Research Article

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Extended-spectrum β-lactamase-producing *Escherichia coli* in wastewaters and refugee camp in Lebanon

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Aim: To evaluate the effects of population influx of refugees on the prevalence of extended-spectrum β-lactamase-producing *Escherichia coli* in wastewater networks in Lebanon. **Materials & methods:** Pulsed-field gel electrophoresis, multilocus sequence typing and antibiotic resistance genes typing were performed. **Results:** 53.1% of isolates recovered from Al-Qaa refugee camp were positive for the tested resistant determinants compared with 49.1% from river effluents. All isolates carried *aac(6)-1b* and/or *aac(3)-II*; none carried *armA, rmtB, ant(4′)-Ia, aph(3′)-Ia* or carbapenemases. CTX-M-15, TEM-1, OXA-1, CMY-2 and SHV-12 were detected. Single and/or double substitutions were detected in GyrA and ParC. Phylogenetic group B2 and ST6470 were the most prevalent. Pulsed-field gel electrophoresis revealed 19 XbaI patterns and 17 pulsotypes. **Conclusion:** The introduction of novel resistance patterns into the wastewater network requires effective control.

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Keywords: antibiotics resistance • ESBLs • Lebanon • MLST • PFGE • population migrations • wastewater

The presence of antibiotic-resistant bacteria in wastewater and sewage networks is a growing public health concern [1]. Resistant bacteria have been previously isolated in soil [2], sewage [3], surface water [4], rural groundwater supplies [5] and municipal drinking water [6]. Aquatic environments provide ideal settings for the horizontal exchange of mobile genetic elements encoding various antibiotic resistance genes (ARGs) [7]. The presence of pollutants, such as heavy metals, also selects for antibiotic resistance [8]. ARGs in water reservoirs originate either as a result of bacterial defense mechanisms [9] or they are transported through runoff processes from human and animal origins [10]. It is accordingly important to determine the risks of the development and spread of resistant bacteria in contaminated environments.

With the absence of adequate wastewater disposal and treatment, the problem of bacteriological contamination in different water sources in Lebanon is extremely aggravated. The Syrian crisis has resulted in the migration of over 900,000 Syrian refugees exerting a major strain on existing resources and services. These are distributed in four major regions in Lebanon with 62% living in the North and Bekaa and the remaining 38% distributed in the Beirut area, Mount Lebanon, Saida and Tyr [11]. The bacterial contamination levels of natural water resources in Lebanon prior to the crisis were variable in rural areas but significantly high in urban areas [12]. Even before the crisis, only 8% of wastewater in Lebanon was treated [11]. The remaining of the population uses cesspools and septic tanks or releases raw sewage directly into the environment [13]. In the absence of efficient community sanitation and sewage disposal and treatment, the risks of transmission of hospital organisms, into the community are extremely high and pose a public health concern [14].

Extended-spectrum β-lactamases (ESBL)-producing *Escherichia coli* were detected in aquatic environments worldwide [15–18] including carriers of $bla_{\text{CTX-M}}$, bla_{OXA}, bla_{SHV} and bla_{TEM} β-lactamases, with some ESBL-genotypes

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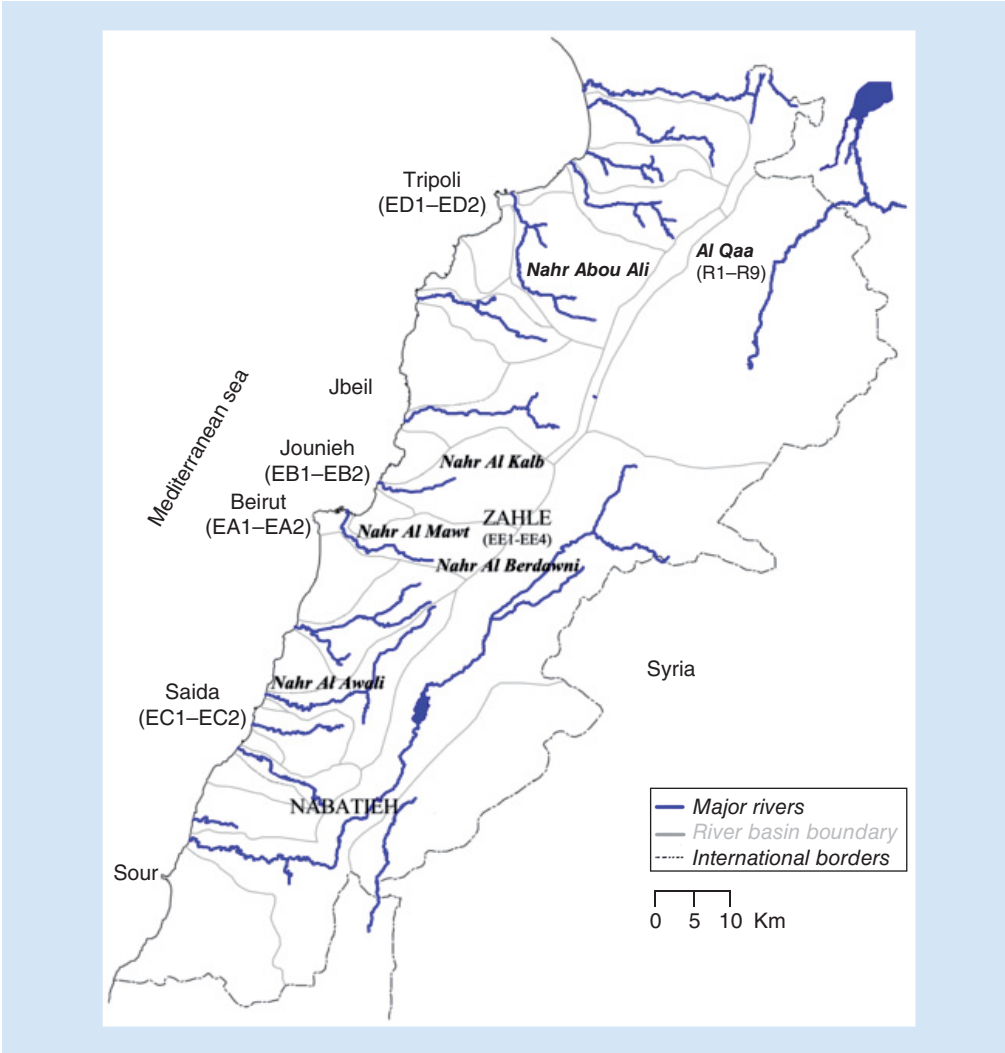


Figure 1. Map of the Lebanese territory depicting the river effluents from where the sewage samples were collected and Al-Qaa refugee camp locations.

showing region-specific patterns in the environment [15]. *E. coli* are used as indicators of fecal contamination in water resources in both urban and rural areas. Despite their diversity, some characters are shared between different strains when exposed to similar environments, which is attributed to selection pressure [19]. Previous studies on the resistance patterns of *E. coli* isolated from sewage showed the highest resistance to tetracycline, ampicillin/clavulanic acid and trimethoprim/sulfamethoxazole [20–21]. Additionally, CTX-M-15 and TEM were among the most common resistance determinants detected in ESBL-producing *E. coli* recovered from water networks [22].

The aim of this study was to investigate and characterize the resistance characteristics of environmental ESBL-producing *E. coli* isolated from river effluents and Al-Qaa refugee camp in Lebanon. The genomic analysis of environmental ESBL-producing bacteria will provide important insights into better understanding of the abundance and spread of resistance determinants. This is a pilot study attempting to explain the increase in clinical cases of infection with multidrug-resistant (MDR) organisms that are being reported in Lebanon.

