Comparison of the Extended-Spectrum Beta-Lactamase (ESBL) *E. coli* Compartment Bag Test Method to the World Health Organization Tricycle Protocol in North Carolina Surface Waters

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Abstract

E. coli **and total coliforms are the most widely used indicator organisms for microbial monitoring of drinking water and recreational freshwater. In many remote and low-resource settings, however, conventional laboratory methods for quantifying these indicators are challenging or infeasible to perform due to limited access to laboratory facilities. The availability of rapid, low-cost methods for quantifying indicator organisms in freshwater samples without the need for laboratory facilities is crucial to facilitate the rapid and robust monitoring of microbial water quality in these types of settings. The global misuse and abuse of antimicrobials have contributed to the rise of antimicrobial resistance. Thus, simple culture methods are needed to detect indicators of such bacteria in freshwaters. In 2021, the World Health Organization released the Tricycle protocol to address this issue by providing guidance for culture-based detection of extended-spectrum beta-lactamase (ESBL)-producing *E. coli* in environmental samples.**

Our research goal was to compare the 100-ml sample volume ESBL *E. coli* quantal and enumerative commercial tests against the more complex Tricycle protocol to detect and quantify ESBL *E. coli* in surface waters. Both commercial tests gave results comparable with the results obtained using the Tricycle protocol, and the quantal and enumerative commercial tests were easier and faster to perform than the Tricycle protocol.

Keywords: *E. coli*, antibiotic resistance, extended-spectrum beta-lactamase, ESBL, field test methods, surface water

Introduction

E. coli and total coliforms are the most widely used indicator organisms for microbial monitoring of drinking water and recreational freshwater. In many remote and low-resource settings, however, conventional laboratory methods for quantifying these bacterial indicators are challenging or infeasible to perform due to the absence of timely access to laboratory facilities within allowed holding times and temperatures (Organisation for Economic Co-operation and Development, 2019; Sargeant et al., 2019). The availability of rapid, low-cost commercial methods for quantifying these indicator organisms in freshwater samples without the need for laboratory facilities provides rapid and convenient monitoring of microbial water quality in such settings.

The compartment bag test (CBT) is one such method that has been validated in the field in a variety of settings as a simple, portable, low-cost, semiquantitative procedure for quantifying *E. coli* in drinking water and surface water samples using ambient temperature incubation (Gronewold et al., 2017; Stauber et al., 2014; Wang et al., 2017). A newer version of this simple culture test for *E. coli* in a plastic bag uses a gel medium to detect and quantify *E. coli* and total coliforms as colonies. Once mixed with a water sample, the gel medium hardens in a short time and *E. coli* colonies then develop and are counted after an overnight incubation.

The increasing global misuse and abuse of antimicrobials in clinical, veterinary, and agricultural settings have contributed to the rise of antimicrobial resistance, which is a stated One Health global concern (World Health Organization [WHO], 2016). This rise created an urgent need to develop and use harmonized culture methods to detect and quantify an *E. coli* indicator of antimicrobial resistance for all settings. In 2021, the World Health Organization (WHO, 2021) released the Tricycle protocol to address this issue by providing guidance on the culture-based detection of extended-spectrum beta-lactamase (ESBL) *E. coli* in environmental, clinical, and animal agriculture samples. To better address the need to detect and quantify ESBL *E. coli* in environmental waters, new versions of these simple commercial tests included the same beta-lactam antibiotic that is used in the Tricycle protocol.

The goal of our research was to compare the two commercial tests—the 100-mL sample volume ESBL *E. coli* MPN (most probable number) CBT test and the GEL ESBL CFU test—to gauge the results against the WHO Tricycle protocol. To our knowledge, this study is the first to compare these methods using field samples of environmental surface waters.

Methods

Sample Collection

A total of 100 samples were collected from May–September 2023 from a variety of surface water sources in central North Carolina. Sample sites included two reservoirs, two lakes (Sunset and Jordan), and three rivers (Cape Fear, Neuse, and Eno). Grab samples of surface waters were collected using sterile polypropylene bottles. Samples were transported and stored on ice at 4 °C and analyzed within 24–48 hr after collection.

Sample Processing and Data Analysis

To address anticipated low concentrations of ESBL *E. coli*, surface water samples were mixed well and analyzed as 100-ml volumes without dilution by all three test methods. Membrane filtration was performed by filtering samples through 0.45-µm cellulose nitrate filters followed by incubation at 44 °C for 24 hr on 100-mm diameter Tryptone Bile X-glucuronide (TBX) agar plates with or without 4 mg/L added cefotaxime (CTX) per the Tricycle protocol (WHO, 2021). We tested duplicate plates for each water sample (n = 200).

