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Special Topic-Antibiotics

Oligotrophic Media Compared with a Tryptic Soy Agar or Broth for the Recovery of *Burkholderia cepacia* Complex from Different Storage Temperatures and Culture Conditions [S]

Youngbeom Ahn[1*], Un Jung Lee[2], Yong-Jin Lee[3], John J. LiPuma[4], David Hussong[5], Bernard Marasa[6], and Carl E. Cerniglia[1]

[1]*Division of Microbiology, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079, USA*

[2]*Division of Biochemical Toxicology, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079, USA*

[3]*Department of Biological Sciences, Albany State University, Albany, GA 31705, USA*

[4]*Department of Pediatrics, University of Michigan, Ann Arbor, MI 48109, USA*

[5]*Eagle Analytical Services, Houston, TX 77099, USA*

[6]*Office of Pharmaceutical Quality, Center for Drug Evaluation and Research, U.S. Food and Drug Administration, Silver Spring, MD 20993, USA*

The *Burkholderia cepacia* complex (BCC) is capable of remaining viable in low-nutrient environments and harsh conditions, posing a contamination risk in non-sterile pharmaceutical products as well as a challenge for detection. To develop optimal recovery methods to detect BCC, three oligotrophic media were evaluated and compared with nutrient media for the recovery of BCC from autoclaved distilled water or antiseptic solutions. Serial dilutions (10^{-1} to 10^{-12} CFU/ml) of 20 BCC strains were inoculated into autoclaved distilled water and stored at 6ºC, 23ºC and 42ºC for 42 days. Six suspensions of *Burkholderia cenocepacia* were used to inoculate aqueous solutions containing 5 µg/ml and 50 µg/ml chlorhexidine gluconate (CHX) and 10 µg/ml benzalkonium chloride (BZK), and stored at 23ºC for a further 199 days. Nutrient media such as Tryptic Soy Agar (TSA) or Tryptic Soy Broth (TSB), oligotrophic media (1/10 strength TSA or TSB, Reasoner's 2nd Agar [R2A] or Reasoner's 2nd Broth [R2AB], and 1/3 strength R2A or R2AB) were compared by inoculating these media with BCC from autoclaved distilled water and from antiseptic samples. The recovery of BCC in water or antiseptics was higher in culture broth than on solid media. Oligotrophic medium showed a higher recovery efficiency than TSA or TSB for the detection of 20 BCC samples. Results from multiple comparisons allowed us to directly identify significant differences between TSA or TSB and oligotrophic media. An oligotrophic medium pre-enrichment resuscitation step is offered for the United States Pharmacopeia (USP) proposed compendial test method for BCC detection.

Keywords: Oligotrophic medium, tryptic soy agar or broth, *Burkholderia cepacia* complex

Introduction

The genus *Burkholderia* is currently comprised of 129 species [1]. Among these are 22 closely related species collectively referred to as the *Burkholderia cepacia* complex (BCC) [2–6]. These bacteria are opportunistic human pathogens that are commonly found in natural environments. They also have been recovered from many contaminated drug products and have become public health concerns [7–12]. A review of the Food and Drug Administration (FDA) pharmaceutical product recalls indicates that BCC isolates are some of the most frequently isolated microorganisms contaminating sterile and non-sterile pharmaceutical products, and several recent outbreaks of infection have been linked to antiseptics that were found to contain BCC [11, 13, 14]. Aqueous products are especially at risk because

of the ability of BCC to remain viable in harsh conditions, including the presence of preservatives [15, 16].

A challenge presented by BCC species lies in their unpredictable capacity to avoid detection [13, 17], ability to grow in low-nutrient conditions [18], resistance to chemical preservatives [19], and the potential to cause disease especially to immunocompromised populations [13, 17]. Some strains are able to grow in distilled water at temperatures as low as 12°C and as high as 48°C [18]. Previous research has shown that some BCC strains are capable of growing in distilled water at 18°C or 23°C for up to 40 days [20] and may be able to grow in the presence of disinfectants and antimicrobial preservatives [16, 21]. The FDA is reminding drug manufacturers to use appropriate acceptance criteria (*e.g.*, United States Pharmacopeia [USP] Chapter <1111> Microbiological examination of non-sterile products: acceptance criteria for pharmaceutical preparations and substances for pharmaceutical use) [22] and test procedures (*e.g.*, USP <61> Microbiological examination of non-sterile products: microbial enumeration tests [23] and USP <62> Microbiological examination of non-sterile products: tests for specified microorganisms [24]) to ensure that drug product components, pharmaceutical water, and finished drug products conform to appropriate quality standards. Pharmaceutical products that are contaminated with BCC may pose serious consequences to vulnerable patients (*e.g.*, persons with compromised immune systems and persons with cystic fibrosis) and yet optimal recovery methods for BCC have not been determined. Preventing BCC contamination in drugs is an important public health goal. This can be achieved by addressing BCC detection during drug manufacturing.

Isolation and correct identification of BCC is challenging [20, 25–27]. A variety of selective and differential media have been proposed for isolating BCC [25–27], but USP methods for detection of *Pseudomonas aeruginosa*, including enrichment using Tryptic Soy Agar (TSA) or Tryptic Soy Broth (TSB), may not be adequate for all strains of BCC [13, 14, 18]. The recovery of microorganisms from pharmaceutical water samples may be increased by using low-nutrient media [16, 20, 28–31]. However, research is needed to compare BCC recovery using existing growth media/ methods as recommended in USP <61> and USP <62> with the recently developed dilute TSA (or TSB) or Reasoner's 2nd Agar (R2A) or Reasoner's 2nd Broth (R2AB) methods [20]. In this study, the 1/10 × TSA/B, 1/3 × R2A/B, and R2A/B is referred to as oligotrophic medium. The aim of this study was to assess the recovery of BCC using oligotrophic media as compared to the TSA or TSB at different storage temperatures in autoclaved distilled water samples as well as at different antiseptic concentrations.

