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Survival and susceptibility of *Burkholderia cepacia* complex in chlorhexidine gluconate and benzalkonium chloride

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Abstract The *Burkholderia cepacia* complex (BCC) includes opportunistic pathogenic bacteria that have occasionally been recovered from various pharmaceutical products, including antiseptics and disinfectants. Plausible reasons for the contamination include intrinsic sources, such as inadequate process controls, especially for water or equipment used during product manufacture, or extrinsic sources, such as improper handling and dilution or distribution in contaminated containers. Because the survival of BCC in antiseptics is a concern to the public health and pharmaceutical industry, we determined minimum inhibitory concentrations (MICs) of 36 BCC strains against the antiseptics, following exposure to chlorhexidine gluconate (CHX) and benzalkonium chloride (BZK) solutions (1–500 µg/ml for each chemical). Susceptibility to CHX and BZK varied across the BCC strains and was recorded as mean 90.3 and 111.1 µg/ml, respectively, at initial inoculation, which was significantly higher than the 46.4 and 61.1 µg/ml levels measured for BCC incubated in water for 40 days. After determining antiseptic MICs of individual BCC strains, BCC recovery was measured on Tryptic Soy Agar (TSA), Reasoner's Second Agar (R2A) and diluted preparations of these media under their sub-MICs. The survival of BCC was monitored for 14 days (336 h) in sub-MICs diluted to less than their antiseptic susceptible concentration value. Diluted TSA and R2A media exhibited greater efficiency of recovery for most BCC strains from the CHX and BZK solutions than full strength TSA or R2A. For BCC survival in antiseptic solutions, the cell number of BCC decreased rapidly within the first 20 min in both antiseptics, but after this, recovery remained constant in CHX and increased in BZK over the 14 day incubation period. The results indicate that BCC in water can remain viable with low susceptibility to antiseptics for 14 days, which suggests the necessity for improved detection methods and control measures to monitor BCC contamination in pharmaceutical products.

Keywords *Burkholderia cepacia* complex · Chlorhexidine gluconate · Benzalkonium chloride · Survival · Susceptibility

Introduction

Antiseptics and disinfectants exhibit a broad spectrum of antimicrobial activity [22]. As defined in Sec 201 of the Federal Food Drug, and Cosmetic Act, an antiseptic is a germicide that destroys microorganisms, especially pathogenic ones. Antiseptics are mainly used in health care to reduce potential infection by transient microbiota on the hands of health care workers and are adjusted for proper skin antisepsis, and disinfectants are used to sterilize medical and industrial devices [36]. Generally alcohols, chlorohexidine gluconate (CHX), iodine and iodophors, quaternary ammonium compounds [e.g., benzalkonium chloride (BZK)], and triclosan are commonly used as antiseptic agents in the United States [4]. They rapidly penetrate into microorganisms and affect proteins, nucleotides, and fatty

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acids, which culminate in cell death [11, 22, 37]. A review of FDA pharmaceutical product recalls indicates that *Burkholderia cepacia* complex (BCC) isolates are some of the most frequently isolated microorganisms contaminating sterile and non-sterile products, and several recent microbial outbreaks were linked to antiseptics that were found to contain BCC [14, 18, 46].

BCC is a group of gram-negative bacteria in the β-Proteobacteria subdivision, recognized as opportunistic pathogens, especially for cystic fibrosis (CF) patients [2, 21]. BCC have recently expanded to 18 genetically distinct species including *B. pseudomultivorans* [30]. BCC are widely distributed in soil, water, and in many other environments, such as hospital equipment and medical devices [2, 44]. BCC bacteria possess a high innate resistance to antimicrobial and biocidal agents, which seems to result from various efflux pumps and their potential to form biofilms [7, 20, 21, 34, 40]. Therefore, clinical isolation methods to detect BCC have used antibiotic combinations, including high concentrations of polymyxin, gentamicin and vancomycin, in the culture media [24]. Rose et al. reported low susceptibility of BCC to various antiseptics, including CHX, cetylpyridinium, triclosan, BZK and povidone. In their study, some BCC strains remained viable in commercial biocide formulations [34].

