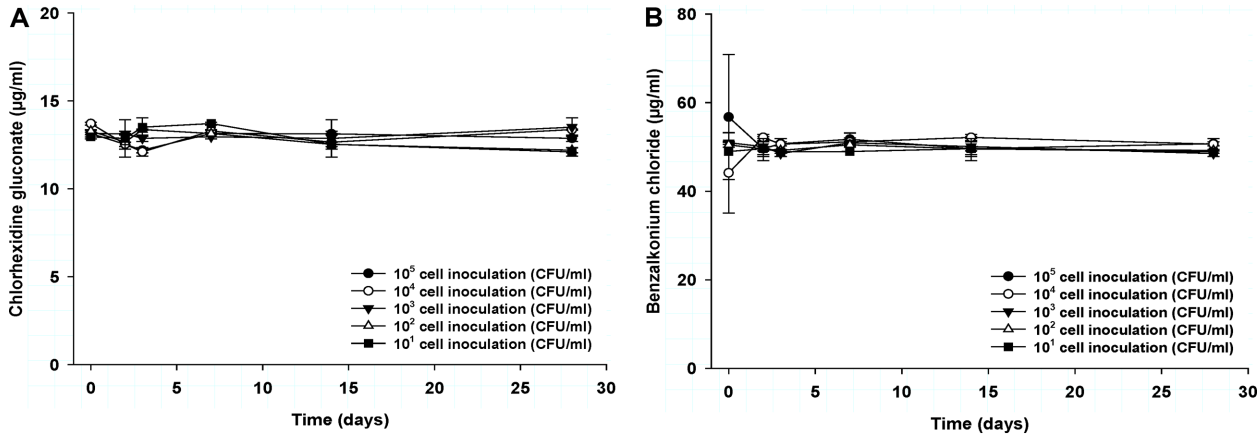


**Fig. 2.** Monitoring of 15 μg/ml chlorhexidine gluconate (**A**) and 50 μg/ml benzalkonium chloride (**B**) concentrations with various cell numbers ($10^5$, $10^4$, $10^3$, $10^2$, and 10 CFU/ml) of *Burkholderia cenocepacia* for 28 days.

Values represent the mean ± standard deviation of six *B. cenocepacia* strains in triplicate.

was inoculated into CHX (5 μg/ml) or BZK (20 μg/ml), incubated for 28 days at 23°C, and then the bacterial cells were removed by sterile filtration. In 5 μg/ml of CHX preincubated with strain HI2976, the maximum growth rate of strain J2315 was similar after 24 h of incubation (Fig. 3A). At a sub-MIC (20 μg/ml) of BZK, strain J2315 showed a substantially shorter lag phase in the solution pre-contaminated with strain HI2976 (T28_BZK_$10^5$ cell) than in the solution not challenged with strain HI2976 (T28_BZK_no cell) in the first 10 h (Fig. 3B). CHX showed slightly higher optical density at 600 nm in all tested microbial challenges than BZK; however, there was no difference between with and without the pre-contamination with strain HI2976 for 28 days.

## Effects of Inoculum Size on the Survival of *B. cenocepacia* in Antiseptic Solutions

The growth recovery profile for each of the six *B. cenocepacia* strains was compared in 1/10× TSA with approximately $10^5$, $10^4$, $10^3$, $10^2$, and 10 CFU/ml inocula (Fig. 4). These data represent averages of the six strains. All tested *B. cenocepacia* strains were shown to survive 28 days in CHX (5 μg/ml) or BZK (20 μg/ml). *B. cenocepacia* strains in diluted CHX or BZK were detected up to $10^2$ CFU/ml of

