

Distribution of Tetracycline-Resistant Bacteria Within the Fort Phantom Hill Reservoir Watershed

Leah M. Sanchez  0009-0008-3367-2475

**Abilene Christian University
Abilene, TX USA**

Jennifer A. Hennigan  0009-0009-2839-7998

**Abilene Christian University
Abilene, TX USA**

Abstract

Tetracycline resistance genes have been reported to be abundant in bacteria in environments impacted by agriculture. The Fort Phantom Hill reservoir watershed in Jones and Taylor counties in Texas includes feedlots, rangeland, and row crop agriculture with manure-treated soils. We hypothesized that tetracycline-resistant coliforms could be present throughout the watershed. To investigate our hypothesis, samples were taken from the sediment of seven sites within the watershed between June 2022-August 2023. Tetracycline-resistant coliforms and non-coliforms were isolated from six out of seven sites. The highest relative abundance of tetracycline-resistant isolates was found at a site at Cedar Creek. Additionally, the highest relative abundance of tetracycline resistance among lactose fermenters was at a site at Elm Creek. From a representative sample of tetracycline-resistant isolates across all sites, the *tetB* genotype was the most common. Additionally, four multi-drug resistant strains of *Escherichia* were identified: CCN-113, CCN-251, CCN-109, and ELM-161. A resistance plasmid was extracted from CCN-251 carrying *dhfr1* and *sulII* resistance genes. Monitoring of the spread of antimicrobial resistance genes through surface water in the Fort Phantom Hill Watershed provides data to support the need for antibiotic stewardship in this region.

Keywords: antimicrobial resistance (AMR); tetracycline; watershed; surface water

Corresponding author email: jah20c@acu.edu

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Introduction

The World Health Organization has declared the spread of antibiotic resistance a leading crisis in medicine (1). Agriculture and human-based activities in urban areas are primary factors in the rise of antimicrobial resistance genes (ARGs) in the environment. ARGs from wastewater treatment plants, agriculture runoff, and animal husbandry can contaminate surface waters, then spread through horizontal gene transfer in the natural environment (2). Application of animal manures to land significantly increases the level of and diversity of ARGs in the soil. Additionally, these ARGs can persist in manure-treated soils for up to 120 days (3). In livestock waste, the most frequently detected classes of ARGs include those that confer resistance to tetracyclines (TET) and sulfonamides (4). Of the medically important drug classes approved for use in food-producing animals in the United States, tetracyclines comprised the highest percentage of total kilograms of antimicrobials sold in 2022 (65%; ~4 million kg) (5). A study in Portugal found that TET resistance genes were present in all multi-drug resistant (MDR) *Enterobacteriaceae* isolated from poultry, swine, and cattle manures (6). Likewise, a study in Korea found that all commensal *E. coli* strains isolated from cattle farms were resistant to tetracyclines (7).

The Fort Phantom Hill reservoir watershed is a mixed-use watershed containing agricultural land and urban areas in Jones and Taylor counties in west Texas in the United States. The area is largely dominated by agribusiness, including feedlots, rangeland, and row crop agriculture with manure-treated soils. Additionally, the land is used by oil industries for exploration, refining, and drilling operations. Ten creeks, three reservoirs, and the Fort Phantom Hill Reservoir make up the watershed. All of these lies within the Brazos River drainage system. Because of topology and urban development, rain in the watershed often produces excessive, rapid runoff and flash floods. Surface water flows from south to north along creeks, ending in the Fort Phantom Hill reservoir. Therefore, nonpoint source pollution poses a

concern for water quality. Row crop agriculture and the expansion of the city of Abilene increases the possibility of anthropogenic compounds entering streams and reservoirs through runoff (8). The goal of this study was to investigate the abundance of TET-resistant bacteria at seven sites throughout Fort Phantom Hill watershed (Figure 1). This study is unique in that it provides evidence of TET-resistance from multiple sites within a mixed-use watershed.

Materials and Methods

Sample collection

Two replicates of 50 grams of sediment and water were obtained from Kirby Lake (32.373,-99.728), Fort Phantom Hill reservoir (32.614, -99.676), Buck Creek (32.541, -99.709), Elm Creek (32.463, -99.777), Cedar Creek (32.471, -99.721 and 32.453, -99.721), and Lytle Creek (32.441, -99.715) using a previously described method (10). All sites were on public property. Samples were obtained at least five days after any rainfall event to reduce potential storm effects on the sites. Each site was sampled on at least two different days. Physical characteristics for sites were obtained using a Vernier LabQuest2 probe (Beavertown, OR), including pH, temperature, conductivity, turbidity, and dissolved oxygen. The metadata for each site was stored using the application Epicollect5 v 7.0.3 (Center for Genomic Pathogen Surveillance, University of Oxford)

Enrichment and selection of TET-resistant coliforms

Enrichment of the samples was completed in buffered peptone water as previously described (10). Serial dilutions were spread onto MacConkey agar (Sigma-Aldrich, USA) and MacConkey agar supplemented with 16 µg/mL tetracycline (IBI Scientific, Road Dubuque, IA) in accordance with the 2022 Clinical Laboratory Standards Institute concentration for tetracycline resistance for *Enterobacteriales* (11). Plates were incubated at 37°C for 18-24 hours before colonies were counted. Isolates were streaked to isolation on

MacConkey agar with 16 µg/mL tetracycline, grown in tryptic soy broth (Neogen, Lansing, MI) supplemented with tetracycline, and stored in 50% glycerol at -80°C.