Parallel 100-ml samples were analyzed using the ESBL CBT for MPN concentrations/100 ml and the GEL ESBL *E. coli* colony test for CFU concentrations/100 ml. All CBT and GEL samples were incubated at 35 °C for 24 hr. If a CBT compartment exhibited a blue-green color after incubation or if a GEL bag had a blue-green colony, that compartment or colony was counted as positive.

Positive and negative control plates (for membrane filtration method) and bags (for CBT and GEL methods) were tested one time per week. A positive control ESBL *E. coli*, a non-ESBL-negative control bacteria, and a negative dilution control (phosphate buffered saline [PBS]) were used for each set of experiments. Additionally, at the beginning of our experiment, all positive control bacteria were compared in a clean matrix (PBS) using each of the methods (Appling et al., 2023) to determine if the methods were comparable to no outside water interactions. The limit of detection for each method is described in the manufacturer's instructions (www.aquagenx.com/).

For the ESBL CBT method, the limit is estimated to be 0.0 MPN, with an upper 95% confidence limit of 2.87 MPN/100 ml. For the GEL bag test, the limit is estimated to be 1.0 CFU/100 ml.

For each method, a subset of presumptive ESBL-positive *E. coli* samples was isolated for further characterization. Overall, one to five presumptive ESBL *E. coli* colonies were selected from each membrane filtration plate, ESBL CBT, or GEL ESBL *E. coli* bag. The colonies were re-streaked for isolation on TBX medium with 4 mg/L CTX as an initial ESBL *E. coli* confirmation step.

Colonies were picked at random from these plates using a sterile loop. The exterior of positive compartments of CBTs were swabbed with 70% ethanol. Next, the compartments were pierced with a sterile syringe and needle, and a drop of medium was spotted onto TBX plates with CTX and streaked as described to obtain individual colonies after incubation. For GEL ESBL *E. coli* positives, the exterior of the GEL bag was swabbed with 70% ethanol, and a large-gauge sterile syringe was used to pierce the GEL medium of the bag. In some instances, it was necessary to use the sterile syringe in combination with a sterile isolation needle to spread the positive colony from the GEL bag.

Colonies isolated from re-streaked TBX plates were picked with a sterile loop, cultured initially on tryptic soy agar (TSA), cultured again overnight in tryptic soy broth (TSB), diluted 1:1 with sterile glycerol, and stored at -80 °C in sterile 2-ml cryovials. Stored isolates were thawed and further characterized by biochemical testing, specifically the indole test, according to the manufacturer's instructions. Isolates were further confirmed as ESBL by Kirby–Bauer susceptibility testing using the criteria defined in the Tricycle protocol (WHO, 2021) for CTX, ceftazidime (CAZ), CTX + clavulanic acid (CLA), and CAZ + CLA as paper discs.

The distributions of presumptive and confirmed ESBL *E. coli* CFU and MPN concentrations were characterized for each method (membrane filtration, ESBL CBT, and GEL ESBL). *E. coli* concentrations were subjected to Shapiro–Wilk normality tests, and the geometric mean, arithmetic standard deviation, and range (minimum and maximum) were calculated. A 0.5 minimum limit of detection was used to reduce bias for nondetects, such that a nondetect for a 100-ml undiluted surface water sample would be calculated as 0.5/100 ml—rather than 0/100 ml—to minimize bias and enable $\log_{10}$-transformation of count data where needed.

The confirmed proportion of ESBL *E. coli* was calculated as the ratio of confirmed ESBL *E. coli* isolates to total isolates tested, adjusted for the number of total isolates collected from each sample type. Differences in $\log_{10}$-transformed concentrations between each test method were evaluated using nonparametric methods. All analyses were conducted in GraphPad Prism version 10.

Results

Concentrations of ESBL *E. coli* in surface water samples were relatively low (<100 CFU or MPN per 100 ml) throughout the study period and the total percentage of presumptively resistant *E. coli* to nonresistant *E. coli* varied between 1.5% and 15.2%. Table 1 presents the occurrence of presumptively positive ESBL *E. coli* by assay method, and Figure 1 displays a box and whisker plot of the presumptively positive concentrations.

To further evaluate the three methods used to detect ESBL *E. coli*, a Friedman test was used because a normal distribution did not adequately represent this data set. At an α level of .05, there was no statistically significant difference between the median detected concentration of the Tricycle protocol membrane filtration method compared with the ESBL CBT (p = .57), the membrane filtration method compared with the GEL ESBL method (p > .99), and the ESBL CBT method compared with the GEL ESBL method (p > .99).

