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Evaluation of liquid and solid culture media for the recovery and enrichment of *Burkholderia cenocepacia* from distilled water

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Abstract *Burkholderia cepacia* complex (BCC) presence has been the cause of recalls of both sterile and non-sterile pharmaceutical products since these opportunistic pathogens have been implicated to cause infections to susceptible individuals. BCC are ubiquitous in nature, but in pharmaceutical settings the most common source is contaminated water systems. Some strains of BCC, previously described as *Pseudomonas cepacia,* were not readily detected by standard culture methods. We have explored different strategies to recover and enrich *Burkholderia cenocepacia* previously cultured in distilled water for 40 days. Enrichment media of varied nutrient concentrations and composition were used, including modified Tryptic Soy Agar or Broth (TSA or TSB), Reasoner's 2nd Agar or Broth (R2A or R2AB), Brain–Heart Infusion Broth (BHIB), Mueller–Hinton Broth (MHB), and Ashdown's (ASH) medium. Of the various broth media tested, cell growth was significantly greater in TSB and R2AB than in BHIB, MHB, or ASH broth. TSB and R2AB were also compared for their recovery efficiency. Generally, there was no significant difference between the numbers of *B. cenocepacia* grown on 15 differently modified TSA and five modified R2A solid media. Overall, however, diluted TSA and TSB media, and R2A and R2AB showed better recovery efficiency than TSA and TSB for inocula containing small numbers of cells. All strains persisted in distilled water for 40 days. Broth media were more effective than solid media for recovery of *B. cenocepacia* from distilled water. These results may assist in improving detection assays with recovery and enrichment strategies to maximize recovery of these fastidious organisms.

Keywords Culture media · Recovery · Enrichment · *Burkholderia cenocepacia* · Distilled water

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Introduction

The *Burkholderia cepacia* complex (BCC) is a group of Gram-negative, non-spore-forming bacilli belonging to the β-*Proteobacteria.* These species are commonly isolated from aquatic and terrestrial ecosystems, plant rhizospheres, and animal surfaces, and are often recovered in clinical settings from persons with cystic fibrosis (CF) [3, 7, 12, 13, 15, 17, 26–29, 33]. The BCC consists of 18 closely related species. Among these, *Burkholderia cenocepacia* and *Burkholderia multivorans* are particularly associated with respiratory tract infections in CF patients [13, 21]. *B. cenocepacia* accounts for approximately 45–90 % of all BCC infections in CF patients worldwide [13, 21], and it has

been associated with fatal necrotizing pneumonia and sepsis (so-called "cepacia syndrome") [4].

In 1981, the FDA discovered a drug product that was contaminated with "*Burkholderia cepacia"*, which was traced back to the failure of the manufacturer's deionized water system. More recently, BCC bacteria have been implicated in a large number of recalls of both sterile and non-sterile pharmaceutical products [10]. In six of these instances, water was the purported source of contamination. The BCC are able to remain viable under harsh conditions, such as in organic solvents, antiseptics, and liquids with low nutrient levels. They also may possess high antimicrobial resistance [25]. There have been reports of *Burkholderia pseudomallei* showing the capability to survive in distilled water for 3–16 years, posing a possible risk for bacterial contamination of pharmaceuticals during manufacturing operations [19, 31]. United States Pharmacopeia (USP) methods for detection of bacteria include enrichment using Tryptic Soy Broth (TSB), which may not be adequate for all strains of BCC [2]. Carson et al. [2] found that *Burkholderia* Strain 1 grew in hot and cold distilled water, and although viable, 99–99.9 % of the cells died when transferred to TSB medium. In recent product-recall investigations that traced BCC strains to contaminated water supplies, the offending organisms had not been detected by standard culture methods using soy casein digest agar cultured at 30–35 °C for 3–5 days [10, 25]. Given these organisms' ability to evade detection, inherent pathogenic potential, resistance to antimicrobials, and adaptability to various nutrient requirements, new detection and culture techniques are required to ensure pharmaceutical product quality and safety.

The purpose of this study was to evaluate various solid and liquid nonselective media for recovery of *B. cenocepacia* strains from distilled water. The results provide useful information on the most suitable media for studies on BCC recovery in pharmaceutical manufacturing operations.

Materials and methods

Bacterial strains

We tested six strains of *B. cenocepacia* obtained from the *B. cepacia* Research Laboratory and Repository at the University of Michigan. Four strains (HI2718, AU1054, AU0222, and AU19236) were from clinical specimens; strains AU0222 and AU19236 did not exhibit lysine decarboxylase activity. Two strains (HI2976 and HI2485) were recovered from environmental sources. These strains were maintained on TSA supplemented with 5 % sheep's blood (Blood Agar; BA).

Bacterial viability assay

For the inoculation of *B. cenocepacia* in sterile distilled water, the bacterial strains were grown on TSA at 30 °C for 48 h. The cultures were washed with autoclaved distilled water, inoculated into 20 ml of autoclaved distilled water (final inoculum dose of approximately 1.1–21 $\times$ 10^6 cells/ml), and incubated at 23 and 18 °C for 40 days. The total cell number in water was determined using flow cytometry (FCM) in an Accuri C6 FCM (Accuri Cytometers) [18]. Duplicate samples were analyzed on days 0, 4, 7, 19, 33 and 40. One-milliliter samples were stained according to the protocol, using the BacLight LIVE/DEAD bacterial viability and counting kit (Invitrogen, Grand Island, NY), and an incubation time of 10 min in the dark. The band pass filters used were 530 $\pm$ 15 nm and 670 nm for SYTO 9 and PI, respectively. The limit of detection of the flow cytometer in this experiment was determined to be 10^5 cells/ml.