Given the ability of BCC to remain viable in difficult to survive environments such as low nutrient levels, biocides, chemical solvents and as contaminants in the pharmaceutical process, their survival in antiseptics may be possible due to improper use of the products and can permit high risk BCC exposure to those susceptible to pulmonary infections. Recently, FDA drug product recalls due to *Burkholderia* spp. contamination were reported in the weekly FDA enforcement report of March 20, 2013 [8]. These events have been caused mainly by using contaminated water for diluting preparations, and over dilution or improper storage of the disinfective solutions. There have been occasional reports of outbreaks associated with CHX and BZK diluted to ineffectively low concentrations using water containing BCC [3, 15, 23, 39, 43]. *Burkholderia* sp. can remain viable for many months in water [13, 32] and pharmaceutical water can also be a source of contamination with these bacteria in industrial settings [46].

Previous investigations demonstrating biocide resistance to BCC strains have been performed mainly on freshly grown cells or biofilms, indicating that biofilm was more resistant to antiseptics than planktonic cells [7, 31, 34, 35]. However, BCC present in distilled water can have different phenotypic properties from cells cultured on nutrient rich media. To understand the mechanisms of potential contamination, it is necessary to model what happens to BCC bacteria in water in the presence of antiseptics. In this study, we determined the susceptibility, survival and bacterial recovery of 36 BCC strains in CHX and BZK solutions.

Materials and methods

Bacterial strains

A collection of 36 BCC strains was obtained from the *Burkholderia cepacia* Research Laboratory and Repository at the University of Michigan (Table 1). This collection included isolates from clinical and environmental habitats. These strains were grown on Tryptic Soy agar (TSA) at 30 °C and stored at −80 °C with 10 % glycerol until used. To prepare the same calculated cell mass for each experiment, these organisms were freshly grown on TSA at 30 °C for 48 h and transferred into sterilized distilled water as described below. All BCC samples were prepared in duplicate.

Determination of antiseptic susceptible concentration

To determine the effects of CHX and BZK on BCC, a modified broth minimum inhibitory concentration (MIC) method [31, 34] was carried out using commercially available CHX (Spectrum Chemical Mfg. Corp.; NJ, USA) and BZK (Acros Organics; NJ, USA). The chemical solutions were diluted to make stock solutions with concentration of 100–10,000 µg/ml and then sterilized by filtration (pore size rating 0.22 µm). For the inoculation of sterile distilled water, the BCC strains were grown on TSA at 30 °C for 48 h, washed with autoclaved distilled water, and transferred into 20 ml autoclaved distilled water [final inoculum of approximately 1–20 $\times$ 10^9 CFU/ml (optical density, $OD_{600} = 0.4$)]. All cell cultures were incubated at 23 °C for 40 days without addition of any nutrients [1]. Initially (on "day–zero"), aliquots of the cell cultures were diluted to an OD_{600} of 0.08–0.1 (approximately 1.1 $\times$ 10^7 CFU/ml) with distilled water, and then more diluted to 1/10 times using an autoclaved distilled water. Each 20 µl of suspended cells and antiseptic stock solutions were added to a 96-well plate with 160 µl of Mueller–Hinton broth (MHB) media (final inoculum dose of approximately 1.1 $\times$ 10^5 CFU/ml). Also, after 40 days, the aliquots of cell cultures were again adjusted to an OD_{600} of 0.1 using distilled water, and then cells and antiseptics were applied in the same way as above. Antiseptics were diluted serially with water to final concentrations of 1, 2, 5, 10, 50, 100, 200 or 500 µg/ml in each well. The 96-well plates were incubated at 23 °C for 72 h and growth was measured by optical density at 600 nm with a Synergy MX spectrophotometer (BioTek Instruments, Winooski, VT). In all experiments, the antiseptic concentration with no cell growth was defined as the MIC or susceptible concentration for each BCC strain. For all experiments, no cell and no antiseptic control wells were prepared. These tests were repeated three times.