Materials and Methods

Bacterial Strains

BCC isolated from either persons with cystic fibrosis or from the environment were obtained from the *Burkholderia cepacia* Research Laboratory and Repository at the University of Michigan [20]. Twenty BCC strains were examined after storage in autoclaved distilled water at different storage temperatures (see Table S1 to S6 in the supplemental material) and six strains of *B. cenocepacia* were examined after storage in chlorhexidine gluconate (CHX) and benzalkonium chloride (BZK) antiseptics (see Tables S7 and S8 in the supplemental material). These strains were grown on TSA or TSB at 30°C for 24 or 48 h and stored at −80°C with 10% glycerol until used.

Media

The four culture media used in the comparison were TSA (or TSB), 1/10 × TSA (*i.e.*, 1/10 strength TSA or 1/10 × TSB), R2A (or R2AB) and 1/3 × R2A (or 1/3 × R2AB) (Table 1) [20]. TSA (or TSB) was used as a control medium. Any positive growth of BCC was further tested using *B. cepacia* selective agar (BCSA) with

Table 1. Formulation of enrichment media used to evaluate the *B. cepacia* complex culture method.

Component	Enrichment media (g/l)			
	TSB	1/10 × TSB	1/3 × R2AB	R2AB
Pancreatic Digest of Casein	17	1.7	-	-
Casein Hydrolysate	-	-	0.16	0.5
Papaic Digest of Soybean	3	0.3	-	-
Casein Peptone	-	-	0.07	0.25
Meat Peptone	-	-	0.07	0.25
Protein (N)	20	2	0.3	1
Glucose Monohydrate	2.5	0.25	-	-
Dextrose	-	-	0.16	0.5
Sodium Pyruvate	-	-	0.1	0.3
Soluble Starch	-	-	0.16	0.5
Carbohydrate (C)	2.5	0.25	0.42	1.3
Disodium Phosphate (Na$_2$HPO$_4$)	2.5	0.25	-	-
Dipotassium Phosphate (K$_2$HPO$_4$)	-	-	0.1	0.3
Phosphate (P)	2.5	0.25	0.1	0.3
Yeast Extract	-	-	0.16	0.5
Sodium Chloride	5	0.5	-	-
Magnesium Sulfate	-	-	0.008	0.024

TSA, R2A, and diluted agar medium (1/10 × TSA and 1/3 × R2A) containing 15 g agar in deionized water were used.

vancomycin, gentamicin, and polymyxin B (Thermo Fisher Scientific, USA) to confirm identity.

In Autoclaved Distilled Water at Different Storage Temperatures

Preparation of sample inocula. For the inoculation of sterile distilled water with BCC, the bacterial strains were grown on TSA at 30°C for 48 h. The cultures were washed three times with autoclaved distilled water, and transferred into 20 ml of autoclaved distilled water. The strains held in water were adjusted to an optical density corresponding to 0.38–0.4 absorbance at a wavelength of 600 nm (approximate cell density 10^{14} colony-forming units per ml (CFU/ml)) in the Synergy MX spectrophotometer from BioTek Instruments, Inc. (USA). Serial dilutions of each BCC suspension were prepared in 20 ml autoclaved distilled water to yield appropriate CFUs (serial dilutions ranging from 10^{-1} to 10^{-12}). Each sample was prepared in triplicate and stored at 6°C (refrigerated incubator, ThermoFisher Scientific), at 23°C (at room temperature, on a lab bench) and at 42°C (incubator, ThermoFisher Scientific) for 42 days. In addition, uninoculated and highly inoculated ($>10^{14}$ CFU/ml) distilled water samples (20 ml) were used as negative and positive controls, respectively.

Growth kinetics at different storage temperatures. To evaluate the effect of nutrient content on growth kinetics, TSB, $1/10 \times$ TSB, R2AB and $1/3 \times$ R2AB broth media were used. *B. cenocepacia* AU19236 was used for these tests and survived at 6°C (serial dilutions ranging from 10^{-4} to 10^{-6}), 23°C (serial dilutions ranging from 10^{-8} to 10^{-10}), and 42°C (serial dilutions ranging from 10^{-1} to 10^{-3}). The 10-μl portions of the dilutions of suspensions held in water after 42 days were transferred into a 96-well plate (containing 90 μl of medium per well) and incubated at 30°C. The kinetics of growth in various broth media were monitored by the Synergy MX spectrophotometer. The kinetic software (Gen5) of the instrument was programmed to measure the absorbance at 600 nm every 2 h at 30°C for 48 h after shaking the microplates for 10 s at an intermediate intensity. Optical density measurements were compared for the cultures growing in TSB, $1/10 \times$ TSB, R2AB, and $1/3 \times$ R2AB. Estimations of the length of the lag phase (in hours; λ) and growth rate (μ) were determined from the mean growth curves generated. Growth curves for each temperature and strain were prepared separately by fitting data to the Baranyi model using DMFit, available at https://www.combase.cc [32].