Growth kinetics in modified TSB and R2AB

To evaluate the effect of nutrient content on growth kinetics, various broth media and diluted preparations of media were used. The strains held in water after 40 days were adjusted to a density corresponding to 0.08–0.1 absorbance at wavelength of 600 nm (approximate cell density = 1.5 $\times$ 10^8 colony-forming units (CFU/ml)) in the Synergy MX spectrophotometer from BioTek Instruments, Inc. (Winooski, VT). The 10-μl suspensions were inoculated into a 96-well plate at 90 μl per well and incubated at 23 °C. The kinetics of growth in various broth media was monitored by the Synergy MX spectrophotometer. The kinetic software (Gen5^{TM}) of the instrument was programmed to measure the absorbance at 600 nm every 2 h at 23 °C for 48 h with 10 s of shaking at intervals. Optical density measurements were compared for the cultures growing in TSB, 1/3 strength TSB, TSB supplemented with 5 % Sheep's Blood, R2AB (i.e., R2A without agar), 1/3 strength R2AB, and R2AB supplemented with 5 % Sheep's Blood.

Evaluation of recovery using broth media

Samples from distilled water cultures were used to compare the recovery efficiency of TSB, R2AB, R2AB with 5 % glycerol (R2AB + Glycerol), BHIB, MHB, and Ashdown's medium (ASH). One hundred microliters of each broth medium were added in the same order to three sets of 96-well plates. Serial dilutions of *B. cenocepacia* were then added to each medium to achieve cell densities of 1,000, 100, 10, and 1 CFU/ml. Uninoculated wells were used as a baseline control. Growth of *B. cenocepacia* strains in

different growth media was determined using the Synergy MX spectrophotometer after subtracting the OD_{600} value of the uninoculated medium. After 72-h incubation at 23 °C, the number of wells in which growth had occurred (as determined by $OD_{600} > 0.045$, where the negative control was $OD_{600} < 0.045$) was recorded. Wells with low OD_{600} (0.040–0.045) were subcultured on TSA to confirm cell growth. Any positive growth was tested using *B. cepacia* selective agar (BCSA) with Vancomycin, Gentamicin, and Polymyxin B (Thermo Fisher Scientific, Waltham, MA, USA) to confirm identity. The absence of cell density after 3 days was interpreted as a failure of recovery.

Comparative recovery on solid media

Bacterial cultures were harvested to determine viable cell counts after 40 days. Serial dilutions of *B. cenocepacia* were placed onto various solid agar media to resuscitate the bacteria from distilled water (Table 1): TSA (full strength, 1/3 strength, 1/10 strength, 1/50 strength, 1/100 strength), R2A (full strength + 20 g peptone, full strength + 6 g peptone, full strength + 2 g peptone, full strength, 1/3 strength), TSA (full strength, 1/3 strength, 1/10 strength, 1/50 strength, 1/100 strength) supplemented with 5 % Sheep's Blood, TSA (full strength, 1/3 strength, 1/10 strength, 1/50 strength, 1/100 strength) heated and supplemented with 5 % Sheep's Blood. Ten or one hundred microliters of each serial dilution (in distilled water) were placed onto R2A and TSA plates for final inocula of approximately 1,000 and 100 CFU.

Table 1 Various combinations of solid and broth media

Solid medium		Broth medium	
TSA	Full strength	TSB	Full strength
	1/3 strength		1/3 strength
	1/10 strength		1/10 strength
	1/50 strength		1/50 strength
	1/100 strength		1/100 strength
			1/1,000 strength
R2A	Full strength + 20 g peptone	R2AB	Full strength
	Full strength + 6 g peptone		1/10 strength
	Full strength + 2 g peptone		1/50 strength
	Full strength		1/100 strength
	1/3 strength		1/1,000 strength
5 % Sheep's Blood TSA	Full strength		
	1/3 strength TSA		
	1/10 strength TSA		
	1/50 strength TSA		
	1/100 strength TSA		
Heat 5 % Sheep's Blood TSA	Full strength TSA		
	1/3 strength TSA		
	1/10 strength TSA		
	1/50 strength TSA		
	1/100 strength TSA		

Comparative recovery in modified TSB and R2AB

Most probable number (MPN) analysis was used for comparison of *B. cenocepacia* recovery efficiency in various dilutions of TSB and R2AB (Table 1). After serial dilution, 100-µl dilutions containing the fewest cells were placed into 96-well microliter plates. The MPN was calculated using a standard MPN equation [1]. Comparison of microbial recovery on solid media to recovery in broth media.

Comparative recovery on solid media to recovery in broth media

To determine whether broth media enhanced recovery of small numbers of BCC, *B. cenocepacia* was prepared initially by inoculating TSB, TSB with 5 % Sheep's Blood, and R2AB with 5 % Sheep's Blood at the 10^{10} dilutions (final cell numbers approximately 10 CFU) at 23 °C for 72 h. The 10-µl suspensions were then subcultured onto 1/3R2A, 1/3TSA, and 5 % Sheep's Blood-TSA.

Diluting solutions

Distilled water containing 0.1 % peptone or 0.1 % yeast extract was appropriately diluted and used for resuscitation of *B. cenocepacia* for 1 h (less than its doubling time) and 12 h at 23 °C. Cultures were incubated for 72 h at 23 °C and compared for their recovery in dilute TSB.

