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Occurrence of extended-spectrum β-lactamase-producing bacteria in urban Clinton River habitat

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ABSTRACT

Objective: The aim of this study was to determine whether Clinton River water is contaminated with antibiotics and is a reservoir of antimicrobial-resistant bacteria.
Methods: Water samples were taken from two sites of Clinton River. Antimicrobial-resistant bacteria were enumerated on agar plates supplemented with six commonly used antibiotics. Extended-spectrum β-lactamase (ESBL)-producing bacteria were identified using a BD Phoenix™ System and by 16S rRNA gene sequencing. Antimicrobial resistance gene transfer was performed by conjugation studies and the location of genes was determined by Southern hybridisation. Virulence properties of ESBL-producing isolates were determined by assessing their biofilm-forming ability, cellular toxicity, and induction of an inflammatory response in intestinal epithelial (Caco-2) cells.
Results: 16S rRNA analysis of water samples showed the presence of potentially pathogenic bacteria (e.g. *Shigella flexneri*, *Klebsiella pneumoniae*, *Aeromonas punctata* and *Pseudomonas aeruginosa*). Among 64 biochemically identified bacterial isolates tested, 42% were resistant to cefotaxime, 34% to chloramphenicol, 9% to tetracycline, 11% to ciprofloxacin and 9% to gentamicin. Of 27 cefotaxime-resistant isolates, 11 (41%) were ESBL-positive and possessed either bla_{CTX-M} ($n = 9$), bla_{TEM} ($n = 1$) or bla_{KPC} ($n = 1$). Comparative analysis of ESBL gene sequences from Clinton River water bacteria showed 98–100% identity with clinical isolates. ESBL-producing isolates from Clinton River water were found to form biofilms, induced inflammatory cytokines and caused toxicity to epithelial cells.
Conclusions: Clinton River water contains isolates with ESBL genes identical to clinical isolates and possessing virulence properties, thus it could be a potential reservoir in causing human infections.

1. Introduction

In the suburban setting, rivers are commonly used for recreational and irrigation purposes and are prone to water pollution, including antibiotics from agricultural runoff and discharge of effluents from wastewater treatment plants. Indeed, antimicrobial-resistant bacteria (ARB) have been isolated from environmental sources, including wastewater effluent [1,2] and river and costal water [3–5]. In recent years, extensive use of third- and fourth-generation cephalosporins for the treatment of infections by ARB has led to the emergence of novel extended-spectrum β-lactamase (ESBL) enzymes that are capable of inactivating extended-spectrum β-lactam antibiotics [6]. Bacteria that are resistant to the newer cephalosporins have evolved due to point mutations originating in variants of common ESBL genes, such as bla_{TEM}, bla_{SHV} and bla_{CTX-M} [7]. The TEM enzymes were the first ESBLs to be described (discovered in a Greek patient named Temoniera), whereas the SHV enzymes were named for their sulfhydryl variable (SHV) groups [8,9]. SHV-type enzymes are more common among *Klebsiella pneumoniae*, whereas TEM-type enzymes are commonly found in ESBL-producing *Escherichia coli* [8]. Cefotaximase (CTX-M) enzymes preferentially cleave cefotaxime over other late-generation cephalosporins [7]. Carbapenems (imipenem, meropenem and ertapenem), another group of antibiotics, are used for the treatment of ESBL-producing bacterial infections and are generally administered intravenously [6].

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Dissemination of ESBL-producing bacterial isolates into the environment is of serious concern, especially in urban water bodies that also receive ARB from watershed [10], storm water [11] and wastewater treatment plants [12], as the environment can become a breeding ground for emerging ARB pathogens. This research is a continuation of a previous study published by the United States Geological Survey (USGS), in co-operation with the Oakland County Health Department, Michigan, which has shown the presence of antimicrobials (erythromycin, azithromycin, ciprofloxacin, trimethoprim and sulfamethoxazole), polychlorinated biphenyls (PCBs) and mercury in the Clinton River [13,14]. Although a few studies from France, India [15] and Switzerland [16] have shown the presence of ESBL-producing bacteria in a river water habitat [4,6,17,18], very few studies have reported such findings in the USA. The presence and dissemination of ESBL-producing bacteria can be alarming as they may transfer resistance genes to other pathogenic bacteria, thus increasing the risk of community infection. The purpose of this pilot study was to assess the occurrence of ESBL (SHV, TEM and CTX-M-1)-producing bacteria and the abundance of gene pollution in urban Clinton River surface water. Furthermore, the virulence properties of ESBL-producing river water bacteria were characterised using in vitro methods.

2. Materials and methods

2.1. Site description, sample collection and processing

The Clinton River watershed extends over 1900 km^2, over the majority of Oakland and Macomb Counties in southeastern Michigan. The majority of this land is covered by forests, with a small fraction being used for agriculture. The remaining areas are mostly urban (residential and commercial). The main branch of the Clinton River is 130 km long and begins in Springfield Township in Oakland County, ending in Lake St Clair, a tributary of the Great Lakes. Two sample sites were chosen for this pilot study, one in Pontiac (site A) and another in Auburn Hills (site B). The sample site at Auburn Hills is located at 42°38′13.0″N, 83°13'12.5″W and has previously been shown to be polluted with mercury and antibiotics. In the previous study, this site contained 0.138 μg/L ciprofloxacin and 0.064 μg/L erythromycin. The sample site in Pontiac is located at 42°37′35.6″N, 83°19′16.2″W and has not been previously shown to contain any antibiotics (Fig. 1). As the State of Michigan recreational water quality standards (used for all surface waters in the state) are in effect from 1 May to 31 October each year, we chose to collect river water samples during these months in 2014. River water samples (three per site) were collected aseptically in sterile Whirl-Pak bags (55 oz.) (Sigma-Aldrich, St Louis, MO) from sites close (ca. 1 m) to the river bank. Samples were collected ca. 10–15 cm below the water surface. River water samples (750 mL) were transported on ice, were stored at 4 °C and were processed within 16 h of collection.

Samples were filtered through 0.45-μm pore glass fibre filters (Millipore, Burlington, MA), followed by 0.22-μm pore sterile polyethersulfone (PES) filters (Corning Inc., Corning, NY). Water samples were spiked with surrogate (carbamazepine-d10) before extraction. Antibiotics were extracted from the sample using a preconditioned Strata X-C18 Giga solid phase extraction (SPE) cartridge (Phenomenex, Torrance, CA) and were eluted with an acetonitrile/methanol solution. Three river water samples from each site were pooled together as one sample for chemical [liquid chromatography–tandem mass spectrometry (LC-MS/MS)] and biological analyses, as summarised in the flow chart in Fig. 2.

2.2. Isolation and biochemical identification of bacteria from river water

Log dilutions of river water samples (100 μL) were spread on tryptic soy agar (TSA) (Becton Dickinson, San Jose, CA) and MacConkey agar (Becton Dickinson) plates. ARB were

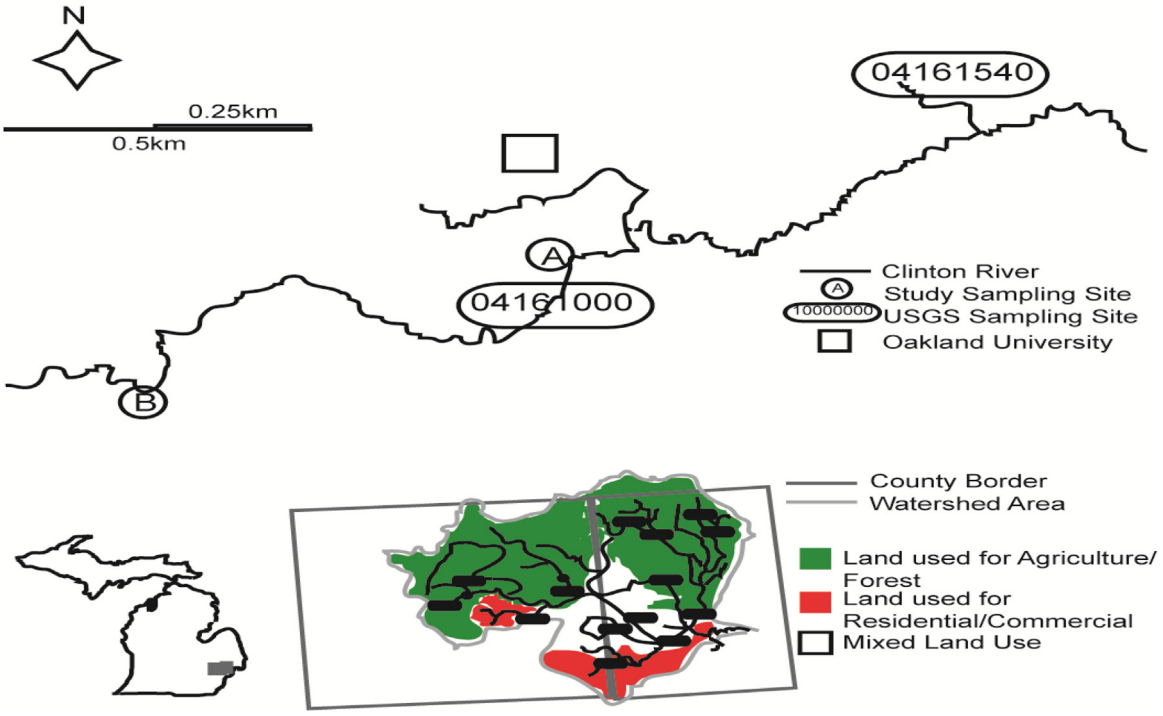

Fig. 1. Site and sampling locations of river water and sediment from urban Clinton River, Michigan (USA). Sample sites used to detect the presence of extended-spectrum β-lactamase (ESBL)-positive pathogenic bacteria in the urban Clinton River were based in two locations, namely Auburn Hills (site B) and Pontiac (site A). Sample site A was located at 42°37'35.6"N, 83°19'16.2"W and sample site B was located at 42°38'13.0"N, 83°13'12.5"W.

