A Comprehensive Account of *Escherichia coli* Sequence Type 131 in Wastewater Reveals an Abundance of Fluoroquinolone-Resistant Clade A Strains

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ABSTRACT In the ten years since its discovery, the *Escherichia coli* clone sequence type 131 (ST131) has become a major international health threat, with the multidrug-resistant and extended-spectrum β-lactamase (ESBL)-producing clade C emerging as the globally dominant form. ST131 has previously been isolated from wastewater; however, most of these studies selectively screened for ESBL-producing organisms, thereby missing the majority of remaining ST131 clades. In this study, we used a high-throughput PCR-based screening strategy to comprehensively examine wastewater for the presence of ST131 over a 1-year period. Additional multiplex PCRs were used to differentiate clades and obtain an unbiased account of the total ST131 population structure within the collection. Furthermore, antimicrobial susceptibility profiles of all ST131-positive samples were tested against a range of commonly used antibiotics. From a total of over 3,762 *E. coli* wastewater samples, 1.86% ($n = 70$) tested positive for ST131, with the majority being clade A isolates. In total, 63% ($n = 44$) were clade A, 29% ($n = 20$) were clade B, 1% ($n = 1$) were clade C0, 6% ($n = 4$) were clade C1, and 1% ($n = 1$) were clade C2. In addition, a very high rate of resistance to commonly used antibiotics among wastewater isolates is reported, with 72.7% ($n = 32$) of clade A resistant to ciprofloxacin and high rates of resistance to gentamicin, sulfamethoxazole-trimethoprim, and tetracycline in clades that are typically sensitive to antibiotics.

IMPORTANCE ST131 is a global pathogen. This clone causes urinary tract infections and is frequently isolated from human sources. However, little is known about ST131 from environmental sources. With the widely reported increase in antibiotic concentrations found in wastewater, there is additional selection pressure for the emergence of antibiotic-resistant ST131 in this niche. The unbiased screening approach reported herein revealed that previously antibiotic-sensitive lineages of ST131 are now resistant to commonly used antibiotics present in wastewater systems and may be capable of surviving UV sterilization. This is the most comprehensive account of ST131 in the wastewater niche to date and an important step in better understanding the ecology of this global pathogen.

KEYWORDS *E. coli* ST131, wastewater, clade distribution, antibiotic resistance

*E*scherichia coli sequence type 131 (ST131) was jointly uncovered in 2008 in North America (1), Europe (2, 3), and Asia (4). This extraintestinal pathogenic *E. coli* (ExPEC) is responsible for millions of urinary tract infections (UTI) and bloodstream infections (BSI) annually (5). Recent bioinformatic and phylogenetic studies have categorized ST131 into five distinct lineages or clades (6). These clades are largely defined by alleles

of the *fimH* gene, as follows: (i) clade A ($fimH_{41}$), (ii) clade B ($fimH_{22}$), and clade C ($fimH_{30}$), further characterized into (iii) clade C0, (iv) clade C1, and (v) clade C2 (also called *H*30, *H*30R1, and *H*30Rx, respectively) based on the absence or presence of bla_{CTX-M} alleles.

In less than a decade, this single clone is now recognized as one of the most successful human pathogens (5), with a global distribution on a pandemic scale (2, 7). A key factor in ST131's global success is due to its ability to acquire resistance to antibiotics (2, 5, 6, 8). The rising prominence of CTX-M15 production and fluoroquinolone resistance among *E. coli* has been attributed to the clonal expansion of clade C ST131 (9). Studies have correlated the strain's evolution of resistance to fluoroquinolones in the 1980s, when this class of antibiotics was used heavily in both human and veterinary medicine (6, 10). Additionally, ST131 has been found to acquire plasmids conferring resistance to β-lactam antibiotics and, more recently, carbapenemases (11, 12).

Despite advances in our knowledge of the genetics and evolution of ST131, progress has been comparatively slower understanding the strain's epidemiology. Phylogenetic analysis of extensive ST131 collections have been unable to identify temporal or geographical clustering (6, 8, 13). ST131 appears to be abundantly distributed across geographical locations (2, 8), displays wide host species range (14–16), and spans potentially vast ecological environments (17), suggesting ST131 is a host generalist pathogen capable of frequent interspecies movement (13). However, large data sets are often overwhelmingly comprised of ST131 strains isolated from human clinical samples (6, 8, 13). The addition of more strains from a wider range of environments is likely to improve strain databases, such that more accurate epidemiological conclusions can be drawn from phylogenetic studies.