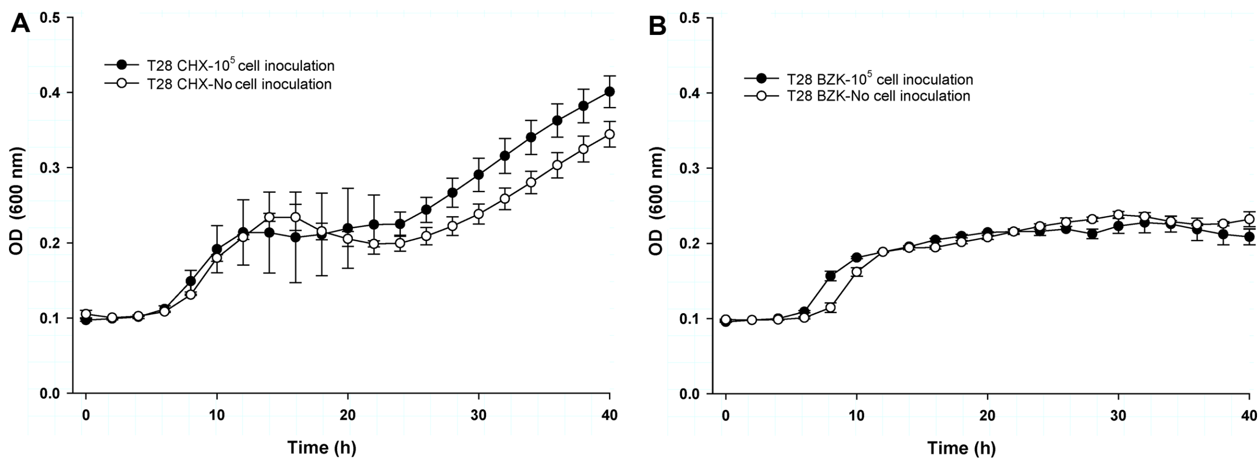


**Fig 3.** Kinetics of the growth of *Burkholderia cenocepacia* J2315 in 5 μg/ml chlorhexidine gluconate (CHX) (**A**) and 20 μg/ml benzalkonium chloride (BZK) (**B**) preincubated with $10^5$ cells of strain HI2976 for 28days.

Symbols represent averages of triplicate samples, and error bars represent the standard deviation. Closed circle: with *B. cenocepacia* HI2976 (approximately $10^5$ CFU/ml) for 28 days at 23°C. Open circle: without *B. cenocepacia* HI2976.