Metabolic profiling and antibiotic screening

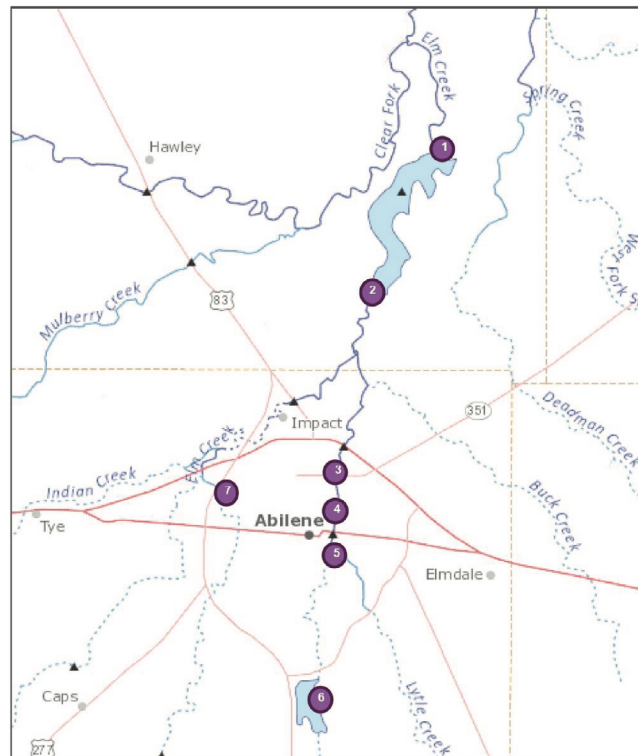
TET-resistant bacteria were inoculated in SIM deeps (HiMedia Laboratories, Kennett Square, PA) and citrate slants (Carolina Biological Supply, Burlington, NC), grown at 37°C for 18-24 hours before analyzing. Isolates were patched on tryptic soy agar (Neogen) supplemented with either 4 µg/mL cefotaxime (MP Biomedicals, Solon, OH) or µg/mL 32 nalidixic acid (Amresco, Solon, OH) and grown at 37°C for 18-24 hours.

Detection of tet resistance genes

Genomic DNA was isolated using a Wizard Genomic DNA purification kit (Promega, Madison, WI). Multiplex PCR of *tetA*, *tetB*, *tetC*, *tetD*, *tetM* and *tetO* genes was conducted using primers as previously described (12) with the following modifications. Briefly, reactions were completed for *tetA*, *tetM*, and *tetO* or *tetB*, *tetD*, and *tetC* genes using Platinum™ PCR Supermix (Thermo Fisher Scientific, USA) or Accuprime Supermix (Thermo Fisher Scientific) for 35 cycles of the following program: 94° for 5 min, 94° for 1 min, 55° for 1 min, 68° for 1:30 min, 68°C for 10 min. Reactions were visualized on 1-1.5% agarose gels using a 100 bp standard ladder (NEB).

Figure 1.

Map of sampling sites in the Fort Phantom Hill watershed



Seven sites were selected from six different lakes and creeks within the Fort Phantom Hill watershed: Fort Phantom Hill Reservoir (1), Buck Creek (2), Cedar Creek (3, North; 4, Central) Lytle Creek (5), Kirby Lake (6) and Elm Creek (7). A creek map was generated using USGS Steamer (9). Solid blue indicated streams and dashed lines indicate intermittent streams. Red solid lines represent roadways. Black triangles show USGS stream gages.