As an ecological niche, wastewater is a potentially rich source of ST131 for a number of reasons. (i) Although ExPEC strains mediate their pathogenicity outside the colon, the habitat of *E. coli* is in the gastrointestinal tract of mammals, the contents of which readily enter urban wastewater systems. (ii) Strains of *E. coli* are known to survive in wastewater for long periods (18–20). (iii) As an environmental niche, wastewater is an open ecosystem with consistent low levels of exposure to long half-life antibiotics, such as fluoroquinolones, that can persist at concentrations as high as the milligram per liter range in wastewater (21–23). This coexistence mediates selective pressure for the evolution of antibiotic-resistant variants of highly adaptable *Enterobacteriaceae* like *Klebsiella* spp. and ST131 in these environments. Unsurprisingly, antibiotic-resistant strains of ST131 have been isolated from wastewater in the Czech Republic (17, 24), Japan (25, 26), and France (27). However, these studies selectively screened for extended-spectrum β-lactamase (ESBL)-producing organisms, thereby omitting antibiotic-sensitive lineages of ST131 like clades A and B from analysis, giving a biased and potentially misleading representation of the true extent of ST131 in the environment.

Therefore, we hypothesized that an unbiased screen of wastewater samples would uncover a more accurate representation of the ST131 population in an urban wastewater system. Consequently, the objectives of this study were to use an unbiased high-throughput PCR-based screen to detect ST131 in wastewater, determine the relative distribution of clades among ST131-positive samples, and determine the resistance profiles to commonly used antibiotics among isolates collected over an entire year.

RESULTS

Total number of samples screened. In total, 4,083 sewage samples containing a mixed population of microorganisms were collected at various points in the treatment process from three different wastewater treatment plants in Calgary, Canada over a 1-year period. Of these, 3,762 were found to contain *E. coli* as visualized on CHROMagar plates, with the remainder consisting of other *Enterobacteriaceae*, including *Klebsiella*,
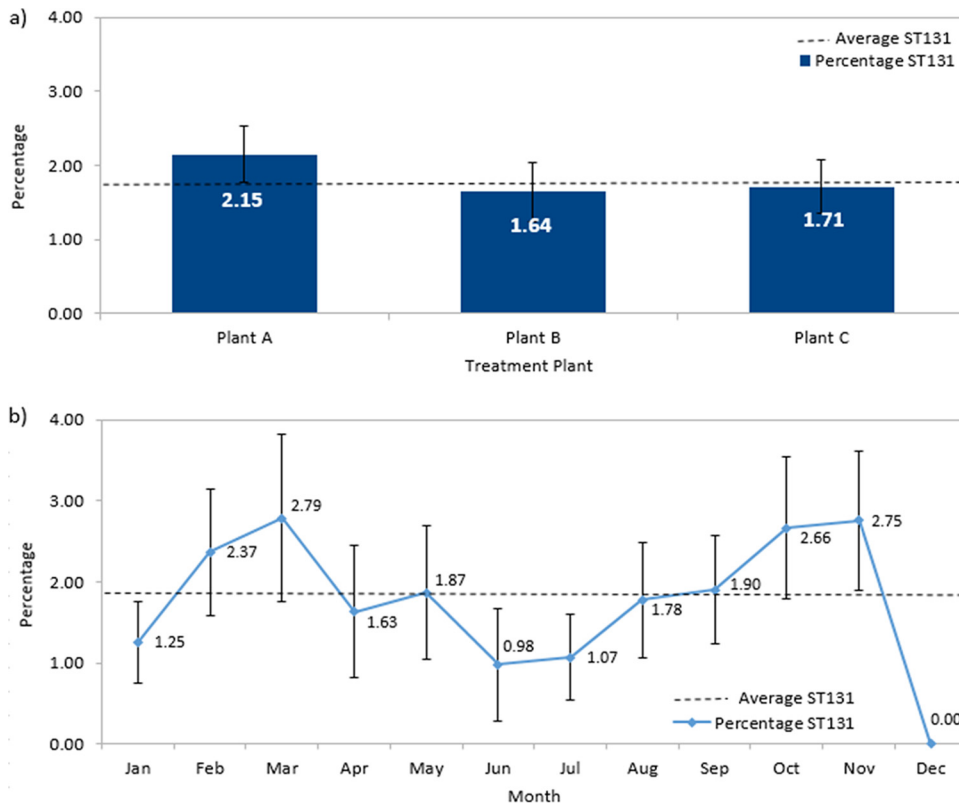
FIG 1 (a) Percentage ST131 of total *E. coli* samples for the three wastewater plants in Calgary. There was no difference in the number of ST131 isolates collected from the three plants. (b) Percentage ST131 of total *E. coli* samples identified in wastewater over time. The average ST131 detection rate is shown as a broken line. Error bars represent standard error in all cases.