Table 1 Susceptible concentrations of antiseptics for *Burkholderia cepacia* complex isolates

Species name	Strain number	Isolation source	CHX (μg/ml)		BZK (μg/ml)	
			Initial[a]	40[b]	Initial[a]	40[b]
B. cepacia	PC783	Onion	100	100	50	50
	AU24442	CF sputum	200	100	50	50
B. multivorans	HI2229	Soil enriched with anthranilate	50	10	50	50
	AU24571	CF sputum	50	10	50	50
B. stabilis	HI2210	CF patient	100	100	100	50
	AU23340	CF sputum	100	100	100	50
B. pyrrocinia	AU11057	CF sputum	50	50	100	50
B. ubonensis	AU7314	Non CF trachial aspirate	100	100	100	50
B. vietnamiensis	HI2212	Rice, rhizosphere soil	50	100	100	50
	AU24694	CF sputum	50	10	100	50
B. dolosa	AU0645	CF sputum	50	10	100	50
	AU22866	CF throat	50	10	100	50
B. ambifaria	HI2468	Pea, rhizosphere	50	10	50	50
	AU23145	CF sputum	50	10	100	50
B. anthina	HI2738	Soil rhizosphere	50	10	50	50
	AU21054	CF throat	50	10	100	50
B. metallica	AU0553	CF sputum	50	10	50	50
	AU16697	CF sputum	50	10	100	50
B. contaminans	HI3429	Sheep with mastitis, milk	100	50	100	50
	AU24637	CF lung	100	50	50	50
B. diffusa	AU1075	CF sputum	50	10	50	50
	AU19637	CF sputum	50	50	100	50
B. arboris	ES0263a	Soil	100	50	100	50
	AU22095	CF sputum	100	50	100	50
B. lata	HI4002	Forest soil	100	50	100	50
B. latens	AU4105	CF sputum	50	50	100	50
	AU22890	CF sputum	50	100	100	50
B. seminalis	AU0475	Environment	100	50	100	50
	AU23436	CF sputum	100	50	100	50
B. cenocepacia	HI2718	CF patient	500	50	500	200
	AU1054	CF blood	100	50	200	100
	AU0222	CF patient	100	50	200	100
	AU19236	CF sputum	100	50	200	100
	HI2976	Environment, sink	100	50	200	100
	HI2485	CDC sample	100	50	200	100
	HI4352	Unknown	100	50	50	50

[a] Initial day (0 day) in distilled water

[b] After 40 days in distilled water

Comparative recovery on solid media

To evaluate viable cell counts on common solid media after 14 days incubation in antiseptic solutions, freshly grown BCC strains were prepared on TSA, transferred to distilled water, and adjusted to approximately 1–20 $\times$ 10^9 CFU/ml (OD_{600} = 0.4). These water samples were incubated at 23 °C for 40 days. BCC strains (approximately 1.4 $\times$ 10^7 to 1.3 $\times$ 10^9 CFU/ml) incubated in water were used for cell recovery comparison and antiseptic concentrations (CHX; 5–50 μg/ml and BZK; 10–50 μg/ml) were adjusted to sub-MICs for each BCC strain based on the above MIC results, because it was considered that these sub-MICs for antiseptics could affect BCC survival, but not kill their population completely. After the BCC strains and antiseptics was incubated for 14 days, serial dilutions of antiseptic samples with BCC strains were used to inoculate five solid media culture plates: TSA (full strength, 1/10 strength, 1/100

strength), R2A (full strength, 1/10 strength). To neutralize antiseptics, a mixture of neutralization solution (0.75 % azolectin and 5 % Tween 80) and sterile distilled water was combined for the first dilution (a 10-fold dilution), and then only sterilized distilled water was used for the second serial dilution [34]. This neutralizer did not affect the growth of BCC strains. All tests were repeated three times. After all test plates were incubated at 23 °C for 48 h, cell recovery efficiency of solid media was evaluated on equally diluted samples of each bacterial strain.

