Title: 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) is a growing threat to human and animal health, and efforts to combat it require widespread, robust and practical monitoring of AMR presence in humans, animals, and the environment. Because early AMR monitoring efforts were cumbersome, costly, and lacked standardization, the WHO Tricycle Protocol (WHO TP) was developed and released in 2021 to standardize and streamline global AMR monitoring by culturing 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 either spread plating or membrane filtration on either MacConkey or TBX agar (supplemented with cefotaxime), which are difficult methods to use in low-resource and field settings, and must be done mostly in lab settings by trained personnel. Therefore, we simultaneously detected and quantified ESBL-Ec in field samples using the WHO TP with membrane filtration (WHO TP MF) and also a simplified method, the compartment bag test (CBT), which quantifies different sample volumes as positive or negative for target bacteria and is easy for anyone to use in the field. 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 WHO TP MF on TBX agar supplemented with cefotaxime, 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 (for antibiotic sensitivity) and EnteroPluri tests (to speciate isolates). The WHO TP MF and the CBT methods were both easy to use, but the MF test 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 in both locations, suggesting that treated wastewater is a source of ESBL-Ec in some surface waters. The results of CBT and WHO TP MF tests were comparable, making the former method suitable as an alternative to the more complex WHO TP MF procedure. Further AMR surveillance using both the WHO TP MF and simpler CBT methods may be useful to further characterize and refine their performance for quantifying AMR occurrence in NC and elsewhere.

Introduction

Antimicrobial resistance (AMR) is a growing threat to human and animal health and the microbial quality of the environment. Current research estimates that AMR related deaths could climb to 10 million annually by 2050 [6–8]. Global, regional, and national efforts have been initiated to prevent and manage AMR hazards through strengthening antibiotic stewardship, infection prevention and control, and interrupting the release of AMR organisms to the environment, among other measures.

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. Global NGOs, governments, and academics have developed monitoring protocols, provided guidance, and enacted legislation aimed at minimizing 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[9]. However, initial monitoring efforts were disparate, fragmented, and costly, failing as a multi-sectoral effort and there was documented evidence of inadequate and unsuitable use in low resource settings [6,10].

The World Health Organization Tricycle Protocol of 2021 was intended to address these inadequacies by adopting a One-Health integrated and streamlined approach. Because human and animal wastes can contaminate surface water and result in human exposure through direct ingestion or in the food chain, a single AMR indicator organism, extended spectrum Beta-

lactamase *Escherichia coli* (ESBL-Ec), was selected for global surveillance and monitoring. A standardized detection method, spread plating or membrane filtration on TBX agar, was developed for analysis of environmental samples. Selecting ESBL-Ec for either direct spread plating or initial membrane filtration for samples with lower AMR bacteria levels reduces the operational cost of the protocol to allow cross-sectoral comparisons. [1,12].

While the Tricycle protocol has been adopted successfully in some countries worldwide, it has not been piloted in the United States previously. This study applied the Tricycle protocol (and for comparison, simpler culture methods) to quantify ESBL-Ec occurrence in surface water, municipal wastewater, and chicken waste in two cities, Raleigh and Chapel Hill, in the Piedmont region of North Carolina.

The occurrence and concentrations of ESBL-Ec in surface water, wastewater, and chicken waste samples across 8 sites in these two cities over an 8-month study period were quantified using. three separate methods: (1) the Tricycle Protocol (membrane filtration [MF]) followed by plating on TBX agar and liquid culture quantal methods on selective media formulations (one proprietary and the other made in-house) utilizing the Compartment Bag Test (CBT) format that gives Most Probable Number as well as presence-absence results [2,3]. The goals of this study were to: 1) Quantify ESBL-Ec occurrence and concentrations in environmental samples, 2) assess the usability of the Tricycle Protocol to monitor ESBL-Ec, and 3) assess the usability and performance of the CBT as an alternate quantification method.

Methods

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 per the WHO Tricycle Protocol. The study areas include several large hospitals in the University of North Carolina (UNC) Medical, Duke Medical, and Wake Medical healthcare systems as well as sources of chicken agriculture.