Statistical analysis

B. cenocepacia recovery data obtained from pure cultures in various broth media were analyzed using Fisher's exact test.

Results

Persistence of *B. cenocepacia* strains in distilled water

Using the flow cytometer, we determined the growth or persistence of *B. cenocepacia* strains in distilled water at 23 and 18 °C over 40 days (Fig. 1). There was no significant difference in growth between the two cultures incubated at these temperatures. Strains AU1054, AU19236, and HI2485 showed an increase of cell number until the

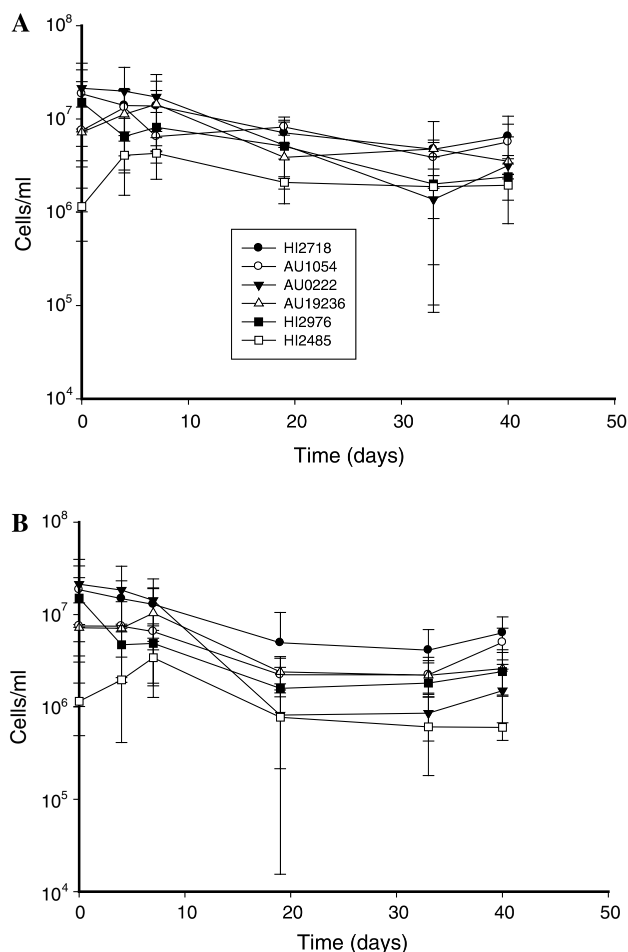

Fig. 1 Growth of *B. cenocepacia* strains in distilled water at 23 °C (**a**) and 18 °C (**b**) measured using the flow cytometer

tenth day, and counts remained stationary thereafter. All strains persisted in distilled water over 40 days.

Growth kinetics of *B. cenocepacia* strains in various broth media

As a preliminary evaluation, *B. cenocepacia* strains were cultured in ten types of broth media inoculated with approximately 1.5 × 10^6 CFU/ml (Fig. 2). The clinical isolates (HI2718, AU1054, AU0222, and AU19236) showed noticeably better growth in media supplemented with blood (Fig. 2a–d). The environmental isolates (HI2976 and HI2485), however, did not grow better in broth media supplemented with blood; instead, they grew better in media containing any form of TSB (Fig. 2e, f). Both clinical and environmental isolates of *B. cenocepacia* showed that optical density was lower when grown on R2AB rather than TSB. We also observed that the environmental isolates of *B. cenocepacia* were recovered more efficiently than the clinical isolates in all liquid media evaluated in this study than on solid media.

Evaluation of recovery using a variety of broth media

The TSB, BHIB, MHB, ASH, R2AB, and R2AB + Gly broth media were evaluated for their ability to recover *B. cenocepacia* at 23 and 18 °C. Growth was detected in all conditions after 3 days of incubation at 23 °C. Compared with TSB and R2AB, recovery was significantly less efficient in ASH, BHIB, and MHB media, with recovery rates of 4/36 ($p < 0.0005$), 13/36 ($p < 0.0466$), and 10/36 ($p < 0.0185$), respectively (Table 2). Evaluation of the six broths inoculated with the fewest cells indicated that TSB and R2AB supported the most efficient recovery of *B. cenocepacia.* TSB and R2AB were selected for further study with smaller inocula.

Comparison of colony counts on various solid media

For evaluation of viable cell counts in solid media, we used several different concentrations of agar media including the traditional full-strength and various dilutions of TSA, R2A, 5 % Sheep's Blood with TSA, heated 5 % Sheep's Blood with TSA (chocolate) agar at 23 and 18 °C (Fig. 3). After 40 days of incubation, all of the agars allowed recovery of the bacteria fairly easily due to the high number of cells that were present in the distilled water at both temperatures. After 72 h of incubation at 23 °C, most of the strains had grown to a point where the number of colonies became too numerous to count.

To evaluate colony recovery on various dilute media, 10 μl of each serial dilution of cells from distilled water was used to inoculate TSA, 1/3TSA, 1/10TSA, 1/50TSA, and 1/100TSA plates (Fig. 3a). Growth was detected under all conditions after 3 days of incubation at 23 °C. All of the agars allowed recovery of the bacteria from inocula of approximately 1,000 CFU/ml. The cell counts were slightly higher and more variable in 1/100TSA (23.5 ± 19.3 × 10^7 CFU/ml) than in TSA (11.8 ± 8.5 × 10^7 CFU/ml). Cell counts were approximately the same in R2A with 20 g peptone, R2A with 6 g peptone, R2A with 2 g peptone, R2A, and 1/3R2A (17.4 ± 13.4 to 24.2 ± 21.2 × 10^7 CFU/ml) (Fig. 3b). In TSA supplemented with 5 % Sheep's Blood and heated 5 % Sheep's Blood, the colony number was 11.5 ± 10.8 to 14.6 ± 7.9 × 10^7 CFU/ml and 10.9 ± 10.6 to 14.1 ± 8.7 × 10^7 CFU/ml, respectively (Fig. 3c, d). In 1/100TSA and 1/3R2A, used as controls, were 14.1 ± 7.2 and 13.3 ± 9.4 × 10^7 CFU/ml, respectively. To establish if the colony recovery was associated with nutrient dilute media, the changes in colony number were plotted against the dilute media. A robust, exponential relationship between colony count and dilute TSA supplemented with heated 5 % Sheep's Blood and dilute TSA was observed (r^2 = 0.64 and 0.49, respectively). Lower correlation

