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Comparison of methods proposed for monitoring cefotaxime-resistant *Escherichia coli* in the water environment

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ABSTRACT *Escherichia coli* is a promising subject for globally coordinated surveillance of antimicrobial resistance (AMR) in water environments due to its clinical relevance and widespread use as an indicator of fecal contamination. Cefotaxime-resistant *E. coli* was recently evaluated favorably for this purpose by the World Health Organization TriCycle Protocol, which specifies tryptone bile x-glucuronide (TBX) medium and incubation at 35°C. We assessed comparability with the U.S. Environmental Protection Agency-approved method for *E. coli* quantification, which uses membrane-thermotolerant *E. coli* (mTEC) agar and incubation at 44.5°C, in terms of recovery of *E. coli* and cefotaxime-resistant *E. coli* from wastewater influent and surface waters. Total *E. coli* concentrations in wastewater influent were 10^6–10^8 CFU/100 mL, while cefotaxime-resistant *E. coli* were ~100-fold lower. Total *E. coli* in surface waters were ~10^2 CFU/100 mL, and cefotaxime-resistant isolates were near the limit of detection (0.4 CFU/100 mL). Total and putative cefotaxime-resistant *E. coli* concentrations did not differ significantly between media or by incubation method; however, colonies isolated on mTEC were more frequently confirmed to species (97.1%) compared to those from TBX (92.5%). Incubation in a water bath at 44.5°C significantly decreased non-specific background growth and improved confirmation frequency on both media (97.4%) compared to incubation at 35°C (92.3%). This study helps to advance globally coordinated AMR in water environments and suggests that the TriCycle Protocol is adaptable to other standard methods that may be required in different locales, while also offering a means to improve specificity by decreasing the frequency of false-positive identification of cefotaxime-resistant *E. coli* by modifying incubation conditions.

IMPORTANCE As antibiotic-resistant bacteria in water environments are increasingly recognized as contributors to the global antibiotic resistance crisis, the need for a monitoring subject that captures antibiotic resistance trends on a global scale increases. The World Health Organization TriCycle Protocol proposes the use of cefotaxime-resistant *Escherichia coli* isolated on tryptone bile x-glucuronide agar. The U.S. Environmental Protection Agency (USEPA) criteria for safe recreational waters also use *E. coli* as an indicator but specify the use of mTEC agar at a higher incubation temperature (44.5°C vs 35°C). We assessed the comparability of these methods for isolating total and cefotaxime-resistant *E. coli*, finding overall good agreement and performance, but significantly higher specificity toward *E. coli* selection with the use of the USEPA incubation protocol and mTEC agar. This study is the first to directly compare these methods and provides evidence that the methods may be used interchangeably for global surveillance of antibiotic resistance in the environment.

KEYWORDS ESBL *E. coli*, environmental AMR, media comparison, performance, specificity, surveillance

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Infections caused by drug-resistant bacteria and fungi contribute to an estimated 5 million deaths globally every year (1). A growing body of evidence indicates that built and natural water environments are important reservoirs of antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARG) (2). Many microbial analytes and protocols have been used to quantify antibiotic resistance in the water environment (3–5). A standardized method for monitoring antimicrobial resistance (AMR) in the environment that is utilized worldwide would greatly facilitate the production of data that are comparable across spatial and temporal spans (6). As the leading cause of death associated with ARB, *Escherichia coli* is highly relevant to human health (1). Its strong potential as a global monitoring target is also related to its persistence in environments such as water and sediments (7, 8) and current monitoring programs as an indicator of fecal contamination in surface waters and treated wastewater (9, 10).

Monitoring cefotaxime-resistant *E. coli* has been identified as an advantageous AMR surveillance target that is relevant across animal, environmental, and human sectors, and the World Health Organization (WHO) has correspondingly developed the "Global Tricycle Surveillance of extended-spectrum beta-lactamase (ESBL) *E. coli* Protocol" (10). Cefotaxime-resistant *E. coli* is a clinically important ARB and is classified as a serious AMR threat by the U.S. Centers for Disease Control and Prevention (11). Cefotaxime is a broad-spectrum, third-generation cephalosporin belonging to the beta-lactam group of antibiotics. Resistance to cefotaxime is typically due to the production of ESBL enzymes which are encoded by ARG such as *blaCTX-M* (12–15). Many ESBL-producing bacteria are also resistant to other antibiotic classes, particularly aminoglycosides, trimethoprim, and quinolones (16, 17). ESBL-producing *E. coli* is resistant to most beta-lactam antibiotics, including penicillins and first- and second-generation cephalosporins, resulting in infections that must be treated by antibiotics of last resort, such as carbapenems (11, 13, 15).

A challenge to global implementation of the WHO Tricycle Protocol is the existence of multiple standard methods for *E. coli* quantification, some of which require regulatory methods by their respective governing bodies. The Tricycle Protocol employs tryptone bile x-glucuronide (TBX) agar amended with 4 µg/mL cefotaxime and growth in a 35°C incubator for 22–24 hours (10). However, the U.S. Environmental Protection Agency (USEPA) utilizes membrane-thermotolerant *E. coli* (mTEC) agar and an incubation method that allows resuscitation of stressed bacteria in a 35°C incubator for 2 hours before transferring plates to a 44.5°C water bath for 20–22 hours to select for thermotolerant *E. coli* (9). Modified mTEC differs from the original formula in that the chromogen 5-bromo-6-chloro-3-indolyl-β-D-glucuronide is included to better distinguish *E. coli* from non-target species (18).