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 at two-week intervals

(Table 1, Figures S1, S2). 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 Orange Water and Sewer Authority's Mason Farm Road Wastewater Treatment Plant in Chapel Hill. Surface water grab samples were collected from the Neuse River (Raleigh) and Morgan Creek (Chapel Hill) up- and downstream of the wastewater treatment plants (WWTPs) using the same kind of sample bottles. These surface waters serve as receiving waters for effluent from their respective WWTPs. Chicken waste/litter samples were collected in sterile 300-mL Whirl-Pak® (Whirl-Pak, Madison, WI) bags from farms in Raleigh and Chapel Hill. 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 1: Environmental Sampling Sites

Type	Raleigh	Chapel Hill
Municipal Raw Sewage	Grab sample taken from screened influent at The City of Raleigh's Neuse River Resource Recovery Facility (NRRRF).	24-hour composite sample taken from Orange Water and Sewer Authority's (OWASA) Mason Farm Road Wastewater Treatment Plant.
Surface Water	Two Sites on the Neuse River; both on the Neuse River Greenway Trail upstream and downstream of NRRRF.	Samples from 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 were collected from the Lake Wheeler Road Test Farm Poultry Unit at NC State University's (NCSU) College of Agriculture and Life Sciences (CALS).	Excreted feces samples from chickens never administered antibiotics were collected from a small-scale, mixed-livestock farm in the Chapel Hill area.

Table 2: Numbers of Samples Collected from Individual Environmental Sampling Sites

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

Sample processing and analysis

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

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. Dilution factors were estimated based on previous results or else based on an initial dilution test 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 prior to dilution and/or analysis. Dilutions were performed immediately before analysis.

Processed samples were analyzed by membrane filtration 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 (n=479 [298 with and 181 without cefotaxime]); In addition, samples were analyzed using the compartment bag test (CBT) without and with 4 mg/L cefotaxime added to the proprietary media (by spiking each sample with 1 mL/100 mL of 100x stock solution to achieve 0.4 mg cefotaxime per 100-mL sample). Also, a new CBT2 (containing proprietary growth media that was pre-formulated to include 4 mg/L cefotaxime) was also used. All 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 [19]. Results were reported as presumptive *E. coli* and ESBL-Ec for assays without/with cefotaxime, respectively. Results from membrane filtration (MF) assays were reported in units of CFU/100 mL, and in units of most probable number (MPN/100 mL) concentrations for CBT assays. The greater number of samples with cefotaxime was used to ensure sufficient presumptive ESBL isolates for further characterization.

A subset of presumptive ESBL organisms were isolated for further characterization. Specifically, 1 to 5 presumptive ESBL-Ec colonies were selected from each positive TBX/cefotaxime plate (and liquid culture aliquots were removed from presumptive positive compartments of cefotaxime-containing CBTs) and were re-streaked for colony isolation on TBX media with 4 mg/L cefotaxime as an initial ESBL-Ec confirmation step. Colonies were picked at random from TBX plates with cefotaxime using a sterile loop. The exterior of positive compartments of CBTs were swabbed with 70% ethanol and the compartments were then pierced with a sterile syringe and needle and a drop of medium was withdrawn, then spotted onto TBX plates with cefotaxime and streaked as described above to obtain individual colonies after incubation. For media drawn from a CBT compartment and streaked onto a plate, it cannot be assumed that all colonies on the streaked plate are clonal. Colony isolates from re-streaked TBX plates were picked with a sterile loop, 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[1]. Stored isolates were

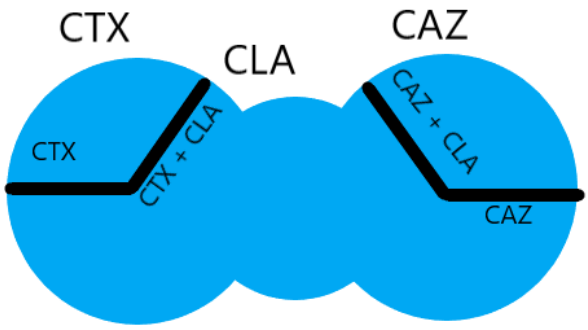
thawed and further characterized by Enteropluri® biochemical testing according to the manufacturer's instructions[5] and by Kirby-Bauer antibiotic sensitivity testing (for cefotaxime [CTX], Imipenem [IMP], ampicillin [AMP], Ceftazidime [CAZ], and vancomycin [VAN]) according to standard methods[1,4]. Isolates were confirmed as ESBL by Kirby Bauer testing using the criteria defined in the Tricycle Protocol using CTX, CAZ, CTX + clauvic acid (CLA), and CAZ + CLA paper discs. Where synergy between CAL and beta lactams was observed (Equations 1-2, Figure 1), strains were scored as ESBL. When the CTX zone of inhibition – the CTX + CLA zone of inhibition was >= 5 mm or the CAZ zone of inhibition – the CAZ + CLA zone of inhibition was >= 5 mm, the isolate was scored as a positive ESBL result [1]

Equation 1: ESBL =1 if (CTX + CLA) – CTX >= 5mm OR if (CAZ + CLA) – CAZ >= 5 mm

Equation 2: ESBL =0 if (CTX + CLA) – CTX < 5mm AND if (CAZ + CLA) – CAZ < 5 mm

Where (CTX + CLA) = the zone of inhibition (in mm) for the area equidistant from the CTX and CLA discs as shown in Figure 1, (CAZ + CLA) = the zone of inhibition (in mm) for the area equidistant from the CAZ and CLA discs as shown in Figure 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.