Materials & methods

Study setting

Sewage water samples were collected from Al-Qaa refugee camp and five major river effluents across Lebanon in which sewage is dumped (Figure 1): Nahr Al-Mawt (Beirut), Nahr Al-Kalb (Jounieh), Nahr Al-Awali (Saida), Nahr Abou Ali (Tripoli) and Al-Berdawni river (Zahle). 5 l per sample was collected in sterile containers, transported to

the laboratory at 5 ± 2°C within 2 h of collection and processed immediately for the enumeration of bacteria. Two samples were collected per site at once during the spring of 2016 and analyzed separately.

All collected samples were diluted at 1/10 with sterile saline and inoculated on MacConkey agar. Lactose fermenters were identified using API 20E gallery (BioMérieux, Inc, MA, USA).

A total of 44 strains were isolated from the water samples. These included a variety of organisms such as *Pseudomonas* spp., *Acinetobacter* spp., *Pasteurella* spp., *Aeromonas* spp., *Proteus* spp., *Klebsiella* spp., *Enterobacter* spp. and *Alcaligenes* spp. *E. coli* comprised the majority of the isolates (n = 34). Upon antimicrobial susceptibility testing, 21 strains were ESBL-producing and thus were selected for further analysis.

Antimicrobial susceptibility testing

Antimicrobial susceptibility profiles of the 21 isolates were determined by the disk-diffusion method, using 30 different antibiotics: amoxicillin, amoxicillin/clavulanic acid, ticarcillin, piperacillin, piperacillin–tazobactam, imipenem, ertapenem, aztreonam, cefalotin, cefuroxime, cefoxitin, cefotaxime, ceftriaxone, ceftazidime, cefepime, cefixime, tobramycin, gentamicin, amikacin, ofloxacin, ciprofloxacin, norfloxacin, tetracycline, levofloxacin, tigecyclin, trimethoprim-sulfamethoxazole, nitrofurantoin, fosfomycin, colistin and meropenem. The results were interpreted according to the Clinical Laboratory Standards Institute guidelines [23].

ESBL-positive isolates were identified by using the double-disk synergy test with third-generation cephalosporins (cefotaxime, ceftazidime and cefixime). All materials were purchased from Bio-Rad Corporation (PA, USA). The results were interpreted according to the Clinical Laboratory Standards Institute guidelines [23].

DNA extraction

DNA extraction was performed on all isolates after inoculation in TSB media and incubation overnight at 37°C, using the NucleoSpin® Tissue Kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions.

Phylogenetic typing

Phylogenetic typing was performed to determine the main phylogenetic groups (A, B1, B2 and D) of the *E. coli* isolates through the PCR amplification of *chuA*, *yjaA* and *tspE4C2* genes, as described previously by Clermont *et al.* [24].

Multilocus sequence typing

All the 21 isolates were typed by multilocus sequence typing (MLST) and their allelic profiles were reported for seven conserved housekeeping genes: *adk, fumC, gyrB, icd, mdh, purA* and *recA*. Allelic-specific PCR was carried under the conditions retrieved from the online available Warwick database [25].

Pulsed-field gel electrophoresis fingerprinting

To analyze the genetic relatedness of the strains, pulsed-field gel electrophoresis (PFGE) fingerprinting was performed on the 21 isolates using XbaI restriction enzyme (Thermo Fisher Scientific, MA, USA), 1% SeaKem agarose gel and the universal laboratory standard *Salmonella enterica* subsp. *enterica* serovar *Braenderup* (ATCC® BAA664™) according to the standard PulseNet protocol [26]. Electrophoresis was performed using the Bio-Rad Laboratories CHEF DR-III system (Bio-Rad Laboratories, Inc, CA, USA) under the conditions set for non-O157 *E. coli* strains [27]. Gels were stained with ethidium bromide. PFGE profiles were analyzed with the BioNumerics software version 7.6.1 (Applied Maths, St-Martens-Latem, Belgium). The pulsotypes were clustered based on the BioNumerics software analysis through dice correlation coefficients with an optimization of 1% and tolerance of 1%. Pulsotypes belonging to a single cluster were 61–77% related to one another. Two isolates were untypable by PFGE (R4 and R5) and required further tests to confirm their identity.

Characterization of PFGE untypable isolates

Two of the isolates (R4 and R5) were untypable by PFGE using both primary and secondary restriction enzymes, XbaI and BlnI/AvrII, respectively. Their identity was further confirmed by 16S rRNA sequencing.

The 16S rRNA gene was amplified using primers SSU-bact-27F (5′-AGAGTTTGATCMTGGCTGAG-3′) and SSU-bact-519R (5′-GWATTACCGCGGCKGCTG-3′) [28]. Amplification was performed in a total volume of 20 µl containing 2-mM deoxynucleoside triphosphates, 10X Taq buffer, 25 mM $MgCl_2$, 20 µM of each primer and 5 U/µl of HotStart AmpliTaq Gold DNA polymerase. The thermal cycling conditions were the following: 95°C

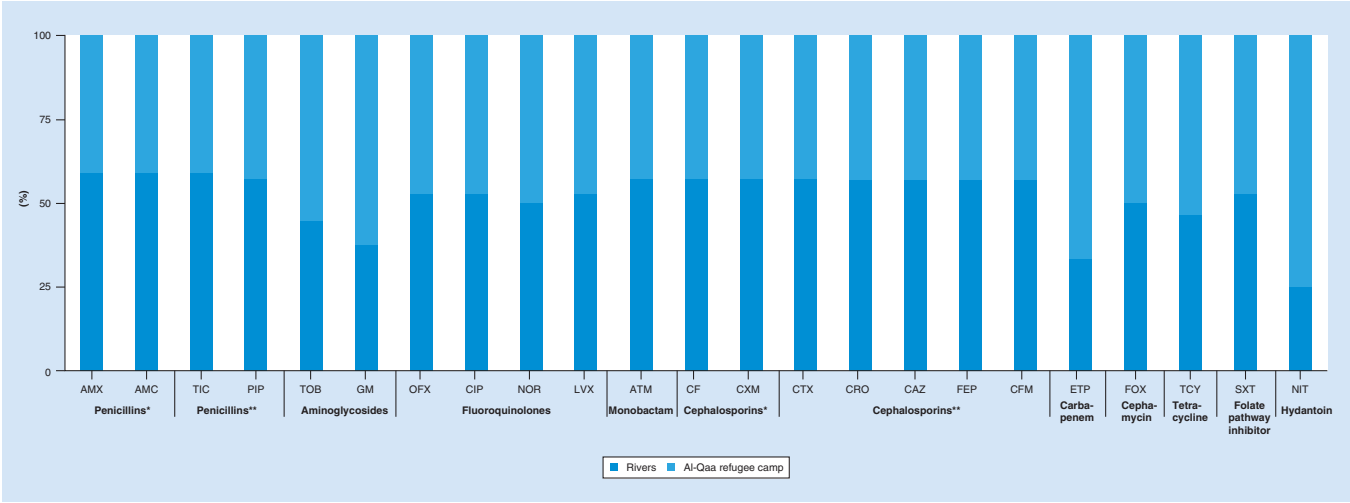


Figure 2. Percentage of resistance against 23 tested antibiotics among extended-spectrum β-lactamases-producing *Escherichia coli* in river effluents and Al-Qaa refugee camp in Lebanon.
AMC: Amoxicillin/clavulanic acid; AMX: Amoxicillin; ATM: Aztreonam; CAZ: Ceftazidime; Cephalosporins*: Non-extended spectrum cephalosporins first- and second-generation; Cephalosporins**: Extended-spectrum cephalosporins third- and fourth-generation; CF: Cefalotin; CFM: Cefixime; CIP: Ciprofloxacin; CRO: Ceftriaxone; CTX: Cefotaxime; CXM: Cefuroxime; ETP: Ertapenem; FEP: Cefepime; FOX: Cefoxitin; GM: Gentamicin; LVX: Levofloxacin; NIT: Nitrofurantoin; NOR: Norfloxacin; OFX: Ofloxacin; Penicillins*: Penicillin + β-lactamase inhibitor; Penicillins**: Antipseudomonal penicillin + β-lactamase inhibitor; PIP: Piperacillin; SXT: Trimethoprime/sulfamethoxazole; TCY: Tetracycline; TIC: Ticarcillin; TOB: Tobramycin.