Solid media comparison at different storage temperatures. BCC strains were directly inoculated from distilled water cultures onto TSA (in USP <61>), $1/10 \times$ TSA, R2A, and $1/3 \times$ R2A for comparison in enumeration tests. Samples were collected on days 0, 1, 2, 3, 4, 7, 9, 14, 22, 28, 35, and 42. BCC survival was tested at 6°C (initial serial dilution 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}), 23°C (initial serial dilution 10^{-8}, 10^{-9}, and 10^{-10}), and 42°C (initial serial dilution 1, 10^{-1}, 10^{-2}, and 10^{-3}) for 42 days. At each time point, 10 μl of serial dilutions of BCC strains were placed on various solid agar media to enumerate the bacteria [33]. The plates were incubated at 30°C for 48 h and the number of colony forming units on each plate was

counted using the ProtoCOL3 automated plate counter (Synbiosis, Frederick, MD). The number of colonies was used to calculate the concentration of bacteria in each medium. Plate assays were performed in three to six replicates and the results were averaged.

Broth media comparison at different storage temperatures. Samples from distilled water cultures were used to compare the recovery efficiency of TSB (in USP<61>), $1/10 \times$ TSB, R2AB, and $1/3 \times$ R2AB. Ninety microliters of each broth medium were added in the same order to three sets of 96-well plates. At each time point, serial dilutions of BCC (10 μl each) after 0, 1, 2, 3, 4, 7, 9, 14, 22, 28, 35, and 42 days were added to each medium. BCC survived at 6°C (initial serial dilution 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}), 23°C (initial serial dilution 10^{-12}), and 42°C (initial serial dilution 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}) for 42 days were determined. Uninoculated wells were used as a baseline control. Growth of BCC strains in different growth media was determined using the Synergy MX spectrophotometer after subtracting the OD_{600} value of the uninoculated medium, as described previously [16, 20]. After 48 h incubation at 30°C, the number of wells in which growth had occurred was recorded. Any positive growth was tested using BCSA to confirm identity. Negative broth cultures (wells with low OD_{600} (0.040–0.045)) were subcultured onto BCSA to confirm a true negative result. The number of positive wells was used to calculate the concentration of bacteria in each medium. Broth assays were performed in six replicates and the results were averaged.

In CHX and BZK Solutions

Preparation of sample inocula. The diluted antiseptics (5 and 50 μg/ml CHX and 10 μg/ml BZK) were prepared using autoclaved distilled water that did not include added nutrients. *B. cenocepacia* strains (approximately 10^8 CFU/ml) were incubated in water at 23°C for 42 days. After incubation, each 1 ml water sample was mixed with the same volume of antiseptic solution (serial dilutions ranging from 10^{-1} to 10^{-6}) or distilled water for control samples, so that the final chemical concentration became 5 and 50 μg/ml for CHX and 10 μg/ml for BZK, respectively, at their sub-MICs mentioned previously [16]. Each sample was prepared in triplicate and stored at 23°C (at room temperature, in a dark environment such as in a closed laboratory cabinet) for 199 days.

Solid media comparison in CHX and BZK. The survival rate of BCC in antiseptics was evaluated after 199 days by introducing *B. cenocepacia* to 5 μg/ml CHX (initial serial dilution 1, 10^{-1}, and 10^{-2}), 50 μg/ml CHX (initial serial dilution 1 and 10^{-1}), and 10 μg/ml BZK (initial serial dilution 1 and 10^{-1}) [16, 21]. Ten μl serial dilutions of antiseptics with BCC strains were placed on TSA, $1/10 \times$ TSA, $1/3 \times$ R2A and R2A to recover the bacteria and incubated at 30°C for 48 h. All counts were performed as described above.

Broth media comparison in CHX and BZK. Broth media were prepared as described in previous broth medium comparisons [16, 20]. After 199 days, serial dilutions of BCC (10 μl each) were added to each broth medium to achieve cell densities of 10^5, 10^4,

and 10^3 CFU/ml. *B. cenocepacia* survived at 5 and 50 µg/ml for CHX and 10 µg/ml BZK CFU/ml (from initial serial dilution 10^{-1}, 10^{-2}, and 10^{-3}) were determined as described above.

Statistical Analysis

The number of colony forming units for BCC was statistically evaluated by the Mann-Whitney Rank Sum Test using SigmaPlot software (GraphPad Software, Inc. USA). The number of positive broth media for BCC was statistically evaluated by the χ^2-test using Social Science Statistics web-based software (https://www.socscistatistics.com/Default.aspx).

The SAS statistical package (version 9.4; SAS Institute Inc.) and R language (version 3.2.3) were used for analyzing data from solid and broth media. All statistical tests were two-tailed, with a significance level of 0.05. For data analysis of solid media, raw data were transformed to stabilize the data variance from the number of observed bacteria to the proportion of observed bacteria. Descriptive statistics, including means with standard deviations

(SDs), were reported for each culture media. Normalization of raw data was calculated by the equation shown below.

$$\text{Normalization} = \frac{\text{number of bacteria}_{it}}{\text{max(number of bacteria}_t)}.$$

where i represents the number of bacteria of different culture media (i = 1, 2, 3, 4), and t represents the number of bacteria of different culture media at trial (t = 1, 2, … 368).

Differences between media groups were analyzed using the one-way analysis of variance (ANOVA) followed by Tukey's HSD test. Univariate analyses were performed using ANOVA for continuous variables and Pearson's χ^2-test for categorical variables to explore significant differences among groups. A Tukey's honest significant difference (HSD) test on ANOVA was applied to examine pairwise differences between groups for continuous variables.