Survival of BCC in antiseptic solutions

The diluted antiseptics were prepared using autoclaved distilled water that did not include added nutrients. BCC strains (approximately 3.2×10^6 to 9.7×10^9 CFU/ml; control sample in distilled water at initial experimental point after incubation) were incubated in water for 40 days. Each 1 ml water sample was mixed with the same volume of antiseptic solution or distilled water for control samples, so that the final chemical concentration became 5–50 and 10–50 µg/ml for CHX and BZK, respectively, as their sub-MICs mentioned previously. The survival of BCC in antiseptics was evaluated after 20 min, 24, 48, 168 and 336 h (14 days). At each time point, 10 µl of serial dilutions of antiseptics with BCC strains were placed on 1/10 TSA to recover the bacteria and incubated at 23 °C for 48 h, because of low resolution of optical density analysis on differentiating BCC survivals using liquid media. The serial dilutions were performed using neutralization solution and sterile water. All counts were performed in triplicate and bacterial colonies were counted as CFU per one milliliter sample after incubation.

Statistical analysis

CFU counts from five solid culture media per individual BCC strain were standardized to compare cell recovery and expressed as a CFU z-score indicating how many standard deviations an element was from the mean on the recovery of each strains (SigmaPlot 11.0 ver.) [38, 49]. The high (CFU z-score >0) and low recovery (CFU z-score <0) of each BCC strain for five solid culture media are indicated in a box plot (Fig. 1). Significant differences ($P < 0.05$) in media type for recovery of BCC were determined using Tukey's test for the raw z-score value from all BCC strains (One way ANOVA; SigmaPlot 11.0 ver.).

Results

Susceptibility of BCC to antiseptics

The susceptible concentrations of freshly prepared BCC strains for CHX and BZK were compared with those of bacteria that had been incubated in distilled water for 40 days (Table 1). The antiseptic susceptibility was assessed by broth dilution MIC assays and defined as cell growth inhibition concentration. For CHX, the mean values at initial day (day 0) were 90.3 (50–500 µg/ml) at initial day (day 0) and 46.4 µg/ml (10–100 µg/ml) at 40 days. BCC bacteria at day 0 had a susceptible concentration twice as high as bacteria incubated in water for 40 days ($P < 0.001$). *Burkholderia cenocepacia* HI2718 was susceptible at the highest CHX concentration (500 µg/ml), unlike the other *B. cenocepacia* strains (100 µg/ml), but after 40 days in water they showed high susceptibility,

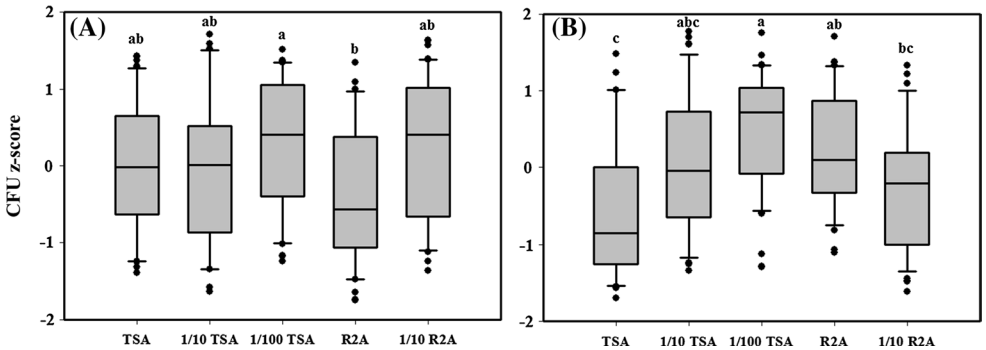

Fig. 1 *Box plots* for comparison of solid media to recover BCC bacteria remaining in chlorhexidine gluconate (**a**; $n = 30$) and benzalkonium chloride (**b**; $n = 35$). Growth of bacteria was measured as CFU on solid media, and expressed as a CFU z-score per individual strain for each medium (SigmaPlot 11.0 ver.). *Boxes* indicate variability of quartiles and the *spaces* of the *box* show the degree of data dispersion. The *line* in the *box* indicates median value of quartiles and outliers are plotted as individual *dots*, maybe meaning variability distant from a range of total measurement. *Vertical lines* from the *boxes* indicate variability outside the upper and lower quartiles. *Positive values* indicate higher growth than the mean for each strain and *negative values* represent lower growth. Different *lowercase letters* indicate statistical significance (ANOVA; $P < 0.05$)

similar to the remaining strains (50 μg/ml). Regardless of the clinical or environmental isolation source, *B. multivorans*, *B. vietnamiensis*, *B. dolosa*, *B. ambifaria*, *B. anthina*, *B. metallica*, and *B. diffusa* maintained in water for 40 days were susceptible to a lower concentration (10 μg/ml) of CHX than at the initial inoculation, except for *B. vietnamiensis* HI2212 and *B. latens* AU22890, which showed decreased susceptibility (100 μg/ml) at 40 days but not on day 0.

