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RESEARCH ARTICLE

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*

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Abstract

Antimicrobial resistance (AMR) threatens human and animal health; effective response requires monitoring AMR presence in humans, animals, and the environment. The World Health Organization Tricycle Protocol (WHO TP) standardizes and streamlines global AMR monitoring around a single indicator organism, extended-spectrum-β-lactamase-producing *Escherichia coli* (ESBL-Ec). The WHO TP culture-based method detects and quantifies ESBL-Ec by spread-plating or membrane filtration on either MacConkey or TBX agar (supplemented with cefotaxime). These methods require laboratories and trained personnel, limiting feasibility in low-resource and field settings. We adapted the WHO TP using a simplified method, the compartment bag test (CBT), to quantify most probable numbers (MPN) of ESBL-Ec in samples. CBT methods can be used correctly in the field by typical adults after a few hours' training. We collected and analyzed municipal wastewater, surface water, and chicken waste samples from sites in Raleigh and Chapel Hill, NC over an 8-month period. Presumptive ESBL-Ec were quantified using MF on TBX agar supplemented with cefotaxime (MF+TBX), as well as using the CBT with chromogenic *E. coli* medium containing cefotaxime. Presumptive ESBL-Ec bacteria were isolated from completed tests for confirmation and characterization by Kirby Bauer disk diffusion tests (antibiotic sensitivity) and EnteroPluri biochemical tests (speciation). Both methods were easy to use, but MF +TBX required additional time and effort. The proportion of *E. coli* that were presumptively ESBL in surface water samples was significantly greater downstream vs upstream of wastewater treatment plant (WWTP) outfalls, suggesting that treated wastewater is a source of ESBL-Ec in some surface waters. The CBT and MF+TBX tests provided similar (but not identical) quantitative results, making the former method suitable as an alternative to the more complex MF+TBX procedure in some applications. Further AMR surveillance using

KCA). In addition, this work was enabled by the generous provision of CBT2 test medium (to MBF), which was made available by the manufacturer at the cost of materials. CBT test kits used in this work were purchased at the discounted rate offered to the University by the manufacturer for all research studies using CBT kits. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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MF+TBX and/or CBT methods may be useful to characterize and refine their performance for AMR monitoring in NC and elsewhere.

Introduction

Antimicrobial resistance (AMR), including the occurrence and proliferation of antimicrobial resistant bacteria (ARBs) is a growing threat to human and animal health and the microbial quality of the environment. Current research estimates that global AMR related deaths could climb to 10 million annually by 2050 [1–4]. Global, regional, and national efforts have been initiated to prevent and manage AMR hazards (such as AMR pathogens and genes) through improving and encouraging ARB and ARG monitoring and surveillance, strengthening antibiotic stewardship, infection prevention and control, and interrupting the release of AMR organisms to the environment.

The implementation of widespread, robust monitoring of AMR hazards in human, animal, and environmental sectors has been identified as critical need to better prevent, manage, and control AMR hazards. Governments, global NGOs, and academics have enacted legislation, developed monitoring protocols, and provided guidance aimed at tracking and controlling AMR occurrence and spread. WHO spearheaded this movement with the creation of the Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR) in 2009. WHO and AGISAR released the Global Action Plan on AMR (GAP-AMR) in 2014 which called for integration of AMR monitoring efforts across clinical, food and animal agriculture (specifically poultry), and environmental sectors as a One Health approach [5]. However, initial monitoring efforts were disparate, fragmented, and costly, making them unsuitable for use in low resource settings [2, 6].

The World Health Organization Tricycle Protocol of 2021 was intended to address these inadequacies by adopting a One-Health integrated and streamlined approach. A single AMR indicator organism, extended spectrum Beta-lactamase *Escherichia coli* (ESBL-Ec), was selected to streamline global surveillance and monitoring. While ESBL-Ec is not a perfect indicator for all human and animal AMR pathogens, its occurrence is likely to be indicative of AMR organisms shed in the feces of humans and/or other mammals, making it a potentially useful indicator for AMR pathogen risk. It is much more feasible to monitor a single ARB target than an assortment of AMR pathogens and genes. A standardized detection method, spread plating or membrane filtration on TBX agar, was developed for analysis of environmental samples. Clinical samples can also be characterized by similar methods. Selecting ESBL-Ec for either direct spread plating or initial membrane filtration for samples with lower predicted ESBL-Ec levels reduces the operational cost of the protocol to allow comparisons across a range of samples and settings that may have very different ESBL-Ec concentrations [7, 8].

While the Tricycle protocol has been adopted successfully in some countries worldwide, it had not been piloted in the United States prior to the outset of this study [9], and has subsequently been piloted once using laboratory-based methods [10]- This work is among the first to pilot the Tricycle protocol in the US and is the first to do so using simple methods that can be conducted in field settings or in laboratory settings with minimal equipment. This study implemented the Tricycle protocol using membrane filtration (MF) and agar medium plating on a differential and selective ESBL *E*, *coli* medium and compared it to the compartment Bag Test (CBT) as a less training- and apparatus-intensive most probable number (MPN) culture method to quantify ESBL-Ec occurrence in environmental samples. Both methods were used

simultaneously for samples of surface water (SW), municipal wastewater (WW), and chicken waste (CW) in two cities, Raleigh and Chapel Hill, in the Piedmont region of North Carolina (NC) USA.

The occurrence and concentrations of ESBL-Ec in surface water, wastewater, and chicken waste samples across eight sites in these two cities over an eight-month study period were quantified using. three separate methods: (1) the WHO TP (MF followed by plating on TBX agar [MF+TBX]) and liquid culture quantal methods on selective media formulations (one proprietary and the other made in-house) utilizing the Compartment Bag Test (CBT) (Aquagenx LLC, Chapel Hill, NC USA format that gives Most Probable Number (MPN) as well as presence-absence results [11, 12]. The goals of this study were to: 1) Quantify ESBL-Ec occurrence and concentrations in environmental samples, 2) assess the feasibility of implementing the WHO TP to monitor ESBL-Ec in NC, USA, and 3) assess the performance of the CBT MPN method as an alternative to MF followed by agar medium plating on TBX agar for colonies (WHO TP).