Enterobacter, *Citrobacter*, or *Serratia*. Of the total *E. coli* population, 1.86% ($n = 70$) tested positive for ST131.

There were no significant differences between the ST131 collected from the three different wastewater treatment plants servicing the city of Calgary (Pearson χ^2 test, $\chi = 1.084$, df $= 2$, $P = 0.582$) (Fig. 1a). When ST131 strains are displayed as a percentage of total *E. coli* collected per month over 2015, ST131 detection remained relatively constant around the mean 1.86% mark throughout the year (Pearson χ^2 test, $\chi = 9.608$, df $= 11$, $P = 0.566$) (Fig. 1b), with the exception of December, where the absence of ST131 is likely due to less frequent sample collection during the holiday season. This is in line with other studies that considered seasonality within their data set when detecting *E. coli* from wastewater (28, 29) but in contrast to studies sampling directly from untreated water (30, 31), potentially highlighting the efficiency of wastewater treatment plants in maintaining a consistent reduced rate of *E. coli* from effluent.

Relative distribution of clades among ST131-positive samples. Among the 70 ST131-positive samples, 62.86% ($n = 44$) were clade A, 28.57% ($n = 20$) were clade B, 1.43% ($n = 1$) were clade C0, 5.71% ($n = 4$) were clade C1, and 1.43% ($n = 1$) were clade C2 (Fig. 2 and Table 1). We compared the relative distribution of ST131 clades isolated from wastewater to an independent study that collected ST131 from bloodstream isolates of infected individuals, conducted in the same geographical location over a similar time period (32). Strains from the clinical study were collected from bloodstream-infected individuals that presented to a centralized laboratory in Calgary over 2016 and were analyzed via whole-genome sequencing. Despite samples being collected from different sources, this is the most appropriate analysis due to the unbiased screening procedure performed in both studies. This study reported a relative distribution of 11.56% ($n = 17$) for clade A, 7.48% ($n = 11$) for clade B, 2.04% ($n = 3$) for
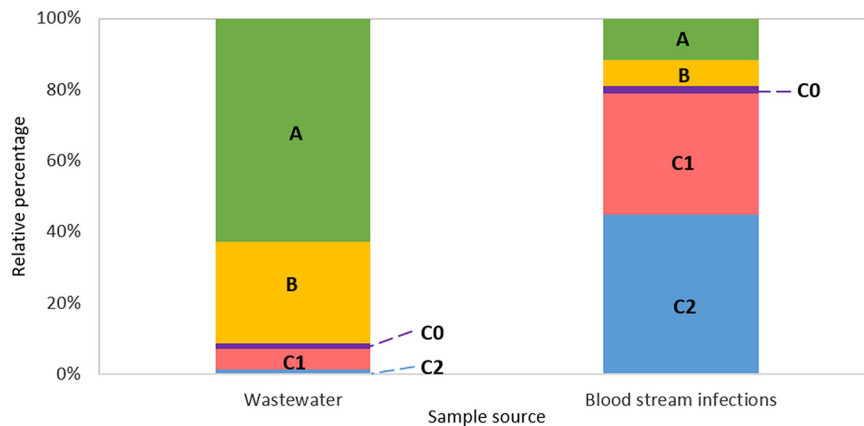
FIG 2 Clade distribution of ST131 isolated from different sources. The ST131 clade distribution obtained from wastewater isolates in this study was compared to the ST131 clade distribution from bloodstream isolates obtained from Peirano et al. (32).

clade C0, 34.01% ($n = 50$) for clade C1, and 44.9% ($n = 66$) for clade C2. These distributions from the two studies differ significantly (Fisher's exact test, $P = 5.13 \times 10^{-25}$). Furthermore, differences between individual clades were significant for all clades except clade C0. This is of note, primarily as clade C is widely recognized as the dominant circulating form of the pathogen (2); however, the unbiased design of our study uncovered a much higher rate of clade A and B strains than clade C from wastewater.

Antimicrobial resistance, ESBL production, and UV survival of ST131 samples isolated from wastewater. (i) Antibiotic resistance. Typically, antibiotic resistance is associated with clade C ST131 (6). As sample sizes for the three C subclades were too small for meaningful comparisons, the majority of analyses on levels of resistance apply to clade A and B strains of ST131. The rates of full resistance to each antibiotic tested in clade A and B strains are indicated in Fig. 3.