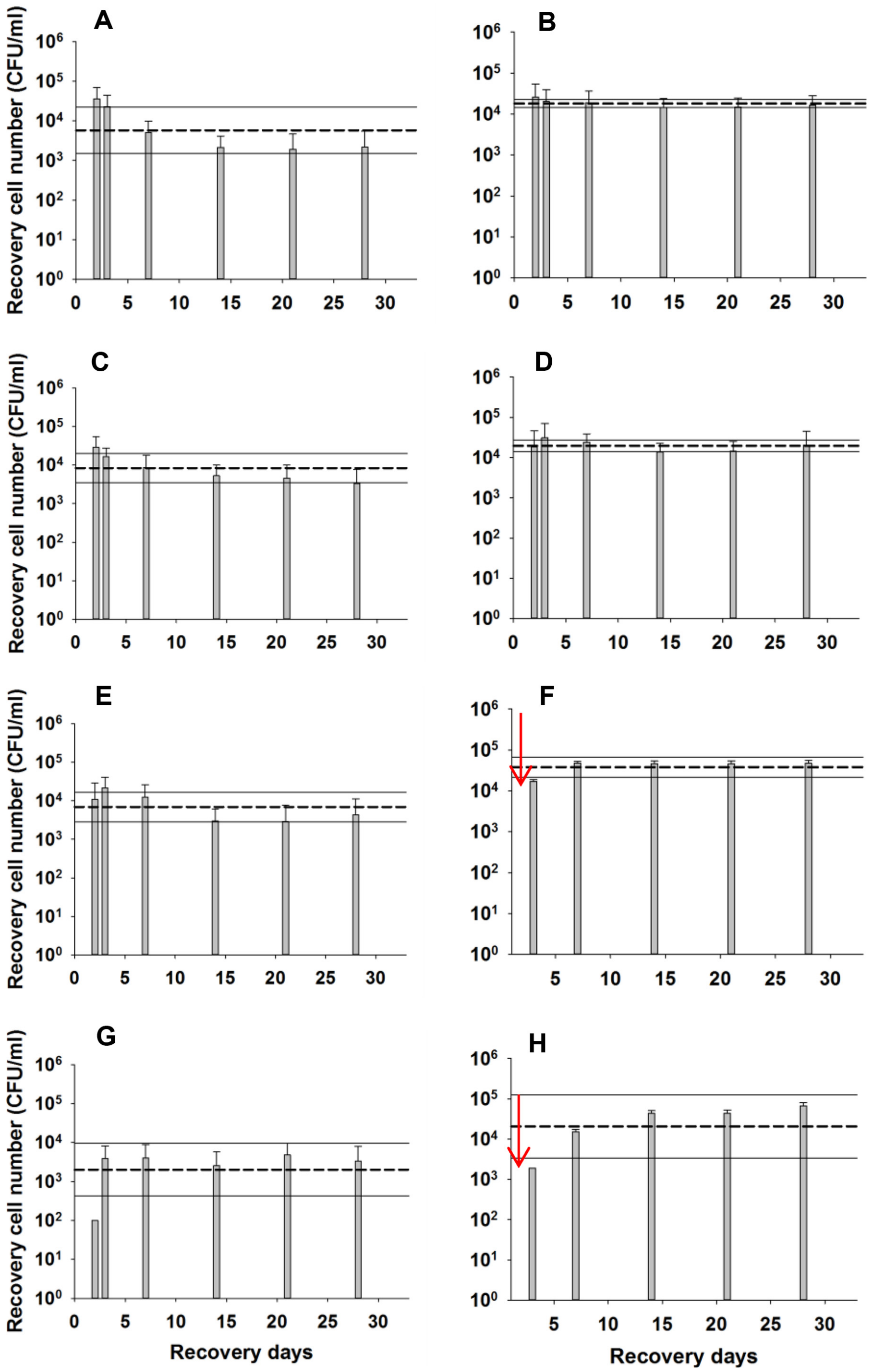


**Fig. 4.** Recovery and detection with various cell numbers of six *Burkholderia cenocepacia* strains during incubation with 5 μg/ml chlorhexidine gluconate (**A**, **C**, **E**, and **G**) and 20 μg/ml benzalkonium chloride (**B**, **D**, **F**, and **H**) for 28 days.

(**A** and **B**) $10^5$ CFU/ml inoculation, (**C** and **D**) $10^4$ CFU/ml inoculation, (**E** and **F**) $10^3$ CFU/ml inoculation, (**G** and **H**) $10^2$ CFU/ml inoculation. Values represent the mean ± standard deviation of six *B. cenocepacia* strains in triplicate. Solid lines show the 95% upper and lower predicted interval. Dashed lines represent the mean. Six *B. cenocepacia* strains were not recovered at 2 days indicated by arrows.

inoculum size, but were not detected at $10^1$ CFU/ml after incubation for 28 days at 23°C. With approximately $10^5$ CFU/ml inoculation on CHX, viable plate counts at 2 and 3 days were $3.5 \pm 3.3 \times 10^4$ and $2.3 \pm 2.2 \times 10^4$ CFU/ml,

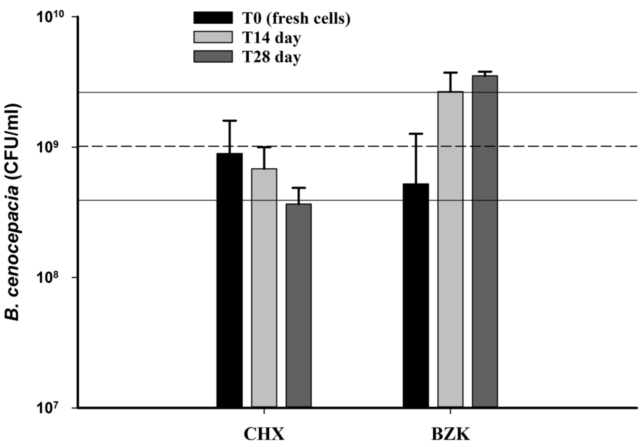


**Fig 5.** Comparison of the recovery of *Burkholderia cenocepacia* in 2 µg/ml chlorhexidine gluconate (CHX) and 10 µg/ml benzalkonium chloride (BZK) after 0, 14, and 28 days preincubation in distilled water.

Values represent the mean ± standard deviation of six *B. cenocepacia* strains in triplicate. Solid lines show the 95% upper and lower predicted interval. The dashed line represents the mean.