Methods

Ethics statement

All farms and wastewater treatment facilities participated voluntarily and gave their informed consent for inclusion before samples were collected. Written permission was obtained to collect samples from the North Carolina State University Lake Wheeler Road Field Laboratory, 3720 Lake Wheeler Rd, Raleigh, NC) (supplementary info), and verbal permission was obtained by the lead author from the farm manager of the privately owned farm, and from the appropriate administrators or staff persons for the utilities visited (Orange Water and Sewer Authority, 400 Jones Ferry Rd, Carrboro, NC; Neuse River Resource Recovery Facility, 8545, 8500 Battle Bridge Rd, Raleigh, NC). Apart from this permission, provided directly by each facility owner or representative, no formal permits were required or obtained for the collection of anonymous environmental samples. Likewise, approval and permits were not required or obtained for sampling publicly accessible surface water in public parks and public lands, such as water samples obtained at the Morgan Creek trailhead. Animal waste samples were collected without any animal contact, from ground-deposited material. No human or animal subjects were included in this work and no data were collected from any human or animal subjects, only data on environmental microbes; as a result, IRB approval was not applicable or sought.

Study location

Sampling was conducted in the NC Piedmont region, in and around a large city (Raleigh; estimated population of 480,000) and a smaller "sentinel" city (Chapel Hill; estimated population ~63,000), as specified in the WHO TP. The study areas include sources of chicken agriculture in addition to urban wastewaters and surface waters.

Sample collection

Samples were collected between July 2021 and March 2022 from surface water, wastewater, and chicken agriculture sites in Raleigh and Chapel Hill, NC on sampling visits conducted approximately every two weeks (Tables 1 & 2, Table A in S1 Text; note that not all sites could be sampled on each visit due to logistical constraints).

Using sterile polypropylene wide-mouth bottles (Thermo-Fisher Scientific, Waltham, MA), 0.5-liter grab samples were collected from Raleigh's Neuse River Resource Recovery (wastewater) Facility and 24-hour composite samples were taken from the autosampler at the Orange

Table 1. Environmental sampling sites.

Type	Raleigh	Chapel Hill
Municipal Raw Sewage	0.5-liter grab samples (n = 17) taken from screened influent at The City of Raleigh's Neuse River Resource Recovery Facility (NRRRF).	0.5-liter aliquots of 24-hour composite samples (n = 21) taken from Orange Water and Sewer Authority's (OWASA) Mason Farm Road Wastewater Treatment Plant.
Surface Water	0.5-liter grab samples (n = 34) taken from two sites on the Neuse River; both on the Neuse River Greenway Trail upstream and downstream of NRRRF.	0.5-liter grab samples (n = 42) taken from two sites upstream and downstream of wastewater treatment plant effluent discharge. Two sites on Morgan Creek; Morgan Creek Trail and NC Botanical Gardens Biological Preserve.
Animal Agriculture	Chicken litter samples (n = 5) were collected from the Lake Wheeler Road Test Farm Poultry Unit at NC State University's (NCSU) College of Agriculture and Life Sciences (CALS) in sterile 300-mL Whirl-Pak® bags.	Excreted feces samples (n = 11) from chickens never administered antibiotics were collected from a small-scale, mixed-livestock farm in the Chapel Hill area using sterile 300-mL Whirl-Pak® bags.

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Water and Sewer Authority's Mason Farm Road Wastewater Treatment Plant in Chapel Hill and transferred to sterile 0.5-liter bottles. Surface water grab samples were collected from the Neuse River in Raleigh and from Morgan Creek in Chapel Hill, both up- and downstream of the respective wastewater treatment plants (WWTPs) in each location using sterile 0.5-liter sample bottles. These surface waters serve as receiving waters for effluent from their respective WWTPs. Chicken waste samples were collected in sterile 300-mL Whirl-Pak® (Whirl-Pak, Madison, WI) bags from farms in Raleigh and Chapel Hill by inverting the sterile bags with gloved hands and collecting samples by directly grabbing with the inverted bag (then un-inverting and sealing) or by scooping with a sterile scoop and depositing in the bag. Approximately 5 chicken droppings per visit were collected at the Chapel Hill site and approximately one cup of fecally soiled chicken litter was collected per visit at the Raleigh site. Samples were stored and transported on ice at 4˚C and analyzed within 24–48 hours of collection. Not every sampling location was visited on each sampling trip due to challenges with regular access to some sites, specifically wastewater and poultry agriculture sites.

Table 2. Sample types and their numbers collected from individual environmental sampling sites.

Site	Abbreviation	Samples Collected	Location
OWASA Mason Farm Wastewater Treatment Plant (Chapel Hill Wastewater Treatment Plant)	CHWW	21	35.896385421957, -79.02412634651
Neuse River Resource Recovery Facility (Raleigh Wastewater Treatment Plant)	RLWW	17	35.71911942915, -78.49133713715
Morgan Creek, Accessed at Morgan Creek Trail (Chapel Hill Surface Water Site- Upstream of WWTP)	CHSW-US	21	35.89636596104, -79.064674647
Morgan Creek, Accessed at NC Botanical Gardens Biological Preserve (Chapel Hill Surface Water Site- Downstream of WWTP)	CHSW-DS	21	35.899616654611, -79.03335485907
Neuse River Greenway Trail- Upper (Upstream Neuse River Surface Water Site- Raleigh)	RLSW-US	17	35.72601358775, -78.51511223741
Neuse River Greenway Trail- Lower (Downstream Neuse River Surface Water Site- Raleigh)	RLSW-DS	17	35.70489807123, -78.47781880433
Chapel Hill-area Farm (Chicken farm, feces)	CHCF	11	Redacted
NCSU Lake Wheeler Road Test Farm Poultry Unit (Raleigh Chicken Farm, litter)	RLCF	5	35.73037987230, -78.67860706562

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Sample processing and analysis

For chicken waste samples collected at the Chapel Hill site, 1 g of chicken feces was suspended in 10 mL of sterile distilled (DI) water and vortex mixed until homogenous. For chicken feces-contaminated litter samples (collected at Raleigh site), 5 grams of each sample were added to 50 mL of sterile DI water, vortex mixed, and the supernatant decanted for analysis. Chicken litter samples contained both bedding and feces, and therefore required a larger mass than chicken feces samples to ensure uniformity of mixed samples.