for 12 min, followed by 30 cycles of 94°C for 30 s, 53°C for 30 s and 72°C for 1 min, and a final extension of 72°C for 10 min. The resulting PCR products were sequenced on the Genetic Analyzer 3500 (Applied Biosystems™, CA, USA). Obtained sequences were extracted using Sequencing Analysis v5.4 software (Applied Biosystems), assembled using CLC Main Workbench v7.0.2 (CLC-bio, Aarhus, Denmark) and blasted against the NCBI 16S rRNA database.

Characterization of ESBL-producing isolates

The presence of β-lactamase-encoding genes was identified through the amplification and sequencing of bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, bla_{OXA} and bla_{ampC} genes. The isolates were screened for gene-encoding carbapenems through the amplification of bla_{VIM1}, bla_{VIM2}, bla_{NDM}, bla_{KPC}, $bla_{KPC\text{-}2}$, bla_{IMP} and $bla_{OXA\text{-}48}$. Aminoglycoside-resistance genes were identified through the amplification of *aac(6′)-1b*, *aac(3′)-II, ant(2′′)-Ia, ant(4′)-Iia, aph(3′)-Ia, armA I* and *rmtB*. Quinolone resistance was screened through the sequencing of *gyrA* and *parC* and searching for amino acid substitutions in the quinolone resistance-determining regions of *gyrA* and *parC* compared with the amino acid sequences retrieved from the genome of *E. coli* K12 (CP002291) used as a reference. Finally, the presence of integrons was detected through the primer set *hep*35 and *hep*36 and class I integrons were further screened using the Intl1 primers set (Table 1).

Results

Antimicrobial susceptibility

According to the definitions proposed by Magiorakos *et al.* [47], all of the isolates were MDR by being nonsusceptible to ≥1 agent in more than three antimicrobial categories. Resistance to penicillins (amoxicillin, tricarcillin and piperacillin) and penicillin/inhibitor combination was detected in all isolates recovered from the refugee camp and river effluents. All the isolates were resistant to at least seven out of the eight tested cephalosporins with EA1, ED1, R3, R5, and R9 being additionally resistant to cephamycin (cefoxitin). All isolates were resistant to the monobactam, aztreonam. The refugee camp isolates showed higher resistance to aminoglycosides, fluoroquinolones and trimethoprim/sulfamethoxazole. Notably, only the latter were resistant to ertapenem (n = 2; 22%; Figure 2). All isolates were susceptible to amikacin, tigecycline, fosfomycin, colistin, meropenem and imipenem.

Table 1. PCR targeted genes and primer sequences used for detection of antibiotic resistance in extended-spectrum β-lactamases-producing *Escherichia coli*.

Sets	PCR target	Primer sequence (5′–3′)	Ta (°C)	Length (bp)	Ref.
I	**β-lactam resistance**				
1	TEM	TemA1: ATAAAATTCTTGAAGAC	42	1075	[29]
		TemB1: TTACCAATGCTTAATCA			
2	SHV	SHV-F: CACTCAAGGATGTATTGTG	49	822	[30]
		SHV-R: TTAGCGTTGCCAGTGCTCG			
3	CTX-M	CTX-C1: ATGTGCAGCACCAGTAAAGT	54	545	[31]
		CTX-C2: ACCGCGATATCGTTGGTGG			
4	OXA	OXA-F: ACACAATACATATCAACTTCGC	61	813	[32]
		OXA-R: AGTGTGTTTAGAATGGTGATC			
5	AmpC	AmpC-F: ATGATGAAAAAATCGTTATGC	56	1143	[33]
		AmpC-R: TTGCAGCTTTTCAAGAATGCGC			
II	**Carbapenem resistance**				
6	VIM1	VIM1-F: AGTGGTGAGTATCCGACAG	52	261	[34]
		VIM1-R: ATGAAAGTGCGTGGAGAC			
7	VIM2	VIM2-F: ATGTTCAAACTTTTGAGTAAG	57	801	[35]
		VIM2-R: CTACTCAACGACTGAGCG			
8	NDM	NDM-F: TTGCCCAATATTATGCACCC	55	420	[36]
		NDM-R: ATTGGCATAAGTCGCAATCC			
9	NDM	preNDM-F: CACCTCATGTTTGAATTCGCC	55	984	[37]
		preNDM-R: CTCTGTCACATCGAAATCGC			
10	OXA-48	OXA-48-F: TTGGTG GCATCGATTATCGG	60	743	[38]
		OXA-48-R: GAGCACTTCTTTTGTGATG GC			
11	IMP	IMP-U-F: GAATAGRRTGGCTTAAYTCTC	52	188	[39]
		IMP-U-R: CCAAACYACTASGTTATC			
12	KPC	KPC-F: GCTACACCTAGCTCCACCTTC	58	990	[38]
		KPC-R: TCCTAGCCTAAATGTGAC			
13	KPC-2	KPC2-F: GCAGCGGCAGCAGTTTGTTGATT	50	800	[40]
		KPC2-R: GTAGACGGCCAACACAATAGGTGC			
III	**Aminoglycoside resistance**				
14	aac(6)-1b	AAC(6)ID: CATGACTGAGCATGACCTT	49	528	[41]
		AAC(6)IU: GAAGGGTTAGGCATCACT			
15	aac(3)-II	ACC3F: CAATAACGGAGGCAATTCG	80	868	[41]
		ACC3R: GATTATCATTGTCGACGG			
16	ant2″-Ia	ant2″-Ia-F: ACGCCGTGGGTCGATGTTTGATGT	60	572	[42]
		ant2″-Ia-R: CTTTTCCGCCCCGAGTGAGGTG			
17	ant4′-Iia	ant4′-Iia-F: CCGGGGCGAGGCGAGTGC	55	423	[42]
		ant4′-Iia-R: TACGTGGGCGGATTGATGGGAACC			
18	aph3′-Ia	aph3′-Ia-F: CGAGCATCAAATGAAACTGC	54	625	[42]
		aph3′-Ia-R: GCGTTGCCAATGATGTTACAG			
19	armA	armA-F: GTGCGAAAACAGTCGTAG	55	846	[43]
		armA-R: GAAGTCAGGATACACCAG			
20	rmtB	rmtB-F: GAGACACCGATGAACATC	55	756	[43]
		rmtB-R: CCTTCTGATTGGCTTATC			
IV	**Quinolone resistance**				
21	gyrA	gyrA-F: CGACCTTGCGAGAGAAAT	58	625	[44]
		gyrA-R: GTTCCATCAGCCCTTCAA			
22	parC	parC-F: CGATTGCCGCCTGAGCCACTT	58	605	[44]
		parC-R: GCGAATAAGTTGAGGAATCAG			

Table 1. PCR targeted genes and primer sequences used for detection of antibiotic resistance in extended-spectrum β-lactamases-producing *Escherichia coli* (cont.).