respectively, and then decreased to 2.2 ± 3.3 × $10^3$ CFU/ml after 28 days (Fig. 4A). Moreover, with $10^4$ and $10^3$ CFU/ml inoculations the highest recovery was observed at 2 and 3 days (1.1–2.9 × $10^4$ CFU/ml) and then decreased to 3.3–4.3 × $10^3$ CFU/ml after 28 days (Figs. 4C and 4E). However, with a $10^2$ CFU/ml inoculum, the greatest recovery was observed at 3 days (3.9 ± 4.3 × $10^4$ CFU/ml) and then remained constant for 28 days (3.3 ± 4.6 × $10^3$) (Fig. 4G). In contrast, $10^2$ and $10^3$ CFU/ml inocula into BZK were not recovered at 2 days, suggesting that the growth of the strain was inhibited more in BZK than in CHX (Figs. 4F and 4H). With $10^5$ or $10^4$ CFU/ml inoculum into BZK, the cultivability of the strain reached a maximum of 2.6 ± 2.9 × $10^4$ CFU/ml at 2 days and 3.1 ± 3.9 × $10^4$ CFU/ml at 3 days, respectively, and then remained constant for 28 days (1.8 ± 0.4 × $10^4$ and 2.0 ± 0.7 × $10^4$ CFU/ml) (Figs. 4B and 4D). Positive controls with different inocula of *B. cenocepacia* strains were recovered at levels of $10^4$ CFU/ml at 2 days and remained constant for 28 days (data not shown). All data fell within the PI95 value. No significant decrease in cultivability compared with $10^5$ CFU/ml inoculation was observed when $10^4$, $10^3$, and $10^2$ CFU/ml inocula into CHX (5 µg/ml) or BZK (20 µg/ml) were grown in 1/10× TSA.

### Effects of Preincubation in Distilled Water on the Survival of *B. cenocepacia* in Antiseptic Solutions

Six *B. cenocepacia* strains have been shown to persist in distilled water for 40 days under energy-restricted conditions. In order to investigate the effects of the length of the preincubation period in distilled water on the survival of *B. cenocepacia* in antiseptic solutions, cells suspended in sterilized distilled water were harvested at days 0, 14, and 28 and then transferred into antiseptic solutions. Average recovery of the six *B. cenocepacia* strains decreased in the CHX solution (8.95 ± 7.01 × $10^8$ to 3.67 ± 1.21 × $10^8$) but increased in BZK (5.22 ± 7.47 × $10^8$ to 3.52 ± 0.27 × $10^9$) as the length of preincubation increased, indicating that the survival rates of preincubated BCC strains are dependent upon the types of antiseptics. Preincubations of 14 and 28 days showed increase in the recovery of *B. cenocepacia* because the data from these two groups did not lie within the PI95 value (Fig. 4). The results obtained clearly indicated that *B. cenocepacia* preincubated in distilled water for 28 days was recovered in higher densities from 20 µg/ml of BZK than from 5 µg/ml of CHX at 0 day and 14 days after inoculation.

## Discussion

The FDA mandated specifying expiration dates on all prescription and over-the-counter medicines in 1979 [40]. Expired preservatives can be less effective or more risky owing to a possible change in chemical concentration or composition. Multiple outbreaks have been linked to contaminated CHX and BZK [3]. Most reported outbreaks caused by CHX and BZK solutions have been traced to improper dilution [17, 18, 25]. Since diluted or opened CHX and BZK may have decreased bactericidal/bacteriostatic potency over a period of time, it is important to adhere to an expiration date. Based on its chemical structure and the presence of an amide functional group, the degradation of CHX is expected under different stress conditions, including autoclaving [41], use in antacid suspensions [42], pH [43], and photocatalytic degradation [44]. On the other hand, BZK is a mixture of alkylbenzyldimethylammonium chlorides that usually contain C-10, C-12, C-14, and C-16 homologs [45]. According to the BZK structure and previous studies [46–48], this chemical could be stable under various stress conditions. In this study, without applying any stresses to CHX and BZK, dilution in distilled water and exposure to ambient air did not result in significant decrease in concentration or bacteriostatic effects over 42 days (Fig. 1). These results provide indirect evidence that CHX and BZK are stable in sterilized distilled water.

It is assumed that medical products are no longer sterile once bottles have been opened. CHX and BZK are used in a variety of commercial products in concentrations ranging from 0.02% to 5% (200–50,000 μg/ml). Concentrations below 0.05% may be insufficient to kill or inhibit the growth of *B. cenocepacia.* As a result, *B. cenocepacia* strains could be resistant to 100–500 μg/ml of CHX and 200–500 μg/ml of BZK in 1/10× TSB. In addition, we previously reported that *B. cenocepacia* can remain viable with low susceptibility to antiseptics for 40 days [32]. We chose in the present study *B. cenocepacia* HI2976 and J2315 to determine the effect of intrinsic contamination of antiseptics on the bacteriostatic activity. *B. cenocepacia* HI2976 was susceptible at the highest CHX and BZK concentration, but after 40 days in water showed high susceptibility (50 μg/ml of CHX or 200 μg/ml of BZK) [32]. In addition, strain J2315 survived in 10 μg/ml of CHX or 30 μg/ml of BZK after 40 days in water incubations (data not shown). *B. cenocepacia* incubated in nutrient-depleted water for a long time can become susceptible to CHX and BZK [32]. In experiments at sub-MIC levels of CHX (5 μg/ml) and BZK (20 μg/ml), strain J2315 showed no differences to strain HI2976 when incubated with and without the antiseptic exposure for 28 days. Because the object of the pre-antiseptic contamination comparison test was to evaluate the kinetics of growth in antiseptic solutions, *B. cenocepacia* strains must survive in and be recovered from antiseptic solutions.