Isolate Analysis for ESBL *E. coli*

Analysis was performed on 306 presumptively positive ESBL *E. coli* isolates detected in the 100 surface water samples (Table 2). There were 117 ESBL *E. coli* isolates analyzed from the membrane filtration method, as well as 91 and 98 isolates analyzed from the ESBL CBT and GEL ESBL methods, respectively. The isolates were initially confirmed by streak plating on TBX agar medium containing CTX. Of the samples first identified as presumptive ESBL *E. coli*, 92.8% were confirmed on the CTX agar plates. By method, 94.0%, 96.7%, and 87.8% of isolates were confirmed using this technique from the membrane filtration, ESBL CBT, and GEL ESBL methods, respectively.

Next, isolates were confirmed as *E. coli* using an indole test. Overall, 85.6% of the

TABLE **1**

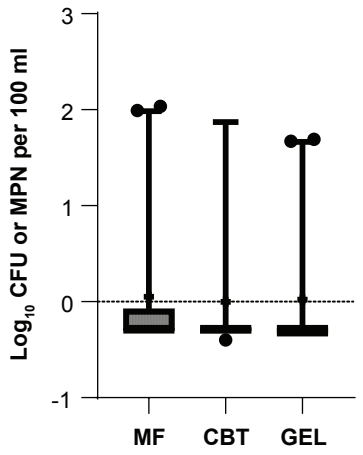
Occurrence of Presumptive Extended-Spectrum Beta-Lactamase (ESBL) *E. coli* by Assay Method

Metric	Measurement	Membrane Filtration (CFU)	ESBL CBT (MPN)	GEL ESBL (CFU)
Geometric mean	CFU or MPN/100 ml	1.12	1.00	1.05
SD	Log_{10} CFU or MPN/100 ml	0.69	0.64	0.69
Nondetect	%	62%	71%	70%
Minimum	Log_{10} CFU or MPN/100 ml	-0.30	-0.40	-0.30
Maximum	Log_{10} CFU or MPN/100 ml	2.03	1.87	1.69

Note. CBT = compartment bag test; CFU = colony forming unit; MPN = most probable number.

FIGURE **1**

Box and Whisker Plot of Presumptive Positive Extended-Spectrum Beta-Lactamase (ESBL) *E. coli* Concentrations



Note. Lines denote median log_{10}-transformed concentrations. Boxes denote first and third quartile log_{10} concentrations. Whiskers denote 95% confidence limits for log_{10} concentration values. Mean values are designated by the dashed line. CBT = ESBL compartment bag test; CFU = colony forming unit; GEL = GEL ESBL; MF = membrane filtration; MPN = most probable number.

isolates were confirmed using this method. Lastly, isolates were evaluated for ESBL positivity using the Tricycle protocol confirmation criteria. Using this technique, 64.4% of the isolates were found to be ESBL-resistant (scored as ESBL-positive). By method, 55.6% of the membrane filtration isolates were resistant, 76.9% of the ESBL CBT isolates were resistant, and 60.2% of the GEL ESBL isolates were resistant.

Discussion

The results of this study indicate that the three methods detect similar concentrations of ESBL *E. coli* in surface water. Based on a Freidman test analysis, there was no statistically significant difference between any of the methods in detecting median concentrations. This result is similar to other evaluations of the *E. coli* CBTs (Stauber et al., 2014; Wang et al., 2017) and initial evaluations of the ESBL CBT method (Appling et al., 2023).

In the surface water samples collected for our study, there were low concentrations of ESBL *E. coli*, ranging from nondetectable amounts to approximately 100 CFU or MPN per 100 ml. The rate of nondetects was between 62% and 71% of each sample set depending on the assay type (Table 1). Other studies conducted on surface waters also showed high numbers of nondetects for ESBL *E. coli* (Blaak et al., 2014), indicating that input into these waters—including wastewater, surface runoff, and animal waste—might not be consistent throughout the year and could be dependent on temperature, weather, and other factors.

In our confirmation analysis of the ESBL *E. coli* isolates, each of the three methods resulted in a similar number of positive isolates, specifically the membrane filtration method (n = 117), ESBL CBT (n = 91), and GEL ESBL (n = 98). Each isolate was then subjected to a series of confirmation tests including a secondary streak plating on ESBL antibiotic-impregnated TBX agar, an indole test, and then antibiotic resistance testing according to the Tricycle protocol. Each method was able to detect a similar percentage of positive ESBL-resistant *E. coli*, adjusted for the number of isolates analyzed.