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



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, agricultural [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). *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 (MLOD) continuity correction was used to reduce bias for non-detects. Thus, a non-detect for a 100 mL undiluted sample would be scored as 0.5/100 mL, rather than 0 per 100 mL, to minimize bias and to enable log-transformation of count data where needed. 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 and location. Presumptive ESBL-Ec results are less robust indicators of ESBL status, but could be calculated for each sample aliquot collected. Confirmed ESBL phenotype is a more robust indicator of resistance, but could only be determined for the smaller subset of aliquots and isolates subjected to further characterization. Both presumptive and confirmed results were included in analyses and reported. Difference in median log-transformed, continuity-corrected concentrations/proportions between sample type and assay type (Table 3) was assessed by non-parametric Wilcoxon signed ranked-pairs tests. All analyses were conducted in GraphPad Prism version 9.5.

Results

Presumptive Proportion of *E. coli* that were ESBL Resistant

Proportions of *E. coli* colonies obtained on selective media that were presumptively ESBL-resistant were quantified. Normality tests did not indicate a good fit for normal distributions, and as a result nonparametric statistics were used (Tables S3 and S4, Figure S3 A&B). Table 4 presents the percentages of *E. coli* presumptively resistant to cefotaxime stratified by environmental 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 resistant was 8.5%. Wastewater had the highest percentage presumptively

resistant at 15%, followed by surface water and agricultural 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 (RLPF) in Raleigh and the Chapel Hill-area small-scale farm (CHPF), with total proportion presumptively resistant less than 1% (0.38% and 0.65% respectively).

Figures 2A-C and Table 3 show the distribution of presumptive ESBL-Ec CFU and MPN concentrations for samples by environmental sample type and analysis method, presented as box and whisker plots. The plots indicate that concentrations of presumptive ESBL-Ec CFU or MPN differed in the order Wastewater > Chicken waste Samples > Surface Water. Across assays, distributions only rarely spanned 2-4 orders of magnitude within each sample type, and most observations (>75%) were clustered within one order of magnitude from the median value.

Differences in the percentage of *E. coli* resistant to antimicrobial compounds between upstream and downstream surface water sampling sites is of considerable interest, as a potential means of investigating sources of ESBL-Ec . However, such differences cannot necessarily be attributed specifically to wastewater outflows[1]. In Raleigh, the Neuse River upper site (RLSW-US) had a percentage presumptively resistant of 5.18% while the Neuse River lower site (RLSW-DS) had a percentage presumptively resistant of 6.23%. In Chapel Hill, the Morgan Creek Trail upstream site (CHSW-US) had a percentage presumptively resistant of 1.89% 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 resulted in P-Statistics of 0.0002 for Raleigh and<0.0001 for Chapel Hill, which are statistically significant. .

Table 3: Occurrence of presumptive ESBL-Ec in environmental samples by sample type and assay method

Assay	Metric	Concentration Unit	WW	SW	AG	Overall
MF+C	Geo Mean	CFU/100 mL	5.57	1.87	0.86	
	Standard Deviation	log CFU/100 mL	1.26	0.648	1.10	
	Non-Detects	%	30.2%	42.1%	64.1%	41.8%
	Min	log CFU/100 mL	-0.30	-0.30	1.70	
	Max	log CFU/100 mL	6.27	2.06	5.70	
CBT +C	Geo Mean	CFU/100 mL	12.44	2.59	1.22	
	Standard	log CFU/100 mL	0.96	0.71	1.01	

	Deviation					
	Non-Detects	%	21.1%	37.4%	61.3%	36.1%
	Min	log CFU/100 mL	1.68	-0.30	2.70	
	Max	log CFU/100 mL	6	2	5.70	
CBT2	Geo Mean	CFU/100 mL	20.84	2.09	1.31	
	Standard Deviation	log CFU/100 mL	0.43	0.71	0.59	
	Non-Detects	%	0%	45.8%	61.5%	35.7%
	Min	log CFU/100 mL	4.98	-0.302	2.70	
	Max	log CFU/100 mL	6	2	4.13	
Overall	Geo Mean	CFU/100 mL	10.33	2,15	1.08	
	Standard Deviation	log CFU/100 mL	0.88	0.69	0.97	
	Non-Detects	%	41.6%	35.2%	35.0%	37.7%
	Min	log CFU/100 mL	-0.30	-0.30	1.70	
	Max	log CFU/100 mL	6.27	2.06	5.70	