Among clade A ST131 strains, there was a very high prevalence of resistance to ciprofloxacin, with 72.7% ($n = 32$) for all A clades strains displaying full resistance. Additionally, there was also a high prevalence of resistance to three other antibiotics tested as follows: 47.7% ($n = 21$), 47.7% ($n = 21$), and 29.5% ($n = 13$) of all clade A strains were resistant to gentamicin, sulfamethoxazole-trimethoprim, and tetracycline, respectively. Strains were also examined to see the extent of multidrug resistance among ST131 isolated from wastewater (Fig. 3b). While 24.3% of strains ($n = 17$) were sensitive to all eight antibiotics tested, 40% ($n = 28$) were resistant to one or two antibiotics and 35.7% ($n = 25$) were multidrug resistant. This includes 2.9% ($n = 2$) of strains that were resistant to six of the eight antibiotics tested, including a clade A strain and a clade C1 strain.

(ii) ESBL production profiles. There was a low rate of ESBL-producing strains found in our collection. Overall, only 8% ($n = 6$) of all ST131 strains isolated from wastewater

TABLE 1 Comparison of frequency distribution of clades between wastewater and human isolates

Clade	Wastewater isolates (%)[a] ($n = 70$)	Human isolates (%)[a] ($n = 147$)	P value	Significance
A	44 (63)	17 (12)	3.93×10^{-15}	<0.001
B	20 (29)	11 (7)	3.33×10^{-5}	<0.001
C0	1 (1)	3 (2)	0.75	NS[b]
C1	4 (6)	50 (34)	6.56×10^{-6}	<0.001
C2	1 (1)	66 (45)	9.21×10^{-11}	<0.001

[a]Percentage of total is given in parentheses.
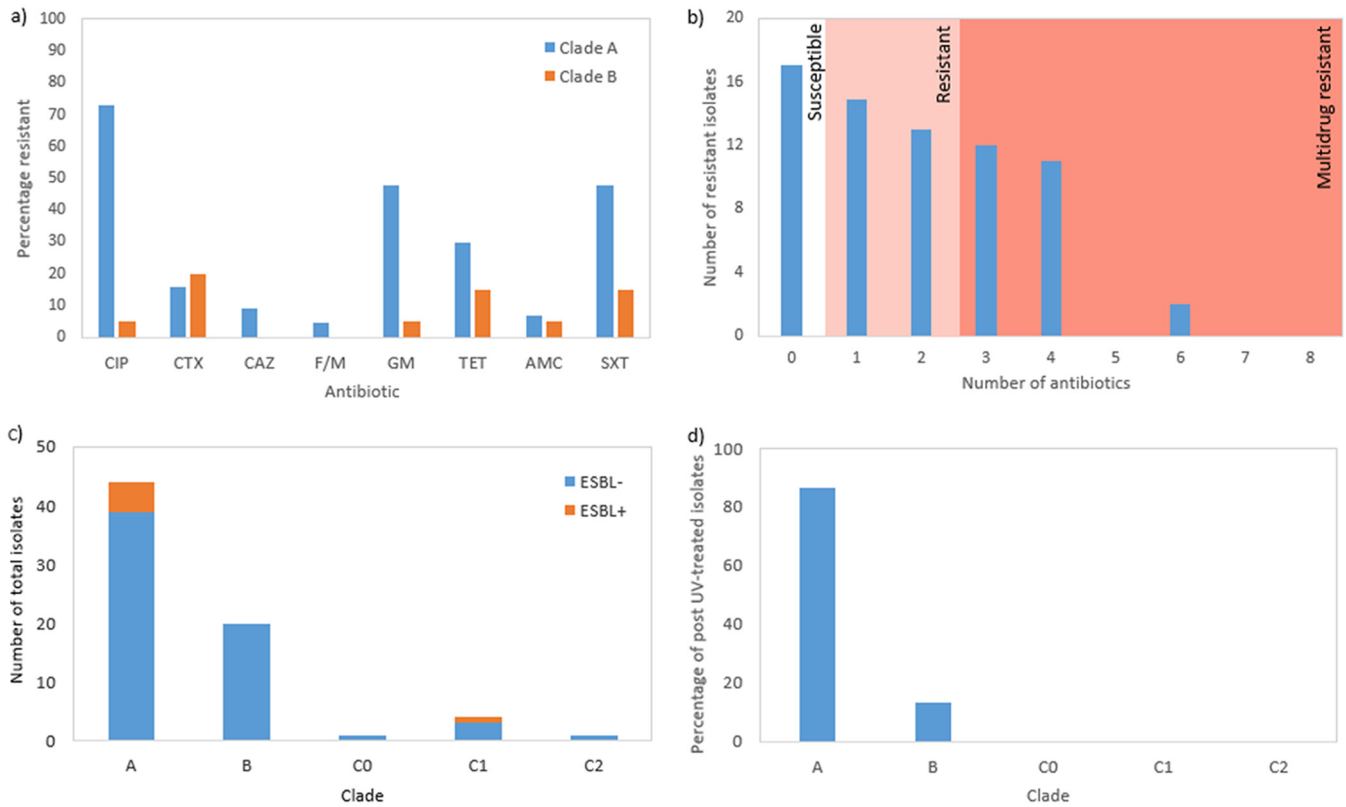[b]NS, not significant.