For data analysis from broth media, a macro, CHISQ_MC, was implemented to perform a Tukey-type multiple comparison on
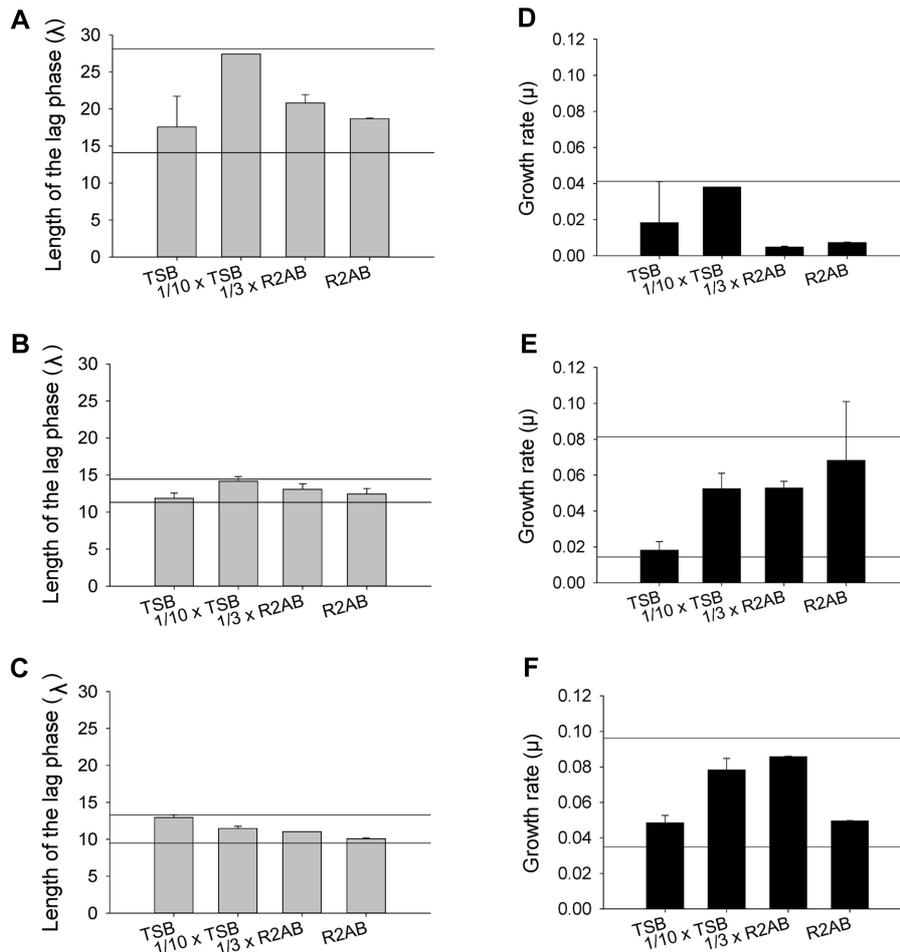

Fig. 1. The lag phase (λ; **A**, **B**, and **C**) and growth rate (μ; **D**, **E**, and **F**) of *B. cenocepacia* AU19236 inoculated in TSB, 1/10 × TSB, 1/3 × R2AB and R2AB medium stored at 6°C (A and D), 25°C (B and E) and 42°C (C and F) for 42 days using the Baranyi growth model. Solid lines show the 95% upper and lower predicted interval.

Pearson's χ^2-test for categorical variables [34]. The calculated χ^2-test statistics and *p*-values are for all pairs of groups.

Results

Compare the Oligotrophic Media to the Current USP <61> Media in Autoclaved Distilled Water