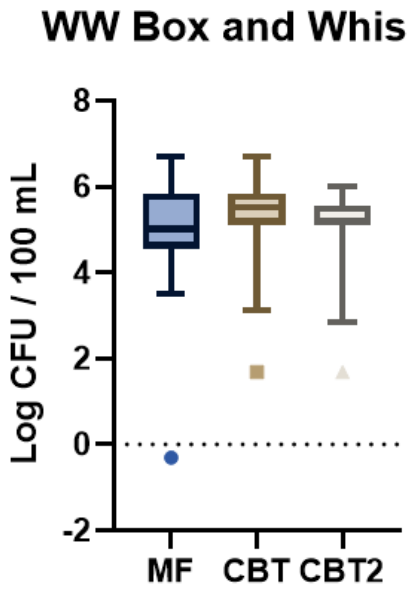


Figure 2A: Box and Whisker Plot of Wastewater Presumptive ESBL-Ec Concentration. Lines denote median log-transformed concentrations; boxes denote 1st and third quartile log concentrations; whiskers denote 95% confidence limits for log concentration values.

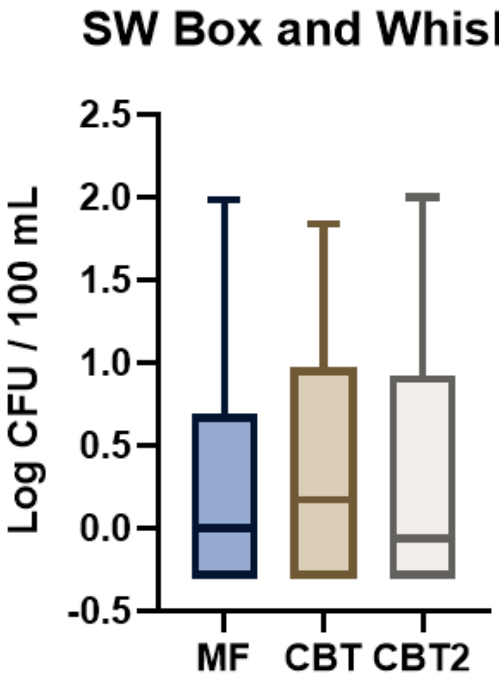


Figure 2B: Box and Whisker Plot of Surface Water Presumptive ESBL-Ec Concentrations. Lines denote median log-transformed concentrations; boxes denote 1st and third quartile log concentrations; whiskers denote 95% confidence limits for log concentration values.