FIG 3 (a) Percentage of clade A and B ST131 isolates resistant to the eight antibiotics used in this study. Antibiotics included are ciprofloxacin (CIP), cefotaxime (CTX), ceftazidime (CAZ), nitrofurantoin (F/M), gentamicin (GM), tetracycline (TET), amoxicillin (AMC), and sulfamethoxazole and trimethoprim (SXT). (b) Extent of multidrug resistance in ST131 samples isolated from wastewater. The background color indicates extent of resistance, where white is susceptible to all antibiotics, pink is resistant to one or two antibiotics, and red is multidrug resistant or resistant to three or more antibiotics. (c) Number of ESBL-positive and ESBL-negative strains from all ST131 isolates. (d) Percentage of post-UV-treated ST131 samples by clade.

were capable of ESBL production according to CLSI guidelines (33) (Fig. 3c). ESBL production is a tightly linked feature of clade C2 ST131 (6). Interestingly, when the clades of the ESBL producers were considered, five were clade A strains and one was a C1 strain.

   (iii) UV treatment survival. Our collection of samples contained isolates from water that were isolated after UV treatment. Overall, 21.4% ($n = 15$) of the ST131-positive samples survived UV treatment. When this is broken down by clade, 86.67% ($n = 13$) were clade A, 13.33% ($n = 2$) were clade B, and there were no clade C samples among post-UV-treated strains (Fig. 3d).

DISCUSSION

   Wastewater in this study refers to all water exiting toilets or drained from domestic bathrooms or kitchens in Calgary in 2015 and does not include agricultural runoff or storm water. Like in most cities, treated wastewater output is returned back to the local river, which is the source of irrigation, drinking water, and recreational activity. Additionally, by-products of the wastewater treatment process are used for biosolid production for fertilizer and composting purposes; thus, it is crucial to know the extent to which wastewater carries human pathogens like ST131. The first objective of this study was to identify ST131 among total *E. coli* in wastewater in Calgary over 2015. ST131 has previously been identified from total *E. coli* in the river Thames and coastal bathing waters (UK) in two separate studies (34, 35), urban sewage and river water in Barcelona (Spain) (36), wastewater treatment plants and hospital wastewater in the Kansai region (Japan) (26), wastewater treatment plants in Brno (Czech Republic) (24), the wastewater treatment network in Besançon (France) (27), and coastal marine sediments in the

Adriatic Sea (Italy) (37). However, our second objective was to comprehensively examine the population structure of ST131 within this environment, which has been overlooked. Given the high proportion of C clades from clinical data reported in Calgary, Canada (32), we expected to uncover a high proportion of ESBL-producing clade C ST131 with our unbiased screening approach but also an increased proportion of antibiotic-sensitive strains. Unexpectedly, we found a low rate of clade C ESBL-producing strains and a high rate of clade A strains. There are a number of reasons to explain the low frequency of clade C and relative abundance of clade A ST131 in wastewater.

Low prevalence of ST131 overall. The low number of clade C strains uncovered in this study is likely due to the low ST131 prevalence in the Calgary wastewater system, with ST131 accounting for only 1.86% from total *E. coli* isolates overall. This rate is low when compared to that of other studies, which range from 73% in Brno, Czech Republic (24) to 2.4% in Besançon, France (27). Both studies sampled from wastewater treatment plants that were smaller than those in Calgary, servicing approximately 400,000 and 120,000 people, respectively, compared to 1,338,022 people in Calgary. Despite differences in the sizes of the plants, sources of samples were collected from municipal wastewater treatment plants that serviced urban and suburban areas, including hospitals and long-term care facilities, and did not include agricultural effluent in all cases. The wide range in ST131 prevalence could also be explained based on geographical location (38) or factors explained below.

Experimental design. Typically, ST131 is isolated in studies attempting to uncover ESBL-producing species or organisms containing various CTX-M alleles present in an environment, with the vast majority of studies employing a selective screening approach to do this (24, 26, 34, 35). Understandably, a high degree of ESBL-producing ST131 is uncovered, but how this breaks down into the different clades of the organism is rarely determined. In our analysis, an unbiased screening approach allowed for the isolation of all clades of ST131, including antibiotic-sensitive strains, which would have been eliminated from analysis if ESBL or ciprofloxacin selective plates were used. This is likely to have accounted for the high rate of clade A and B strains, which are typically not associated with ESBL production.