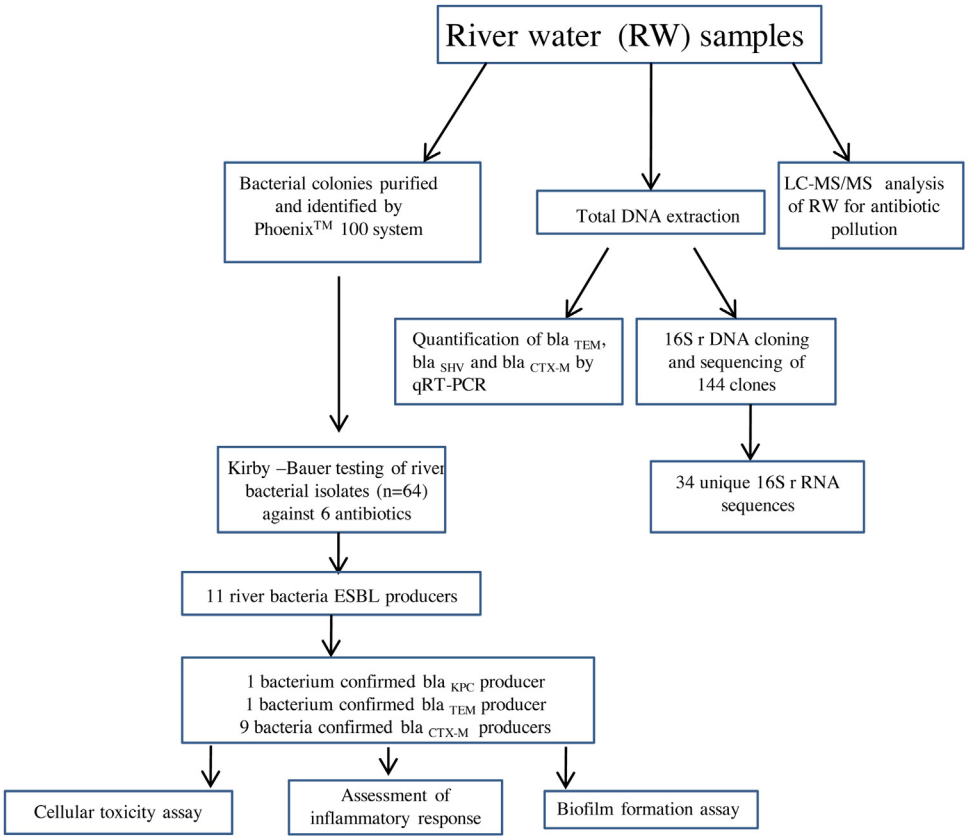

Fig. 2. Flowchart of Clinton River water sample analysis. LC-MS/MS, liquid chromatography–tandem mass spectrometry.

enumerated on agar plates supplemented with the appropriate antibiotics. All antibiotic powders used in the agar plates were purchased from Sigma-Aldrich, including ampicillin (32 μg/mL), cefotaxime (64 μg/mL), ciprofloxacin (4 μg/mL), chloramphenicol (25 μg/mL), gentamicin (16 μg/mL) and tetracycline (16 μg/mL). For isolation of *Salmonella* and *Shigella* spp. isolates, river water samples were cultured in enrichment medium (Selenite F broth; Becton Dickinson) for 48 h and were plated on *Salmonella–Shigella* agar (Becton Dickinson) and eosin methylene blue agar (Becton Dickinson) plates. *Escherichia coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as quality control strains. ESBL-producing bacteria were identified using a BD Phoenix™ system (Becton Dickinson) and by 16S rRNA sequencing. The BD Phoenix™ System utilises both fluorometric and colorimetric signals for biochemical identification. Briefly, BD Phoenix™ ID Broth was inoculated and the suspension was transferred to BD Phoenix™ ATS broth followed by loading the panels in the BD Phoenix™ System.

2.3. Quantification of genes in river water by real-time quantitative PCR (qPCR)

The quantity of target DNA in river water samples was calculated based on a standard curve generated using known quantities of template DNA. Standards for qPCR were prepared by PCR amplification of genes from positive controls, followed by ligation into a pGEM®-T Easy Cloning Vector I system (Promega, Madison, WI) and transformation into *E. coli* JM109. Hybrid plasmids were purified using a plasmid isolation kit (QIAGEN, Germantown, MD). Ten-fold serial dilutions of plasmid DNA were

Table 1
PCR primer sequences used for real-time quantitative PCR.

Target gene	Primer sequence	T_m (°C)	Expected amplicon size (bp)
bla_{SHV}	F: GGTTATGCGTTATATTCGCC R: TTAGCGTTGCCAGTGCTC	60	867
bla_{TEM}	F: CCGTG TCGCCCTTATTCC R: AGGCACCTATCTCAGCGA	56	800
bla_{CTX-M}	F: TTTGCGATGTGCAGTACCAGTAA R: TCCGCTGCCGGTTTTATC	56	500
$bla_{CTX-M-1}$	F: GGACGTACAGCAAAAACTTGC R: CGGTTCGCTTTCACTTTTCTT	55	624
bla_{KPC}	F: CATTCAAGGGCTTTCTTGCTGC R: ACGACGGCATAGTCATTTGC	60	538
16S rRNA	F: AGAGTTTGATCMTGGCTCAG R: AAGGAGGTGATCCAGCC	60	1400

T_m, melting temperature.

prepared and were run on a StepOne™ thermal cycler (Applied Biosystems, Foster City, CA) to generate standard curves ($R^2 > 0.99$). Absolute quantification of three genes (bla_{SHV}, bla_{TEM} and $bla_{CTX\text{-}M\text{-}1}$) was performed and total river water DNA was extracted using a PowerWater DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA) according to the manufacturer's instructions. Quantitative assessment of the genes was carried out using SYBR™ Green-based qPCR using specific primers on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). The real-time primers are summarised in Table 1. The copy number of the gene per mL of river water sample was calculated using the $\Delta\Delta C_T$ method. The resistance gene copy number was normalised against the 16S rRNA gene.

2.4. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of river water bacteria was performed for all isolates by the disk diffusion test (Table 2) following Clinical and Laboratory Standards Institute (CLSI) guidelines [19]. A bacterial strain was considered ESBL-positive if the cefotaxime/clavulanic acid and ceftazidime/clavulanic acid disks showed an inhibition zone ≥5 mm larger compared with the inhibition zone produced by the cefotaxime or ceftazidime disk alone. *Klebsiella pneumoniae* ATCC 700603 and *Pseudomonas aeruginosa* ATCC 27853 served as positive control strains and *E. coli* ATCC 25922 as a negative control strain. Antimicrobial susceptibility testing was performed using imipenem (10 μg), ertapenem (10 μg) and meropenem (10 μg) for the carbapenemase-producing isolates.

2.5. Biofilm assay

ESBL-positive river water bacterial isolates were tested for their ability to form biofilms by staining bacterial cells on a glass coverslip using a LIVE/DEAD *Bac*Light™ Bacterial Viability Kit (Invitrogen, Carlsbad, CA) as described previously [20]. ImageJ software was used to determine the relative fluorescence intensity of live and dead bacterial cells on the biofilm [21]. *Staphylococcus epidermidis* ATCC 12228 was used as a biofilm-negative control, whilst the uropathogen *K. pneumoniae* ATCC 700603 was used as a biofilm-positive control.

Table 2
Minimum inhibitory concentrations (MICs) of bacteria ($n = 64$) isolated from Clinton River water.

Antimicrobial agent	MIC (μg/mL)		
	Range	MIC_{50}	MIC_{90}
Amikacin	2–64	8	>64
Ampicillin	2–32	16	>16
Aztreonam	0.25–32	<1	>16
Cefepime	0.625–32	<1	>16
Cefoxitin	8–32	16	>16
Cefazolin	8–32	16	>16
Ceftazidime	2–32	8	>16
Ceftriaxone	0.625–32	<1	>16
Cefotaxime	8–64	16	>32
Cefuroxime	8–32	16	>16
Ciprofloxacin	4–64	>0.5	>32
Chloramphenicol	2–512	64	>256
Ertapenem	2–16	8	>8
Gentamicin	0.25–16	<2	>8
Imipenem	2–16	8	>8
Levofloxacin	0.5–128	0.5	>64
Meropenem	2–16	8	>8
Nitrofurantoin	2–512	<16	>256
Tetracycline	2–512	128	>256

$MIC_{50/90}$, MIC required to inhibit 50% and 90% of the isolates ($n = 64$), respectively.