CHX and BZK are bacteriostatic or bactericidal depending on their concentrations. Although antimicrobial activity of CHX and BZK was not changed by abiotic and biotic conditions, both the bacterial inoculum size and intrinsic contamination may pose a potential risk. Thus, it is important to ensure the efficient detection of BCC in industrial settings and in pharmaceutical products [32]. In this study, we chose 1/10 TSA to monitor the recovery and survival of six *B. cenocepacia* strains. As a result, the *B. cenocepacia* strains were detected with an inoculum size as low as $10^2$ CFU/ml after 3 days at 23°C and even after 28 days of extended incubation in antiseptic solutions. These results are in agreement with previous studies demonstrating that BCC can survive and remain viable in CHX and BZK solutions for long periods of time [32]. It has been reported that *Pseudomonas aeruginosa*, with the genome size of 6.3 Mb, can adapt rapidly to multiple stressful environmental conditions, including starvation, temperature shock, desiccation, and antibiotic treatments [49−51]. Moreover, Chen *et al*. [52] reported that the starvation of *P. aeruginosa* ATCC 27853 in various water media enhanced its resistance to beta-lactam antibiotics. Therefore, that BCC strains adapt to adverse conditions may be due to their large genomes (5.5–10 Mb) [53, 54], which may support their enormous metabolic versatility and thus their adaptability to almost any challenging environmental condition.

General resistance mechanisms against antiseptics in bacteria may include adaptive phenotypic changes, efflux pumps [55], metabolic inactivation of biocides [56], and alterations of the target site [1, 36]. For example, *Pseudomonas* sp. strain A-3 isolated from sludge was able to degrade CHX [56]. Recently, we reported that the presence of additional nutrient was necessary for the degradation of BZK and its alkyl derivatives by BCC [36]. *B. cenocepacia* could not degrade BZK in distilled water without nutrients. Because BZK enters BCC cells quickly by simple diffusion, the ability of BCC strains to increase efflux pump activity may partly explain their resistance against BZK [36]. In addition, the toxicity of antiseptic solutions may induce a viable but nonculturable state of BCC in BZK solutions [32]. When 1/10× TSB was added to distilled water on the $28^{th}$ day, *B. cenocepacia* grew well in 10 μg/ml of CHX and 50 μg/ml of BZK (data not shown). Our data showed that there was no net change in the concentrations of CHX or BZK (Fig. 2). In addition, the difference in bacteriostatic or bactericidal activity between contaminated antiseptics and non-contaminated antiseptics was not significant (Fig. 3). However, *B. cenocepacia* strains remained viable and were well recovered from antiseptic solutions, suggesting that *B. cenocepacia* strains in contaminated antiseptics could be transmitted to patients and cause problems subsequently.

In this study, we compared the bacteriostatic effects of extended storage of opened or diluted solutions of CHX and BZK at sublethal concentrations on six *B. cenocepacia* strains using chemical and microbiological assays. Abiotic and biotic changes in the toxicity of CHX and BZK were not observed for 28 days at 23°C. The six *B. cenocepacia* strains in CHX and BZK remained viable with low susceptibility to the antiseptics and were recovered from solutions inoculated with $10^2$ CFU/ml and above in CHX (5 μg/ml) or BZK (20 μg/ml). The six *B. cenocepacia* strains preincubated in distilled water for 14 and 28 days were recovered from 5 μg/ml of CHX and 20 μg/ml of BZK, suggesting that *B. cenocepacia* strains could cause intrinsic contamination of antiseptics. To assure public safety, opened drug products containing these preservatives or diluted antiseptics will need the safe-use period (shelf-life) warning.