Municipal wastewater and chicken waste suspension/supernatant samples were diluted with sterile DI water to bring concentrations to quantifiable levels of 10–200 CFU/100mL or MPN/100 mL. Suitable dilution factors were estimated based on previous results or else based on an initial dilution test (preparing and culturing serial dilutions of sample to determine the most suitable dilution factor) performed on the day of sample collection, prior to full sample analysis the following day. Surface water samples were typically analyzed undiluted or diluted 1:10 (10^{-1} dilution), while wastewater and chicken waste samples were typically diluted to between 10^{-3} and 10^{-5}. Samples were mixed and diluted immediately before analysis, and dilution factors were recorded. Results were then normalized to initial undiluted sample volume or mass prior to data analysis.

Replicate 100-mL aliquots of processed samples were analyzed by membrane filtration (MF) followed by incubation on TBX agar and using the compartment bag test (CBT), both with and without added cefotaxime (4 mg/L). MF samples (100-mL) were filtered through 0.45-μm cellulose nitrate filters followed by incubation (at 44˚C for 24 hours) on 47-mm diameter TBX agar plates with or without 4 mg/L added Cefotaxime (CTX) according to the Tricycle protocol. Blue-green colonies were counted as presumptively positive ESBL-Ec. CBT samples (100-mL) were analyzed according to the manufacturer's instructions. For CBT assays with added cefotaxime, 4 mg/L cefotaxime was added to the proprietary media by spiking each sample with 1 mL/100 mL of 100x stock solution. Apart from this modification, CBT assays were performed as described in the manufacturer's instructions (Aquagenx LLC n.d.). A new CBT2 (containing the same proprietary growth medium as the CBT but pre-formulated to include 4 mg/L cefotaxime) was also used; apart from the differerence in media formulation, the method was performed identically to the standard CBT. All 100-mL CBT samples were incubated at 35˚C for 24 hours. If a CBT compartment exhibited a blue-green color after incubation, that compartment was counted as positive [13]. Results were reported as presumptive *E. coli* and ESBL-Ec for assays without/with cefotaxime, respectively. Results from membrane filtration (MF) and agar medium plating assays for colonies on TBX were reported in units of CFU/100 mL, and in units of most probable number (MPN/100 mL) concentrations for CBT assays. For MF and agar medium plating assays, all colonies meeting standard assay criteria for *E. coli* colony appearance were counted as presumptively positive [7].

A subset of presumptive ESBL organisms were isolated from MF+TBX agar medium plates for further characterization. Specifically, 1 to 5 presumptive ESBL-Ec colonies (identified as described above) were selected from positive TBX/cefotaxime plates using a sterile inoculating loop, and streaked to isolation on TBX media with 4 mg/L cefotaxime (isolation plates) before incubation (as described above). Following incubation, a single presumptive positive colony was picked with a sterile loop from each isolation plate, cultured overnight in Tryptic Soy Broth (TSB), diluted 1:1 with sterile glycerol, and stored at -20˚C or -80˚C in sterile 2mL cryovials [7]. Presumptive ESBL-Ec organisms were isolated from CBTs by a similar method. The exteriors of 1–5 positive compartments of each CBT were swabbed with 70% ethanol, the compartments were then pierced using a sterile syringe and needle and a drop (approximately 20–50 μL) of medium was withdrawn, then spotted onto a TBX plate with cefotaxime, streaked to

isolation, and then incubated at 44˚C for 24 hours. Following incubation, a single presumptive target colony was picked from each such isolation plate with a sterile loop, incubated overnight in TSB at 35˚C, diluted with glycerol, and stored as described above.

Stored isolates were thawed and further characterized by Enteropluri® (Liofilchem, Roseto degli Abruzzi (TE), Italy) biochemical testing according to the manufacturer's instructions [14] and by Kirby-Bauer antibiotic sensitivity testing (for cefotaxime [CTX], Imipenem [IMP], ampicillin [AMP], Ceftazidime [CAZ], and vancomycin [VAN]) using Oxoid™ antimicrobial susceptibility testing disks (Thermo Scientific™, Waltham, MA) according to standard methodshttps://sciwheel.com/work/citation?ids=12673339,11872598&pre=&pre=&suf=&suf=&sa=0,0 [7, 15]. Isolates were confirmed as ESBL by Kirby Bauer testing using the criteria defined in the Tricycle Protocol using CTX, CAZ, CTX + clavulanic acid (CLA), and CAZ + CLA paper discs. Where synergy between CLA and beta lactams was observed (Eqs 1–2, Fig 1), strains were scored as ESBL. When the CTX zone of inhibition minus the CTX + CLA zone of inhibition was > = 5 mm or the CAZ zone of inhibition minus the CAZ + CLA zone of inhibition was > = 5 mm, the isolate was scored as a positive ESBL result [7].

$$\text{ESBL} = 1 \text{ if} (\text{CTX} + \text{CLA}) - \text{CTX} >= 5\text{mm OR if} (\text{CAZ} + \text{CLA}) - \text{CAZ} >= 5 \text{ mm} \quad \text{Eq1}$$

$$\text{ESBL} = 0 \text{ if} (\text{CTX} + \text{CLA}) - \text{CTX} < 5\text{mm AND if} (\text{CAZ} + \text{CLA}) - \text{CAZ} < 5 \text{ mm} \quad \text{Eq2}$$

Where (CTX + CLA) = the zone of inhibition (in mm) for the area equidistant from the CTX and CLA discs as shown in Fig 1, (CAZ + CLA) = the zone of inhibition (in mm) for the area equidistant from the CAZ and CLA discs as shown in Fig 1, (CTX) = the zone of inhibition (in mm) for the area surrounding the CTX paper disc but furthest from the CLA paper disc, and (CAZ) = the zone of inhibition (in mm) for the area surrounding the CAZ paper disc and furthest from the CLA paper disc.

The distributions of presumptive and confirmed *E. coli* and ESBL-Ec CFU and MPN concentrations were characterized separately for each environmental sample type (surface water, wastewater, chicken waste), each assay format (MF, CBT), and antibiotic absence or presence (without CTX, on media amended with CTX, or on proprietary media formulated with CTX).