2.6. Cytotoxicity assay [lactate dehydrogenase (LDH) assay]

Induction of cell cytotoxicity by environmental bacteria on intestinal epithelial (Caco-2) cells was determined using a Cytotoxicity Detection Kit (LDH) (Roche, Basel, Switzerland) as described previously [20]. *Klebsiella pneumoniae* ATCC 700603 was used as the positive control and *E. coli* HB101 as the negative control.

2.7. Nucleotide accession nos

Partial coding sequences of bla_{KPC} from *K. pneumoniae* strain OU11 and bla_{TEM} from *Streptococcus porcinus* strain OU10 that were <100% identical to previously identified genes have been deposited in GenBank under the accession nos. KF444271 and KF444270, respectively.

2.8. Statistical analysis

Statistical analysis of viable bacterial counts was performed using SAS statistical software v.9.2. The significance of the effect of antibiotic pollution and the effect of antibiotics in growth media was determined by Dunnett's *t*-test, where statistical significance was determined at a *P*-value of <0.05 and a 95% confidence interval (CI).

3. Results

3.1. Detection of antibiotics in Clinton River water

The target compounds in this study included cefotaxime, ciprofloxacin, ceftazidime and tetracycline. Among the four target compounds, 10 μg/L tetracycline (parent ion m/z, 445.1; product ion m/z, 410.2) and 20 μg/L ciprofloxacin (parent ion m/z, 332.2; product ion m/z, 314.2) was detected in river water from site A (Pontiac). The retention time of ciprofloxacin was 2.6 min and that of tetracycline was 1.3 min. Carbamazepine (parent ion m/z, 247.100; product ion m/z, 204.161), the surrogate extraction control, was detected (retention time, 14.129 min.), as was caffeine-C3 (parent ion m/z, 198.000; product ion, 140.073), the internal control (retention time, 2.34 min). None of the target compounds were detected in river water from site B (Auburn Hills). The standards for cefotaxime (parent ion m/z, 456.08; product ion m/z, 167.1; retention time, 4.3 min) and ceftazidime (parent ion m/z, 631.4; product ion m/z, 442.1; retention time, 9.6 min) were detected in spiked samples.

3.2. Isolation and identification of antimicrobial-resistant bacteria

Clinton River water showed the presence of $3.93 \times 102 \pm 1.46 \times 103$ CFU/mL (mean ± standard deviation) on TSA plates. The cultivable bacterial community was highly diverse, containing groups of pathogens and other infectious bacteria. Of a total of 136 isolated bacterial colonies from river water samples, 62 (46%) isolated colonies were resistant to cefotaxime, of which 18% (11/62) were found to be ESBL-producers (Table 3). Other than cefotaxime, 34% of isolates were resistant to chloramphenicol, 9% to tetracycline, 11% to ciprofloxacin and 9% to gentamicin. ESBL activity was confirmed double-disk synergy test using cefotaxime and amoxicillin/clavulanic acid disks (CLSI) and minimum inhibitory concentration (MIC) determination (BD Phoenix™ system). In addition, ESBL genes were identified and sequenced (Supplementary Fig. S1). Colistin susceptibility testing by Etest (bioMérieux, Durham, NC) of ESBL-producing bacteria showed no resistance to colistin. Biochemical analysis of the cultivable bacteria revealed the presence of potentially pathogenic bacteria,

Table 3
Characteristics of extended-spectrum β-lactamase (ESBL)-producing bacteria isolated from Clinton River water.

Isolate ID	Antimicrobial resistance pattern	ESBL gene amplified	Biofilm formation	Cytotoxicity in Caco-2 cells
Rahnella aquatilis (strain OU1)	CTX/CHL/CZO/CXM/CAZ	$bla_{CTX\text{-}M\text{-}1}$	Medium	Yes
R. aquatilis (strain OU2)	CTX/CZO/CXM/CAZ	$bla_{CTX\text{-}M\text{-}1}$	Medium	Yes
Aeromonas sobria (strain OU3)	CTX/GEN/AMP/CZO/FOX/CXM/ETP/SXT/CAZ	$bla_{CTX\text{-}M\text{-}1}$	Medium	Yes
Aeromonas caviae (strain OU4)	CTX/CHL/GEN/AMP/CZO/FOX/CXM/ETP/CAZ	$bla_{CTX\text{-}M\text{-}1}$	Medium	Yes
A. sobria (strain OU5)	CTX/CHL/GEN/AMP/CZO/FOX/CXM/ETP/SXT/TZP/CAZ	$bla_{CTX\text{-}M\text{-}1}$	Medium	Yes
Bacillus megaterium (strain OU6)	CTX/CAZ	$bla_{CTX\text{-}M\text{-}1}$	Low	Yes
Leifsonia aquatic (strain OU7)	CTX/CAZ	$bla_{CTX\text{-}M\text{-}1}$	Low	Yes
Flavobacterium meningosepticum (strain OU8)	–[a]	$bla_{CTX\text{-}M\text{-}1}$	Medium	Yes
Pseudomonas sp. (strain OU9)	CTX/CHL/CAZ	$bla_{CTX\text{-}M\text{-}1}$	Medium	Yes
Streptococcus porcinus (strain OU10)	CTX/GEN/CAZ	bla_{TEM}	Low	Yes
Klebsiella pneumoniae (strain OU11)	CTX/AMP/ATM/CZO/FEP/FOX/CRO/CXM/CIP/ETP/LVX/CAZ/IPM/ETP/MEM/TZP/CHL/CIP/GEN	bla_{KPC}	High	Yes

ESBL, extended-spectrum β-lactamase; Caco-2, human carcinoma of the colon cells; CTX, cefotaxime; CHL, chloramphenicol; CZO, cefazolin; CXM, cefuroxime; CAZ, ceftazidime; GEN, gentamicin; AMP, ampicillin; FOX, cefoxitin; ETP, ertapenem; SXT, trimethoprim/sulfamethoxazole; TZP, piperacillin/tazobactam; ATM, aztreonam; NIT, nitrofurantoin; FEP, cefepime; CRO, ceftriaxone; CIP, ciprofloxacin; LVX, levofloxacin; IPM, imipenem; MEM, meropenem; TET, tetracycline; $bla_{CTX\text{-}M\text{-}1}$, cefotaximase-type ESBL; bla_{TEM}, Temoniera-type ESBL; bla_{KPC}, *K. pneumoniae* carbapenemase-type ESBL.
[a] Inconclusive susceptibility data.

including *Rahnella aquatilis*, *Burkholderia cepacia*, *Aeromonas veronii*, *Pseudomonas oryzihabitans*, *Pseudomonas putida*, *Salmonella typhi*, *Streptococcus gordonii*, *Streptococcus agalactiae*, *Leifsonia aquatica*, *Aeromonas caviae*, *Staphylococcus aureus*, *Dermacoccus nishinomiyaensis*, *Shewanella putrefaciens*, *Shigella dysenteriae* and *Yersinia ruckeri*. Their identity was further confirmed by 16S rRNA sequencing.

3.3. 16S rRNA typing of Clinton River water samples

Total DNA was extracted from river water samples using a PowerWater DNA Isolation Kit. PCR was performed using a 16S rRNA primer and the product was then cloned into a pGEM®-T Easy Cloning Vector I system and sequenced using an ABI Prism® 3730 DNA Analyzer (Applied Biosystems) at Applied Genomics Technology Center (Wayne State University, Detroit, MI). A phylogenetic tree was constructed using BioEdit software. A total of 144 clones were sequenced, resulting in 34 unique sequences (Fig. 3). The majority of these sequences belonged to *Pseudomonas*, *Aeromonas* and enteric bacteria such as *Klebsiella*, *Escherichia*, *Shigella* and *Enterobacter*. Four sequences were identified as uncultured bacteria by the BLASTn search.