16S PCR and Sanger sequencing

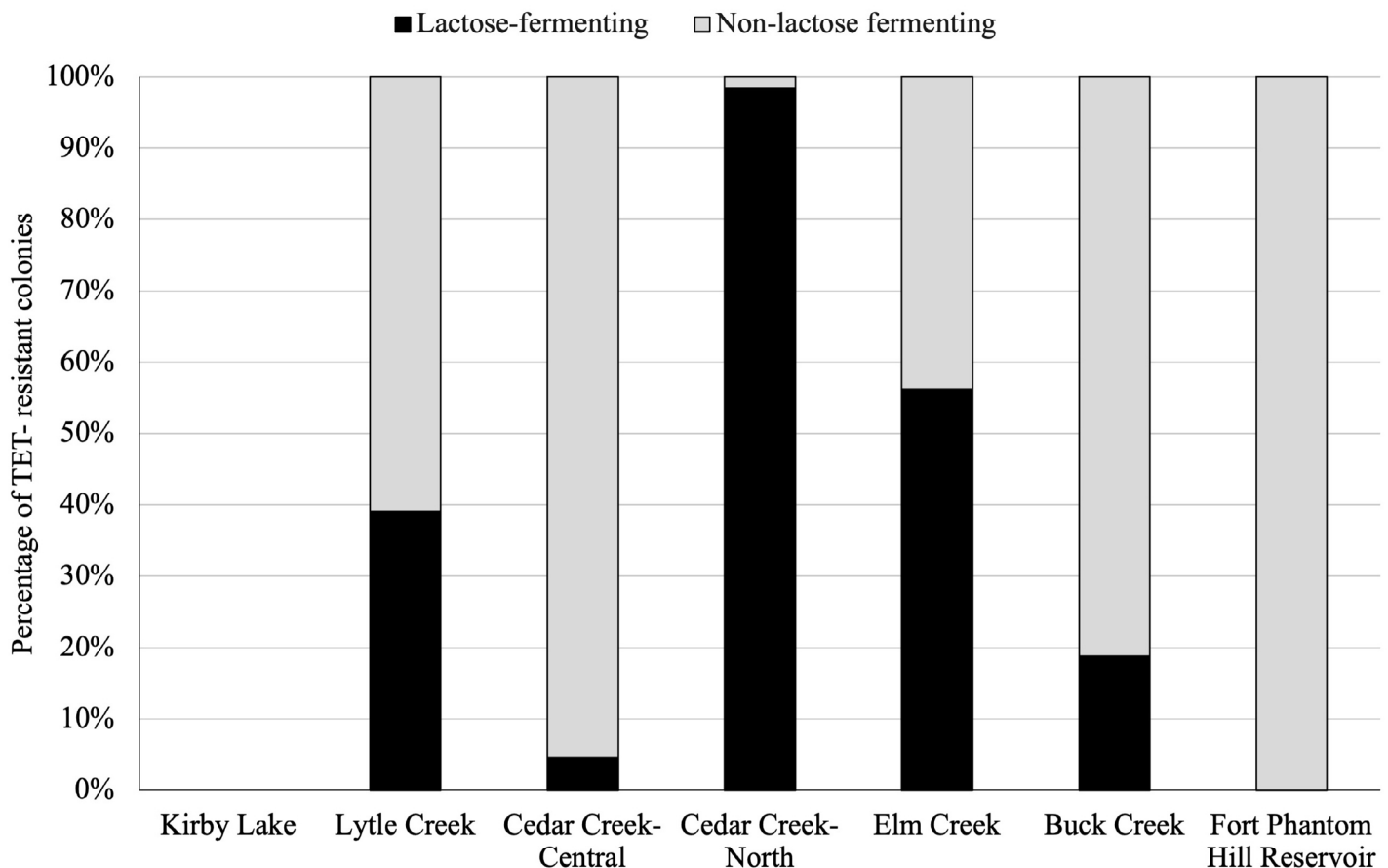
16S rDNA was amplified using 27F and 1492R primers as previously described (13). Amplicons were purified and concentrated using a Monarch® PCR & DNA Cleanup kit (NEB, Ipswich, MA) and submitted to MC BioLabs (San Francisco, CA) for Sanger sequencing. Sequences were analyzed and edited in Sequencher 5.4.6 (Gene Codes Corporation, Ann Arbor, MI) and queried in the NCBI 16S ribosomal RNA sequence database using Megablast. Genus level identities were assigned to isolates with consistent percent identities of no less than 95%.

96 well plate antibiotic susceptibility testing

Environmental TET-resistant isolates and *Escherichia coli* ATCC 25922 were inoculated in Mueller-Hinton broth (Sigma-Aldrich) and grown overnight at 37°C. Cultures were standardized to 0.12–0.14 OD₆₀₀ using a NanoDrop One (Thermo Scientific), equivalent to 0.5 McFarland Standard. In triplicate wells of a 96-well plate (CELLTREAT, USA), 100 uL of standardized cultures were added to 200 uL of Mueller-Hinton broth supplemented with antibiotics to produce the following final concentrations at 300 uL: 4 µg/mL cefotaxime, 16 µg/mL tetracycline, 32 µg/mL nalidixic acid, 64 µg/mL kanamycin (Alfa Aesar, Ward Hill, MA), 32

Figure 2.

Composition of the TET-resistant population per site



The proportion of TET-resistant lactose-fermenting (black) and non-lactose fermenting colonies (gray) was calculated as a percentage of total TET-resistant CFUs. The bars represent the average percentage of four samples for each site.

$\mu\text{g/mL}$ ampicillin (Sigma Aldrich), and $16 \mu\text{g/mL}$ trimethoprim (Sigma Aldrich). Concentrations are consistent with the 2022 Clinical Laboratory Standards Institute concentration for resistances for *Enterobacteriales* (Clinical and Laboratory Standards Institute (11). Plates were statically incubated at $37 \text{ }^\circ\text{C}$ for 24 ± 2 hours of growth and read at OD_{630} using a SmartReader™ 96 (Accuris, Edison, NJ). OD_{630} readings ≥ 0.25 were classified as resistant.

Plasmid isolation and sequencing

The plasmids from CCN-251 and CCN-113 were isolated using a Monarch™ Plasmid Miniprep Kit (NEB) and visualized on a 1.5% gel using a 10 kB supercoiled DNA ladder (NEB). Whole plasmid sequencing was performed by Plasmidsaurus (Eugene, OR) using Oxford Nanopore Technology with custom analysis and annotation.

Results

To determine the relative abundance of TET resistance in the Fort Phantom Hill Reservoir watershed, sites were selected to represent the ten creeks and three reservoirs that comprise the watershed (Figure 1). Not all creeks could be sampled due to lack of accessibility and drought conditions. Sites at Indian Creek to the northwest and Catclaw Creek were dry, except immediately following rainfall. Because bacterial and ARG loads have been shown to increase with rainfall events, these sites were excluded from this study (14, 15). Of the seven sites investigated, TET-resistant isolates were found at six sites; however, lactose-fermenting TET-resistant isolates were only isolated at five sites (Figure 2).

No TET-resistant isolates were obtained from the southernmost site, Kirby Lake, even though the number of colony forming units (CFUs) on MacConkey agar plates for the same samples did not differ from other sites. Only non-lactose fermenting isolates were cultured from the Fort Phantom Hill Reservoir. TET-resistant lactose-fermenting bacteria composed the majority of the TET-resistant population at two sites, Cedar Creek-North (98.4%) and Elm Creek

Table 1.