Here, we compare the methodology of the WHO TriCycle Protocol (10) for surveillance of cefotaxime-resistant *E. coli* to an adaptation of USEPA Method 1603 (9) with cefotaxime added in terms of the number of presumptive *E. coli* recovered from wastewater influent and surface waters (sensitivity) as well as the confirmation frequency of colonies as *E. coli* (specificity, i.e., the rate of false-positive observations). We crossed media and incubation conditions from the TriCycle protocol (TBX agar using the "incubator method": 35°C incubator for 22–24 hours) and USEPA Method 1603 (mTEC agar using the "water bath method": 35°C incubator for 2 hours followed by 44.5°C water bath for 20–22 hours) on media with and without 4 µg/mL cefotaxime. The findings facilitate the global adoption of cefotaxime-resistant *E. coli* for AMR monitoring in water environments.

MATERIALS AND METHODS

Media preparation

TBX and mTEC were prepared according to the manufacturer's instructions. Cefotaxime sodium salt (Fisher Sci Cat No. AC454950010) was dissolved in nuclease-free water to create a stock solution containing 10 mg/mL cefotaxime and sterilized by passage through a 0.2 µm syringe filter. Cefotaxime was added to media tempered to 50°C

to obtain antibiotic-amended media containing 4 µg/mL cefotaxime. Media with or without cefotaxime were poured into 50 mm plates and allowed to cool on a bench, shielded from light, for 2–4 hours and transferred to a refrigerator for use within 1 week. Antibiotic-sensitive *E. coli* ATCC 25922 and cefotaxime-resistant control ATCC BAA-199 were used as controls for media with and without antibiotic supplementation. The cefotaxime-sensitive strain never grew on plates amended with cefotaxime, while the cefotaxime-resistant strain always grew. All media batches produced colonies of the appropriate morphology from both control strains. A media blank was used throughout for each condition.

Sample collection

Two replicate wastewater influent samples (hereafter referred to as "influent") were collected from each of the four different water treatment facilities that produce wastewater across the United States (Georgia, Nevada, California, and Virginia). Samples were collected by utility partners and were shipped on ice and processed within 30 hours of collection. Two surface water samples were collected from each of three locations in the Tampa Bay area, designated as FL-1, FL-2, and FL-3 one from a brackish estuary, Ben T. Davis (BTD; 27.9700394, –82.5794991) and two from freshwater rivers, Bullfrog Creek (BFC; 27.7925882, –82.3519616) and Hillsborough River (HR; 28.0716617,–82.3778620).

Sample processing

Influent samples were concentrated by membrane filtration onto a 47 mm filter (0.45 µm pore size) by filtering 1 mL at three dilutions in phosphate-buffered saline (10^{-2}, 10^{-3}, or 10^{-4} for total *E. coli* and 10^{-1}, 10^{-2}, or 10^{-3} for cefotaxime-resistant *E. coli*). Filter funnels were rinsed with 20–30 mL sterile buffered water immediately following filtration. Four technical replicate membranes were counted for each medium (TBX, TBX +4 µg/mL cefotaxime, mTEC, mTEC +4 µg/mL cefotaxime). The limit of detection was calculated by taking the fewest number of colonies observable (1) and dividing it by the largest volume filtered to obtain the lower limit in CFU/mL. This number was then multiplied by 100 to obtain the lower limit of detection as CFU/100 mL. The lower limit of detection for total *E. coli* cultured from influent was 104 CFU/100 mL [(1/0.01) × 100], and it was 103 CFU/100 mL [(1/0.1) × 100] for cefotaxime-resistant *E. coli* in influent. Surface water samples were concentrated by membrane filtration by filtering three volumes (1, 10, or 100 mL for total *E. coli* and 10, 100, or 250 mL for cefotaxime-resistant *E. coli*) in four technical replicates for each media. The lower limit of detection for total *E. coli* cultured from surface water was 1 CFU/100 mL [(1/100) × 100], and it was 0.4 CFU/100 mL [(1/250) × 100] for cefotaxime-resistant *E. coli* in surface water. Two technical replicates for each dilution on each media were incubated using the water bath method: resuscitation in a 35°C ± 0.5°C incubator for 2 hours and then transferred and fully submerged in a 44.5°C ± 0.2°C water bath for 20–22 hours (9). Plates that were submerged in a water bath were first placed inside a gallon Ziploc bag in stacks of 3–5 plates and as much air as possible was removed before sealing the bag. The Ziploc bag was then placed into a secondary, larger plastic bag, which was sealed and submerged. Weighted rings were used to hold the bag under water, and the opening of the secondary bag was taped to the outside of the water bath to prevent water from entering the bag. The remaining two technical replicates for each dilution on each media were incubated using the incubator method: growth in a 35°C ± 0.5°C incubator for 22–24 hours (10).

Isolation and confirmation

Whenever possible, 20 typical colonies (purple colonies on mTEC and blue colonies on TBX) from each condition for each site were isolated and confirmed to species by a SYBR Green quantitative PCR (qPCR) assay using the *uidA* gene, adapted from references (6, 19, 19). All colonies were tested for *uidA* when fewer than 20 were available for a given sample, which only occurred in the case of cefotaxime-resistant isolates from surface waters. Each isolated colony was picked off of the medium using a sterile 0.2 mL pipette

tip and was resuspended in 50 µL of nuclease-free water, vortexed at high speed for 1 minute, and heated at 95°C for 15 minutes to lyse cells for the template DNA. The primers used were as follows: uidA405f 5′-CAACGAACTGAACTGGCAGA-3′ and uidA405r 5′-CATT ACGCTGCGATGGAT-3′ (6). The reaction mixture consisted of 12.5 µL SYBR Green qPCR Master Mix, 1 µL of each primer at 10 µM, 5.5 µL nuclease-free water, and 5 µL template DNA. Conditions for qPCR on the Bio-Rad CFX96 thermocycler were: 50°C for 2 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, and a final step of 95°C for 10 minutes. *E. coli* ATCC 25922 was used as a positive control, and nuclease-free water was used as a no-template control. Both controls were used in each assay run; the positive control was always amplified, while the no template control was never amplified. The results were analyzed in binary format, without a standard curve. Figure 1 depicts a typical melt curve; the typical C_T value for a confirmed result was between 18 and 24, and isolates with C_T values greater than 30 were considered false positives.