Sets	PCR target	Primer sequence (5′–3′)	Ta (°C)	Length (bp)	Ref.
V	**Integron-encoded integrase**				
23	Intergrons	hep35: TGCGGGTYAARGATBTKGATTT	49	Variable	[45]
		hep36: CARCACATGCGTRTARAT			
24	IntI1	IntI1-F: ACATGTGATGGCGACGCACGA	60	569	[46]
		IntI1-R: ATTTCTGTCCTGGCTGGCGA			

Detection of ARGs

On average, in the 21 *E. coli* isolates identified as being ESBL producers, 53.1% (average = 4.78) of the isolates recovered from the Al-Qaa refugee camp were positive for eight of the tested resistance determinants (CTX-M-14, OXA-1, SHV-12, CMY-2*, aac(6)-Ib, acc(3)-II, acc(3)-II, Int-I1*), compared with 49.1% (average = 4.41) of those from river effluents. ESBL prevalence in the refugee camp and river effluents was comparable and no significant difference was detected. R5, R9 and EE3 carried the bla_{ampC} gene, which upon sequencing was identified as CMY-2.

All the isolates carried at least one of the two genes encoding for aminoglycoside resistance *aac(6)-1b* and/or *aac(3)-II*, while remained negative for *armA, rmtB, ant(4′)-Iia* and *aph(3′)-Ia*. On the other hand, CTX-M, TEM and SHV were detected at a higher (bla_{CTX-M}, bla_{TEM}) or similar (bla_{SHV}) percentages in the isolates from the river effluents compared with those from the refugee camp. We did not detect any of the following carbapenemase genes: bla_{VIM1}, bla_{VIM2}, bla_{NDM}, bla_{KPC}, bla_{KPC-2}, bla_{IMP} and bla_{OXA-48}.

Alterations in the target proteins of fluoroquinolones in some of the isolates as single or double substitutions were detected upon sequencing of *gyrA* and *parC* (Table 2).

Phylogenetic typing

Overall, *E. coli* phylogenetic group B2 was the most prevalent representing 38.1% (n = 8) of all isolates, followed by type A (n = 7; 33.3%) and type D (n = 6; 28.6%). This order of prevalence was also seen among isolates collected from river effluents being: 50.0% (n = 5), 33.3% (n = 4) and 16.7% (n = 2) for types B2, A and D, respectively. Within the refugee camp isolates, phylogroup D was the most prevalent (n = 4; 44.4%), followed by A (n = 3; 33.3%) and B2 (n = 2; 22.2%). The average resistance detected within phylogroup B2 isolates recovered between river effluents and the refugee camp (rivers: 52%; refugee camp: 58%), and phylogroup D (rivers: 67%; refugee camp: 69%) was comparable, while it was higher for isolates of phylogroup A from the refugee camp (67%) compared with the ones from river effluents (43%).

Multilocus sequence typing

In total 11 sequence types (STs) were identified. ST6470 was the most commonly detected (n = 6; 28.6%), followed by ST90 (n = 4; 19.1%) and ST4144 (n = 2; 9.52%). ST4608, ST6480 and ST6894 were only recovered from the Al-Qaa refugee camp, while four other STs (ST617, ST38, ST6222 and ST6450) were unique to the river effluents. In general, high diversity in the ST populations was observed in all the sites except for Nahr El-Mawt having only ST617 (n = 2; Table 3).

PFGE analysis

Genomic DNA fingerprinting of the isolates using PFGE revealed 19 distinct XbaI restriction patterns producing 17 different pulsotypes according to previously defined standards [47] (Figure 3). Two of the isolates recovered from Al-Qaa refugee camp effluent (R4 and R5) were untypable, belonged to ST4608 and ST90, and were citrate-positive variants. Both were confirmed to be *E. coli* using API 20E (BioMérieux, Inc), RAPID*E. coli* 2 Agar (Bio-Rad) and 16S rRNA sequencing.

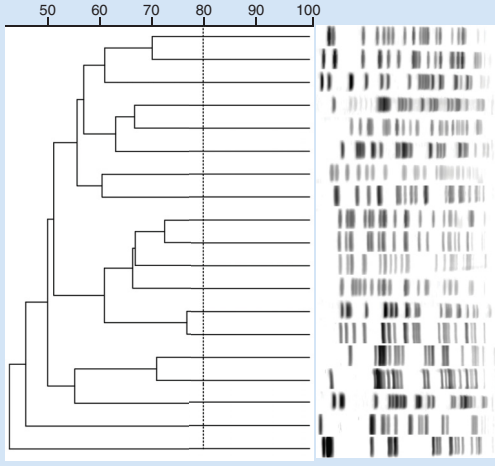
Six isolates that had the same MLST sequence type ST6470, were recovered from all sites except from Nahr El-Mawt, and belonged to phylogroup B2 (except EB2 that belonged to group D), clustered together and represented pulsotypes 9 (EB2 and EE1), 10 (ED2), 11 (EC2) and 12 (R1and R2).

Studying the clonal relationship of all the isolates using PFGE, MLST and phylogenetic typing revealed that there was no significant association between the MLST type, the phylogenetic group and the pulsotype. However, PFGE correctly segregated *E. coli* isolates belonging to different STs as different types.

Table 2. Prevalence of PCR-detected antibiotic resistance genes among extended-spectrum β-lactamases-producing *Escherichia coli* collected from river effluents and Al-Qaa refugee camp in Lebanon and amino acid substitutions in *gyrA* and *parC*.

Region	River source	Key	bla_{CTX-M}	bla_{TEM-1}	bla_{SHV-12}	bla_{OXA-1}	*aac(6)-1b*	*acc3-II*	CMY-2	*intI1*	*gyrA*	*parC*
Beirut	Nahr Al-Mawt	EA1	CTX-M-15	+	+	–	+	+	–	+	–	–
		EA2	CTX-M-15	+	–	+	+	+	–	+	–	–
Jounieh	Nahr Al-Kalb	EB1	CTX-M-15	+	–	–	–	+	–	+	–	–
		EB2	CTX-M-15	–	+	+	+	+	–	+	–	S80I
Saida	Nahr Al-Awali	EC1	–	–	–	–	+	+	–	–	–	–
		EC2	–	–	–	–	+	–	–	–	–	E84K
Tripoli	Abou-Ali	ED1	CTX-M-27	+	–	–	+	+	–	+	–	–
		ED2	CTX-M-15	+	–	–	+	+	–	–	D87V	S80I
Zahle	Berdawni	EE1	CTX-M-15	–	+	+	+	+	–	–	–	S80I & E84V
		EE2	–	–	+	+	+	+	–	–	–	S80I
		EE3	CTX-M-15	–	–	–	+	+	+	+	–	S80I
		EE4	CTX-M-9	–	–	–	+	+	–	–	–	–
%			75.0	41.7	33.3	33.3	91.7	91.7	8.3	50.0		
Refugee camp	Al-Qaa	R1	CTX-M-15	–	–	+	+	+	–	–	–	S80I & E84V
		R2	CTX-M-15	–	–	–	+	+	–	–	S83T	–
		R3	CTX-M-15	–	+	+	+	+	–	+	–	–
		R4	CTX-M-15	–	–	–	+	+	–	+	–	–
		R5	–	+	–	–	+	+	+	+	–	–
		R6	–	+	+	–	+	+	–	+	–	–
		R7	CTX-M-15	–	–	+	+	+	–	+	–	–
		R8	–	+	–	–	+	+	–	+	–	–
		R9	CTX-M-14	–	+	+	+	+	+	+	–	–
%			66.7	33.3	33.3	44.4	100.0	100.0	22.2	77.8		

–: Absence; +: Presence.