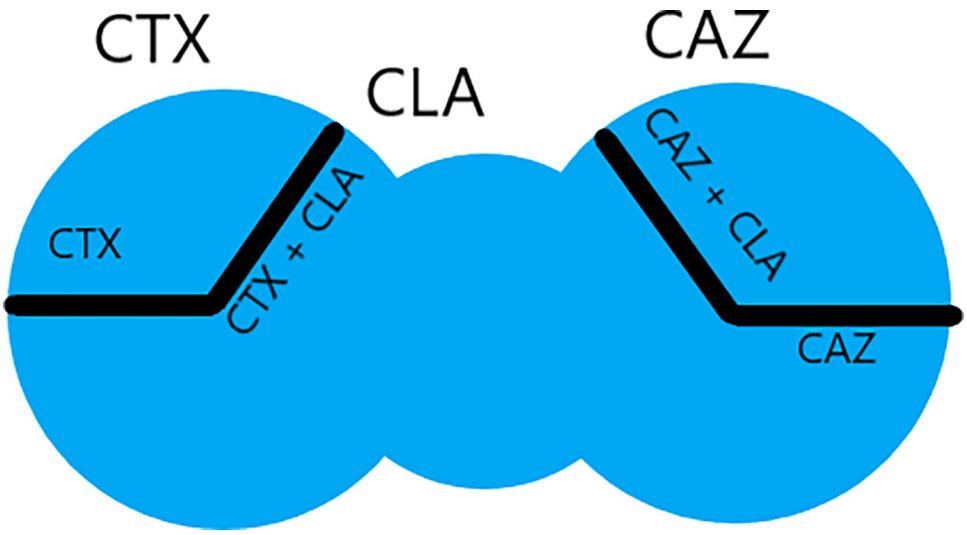


Fig 1. ESBL characterization of isolates using zones of inhibition for CTX, CLA, and CAZ discs in the Kirby-Bauer assay.

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E. coli concentrations were subjected to Shapiro–Wilk normality tests and the geometric mean, arithmetic standard deviation, and range were calculated. As with all culture-based methods, the minimum limit of detection (MLOD) of all methods used corresponded to the detection of one colony forming unit or MPN in the sample volume analyzed (100 mL). Because non-detects may represent any concentration between this MLOD and 0, it is useful to introduce a continuity correction to minimize bias in calculations and enable the inclusion of non-detects in calculations relying on log-transformed values. Non-detects were therefore assigned a nominal value of one half the minimum limit of detection (MLOD/2) for all calculations. Thus, a non-detect for a 100 mL undiluted sample would be scored as 0.5/100 mL. Presumptive resistance proportion was calculated as the ratio of ESBL-Ec/total Ec quantified in each sample. Confirmed ESBL-Ec proportion was calculated as the ratio of confirmed ESBL-Ec isolates to total isolates tested, adjusted for the numbers of isolates collected from each sample type (wastewater/ surface water/chicken waste) and location. Both presumptive and confirmed results were included in analyses and reported. Presumptive ESBL-Ec results are considered less robust indicators of ESBL status than confirmed ESBL phenotype, but have the advantage of quantifying all culturable organisms in each sample aliquot capable of growing on the differential and selective culture media and additives used. By contrast, confirmed ESBL phenotype is a more robust indicator of resistance, but was only able to be determined at the isolate level for the smaller subset of isolates subjected to further characterization. Difference in median log-transformed, continuity-corrected values between sample type and assay type (Table 3) was assessed by non-parametric Wilcoxon signed ranked-paired tests. All analyses were conducted in GraphPad Prism version 9.5 (Graphpad Software, Boston, MA).

Table 3. Occurrence of presumptive ESBL-Ec in environmental samples by sample type and assay method (WW = Wastewater; SW = Surface Water; CW = Chicken Waste).

Assay	Metric	Concentration Unit	WW	SW	CW	Overall
MF-TBX +CTX	Mean	log CFU/100 mL	3.58 (n = 84)	0.51 (n = 48)	1.01 (n = 39)	2.09 (n = 174)
	Standard Deviation	log CFU/100 mL	2.54	0.76	1.79	2.46
	Non-Detects	%	30.2%	42.1%	64.1%	41.8%
	Min	log CFU/100 mL	-0.30	-0.30	-0.30	-.30
	Max	log CFU/100 mL	6.58	2.06	4.00	6.58
CBT +CTX	Mean	log MPN/100 mL	4.17 (n = 73)	1.00 (n = 32)	1.25 (n = 31)	2.76 (n = 136)
	Standard Deviation	log MPN/100 mL	2.34	0.62	2.00	2.49
	Non-Detects	%	21.1%	37.4%	61.3%	36.1%
	Min	log MPN/100 mL	-0.30	-0.30	-0.30	-0.30
	Max	log MPN/100 mL	6	2	4.68	6.00
CBT2	Mean	log MPN/100 mL	5.29 (n = 63)	0.99 (n = 32)	1.16 (n = 31)	3.18 (n = 126)
	Standard Deviation	log MPN/100 mL	0.48	0.65	2.02	2.38
	Non-Detects	%	0%	45.8%	61.5%	35.7%
	Min	log MPN/100 mL	-0.30	-0.302	-0.30	-0.30
	Max	log MPN/100 mL	6	2	4.13	6
Overall	Mean	log CFU or MPN/100 mL	4.27 (n = 220)	0.79 (n = 112)	1.13 (n = 101)	2.61 (n = 436)
	Standard Deviation	log CFU or MPN/100 mL	2.19	0.73	1.91	2.49
	Non-Detects	%	41.6%	35.2%	35.0%	37.7%
	Min	log CFU or MPN/100 mL	-0.30	-0.30	-0.30	-0.30
	Max	log CFU or MPN/100 mL	6.58	2.06	4.68	6.58