Statistical analysis

E. coli concentrations were calculated as CFU/100 mL and $\log_{10}$-transformed for statistical analyses and data visualization. When *E. coli* was not detected in assays targeting cefotaxime-resistant *E. coli* in surface water samples, the value was entered at half the limit of detection for statistical purposes. No other assay/sample type combinations yielded observations of CFU below the limit of detection. Statistical significance was designated at $P < 0.05$ for all tests. Differences in *E. coli* concentrations by medium (mTEC vs TBX) were tested by pooling data regardless of incubation conditions and antibiotic presence ($n = 14$). Differences in *E. coli* concentrations by incubation method (incubator vs water bath) were tested by pooling data regardless of media and antibiotic presence ($n = 14$). The non-parametric Wilcoxon signed-rank test and Kruskal-Wallis one-way analysis of variance (ANOVA) were used to analyze the non-normally distributed data. The specificity of methods (confirmation frequency) was calculated as 1-the frequency of false-positive observations and was compared by testing differences in confirmation frequency by χ^2 analysis. The influence of the incubation method and media within each sample type (influent and surface water) on the frequency of confirmation was measured using Fisher's Exact test. Statistical analyses were performed using RStudio 4.2.2 base commands. Fig. 2 and 3 were generated in RStudio 4.2.2 using the ggplot2 package.

RESULTS

Effect of medium and culture method on *E. coli* concentrations

E. coli concentrations with and without cefotaxime were compared on mTEC and TBX agar under each incubation method. Neither growth medium (TBX vs mTEC) nor

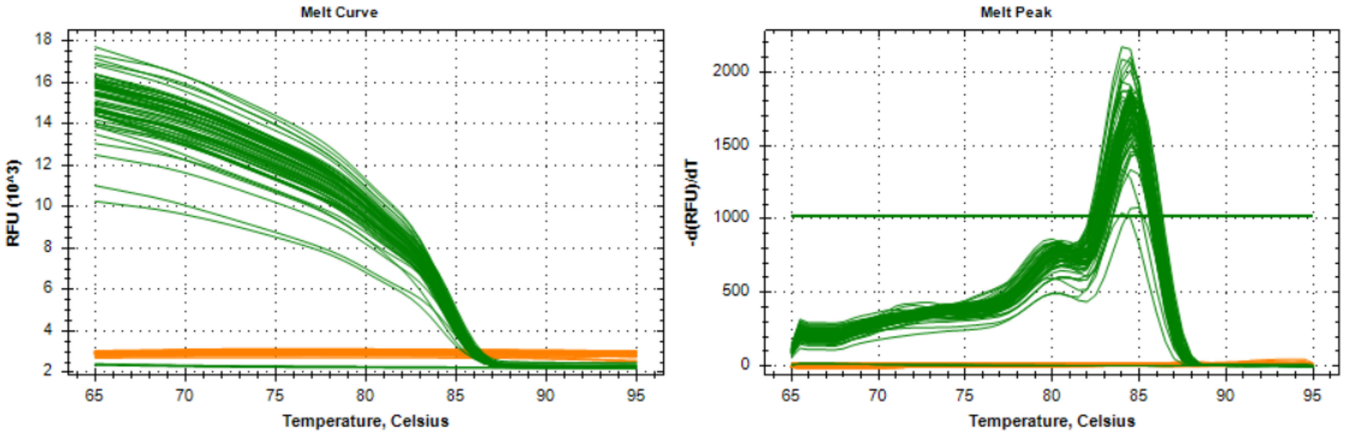

FIG 1 Representative melt curve analysis of *uid*A SYBR Green PCR assay used to confirm isolates as *E. coli*.