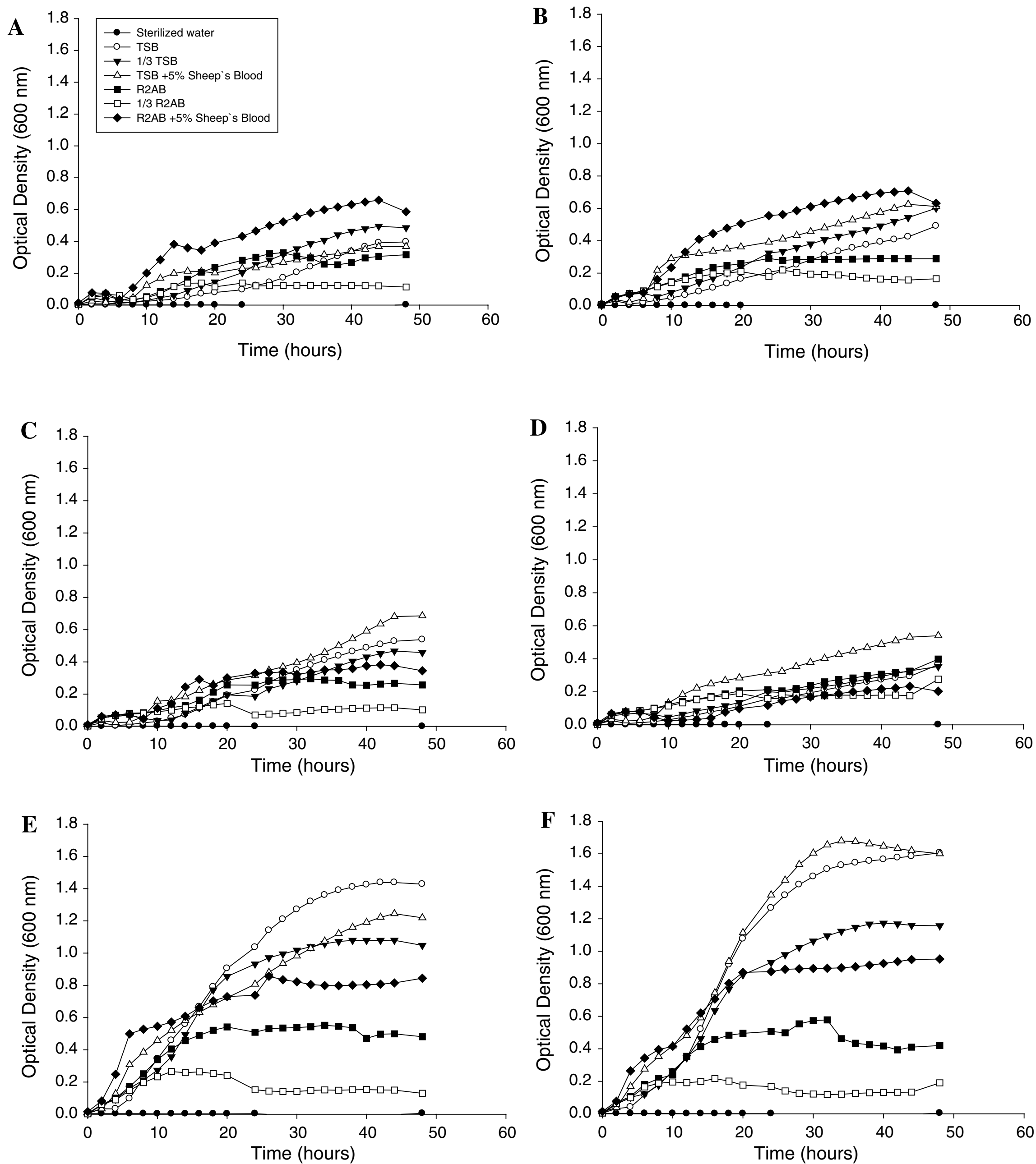

Fig. 2 Changes in optical density (600 nm) of *B. cenocepacia* cultured at 28 °C for 44 h in various broth media, using 96-well plates. The clinical isolates; HI2718 (**a**), AU1054 (**b**), AU0222 (**c**), AU19236 (**d**) and the environmental isolates; HI2976 (**e**) and HI2485 (**f**)

coefficients were observed for dilute TSA with supplemented 5 % Sheep's Blood ($r^2 = 0.14$).