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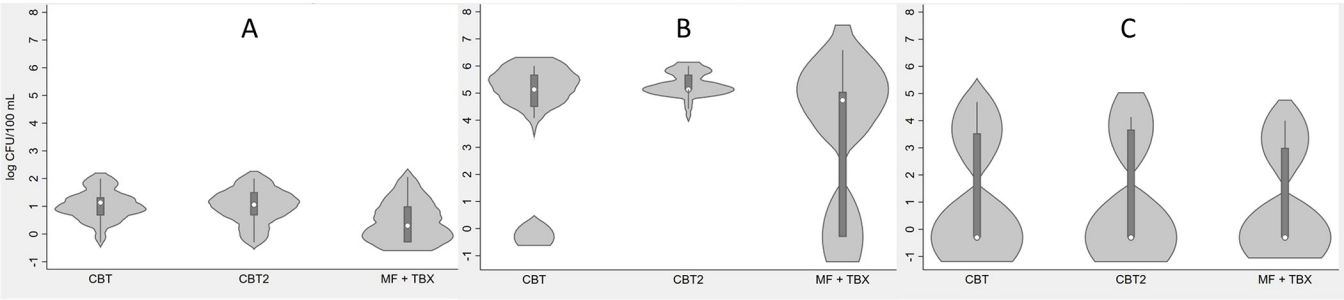


Fig 2. Violin Plots of Log-transformed Presumptive ESBL-Ec Concentrations in A) Surface Water; B) Wastewater, and C) Chicken Waste Samples. Central dots denote median log-transformed concentrations; boxes denote 1st and third quartile log concentrations; whiskers denote 95% confidence limits for log concentration values.

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Results

Presumptive concentrations of *E. coli* that were ESBL resistant

Fig 2A–2C and Table 3 show the distribution of presumptive ESBL-Ec CFU and MPN concentrations for samples by environmental sample type and analysis method. ESBL-Ec were detected in all environmental media tested. Concentrations of presumptive ESBL-Ec CFU or MPN were greatest in wastewater samples, followed by chicken waste samples, and were lowest in surface water samples. Across assays, distributions spanned 2–4 orders of magnitude, and most observations (>75%) were clustered within one order of magnitude from the median value. A non-trivial proportion of samples from each environmental medium and for each test method produced nondetects; as a result, the minimum value of the log-transformed, continuity-corrected CFU or MPN concentration was -0.30 for each sample type and test method (Table 3).

Presumptive proportion of *E. coli* that were ESBL resistant

Proportions of colonies obtained on selective *E. coli* media that were presumptively ESBL-resistant (ESBL, based on growth in presence of 4 mg/L added CTX) were quantified. Normality tests did not indicate a good fit for normal distributions, and as a result, nonparametric statistics were used (Tables C, D, and E, Figs A1 and A2 in S1 Text). Table 4 presents the percentages of *E. coli* that were presumptively ESBL stratified by sample type, as well as by each individual sampling location in the study. Throughout the study period and across all environmental sample types, the total percentage presumptively ESBL was 8.5%. Wastewater had the highest percentage presumptively resistant at 15%, followed by surface water and chicken waste samples at 7.2% and 0.5% respectively. In wastewater, the NRRRF (RLWW) in Raleigh had a proportion presumptively resistant of 18.7% while OWASA (CHWW) in Chapel Hill had a proportion presumptively resistant of 12.3%. In chicken waste samples, proportion presumptively resistant was much lower in both the Lake Wheeler Road Poultry Facility

Table 4. Percentage of *E. coli* that were presumptively ESBL resistant. in culture-based tests from environmental samples (stratified by site: WW = Wastewater; SW = Surface Water upstream or downstream of wastewater treatment plant outfall; CW = Chicken Waste).

	WW	SW (upstream)	SW (downstream)	CW	Upstream vs Downstream (p value)
Raleigh	18.7% (n = 64)	5.2% (n = 62)	6.2% (n = 62)	0.3% (n = 17)	.0002***
Chapel Hill	12.3% (n = 77)	1.9% (n = 77)	15.0% (n = 77)	0.7% (n = 43)	.0001****
Overall	15.2% (n = 141)	4.4% (n = 139)	7.9% (n = 139)	0.5% (n = 60)	N/A

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(RLPF) in Raleigh and the Chapel Hill-area small-scale farm (CHPF), with less than 1% (0.38% and 0.65% respectively) presumptively ESBL.

Differences in the percentage of *E. coli* resistant to antimicrobial compounds between upstream and downstream surface water sampling sites is of considerable interest because such differences may shed light on the relative importance of wastewater treatment plant discharges and other environmental sources reaching surface waters as potential sources of ESBL-Ec. However, despite this illustrative value, observed differences between upstream and downstream samples cannot be definitively attributed specifically and entirely to wastewater outflowshttps://sciwheel.com/work/citation?ids=11872598&pre=&suf=&sa=0 [7]. In Raleigh, the Neuse River upper site (RLSW-US) had a percentage presumptively resistant of 5.2% while the Neuse River lower site (RLSW-DS) had a percentage presumptively resistant of 6.2%. In Chapel Hill, the Morgan Creek Trail upstream site (CHSW-US) had a percentage presumptively resistant of 1.9% while the Mason Farm Biological Preserve site (CHSW-DS) downstream site had a percentage presumptively resistant of 15.4% (Table 4). A one-sample Wilcoxon Signed Rank test on each upstream versus downstream pairing indicated that downstream samples had significantly higher proportions of presumptively resistant organisms than upstream samples (p value = 0.0002 for Raleigh and $p<0.0001$ for Chapel Hill).

Differences in antibiotic resistance proportion of *E. coli*

A Wilcoxon Signed Rank test of the log-transformed concentrations of *E. coli* in each sample exhibiting presumptive ESBL resistance for the three assay methods used in this work indicates that the CBT and CBT2 produced results significantly different from those obtained by MF on TBX + cefotaxime ($p = 0.008$ and $p<0.0001$, respectively). The two CBT methods do not significantly differ from each other ($p = 0.12$). Visual inspection of paired log-log plots of the results from each method suggests that the three methods produced broadly similar results at the order-of-magnitude level for most samples tested (Fig 3A–3C).

ESBL *E. coli* isolate analysis

Kirby Bauer antibiotic sensitivity test. *Wastewater*. Of Kirby Bauer analyses conducted on 142 wastewater isolates from 16 samples, 17.6% of isolates were confirmed ESBL-Ec positive by the Tricycle Protocol criteria (Table 6). There were no CTX-negative isolates meeting this definition, while 23.4% of CTX+ isolates met this definition. Isolates resistant to 3 or more of the antibiotics tested were considered multidrug resistant (MDR). Overall, 78.2% of isolates were MDR: 97.2% of isolates from CTX+ assays and 20% from CTX- assays, respectively.