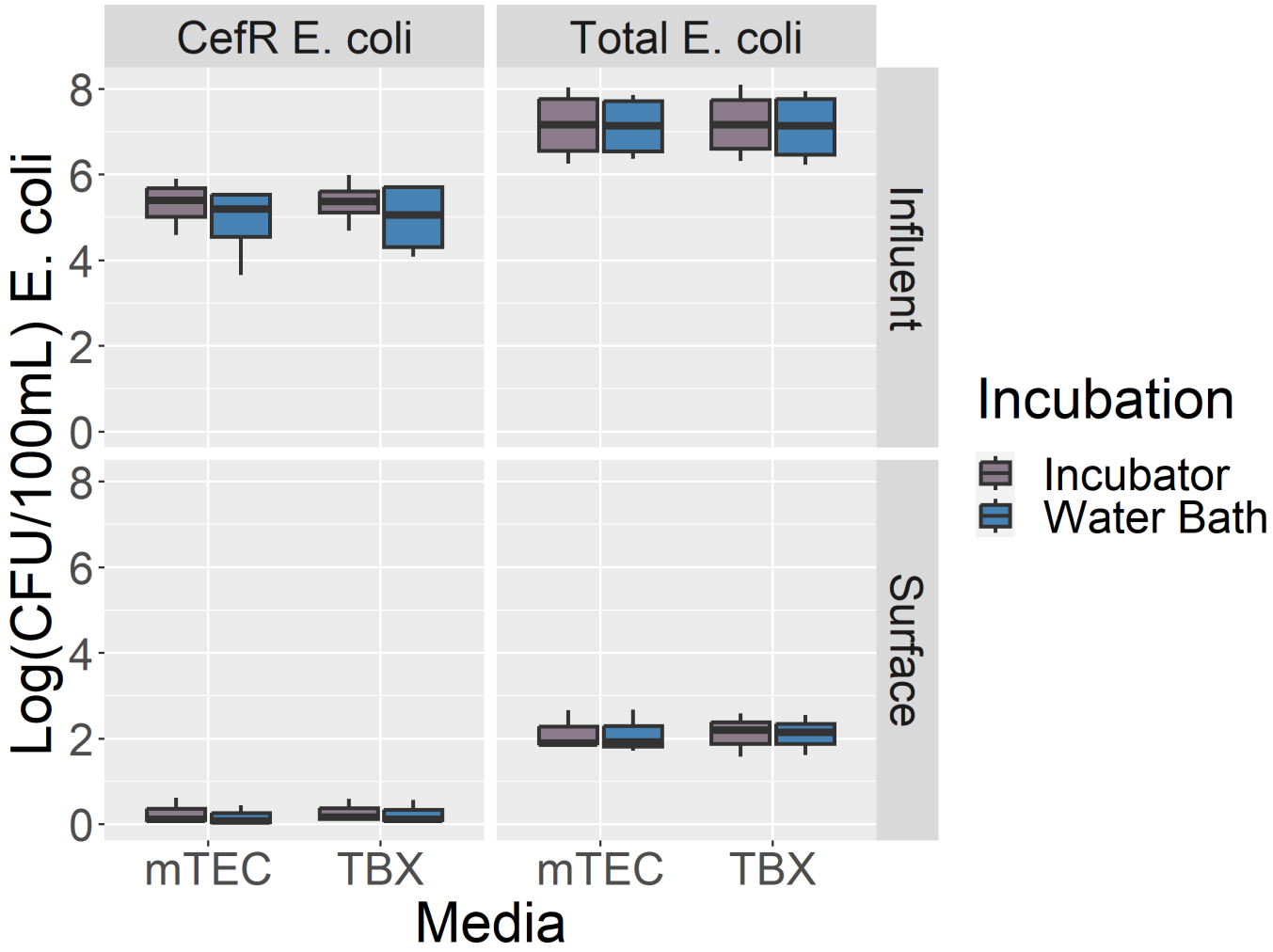

FIG 2 Comparison of media type (mTEC vs TBX) and incubation method for cefotaxime-resistant (CefR; left) and total (right) *E. coli* concentrations (log_{10} CFU/100 mL) measured in wastewater influent (top) and surface water (bottom). Data obtained using the incubator method are shown in purple, and incubation using the two-step water bath method is shown in blue.

incubation method (incubator vs water bath) significantly affected total or cefotaxime-resistant *E. coli* concentrations (Fig. 2; Table 1). Total *E. coli* concentrations ranged from 6.2 to 8.1 log_{10} CFU/100 mL in influent and 1.6 to 2.7 log_{10} CFU/100 mL in surface water (Fig. 2). Cefotaxime-resistant *E. coli* comprised 0.2%–3.6% of the *E. coli* population in influent and 0.1%–1.0% of the *E. coli* population in surface water, with concentrations ranging from 3.7 to 6.0 log_{10} CFU/100 mL in influent and below the limit of detection to 0.6 log_{10} CFU/100 mL in surface water (Fig. 2; Table 2). Cefotaxime-resistant *E. coli* was not detected in the HR site on TBX (incubator or water bath method) or on mTEC (incubator method only; Table 2) but was detected under all other conditions at all sites, albeit at much lower concentrations compared to influent samples (Fig. 2). Putative cefotaxime-resistant *E. coli* colonies isolated from surface water samples ranged from 0 (HR) to 4 (BTD; Fig. 3).

Confirmation frequency

E. coli isolated from media with (cefotaxime-resistant *E. coli*) and without (total *E. coli*) cefotaxime was pooled into one group for an overarching analysis of the effect of media and incubation protocol on confirmation frequency (Table 1). Confirmation frequency of colonies isolated from influent (20 colonies × 4 influent sites × 2 incubation methods ×