Growth kinetics in TSB and R2AB at different storage temperatures. To understand the effects of temperature on survival, the 20 BCC strains were stored at three temperatures (6°C, 23°C, and 42°C) for 42 days and recovered in various media. In general, survival was observed at 6°C, 23°C, and 42°C, and no species-specific trend was noted. Growth of *B. cenocepacia* AU19236 was observed when media were inoculated with initial concentrations between 10^2 and 10^0 CFU/ml in distilled water stored at 6°C and 23°C, and between 10^8 and 10^6 CFU/ml in distilled water stored at 42°C for 42 days. Growth was observed in TSB, $1/10 \times$ TSB, R2AB and $1/3 \times$ R2AB broth media after incubation for 24 h at 30°C for *B. cenocepacia* AU19236 and kinetics were described using the Baranyi model [32]. The average R^2 values for growth curves of *B. cenocepacia* AU19236 inoculated on TSB, $1/10 \times$ TSB, R2AB and $1/3 \times$ R2AB and fitted to the Baranyi model, were > 0.98. The high R^2 values were obtained in average growth curves because the regression line approximated the observed data points. Cultures in distilled water stored at 6°C showed a much longer lag time (λ), ranging between 17.6 and 27.4 h (Fig. 1A). No significant differences were observed between the lag times estimated for different media at 23°C and 42°C (10.1 and 14.2 h, respectively) (Figs. 1B and 1C). The lag time values (λ) were greater in $1/10 \times$ TSB, R2AB and $1/3 \times$ R2AB than in TSB at 6°C and 23°C, but not at 42°C. However, lag time fell within the 95% confidence interval, irrespective of the media used ($p > 0.05$), although the lag time was a bit longer for $1/10 \times$ TSB. Furthermore, growth rate (μ) did not significantly differ regardless of the media used ($p > 0.05$), although the growth rate was greater for more favorable growth media, such as $1/10 \times$ TSB, R2AB and $1/3 \times$ R2AB, compared to TSB at 23°C and 42°C, but not at 6°C (Figs. 1D–1F).
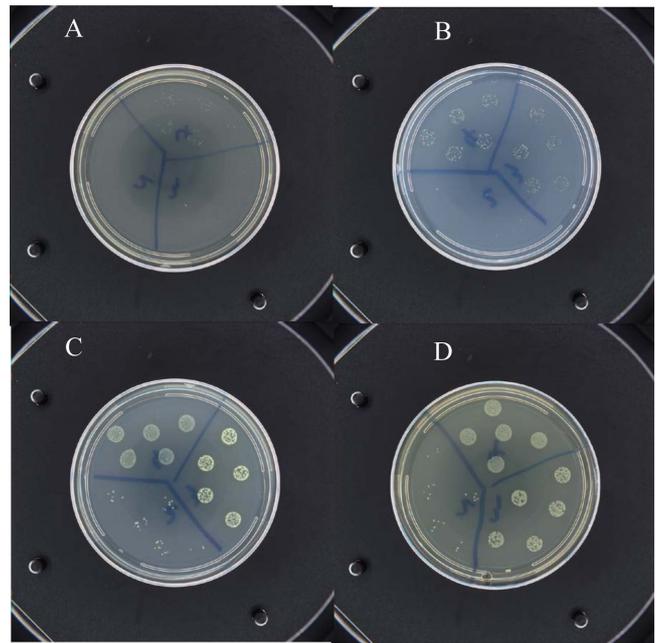

Fig. 2. Plate images of *B. cenocepacia* AU19236 grown on TSA (**A**), $1/10 \times$ TSA (**B**), $1/3 \times$ R2A (**C**), and R2A (**D**) media stored at 42°C for 42 days.

For comparison with TSA, a series of three dilutions was plated (five times) for 20 strains, and imaged using the ProtoCOL3 automated plate counter.

TSA and oligotrophic solid media comparison at different storage temperatures. For evaluation of viable colony counts in solid media, each plate was counted using the ProtoCOL3 automated plate counter (Fig. 2). After 42 days of incubation, all of the agars allowed recovery of bacteria fairly easily due to the high number of colonies that were present in the distilled water at 6°C (10^4-10^8 CFU/ml) (see Table S1), and 23°C (10^7-10^{12} CFU/ml) (see Table S2) (Table 2). However, when 20 BCC strains were plated on solid medium, only eight (*B. arboris* AU22095, *B. lata* HI4002 256, *B. cenocepacia* AU1054, *B. cenocepacia* AU0222, *B. cenocepacia* AU19236, *B. cenocepacia* HI2976, *B. cenocepacia* HI2485 and *B. cenocepacia* J2315) grew after 42 days at 42°C (10^2-10^6 CFU/ml) in distilled water (see Table S3). The remaining

Table 2. Colony counts of *B. cepacia* complex at 1-10^{-10} dilutions in nonselective solid media at 6°C, 23°C, and 42°C for 42 days.

Temperature (°C)	Tested initial serial dilution	Number of recovery (CFU/ml)	TSA	$1/10 \times$ TSA	$1/3 \times$ R2A	R2A
6	10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}	10^8	1.67 ± 6.41	1.49 ± 5.93	1.79 ± 6.81	1.95 ± 7.31
23	10^{-10}, 10^{-9}, 10^{-8}	10^{12}	3.44 ± 5.09	4.18 ± 6.17	4.17 ± 5.62	$4.46 \pm 5.78^*$
42	10^{-3}, 10^{-2}, 10^{-1}, 1	10^5	0.57 ± 1.39	$2.01 \pm 4.17^*$	$6.56 \pm 15.05^*$	$6.53 \pm 14.72^*$

*There is a statistically significant difference between TSA and other medium groups ($p < 0.05$).

strains did not grow on solid medium after nine days at 42°C despite longer incubation times of up to 96 h.

Under 6°C storage conditions after 42 days, all of the agars allowed recovery of approximately 10^4-10^8 CFU/ml (Table 2, see Table S1). *B. lata* HI4002, *B. cenocepacia* HI2976 and *B. cenocepacia* HI2485 were among the BCC which exhibited a high colony count of 10^8 CFU/ml. The colony counts were slightly higher and more variable on $1/3 \times$ R2A ($1.79 \pm 6.81 \times 10^8$ CFU/ml) and R2A ($1.95 \pm 7.31 \times 10^8$ CFU/ml) than in TSA ($1.67 \pm 6.41 \times 10^8$ CFU/ml). The Mann-Whitney Rank Sum Test was performed to compare the difference between TSA and $1/10 \times$ TSA, $1/3 \times$ R2A, and R2A, and a significant difference was not found ($p <$ 0.05). In distilled water at 23°C, an initial population of 10^{12} CFU/ml of BCC remained unchanged on various media. The colony counts were higher and more variable in $1/10 \times$ TSA ($4.18 \pm 6.17 \times 10^{12}$ CFU/ml), $1/3 \times$ R2A ($4.17 \pm 5.62 \times 10^{12}$ CFU/ml) and R2A ($4.46 \pm 5.78 \times 10^{12}$ CFU/ml) than in TSA ($3.44 \pm 5.09 \times 10^{12}$ CFU/ml) (Table 2, see Table S2). The Mann-Whitney Rank Sum Test was performed to compare the difference between TSA and R2A, and a significant difference was found ($p < 0.041$). At 42°C, only eight strains grew to approximately 10^5 CFU/ml (Table 2, see Table S3). The colony counts were higher and more variable in $1/10 \times$ TSA ($2.01 \pm 4.17 \times 10^5$ CFU/ml), $1/3 \times$ R2A ($6.56 \pm 15.05 \times 10^5$ CFU/ml) and R2A ($6.53 \pm 14.72 \times 10^5$ CFU/ml) than in TSA ($0.57 \pm 1.39 \times 10^5$ CFU/ml). The Mann-Whitney Rank Sum Test was performed to compare the difference and showed significant differences between TSA and oligotrophic media, such as $1/10 \times$ TSA ($p = 0.008$), $1/3 \times$ R2A ($p < 0.001$), and R2A ($p < 0.001$).