Larger amounts of BZK than CHX were required for inhibiting BCC strains from water. Mean susceptible concentration for BZK at the initial day was 111.1 μg/ml (50–500 μg/ml), which was significantly higher than 61.1 μg/ml (50–200 μg/ml) among 40 day-incubated BCC ($P < 0.001$) (Table 1). *B. cenocepacia* strains showed higher susceptibilities (50–500 μg/ml) to BZK and also had high (50–200 μg/ml) susceptible concentrations after 40 days incubation, compared with the remaining 29 BCC strains (50–100 and 50 μg/ml at day 0 and 40 days in water, respectively). Similar to CHX, BCC at the 40 day test point did not present apparent differences between or within species for susceptibility to BZK, regardless of clinical or environmental origins. *B. cenocepacia* HI2718, strains isolated from CF patients, could grow in the highest BZK concentration among BCC and showed susceptible concentrations of 500 and 200 μg/ml in water at initial and 40 day incubations, respectively. The other 30 strains showed the same BZK susceptible concentration (50 μg/ml) after 40 days in water, which was different from the initial day samples, showing various susceptibilities among the bacterial strains.

Comparison of various solid media for recovering BCC

After 14 days of incubation in antiseptic solutions, recovery of BCC was evaluated on various solid culture media (Fig. 1). Each 10 μl serial dilution from antiseptic solutions was used to inoculate plates of TSA, 1/10 TSA, 1/100 TSA, R2A and 1/10 R2A media. Colony counts on solid media showed large variations among bacterial species and strains in antiseptic solutions, and the mean values were 6.7×10^6 to 4.7×10^8 and 1.3×10^7 to 3.9×10^8 CFU/ml on five solid media for CHX and BZK, respectively. We transformed the CFU count data as a standardized z-score for each solid medium type and individual strain and then compared bacterial recovery on five solid media (Fig. 1). Among 30 strains from CHX solutions, 9 strains showed the highest CFU *z*-scores on 1/100 TSA medium, while the CFU *z*-scores of 3 strains were the highest on R2A. Six strains including *B. pyrrocinia* AU1057, *B. contaminans* AU24637, *B. arboris* AU22095, *B. lata* HI4002, *B. seminalis* AU0475 and *B. seminalis* AU23436 could not be recovered on any of the five solid culture media after 14 days incubation in CHX solutions.

For the 35 strains in BZK solutions, 11 strains showed the highest CFU *z*-scores on 1/100 TSA, but TSA medium induced the best recovery for 5 strains. *B. lata* HI4002 did not grow after 14 days of incubation in BZK. The 1/100 TSA medium (mean CFU *z*-scores 0.26 ± 0.81 and 0.49 ± 0.76 on CHX and BZK, respectively) could support the highest recovery of BCC strains from antiseptic solutions; and R2A (-0.39 ± 0.84) and TSA (-0.54 ± 0.90) had the lowest recovery values in CHX and BZK, respectively (Fig. 1). These results indicate that the diluted TSA media brought about an efficient recovery of BCC bacteria from antiseptic solutions. Even though 1/100 TSA could support the highest recovery of BCC, colony size was affected and appeared smaller and light colored. Therefore, we chose 1/10 TSA to monitor the recovery and survival of BCC.