Comparison of viable cell counts in modified TSB and R2AB

Cultures were compared for their recovery on the traditional full-strength and various dilutions (1/3, 1/10, 1/50, and 1/100) of TSB and R2AB. Optical density results (OD_{600nm}) in 1/100TSB and 1/100R2AB were higher (0.067 and 0.051) for all the strains at the 10^8 dilution (final cell number approximately 10^3 CFU/ml) inoculum, compared to the negative control (0.036). The highest OD_{600nm} was observed with the environmental isolates (HI2976 and HI2485) in TSB. Both clinical isolates with unusual phenotypes (AU0222 and AU19236) and the environmental isolates (HI2976 and HI2485) showed similar OD values at 600 nm. At the 10^7 dilution with the TSB dilutions (Table 3), the cell count was estimated to be 1.02 ± 0.58 to $1.49 \pm 1.49 \times 10^7$ CFU/ml. MPN counts

Table 2 Number of positive wells (number of wells set up) of *B. cenocepacia* at 10^8 dilution in nonselective broth media after 44 days

Strain	TSB	BHIB	MHB	ASH	R2AB	R2AB + glycerol
HI2718	5/6[a]	3/6	2/6	0/6	4/6	3/6
AU1054	4/6	1/6	1/6	0/6	4/6	2/6
AU0222	4/6	2/6	1/6	0/6	5/6	3/6
AU19236	5/6	2/6	1/6	1/6	4/6	3/6
HI2976	3/6	1/6	3/6	1/6	3/6	4/6
HI2485	4/6	4/6	2/6	2/6	4/6	4/6
Total	25/36 A[b]	13/36 B ($p < 0.0466$)	10/36 B ($p < 0.0185$)	4/36 B ($p < 0.0005$)	24/36 A	19/36 AB[c] ($p < 0.1184$)

[a] Number of recovery/number of tests

[b] Significant difference derived from Fisher's exact test between A and B ($p < 0.05$)

[c] Significant difference between AB and A or B ($p > 0.05$)

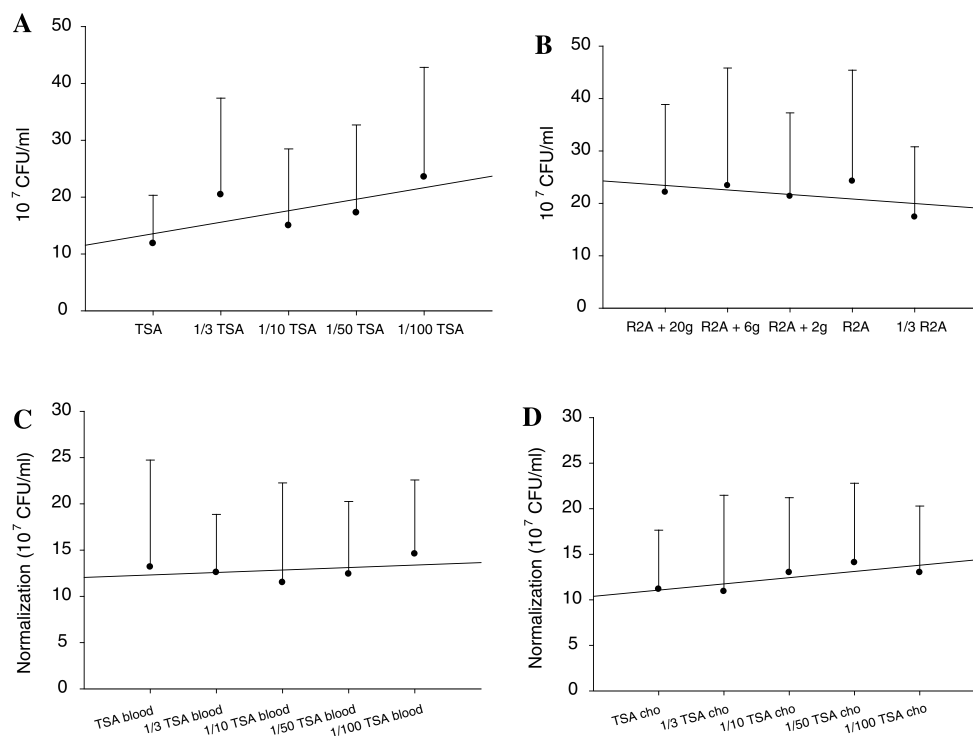

Fig. 3 Comparative recovery of *B. cenocepacia* on various agar plates: various dilutions of TSA (1/3, 1/10, 1/50, and 1/100, $r^2 = 0.4909$) (**a**), modified R2A (R2A with 20 g peptone, R2A with 6 g peptone, R2A with 2 g peptone, R2A, and 1/3R2A, $r^2 = 0.2635$) (**b**), supplemented with 5 % Sheep's Blood (1/3, 1/10, 1/50, and 1/100 TSA, $r^2 = 0.1371$) (**c**) and with heated 5 % Sheep's Blood (1/3, 1/10, 1/50, and 1/100 TSA, $r^2 = 0.6423$) (**d**). *Error bars* represent at least three replicates

using R2AB diluents (Table 3) estimated the cell count to be 2.83 ± 0.59 to $4.47 \pm 4.01 \times 10^7$ CFU/ml. TSB and R2AB media showed no significant difference in the recovery by MPN counts.

Comparison of microbial recovery on solid media to recovery in broth media

With inocula of the 10^{10} dilutions from TSB culture at 23 °C, all strains failed to grow on 1/3TSA, 1/3R2A, and 5 % Sheep's Blood-TSA agar plates (Table 4). With inocula of approximately 10 CFU/ml from 5 % Sheep's Blood-TSB and 5 % Sheep's Blood—R2AB at 23 °C, all strains grew poorly on 1/3TSA agar plates.