TSB and oligotrophic broth media comparison at different storage temperatures. The TSB, $1/10 \times$ TSB, $1/3 \times$ R2AB, and R2AB broth media were evaluated for their ability to recover BCC at 6°C, 23°C, and 42°C (Table 3). At 6°C storage, compared with $1/10 \times$ TSB, $1/3 \times$ R2AB, and R2AB, recovery was less efficient in TSB media, with the

portion of recovery wells of 351/480 (number of recovery/ number of tests), 345/480, and 353/480, respectively (Table 3, see Table S4). The χ^2 test on absolute numbers tested for significant differences in the frequency of growth between broth media. Significance was set at $p < 0.05$. The statistical analysis of data reveals that there was significant difference between TSB and $1/10 \times$ TSB (χ^2 statistic = 9.3528, $p = 0.0022$), $1/3 \times$ R2AB (χ^2 statistic = 6.093, $p = 0.0086$), R2AB (χ^2 statistic = 10.2594, $p = 0.00136$) at 6°C for 42 days storage. Evaluation of the four broths inoculated with the lowest colony numbers (10^{12}) indicated that $1/10 \times$ TSB, $1/3 \times$ R2AB, and R2AB supported the most efficient recovery of BCC from 23°C distilled water for 42 days. Differences in recovery of BCC among the TSB, $1/10 \times$ TSB, $1/3 \times$ R2AB, and R2AB media occurred in 39, 94, 107, 79 of the 480 experimental trials, respectively (Table 3, see Table S5). Statistical analyses showed that TSB was less effective than $1/10 \times$ TSB (χ^2 statistic = 26.4022, $p < 0.05$), $1/3 \times$ R2AB (χ^2 statistic = 37.3518, $p < 0.05$), and R2AB (χ^2 statistic = 15.4596, $p < 0.05$) media ($p < 0.05$). The recovery of BCC after 42 days of storage at 42°C is shown in Table 3 and Table S6 in the supplemental material. The χ^2 statistic test was performed to compare the difference between TSA and $1/10 \times$ TSB, $1/3 \times$ R2AB, and R2AB media, and no significant difference was found ($p > 0.05$) (Table 3, see Table S6).

Recovery of *B. cenocepacia* from CHX and BZK

TSA and dilute solid media comparison at different concentration of antiseptics. Water–adapted 6 *B. cenocepacia* strains were suspended in 5 and 50 μg/ml of CHX or 10 μg/ml of BZK. In 5 and 50 μg/ml of CHX solutions, colony counts were slightly higher in $1/10 \times$ TSA ($7.06 \pm 6.5 \times 10^4$ CFU/ml, $3.73 \pm 3.93 \times 10^3$ CFU/ml) than in TSA ($6.59 \pm 5.12 \times 10^4$ CFU/ml, $3.03 \pm 3.42 \times 10^3$ CFU/ml), respectively (Table 4, see Table S7). The Mann-Whitney Rank Sum Test was performed to compare the difference between TSA and $1/10 \times$ TSA, and a significant difference

Table 3. Number of positive wells (number of wells set up) of *B. cepacia* complex at 10^{-2}-10^{-12} dilutions in nonselective broth media at 6°C, 23°C, and 42°C for 42 days.

Temperature (°C)	Tested initial serial dilution	TSB	$1/10 \times$ TSB	$1/3 \times$ R2AB	R2AB
6	10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}	307/480[a]	351/480[b]	345/480[b]	353/480[b]
23	10^{-12}	39/480	94/480[b]	107/480[b]	79/480[b]
42	10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}	246/288	241/288	241/288	244/288

[a]Number of recovery/number of tests.

[b]There is a statistically significant difference between TSA and other medium groups ($p < 0.05$).

Table 4. Colony counts of 6 *B. cenocepacia* strains at 1-10^{-2} dilutions in nonselective solid media at 23°C for 199 days

Antiseptics (concentration)	Tested initial serial dilution	Number of recovery (CFU/ml)	TSA	1/10 × TSA	1/3 × R2A	R2A
CHX (5 μg/ml)	10^{-2}, 10^{-1}, 1	10^4	6.59 ± 5.12	7.06 ± 6.5	6.36 ± 5.96	6.67 ± 6.54
CHX (50 μg/ml)	10^{-1}, 1	10^3	3.03 ± 3.42	3.73 ± 3.93	2.88 ± 3.00	3.03 ± 3.42
BZK (10 μg/ml)	10^{-1}, 1	10^3	1.27 ± 1.28	1.51 ± 1.77	1.58 ± 1.73	1.79 ± 2.24

Table 5. Number of positive wells (number of wells set up) of *B. cenocepacia* at 10^{-1} -10^{-3} dilutions in nonselective broth media at 23°C for 199 days.