3.4. Real-time quantitative PCR of extended-spectrum β-lactamase genes

Total DNA from river water was subjected to q-PCR analysis for detection of pollution by ESBL genes (bla_{SHV}, bla_{TEM} and $bla_{CTX\text{-}M}$). A standard curve was plotted using ten-fold serial dilutions (10^9 to 10^{10}) of a plasmid bearing the $bla_{CTX\text{-}M\text{-}1}$, bla_{SHV} and bla_{TEM} genes ligated to a pGEM®-T Easy Cloning Vector. Copy numbers for the $bla_{CTX\text{-}M\text{-}1}$, bla_{SHV} and bla_{TEM} genes were calculated per mL of water sample based on values from the standard curve. The amount of bacterial biomass in the Clinton River water was quantified by extracting total DNA and amplifying 16S rRNA gene fragments by qPCR. The number of bacteria per mL of river water was estimated

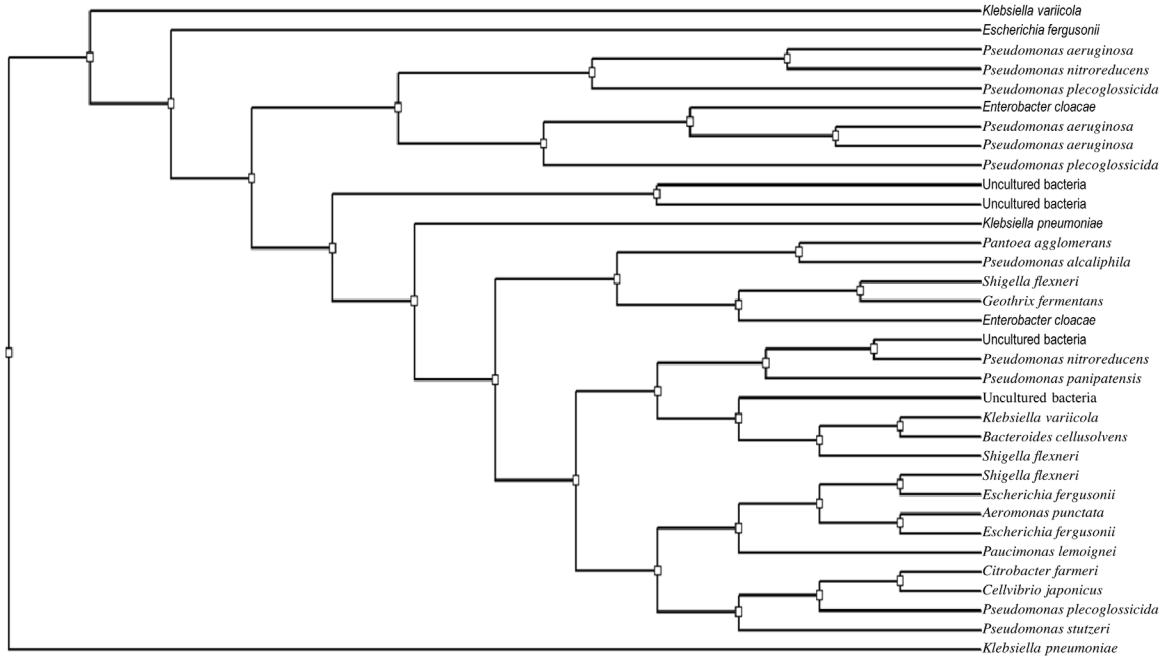

Fig. 3. 16S rDNA (rRNA gene) sequence-based rooted dendrogram showing clustering of bacteria from Clinton River water.

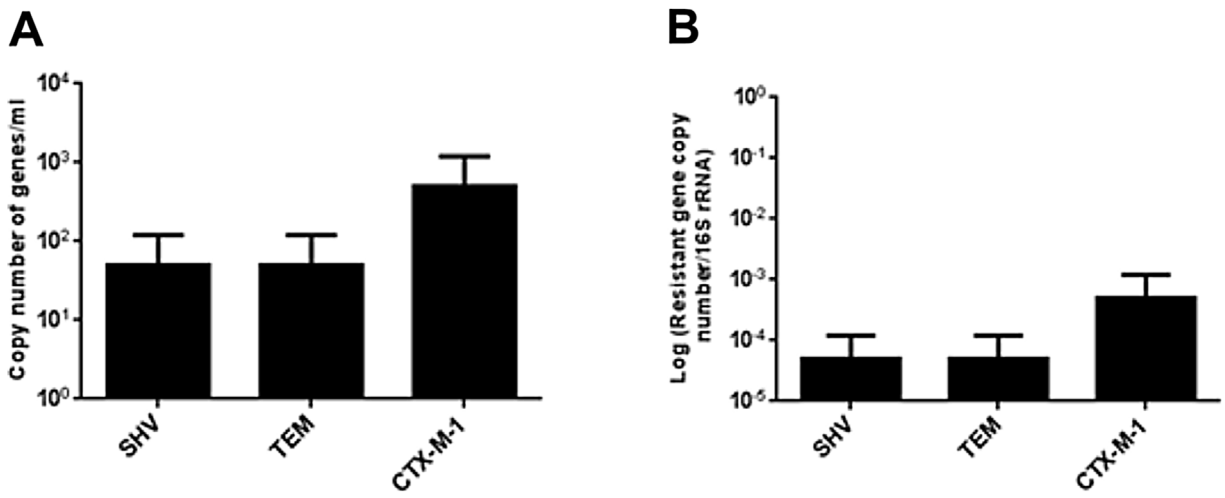

Fig. 4. Assessment of the concentration of antimicrobial resistance genes in Clinton River water: (A) absolute concentration of bla_{SHV}, bla_{TEM} and $bla_{CTX\text{-}M\text{-}1}$ genes/mL of Clinton River water; and (B) gene abundances normalised to ambient 16S rRNA gene level. Values shown are the arithmetic mean; error bars show the standard deviation.

to be 8.53×10^4. The copy number (gene/mL) of river water was 1.19×10^2, 1.21×10^2 and 1.35×10^3 for bla_{SHV}, bla_{TEM} and $bla_{CTX\text{-}M\text{-}1}$, respectively (Fig. 4A). The copy numbers for bla_{SHV}, bla_{TEM} and $bla_{CTX\text{-}M\text{-}1}$ were normalised against the 16S rRNA gene and are shown in Fig. 4B.

3.5. Bacteriological analysis of antibiotic-polluted versus non-antibiotic-polluted river water

As the results using non-cultivable methods revealed the presence of *Enterobacter* and ESBL genes, we built off of this work by further investigating the nature of the bacterial composition using conventional cultivable methods. The viable bacterial count (VBC) of antibiotic-polluted (Pontiac) and non-antibiotic-polluted (Auburn Hills) sample sites were compared and no significant difference in VBC between the two sites was found (Dunnett's *t*-test, $P > 0.05$). In addition, the difference between the VBC on antibiotic-supplemented and non-supplemented TSA plates was compared. The VBC on the non-supplemented TSA plates (control) showed a significant difference compared with supplemented TSA plates for ciprofloxacin (Dunnett's *t*-test, $P < 0.05$, 95% CI), cefotaxime (Dunnett's *t*-test, $P < 0.05$, 95% CI), gentamicin (Dunnett's *t*-test, $P < 0.05$, 95% CI) and tetracycline (Dunnett's *t*-test, $P < 0.05$, 95% CI). There was no significant difference in VBC

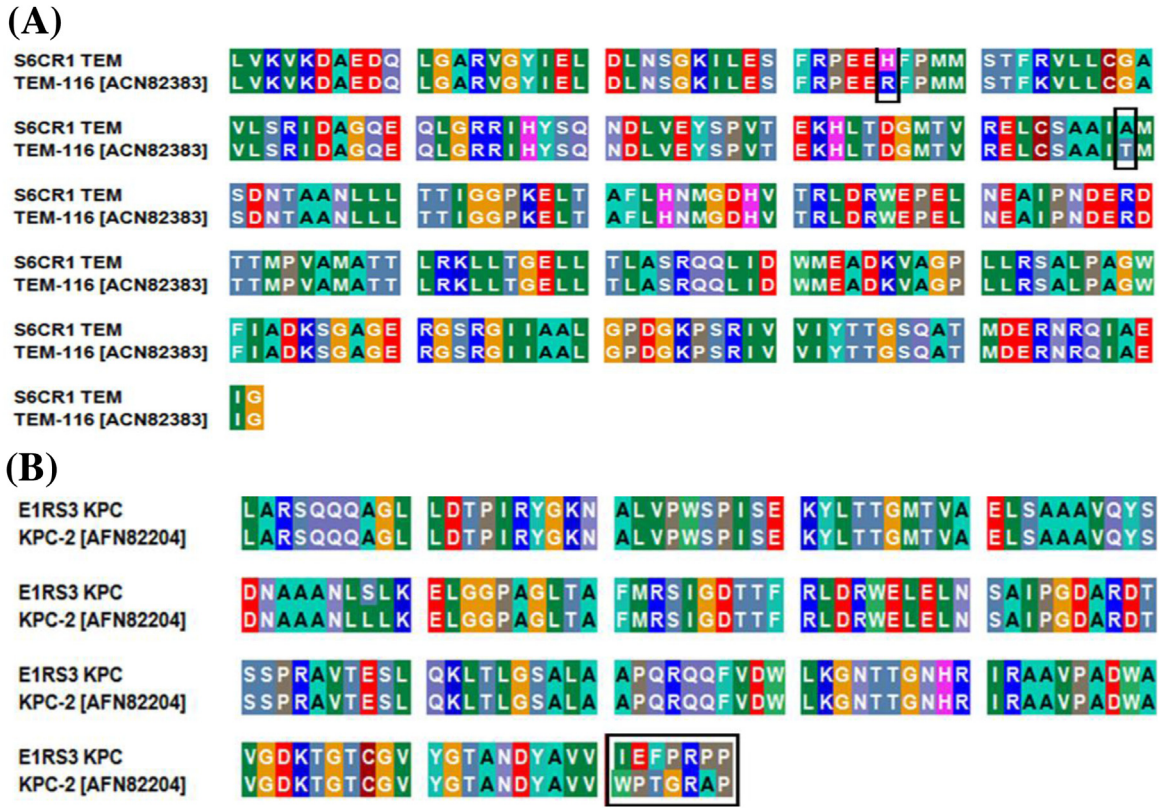

Fig. 5. Amino acid sequence comparison of (A) the bla_{TEM} gene sequence amplified from *Streptococcus porcinus* S6CR1 and (B) the bla_{KPC} gene sequence amplified from *Klebsiella pneumoniae* E1RS3 compared against Clinton River water isolates using BLAST.

between ampicillin-supplemented TSA plates and the control (Dunnett's *t*-test, $P < 0.05$, 95% CI). Overall, the plates supplemented with antibiotics had significantly lower bacterial counts than those that were not supplemented.