Additionally, sampling strategy is an important consideration, as many previous studies focused on untreated water (24, 26, 27, 34–37). In our study, samples were collected before and after UV treatment to determine whether ST131 isolates were capable of surviving the entire wastewater treatment process. It is currently unknown whether the isolation of ST131 after UV treatment is due to technical limitations of the UV sterilization protocol used by treatment plants or whether UV nonsusceptibility was achieved through biological means, such as upregulated UV repair mechanisms (39) or mechanisms of UV tolerance (40).

Underestimated clade A prevalence. Clade C may not be the most dominant form, but it is the most frequently isolated. As a consequence, there is an underestimation in the prevalence of clade A in the environment. Among nonenvironmentally focused studies, ST131 isolates are predominantly selectively screened from human or animal (either companion, farm, or wildlife) sources where only sick individuals are screened. A very limited number of studies have focused on isolation of ST131 from asymptomatic carriers potentially harboring less-pathogenic clades (41). Thus, a potentially large source of nonpathogenic and non-ESBL-producing ST131 may be underrepresented and unrealized in the current literature.

Adaptation of clade A to the environment. Adaptive diversification among *E. coli* isolates is well documented (42, 43). For ST131, a picture is beginning to emerge of each clade specializing to different niches. Many consider the evolutionary trajectory of clade C as a nascent human host specialist (2). More recently, Liu et al. investigated ST131 isolated from meat products and found that almost all samples were clade B isolates (44). If we assume that clades B and C have adapted to food animal and human hosts, respectively, the abundance of clade A in wastewater presented herein could

suggest evidence of adaptation to this environment. Zhi et al. identified naturalized stress-tolerant environmental *E. coli* with a greater ability to survive in wastewater due to the presence of an insertion element (IS*30*) that upregulated *uspC*, producing improved motility and enhanced UV resistance in affected strains (45). PCR screens for this mutation were performed on all 70 ST131 strains isolated in our study, but only two were positive for the IS*30* insertion, suggesting that there may be a variety of mutations responsible for adaptation to this particular niche. The collection of more environmental isolates in an unbiased manner and their addition to large-scale phylogenetic analyses could increase the power of epidemiological studies.

The third and final objective of this study was to determine the antibiotic susceptibilities of all of the ST131 wastewater isolates. Given the clade distribution and what is known about the resistance profiles of the different clades (6, 8), we expected antibiotic resistance to be limited to fluoroquinolone-resistant and ESBL-producing clade C isolates. Unexpectedly, there was a high rate of fluoroquinolone resistance from the clade A isolates and not from the clade C isolates.

Since the 1980s and 1990s, fluoroquinolones were the antibiotics of choice for UTI treatment in Europe and North America (46). However, up to 62% of administered fluoroquinolones are excreted nonmetabolized in urine (47), and as a result, these antibiotics are an emerging pollutant of water and soil environments (48), with high environmental concentrations promoting the evolution of resistant forms. In *E. coli*, resistance to fluoroquinolones arises due to the presence of point mutations in *gyrA* and *parC* (49), first observed in the 1980s when these antibiotics were prescribed heavily in human and veterinary medicine. These are defining features of the C clades of ST131 (6) and have since been linked to the rise of that clade. Our observation of fluoroquinolone resistance in clade A strains could suggest independent acquisition from resistance in clade C strains, as fluoroquinolone-resistant mutations are typically located on the ST131 chromosome rather than plasmids. However, fluoroquinolone resistance can be mediated through the acquisition of plasmids containing the *aac(6′)-lb-cr* gene in some cases (50). Regardless of the underlying molecular mechanisms involved, this resistant phenotype is likely to account for the high rate of clade A ST131 in wastewater.

The higher number of ESBL producers in clade A than clade C2 suggests that ESBL production in clades other than C2 is apparent, most likely as a result of horizontal transmission of plasmids among organisms in similar niches. Recent quantitative studies enumerating bacteria in water samples have found ST131 to be among the most ubiquitous ESBL-producing organisms in this niche (24, 26, 35), but this is not always the case. Dhanji et al. uncovered a high rate of fluoroquinolone-resistant but ESBL-negative ST131 isolates using a ciprofloxacin selective screening approach to uncover ST131 in the Thames river, United Kingdom (34).