Of the 117 isolates detected from the membrane filtration method, 65 (55.6%) were confirmed by the methods previously described and identified as ESBL-positive *E. coli*. For the ESBL CBT method, 70 of the 91 initial isolates identified (76.9%) were confirmed as ESBL *E. coli*. For the GEL ESBL method, 59 of the 98 isolates (60.2%) were confirmed as ESBL *E. coli*. These resistance percentages are higher than the percentages reported in the initial evaluation of the ESBL CBT (Appling et al., 2023); however, our results are similar to other evaluations of isolates in surface waters in North America (Haberecht et al., 2019).

Although not directly considered in the methods comparison presented in our evaluation, previous published work has compared CBT to standard laboratory methods, including the membrane filtration method considered here (Bain et al., 2012). When factoring in the cost of agar and petri dishes, the estimated cost of the standard CBT method is approximately the same amount per sample. The ease of use for the CBT, however, which allows a user to process the sample without the need for an incubator, specialized pipette, or other laboratory equipment, greatly reduces the cost of this test itself. For limited-resource settings, field tests such as the CBT or GEL method offer an opportunity to test for pathogens that might not be considered within standard monitoring practice due to a lack of available facilities or laboratory equipment.

The three methods we evaluated are comparable in terms of the detection of ESBL *E. coli* concentrations and the overall confirmation of ESBL *E. coli* isolates. There were several limitations. Specifically, while the ESBL CBT detects MPN concentrations and membrane filtration and GEL ESBL methods detect CFU concentrations, we compared

TABLE **2**

Results of Isolate Confirmation Testing by Assay Method

Method	CTX Streak Plating # (%)	Indole Positive # (%)	ESBL Positive # (%)
Membrane filtration (*n* = 117)	110 (94.0)	97 (82.9)	65 (55.6)
ESBL CBT (*n* = 91)	88 (96.7)	83 (91.2)	70 (76.9)
GEL ESBL (*n* = 98)	86 (87.8)	82 (83.7)	59 (60.2)
Total (*N* = 306)	287 (92.8)	262 (85.6)	197 (64.4)

Note. CBT = compartment bag test; CTX = cefotaxime; ESBL = extended-spectrum beta-lactamase.

the three methods directly for our evaluation. This method of direct comparison has been previously evaluated and although concentrations differ when microorganisms are detected by each method for a variety of reasons, when these methods are used on field samples or in field settings, the results appear to be equivalent (Eckner, 1998; Gronewold & Wolpert, 2008). As such, in our evaluation, microbial concentrations based on CFU and MPN units are treated as equivalent, as previously documented by Bailey et al. (2017).

The ESBL CBT and GEL ESBL methods are portable and easy to use and would be particularly applicable when used in field conditions. For the GEL ESBL method, however, there is a learning curve and the manufacturer's instructions are cumbersome to a new user. Therefore, for more consistent results, it could be helpful to provide additional visual aids for the use of this method.

These methodological limitations, in addition to the limited number of samples (*n* = 100), are important considerations when comparing the various experimental methods. Despite these limitations, the results from our evaluation of 306 presumptive ESBL *E. coli* isolates examined across the three methods would be comparable with results one would expect with testing using the Tricycle protocol. Additionally, although it was not a direct focus of our evaluation, the quality of the surface water included in our comparison of the three methods is a relevant variable that would be interesting to consider in future comparisons.

Conclusion

Our evaluation provides quantitative evidence that the three different culture methods we compared can detect statistically similar levels of ESBL *E. coli* in surface water samples. We found no statistically significant difference in the three methods for detecting ESBL *E. coli*. For the membrane filtration method, 55.6% of the presumptively positive isolates were confirmed as ESBL *E. coli*. For the ESBL CBT method, 76.9% of the presumptively positive isolates were confirmed as ESBL *E. coli*. Lastly, for the GEL ESBL method, 60.2% of the presumptively positive isolates were confirmed as ESBL *E. coli*. These field methods likely are suitable for field applications in settings with limited resources or infrastructure, as they gave comparable results to the standard method, which is not easily usable in the field because it requires additional materials and equipment.

Continued and widespread monitoring of ESBL *E. coli* in environmental waters is a useful monitoring and surveillance approach for antimicrobial resistance, and as such is recommended by WHO. Our research suggests the need, however, to further adapt and simplify the current Tricycle protocol to more easily and broadly detect ESBL *E. coli* in environmental waters by field testing.