To study the phenotypes of cultivable bacteria in river water, morphologically distinct bacterial colonies were selected from the culture plates and were purified by streaking on TSA plates. Purified colonies were identified based on biochemical tests using a BD Phoenix™ System [22]. The identified bacteria were *R. aquatilis* (*n* = 2), *B. cepacia* (*n* = 1), *A. veronii* (*n* = 4), *Aeromonas sobria* (*n* = 17), *A. caviae* (*n* = 9), *Pseudomonas fluorescens* (*n* = 1), *P. oryzihabitans* (*n* = 1), *Pseudomonas pseudoalcaligenes* (*n* = 1), *P. putida* (*n* = 5), *Pseudomonas mendocina* (*n* = 1), *Pseudomonas* sp. (*n* = 3), *Salmonella* Typhi (*n* = 1), *S. gordonii* (*n* = 2), *S. porcinus* (*n* = 1), *L. aquatica* (*n* = 1), *Staphylococcus gallinarum* (*n* = 1), *S. aureus* (*n* = 1), *D. nishinomiyaensis* (*n* = 1), *S. putrefaciens* (*n* = 2), *K. pneumoniae* (*n* = 1), *Y. ruckeri* (*n* = 1), *Enterococcus casseliflavus* (*n* = 1), *Arcanobacterium pyogenes* (*n* = 1), *Bacillus megaterium* (*n* = 2), *Citrobacter braakii* (*n* = 1), *Moraxella* sp. (*n* = 1), and *Macrococcus caseolyticus* (*n* = 1).

3.6. Antimicrobial susceptibility testing of river water bacteria

Antimicrobial susceptibility testing was performed by the Kirby–Bauer disk diffusion method on the 64 biochemically identified bacteria against a panel of six antibiotics (cefotaxime, chloramphenicol, ciprofloxacin, tetracycline, gentamicin and ampicillin). Of the 64 isolates tested, 27 (42%) were resistant to cefotaxime, 34% to chloramphenicol, 11% to ciprofloxacin, 9% to tetracycline and 9% to gentamicin. Of the 27 cefotaxime-resistant bacteria, 11 (41%) were ESBL-producers, among which 4 (36%) were also resistant to aminoglycosides and carbapenems. The characteristics of the 11 ESBL-producing isolates are summarised in Table 3.

3.7. DNA sequence analysis of extended-spectrum β-lactamase genes

Of the 11 ESBL-positive bacteria, 9 amplified for the $bla_{\text{CTX-M-1}}$ gene sequence, *S. porcinus* amplified for the bla_{TEM} gene sequence and *K. pneumoniae* amplified for the bla_{KPC} gene sequence (Supplementary Fig. S1). PCR products were purified and were ligated into a pGEM®-T Easy Cloning Vector and the clones were selected on ampicillin-containing plates. Plasmids were isolated and their sequences were compared against known ESBL sequences. Amplicons for $bla_{\text{CTX-M-1}}$ and bla_{KPC} (accession no. KF444271) showed 100% sequence similarity, whereas the bla_{TEM} (accession no. KF444270) sequence showed 99% similarity to published ESBL sequences (NCBI) both from clinical and environmental bacteria. Amino acid sequence comparison of Clinton River water bla_{TEM} (Fig. 5A) and bla_{KPC} (Fig. 5B) with clinical bacteria showed significant identity.

3.8. Conjugal transfer of antimicrobial resistance

To test whether multidrug-resistant (MDR) ESBL-producing bacteria can transfer antimicrobial resistance traits to other bacteria, conjugal transfer experiments were performed. Among the ESBL-positive bacteria from river water, only *K. pneumoniae* harbouring the $bla_{\text{KPC-2}}$ gene transferred to *E. coli* J53 Azr, which was confirmed by susceptibility testing and PCR of the transconjugants. The transconjugants were found to be resistant to cefotaxime, ertapenem, meropenem and imipenem. Overall, the frequency of transfer was 10^2. The plasmids are self-transmissible as the donor and the transconjugant harboured plasmids of the same size. Furthermore, Southern hybridisation analysis (Fig. 6) and PCR amplification of the resistance genes confirmed the transfer and plasmid location of the bla_{KPC} and $bla_{\text{CTX-M-1}}$ genes in the transconjugants.

3.9. Analysis of the pathogenicity of river water bacteria

Since the sequence analysis of ESBL genes of river water bacteria was similar to that reported in human clinical isolates, we hypothesised that the river water bacteria possess the ability to cause disease. Therefore, we choose to examine the pathogenicity of MDR ESBL-producing river water bacteria. The data showed that these bacteria have the ability to form biofilms (Fig. 7), a critical step in the pathogenesis of bacterial infections. The majority of the ESBL-producing bacteria were moderate biofilm producers, whilst three were low producers and one isolate (*K. pneumoniae* OU11) was classified as a high biofilm producer (Table 3).

Since mucosal epithelial cells provide the first line of defence against invading micro-organisms, we tested whether river water

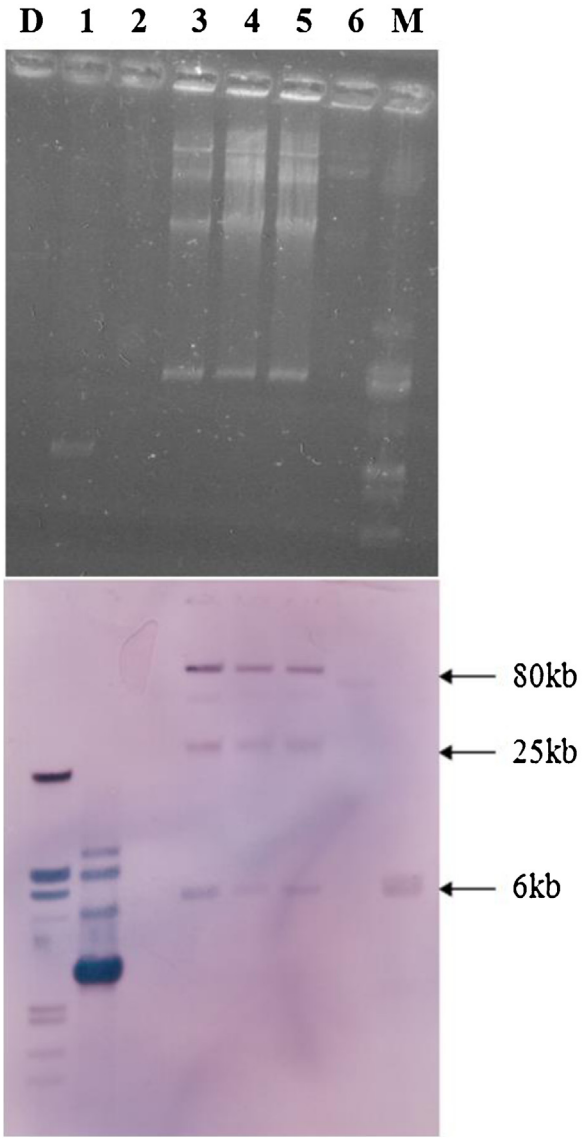

Fig. 6. Gel electrophoresis and Southern hybridisation of donors and transconjugants. Top: agarose gel electrophoresis of *Klebsiella pneumoniae* strain OU11 and transconjugant plasmids. Bottom: Southern blot of *K. pneumoniae* strain OU11 and transconjugant plasmids using bla_{KPC} probe. Lane D, DIG Marker III; lane 1, *Escherichia coli* clone; lane 2, water control; lane 3, *K. pneumoniae* strain OU11; lane 4, *Salmonella typhimurium* transconjugant; lane 5, *Shigella flexneri* transconjugant; lane 6, *K. pneumoniae* ATCC 700603; lane M, *E. coli* V517.