Enrichment in various types of diluting solutions

Distilled water, 0.1 % peptone and 0.1 % yeast extract were used to dilute and enrich the environmental isolates (HI2976 and HI2485). After 1 h of incubation, the

Table 3 MPN estimates of the number of cells of *B. cenocepacia* recoverable on dilute TSB and R2AB from 23 °C distilled water after 44 days

Medium	No. of positive wells (no. of wells set up) at each dilution			MPN (CFU/ml) at 10^7 dilution
	10^6	10^7	10^8	
TSB				
TSB	60 (60)	44 (60)	6 (60)	1.09 ± 0.51
1/3TSB	60 (60)	39 (60)	8 (60)	1.31 ± 0.64
1/10TSB	60 (60)	36 (60)	1 (60)	1.02 ± 0.58
1/50TSB	60 (60)	36 (60)	5 (60)	1.43 ± 1.46
1/100TSB	60 (60)	38 (60)	6 (60)	1.49 ± 1.49
R2AB				
R2AB	60 (60)	60 (60)	8 (60)	3.13 ± 0.45
1/10R2AB	60 (60)	60 (60)	8 (60)	3.27 ± 0.95
1/50R2AB	60 (60)	60 (60)	11 (60)	4.47 ± 4.01
1/100R2AB	60 (60)	60 (60)	4 (60)	2.83 ± 0.59
1/100TSB	60 (60)	60 (60)	8 (60)	3.49 ± 1.23

highest CFU (1.29 ± 0.9 × 10^8 CFU/ml) were observed from HI2485 using 0.1 % peptone water as the diluent (Fig. 4a). After 12-h enrichment, this value increased to 24 ± 0.0 × 10^8 CFU/ml (Fig. 4b). Also, this number was observed on 0.1 % yeast extract water used for enrichment.

Discussion

Water has been reported to be an important environmental source of BCC [2, 6, 16, 19, 28, 33]. In this study, we assessed the recovery of *B. cenocepacia* strains from distilled water by using various enrichment techniques. BCC species have been reported as being capable of survival in water for prolonged periods [2, 6, 16, 19, 22]. Several studies also found that *B. pseudomallei* inoculated in distilled water was culturable for at least 30 days, with some isolates surviving up to 200 days or 16 years [6, 16, 19, 22]. *B. pseudomallei* can endure nutrient-depleted environments as well as a wide range of pH, salt concentrations, and temperatures for periods of up to 28 days [22]. Our results indicate that *B. cenocepacia* can grow or persist in distilled water over 40 days (Fig. 1). Bacterial survival in water might depend on physiological state, tolerance of nutrient-depleted environments, interactions with other bacteria, and fluctuations in temperature [2, 23].

The clinical isolates (HI2718, AU1054, AU0222, and AU19236) showed more rapid growth in TSB or R2AB broth (Fig. 2) supplemented with blood. Broth enrichment media that were supplemented with blood produced better recovery of *B. cenocepacia* than broth media without blood. This may be explained by pathogenic bacteria having become adapted to nutrients in blood [30]. *B. cenocepacia* isolates from the environment (HI2976 and HI2485)

Table 4 Recovery of *B. cenocepacia* from 96-well plates to three agar media after 44 days at 23 °C

Cultured initially in broth	10^{10} dilution (final cell number approximately 10 CFU/ml)								
	TSB			TSB + 5 % Sheep Blood			R2AB + 5 % Sheep Blood		
Subcultured onto agar plate	1/3 strength TSA	1/3 strength R2A	5 % Sheep Blood TSA	1/3 strength TSA	1/3 strength R2A	5 % Sheep Blood TSA	1/3 strength TSA	1/3 strength R2A	5 % Sheep Blood TSA
Strain									
HI2718	−−a	−−	−−	+−	++	++	++	++	++
	−−b	−−	−−	++	++	++	++	++	++
AU1054	−−	−−	−−	+−	++	++	+−	++	++
	−−	−−	−−	+−	++	++	+−	++	++
AU0222	−−	−−	−−	+−	++	++	+−	++	++
	−−	−−	+−	++	++	++	++	++	++
AU19236	−−	−−	−−	++	++	++	−−	++	++
	−−	−−	+−	++	++	++	+−	++	++
HI2976	−−	−−	−−	−−	++	++	+−	++	++
	−−	−−	+−	++	++	++	++	++	++
HI2485	−−	−−	+−	+−	++	++	+−	++	++
	++	−−	+−	++	++	++	++	++	++

++: All growth, +− : some growth,−−: no growth

[a] Growth on agar plate when inoculated at 0.7 to 1.5 × 10^8 CFU/ml from cultures at 23 °C

[b] Growth on agar plate when inoculated at 0.7 to 1.5 × 10^8 CFU/ml from cultures at 18 °C

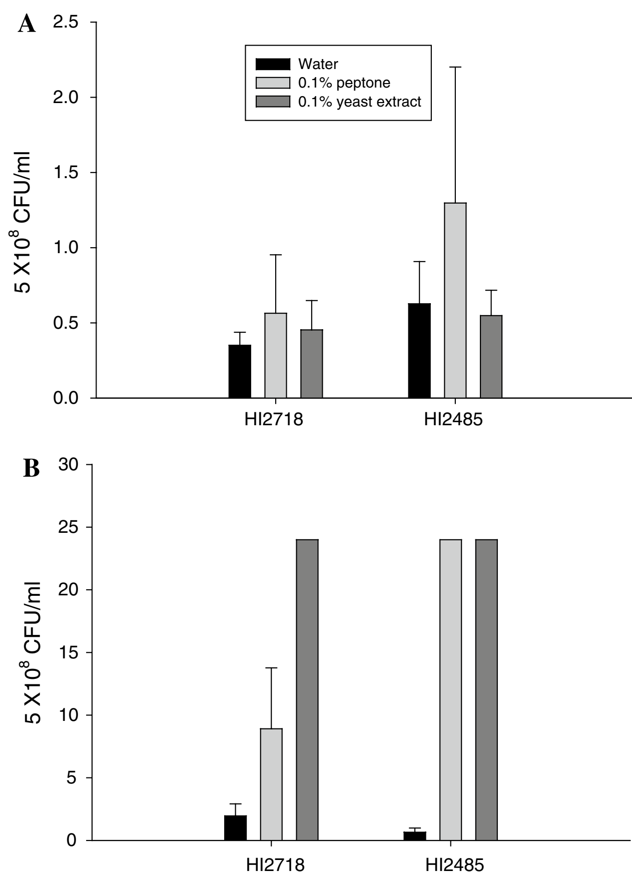

Fig. 4 Comparative recovery of *B. cenocepacia* HI2718 and HI2485 enriched in various diluents incubated for 1 h (**a**) and 12 h (**b**)

grew better in full-strength media made with any form of TSB other than 1/3TSB, and in R2AB (Fig. 2e, and f). Also, environmental isolates grew much better than clinical isolates in all media used in this study (Fig. 2). This may be due to the environmental isolates being adapted to harsh environments, with the capacity to adjust more easily to the low nutrient sterilized water than the clinical isolates.