Surface water. Of Kirby Bauer analyses conducted on 233 surface water isolates from 31 samples, 33.9% of all isolates were ESBL-Ec positive by Tricycle Protocol confirmation criteria; 44.3% of isolates from CTX+ assays and 1.8% of isolates from CTX- negative assays (Table 6). Overall, 74.2% of surface water isolates were MDR: 94.9% of isolates from CTX+ assays and 10.5% of isolates from CTX- negative assays. When results were disaggregated by location (Raleigh and Chapel Hill) and stratified by site position (upstream vs downstream), some differences in antibiotic sensitivity characteristics were observed; Chi-square tests indicated that a higher proportion of downstream isolates were ESBL and MDR than upstream isolates ($p<0.01$ and $p = 0.03$, respectively; Table 5, Table F in S1 Text).

Chicken waste. Of Kirby Bauer analyses conducted on 52 chicken waste isolates from 7 samples, 50% of isolates were ESBL positive by WHO TP confirmation criteria; 100% of isolates from CTX+ assays and no isolates from CTX- negative assays (Table 5). Approximately 54% of chicken waste isolates were MDR: 100% of isolates from CTX+ assays and 8% of isolates from CTX- negative assays.

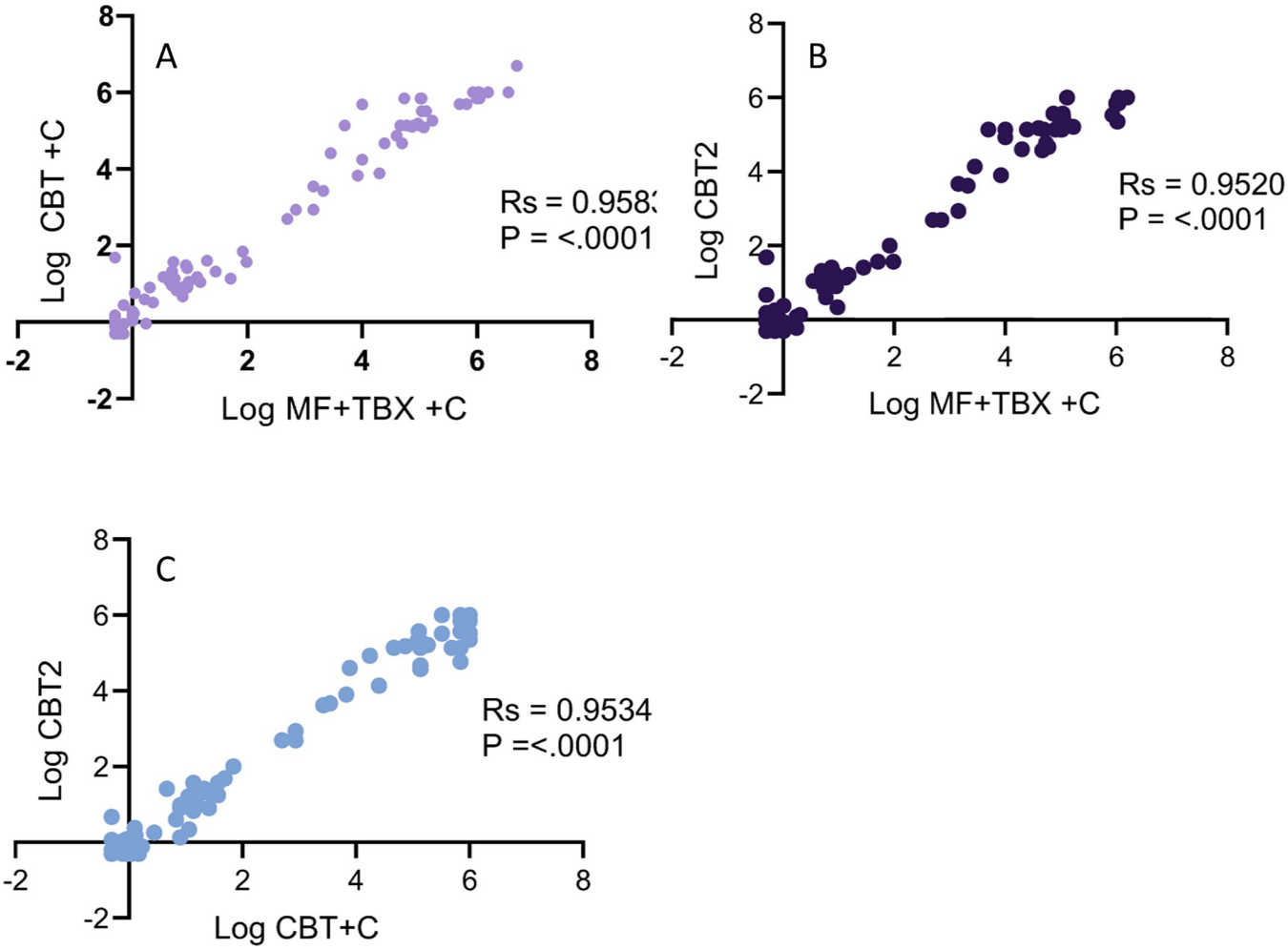


Fig 3. Log-Log Plots of A) MF-TBX vs. CBT, B) MF-TBX vs. CBT2, and C) CBT vs. CBT2. Rs and P values represent fit parameters for regression lines of best fit for each log-log plot.

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Speciation by EnteroPluri testing

Of 209 isolates characterized by EnteroPluri (EP) biochemical testing, 87% were confirmed as *E. coli*, with 13% classified as other organisms (Table 6) [14]. All of the other organisms identified were Gram-negative enterobacteriaceae: *Kluyvera ascorbata*, *Escherichia vulneris*, *Pantoea agglomerans*, *Shighella flexneri*, *Klebsiella oxytoca*, *Citrobacter koseri*, and *Citrobacter freundii*. Results were stratified by sample type and assay type (MF + TBX agar medium colonies, CBT, and CBT2). However, no data are available for CTX- negative isolates from CBT2 tests because cefotaxime is always a component of the CBT2 test medium. The proportion of isolates classified as *E. coli* was not significantly different by test type.