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References

Appling, K.C., Sobsey, M.D., Durso, L.M., & Fisher, M.B. (2023). Environmental monitoring of antimicrobial resistant bacteria in North Carolina water and wastewater using the WHO Tricycle protocol in combination with membrane filtration and compartment bag test methods for detecting and quantifying ESBL *E. coli*. *PLOS Water*, *2*(9), e0000117. https://doi.org/10.1371/journal.pwat.0000117

Bailey, E.S., Price, M., Casanova, L.M., & Sobsey, M.D. (2017). *E. coli* CB390: An alternative *E. coli* host for simultaneous detection of somatic and F+ coliphage viruses in reclaimed and other waters. *Journal of Virological Methods*, *250*, 25–28. https://doi.org/10.1016/j.jviromet.2017.09.016

Bain, R., Bartram, J., Elliott, M., Matthews, R., McMahan, L., Tung, R., Chuang, P., & Gundry, S. (2012). A summary catalogue of microbial drinking water tests for low and medium resource settings. *International Journal of Environmental Research and Public Health*, *9*(5), 1609–1625. https://doi.org/10.3390/ijerph9051609

Blaak, H., de Kruijf, P., Hamidjaja, R.A., van Hoek, A.H.A.M., de Roda Husman, A.M., & Schets, F.M. (2014). Prevalence and characteristics of ESBL-producing *E. coli* in Dutch recreational waters influenced by wastewater treatment plants. *Veterinary Microbiology*, *171*(3–4), 448–459. https://doi.org/10.1016/j.vetmic.2014.03.007

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References *continued from page 11*

Eckner, K.F. (1998). Comparison of membrane filtration and multiple-tube fermentation by the Colilert and Enterolert methods for detection of waterborne coliform bacteria, *Escherichia coli*, and enterococci used in drinking and bathing water quality monitoring in southern Sweden. *Applied and Environmental Microbiology*, *64*(8), 3079–3083. https://doi.org/10.1128/AEM.64.8.3079-3083.1998

Gronewold, A.D., Sobsey, M.D., & McMahan, L. (2017). The compartment bag test (CBT) for enumerating fecal indicator bacteria: Basis for design and interpretation of results. *Science of the Total Environment*, *587–588*, 102–107. https://doi.org/10.1016/j.scitotenv.2017.02.055

Gronewold, A.D., & Wolpert, R.L. (2008). Modeling the relationship between most probable number (MPN) and colony-forming unit (CFU) estimates of fecal coliform concentration. *Water Research*, *42*(13), 3327–3334. https://doi.org/10.1016/j.watres.2008.04.011

Haberecht, H.B., Nealon, N.J., Gilliland, J.R., Holder, A.V., Runyan, C., Oppel, R.C., Ibrahim, H.M., Mueller, L., Schrupp, F., Vilchez, S., Antony, L., Scaria, J., & Ryan, E.P. (2019). Antimicrobial-resistant *Escherichia coli* from environmental waters in Northern Colorado. *Journal of Environmental and Public Health*, Article 3862949. https://doi.org/10.1155/2019/3862949

Organisation for Economic Co-operation and Development. (2019). *Antimicrobial resistance—Tackling the burden in the European Union.* https://www.oecd.org/en/publications/antimicrobial-resistance-tackling-the-burden-in-the-european-union_33cbfc1c-en.html

Sargeant, J.M., O'Connor, A.M., & Winder, C.B. (2019). Editorial: Systematic reviews reveal a need for more, better data to inform antimicrobial stewardship practices in animal agriculture. *Animal Health Research Reviews*, *20*(2), 103–105. https://doi.org/10.1017/S1466252319000240

Stauber, C., Miller, C., Cantrell, B., & Kroell, K. (2014). Evaluation of the compartment bag test for the detection of *Escherichia coli* in water. *Journal of Microbiological Methods*, *99*, 66–70. https://doi.org/10.1016/j.mimet.2014.02.008

Wang, A., McMahan, L., Rutstein, S., Stauber, C., Reyes, J., & Sobsey, M.D. (2017). Household microbial water quality testing in a Peruvian demographic and health survey: Evaluation of the compartment bag test for *Escherichia coli*. *The American Journal of Tropical Medicine and Hygiene*, *96*(4), 970–975. https://doi.org/10.4269/ajtmh.15-0717

World Health Organization. (2016). *Global action plan on antimicrobial resistance*. https://www.who.int/publications/i/item/9789241509763

World Health Organization. (2021). *WHO integrated global surveillance on ESBL-producing* E. coli *using a "One Health" approach: Implementation and opportunities*. https://iris.who.int/bitstream/handle/10665/340079/9789240021402-eng.pdf

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