Mean relative abundance of TET-resistance at each site

	TET-Resistance in Gram-negatives	TET-resistance in lactose-fermenters
Kirby Lake	0%	0%
Lytle Creek	0.00019%	0.0005%
Cedar Creek-Central	0.14676%	0.01829%
Cedar Creek-North	0.00347%	0.06531%
Elm Creek	0.04718%	3.38440%
Buck Creek	0.00275%	0.00879%
Fort PhantomHill Reservoir	0.00012%	0%

The mean relative abundance of TET-resistant lactose-fermenting and non-lactose fermenting colonies was calculated as a percentage of resistant CFUs to total CFUs on MacConkey agar lacking tetracycline for four samples for each site.

(56.1%) (Figure 2). The site with the highest overall TET-resistance was Cedar Creek-Central (0.146%), followed by Elm Creek (0.047%) (Table 1).

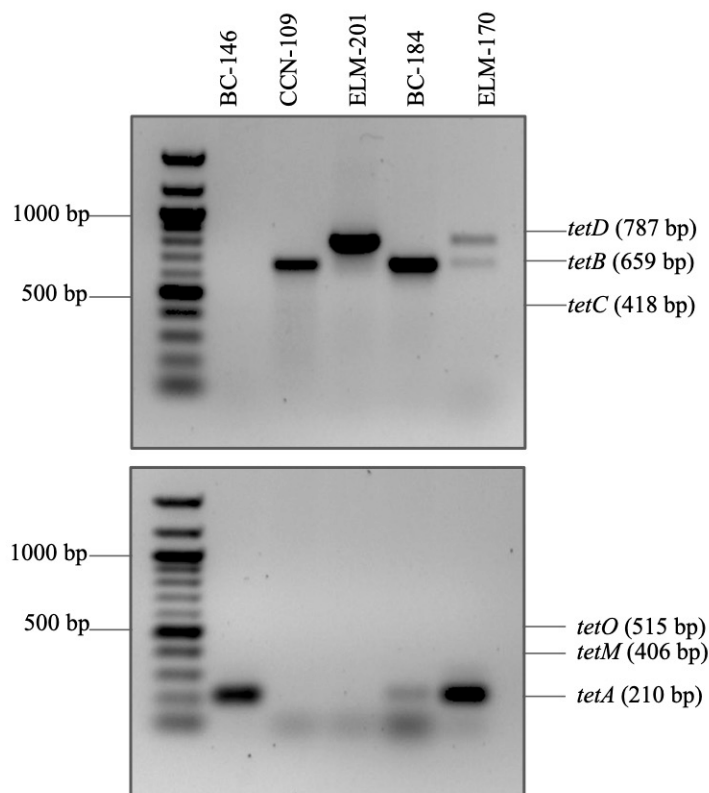
However, the site with the highest TET resistance in lactose-fermenters was Elm Creek (3.384%), followed by Cedar Creek-North (0.065%). Since the total number of TET-resistant potential coliform colonies for all sites was over 3000, every isolate could not be tested further. Instead, a random sample of up to ten well isolated colonies from each replicate was selected and metabolic profiles obtained for 148 TET-resistant isolates using the following criteria: nalidixic acid resistance, cefotaxime resistance, H₂S production, motility, indole production, lactose fermentation, and citrate utilization. Using these criteria, 23

different metabolic types were found throughout the watershed and 16S sequencing was performed to determine the genera of 37 representative strains for each metabolic type at each site (Table 2).

The most prominent genus identified throughout the watershed was *Escherichia*; however, TET-resistant strains of *Pseudomonas*, *Klebsiella*, *Providencia*, *Serratia*, *Enterobacter* and *Raoultella* were also identified. To determine the *tet* genotypes for the representative strains of each metabolic type, multiplex PCR was used to detect the presence of the *tetA*, *tetB*, *tetC*, *tetD*, *tetM*, and *tetO* genes. Of the 37 representative TET-resistant strains genotyped, isolates with the genotypes *tetA*, *tetB*, *tetD*, *tetA tetB*, and *tetA tetB tetD* were identified (Figure 3).

Figure 3.

tet genotypes represented in the Fort Phantom Hill Watershed



Multiplex PCRs for *tet* genes *tetB*, *tetD*, and *tetC* (top panel) and *tetO*, *tetM*, and *tetA* (bottom) panels are shown on a 1% agarose gel. The band sizes for each gene product are indicated. A 100 bp ladder (NEB) is included in the first lane for comparison.

Table 2.

Metabolic characteristics of representative TET-resistant strains from Fort Phantom Hill watershed sites.

Site	Genus	# of isolates	Rep Strain #	tetracycline resistance	nalidixic acid resistance	cefotaxime resistance	H ₂ S production	motility	Indole production	lactose fermentation	citrate utilization
Elm Creek (ELM)	<i>Klebsiella</i>	3	201								
	<i>Klebsiella</i>	10	218								
	<i>Escherichia</i>	26	227								
	<i>Escherichia</i>	1	237								
	<i>Escherichia</i>	1	170								
	<i>Enterobacter</i>	1	152								
	<i>Providencia</i>	2	164								
	<i>Escherichia</i>	1	161								
	<i>Pseudomonas</i>	1	215								
Cedar Creek - Central (CCC)	<i>Escherichia</i>	2	112								
	<i>Klebsiella</i>	3	268								
	<i>Providencia</i>	1	272								
	<i>Providencia</i>	4	ST-F4								
	<i>Providencia</i>	4	ST-F10								
	<i>Serratia</i>	17	275								
	<i>Klebsiella</i>	3	267								
	<i>Providencia</i>	1	271								

Positive characteristics are indicated by black squares, negative results are indicated by white squares, and ambiguous results are indicated by gray squares. Genus of representative strain for each metabolic type is listed. FP, Fort Phantom Hill Reservoir

Table 2 continues "Serratia" on page 3131.