Key	Type	Phylo	ST	Genotype	Integron
EB1	1	A	ST4144	CTX-M-15, TEM-1, acc(3)-II	Int-I1
EE4	2	A	ST38	CTX-M-9, aac(6)-Ib, acc(3)-II	–
R3	3	D	ST4608	CTX-M-15, OXA-1, SHV-12, aac(6)-Ib, acc(3)-II	Int-I1
R7	4	D	ST6894	CTX-M-15, OXA-1, aac(6)-Ib, acc(3)-II	Int-I1
R9	5	D	ST4144	CTX-M-14, OXA-1, SHV-12, CMY-2, aac(6)-Ib, acc(3)-II, acc(3)-II	Int-I1
R6	6	A	ST6480	TEM-1, SHV-12, aac(6)-Ib, acc(3)-II	Int-I1
ED1	7	B2	ST6222	CTX-M-15, TEM-1, aac(6)-Ib, acc(3)-II	–
EE3	8	A	ST90	CTX-M-15, OXA-1, CMY-2, aac(6)-Ib, acc(3)-II	Int-I1
EB2	9	D	ST6470	CTX-M-15, OXA-1, SHV-12, aac(6)-Ib, acc(3)-II	Int-I1
EE1	9	B2	ST6470	CTX-M-15, OXA-1, SHV-12, aac(6)-Ib, acc(3)-II	–
ED2	10	B2	ST6470	CTX-M-27, TEM-1, aac(6)-Ib, acc(3)-II	Int-I1
EC2	11	B2	ST6470	aac(6)-Ib	–
R1	12	B2	ST6470	CTX-M-15, OXA-1, aac(6)-Ib, acc(3)-II	–
R2	12	B2	ST6470	CTX-M-15, aac(6)-Ib, acc(3)-II	–
EA1	13	B2	ST617	CTX-M-15, TEM-1, OXA-1, aac(6)-Ib, acc(3)-II	Int-I1
EA2	14	B2	ST617	CTX-M-15, TEM-1, SHV-12, aac(6)-Ib, acc(3)-II	Int-I1
R8	15	A	ST90	TEM-1, aac(6)-Ib, acc(3)-II	Int-I1
EE2	16	D	ST90	OXA-1, SHV-12, ant(2")-Ia, aac(6)-Ib, acc(3)-II	–
EC1	17	A	ST6450	aac(6)-Ib, acc(3)-II	–

Figure 3. Pulsed-field gel electrophoresis patterns, STs, phylogenetic groups and genotype of extended-spectrum β-lactamases-producing *Escherichia coli*.
Phylo: Phylogenetic type; ST: Sequence type.

Table 3. Distribution of PCR detected antibiotic resistance genes among extended-spectrum β-lactamases-producing *Escherichia coli* collected from river effluents and Al-Qaa refugee camp in Lebanon and correlation with phylogenetic group ST and phenotype.

City	River source	Key	Phylo	ST	Phenotype	Genotype	Integron
Beirut	Nahr Al-Mawt	EA1	B2	ST617	AMX-AMC-TIC-TZP-ATM-CF-CXM-FOX-CTX-CRO-CAZ-FEP-CFM-TOB-OFX-CIP-NOR-LVX-TCY-SXT	CTX-M-15, TEM-1, SHV-12, *aac(6)-Ib, acc(3)-II*	Int-I1
		EA2	B2	ST617	AMX-AMC-TIC-TZP-ATM-CF-CXM-CTX-CRO-CAZ-FEP-CFM-OFX-CIP-NOR-LVX-TCY-SXT	CTX-M-15, TEM-1, OXA-1, *aac(6)-Ib, acc(3)-II*	Int-I1
Jounieh	Nahr Al-Kalb	EB1	A	ST4144	AMX-AMC-TIC-TZP-ATM-CF-CXM-CTX-CRO-CAZ-FEP-CFM-SXT	CTX-M-15, TEM-1, *acc(3)-II*	Int-I1
		EB2	D	ST6470	AMX-AMC-TIC-TZP-ATM-CF-CXM-CTX-CRO-CAZ-FEP-CFM-TOB-GM-OFX-CIP-NOR-LVX-TCY-SXT	CTX-M-15, OXA-1, SHV-12, *aac(6)-Ib, acc(3)-II*	Int-I1
Saida	Nahr Al-Awali	EC1	A	ST6450	AMX-AMC-TIC-PIP-TZP-ATM-CF-CXM-CTX-CRO-CAZ-FEP-CFM-NOR	*aac(6)-Ib, acc(3)-II*	
		EC2	B2	ST6470	AMX-AMC-TIC-PIP-TZP-ATM-CF-CXM-CTX-CRO-CAZ-FEP-CFM-OFX-CIP-NOR-LVX	*aac(6)-Ib*	
Tripoli	Abou-Ali	ED1	B2	ST6222	AMX-AMC-TIC-TZP-ATM-CF-CXM-FOX-CTX-CRO-CAZ-FEP-CFM-OFX-CIP-NOR-LVX	CTX-M-27, TEM-1, *aac(6)-Ib, acc(3)-II*	Int-I1
		ED2	B2	ST6470	AMX-AMC-TIC-TZP-ATM-CF-CXM-CTX-CRO-CAZ-FEP-CFM-OFX-CIP-NOR-LVX-TCY-SXT	CTX-M-15, TEM-1, *aac(6)-Ib, acc(3)-II*	
Zahle	Berdawni	EE1	B2	ST6470	AMX-AMC-TIC-TZP-ATM-CF-CXM-CTX-CRO-CAZ-FEP-CFM-TOB-GM-OFX-CIP-NOR-LVX-TCY-SXT	CTX-M-15, OXA-1, SHV-12, *aac(6)-Ib, acc(3)-II*	
		EE2	D	ST90	AMX-AMC-TIC-TZP-ATM-CF-CXM-CTX-CRO-CAZ-FEP-CFM-TOB-GM-OFX-CIP-NOR-LVX-TCY-SXT	OXA-1, SHV-12, *ant(2")-Ia, aac(6)-Ib, acc(3)-II*	
		EE3	A	ST90	AMX-AMC-TIC-TZP-ATM-CF-CXM-CTX-CRO-CAZ-FEP-CFM-TOB-GM-OFX-CIP-NOR-LVX-TCY-SXT	CTX-M-15, OXA-1, CMY-2, *aac(6)-Ib, acc(3)-II*	Int-I1
		EE4	A	ST38	AMX-AMC-TIC-TZP-ATM-CF-CXM-CTX-CRO-CAZ-FEP-CFM-SXT	CTX-M-9, *aac(6)-Ib, acc(3)-II*	
Bekaa	Al-Qaa	R1	B2	ST6470	AMX-AMC-TIC-TZP-ATM-CF-CXM-CTX-CRO-CAZ-FEP-CFM-OFX-CIP-NOR-LVX-TCY-SXT	CTX-M-15, OXA-1, *aac(6)-Ib, acc(3)-II*	
		R2	B2	ST6470	AMX-AMC-TIC-TZP-ETP-ATM-CF-CXM-CTX-CRO-CAZ-FEP-CFM-NOR-TCY-SXT-NIT	CTX-M-15, *aac(6)-Ib, acc(3)-II*	
		R3	D	ST4608	AMX-AMC-TIC-TZP-ATM-CF-CXM-FOX-CTX-CRO-CAZ-FEP-CFM-TOB-GM-OFX-CIP-NOR-LVX-TCY-SXT-NIT	CTX-M-15, OXA-1, SHV-12, *aac(6)-Ib, acc(3)-II*	Int-I1
		R4	D	ST4608	AMX-AMC-TIC-TZP-ATM-CF-CXM-CTX-CRO-CAZ-FEP-CFM-TOB-GM-OFX-CIP-NOR-LVX-TCY-SXT	CTX-M-15, *aac(6)-Ib, acc(3)-II*	Int-I1
		R5	A	ST90	AMX-AMC-TIC-TZP-ATM-CF-CXM-FOX-CTX-CRO-CAZ-FEP-CFM-TOB-GM-OFX-CIP-NOR-LVX-TCY-SXT-NIT	TEM-1, CMY-2, *aac(6)-Ib, acc(3)-II*	Int-I1
		R6	A	ST6480	AMX-AMC-TIC-TZP-ATM-CF-CXM-CTX-CRO-CAZ-FEP-CFM-OFX-CIP-NOR-LVX-TCY-SXT	TEM-1, SHV-12, *aac(6)-Ib, acc(3)-II*	Int-I1
		R7	D	ST6894	AMX-AMC-TIC-TZP-ATM-CF-CXM-CTX-CRO-CAZ-FEP-CFM-TOB-GM-OFX-CIP-NOR-LVX-TCY-SXT	CTX-M-15, OXA-1, *aac(6)-Ib, acc(3)-II*	Int-I1
		R8	A	ST90	AMX-AMC-TIC-TZP-ATM-CF-CXM-CTX-CRO-CAZ-FEP-CFM-TOB-GM-OFX-CIP-NOR-LVX-TCY-SXT	TEM-1, *aac(6)-Ib, acc(3)-II*	Int-I1
		R9	D	ST4144	AMX-AMC-TIC-TZP-ETP-ATM-CF-CXM-FOX-CTX-CRO-CAZ-FEP-CFM-OFX-CIP-NOR-LVX	CTX-M-14, OXA-1, SHV-12, CMY-2, *aac(6)-Ib, acc(3)-II, acc(3)-II*	Int-I1