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## References

1. Gnanadhas DP, Marathe SA, Chakravortty D. 2013. Biocides – resistance, cross-resistance mechanisms and assessment. *Expert Opin. Investig. Drugs* **22:** 191-206.
2. Gilbert P, Moore LE. 2005. Cationic antiseptics: diversity of action under a common epithet. *J. Appl. Microbiol.* **99:** 703-715.
3. Weber DJ, Rutala WA, Sickbert-Bennett EE. 2007. Outbreaks associated with contaminated antiseptics and disinfectants. *Antimicrob. Agents Chemother.* **51:** 4217-4224.
4. D'Arcy PF, Taylor EP. 1962. Quaternary ammonium compounds in medicinal chemistry. I. *J. Pharm. Pharmacol.* **14:** 129-146.
5. D'Arcy PF, Taylor EP. 1962. Quaternary ammonium compounds in medicinal chemistry. II. *J. Pharm. Pharmacol.* **14:** 193-216.
6. Dabbah R, Chang WW, Cooper MS. 1996. The use of preservatives in compendial articles. *Pharmacopeial Forum* **22:** 2696-2704.
7. Brambilla E, Cagetti MG, Fadini L, Pariset P, Strohmenger L, Twetman S. 2004. Chlorhexidine concentration in saliva after topical treatment with an antibacterial dental varnish. *Am. J. Dent.* **17:** 196-198.
8. Russell AD. 2003. Biocide use and antibiotic resistance: the relevance of laboratory findings to clinical and environmental situations. *Lancet Infect. Dis.* **3:** 794-803.
9. Tandukar M, Oh S, Tezel U, Konstantinidis KT, Pavlostathis SG. 2013. Long-term exposure to benzalkonium chloride disinfectants results in change of microbial community structure and increased antimicrobial resistance. *Environ. Sci. Technol.* **47:** 9730-9738.
10. To MS, Favrin S, Romanova N, Griffiths MW. 2002. Postadaptational resistance to benzalkonium chloride and subsequent physicochemical modifications of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* **68:** 5258-5264.
11. Mahenthiralingam E, Urban TA, Goldberg JB. 2005. The multifarious, multireplicon *Burkholderia cepacia* complex. *Nat. Rev. Microbiol.* **3:** 144-156.
12. Govan JRW, Hughes JE, Vandamme P. 1996. *Burkholderia cepacia*: medical, taxonomic and ecological issues. *J. Med. Microbiol.* **45:** 395-407.
13. Mahenthiralingam E, Vandamme P. 2005. Taxonomy and pathogenesis of the *Burkholderia cepacia* complex. *Chron. Respir. Dis.* **2:** 209-217.
14. Dixon RE, Kaslow RA, Mackel DC, Fulkerson CC, Mallison GF. 1976. Aqueous quaternary ammonium antiseptics and disinfectants – use and misuse. *J. Am. Med. Assoc.* **236:** 2415-2417.
15. FDA U. 2013. Enforcement report – week of March 20, 2013. Available from https://www.accessdata.fda.gov/scripts/ires/index.cfm. Accessed 9 May 2017.
16. Fox JG, Beaucage CM, Folta CA, Thornton GW. 1981. Nosocomial transmission of *Serratia marcescens* in a veterinary hospital due to contamination by benzalkonium chloride. *J. Clin. Microbiol.* **14:** 157-160.
17. Guinness M, Levey J. 1976. Contamination of aqueous dilutions of resiguard disinfectant with *Pseudomonas*. *Med. J. Aust.* **2:** 392-392.
18. Kaslow RA, Macel DC, Mallison GF. 1976. Nosocomial pseudobacteremia. Positive blood cultures due to contaminated benzalkonium antiseptic. *J. Am. Med. Assoc.* **236:** 2407-2409.
19. Lee JC, Fialkow PJ. 1961. Benzalkonium chloride – source of hospital infection with gram-negative bacteria. *J. Am. Med. Assoc.* **177:** 708-710.
20. Malizia WF, Gangarosa EJ, Goley AF. 1960. Benzalkonium chloride as a source of infection. *N. Engl. J. Med.* **263:** 800-802.
21. Nakashima AK, Mccarthy MA, Martone WJ, Anderson RL. 1987. Epidemic septic arthritis caused by *Serratia marcescens* and associated with a benzalkonium chloride antiseptic. *J. Clin. Microbiol.* **25:** 1014-1018.
22. Nasser RM, Rahi AC, Haddad MF, Daoud Z, Irani-Hakime N, Almawi WY. 2004. Outbreak of *Burkholderia cepacia* bacteremia traced to contaminated hospital water used for dilution of an alcohol skin antiseptic. *Infect. Control Hosp. Epidemiol.* **25:** 231-239.
23. Plotkin SA, Austrian R. 1958. Bacteremia caused by *Pseudomonas* sp. following the use of materials stored in solutions of a cationic surface active agent. *Am. J. Med. Sci.* **235:** 621-627.
24. Sautter RL, Mattman LH, Legaspi RC. 1984. *Serratia marcescens* meningitis associated with a contaminated benzalkonium chloride solution. *Infect. Control Hosp. Epidemiol.* **5:** 223-225.
25. Sobel JD, Hashman N, Reinherz G, Merzbach D. 1982. Nosocomial *Pseudomonas cepacia* infection associated with chlorhexidine contamination. *Am. J. Med.* **73:** 183-186.
26. Tiwari TSP, Ray B, Jost KC, Rathod MK, Zhang YS, Brown-Elliott BA, *et al.* 2003. Forty years of disinfectant failure: outbreak of postinjection *Mycobacterium abscessus* infection caused by contamination of benzalkonium chloride. *Clin. Infect. Dis.* **36:** 954-962.
27. CDC. 2017. Multistate outbreak of *Burkholderia cepacia* bloodstream infections associated with contaminated prefilled saline flush syringes. Available from https://www.cdc.gov/hai/outbreaks/b-cepacia-saline-flush/index.html. Accessed 9 May 2017.
28. FDA. 2016. FDA updates on multistate outbreak of *Burkholderia cepacia* infections. Available from https://www.fda.gov/drugs/drugsafety/ucm511527.htm. Accessed 9 May 2017.