Table 3.*tet* genotypes identified at each site

	<i>tetA</i>	<i>tetB</i>	<i>tetD</i>	<i>tetA tetB</i>	<i>tetA tetB tetD</i>	Undetermined
Elm (n=9)	0.00%	22.22%	22.22%	33.33%	11.11%	11.11%
Cedar Creek- Central (n=8)	0.00%	100.00%	0.00%	0.00%	0.00%	0.00%
Fort Phantom Hill Reservoir (n=1)	0.00%	100.00%	0.00%	0.00%	0.00%	0.00%
Buck (n = 9)	33.33%	11.11%	0.00%	44.44%	11.11%	0.00%
Cedar Creek-North (n=5)	20.00%	80.00%	0.00%	0.00%	0.00%	0.00%
Lytle (n=5)	0.00%	20.00%	0.00%	80.00%	0.00%	0.00%
Total (n=37)	10.81%	45.95%	5.41%	29.73%	5.41%	2.70%

The percentage of *tet* genotypes for representatives of each metabolic group at each site are shown (n=37).

Genotypes including *tetC*, *tetM*, or *tetO* genes were not detected in any isolate. None of the six genes were detected in one isolate, ELM-164, indicating that it harbors a different *tet* gene. Of the represented group, the *tetB* genotype was the most abundant at 45.95% (17/37) and was present at all sites (Table 3).

The *tetA tetB* genotype was the second most abundant at 29.73% (11/37). Two isolates harboring *tetD* only were also detected at Elm Creek; *tetD* was found in combination with *tetA* and *tetB* at Elm Creek and Buck Creek. Altogether, 35.14% (13/37) of the representative isolates carried more than one *tet* resistance gene. The genotype found in the most different genera was *tetB*, being detected in *Escherichia*, *Enterobacter*, *Klebsiella*, *Providencia*, *Serratia*, and *Raoultella*. In contrast, the *tetD* genotype was only found in *Klebsiella*.

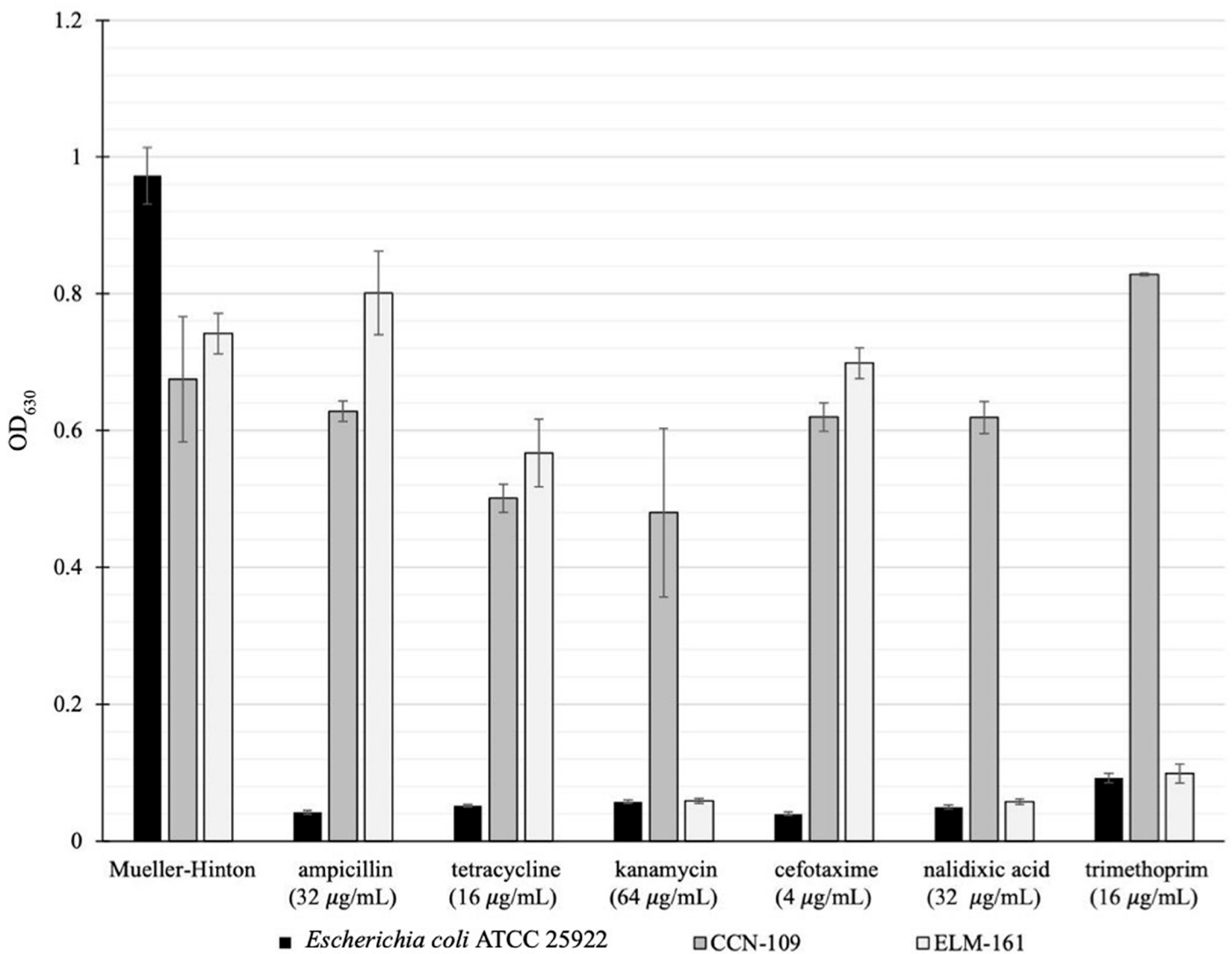
When the 148 TET-resistant isolates were screened for multi-drug resistance, seven isolates grew on MacConkey agar supplemented with cefotaxime and nalidixic acid: one *Escherichia* isolate (CCN-109) and six *Pseudomonas*. Additionally, one *Escherichia* isolate (ELM-161) showed resistance to cefotaxime only. The resistance profiles of these two isolates were further investigated using antibiotic sensitivity microdi-