Several studies describe attempts to isolate BCC bacteria from human specimens, surface waters, and soil by using selective media [3, 7, 12, 15, 17, 27, 28, 33]. However, culture–based recovery of BCC by using selective media represented about 15 % of sample sites, which is an underestimate of environmental populations [15]. Vermis and colleagues [28] isolated *B. cepacia*, *B. cenocepacia* and *Burkholderia vietnamiensis* from European rivers, and Olapade et al. [17] recovered these species from several streams in the USA. *B. cepacia* and *B. pseudomallei* were recovered from 3.5 to 7 % of the 85 drinking water samples analyzed [33]. Kaper et al. [11] demonstrated that a primary, nonselective, ambient-temperature enrichment was superior to selective enrichment strategies for the detection and enumeration of *Salmonella* spp. in estuarine waters using TSB. Our comparison of TSB, BHIB, MHB, ASH, and R2AB enrichment broths demonstrated that enrichment in TSB and R2AB resulted in a significantly higher recovery rate of *B. cenocepacia* in low numbers from distilled water than enrichment in the other three media, including ASH (Table 2). TSB was developed for use as a general purpose medium, providing enough nutrients to allow for a wide variety of microorganisms to grow.

Peptone is used as an organic nitrogen source in microbiological culture media for cultivation of a variety of bacteria. Based on the weight of peptone, R2AB contains only 0.5 g/l proteose peptone, which is 40-fold less than TSB. Carson et al. [2] described "Strain 1" as growing in commercially supplied sterile distilled water at 47 °C and declining or not surviving in TSB. It is possible that the richness of TSB placed too much metabolic stress on nutrient-limited water populations of BCC. Jannasch [9] proposed that organisms had the ability to become temporarily inactive below a threshold substrate concentration. These bacteria lose the ability to multiply as a result of certain stresses but remain otherwise functional, or viable, as individual organisms. As species of the BCC are nutritionally diverse, some organisms might be able to use dissolved organic matter present in oligotrophic aquatic environments [17]. Reasoner and coworkers [20] described a new medium (R2A) for the plate count analysis and cultivation of bacteria from potable water supplies. R2A supported slower-growing oligotrophic species rather than faster-growing heterotrophs. The 1/10TSA, R2A, and 1/3R2A provided better recovery than TSA at the lowest inoculum level (approximately 10^2 CFU/ml). This result is in agreement with findings that soil microorganisms living in an oligotrophic environment may not grow well on rich media [32]. Also, higher recovery frequencies and larger population densities of BCC have been reported from soil and crop rhizospheres using low-nutrient, nonselective media, including 1/10TSA [8].

Gibb [5] reported that agar media were better than broth media for recovery of fastidious organisms from samples with small populations. However, our results showed that agar plate cultures generally failed to recover *B. cenocepacia* when inoculated with $\leq 10^3$ CFU, demonstrating that the broth media used were about tenfold more sensitive than any solid medium tested. This result has also been shown in the recovery of *Propionibacteria* and coagulase-negative *Staphylococci* [14]. The superiority of broth over solid media is also supported by subculture studies that failed to recover consistently 10–100 CFU of *B. cenocepacia* from agar plates (Table 4). Enrichment of water samples in broth prior to plating seemed useful. 0.1 % peptone provided essentially full protection of bacteria for fast doubling time. This result agrees with findings of Straka and Stokes [24] that the loss of viability during dilution

can be avoided by using peptone water as the diluent. The most important role of pre-enrichment (12-h incubation in 0.1 % peptone and yeast extract) is in supporting the recovery of *B. cenocepacia* that may be in a debilitated state [8].

In summary, we found by flow cytometric measurements that *B. cenocepacia* in distilled water retained viability over 40 days. Concerning the effectiveness of the various broth media tested, the recovery was significantly greater using TSB and R2AB than BHIB, MHB, and ASH medium. R2A and dilutions of TSA were better enrichment media for recovery of *B. cenocepacia* from distilled water. On the basis of our experiments, direct culture procedures were not sufficient to recover the organisms from distilled water. Standard culturing methods may not reliably recover these microorganisms. We recommend the use of dilute TSB broth or R2AB broth enrichment, which allows for improved recovery of BCC organisms present in pharmaceutical water.