29. LiPuma JJ, Spilker T, Gill LH, Campbell PW 3rd, Liu L, Mahenthiralingam E. 2001. Disproportionate distribution of *Burkholderia cepacia* complex species and transmissibility markers in cystic fibrosis. *Am. J. Respir. Crit. Care Med.* **164:** 92-96.
30. Reik R, Spilker T, Lipuma JJ. 2005. Distribution of *Burkholderia cepacia* complex species among isolates recovered from persons with or without cystic fibrosis. *J. Clin. Microbiol.* **43:** 2926-2928.
31. Shehabi AA, Abu-al-Soud W, Mahafzah A, Khuri-Bulos N, Khader IA, Ouis IS, *et al.* 2004. Investigation of *Burkholderia cepacia* nosocomial outbreak with high fatality in patients suffering from diseases other than cystic fibrosis. *Scand. J. Infect. Dis.* **36:** 174-178.
32. Kim JM, Ahn Y, LiPuma JJ, Hussong D, Cerniglia CE. 2015. Survival and susceptibility of *Burkholderia cepacia* complex in chlorhexidine gluconate and benzalkonium chloride. *J. Ind. Microbiol. Biotechnol.* **42:** 905-913.
33. Rose H, Baldwin A, Dowson CG, Mahenthiralingam E. 2009. Biocide susceptibility of the *Burkholderia cepacia* complex. *J. Antimicrob. Chemother.* **63:** 502-510.
34. Vigeant P, Loo VG, Bertrand C, Dixon C, Hollis R, Pfaller MA, *et al.* 1998. An outbreak of *Serratia marcescens* infections related to contaminated chlorhexidine. *Infect. Control Hosp. Epidemiol.* **19:** 791-794.
35. Ahn Y, Kim JM, Ahn H, Lee YJ, LiPuma JJ, Hussong D, *et al.* 2014. Evaluation of liquid and solid culture media for the recovery and enrichment of *Burkholderia cenocepacia* from distilled water. *J. Ind. Microbiol. Biotechnol.* **41:** 1109-1118.
36. Ahn Y, Kim JM, Kweon O, Kim SJ, Jones RC, Woodling K, *et al.* 2016. Intrinsic resistance of *Burkholderia cepacia* complex to benzalkonium chloride. *MBio.* **7:** e01716-16.
37. Hajaya MG, Pavlostathis SG. 2012. Fate and effect of benzalkonium chlorides in a continuous-flow biological nitrogen removal system treating poultry processing wastewater. *Bioresour. Technol.* **118:** 73-81.
38. Hazan R, Que Y-A, Maura D, Rahme LG. 2012. A method for high throughput determination of viable bacteria cell counts in 96-well plates. *BMC Microbiol.* **12:** 259.
39. Cassidy MB, Leung KT, Lee H, Trevors JT. 2000. A comparison of enumeration methods for culturable *Pseudomonas fluorescens* cells marked with green fluorescent protein. *J. Microbiol. Methods* **40:** 135-145.
40. FDA. 2015. Expiration dating and stability testing for human drug products. Available from https://www.fda.gov/ICECI/Inspections/InspectionGuides/InspectionTechnicalGuides/ucm072919.htm. Accessed 17 May 2017.
41. Dolby J, Gunnarsson B, Kronberg L, Wikner H. 1972. Stability of chlorhexidine when autoclaving. *Pharm. Acta Helv.* **47:** 615-620.
42. Franck M, Schmidt PC. 1993. Degradation of chlorhexidine in antacid suspensions – a novel approach to describe degradation kinetics. *Eur. J. Pharm. Biopharm.* **39:** 19-24.