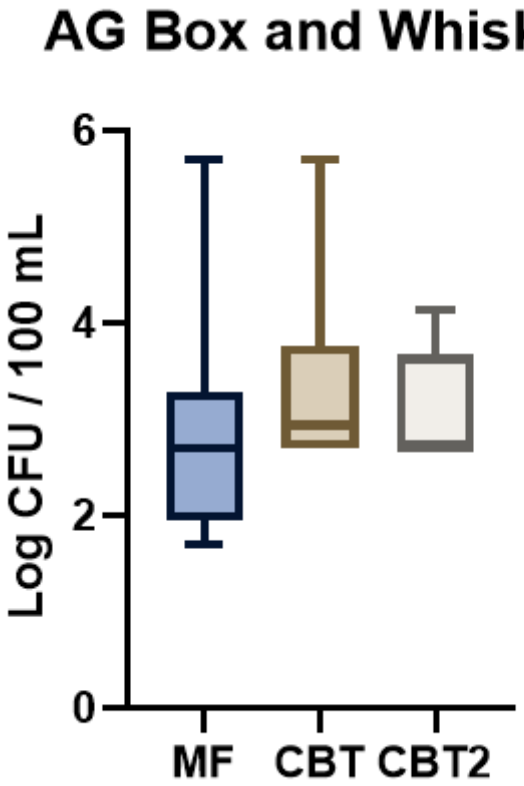


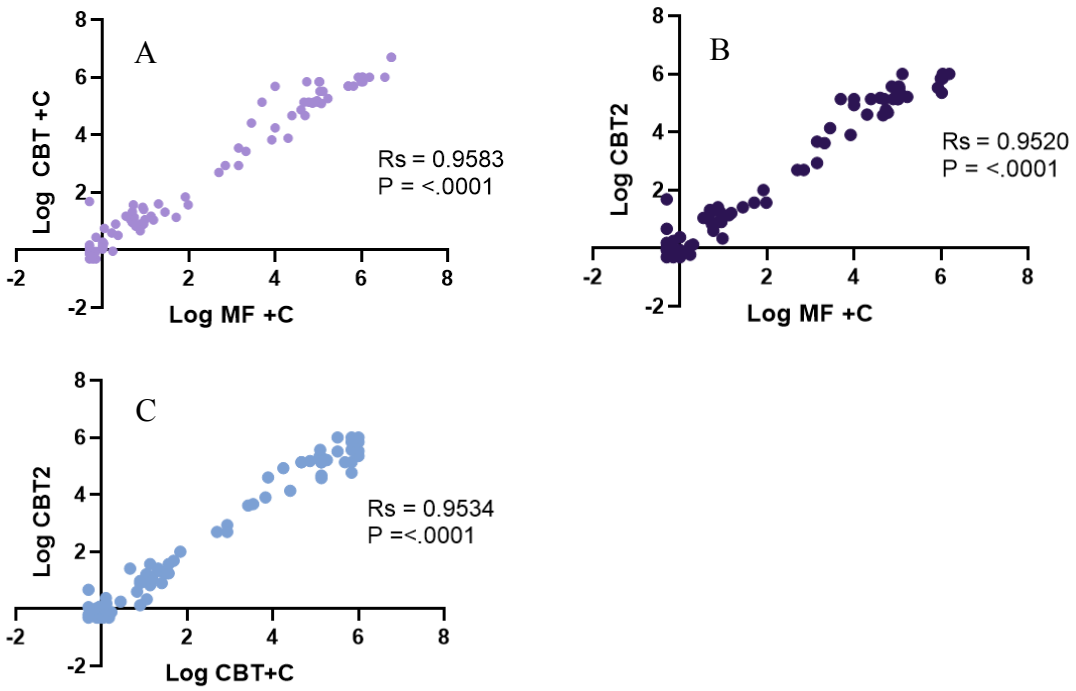
Figure 2C: Box and Whisker Plot of Poultry Waste Sample Presumptive ESBL-Ec Concentrations. Lines denote median log-transformed concentrations; boxes denote 1st and third quartile log concentrations; whiskers denote 95% confidence limits for log concentration values.

Table 4: Percentage of *E. coli* ESBL resistant in environmental samples (stratified by site)

	WW	SW (upstream)	SW (downstream)	AG	Up vs Down (P)
Raleigh	18.7%	5.2%	6.2%	0.3%	.0002***
Chapel Hill	12.3%	1.9%	15.0%	0.7%	.0001****
Overall	15.2%	4.4%	7.9%	0.5%	N/A

Differences in antibiotic resistance proportion of *E. coli*

The proportion of *E. coli* in each sample exhibiting 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 ($p<0.0001$ and $p=0.004$, respectively), but the two CBT methods do not significantly differ from each other ($p=0.090$). However, inspection of the results from each method suggests that the overall performances of the three methods are broadly comparable, as shown in Figure 3 A-C), with Rs values of 0.95.



Figures 3 A-C: Log-Log Plots of A) MF vs. CBT, B) MF vs. CBT2, and C) CBT vs. CBT2

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 described above (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 antimicrobials 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). Most (74.2%) 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 but there were no clear trends across both locations (Table S5).

Chicken Waste

Of Kirby Bauer analyses conducted on 52 chicken farm isolates from 7 samples, 50% of isolates were ESBL positive by Tricycle Protocol confirmation criteria; 100% of isolates from CTX+ assays and no isolates from CTX- negative assays (Table 6). Approximately 54% of chicken waste isolates were MDR: 100% of isolates from CTX+ assays and 8% of isolates from CTX- negative assays.

Table 5: Kirby Bauer antibiotic sensitivity results for presumptive *E. coli* isolates from environmental samples

Type	N (samples)	Media	n (isolates)	Resistance Characteristics						
				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%
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%

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 7)[5]. All of the other

organisms identified were Gram-negative: *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, 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.

Table 6. Enteropluri Isolate speciation results

Type	**N (samples)**	**Media**	**Confirmed *E. coli* (%)**				**n (isolates)**			
			Overall	MF	CBT	CBT2	Overall	MF	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

Discussion

Tricycle Protocol Adaptation and Evaluation

The Tricycle Protocol, 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 highly feasible to implement and sustain in this study. Because the Tricycle Protocol was designed largely for implementation in low- and middle- income countries (LMICs), some adaptations were required[1]. The Protocol calls for poultry 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 operation.

ESBL-Ec Occurrence

Overall, ESBL-Ec were found to be prevalent across waste sources. As expected, ESBL-Ec concentratrations were greatest in municipal wastewater and decreased from WW to poultry waste to surface waters. ESBL-Ec 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.

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, even though most of these isolates were not confirmed ESBL-positive in surface water and wastewater 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 the relatively fewer poultry waste samples analyzed.