There was a high occurrence of resistance to antibiotics that are commonly found at high concentrations in wastewater (51). Sulfamethoxazole-trimethoprim and gentamicin are used to treat UTIs either for uncomplicated infection (52) or intravesically for prophylaxis or recurrent infection (53), respectively. All three are found nondegraded and at high concentrations in aquatic environments (54–56), imposing selection pressure for the evolution of resistant forms. Resistance mechanisms to all three drugs are reported and understood at a molecular level (57–59). Furthermore, the antibiotics included in this study were of five different classes, meaning the evolution of resistance to one antibiotic was unlikely to mediate cross-resistance to antibiotics of other classes, and these resistance profiles are likely independent acquisition events. Intuitively, resistance profiles of isolates correlated with antibiotics that are either most commonly used to treat UTIs and/or most frequently detected in water. Overall, the presence of these antibiotics in wastewater is likely driving the high resistance levels from all clades of ST131, as indicated by our data.

Conclusion. There were limitations to this study. First, this is a PCR-based screening approach, so we were only able to detect already known clades of ST131. No new or

emerging clades could be detected in this screen. Second, genomic analysis has not yet been performed on the 70 wastewater isolates, as this is the focus of a future study. However, our aim here was to perform a high-throughput screening, and this was possible and successful with PCR. This study confirmed the presence of ST131, albeit a low one, in wastewater in Calgary, Canada. Nevertheless, a large sample size and a comprehensive unbiased screening approach uncovered the largest collection of ST131 isolated from wastewater in a single study. In-depth analysis of the population structure revealed that previously overlooked ESBL-negative clade A and B strains are the highest contributor to this contamination. These previously nonresistant clades are actively acquiring new resistances, most likely due to selection pressure from the presence of consistent low levels of commonly used antibiotics. Additionally, clade A strains of ST131 may have inherent resistances to UV sterilization. Genome sequencing and comparative genomics of these strains are logical next steps to uncover the underlying molecular mechanisms for these findings.

Wastewater treatment plants are already identified as key hot spots for emerging resistances among ST131. The addition of these samples to large-scale epidemiology studies will be invaluable in developing a more complete epidemiological understanding of this emerging global pathogen.

MATERIALS AND METHODS

Wastewater treatment plant overview. There are three urban wastewater treatment plants (treatment plants A, B, and C) located in Calgary, AB, Canada (60), which service 966,337, 280,788, and 90,897 inhabitants, respectively, with a combined total of 1,338,022 inhabitants in 2015 overall. The three treatment plants range in size but collectively processed 465,068 m^3 of wastewater per day in 2015. In all cases, treatment processes were identical. Briefly, raw wastewater (water from sinks, drains, and toilets from industrial complexes, health care facilities, and domestic households) is collected via one of three collection systems for each treatment facility. The inflow passes through headwork gates to remove large solids and then primary clarifiers to remove smaller solids. For microbial disinfection, wastewater is pumped through cloth media disks, passes through a UV disinfection step, and is released into the Bow River. The UV disinfection step uses UV lamps, ranging from 65 to 2,800 W, allowing for an average UV dose of 26 mJ/cm^2 as per Alberta Environment Protection standards. The average final effluent of total suspended solids from all three wastewater plants was 6 mg/liter in 2015.

Collection of *E. coli* wastewater samples. Samples were collected every week over the entire 2015 calendar year from the three different urban wastewater treatment plants in Calgary. Samples were collected before and after UV treatment by filtering water through Büchner flasks containing EZ-Pak 0.45-μm, 47-mm white gridded filters (Millipore, Burlington, MA, USA). Filter membranes were then removed and placed on m-FC agar plates, containing bile salts and rosolic acid for selective growth fecal coliforms, on which *E. coli* are identified as blue colonies after overnight incubation at 44.5°C. *E. coli* colonies are picked and grown in liquid Luria-Bertani (LB) broth in a 96-well plate format overnight at 37°C. The following day, 100 μl of 50% glycerol are added to the cultures and stored at −80°C.

High-throughput ST131 detection among *E. coli* wastewater samples. For the detection of ST131 among *E. coli* wastewater samples, a high-throughput PCR-based screening assay was developed and applied to all 3,762 sewage samples containing *E. coli* collected from the three treatment plants. Cultures were established by inoculating 100 μl of LB liquid broth with frozen glycerol stocks in 96-well plates, sealed with breathable membranes (Nunc, Rochester, New York, USA), and incubated overnight at 37°C at 200 rpm. DNA was prepared by transferring 50 μl of overnight culture into another 96-well plate, adding 50 μl of Milli-Q, and sealing the plate with a 96-well PCR plate seal (Bio-Rad, Hercules, CA, USA). Heat lysis on all samples was performed in a T100 Bio-Rad thermocycler machine and consisted of heating the samples to 95°C for 10 min, followed by cooling at 4°C for 5 min. Samples were then centrifuged for 4,000 rpm for 15 min to remove cell debris, and 2 μl of supernatant was used as DNA for downstream processes.