lution assays. Isolate CCN-109 shows additional phenotypic resistance to ampicillin, kanamycin, and trimethoprim (Figure 4).

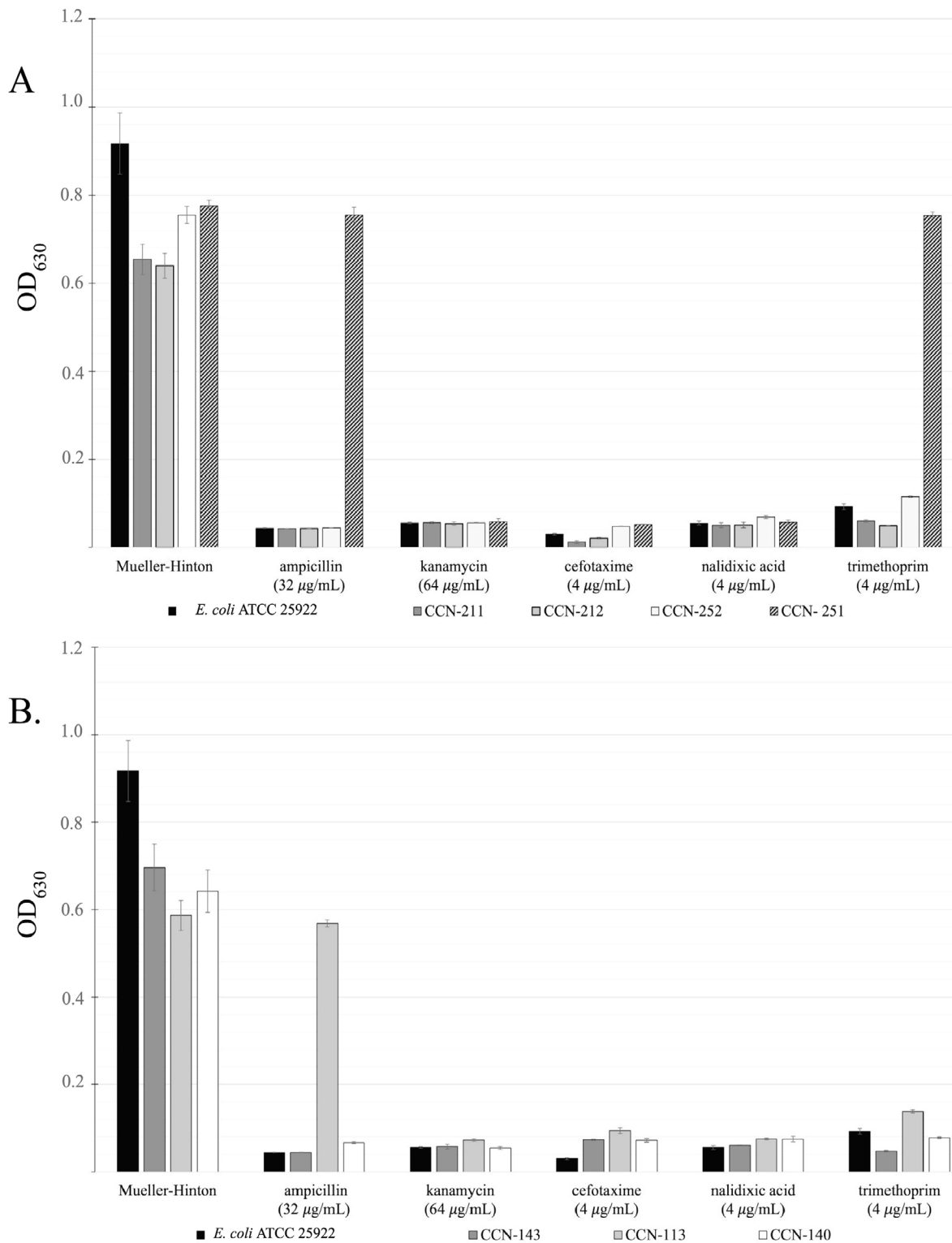
ELM-161 was further determined to be resistant to ampicillin only. Since CCN-109 appeared to have a unique resistance profile, other *Escherichia* isolates from the Cedar Creek-North site were further investigated to determine if others showed MDR phenotypes. CCN-251 showed additional resistances to ampicillin and trimethoprim, and CCN-113 showed an additional resistance to ampicillin only (Figure 5). Other *Escherichia* isolates were not phenotypically resistant to the other antibiotics tested.

Because ARGs can be transmitted horizontally, we investigated whether CCN-109, CCN-113, and CCN-251 strains harbored small plasmids that are responsible for these phenotypes. Using a plasmid isolation kit, we found that CCN-251 carried a 6.7 kb plasmid and CCN-113 carried a 4.6 kb plasmid (Figure 6A and B, respectively), but no plasmid was detected in CCN-109 using these methods.