The reason for the low ESBL confirmation rates using 4 mg/L cefotaxime are 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 controls 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[26]. 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 Tricycle Protocol 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 a standard WHO Tricycle Protocol. The CBT achieved comparable performance to MF and agar media culture for colonies to quantify presumptive ESBL-Ec in environmental samples. While results for a paired Wilcoxon signed rank test between the two culture tests were significantly different, log-log plots showed a high correlation of 95% ($p<0.05$) as evidence that the methods produce largely comparable results for the samples analyzed. Therefore, the CBT-based methods and media may be suitable alternatives to the more cumbersome MF methods for ESBL-Ec quantitation in environmental field applications currently implemented under the WHO TP.

The Tricycle Protocol is designed to be feasible in LMICs, but current MF methods require substantive infrastructure and capacity in addition to more time for preparation and analysis. MF media must be prepared in advance, sterilized, and poured into plates prior to sample analysis. Filtration requires a source of vacuum, a filtration assembly, and the means to sterilize it. Incubation at 44^{o}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 space[3,19]. 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 Tricycle Protocol in remote and/or low-resource settings[3,23,24].

Study Limitations

A limitation of this study was the adaptation of the Tricycle Protocol 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 *E. coli* isolates collected per sample vs. Tricycle protocol specifications is not likely to substantively 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 Tricycle protocol. However, the temporal and source (human/animal/environment) distribution of samples and isolates was more skewed than that prescribed in the Tricycle Protocol, 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 demonstrated that the Tricycle Protocol for ESBL *E. coli* detection, quantification and characterization in environmental samples can be successfully adapted to a North Carolina, USA context. Overall, the Tricycle Protocol 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: municipal wastewater>poultry fecal wastes>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 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.

The two novel candidate ESBL-Ec quantitation methods, the adapted CBT with added cefotaxime and the CBT2 (containing cefotaxime in its prepackaged medium), showed good overall agreement with the standard Tricycle Protocol MF agar medium method. These alternative methods appear suitable for use in the Tricycle Protocol 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 Tricycle Protocol 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.

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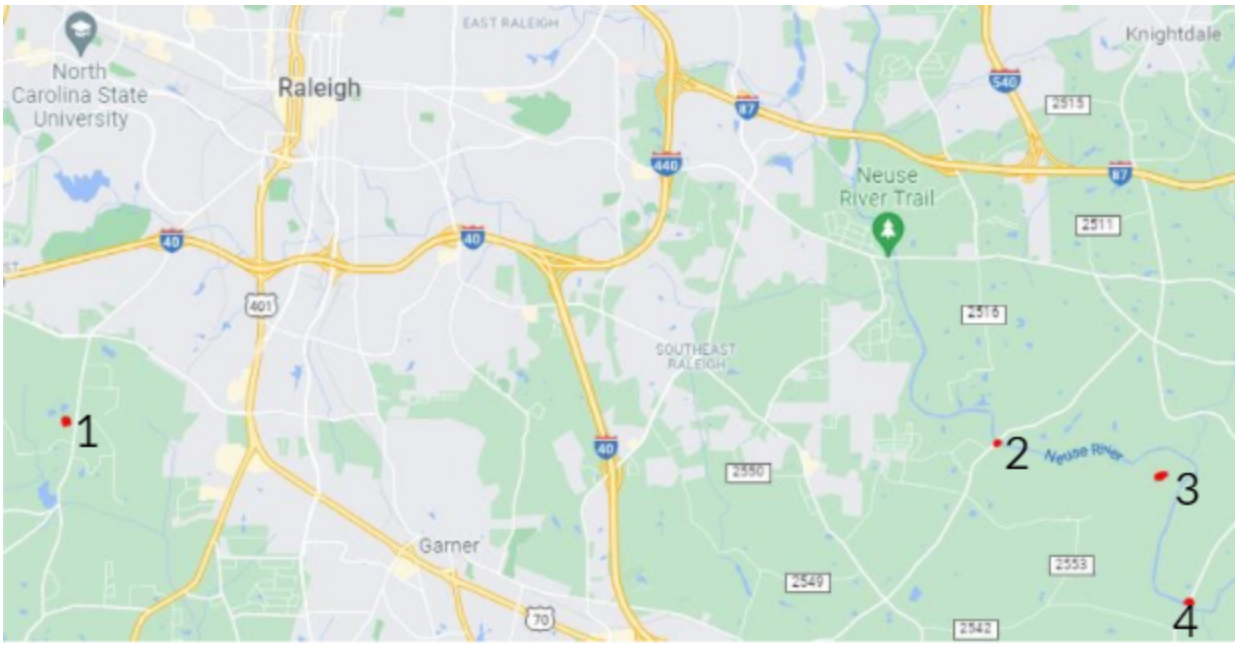
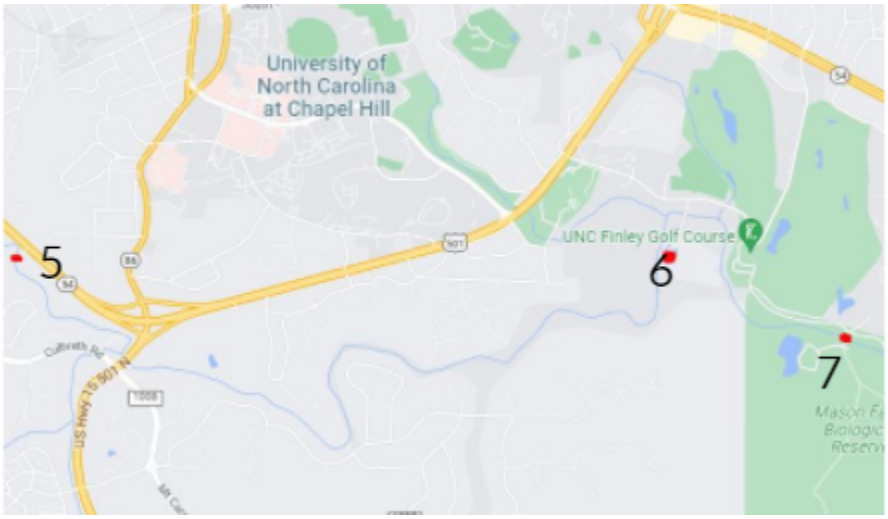
Supporting Information