For the PCR detection of ST131 among wastewater samples, previously published primers (Table 2) were used. PCR reagents were scaled up to screen 96 samples in each assay. All PCRs were performed with ThermoPol *Taq* (New England Biolabs, Ipswich, MA, USA) and consisted of an initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 57°C for 20 s, and amplification at 68°C for 40 s, with a final amplification step at 68°C for 1 min. To determine the limits of ST131 DNA detection, PCRs were performed as above on a dilution series of purified genomic DNA extracted from *E. coli* CD306. Visible amplification was observed with DNA concentrations as low as 2.8 pg/μl or 8.5×10^{-10} pmol of genomic DNA.

Sample purification. To ensure all samples that were found to contain ST131 from the wastewater treatment plants were pure, all ST131-positive samples were streaked to purity on nonselective CHRO-Magar orientation plates (bioMérieux, Marcy-l'Étoile, France), where *E. coli* colonies grow as dark pink to red colonies. After purity was confirmed, single colonies were used to inoculate 3 ml of LB liquid broth and incubated overnight at 37°C at 200 rpm, and 500-μl aliquots of pure cultures were cryogenically preserved in 50% glycerol at −80°C.

TABLE 2 Primers used in this study

Name	Working concn (μM)	Sequence (5′ to 3′)	Identified element	Reference
ST131_R19-YF1	0.25	AGCAACGATATTTGCCCATT	ST131	62
ST131_R19-YR1	0.25	GGCGATAACAGTACGCCATT		
CladeAsp4-F	0.25	TGACGGGACGTGAGCAAATTA	Clade A	
CladeAsp4-R	0.25	AGTCAGACCTAGCCACCCTT		
prfC-F	0.25	CAACGTTGAAGCAGTGTATGAG	Clade B	
prfC-R	0.25	TGACAATCGACGGCTTTAGA		
C-SNP-1-F	0.15	CGCTGGCCAGTTATCTGAAAT	Clade C0	
C-SNP-1-R	0.15	CCTTTCACCAACTGGGTTACT		
C1-F	0.10	GGCCCCACAAATTGCTT	Clade C1	
C1-R	0.10	CGCACCTCCGATACCAAA		
C2-F	0.20	ACGGATTCAGGTAGACGATT	Clade C2	
C2-R	0.20	CCTCACCAAAGTTGCGATTAC		
flh-IS-F	10	CGGGGAACAAATGAGAACAC	IS*30* insertion	45
fllh-IS-R	10	TGGAGAAACGACGCAATC		

Clade identification of purified ST131-positive samples. DNA from ST131-positive samples was used in multiplex PCRs with clade-specific primers to identify the clade to which the purified ST131-positive cultures belonged (Table 2). The clade-specific PCR program involved initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 40 s, annealing at 57°C for 40 s, and amplification at 68°C for 40 s, with a final amplification step at 68°C for 7 min. Samples were run on a 2.5% agarose gel.

Antimicrobial susceptibility testing of ST131-positive samples. The antimicrobial resistance profiles of all ST131-positive isolates were determined. Samples were subjected to disk diffusion assays with eight antibiotics (Table 3). ESBL classification was based on the addition of β-lactam inhibitor clavulanic acid (10 μg) to cefotaxime and ceftazidime. Resistance profiles were assessed based on CLSI guidelines (33) and multidrug resistance classifications were based on Magiorakos et al. (61).

Statistical analysis. Comparisons of proportions of ST131 isolated from treatment plant or by season were analyzed by a Pearson χ^2 test. Comparison of proportions of ST131 clades between clinical and wastewater samples were analyzed via Fisher's exact test. In all cases, significance was designated for a P value of <0.05. All statistical analyses were performed in SPSS Statistics 25 (IBM Corp., Armonk, NY, USA).

TABLE 3 List of antimicrobial compounds used in this study

Antibiotic	Classification	Concn (μg)
Ciprofloxacin	Fluoroquinolone	5
Cefotaxime	β-Lactam, third-generation cephalosporin	30
Ceftazidime	β-Lactam, third-generation cephalosporin	30
Gentamycin	Aminoglycoside	10
Sulfamethoxazole and trimethoprim	Sulfanilamide and dihydrofolate reductase inhibitor	23.75/1.25
Nitrofurantoin	Nitrofuran	300
Amoxycillin	β-Lactam	20
Tetracycline	Tetracycline	30

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