Antiseptics (concentration)	Tested initial serial dilution	TSB	1/10 × TSB	1/3 × R2AB	R2AB
CHX (5 μg /ml)	10^{-3}, 10^{-2}, 10^{-1}	116/144[a]	107/144	116/144	134/144[b]
CHX (50 μg /ml)	10^{-3}, 10^{-2}, 10^{-1}	94/144	78/144	85/144	114/144[b]
BZK (10 μg /ml)	10^{-3}, 10^{-2}, 10^{-1}	67/144	62/144	95/144[b]	113/144[b]

[a]Number of recovery/number of tests.

[b]There is a statistically significant difference between TSA and other medium groups ($p < 0.05$).

was not found ($p < 0.05$). Also, in 10 μg/ml BZK solutions, the population counts were slightly higher in 1/10 × TSA (1.51 ± 1.77 × 10^3 CFU/ml), 1/3 × R2A (1.58 ± 1.73 × 10^3 CFU/ml), R2A (1.79 ± 2.24 × 10^3 CFU/ml) than in TSA (1.27 ± 1.28 × 10^3 CFU/ml), respectively. There was no difference in recovery by Mann-Whitney Rank Sum Test.

TSB and oligotrophic broth media comparison at different concentration of antiseptics. The TSB, 1/10 × TSB, 1/3 × R2AB, and R2AB broth media were evaluated for their ability to recover *B. cenocepacia* in 5 and 50 μg/ml of CHX or 10 μg/ml of BZK. Recovery was more efficient in R2AB media, with the portion of recovery wells of 134/144 (number of recovery/number of tests, from 5 μg/ml of CHX), 114/144 (50 μg/ml of CHX), and 113/144 (10 μg/ml of BZK), respectively (Table 5, see Table S8). The statistical analysis of data reveals that there was significant difference between TSB and R2AB of 5 μg/ml of CHX (χ^2 statistic = 6.9231, $p = 0.0085$), 50 μg/ml of CHX (χ^2 statistic = 9.8223, $p = 0.0017$), and 10 μg/ml of BZK (χ^2 statistic = 31.3481, $p < 0.05$).

Comparison by Statistical Analysis

TSA and oligotrophic solid media pairwise comparison. A Tukey's HSD test on ANOVA was performed to examine pairwise differences of the proportion of the colony counts between different agar media. R2A had the highest mean of BCC recovery (0.84, standard deviation = 0.20), which was similar to the growth rate of 1/3 × R2A (0.82, standard deviation = 0.22) (Table 6A). Both recovery of BCC in R2A and 1/3 × R2A media were significantly different from recovery of BCC in TSA media ($p < 0.05$). 1/10 × TSA was more likely to have recovery (0.69, standard deviation = 0.28) compared with TSA (0.61, standard deviation = 0.30) ($p < 0.05$).

TSB and oligotrophic broth media pairwise comparison. A Tukey-type multiple comparison on Pearson's χ^2 -test was performed to evaluate pairwise difference of the recovered BCC between different broth media. After adjusting for multiple comparisons, the recovery ability of BCC in TSB media was significantly different from 1/3 ×

Table 6. Summary of descriptive statistics and results of post hoc tests using Tukey's HSD test on ANOVA (A) and Tukey-type multiple comparison on Pearson's χ^2 test (B).

(A) Solid media

	TSA	1/10 × TSA	1/3 × R2A	R2A
Mean of rate (standard deviation)	0.61 (0.30)	0.69 (0.28)*	0.82 (0.22)*	0.84 (0.20)*

*$p < 0.05$ significantly different from TSA.

(B) Broth media

	TSB	1/10 × TSB	1/3 × R2AB	R2AB
Number of positives/number of negatives	1383/873	1477/809	1506/750*	1557/669*

*$p < 0.05$ significantly different from TSA.

R2AB and R2AB, separately. The TSB media was observed to have lower counts of BCC compared with 1/10 × TSB, but this finding did not reach statistical significance (Table 6B).

Discussion

Species in the BCC are able to remain viable under harsh conditions such as in water, organic solvents, antiseptics, and liquids with low nutrient levels for prolonged periods [13, 15, 16, 18, 35–39]. The survival of BCC at temperatures ranging from 10°C to 50°C has been previously reported [18]. *Burkholderia pseudomallei* (not a species in the BCC) remained viable when incubated at 8°C for more than 1133 days, and at above 40°C for less than one month [40]. Also, *B. pseudomallei* survived in distilled water for 200 days or 16 years [36, 38]. Previously, we reported that *B. cenocepacia* can persist in distilled water at 18°C, and 23°C for 40 days [20]. In this investigation, the survival of 20 BCC strains in distilled water samples was observed at 6°C and 23°C over a 42-day period, which slightly surpasses the previously examined 40-day period (see Table S1 to S6). However, 12 BCC strains did not grow when incubated at 42°C during the same time period. Although, we observed that six *B. cenocepacia* strains endured nutrient-depleted environments in a wide range of temperatures (6°C to 42°C) for periods of up to 42 days, there was a significant decrease (10 to 10^4 fold) in the recovered viable count for all strains. Furthermore, all six *B. cenocepacia* strains survived for at least 199 days– which is 5 times the 40-day period – in the presence of 5-50 μg/ml CHX and BZK. These results are consistent with our previous studies in which *B. cenocepacia* strains were intrinsically resistant to a wide range of antiseptics and were shown to survive for at least 28 days in the presence of CHX and BZK [3, 16, 21].