Survival of BCC in antiseptics

To monitor the survival of BCC in sub-MICs of CHX or BZK, 36 strains previously incubated in water for 40 days were placed in water-diluted antiseptic solutions without any additional nutrients and then their survival was evaluated on 1/10 TSA during 336 h (14 days). After 40 days of incubation in water, BCC strains from clinical and environmental sources showed mean cell counts of $4.36–4.87 \times 10^8$ CFU/ml, respectively. Water–adapted BCC strains were suspended in 5–50 μg/ml of CHX or 10–50 μg/ml of BZK based on their susceptibility to the antiseptics (Table 1). Contact with antiseptics generally inhibited the recovery of bacteria, but temporal detection of the surviving cells after 336 h was different in CHX and BZK solutions (Fig. 2). In CHX solutions, the clinical and environmental isolates showed similar cell counts ($4.44–5.81 \times 10^7$ CFU/ml, respectively) until 24 h, but showed different survival patterns after that time. The survival of clinical isolates remained stationary after 24 h and finally reached to $3.85 \pm 6.26 \times 10^7$ CFU/ml at 336 h. Environmental isolates consistently decreased and showed relatively low survival, as $1.83 \pm 1.37 \times 10^7$ CFU/ml at 336 h (Fig. 2a, inner line graph). BZK solution exposure quickly reduced the recovery of most BCC strains ($2.53 \pm 3.80 \times 10^7$ and $3.05 \pm 4.81 \times 10^7$ CFU/ml from clinical and environmental isolates, respectively) within 20 min. After that, BCC numbers were largely increased ($1.41 \pm 0.91 \times 10^8$ and $1.37 \pm 0.57 \times 10^8$ CFU/ml from clinical and environmental isolates, respectively) for 14 days (336 h). There was no difference in recovery by bacterial isolation source (Fig. 2b, inner line graph).

Although the survival of all BCC strains was reduced by antiseptic compounds, they exhibited different recoveries