AMC: Amoxicillin/clavulanic acid; AMX: Amoxicillin; ATM: Aztreonam; CAZ: Ceftazidime; CF: Cefalotin; CFM: Cefixime; CIP: Ciprofloxacin; CRO: Ceftriaxone; CTX: Cefotaxime; CXM: Cefuroxime; ETP: Ertapenem; FEP: Cefepime; FOX: Cefoxitin; GM: Gentamicin; LVX: Levofloxacin; NIT: Nitrofurantoin; NOR: Norfloxacin; OFX: Ofloxacin; Phylo: Phylogenetic group; PIP: Piperacillin; ST: Sequence type; SXT: Trimethoprime/sulfamethoxazole; TCY: Tetracycline; TIC: Ticarcillin; TOB: Tobramycin; TZP: Piperacillin/tazobactam.

Discussion

Antibiotic-resistant strains can reach the environment through human excretions and manure of animals [10]. The presence of antibiotic-resistant bacteria in surface waters in Lebanon is a growing public health concern. Water constitutes an important route by which resistance genes are introduced, dispersed and evolved in natural bacterial ecosystems [48]. In such ecosystems, environmental bacteria act as reservoirs of resistance genes. The increasing concentrations of antimicrobial agents originating from household products, industrial waste, heavy metals, animal farming and agriculture introduced into water, favor the evolution and spread of resistance [49]. Wastewater management requires collection, treatment and disposal of treated effluent. In Lebanon and in the absence of operational wastewater treatment plants, effluents from inland communities are disposed in rivers, streams, on open land or underground [12]. This problem, however, was further aggravated following the Syrian war crisis with thousands fleeing their homes and seeking refuge in Lebanon, with one of the key challenges being safe management of wastewater in refugee camps [11].

In this pilot study, we attempt to understand the reasons for the increase in clinical cases of infection with MDR organisms that are being reported in Lebanon. Sewage samples from river effluents and from the Al-Qaa refugee camp were collected, susceptibility testing was done against antimicrobials used to treat *E. coli* infections, and all recovered ESBLs were further characterized. 21 ESBL-producing isolates were recovered from the five river effluents (n = 12) and Al-Qaa refugee camp (n = 9). The ESBL-producing *E. coli* detected were additionally MDR, which was in harmony with Luczkiewicz *et al.*, findings that also reported on MDR ESBLs in treated wastewater treatment plant [20]. On the other hand, resistance to penicillins (amoxicillin, tricarcillin and piperacillin), penicillin/inhibitor combination (amoxicillin–calvulanate [AMC] and piperacillin–tazobacatam), quinolones and cephalosporins was very common among the ESBLs undertaken in this study. Mechanisms of AMC resistance of *E. coli* include: hyperproduction of class A β-lactamase (TEM-1 and SHV-1) [50–51], plasmid or chromosomal-encoded AmpC β-lactamase [52], production of inhibitor-resistant TEM β-lactamase [53–54], plasmid-mediated β-lactamase OXA-1 or modified outer membrane proteins [55]. AmpC production by *E. coli* normally occurs at a low level and clinically not significant; however, plasmid-derived AmpC production is an emerging problem [56], and could along with other resistance determinants account for the high prevalence of AMC resistance observed in this study.

CTX-M, TEM and SHV were detected at a higher ($bla_{\text{CTX-M}}$, bla_{TEM}) or similar (bla_{SHV}) percentages in the isolates from the river effluents compared with those from the refugee camp. This was in accordance with Bréchet *et al.* that reported that ESBL-producing *E. coli* were recovered from environmental samples and accounted for 0.3% of *E. coli* in untreated water upstream of a sewage treatment plant [57]. CTX-M was the most common detected enzyme by Bréchet *et al.* [57], which was in agreement with our results.

All the isolates in this study were resistant to third-generation cephalosporins and aztreonam. ESBLs are plasmid-mediated enzymes that can hydrolyze penicillins, third-generation cephalosporins and azetreonam but not cephamycins [58]. However, EA1, ED1, R3, R5 and R9 were additionally resistant to cephamycin (cefoxitin). Inhibition by β-lactamase inhibitors such as clavulanic acid and inability to hydrolyze cephamycins differentiates the ESBLs from the AmpC-type β-lactamases [59]. AmpC coproduction, which makes ESBLs resistant to β-lactamase inhibitors, made the observed ESBL phenotype in this study more complex.

The observed ertapenem resistance in some of the samples is in accordance with previous findings by Meir-Gruber *et al.* [60], examining the levels of antimicrobial resistance in hospitals and community sewage water. Previously, ertapenem resistance without the production of a carbapenemase enzyme has been reported [61]. This can be due to either the overexpression of efflux pumps or to the coupling of a permeability defect and the overexpression of a CTX-M-type enzyme [61]. Samples which were ertapenem resistant were also positive for CTX-M that could explain their phenotype.

Two of the isolates R1 and R2 were citrate-positive variants and were untypable by PFGE. The incidence of citrate-positive *E. coli* has been very low. Washington II and Timm previously reported on three citrate-positive variants isolated from cultures of human origin [62], while Ishiguro and Sato isolated 67 isolates of citrate-positive variants of *E. coli* from human, domestic animal, feral bird and environmental sources [63]. The structural gene responsible for citrate-positive variants in *E. coli* was located on a conjugative [63], which could be the case with R1 and R2.