The plasmid isolated from CCN-251 contains *sulII* and *dfrI* genes, which encode sulfonamide-resistant dihydropteroate synthase and

Figure 4.*Antibiotic susceptibility of CCN-109 and ELM-161*

OD₆₃₀ readings are shown for *E. coli* ATCC 25922, CCN-109, and ELM-161 grown in Mueller-Hinton with and without antibiotics at concentrations in accordance with CLSI breakpoints for *Enterobacteriales* (11). Standard deviations for three replicates are indicated.

Figure 5.*Antibiotic susceptibility of Escherichia isolates from Cedar Creek-North*

OD₆₃₀ readings are shown for *E. coli* ATCC 25922 and *Escherichia* isolates from Cedar Creek- North. All were grown in Mueller-Hinton broth with or without antibiotics at concentrations in accordance with CLSI break-points for *Enterobacteriales* (11). Standard deviations for three replicates are indicated.

trimethoprim-resistant dihydrofolate reductase enzymes, respectively. The *aphE* gene on the plasmid is incomplete, but encodes an aminoglycoside phosphotransferase which would inactivate aminoglycoside antibiotics like kanamycin. The plasmid from CCN-113 harbors the *fepE* gene, but no ARGs.

Conclusions

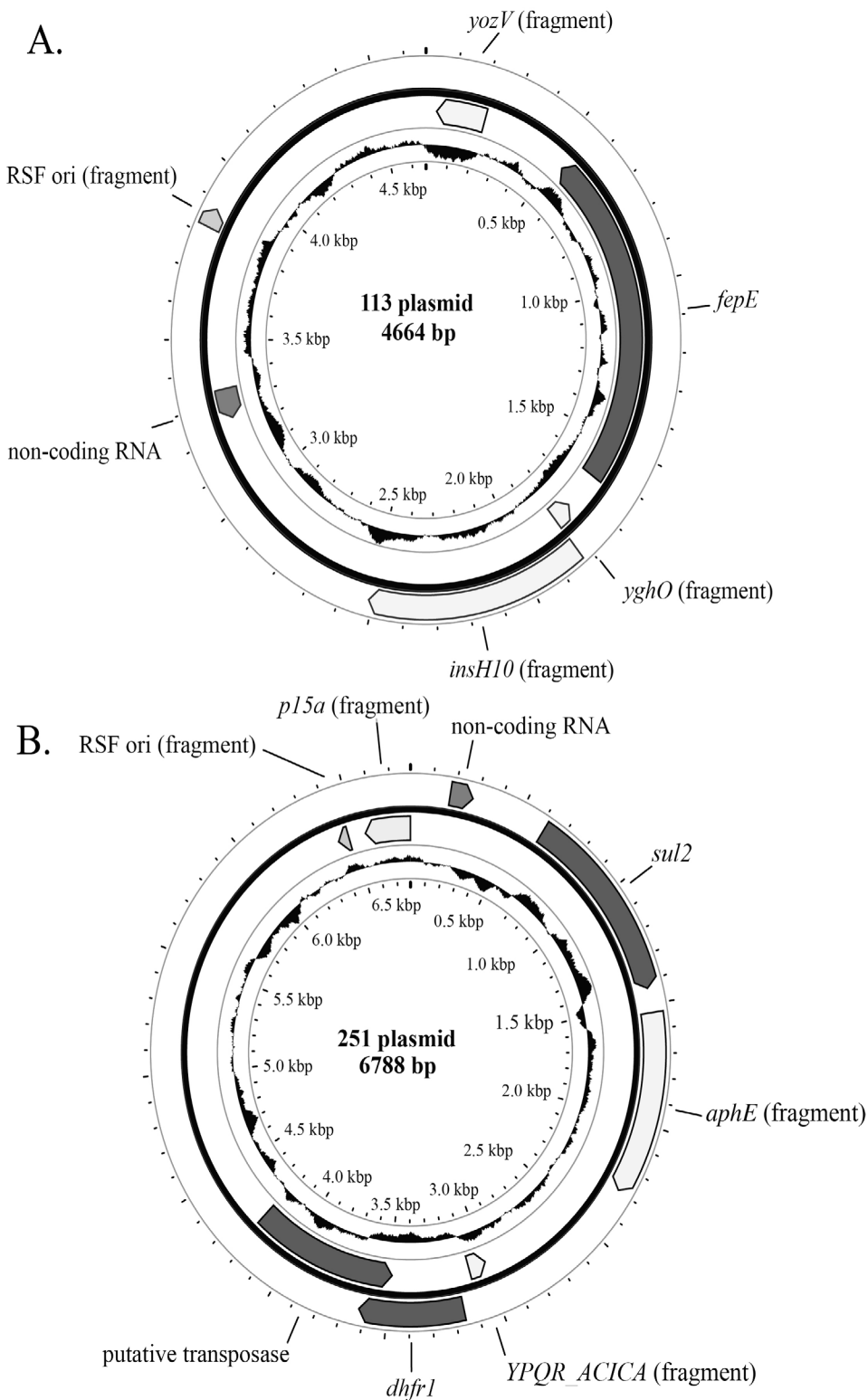
Our investigation of the Fort Phantom Hill Reservoir watershed found a low level of TET resistance throughout, consistent with our hypothesis. The highest relative percentage of TET-resistant lactose-fermenters was 3.38% at a central location where Elm Creek flows through a neighborhood. A correlation was not observed between greater TET-resistance and the direction of waterflow in the watershed (Figure 2). Reported frequencies of TET resistance in environmental surface water samples varies; for example, in *E. coli* from the water and sediments of two rivers in Austria, the frequency was between 1% and 11% of isolates (17). Therefore, TET-resistance in the Fort Phantom Hill watershed was similar to or less than that found in other locations. While no sites were on active farmland, all sites were no more than 8 km downstream of land zoned as agriculture-open space by the city of Abilene. Future studies comparing TET-resistance in sediments from ponds on nearby ranchland would provide a valuable comparison for our data set.