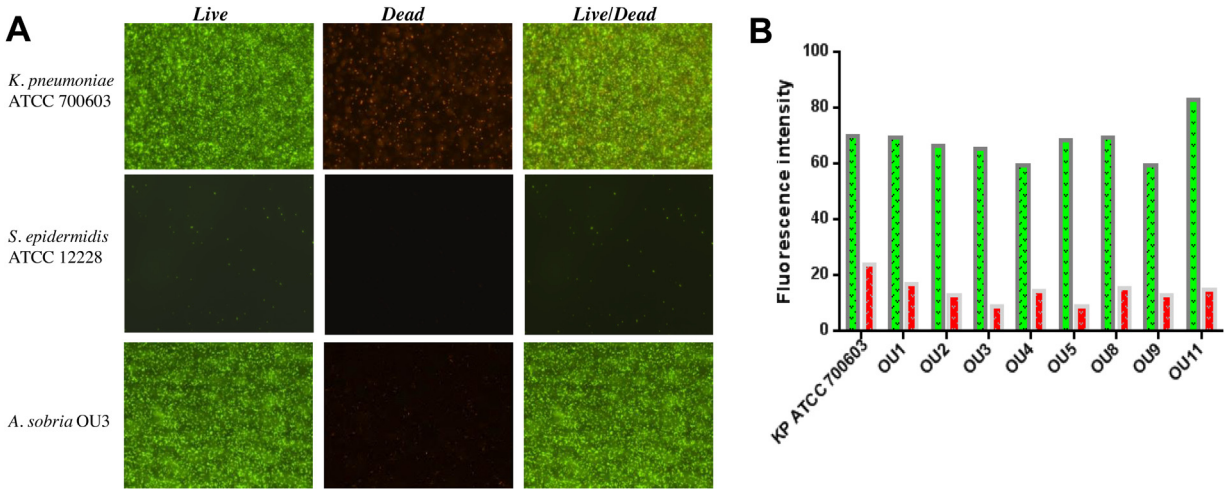

Fig. 7. Determination of biofilm formation by river water bacteria. (A) The ability of bacteria to form biofilms was tested using a LIVE/DEAD *Bac*Light™ Bacterial Viability Kit. Bacteria were labelled with a LIVE/DEAD stain where live bacteria fluoresce green and dead bacteria fluoresce red. (B) Florescence intensity was measured using ImageJ software. The graph represents the intensity of florescence in live and dead bacteria.

bacteria can induce an innate inflammatory response in human intestinal epithelial (Caco-2) cells. To this end, the data showed that exposure of Caco-2 cells to ESBL-producing river water bacteria resulted in the expression of various inflammatory mediators [tumour necrosis factor-alpha (TNFα), interleukin-1-beta (IL-1β), IL-6 and IL-8] both at the mRNA level (assessed by qPCR) and at the protein level [accessed by enzyme-linked immunosorbent assay (ELISA)]. All isolates induced upregulation of IL-1β and IL-8 (ranging from 2–8-fold), whereas the expression pattern of other inflammatory mediators varied among the isolates. The increased production of inflammatory mediators can lead to host cell death. The cytotoxicity analysis revealed that ESBL-positive river water bacteria cause significant cell death upon exposure of Caco-2 cells as evidenced by increased levels of LDH. The percentage cytotoxicity at 8-h post-infection varied among the isolates, ranging from 35% to 65% (Fig. 8). As anticipated, the non-pathogenic cloning strain *E. coli* HB101 caused minimal cytotoxicity (<1%).

4. Discussion

The widespread use of antibiotics has led to the evolution of MDR bacterial strains, with aquatic ecosystems being the primary niche [23]. Exposure to antibiotics can cause selective pressure that favours the occurrence and dissemination of ARB [24]. This is an emerging problem worldwide both in community and hospital settings. Industrial and anthropogenic activities further add to the existing issues. This study was designed to assess ESBL-producing bacteria in urban Clinton River in Michigan and to investigate whether these bacteria possess pathogenic properties necessary to cause bacterial infections. Here we report the presence of MDR ESBL-producing bacteria, including a carbapenemase-producing *K. pneumoniae* strain OU11 [US Centers for Disease Control and Prevention (CDC) classification, urgent threat level] as well as other bacteria of serious health impact (SHV, TEM and CTX-M) (CDC) in water from the Clinton River.

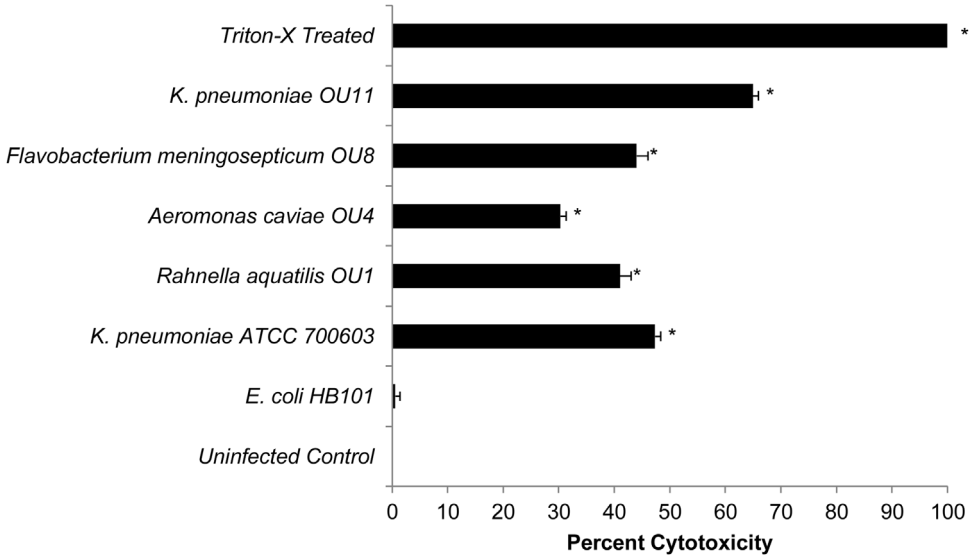

Fig. 8. Assessment of cytotoxicity of river water bacteria on epithelial cells. The conditioned medium (100 μL) from wells containing human intestinal epithelial (Caco-2) cells was collected at 8 h post-infection with river water bacterial isolates and was used to assess cytotoxicity using a Cytotoxicity Detection Kit (LDH). Medium from uninfected cells served as the low control, and cells lysed with Triton-X served as the high control. Data presented are the percentage toxicity compared with the control. Each experiment was performed in triplicate. Values shown are the arithmetic mean; error bars show the standard deviation. * $P < 0.05$.

The degree of ESBL gene (bla_{SHV}, bla_{TEM} and $bla_{CTX-M-1}$) pollution in the Clinton River was assessed by targeting non-cultivable bacteria in the river water and using these strains for amplification of ESBL genes by qPCR. The results showed an abundance of ESBL genes, many of which are highly prevalent in clinical settings [7,25]. However, the current results differed from other studies as the copy number of TEM-type and SHV-type β-lactamase genes was lower in the Clinton River compared with rivers in China [26] and Europe [27]. The current results on the abundance and diversity of ESBL genes in the Clinton River are in line with other studies in China [18] and the UK [28]. In terms of the relative abundance of β-lactamase genes in the bacterial population, the *bla* gene to 16S rRNA gene ratio was found to be of the order of 10^{-3} genes/mL for $bla_{CTX-M-1}$ and 10^{-4} genes/mL for bla_{TEM} and bla_{SHV}. The 16S rRNA gene copy number per mL from Clinton River water was similar to the copy number extracted from Rocky Mountain lakes [29] and from lake water collected from Lausanne's drinking water supply in Switzerland [16] and was of the order of 10^{6} genes/mL.

These ESBL sequences were compared against known $bla_{CTX-M-1}$ and bla_{TEM} sequences using the NCBI BLASTn database. All of the $bla_{CTX-M-1}$ gene sequences from the water isolates were found to be 100% identical to those of a clinical strain of *K. pneumoniae*, designated 516, first isolated in China (GenBank accession no. EU202673). The bla_{TEM} gene sequence of *S. porcinus* showed 99% similarity to a plasmid harbouring the bla_{TEM-1} gene, characteristic of an *E.coli* O25:H4-ST131 clone first isolated in the UK (GenBank accession no. NC_013121). These results suggest that these ESBL sequences are widespread around the globe and that the dissemination of these genes is relevant to public health. The bla_{KPC-2} gene present in *K. pneumoniae* strain OU11 from Clinton River water was shown to be 100% identical to a clinical strain of *K. pneumoniae* containing a carbapenem-hydrolysing β-lactamase gene (bla_{KPC-2}) first isolated in Maryland, USA (GenBank accession no. AY034847). The presence of this KPC-producing bacterium in the Clinton River is alarming as this gene is located on a conjugative plasmid and can mediate conjugal gene transfer to pathogenic and saprophytic bacteria.