E. coli strains mainly fall into four phylogroups (A, B1, B2 and D). Group A and B1 are sister groups, strains from phylogroups B2 and D contained more virulence factors than A and B1 [64,65]. The extraintestinal pathogenic strains belong to B2 and D, intestinal pathogenic strains group belong to A, B1 and D, and the commensal strain

usually belongs to A and B1 groups [65–68]. In this study, *E. coli* phylogroup B2 was the most prevalent representing 38.1% (n = 8) of all isolates, followed by type A (n = 7; 33.3%) and type D (n = 6; 28.6%). Within the refugee camp isolates, however, phylogroup D was the most prevalent. Phylogroups B2 and D exhibit in general less antimicrobial resistance compared with A and B1 phylogroups [69], which was in contrast to our results with resistance being most prevalent in phylogroup D and comparable in phylogroups A and B1. It is noteworthy that the isolates from river effluents typed as belonging to phylogroup A were the most susceptible. The modified resistance patterns observed within the different phylogroups in this study, revealed the impact of the overuse of antibiotics and the lack of proper wastewater management on the dynamics and characteristics of ESBL-producing *E. coli.*

We used PFGE and MLST to subtype the isolates and determine epidemiological associations. Based on that, we did not detect any significant association between the MLST type, the phylogenetic group and the pulsotype. However, PFGE did correctly segregate *E. coli* isolates belonging to different STs as different types. PFGE shows high discriminative power in grouping *E. coli* isolates belonging to different STs [70], while MLST has clear advantage in studies involving ESBL-producing *E. coli*, due to its greater discriminatory ability and reproducibility than PFGE and the ability to define genetically related bacterial strains [61]. ST6470 (allelic profile: 53-40-47-13-36-486-29), which differed from ST3948, part of the ST131 complex, by only a single locus (53-40-47-13-36-10-29), is a newly emerging ST.

The isolates yielded 19 different PFGE restriction patterns and 17 distinct pulsotypes. A remarkable diversity of PFGE pulsotypes has been previously reported in *E. coli* isolated from natural surface waters [71]. A great variety of pulsotypes has been also observed among ST131 isolates from single water samples collected from raw sewage and river water [51] and among clinical samples of CTXM-15-producing *E. coli* [72]. These findings suggest that water bodies are inhabited by genetically diverse *E. coli* rather than environmentally adapted lineages and imply an association with clinical isolates.

The epidemiology of ESBL-producing *E. coli* is a complex and evolving phenomenon attributed to the horizontal transfer of genetic elements and clonal spread of major clones. ESBL production rates are specifically high in wastewater due to selective pressure and genetic exchange in sludge [22]. In this study, class 1 integrons were common among ESBL-producing isolates, which was in harmony with the findings from Algeria reported by Alouache *et al.* [73]. CTX-M-15 was found to be the most prevalent ESBL genotype in wastewater and sewage studies in most regions worldwide including the Americas, Europe and the Middle East [74]. Additionally, the CTX-M-15 was previously detected in Enterobacteriaceae recovered from seawater, rivers and effluents of wastewater treatment plants and hospitals [75], and similar to our findings, was associated with TEM-1 and/or OXA-1 [73]. Additionally, sequencing of *gyrA* and *parC* genes in this study revealed a single substitution D87V and S83T in GyrA and a single S80I or double substitutions S80I and E84V in ParC conferring resistance to fluoroquinolones [73].

This pilot study was initiated in an attempt to understand the increase in clinical cases of infection with such resistant organisms that are being reported in our country. The small numbers collected are a limitation, yet patterns of genetic resistance were interesting to motivate us to complete our investigation by doing a more comprehensive study in the near future.

Conclusion

Although effluents are diluted once they reach the river, they still exert a selective pressure and alter the population dynamics of microorganisms. ESBL-producing *E. coli* strains are emerging pathogens and their molecular typing is useful for surveillance purposes. To the best of our knowledge, this is the first pilot study to characterize ESBL-producing *E. coli* in river effluents and a refugee camp in Lebanon. Further investigations are required concerning the effects of antibiotic-resistant strains released from sewage on the natural ecosystem, on selection of resistance and its impact on human health.

Future perspective

The continuous contamination of rivers over the years without proper handling has worsened bacterial contamination and their resistome in the Lebanese wastewater networks. The problem was aggravated by the influx of refugees often living in poor hygiene conditions and putting strain on the available resources. Thus, for future studies, a larger number of isolates will be studied and multiple ESBL-producing coliforms such as *Enterococcus* spp. will be also considered through plasmid typing and genome analysis needed to give an insight of the current situation of resistance prevalence and dissemination between organisms. Another approach would be to attempt

to correlate environmental and clinical isolates through the evaluation of urine and/or stool cultures from urinary tract infections, and particularly ESBL-producing isolates recovered from pediatric infections, among the refugee population.

Summary points

Influx of population has aggravated the problem of bacteriological environmental contamination in Lebanon

- More isolates on average (53.1%) recovered from the Al-Qaa refugee camp were positive for the tested resistant determinants, compared with river effluents (49.1%).

High prevalence of antibiotic-resistance genes

- All the isolates carried at least one of the two genes encoding for aminoglycoside resistance *aac(6)-1b* and/or *aac(3)-II*.
- CTX-M-15 was the most common among all the isolates (57.4%; n = 12).
- CTX-M-27, CTX-M-14 and CTX-M-9 were additionally detected.
- TEM-1, OXA-1, CMY-2 and SHV-12 were among the detected subtypes.
- In GyrA, a single substitution was detected D87V or S83T in two of the isolates.
- In ParC, single S80I or double substitutions S80I and E84V were detected in some of the isolates.

Absence of carbapenemases & other resistance determinants

- None of the following carbapenemase genes: *VIM1, VIM2, NDM, KPC, IMP* and *OXA-48* were detected.
- All isolates remained negative for *armA, rmtB, ant(4′)-Iia* and *aph(3′)-Ia.*

Diversity in phylogenetic groups, sequence types (STs) & pulsotypes

- Overall, *E. coli* phylogenetic group B2 was the most prevalent (38.1%), followed by type A and type D.
- In total 11 STs were identified.
- ST6470 was the most commonly detected followed by ST90 and ST4144. ST6470, which differed from ST3948, part of the ST131 complex, by only a single locus, is a newly emerging ST.
- Pulsed-field gel electrophoresis genomic DNA fingerprinting revealed 19 distinct XbaI restriction patterns, grouped into 17 pulsotypes.
- A significant association between the multilocus sequence typing, the phylogenetic group and the pulsotype was not detected.
- Pulsed-field gel electrophoresis did correctly segregate *Escherichia coli* isolates belonging to different STs as different types.

Conclusion

- This is the first pilot study to characterize extended-spectrum β-lactamases-producing *E. coli* in river effluents and a refugee camp in Lebanon.
- The introduction of novel resistance patterns into the Lebanese wastewater network requires effective control measures.