Discussion

Tricycle protocol adaptation, site selection, and feasibility assessment

The WHO TP, which specifies sampling for each site and sample type 8–12 times per calendar year with at least one sample collection in each major season, was feasible to implement and sustain in this study. Because the WHO TP was designed largely for implementation in low-

Table 5. Kirby Bauer antibiotic sensitivity results for presumptive *E. coli* isolates from environmental samples (stratified by site: WW = Wastewater; SW = Surface Water upstream or downstream of wastewater treatment plant outfall; CW = Chicken Waste).

Type	N (samples)	Media	n (isolates)	Resistance Characteristics (% of isolates resistant)						
				ESBL	MDR	CTX	IMP	AMP	CAZ	VAN
WW	16	Both	142	17.6%	78.2%	78.9%	0%	78.9%	46.5%	100.0%
		CTX+	107	23.4%	97.2%	98.1%	0%	98.1%	58.9%	100.0%
		CTX-	35	0%	20.0%	20.0%	0%	20.0%	8.6%	100.0%
SW	31	Both	233	33.9%	74.2%	62.7%	0%	77.7%	51.1%	100.0%
		CTX+	176	44.3%	94.9%	81.3%	0%	98.9%	64.2%	100.0%
		CTX-	57	01.8%	10.5%	5.3%	0%	12.3%	10.5%	100.0%
SW (Upstream)		Both	106	29.2%	72.6%	54.7%	0.0%	75.5%	42.5%	100.0%
		CTX+	76	39.5%	96.1%	75.0%	0.0%	100.0%	53.9%	100.0%
		CTX-	30	3.3%	13.3%	3.3%	0.0%	13.3%	13.3%	100.0%
SW (Downstream)		Both	127	37.8%	75.6%	69.3%	0.0%	79.5%	58.3%	100.0%
		CTX+	100	48.0%	94.0%	86.0%	0.0%	98.0%	72.0%	100.0%
		CTX-	27	0.0%	7.4%	7.4%	0.0%	11.1%	7.4%	100.0%
CW	7	Both	52	50.0%	53.8%	55.8%	0%	67.3%	25.0%	100.0%
		CTX+	26	100.0%	100.0%	100.0%	0%	100.0%	50.0%	100.0%
		CTX-	26	0%	7.7%	11.5%	0%	34.6%	0%	100.0%

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and middle- income countries (LMICs), some adaptations were requiredhttps://sciwheel.com/work/citation?ids=11872598&pre=&suf=&sa=0 [7]. The protocol calls for poultry, specifically chicken, agricultural sampling of wet market runoff or slaughter facility runoff. While wet markets and slaughter facilities exist in NC, access to them was not possible. Gaining access to slaughter facilities through longstanding research relationships or through USDA partnerships may facilitate sampling of such sites in the future. However, for this study the protocol was adapted to the collection of chicken feces and/or litter from chicken farms willing to allow access. The anonymous Chapel Hill farm is a small commercial operation with fewer than 500 chickens and may not be representative of larger operations. The Raleigh farm is a university facility with multiple chicken houses that is more representative of a typical commercial

Table 6. Enteropluri isolate speciation results (stratified by site: WW = Wastewater; SW = Surface Water upstream or downstream of wastewater treatment plant outfall; CW = Chicken Waste and test type: MF-TBX = Membrane filtration + TBX agar plating; CBT and CBT2 denote the compartment bag test and modified compartment bag test as defined previously; and media type: CTX+ denotes media containing 4 mg/L CTX; CTX- denotes media without CTX; "Both" denotes combined results for media with- and without CTX).

Type	N (samples)	Media	Confirmed *E. coli* (% of isolates)				n (isolates)			
			Overall	MF-TBX	CBT	CBT2	Overall	MF-TBX	CBT	CBT2
Overall	54	Both	87	92	83	85	209	94	69	46
		CTX+	90	97	87	85	160	60	54	46
		CTX-	78	82	67	-	49	34	15	0
WW	16	Both	77	76	81	75	69	25	16	28
		CTX+	78	91	75	75	51	11	12	28
		CTX-	72	57	100	-	18	14	4	0
SW	31	Both	94	97	89	100	117	61	44	12
		CTX+	96	98	92	100	95	45	38	12
		CTX-	86	94	67	-	22	16	6	0
CW	7	Both	83	100	56	100	23	8	9	6
		CTX+	93	100	75	100	14	4	4	6
		CTX-	67	100	40	-	9	4	5	0

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operation. It is worth noting that antibiotic usage in small commercial farms and research facilities may be more limited than on larger commercial farms, and the density of chickens may be different (i.e. lower, particularly for free-range vs caged facilities). As a result, selective pressures for AMR occurrence and transmission may be less acute in the studied farms than in some larger commercial operations.

ESBL-Ec occurrence

Overall, ESBL-Ec were found to be prevalent across sample types. As expected, ESBL-Ec concentratrations were greatest in municipal wastewater and decreased from WW to chicken waste to surface waters. ESBL-Ec levels in surface water were higher in samples collected downstream of wastewater treatment plant outfalls than in upstream samples. While this difference cannot be directly and causally attributed to the presence of the wastewater outfalls, the results suggest that treated wastewater may be an important source of culturable ESBL-Ec in surface waters. Future efforts may include monitoring of streamflow, rainfall, and wastewater discharge outflows to support calculations that can better track contributions of wastewater, stormwater, and/or other sources over time.

Confirmation testing of presumptive ESBL *E. coli*

Agreement between presumptive isolates from tests containing cefotaxime and CTX-resistance in Kirby Bauer assays was 98% in wastewater samples, 100% in poultry samples, and 81% in surface water samples. Confirmation testing indicated that the culture-based methods used were highly selective for *E. coli*, as most presumptive *E. coli* isolates were confirmed as *E. coli*. The ESBL confirmation of presumptive *E. coli* isolates using the Kirby Bauer test confirmed 23% of presumptive isolates from wastewater samples, 44% surface water samples, and 100% of isolates from chicken waste samples.

The reason for the low ESBL confirmation rates using 4 mg/L cefotaxime is not known and could be caused by deficiencies in the culture media used. In a previous study that identified 4 mg/L cefotaxime as the optimum concentration based on positive control strains of *E. coli*, the application of this concentration to field samples of water and poultry ceca samples gave ESBL confirmation rates of only 45% and 16.6% of phenotypically expressed ESBL production [16]. It is possible that current Tricycle Protocol criteria for confirming ESBL production of *E. coli* may not be optimal for environmental *E. coli* in our NC study setting and its samples. Therefore, further studies are needed to determine the reasons for low ESBL *E. coli* confirmation rates from field environmental samples.