Most of the representative TET-resistant isolates that were genotyped in this study carried *tetA* and/or *tetB* genes (Table 3), which is consistent with a previous report that found *tetB* was the most frequent resistance gene in non-clinical *E. coli* isolates from animal and human sources (63%), and *tetA* was the second most frequent (35%) (18). Other studies also found that *tetA* and *tetB* were the most common *tet* resistance genes in commensal *E. coli* isolates from cattle (7) and *E. coli* from meat and meat products (19). The genotypes *tetA*, *tetB*, *tetC*, *tetO*, *tetW*, and *tetM* have all been detected in hospital wastewater collected from wastewater treatment plants, and *tetA*, *tetQ*, and *tetW* have been detected in municipal wastewater

(2). Other studies show the average abundance of *tetM* to be higher in manure and wastewater samples than *tetA* or *tetB* (20). Perez-Valera and colleagues found that the treatment of soils with manure increased the abundance of *tetM*, and, moreover, *tetM* most likely originated from the manure (21). In a study that compared the presence of genes in the outlets of manure applied catchments, *tetM* was significantly higher than non-manure catchments (22). The *tetM* gene was not detected in any isolate in our study.

Of the isolates that were genotyped in this study, 35.14% (n=13) carried more than one *tet* gene (Table 3). In contrast, another study found that only 8.7% of TET-resistant *E. coli* cattle commensals carried more than one *tet* gene. The presence of more than one gene for TET resistance was thought to indicate selective pressure due to the high level of tetracycline in an environment (7). Further investigation is needed to determine concentration of tetracycline in the Fort Phantom Hill surface water and if the isolates with more than one *tet* gene are more resistant to tetracycline; these isolates were not challenged with tetracycline concentrations greater than 16 µg/mL. Additionally, since the methods in this study only tested viable isolates that were phenotypically TET-resistant, it is possible that additional non-functional TET genes in the populations were missed.

In addition to TET resistance, MDR resistant *E. coli* were also recovered, including two isolates that carried small non-conjugative plasmids (Figure 6). The *dhfr* gene encoded in the plasmid recovered from CCN-251 provides an explanation for its phenotypic trimethoprim resistance (Figures 5 & 6). Likewise, the presence of *sulIII* would be expected to confer resistance to other sulfonamides but must be tested experimentally. Plasmids encoding both *sul* and *dhfr* genes have been readily isolated from sulfamethoxazole and trimethoprim-resistant *E. coli* in stream water. Moreover, these *E. coli* isolates were found to harbor multiple *sul* and *dhfr* genes, which is likely due to the influence of sub-inhibitory concentrations of trimethoprim and sulfamethoxazole

Figure 6.*Plasmid maps of plasmids isolated from Escherichia isolates*

Plasmid maps generated from plasmids isolated from *Escherichia* isolates 113 (A) and 251 (B). GC content is mapped in black, genes in dark gray, gene fragments in white, and origins of replication in gray. Plasmid maps were generated using PlasMapper 3.0 (16).

found in the water (23). The association of *sul* and *dhfr* genes on mobile elements, such as the CCN-251 plasmid isolated in this study, is highly relevant to the spread of resistance in aquatic environments like the Fort Phantom Hill Watershed and has a potential impact on human health and agriculture.

In contrast, sequencing of the plasmid recovered from CCN-113 did not reveal any ARGs, only one full-length coding sequence for *fepE*. In pathogenic *E. coli* O157:H7, the FepE protein (also called Wzz_{FepE}) is responsible for very long O-antigen chain lengths in lipopolysaccharide (>80 repeat units) (24). In both *Salmonella typhimurium* and *Shigella flexneri*, Wzz_{FepE} and homologue clD_{pHS-2} , respectively, were found to be essential for serum resistance (25, 26). Importantly, FepE was found to be positively selected in uropathogenic *E. coli* (UPEC) clinical isolates (27). More investigation of CCN-113 is necessary to determine the contribution of the plasmid-borne *fepE* to its potential virulence and to conduct core genome multilocus sequence typing analysis. MIC values for all isolates with the antimicrobial agents tested must be determined to follow-up on MDR isolates, particularly CCN-109 and ELM-161.

Of notable interest is the mechanism of cefotaxime resistance in these isolates since resistance is often due to the production of extended-spectrum beta-lactamases (ESBLs). Certain ESBLs, such as bla_{CTX-M} , bla_{TEM} and bla_{SHV} are associated with clinical infections caused by Enterobacterales, with bla_{CTX-M} being the most prevalent type. The highly successful mobilization of ESBL genes on conjugative plasmids has led to the rapid spread of ESBL-producing Enterobacterales globally over the last ten years (28). ESBL-producing Enterobacterales are not limited to the clinic, but have been detected in livestock, wildlife, companion animals, wastewater, environmental waters, and healthy carriers. Considering the data on the presence of ESBLs in aquatic environments are lacking in North America, more research should be conducted to examine the prevalence of ESBL-producing Enterobacterales in surface waters (29). We recommend additional studies

be completed on the prevalence of ESBLs within the Lake Fort Phantom Hill Reservoir watershed, particularly in areas impacted by agriculture, to further elucidate their impact on the region.

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