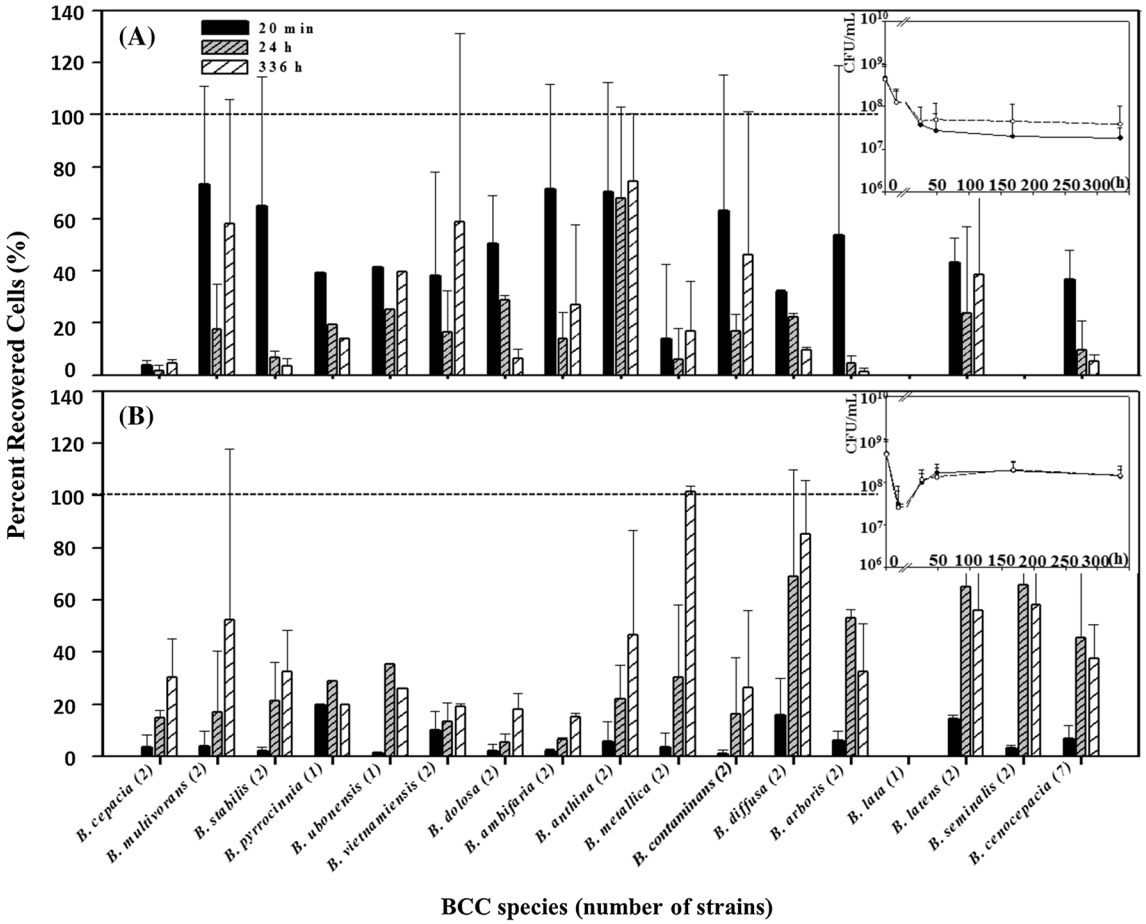

Fig. 2 Recovery of BCC strains during incubation in chlorhexidine gluconate (**a**) and benzalkonium chloride (**b**). The *dashed line* indicates the initial cell portion (100 % at 0 h, approximately 4.36 to 4.87 × 10^8 CFU/ml). Antiseptic treatment concentration was adjusted as their sub-MIC values, 5–50 and 10–50 μg/ml for CHX and BZK, respectively, (Table 1). *Inner line graph* shows an average fraction (CFU/ml) of BCC bacteria surviving in chlorhexidine gluconate (**a**) and benzalkonium chloride (**b**). The first time point (0 h) was from samples without chemicals; the recovery of samples with antiseptics was initially evaluated at 20 min after adding chemicals. *Open circles* with *broken lines* indicate clinical isolates the mean values of clinical isolates and *closed circles* with *solid lines* represent the mean values of environmental isolates on both graphs

from CHX and BZK for the test period (Fig. 2). The mean survival of BCC strains was largely reduced to 17.1 ± 18.7 % (n = 36) after 24 h following contact with CHX solution, and then slightly increased to 23.6 ± 32.3 % (n = 36) after 336 h, compared to initial water samples without antiseptic (Fig. 2a). Of particular note, after 336 h (14 days) incubation, *B. multivorans* AU24571, *B. anthina* HI2738 and *B. vietnamiensis* AU24694 showed relatively high survival of >90 %, and *B. vietnamiensis* AU24694 showed a higher recovery of 110 % than the control sample, but five strains (*B. arboris* AU22095, *B. lata* HI4002, *B. latens* AU4105, *B. seminalis* AU0475 and AU23436) were not detected from CHX solutions at 336 h (14 days).

BZK solutions sharply reduced the mean recovery rates of BCC to 6.3 ± 6.3 % (n = 36) after the initial 20 min incubation; thereafter the survival of bacteria increased to 57.4 ± 36.2 % (n = 36) at 168 h and finally decreased to 41.7 ± 28.8 % (n = 36) at 336 h (14 days) against control samples from water without BZK (Fig. 2b). When analyzing individual strains, whereas *B. multivorans* HI2229, two *B. metallica* strains (AU0553 and AU16697) and *B. diffusa* AU1075 had high survival in BZK (above 98 %), the survival of *B. contaminans* AU24637 was 5.9 % at 336 h. *B. arboris* AU22095 and *B. latens* AU4105, which could not grow in CHX solutions at 336 h, were consistently detected from BZK solutions and their survival values were 20 and 30 %, respectively. *B. lata* HI4002 could not be detected in BZK solutions after 336 h, as well.

Discussion

Water is a known habitat for many *Burkholderia* spp. in natural environments and also has been linked to BCC

contamination of industrial products [5, 9, 20, 25, 32, 33, 45, 46]. In this study, BCC strains maintained in water for 40 days showed different susceptible concentrations in antiseptic solutions. Most strains incubated in water for 40 days were more susceptible to CHX and BZK than strains at the initial inoculation. These results show that BCC incubated in nutrient-depleted water for a long time can become vulnerable to antiseptics but some strains of *B. cenocepacia* can have relatively low susceptibility to antiseptics after 40 days incubation in water. BCC had susceptible concentrations of 50–500 μg/ml (CHX) and 10–200 μg/ml (BZK) at initial inoculation and after 40 days in water, respectively. Considering that CHX and BZK are used in a variety of commercial products in concentrations ranging from 0.02 % to 5 % (200–50,000 μg/ml), this might be insufficient to kill *B. cenocepacia* HI2718 at the 0.02 % (200 μg/ml) concentration. These results are in agreement with previous studies demonstrating MIC values of *B. cenocepacia* clinical isolates exposed to antimicrobial agents, including CHX and BZK [7, 34]. Our data show the importance of proper antiseptic dilution levels in pharmaceutical water and products to be effective as a disinfectant.