USP <62> tests allow the detection or limited occurrence of specified microorganisms that may be present under the conditions described [24]. The bacterial test strains were grown in culture vessels containing TSB or TSA at 30°C to 35°C for 18 to 24 h. We compared the currently accepted TSA (or TSB) media methods in USP <61> and USP <62> to the diluted media methods for the detection of BCC [20, 23, 24]. TSA (or TSB) media was developed for use as a general purpose medium, providing enough nutrients to allow for a wide variety of microorganisms to grow [23, 24]. The ability to grow and persist in multiple environments appears to be the key factor that promotes BCC persistence. Lag phase depends on the physiological state of the inoculum, as well as on growth conditions. Temperature-dependent and nutrient-depleted environments could alter the lag

phase of growth. *B. cenocepacia* AU19236 had a significantly longer lag phase at 6°C (17.6–27.4 h) than at 23°C and 42°C (10.1–14.2 h). However, no significant differences in lag times were observed between TSB and oligotrophic media.

R2A seemed the most appropriate medium to detect bacteria from water samples, and is recommended by the American Society for Testing and Materials (ASTM) for testing of water quality [41]. Our comparison of TSA/B and oligotrophic medium (1/10 × TSA/B, 1/3 × R2A/B, and R2A/B) demonstrated that samples recovered in oligotrophic medium resulted in significantly higher recovery rates of BCC in autoclaved distilled water and in antiseptics at 23°C, 6°C and 42°C storage for 42 days than recovered in TSA (Tables 2, 3, and 4). These findings are consistent with our previous studies evaluating the recovery of BCC from distilled water and antiseptic solution at 23°C [16, 20]. This result is in agreement with findings that higher recovery frequencies and larger population densities of BCC have been reported from water, soil and crop rhizospheres using low-nutrient, nonselective media [20, 28–31, 42, 43]. Furthermore, Peeters et al. [43] reported that a higher number of distinct colony morphologies were present on the ten-fold diluted formulation of *Pseudomonas cepacia* azelaic acid tryptamine medium (1/10 × PCAT). It is possible that the nutrient-rich conventional growth media may favor the growth of faster-growing bacteria at the expense of slow-growing ones. This media may also inhibit or place metabolic stress on nutrient-limited water populations of BCC. Although TSA/B is generally considered the medium of choice, technical specifications for the USP survey of BCC in non-sterile pharmaceutical materials allow for different oligotrophic agar to be used.

USP <62> "*Microbiological examination of non-sterile products: tests for specified microorganisms*" is performed routinely to test for the presence of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, bile-tolerant Gram-negative bacteria, *Clostridia* species, *Salmonella* species and/or *Candida albicans* [24]. A test sample is first enriched by inoculation into soybean casien digest broth (TSB), then streaking onto selective agars to determine the presence of specified microorganisms [24]. The pre-enrichment of samples is used as a means of increasing sensitivity and test reliability. In this study, by direct culture on TSA, BCC was not detected in water samples at 23°C (initial serial dilution 10^{-10}, 10^{-11}, and 10^{-12}). However, broth media used were about ten-fold to a hundred-fold more sensitive (one to two order of magnitude greater) than any solid medium tested (Tables 2 and 3). Previously, Vermis *et al.* [26] reported that liquid enrichment prior to plating may enhance the isolation of

BCC from water samples, suggesting that liquid enrichment is necessary in support of BCC recovery. Moreover, we reported that the superiority of broth over solid media is also supported by recovery of *B. cenocepacia* from distilled water [20]. The kind of agar used as a solidifying agent may interfere in the early stages of growth. The growth of motile species such as *P. aeruginosa, L. monocytogenes, S. typhimurium* and *E. coli* is affected by agar concentration (reduced water activity); these species typically do not grow on media with elevated agar concentrations [44]. The need for a stress recovery phase to recover microorganisms was demonstrated by longer incubation times and consistently higher recovery on low-nutrient media and temperatures below 35°C. The culture method and medium used influenced the recovery of some BCC samples from distilled water and antiseptic samples. Low nutrient media as a pre-enrichment step are strongly supported for the compendial test method for BCC detection.

The use of the Tukey's HSD test showed that oligotrophic solid media were observed to have higher BCC recovery rates compared with TSA. In addition, the number of bacteria recovered with different broth media indicated that oligotrophic broth media provide for the highest recovery of BCC. The differences between oligotrophic media and TSB or TSA medium for growth or survival in this study were due to the favorable nutritional composition of oligotrophics, compared to a high nutrition medium. On the basis of our experiments, R2A and dilutions of TSA constituted better enrichment media for the recovery of BCC from distilled water and antiseptics. In addition, the recovery of microorganisms from distilled water samples and antiseptics increased by using a pre-enrichment medium. Results in this study provide confirmatory data for USP in support of its proposed compendial chapter for BCC detection. Results from this study clearly demonstrate the utility of using diluted media and enriched cultures with a pre-enrichment step as a superior strategy in the compendial test method USP <60> for BCC recovery and detection [45]. Furthermore, this study provides a comprehensive data set that supports the USP inclusion of lower nutrient, resuscitative media as suggested previously [18, 20].

Acknowledgments

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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