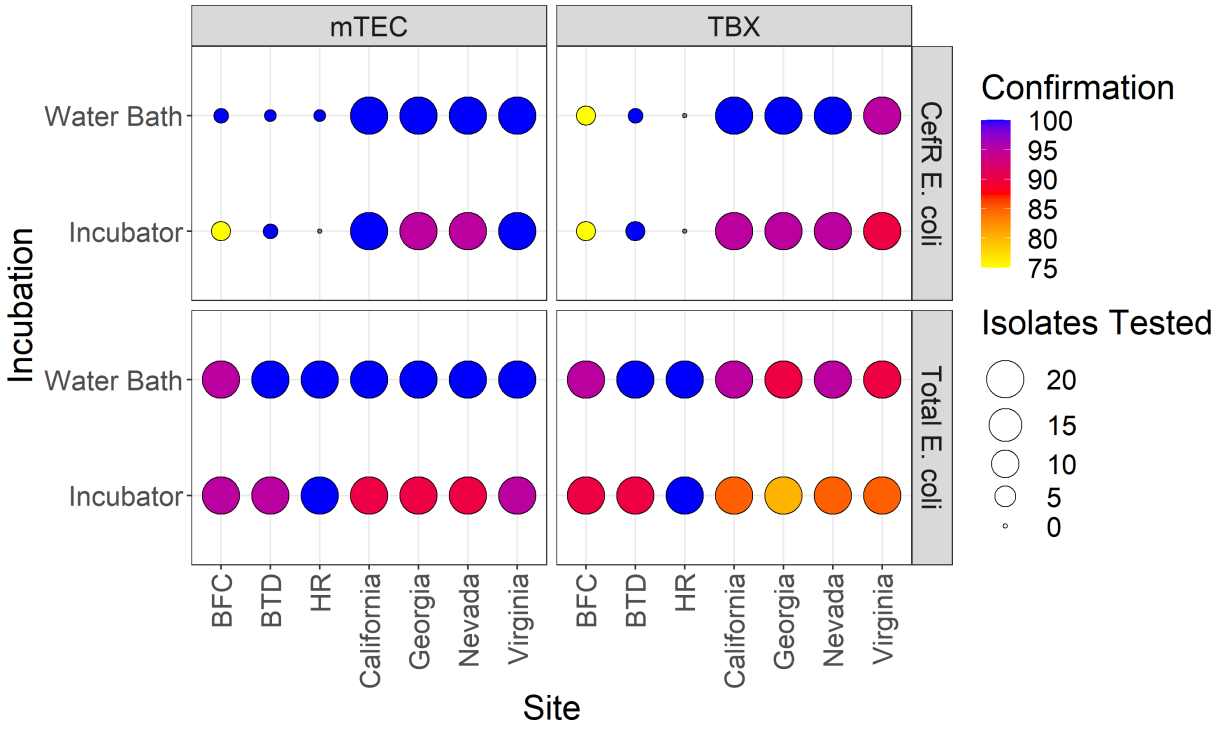

FIG 3 Frequency of confirmation of colonies as *E. coli* (% *uidA*-positive). Data are shown by medium [mTEC (left) vs TBX (right)] with (top) or without (bottom) antibiotics and cultured following the two-step water bath or incubator protocol. BFC, BTD, and HR represent surface water sites; Georgia, Virginia, California, and Nevada represent wastewater influent sites. Cefotaxime-resistant *E. coli* at HR was below the limit of detection on mTEC agar (incubator method) and TBX (incubator and water bath method).

2 media × 2 antibiotic conditions = 640 colonies) was significantly higher (P = 0.0019) when the water bath incubation method was utilized (97.8%) compared to the incubator method (91.9%), and the comparison of media also resulted in a significantly higher (P = 0.001) confirmation frequency of influent isolates on mTEC (96.9%) vs TBX agar (91.6%; Fig. 3; Table 1). In both comparisons, each group contained an equal number (n = 320) of colonies. However, the incubation method did not significantly affect the confirmation frequency of the 66 cefotaxime-resistant *E. coli* colonies isolated from surface water (97.0% in water bath vs 93.2% in an incubator) nor did the medium (96.9% for mTEC vs 93.3% for TBX), although the trends followed that of influent data. Figure 4 illustrates the crowding and overgrowth of atypical colonies frequently observed on mTEC and TBX agar inoculated with influent and incubated at 35°C in a conventional incubator.

Confirmation frequency for cefotaxime-resistant and total *E. coli* colonies was also analyzed in separate pools by medium and incubation protocol, with data for influent and surface water isolates combined (Table 3). Total *E. coli* confirmation frequency was significantly different by method (P = 0.0009; Tables 1 and 3), i.e., the mTEC-water bath protocol provided the highest confirmation frequency (99.3%), while the TBX-incubator protocol provided the lowest (86.2%). In contrast, confirmation frequencies of cefotaxime-resistant *E. coli* were not significantly different by method, although the trend was similar to the total *E. coli* data. Confirmation frequency was highest for the mTEC-water bath combination (100%) and lowest for the TBX-incubator protocol (92.6%).

DISCUSSION

The Tricycle Method evaluated in this manuscript is intended for use in all sectors in global surveillance of cefotaxime-resistant *E. coli*, whereas USEPA Method 1603 is utilized in the U.S. for recreational water quality and wastewater monitoring (environmental sector). The specificity of USEPA Method 1603 toward *E coli* lends itself to applications

TABLE 1 *P*-values for statistical tests[b]