It is important to ensure the efficient detection of BCC in industrial settings and in pharmaceutical products because they can cause high patient risks. Despite the many molecular assays that can provide reliable and accurate detection of different microbial populations, it remains critical to understand the efficacy of bacterial detection media because many CF facilities and industrial environments do not have easy access to molecular techniques on-site. Moreover, the conventional culture methods for recovery of various pathogen populations enable the isolation of cultures for purification and confirmatory biochemical tests. Previous studies have developed various selective media for the rapid and accurate isolation of BCC [6, 10, 12]. Recently, we reported that six *B. cenocepacia* strains were recovered better using diluted TSA/TSB and R2A/R2AB than using full strength TSA and TSB from inocula containing low numbers of cells following pre-incubation in water for 40 days [1]. In the current study, five solid culture media (TSA, 1/10TSA, 1/100TSA, R2A and 1/10R2A) were evaluated for recovering BCC cells from antiseptic solutions. Although all BCC strains from CHX and BZK solutions could grow well on all tested media, many strains were recovered better on dilute TSA than on full strength TSA. Dilute TSA or R2A are not able to selectively recover BCC strains, unlike media containing greater concentrations of nutrients and combinations of antibiotics [12, 47]. However, it may be modified for detection of BCC strains from the diluted antiseptic samples contaminated with other organisms.

During 336 h, the survival of BCC was monitored in CHX and BZK solutions diluted to less than their susceptible concentration. The survival of most BCC strains showed a sharp reduction in CHX and BZK solutions at 20 min incubation, which means that they were affected considerably by the adjusted sub-MICs of chemical solutions. CHX mainly acts by cell membrane disruption, respiratory inhibition, and cytoplasmic coagulation [42]. *Pseudomonas* sp. strain A-3 isolated from sludge can degrade CHX [16], but CHX resistance of BCC has been reported to mainly be due to efflux pump mechanisms [7, 21]. *B. multivorans* AU24571, *B. vietnamiensis* AU24694, *B. ambifaria* AU23145 and *B. contaminans* AU24637 strains from clinical isolates were better recovered after 14 days than after only 24 h exposure to CHX, although they had low susceptible concentrations of CHX (10–100 μg/ml). This phenomenon was conspicuous around BCC strains on BZK. These results suggest two previously reported scenarios, namely, biodegradation of BZK to inactive products [29, 50], or the induction of a viable but nonculturable (VBNC) state of BCC in BZK solutions [48]. Biodegradation of BZK has been reported for bacteria isolated from activated sludge, and it has been suggested that *Aeromonas hydrophila* strain K may utilize BZK as an energy source [29, 50]. Some *Burkholderia* species use pollutants and aromatic substrates as carbon sources [21, 41]. Therefore, many BCC strains might also biodegrade and metabolize BZK for cell growth, but demonstrating these possibilities requires further study. On the other hand, since Xu et al. [48] originally described the VBNC phase of bacteria, many other studies have documented that state in a great variety of microorganisms [19, 26–28]. The VBNC phase of bacteria can be induced by starvation or harsh environmental conditions that produce sublethal injury. Under these conditions, bacteria were not able to multiply in growth media. The toxicity of antiseptic solutions may have induced a VBNC state leading to low CFU counts of BCC on solid culture media shortly after exposing BCC to antiseptics. However, BCC strains were subsequently recovered, as evidenced by increased CFU, possibly due to decrease of the toxicity by degradation of antiseptic molecules or an adaptation accompanying a regulation of relevant gene expression [17]. The possibility of these strategies on BCC strains needs to be evaluated through further investigations.

To assure public safety, effective detection and control of BCC contamination is critical for manufacturing pharmaceutical products to assure manufacturing process control. The results of this study demonstrate survival of BCC strains in antiseptic solutions and show the need for more investigations concerning survival strategies of BCC exposed to various antiseptic solutions.

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