Figure S1: Raleigh, NC Sites



1. Lake Wheeler Road Test Farm, 2. Upstream Neuse River Greenway Trail, 3. Neuse River Resource Recovery Facility, 4. Downstream Neuse River Greenway Trail

Figure S2: Chapel Hill, NC Sites



5. Upstream Morgan Creek Trailhead, 6. OWASA Mason Farm WWTP, 7. Downstream Morgan Creek Mason Farm Biological Reserve, Chapel Hill Farm Location not included

Figures S3 A and B: QQ plots of Continuous and Log-Transformed Data

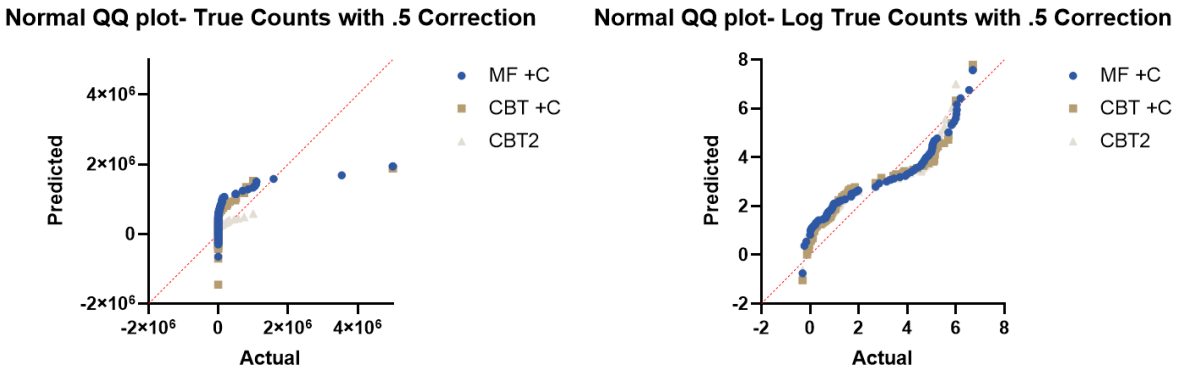


Table S1: Antibiotic Disk and Zone of Inhibition Breakpoints for E. coli

Antibiotic	Disk Concentration (ug)	Resistant (mm)	Intermediate (mm)	Susceptible (mm)
Imipenem (IMP)	10	<13	13-16	>16
Vancomycin (VAN)*	30	<12*	*	*
Ampicillin (AMP)	10	≤13	14-16	≥17
Cefotaxime (CTX)	30	<20**	**	>20**
Ceftazidime-clavulanate (CAZ CLA)	30-10	**	**	**
Cefotaxime-clavulanate (CTX CLA)	30-10	**	**	**
Ceftazidime (CAZ)	30	≤19**	20-22**	>22**

* *E. coli* has an intrinsic resistance to Vancomycin. In this test, Vancomycin serves as an additional phenotypic confirmation that the sample isolate is *E. coli*. [19]