A series of BD Phoenix™ System-based biochemical tests was used to identify environmental bacteria isolated from Clinton River water. In previous studies, the BD Phoenix™ System has been successfully used to identify environmental bacteria, including bacteria isolated from river water. All of the isolates tested were identified to species level. To this end, the results indicated that 41% (11/27) of the cefotaxime-resistant Clinton River water bacteria were ESBL-positive, with a predominance (82%) of the $bla_{CTX-M-1}$ gene. Previous studies have also reported a high percentage of bla_{CTX-M} genes in aquatic isolates [6,30]. In summary, the antibiotics tested against environmental bacteria showed a similar effective range to those of clinical isolates in local hospitals. The antimicrobial resistance profile of environmental bacteria isolated from the Clinton River showed similar resistance patterns to those reported for river isolates from the Seine River in France [17] and the Yangtze River basin in China [31]. It is also worrisome that most ESBL-producing strains are frequently cross-resistant to other classes of antimicrobial agents. This is due to the fact that ESBL genes are commonly located on conjugative plasmids that also harbour genes conferring resistance to other classes of antibiotics, such as quinolones and aminoglycosides. It is of particular concern that all 11 ESBL-producing isolates described in this study expressed a MDR phenotype. The MDR phenotype of the isolates could be explained in part due to coexistence of *qnrS*, *aac(6′)*, *tetB*, *tetD* and *tetE* genes in Clinton River water bacteria. However, a more detailed investigation is required to understand the mechanisms of evolution of multidrug resistance in bacteria. Recent studies have shown the occurrence of colistin-resistant bacteria in water and vegetables [32,33], posing a threat to the treatment of bacterial infections caused by carbapenem-, aminoglycoside- and fluoroquinolone-resistant bacteria [34]. However, colistin resistance was not observed in the current study. The spreading potential of plasmid-mediated colistin resistance is worrisome [35] and continued environmental surveillance is needed.

Interestingly, in this study we did not detect the presence of bla_{SHV} genes in the ESBL-positive bacteria by cultivable methods, although other studies have shown the presence of SHV-producing bacteria in the river. This could be due to the dominance of cefotaxime-

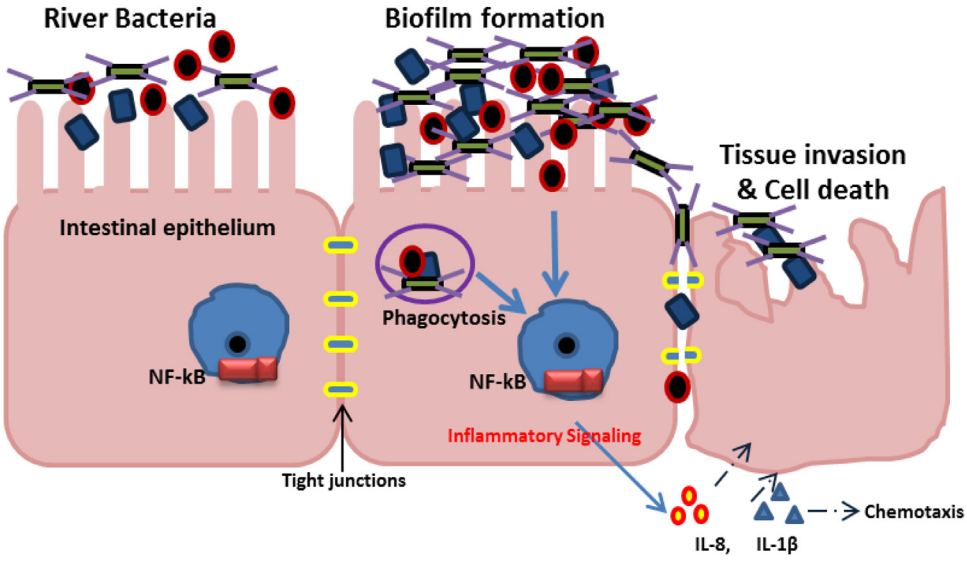

Fig. 9. Proposed mechanisms for inflammatory response and cell death induced by river water bacteria. Mucosal surfaces such as the intestinal epithelium provide the first line of innate host defence against invading pathogens. In immunocompetent individuals, ingestion of an environmental pollutant such as river water bacteria is quickly removed due to strong innate defences characterised by the generation of inflammatory mediators (IL-8 or IL-1β), which attract (chemotaxis) other innate cells such as neutrophils to kill the pathogens. However, ingestion of river water bacteria in immunocompromised individuals (e.g. patients with diabetes, AIDS or cancer) can lead to bacterial proliferation and biofilm formation, triggering persistent inflammation that causes cellular toxicity or cell death and facilitates the invasion of bacteria to deeper tissue layers leading to life-threatening infections. IL, interleukin; AIDS, acquired immune deficiency syndrome; NF-kB, nuclear factor kappa-B.

resistant bacteria in the Clinton River water habitat. To the best of our knowledge, this is the first study to report carbapenemase-producing bacteria in the Clinton River. The emergence of carbapenemase-producing bacteria in Great Lakes Water Bodies (GLWB) is worrisome and studies are currently in progress in our laboratory to assess the diversity of carbapenemase genes in GLWB. bla_{KPC-2} gene pollution was detected in an aquatic environment in Portugal [36], and bla_{IMI-2} gene pollution has been reported in US rivers [3]. Constant monitoring of the dissemination, abundance and diversity of these genes is required to combat the emergence of antimicrobial-resistant micro-organisms in rivers.

Most environmental isolates are saprophytic bacteria and are traditionally labelled as non-pathogenic. However, the presence of pathogenic bacteria in urban river water bodies has been reported [18]. Rivers are used both for recreational [37] and irrigation purposes [27] and could disseminate MDR pathogenic bacteria. Often environmental bacteria do not carry pathogenic traits and are generally considered harmless. To establish that environmental bacteria are indeed non-pathogenic even after acquiring ESBL genes, we examined the pathogenic properties of MDR ESBL-producing bacteria isolated from Clinton River water. Biofilm formation is one of the known virulence properties of pathogenic bacteria causing nosocomial [38] and intestinal [39] infections. The ability of bacteria to form biofilms greatly increases their tolerance to antibiotics and also helps them resist clearance by the host immune system [38], thus inducing a state of chronic inflammation [40]. The results of the current study show that ESBL-producing environmental bacteria (*Aeromonas*, *Rahnella*, *Pseudomonas*, *Flavobacterium* and *Klebsiella*) are capable of forming biofilms and therefore have the potential to be pathogenic and to colonise the host intestinal epithelium. In addition, infected cells were found to be cytotoxic both at 4 h and 8 h post-infection. To test our hypothesis that environmental bacteria could evoke an immune response in the host, Caco-2 cells were infected with MDR river water isolates. The results showed the induction of significantly higher IL-8 levels, especially when Caco-2 cells were infected with river water bacteria. Significantly lower levels of TNFα and IL-1β were detected, which is to be expected as these cytokines govern the expression of other cytokines, including IL-8 and IL-6, and are likely to be secreted during the later stages of infection [41]. As depicted in Fig. 9, although the production of these inflammatory mediators is critical to evoke the innate host defence, their excessive or chronic production could lead to cell death or tissue damage, particularly in immunocompromised individuals.

In summary, this study demonstrates the presence of ESBL-positive bacteria in the Clinton River and showed that these strains possess multiple antimicrobial resistance patterns similar to those of clinical isolates. Moreover, these river water bacteria were found to exhibit virulence traits, suggesting a potential health risk to tourists using Michigan water bodies for recreational activities as well as to aquatic animals (Fig. 9). It is very troublesome that the ESBL-producing strains were co-resistant to gentamicin and ciprofloxacin as this greatly limits possible treatment options with three important classes of antibiotics (β-lactams, amino-glycosides and quinolones). The emergence of carbapenem resistance on conjugal plasmids in the aquatic environment is also worrisome. Further systematic studies are warranted to establish the source and dissemination of resistant bacteria in GLWB as this will no doubt impact both Canadian and American populations.

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Competing interests

None declared.

Ethical approval

Not required.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jgar.2018.10.007.