Metric	Comparison	Data disposition[a]	Test	*P*-value[c] and direction
Concentration	mTEC (*n* = 14) vs TBX (*n* = 14)	Total and cefR pooled; incubator and water bath pooled; surface and influent pooled	Wilcoxon rank sum	0.95
Concentration	Incubator (*n* = 14) vs water bath (*n* = 14)	Total and cefR pooled; mTEC and TBX pooled; surface and influent pooled		0.80
Concentration	CefR on mTEC: incubator (*n* = 14) vs water bath (*n* = 14)	Surface and influent pooled		0.036*
Concentration	CefR on TBX: incubator (*n* = 14) vs water bath (*n* = 14)	Surface and influent pooled		0.1422
Concentration	Total on mTEC: incubator (*n* = 14) vs water bath (*n* = 14)	Surface and influent pooled		0.688
Concentration	Total on TBX: incubator (*n* = 14) vs water bath (*n* = 14)	Surface and influent pooled		0.297
Concentration	CefR: mTEC incubator (*n* = 14) vs mTEC water bath (*n* = 14) vs TBX incubator (*n* = 14) vs TBX water bath (*n* = 14)	Surface and influent pooled	Kruskal-Wallis one-way ANOVA	0.918
Concentration	Total: mTEC incubator (*n* = 14) vs mTEC water bath (*n* = 14) vs TBX incubator (*n* = 14) vs TBX water bath (*n* = 14)	Surface and influent pooled		0.998
Confirmation frequency	TBX (*n* = 320) vs mTEC (*n* = 320), influent	Incubator and water bath pooled; total and cefR pooled	Fisher's exact	0.0019* TBX < mTEC
Confirmation frequency	Incubator (*n* = 320) vs water bath (*n* = 320), influent	mTEC and TBX pooled; total and cefR pooled		0.001* Incubator < water bath
Confirmation frequency	TBX vs mTEC (*n* = 130) and surface water (*n* = 134; total + cefR)	Total and cefR pooled; incubator and water bath pooled		0.26
Confirmation frequency	Incubator (*n* = 134) vs water bath (*n* = 130) and surface water	mTEC and TBX pooled; total and cefR pooled		0.26
Confirmation frequency	CefR *E. coli* (*n* = 344): mTEC incubator vs mTEC water bath vs TBX incubator vs TBX water bath	Surface and influent pooled	Multiple χ^2	0.28
Confirmation frequency	Total *E. coli* (*n* = 560): mTEC incubator vs mTEC water bath vs TBX incubator vs TBX water bath	Surface and influent pooled		0.0009*

[a]Measurements were pooled across conditions as shown in the "Data disposition" column.
[b]Media with no antibiotic amendment measured total *E. coli*. CefR, cefotaxime resistant. Data were pooled for some analyses; see the "Data disposition" column.
[c]Statistical significance ($P < 0.05$) is designated with an asterisk (*).

in other sectors, including the isolation of antibiotic-resistant *E. coli* from water and wastewater. Increasing comparability among research and surveillance efforts is an identified need for analysis of the spread of AMR worldwide (6). This is the first study to compare both media and incubation protocol of proposed methods for monitoring total and cefotaxime-resistant *E. coli* in the water environment, focusing on method specificity as well as recovery of putative cefotaxime-resistant isolates from wastewater and surface water. This study provides a better understanding of the factors that limit the comparability of research and monitoring efforts that focus on AMR in the environment.

Cefotaxime-resistant *E. coli* in wastewater and surface water

Cefotaxime-resistant *E. coli* in influent and surface water was assessed in this study as an approximation of ESBL *E. coli*. ESBL genes (i.e., CTX-M-type β-lactamases) confer resistance to beta lactams, including cefotaxime; however, not all cefotaxime-resistant *E. coli* possess ESBL genes (20). Regardless of the methods used to isolate cefotaxime-resistant *E. coli* influent concentrations in this study were ~10^4–10^6 CFU/100 mL, and two orders of magnitude were below total *E. coli*. Cefotaxime-resistant *E. coli* was detected in both of the freshwater bodies (BFC and HR) and in the estuarine sample (BTD), although it was only detected on mTEC using the water bath protocol in the (HR) sample. Other U.S. studies have found similar resistance frequency. In surface water and reclaimed water from the mid-Atlantic, 1.4% of *E. coli* strains were cefotaxime resistant (21). A

TABLE 2 Median *E. coli* concentrations observed for each combination of media, incubation method, and antibiotic level

Sample type (*n*)	Media type	Cefotaxime amendment	Incubation method	Concentration range (log_{10} CFU/100 mL)	Frequency of detection
Influent (*n* = 8)	mTEC	4 mg/mL	Incubator (35°C)	4.59–5.9	100%
			Water bath (44.5°C[a])	3.7–5.6	100%
		None	Incubator (35°C)	6.3–8.0	100%
			Water bath (44.5°C)	6.4–7.9	100%
	TBX	4 mg/mL	Incubator (35°C)	4.7–6.0	100%
			Water bath (44.5°C)	4.1–5.7	100%
		None	Incubator (35°C)	6.3–8.1	100%
			Water bath (44.5°C)	6.2–8.0	100%
Surface water (*n* = 6)	mTEC	4 mg/mL	Incubator (35°C)	0.1–0.6	66.6%
			Water bath (44.5°C)	0.03–0.5	100%
		None	Incubator (35°C)	1.9–2.7	100%
			Water bath (44.5°C)	1.7–2.7	100%
	TBX	4 mg/mL	Incubator (35°C)	0.1–0.6	66.6%
			Water bath (44.5°C)	0.1–0.6	66.6%
		None	Incubator (35°C)	1.6–2.6	100%
			Water bath (44.5°C)	1.6–2.6	100%

[a]The water bath incubation included resuscitation in a 35°C incubator for 2 hours and then transferred and fully submerged in a 44.5°C water bath for 20–22 hours.

lower percentage of *E. coli* isolates (0.4%) from primary clarifier effluent collected across seven geographically distinct wastewater treatment plants were cefotaxime-resistant (22). Cefotaxime resistance frequencies of 1% or less were also observed in the influent across a recent survey of six wastewater treatment plants in the United States (23).

Although we were able to test 20 colonies per treatment (media × incubation) for species confirmation and count at least 10 colonies per plate for each influent sample, and for total *E. coli* in all samples, the relatively low concentration of cefotaxime-resistant *E. coli* in surface water samples produced low colony counts (0–4) in spite of relatively large sample sizes (250 mL). Counting a small number of colonies to calculate concentrations and to assess confirmation frequency can introduce error and complicate statistical analysis. In many cases, we chose to pool surface water and influent data and use non-parametric statistical methods to avoid artifacts from small sample sizes and calculations made from plates with only 1–4 colonies. In cases where influent data showed significant differences between media and incubation conditions, but analysis of surface water results produced similar trends but no significant differences, it is apparent that low numbers of surface water colonies could have contributed to the failure to find significant differences. Future studies could employ larger plates and/or more pseudoreplicate plates to obtain more colonies on cefotaxime-amended media.