** The bottom four antibiotics on the table are used as a combination disk diffusion method to test phenotypically for ESBL status. If the zone of inhibition for a combined disk (CAZ CLA or CTX CLA) is greater than or equal to 5 mm larger than their single antibiotic counterpart (CAZ and CTX respectively), then that sample is considered ESBL. [1]

Table S2: Analytical Methods - Sample Level

Normality Testing	Normality testing of raw data and log-transformed data with a correction* for non-detects by the Shapiro-Wilk Test. Generated QQ plots from both raw and log-transformed data. Analysis performed in Graphpad

	Prism
Hypothesis Testing- Methods Comparison	Wilcoxon signed ranked-pairs test on Log-Transformed data with a correction* for non-detects. Spearman Correlation Coefficients calculated and Log-Log plots generated. Analysis performed in GraphpadPrism.
Proportion Resistance	Proportion resistance by sample type and site calculated from average counts of membrane filtration followed by plating on TBX medium without or with with added cefotaxime and from CBT MPN results on chromogenic *E. coli* broth culture medium without and with cefotaxime . One-Sample Wilcoxon Test performed to test the significance of differences in proportion of ESBL-Ec resistant between sites.

562 ** Non-Detect Correction: Transform non-detect values from 0 to another value to allow for log-*
563 *transformation. Correction was recorded value (0) + 0.5 * Method limit of detection (MLOD).*
564 *In the case of all three tests MLOD was 1 CFU / 100 mL.*
565
566 *Table S3: Determination of Normality*

True Count with 0.5 Correction	MF + TBX agar with cefotaxime	CBT + cefotaxime	CBT2
Probability normal (Gaussian)	0%	0%	0%
Probability lognormal	100%	100%	100%
Likelihood ratio (LR)	0.000	1.133e-310	1.830e-231
1/LR	+infinity	+infinity	5.464e+230
Which distribution is more likely?	Lognormal	Lognormal	Lognormal

567
568 *Table S4: Normality Testing - Shapiro-Wilk Test results*

Log-Transformed with 0.5 MLOD Correction	MF +C	CBT +C	CBT2
W	0.8377	0.8440	0.8326
P value	<0.0001	<0.0001	<0.0001

Passed normality test (alpha=0.05)?	No	No	No
P value summary	****	****	****

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571 *Table S5: Kirby Bauer antibiotic sensitivity results for surface water isolates by site location and*
572 *position*

Location	Position	N (samples)	Media	n (isolates)	Resistance Characteristics						
					ESBL	MDR	CTX	IMP	AMP	CAZ	VAN
Raleigh	**All**	**14**	**Both**	**113**	**40.7%**	**73.5%**	**58.4%**	**0.0%**	**77.0%**	**61.1%**	**100.0%**
			CTX+	84	53.6%	94.0%	77.4%	0.0%	97.6%	77.4%	100.0%
			CTX-	29	3.4%	14.0%	3.4%	0.0%	17.2%	13.8%	100.0%
	Upstream	**7**	**Both**	**54**	**24.1%**	**77.8%**	**48.1%**	**0.0%**	**77.8%**	**59.3%**	**100.0%**
			CTX+	38	31.6%	100.0%	65.8%	0.0%	100.0%	73.7%	100.0%
			CTX-	16	6.3%	25.0%	6.2%	0.0%	25.0%	25.0%	100.0%
	Downstream	**7**	**Both**	**59**	**55.9%**	**69.5%**	**67.8%**	**0.0%**	**76.3%**	**62.7%**	**100.0%**
			CTX+	46	71.7%	89.1%	87.0%	0.0%	95.7%	80.4%	100.0%
			CTX-	13	0.0%	0.0%	0.0%	0.0%	7.7%	0.0%	100.0%
Chapel Hill	**All**	**17**	**Both**	**120**	**27.5%**	**75.0%**	**66.7%**	**0.0%**	**78.3%**	**41.7%**	**100.0%**
			CTX+	92	35.9%	95.7%	84.8%	0.0%	100.0%	52.2%	100.0%
			CTX-	28	0.0%	7.1%	7.1%	0.0%	7.1%	7.1%	100.0%
	Upstream	**8**	**Both**	**52**	**34.6%**	**67.3%**	**61.5%**	**0.0%**	**73.1%**	**25.0%**	**100.0%**
			CTX+	38	47.4%	92.1%	84.2%	0.0%	100.0%	34.2%	100.0%
			CTX-	14	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%
	Downstream	**9**	**Both**	**68**	**22.1%**	**80.9%**	**70.6%**	**0.0%**	**82.4%**	**54.4%**	**100.0%**
			CTX+	54	27.8%	98.1%	85.2%	0.0%	100.0%	64.8%	100.0%
			CTX-	14	0.0%	14.3%	14.3%	0.0%	14.3%	14.3%	100.0%

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