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References

1. Blodgett R (2010) Bacteriological analytical manual Appendix 2: Most probable number from serial dilutions. http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm109656.htm. Accessed 31 Feb 2014
2. Carson LA, Favero MS, Bond WW, Petersen NJ (1973) Morphological, biochemical, and growth characteristics of *Pseudomonas cepacia* from distilled water. Appl Microbiol 25:476–483
3. Carson LA, Tablan OC, Cusick LB, Jarvis WR, Favero MS, Bland LA (1988) Comparative-evaluation of selective media for isolation of *Pseudomonas cepacia* from cystic-fibrosis patients and environmental sources. J Clin Microbiol 26:2096–2100
4. Drevinek P, Mahenthiralingam E (2010) *Burkholderia cenocepacia* in cystic fibrosis: epidemiology and molecular mechanisms of virulence. Clin Microbiol Infect 16:821–830
5. Gibb AP (1999) Plates are better than broth for recovery of fastidious organisms from some specimen material. J Clin Microbiol 37:875
6. Gilbert SE, Rose LJ (2012) Survival and persistence of nonspore-forming biothreat agents in water. Lett Appl Microbiol 55:189–194
7. Glass MB, Beesley CA, Wilkins PP, Hoffmaster AR (2009) Comparison of four selective media for the isolation of *Burkholderia mallei* and *Burkholderia pseudomallei*. Am J Trop Med Hyg 80:1023–1028
8. Hagedorn C, Gould WD, Bardinelli TR, Gustavson DR (1987) A selective medium for enumeration and recovery of *Pseudomonas cepacia* biotypes from soil. Appl Environ Microbiol 53:2265–2268
9. Jannasch HW (1967) Growth of marine bacteria at limiting concentrations of organic carbon in seawater. Limnol Oceanogr 12:264–271
10. Jimenez L (2007) Microbial diversity in pharmaceutical product recalls and environments. PDA J Pharm Sci Technol 61:383–399
11. Kaper JB, Sayler GS, Baldini MM, Colwell RR (1977) Ambient temperature primary nonselective enrichment for isolation of *Salmonella* spp. from an estuarine environment. Appl Environ Microbiol 33:829–835
12. Limmathurotsakul D, Wuthiekanun V, Amornchai P, Wongsuwan G, Day NPJ, Peacock SJ (2012) Effectiveness of a simplified method for isolation of *Burkholderia pseudomallei* from soil. Appl Environ Microbiol 78:876–877
13. Mahenthiralingam E, Urban TA, Goldberg JB (2005) The multifarious, multireplicon *Burkholderia cepacia* complex. Nat Rev Microbiol 3:144–156
14. Meredith FT, Phillips HK, Reller LB (1997) Clinical utility of broth cultures of cerebrospinal fluid from patients at risk for shunt infections. J Clin Microbiol 35:3109–3111
15. Miller SCM, LiPuma JJ, Parke JL (2002) Culture-based and non-growth-dependent detection of the *Burkholderia cepacia* complex in soil environments. Appl Environ Microbiol 68:3750–3758
16. Moore RA, Tuanyok A, Woods DE (2008) Survival of *Burkholderia pseudomallei* in water. BMC Res Notes 1:11–16
17. Olapade OA, Gao X, Leff LG (2005) Abundance of three bacterial populations in selected streams. Microb Ecol 49:461–467
18. Pan HM, Feng JH, Cerniglia CE, Chen HZ (2011) Effects of Orange II and Sudan III azo dyes and their metabolites on *Staphylococcus aureus*. J Ind Microbiol Biotechnol 38:1729–1738
19. Pumpuang A, Chantratita N, Wikraiphat C, Saiprom N, Day NPJ, Peacock SJ, Wuthiekanun V (2011) Survival of *Burkholderia pseudomallei* in distilled water for 16 years. Trans R Soc Trop Med Hyg 105:598–600
20. Reasoner DJ, Geldreich EE (1985) A new medium for the enumeration and subculture of bacteria from potable water. Appl Environ Microbiol 49:1–7
21. 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
22. Robertson J, Levy A, Sagripanti JL, Inglis TJJ (2010) The survival of *Burkholderia pseudomallei* in liquid media. Am J Trop Med Hyg 82:88–94
23. Schaffter N, Parriaux A (2002) Pathogenic-bacterial water contamination in mountainous catchments. Water Res 36:131–139
24. Straka RP, Stokes JL (1957) Rapid destruction of bacteria in commonly used diluents and its elimination. Appl Microbiol 5:21–25
25. Torbeck L, Raccasi D, Guilfoyle DE, Friedman RL, Hussong D (2011) *Burkholderia cepacia*: this decision is overdue. PDA J Pharm Sci Technol 65:535–543
26. Vandamme P, Holmes B, Coenye T, Goris J, Mahenthiralingam E, Lipuma JJ, Govan JR (2003) *Burkholderia cenocepacia* sp. nov—a new twist to an old story. Res Microbiol 154:91–96
27. Vanlaere E, Coenye T, Samyn E, Van den Plas C, Govan J, de Baets F, de Boeck K, Knoop C, Vandamme P (2005) A novel strategy for the isolation and identification of environmental *Burkholderia cepacia* complex bacteria. FEMS Microbiol Lett 249:303–307
28. Vermis K, Brachkova M, Vandamme P, Nelis H (2003) Isolation of *Burkholderia cepacia* complex genomovars from waters. Syst Appl Microbiol 26:595–600
29. Vial L, Chapalain A, Groleau MC, Deziel E (2011) The various lifestyles of the *Burkholderia cepacia* complex species: a tribute to adaptation. Environ Microbiol 13:1–12
30. Washington JA (1989) Blood cultures—an overview. Eur J Clin Microbiol Infect Dis 8:803–806

31. Wuthiekanun V, Smith MD, White NJ (1995) Survival of *Burkholderia pseudomallei* in the absence of nutrients. Trans R Soc Trop Med Hyg 89:491
32. Young CS, Burns RG (1993) Detection, survival, and activity of bacteria added to soil. In: Bollag J-M, Stotzky G (eds) Soil biochemistry, vol 8., Marcel Dekker, Inc., New York, USA, pp 1–41
33. Zanetti F, De Luca G, Stampi S (2000) Recovery of *Burkholderia pseudomallei* and *B. cepacia* from drinking water. Int J Food Microbiol 59:67–72