Comparison of method performance

We found that the incubation conditions impacted method performance more than the media, i.e., water bath incubation significantly lowered the rate of false-positive observations of *E. coli* on mTEC and TBX. The first use of the two-step water bath incubation method was in the initial evaluation of mTEC performance for isolating *E. coli* in recreational waters by the USEPA. The water bath method was accepted for use in USEPA Method 1603 (9). The multi-laboratory validation of mTEC agar using the water bath incubation method found that the sensitivity was 94.1%–96.1% and the specificity was 94.0%–98.5% for samples from marine, freshwater, secondary wastewater, and disinfected wastewater (9, 18). TBX agar was evaluated in the literature using the incubator method, which reported the sensitivity and specificity as 90% and 89.1%, respectively (24). The effect of the incubation method on TBX agar performance has not previously been systematically studied; however, one study modified TBX agar and the incubation protocol with a two-step incubation method (25). TBX agar was overlaid with a non-specific minerals-modified glutamate agar to improve media sensitivity and

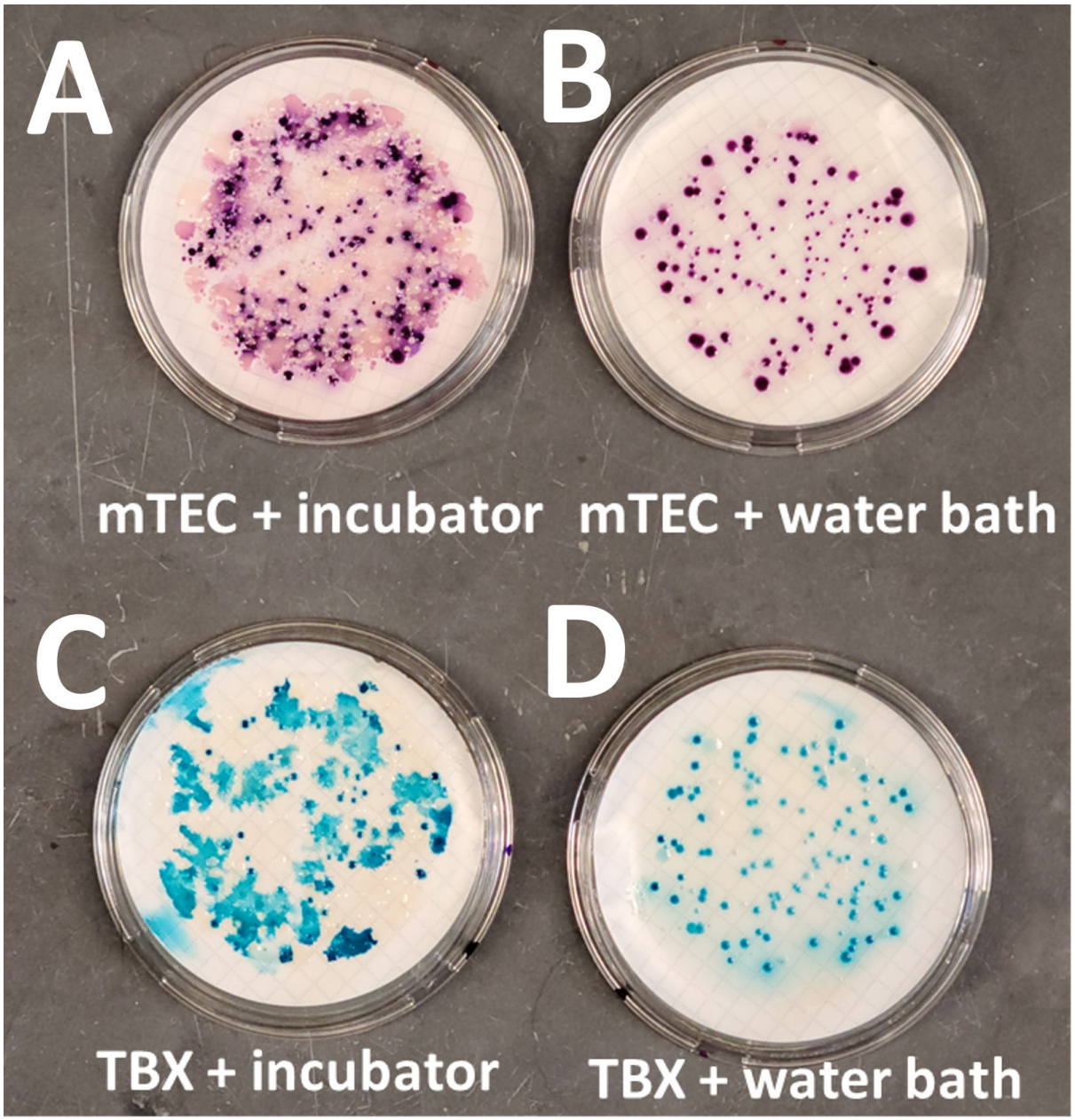

FIG 4 Appearance of colonies isolated from 0.001 mL wastewater on mTEC (A and B) and TBX (C and D) plates without cefotaxime using the incubator (A and C) and water bath (B and D) incubation methods. Typical colonies on mTEC are a deep purple color and typical colonies on TBX are a teal blue color. Crowding and overgrowth on plates that is typically indicative of non-target organisms (beige/white and lavender colonies) was observed when incubation was carried out using the incubator method.

incubated at 36°C for 6 hours followed by 44.5°C for 18 hours in a conventional incubator (25). The frequency of false-positive observations was 2.4% in coastal water and 3.0% in inland water, which is comparable to the 3.1% false-positive rate observed in this study for TBX using the water bath incubation method (frequency of confirmation ~97%).