References

[1] Brown KD, Kulis J, Thomson B, Chapman TH, Mawhinney DB. Occurrence of antibiotics in hospital, residential, and dairy effluent, municipal wastewater, and the Rio Grande in New Mexico. Sci Total Environ 2006;366:772–83.
[2] Karthikeyan KG, Meyer MT. Occurrence of antibiotics in wastewater treatment facilities in Wisconsin, USA. Sci Total Environ 2006;361:196–207.
[3] Aubron C, Poirel L, Ash RJ, Nordmann P. Carbapenemase-producing Enterobacteriaceae, US. rivers. Emerg Infect Dis 2005;11:260–4.
[4] Jang J, Suh YS, Di DY Unno T, Sadowsky MJ, Hur HG. Pathogenic *Escherichia coli* strains producing extended-spectrum β-lactamases in the Yeongsan River basin of South Korea. Environ Sci Technol 2013;47:1128–36.
[5] Montezzi LF, Campana EH, Corrêa LL, Justo LH, Paschoal RP, da Silva ILVD, et al. Occurrence of carbapenemase-producing bacteria in coastal recreational waters. Int J Antimicrob Agents 2015;45:174–7.
[6] Zurfluh K, Hachler H, Nuesch-Inderbinen M, Stephan R. Characteristics of extended-spectrum β-lactamase- and carbapenemase-producing Enterobacteriaceae isolates from rivers and lakes in Switzerland. Appl Environ Microbiol 2013;79:3021–6.
[7] Bush K, Jacoby GA. Updated functional classification of β-lactamases. Antimicrob Agents Chemother 2010;54:969–76.
[8] Paterson DL, Bonomo RA. Extended-spectrum β-lactamases: a clinical update. Clin Microbiol Rev 2005;18:657–86.
[9] Jain A, Mondal R. TEM & SHV genes in extended spectrum β-lactamase producing *Klebsiella* species beta their antimicrobial resistance pattern. Indian J Med Res 2008;128:759–64.
[10] Maal-Bared R, Bartlett KH, Bowie WR, Hall ER. Phenotypic antibiotic resistance of *Escherichia coli* and *E coli* O157 isolated from water, sediment and biofilms in an agricultural watershed in British Columbia. Sci Total Environ 2013;443:315–23.
[11] Nagachinta S, Chen J. Transfer of class 1 integron-mediated antibiotic resistance genes from Shiga toxin-producing *Escherichia coli* to a susceptible *E. coli* K-12 strain in storm water and bovine feces. Appl Environ Microbiol 2008;74:5063–7.
[12] Gatica J, Cytryn E. Impact of treated wastewater irrigation on antibiotic resistance in the soil microbiome. Environ Sci Pollut Res Int 2013;20:3529–38.
[13] Fogarty LR, Duris JW, Crowley SL, Hardigan N. Antibiotic-resistant fecal bacteria, antibiotics, and mercury in surface waters of Oakland County, Michigan, 2005–2006: US Geological Survey Scientific Investigations Report 2007-5242. 2007.
[14] Walia S, Murleedharn C, Band J, Kanwar M, Kumar A. Quantitation of antibiotic resistance genes pollution in hospital waste water effluent and urban Clinton River water, Michigan, USA. Curr Med Res Pract 2016;6:149–51.
[15] Bajaj P, Singh NS, Kanaujia PK, Virdi JS. Distribution and molecular characterization of genes encoding CTX-M and AmpC β-lactamases in *Escherichia coli* isolated from an Indian urban aquatic environment. Sci Total Environ 2015;505:350–6.
[16] Czekalski N, Berthold T, Caucci S, Egli A, Buergmann H. Increased levels of multiresistant bacteria and resistance genes after wastewater treatment and their dissemination into Lake Geneva, Switzerland. Front Microbiol 2012;3:106.
[17] Girlich D, Poirel L, Nordmann P. Diversity of clavulanic acid-inhibited extended-spectrum β-lactamases in *Aeromonas* spp. from the Seine River, Paris, France. Antimicrob Agents Chemother 2011;55:1256–61.
[18] Lu SY, Zhang YL, Geng SN, Li TY, Ye ZM, Zhang DS, et al. High diversity of extended-spectrum β-lactamase-producing bacteria in an urban river sediment habitat. Appl Environ Microbiol 2010;76:5972–6.

[19] Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; nineteenth informational supplement. CLSI document M100-S19. Wayne, PA: CLSI; 2009.
[20] Talreja D, Muraleedharan C, Gunathilaka G, Zhang YF, Kaye KS, Walia SK, et al. Virulence properties of multidrug resistant ocular isolates of *Acinetobacter baumannii*. Curr Eye Res 2014;39:695–704.
[21] Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 2012;9:671–5.
[22] Bhutani N, Muraleedharan C, Talreja D, Rana SW, Walia S, Kumar A, et al. Occurrence of multidrug resistant extended spectrum (-lactamase-producing bacteria on iceberg lettuce retailed for human consumption. Biomed Res Int 2015;2015:547547.
[23] Kummerer K. Antibiotics in the aquatic environment—a review—part I. Chemosphere 2009;75:417–34.
[24] Tian BY, Fadhil NH, Powell JE, Kwong WK, Moran NA. Long-term exposure to antibiotics has caused accumulation of resistance determinants in the gut microbiota of honeybees. MBio 2012;3:, doi:http://dx.doi.org/10.1128/mBio.00377-12 pii: e00377–12.
[25] Falagas ME, Karageorgopoulos DE. Extended-spectrum β-lactamase-producing organisms. J Hosp Infect 2009;73:345–54.
[26] Jiang L, Hu XL, Xu T, Zhang HC, Sheng D, Yin DQ. Prevalence of antibiotic resistance genes and their relationship with antibiotics in the Huangpu River and the drinking water sources, Shanghai, China. Sci Total Environ 2013;458:267–72.
[27] Bockelmann U, Dorries HH, Ayuso-Gabella MN, de Marcay MS, Tandoi V, Levantesi C, et al. Quantitative PCR. monitoring of antibiotic resistance genes and bacterial pathogens in three European artificial groundwater recharge systems. Appl Environ Microbiol 2009;75:154–63.
[28] Dhanji H, Murphy NM, Akhigbe C, Doumith M, Hope R, Livermore DM, et al. Isolation of fluoroquinolone-resistant O25b:H4-ST131 *Escherichia coli* with CTX-M-14 extended-spectrum β-lactamase from UK river water. J Antimicrob Chemother 2011;66:512–6.
[29] Yang H, Byelashov OA, Geornaras I, Goodridge LD, Nightingale KK, Belk KE, et al. Presence of antibiotic-resistant commensal bacteria in samples from agricultural, city, and national park environments evaluated by standard culture and real-time PCR methods. Can J Microbiol 2010;56:761–70.
[30] Tacao M, Correia A, Henriques I. Resistance to broad-spectrum antibiotics in aquatic systems: anthropogenic activities modulate the dissemination of $bla_{\text{CTX-M-like}}$ genes. Appl Environ Microbiol 2012;78:4134–40.
[31] Chen H, Shu WQ, Chang XS, Chen JA, Guo YB, Tan Y. The profile of antibiotics resistance and integrons of extended-spectrum β-lactamase producing thermotolerant coliforms isolated from the Yangtze River basin in Chongqing. Environ Pollut 2010;158:2459–64.
[32] Caltagirone M, Nucleo E, Spalla M, Zara F, Novazzi F, Marchetti VM, et al. Occurrence of extended spectrum β-lactamases, KPC-type, and MCR-1.2-producing Enterobacteriaceae from wells, river water, and wastewater treatment plants in Oltrepò Pavese Area, Northern Italy. Front Microbiol 2017;8:2232.
[33] Zurfuh K, Poirel L, Nordmann P, Nuesch-Inderbinen M, Hachler H, Stephan R. Occurrence of the plasmid-borne *mcr-1* colistin resistance gene in extended-spectrum-β-lactamase-producing Enterobacteriaceae in river water and imported vegetable samples in Switzerland. Antimicrob Agents Chemother 2016;60:2594–5.
[34] Marti E, Huerta B, Rodriguez-Mozaz S, Barcelo D, Jofre J, Balcazar JL. Characterization of ciprofloxacin-resistant isolates from a wastewater treatment plant and its receiving river. Water Res 2014;61:67–76.
[35] Marchaim D, Chopra T, Pogue JM, Perez F, Hujer AM, Rudin S, et al. Outbreak of colistin-resistant, carbapenem-resistant *Klebsiella pneumoniae* in Metropolitan Detroit, Michigan.Antimicrob Agents. Chemother 2011;55:593–9.
[36] Poirel L, Barbosa-Vasconcelos A, Simoes RR, Da Costa PM, Liu W, Nordmann P. Environmental KPC-producing *Escherichia coli* isolates in Portugal. Antimicrob Agents Chemother 2012;56:1662–3.
[37] Hamelin K, Bruant G, El-Shaarawi A, Hill S, Edge TA, Bekal S, et al. A virulence and antimicrobial resistance DNA microarray detects a high frequency of virulence genes in *Escherichia coli* isolates from Great Lakes recreational waters. Appl Environ Microbiol 2006;72:4200–6.
[38] Sambanthamoorthy K, Gokhale AA, Lao WW, Parashar V, Neiditch MB, Semmelhack MF, et al. Identification of a novel benzimidazole that inhibits bacterial biofilm formation in a broad-spectrum manner. Antimicrob Agents Chemother 2011;55:4369–78.
[39] Bommarius B, Anyanful A, Izrayelit Y, Bhatt S, Cartwright E, Wang W, et al. A family of indoles regulate virulence and Shiga toxin production in pathogenic *E coli*. PLoS One 2013;8:e54456.
[40] Wolcott RD, Rhoads DD, Bennett ME, Wolcott BM, Gogokhia L, Costerton JW, et al. Chronic wounds and the medical biofilm paradigm. J Wound Care 2010;19:45–53.
[41] Xue ML, Thakur A, Lutze-Mann L, Willcox MDP. Pro-inflammatory cytokine/chemokine gene expression in human corneal epithelial cells colonized by *Pseudomonas aeruginosa*. Clin Exp Ophthalmol 2000;28:197–200.