One factor that we did not explore in this study is the presence of ESBL genes in isolates. Characterizing the genotypes linked to ESBL production with phenotypic Kirby Bauer test results could help determine whether the current operational definition for confirming ESBL phenotype is aligned with molecular data. A comparison of resistance phenotypes to common and relevant extended-spectrum beta lactam drugs compared to genotypes would be informative and could improve understanding of the extent and genetic basis of Beta-lactam resistance. If such studies indicate that current phenotypic and genotypic criteria are suitable, future efforts should perhaps focus on improving the selectivity and specificity of culture media for ESBL-Ec surveillance in NC.

Comparison of the WHO TP and the CBT as culture-based test methods for ESBL-Ec

This study is the first systematic evaluation of the Compartment Bag Test (CBT) for ESBL-Ec quantitation in comparison with the standard WHO TP. The CBT achieved comparable but

not identical performance to MF and agar media culture for colonies to quantify presumptive ESBL-Ec in environmental samples. Results for a paired Wilcoxon signed rank test between the two culture tests were significantly different, with a tendency for slightly higher estimates of presumptive ESBL-Ec using CBT tests and media. However, in many applications of environmental monitoring and surveillance data, management decisions and actions may be taken based on order-of-magnitude quantitation; e.g., a similar action will often be taken when presumptive ESBL-Ec concentrations are estimated to be 10^3.5 CFU/100 mL vs when they are estimated to be 10^4 CFU/100 mL in a given sample. An inspection of log-log plots of presumptive ESBL-Ec concentrations determined using the methods evaluated in this study suggests that results varied by less than one order of magnitude between paired tests in most cases. It is therefore reasonable to speculate that similar management decisions might be taken in many cases regardless of which of the three tests was used, and that in such cases the CBT and CBT2 methods may present simpler alternatives that are fit-for-purpose in settings without ready access to a lab where MF is practical. CBT-based methods and media may be suitable alternatives to (if not exact substitutes for) the more skill- and equipment-intensive MF + TBX agar plating methods for presumptive ESBL-Ec quantitation in many environmental field applications, including surveillance under the WHO TP in remote and low-resource settings.

The WHO TP is designed to be feasible in LMICs, but current MF+TBX agar plating methods require non-trivial infrastructure and capacity in addition to more time for preparation and analysis. MF agar media must be prepared in advance, sterilized, and poured into plates prior to sample analysis, then refrigerated unless used immediately. Filtration requires a source of vacuum, a filtration assembly, and the means to sterilize it. Incubation at 44˚C generally requires a reliable source of electricity. By contrast, the CBT is a field-ready, self-contained and portable *E. coli* and total coliform test that is easy to use that does not require electricity, additional materials or equipment or dedicated laboratory spacehttps://sciwheel.com/work/citation?ids=12673369,9538203&pre=&pre=&suf=&suf=&sa=0,0 [12, 13]. Users can often be trained in a few hours. Easy-to-use, infrastructure-independent ESBL-Ec detection and quantitation methods such as the CBT methods evaluated in this study can improve and enhance the feasibility of AMR surveillance using the WHO TP in remote and/or low-resource settingshttps://sciwheel.com/work/citation?ids=9538203,6383526,10861197&pre=&pre=&pre=&suf=&suf=&suf=&sa=0,0,0 [12, 17, 18].

Study limitations

A limitation of this study was the adaptation of the WHO TP to local conditions and study constraints for poultry sample access. Only 16 samples were collected from chicken waste sites, with 5 from the Raleigh site and 11 from the Chapel Hill site. This was considerably fewer samples than collected from municipal wastewater (38 samples) and surface water sites (76 samples).

The smaller number of ESBL-Ec isolates collected per sample vs. WHO TP specifications is not likely to substantially impact the overall findings of this work. This is because isolate collection was random and representative of sampling throughout the study, and the total number of isolates characterized (209) is comparable to what might be produced in one year using the unmodified WHO TP. However, the temporal and source (human/animal/environment) distribution of samples and isolates was more skewed than that prescribed in the WHO TP, and therefore, results are less representative than what might have been obtained using a strict implementation of the protocol at scale, particularly for isolate-level outcomes. Further work is recommended to increase sample size and representativeness across seasons and sample types.

Conclusion

This study found that the WHO TP for ESBL-Ec detection, quantification and characterization in environmental samples can be successfully adapted to a North Carolina, USA context. Overall, the WHO TP was easy to use and provided relevant data on ESBL-Ec occurrence in the environmental samples of municipal wastewater, ambient surface water and chicken fecal wastes. Frequencies of presumptive and confirmed ESBL-Ec were detected in environmental samples in the expected order: highest in municipal wastewater, followed by chicken waste, and lowest in surface water. Notably, the proportion of *E. coli* that were identified as presumptively ESBL-Ec were significantly higher in surface water samples collected downstream vs upstream of WWTP outfalls, suggesting that treated wastewater may be an important source of culturable ESBL-Ec and other AMR organisms in such surface waters. Confirmation testing of ESBL status by Kirby Bauer antibiotic susceptibility testing was often not in agreement with presumptive results of MF and TBX agar medium plating or CBT-based tests using the recommended ESBL-selective media containing 4 mg/L cefotaxime. Further analysis is recommended to understand the reasons for the low agreement observed in this study.

Two novel candidate ESBL-Ec quantitation methods, the adapted CBT with added cefotaxime and the CBT2 (containing cefotaxime in its prepackaged medium), showed overall similarity to (if not perfect agreement with) results obtained using the standard WHO TP MF +TBX agar medium method. These alternative methods appear suitable for use in the WHO TP in many cases, and may enhance the feasibility and accessibility of implementing the protocol in settings with limited resources and infrastructure, and/or limited access to highly trained personnel. Continued monitoring in North Carolina using the adapted WHO TP is recommended to further validate the findings of this work and provide new opportunities to further adapt and refine the protocols and their use in NC and perhaps elsewhere in the USA.

Supporting information

S1 Text.
(DOCX)

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