Both media performed well under the water bath incubation; however, mTEC agar consistently performed better than TBX agar in all conditions. The confirmation frequency for mTEC agar with all colonies pooled was significantly higher than that of TBX agar (P = 0.0019). Under all conditions, colonies from mTEC were more frequently confirmed than colonies from TBX. The largest difference in confirmation frequency between the two media was observed for total *E. coli* using the incubator method

TABLE 3 Frequency of confirmation (%) of isolates confirmed as *E. coli* for each media/incubation treatment with data from influent and surface water pooled[a]

Resistance status	Protocol	Percent confirmed (*n*)
Total	mTEC-incubator	93.1% (140)
Total	mTEC-water bath	99.3% (140)
Total	TBX-incubator	86.2% (140)
Total	TBX-water bath	94.7% (140)
Cefotaxime resistant	mTEC-incubator	96.4% (86)
Cefotaxime resistant	mTEC-water bath	100% (86)
Cefotaxime resistant	TBX-incubator	92.6 (86)
Cefotaxime resistant	TBX-water bath	95.2% (86)

[a]Confirmation was completed using SYBR green qPCR and melt curve analysis.

(difference of 6.9%), followed by cefotaxime-resistant *E. coli* using the water bath method (difference of 4.8%), total *E. coli* using the water bath method (difference of 4.6%), and cefotaxime-resistant *E. coli* using the incubator method (difference of 3.8%). Amending the media with antibiotics added an additional layer of selection that resulted in less background growth, but confirmation frequencies were still lower when incubated using the incubator method (96.4% for mTEC and 92.6% for TBX) vs the water bath method (100% for mTEC and 95.2% for TBX). All confirmation frequencies were >90% with the exception of total *E. coli* using the incubator method, which had a confirmation frequency of 86.2%. The water bath method brought the confirmation frequency of total *E. coli* on TBX to comparable, albeit still lower (difference of 4.6%), levels compared to mTEC.

The water bath method consists of three components that we believe influence the media performance: the two-hour incubation at 35°C (±0.5°C), the 20–22 hour incubation at 44.5°C (±0.2°C), and the use of the water bath for the second phase of incubation. The two-hour, 35°C incubation step was added to the USEPA protocol (Method 1603) to encourage the growth of stressed *E. coli* cells (9). This would be a particularly important step when isolating from high-stress environments such as post-disinfection steps in the wastewater treatment process. The higher incubation temperature (44.5°C) targets thermotolerant *E. coli* and reduces background growth. Incubation in a water bath improves temperature stability compared to a conventional incubator, which is required for the narrow incubation range of 44.5°C ± 0.2°C (26). These factors together bring the sensitivity and specificity of both media to more comparable levels.

Effect of cefotaxime on confirmation frequency

Although at least 75% of typical *E. coli* colonies on mTEC and TBX were confirmed under all conditions where colonies were isolated, significant differences among the media were observed for confirmation frequency of total *E. coli* (no antibiotics added) but not for cefotaxime-resistant *E. coli*. The water bath incubation protocol consistently produced the highest confirmation frequency of total *E. coli*, and differences were significant when influent isolates were analyzed alone, or when influent and surface water isolates were pooled. The observed difference in method specificity attributable to incubation protocol was due to the nonspecific growth of species other than *E. coli* on plates incubated at 35°C in a conventional incubator. Crowding of plates by white/beige and lavender non-target organisms over the top of the filter prevented accurate picking of isolated colonies. Several additional subcultures were needed to produce pure cultures from colonies on plates processed by the incubator protocol. The addition of cefotaxime to the medium reduced the background growth and associated issues.

Benefits and drawbacks of each method

The prospective methods for standardized monitoring of cefotaxime-resistant *E. coli* in the water environment tested in this study possess benefits and drawbacks. The method

for cefotaxime-resistant *E. coli* proposed in this study was developed for compatibility with wastewater utility implementation and recreational water quality monitoring in the United States and thus utilized mTEC agar and USEPA Method 1603 for *E. coli* (9). Many agencies in the United States already use mTEC agar for *E. coli* monitoring in treater wastewater, treated recycled water, discharge testing, and surface water, making it a readily available option for these agencies. However, mTEC agar is quite costly, at >$2000 per 500 g in 2022. This cost is over three times that of TBX agar, as recommended by the WHO Tricycle Protocol (10). USEPA Method 1603 also requires an incubator and a water bath, while the Tricycle Protocol requires only an incubator. The high cost of mTEC and the need for additional equipment could affect affordability in some countries and hinder efforts to implement monitoring on a global basis.

We found that utilizing the water bath incubation method is a beneficial step for increasing the comparability of studies that use TBX or mTEC agar to isolate *E. coli* from influent and aquatic environments. A One Health approach to AMR monitoring and mitigation emphasizes the roles of interconnected sectors (human, animal, and environmental) in emergence and dissemination of ARB and ARG. The environmental dimension of One Health has been recognized as one that merits coordinated surveillance efforts (4, 27, 28). These findings will increase accessibility to comparable methods and therefore will facilitate efforts toward global surveillance of antibiotic-resistant *E. coli*.

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Jeanette Calarco, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review and editing | Amy Pruden, Conceptualization, Funding acquisition, Project administration, Writing – review and editing | Valerie J. Harwood, Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review and editing

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