43. Zong Z, Kirsch LE. 2012. Studies on the instability of chlorhexidine, part I: kinetics and mechanisms. *J. Pharm. Sci.* **101:** 2417-2427.
44. Das R, Sarkar S, Chakraborty S, Choi H, Bhattacharjee C. 2014. Remediation of antiseptic components in wastewater by photocatalysis using $TiO_2$ nanoparticles. *Ind. Eng. Chem. Res.* **53:** 3012-3020.
45. Rucker RR, Johnson HE, Ordal EJ. 1949. An investigation of the bactericidal action and fish toxicity of two homologous series of quaternary ammonium compounds. *J. Bacteriol.* **57:** 225-234.
46. Jovovic M, Kostic N, Jancic-Stojanovic B, Malenovic A. 2012. Investigation of tropicamide and benzalkonium chloride stability using liquid chromatography. *J. Liq. Chromatogr. Relat. Technol.* **35:** 231-239.
47. Parhizkari G, Delker G, Miller RB, Chen C. 1995. A stability indicating HPLC method for the determination of benzalkonium chloride in 0.5 percent tramadol ophthalmic solution. *Chromatographia* **40:** 155-158.
48. Parhizkari G, Miller RB, Chen C. 1995. A stability indicating HPLC method for the determination of benzalkonium chloride in phenylephrine HCl 10 percent ophthalmic solution. *J. Liq. Chromatogr.* **18:** 553-563.
49. Cheriaa J, Rouabhia M, Maatallah M, Bakhrouf A. 2012. Phenotypic stress response of *Pseudomonas aeruginosa* following culture in water microcosms. *J. Water Health* **10:** 130-139.
50. Goh EB, Yim G, Tsui W, McClure J, Surette MG, Davies J. 2002. Transcriptional modulation of bacterial gene expression by subinhibitory concentrations of antibiotics. *Proc. Natl. Acad. Sci. USA* **99:** 17025-17030.
51. Ramos JL, Gallegos MT, Marques S, Ramos-Gonzalez MI, Espinosa-Urgel M, Segura A. 2001. Responses of gram-negative bacteria to certain environmental stressors. *Curr. Opin. Microbiol.* **4:** 166-171.
52. Chen HY, Yuan M, Livermore DM. 1995. Mechanisms of resistance to beta-lactam antibiotics amongst *Pseudomonas aeruginosa* isolates collected in the UK in 1993. *J. Med. Microbiol.* **43:** 300-309.
53. Jung JY, Ahn Y, Kweon O, LiPuma JJ, Hussong D, Marasa BS, Cerniglia CE. 2017. Improved high-quality draft genome sequence and annotation of *Burkholderia contaminans* LMG $23361^T$. *Genome Announc.* **5:** e00245-e00217.
54. Ong HS, Mohamed R, Firdaus-Raih M. 2012. Comparative genome sequence analysis reveals the extent of diversity and conservation for glycan-associated proteins in *Burkholderia* spp. *Comp. Funct. Genomics* **2012:** 752867.
55. Guglierame P, Pasca MR, De Rossi E, Buroni S, Arrigo P, Manina G, *et al.* 2006. Efflux pump genes of the resistance nodulation division family in *Burkholderia cenocepacia* genome. *BMC Microbiol.* **6:** 66.
56. Kido Y, Kodama H, Uraki F, Uyeda M, Tsuruoka M, Shibata M. 1988. Microbial degradation of disinfectants. II. Complete degradation of chlorhexidine. *Eisei Kagaku* **34:** 97-101.