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Author contributions

Conception and design of the study: R Moghnieh, R Husni, S Tokajian and E Abboud. Sample collection and isolation: E Abboud, R Husni and S Youssef. Experimental work and data analysis: S Tokajian, T Salloum, H Arabaghian, J Moussa and S Alousi. Data interpretation and discussion: S Tokajian, T Salloum, H Arabaghian and S Alousi. Manuscript writing: S Tokajian, T Salloum, H Arabaghian and S Alousi. Funding: S Tokajian, R Husni and R Moghnieh.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1. Reinthaler FF, Posch J, Feierl G *et al.* Antibiotic resistance of *E. coli* in sewage and sludge. *Water Res.* 37(8), 1685–1690 (2003).
2. Udikovic-Kolic N, Wichmann F, Broderick NA, Handelsman J. Bloom of resident antibiotic-resistant bacteria in soil following manure fertilization. *Proc. Natl Acad. Sci. USA* 42(111), 15202–15207 (2014).
3. Kümmerer K. Drugs in the environment: emission of drugs, diagnostic aids and disinfectants into wastewater by hospitals in relation to other sources – a review. *Chemosphere* 45(6–7), 957–969 (2001).
4. Schwartz T, Kohnen W, Jansen B, Obst U. Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. *FEMS Microbiol. Ecol.* 43(3), 325–335 (2003).
5. Chee-Sanford JC, Aminov RI, Krapac IJ, Garrigues-Jeanjean N, Mackie, RI. Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Appl. Environ. Microbiol.* 67(4), 1494–1502 (2001).
6. Khan S, Knapp CW, Beattie TK. Antibiotic resistant bacteria found in municipal drinking water. *Environ. Process* 3, 541–552 (2016).
7. Taylor NGH, Verner-Jeffreys DW, Baker-Austin C. Aquatic systems: maintaining, mixing and mobilizing antimicrobial resistance? *Trends Ecol. Evol.* 26(6), 278–284 (2011).
8. Wright MS, Baker-Austin C, Lindell AH, Stepanauskas R, Stokes HW, McArthur JV. Influence of industrial contamination on mobile genetic elements: class 1 integron abundance and gene cassette structure in aquatic bacterial communities. *ISME J.* 2(4), 417–428 (2008).
9. Gaze WH, Krone SM, Larsson DGJ *et al.* Influence of humans on evolution and mobilization of environmental antibiotic resistome. *Emerg. Infect. Dis.* 19(7) e120871 (2013).
10. Pruden A, Arabi M, Storteboom HN. Correlation between upstream human activities and riverine antibiotic resistance genes. *Environ. Sci. Technol.* 46(21), 11541–11549 (2012).
11. UNHCR. 2014 Syria Regional Response Plan. (2014). http://data.unhcr.org/syrianrefugees/regional.php

• **One of the few reports tackling the issue of wastewater treatment in Lebanon and influx of refugees.**

12. Khouri R, Yassin N, Farjallah N, El Hajj R, Mahmassani R, Rajeh D. Impact of population growth and climate change on water scarcity, agricultural output and food security. American University of Beirut, Beirut, Lebanon (2014). www.aub.edu.lb/ifi/

• **Case studies discussing the issues of population growth and water security in Lebanon before and after the refugee crisis.**

13. World Bank. *Republic of Lebanon – water sector: public expenditure review.* World Bank, Washington, DC, USA (2010). http://documents.worldbank.org/curated/en/965931468265767738/Republic-of-Lebanon-Water-sector-public-expenditure-review
14. Finley R, Glass-Kaastra SK, Hutchinson J, Patrick DM, Weiss K, Conly J. Declines in outpatient antimicrobial use in Canada (1995–2010). *PLoS ONE* 8(10), e76398 (2013).
15. Blaak H, Hamidjaja RA, Van Hoek AHAM, De Heer L, De Roda Husman AM, Schets FM. Detection of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* on flies at poultry farms. *Appl. Environ. Microbiol.* 80(1), 239–246 (2014).
16. Chen H, Shu W, Chang X, Chen JA, Guo Y, Tan Y. The profile of antibiotics resistance and in tegrons of extended-spectrum beta-lactamase producing thermotolerant coliforms isolated from the Yangtze River basin in Chongqing. *Environ. Pollut.* 158(7), 2459–2464 (2010).
17. Dhanji H, Patel R, Wall R *et al.* Variation in the genetic environments of bla(CTX-M-15) in *Escherichia coli* from the feces of travelers returning to the United Kingdom. *J. Antimicrob. Chemother.* 66(5), 1005–1012 (2011).
18. Hong HJ, Hutchings MI, Neu JM, Wright GD, Paget MSB, Buttner MJ. Characterization of an inducible vancomycin resistance system in *Streptomyces coelicolor* reveals a novel gene (*vanK*) required for drug resistance. *Mol. Microbiol.* 52(4), 1107–1121 (2004).
19. Ishii S, Sadowsky MJ. *Escherichia coli* in the environment: implications for water quality and human health. *Microb. Env.* 23(2), 101–108 (2008).
20. Luczkiewicz A, Kotlarska E, Artichowicz W, Tarasewicz K, Fudala-Ksiazek S. Antimicrobial resistance of *Pseudomonas* spp. isolated from wastewater and wastewater-impacted marine coastal zone. *Environ. Sci. Pollut. Res. Int.* 22(24), 19823–19834 (2015).

21. Hölzel CS, Schwaiger K, Harms K *et al.* Sewage sludge and liquid pig manure as possible sources of antibiotic resistant bacteria. *Env. Res.* 110(4), 318–326 (2010).

• **Describes the possibility of sewage water being an important reservoir of resistance determinants in *Escherichia coli.***

22. Reinthaler FF, Feierl G, Galler H *et al.* ESBL-producing *E. coli* in Austrian sewage sludge. *Water Res.* 44(6), 1981–1985 (2010).

•• **Describes the most common resistance determinants detected in extended-spectrum β-lactamase (ESBL)-producing *E. coli* recovered from water networks.**

23. Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing; 20th Informational Supplement*. CLSI Document, CLSI M100, S26, Wayne, PA, USA (2015).

24. Clermont O, Bonacorsi S, Bingen E. Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Appl. Environ. Microbiol.* 66(10), 4555–4558 (2000).

25. EnteroBase. http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/documents/primersColi_html

26. PulseNet International. www.pulsenetinternational.org

27. Centers for Disease Control and Prevention. PulseNet. www.cdc.gov/pulsenet/

28. Schmidt TM, DeLong EF, Pace NR. Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. *J. Bacteriol.* 173(14), 4371–4378 (1991).

29. Speldooren V, Heym B, Labia R, Nicolas-Chanoine MH. Discriminatory detection of inhibitor-resistant β-Lactamases in *Escherichia coli* by single-strand conformation polymorphism-PCR. *Antimicrob. Agents Chemother.*42(4), 879–884 (1998).

30. Pitout JD, Thomson KS, Hanson ND, Ehrhardt AF, Moland ES, Sanders CC. β-lactamases responsible for resistance to expanded-spectrum cephalosporins in *Klebsiella pneumoniae*, *Escherichia coli*, and *Proteus mirabilis* isolates recovered in South Africa. *Antimicrob. Agents Chemother.*42(6), 1350–1354 (1998).

31. Armand-Lefevre L, Leflon-Guibout V, Bredin J *et al.* Imipenem resistance in *Salmonella enterica* serovar Wien related to porin loss and CMY-4 β-lactamase production. *Antimicrob. Agents Chemother.* 47(3), 1165–1168 (2003).

32. Saenz Y, Briñas L, Domínguez E *et al.* Mechanisms of resistance in multiple-antibiotic-resistant *Escherichia coli* strains of human, animal, and food origins. *Antimicrob. Agents Chemother.* 48(10), 3996–4001 (2011).

33. Lob SH, Kazmierczak KM, Badal RE *et al.* Trends in susceptibility of *Escherichia coli* from intra-abdominal infections to ertapenem and comparators in the United States according to data from the SMART program, 2009 to 2013. *Antimicrob. Agents Chemother.*59(6), 3606–3610 (2015).

34. Tsakris A, Pournaras S, Woodford N *et al.* Outbreak of infections caused by *Pseudomonas aeruginosa* producing VIM-1 carbapenemase in Greece. *J. Clin. Microbiol.* 38(3), 1290–1292 (2000).

35. Poirel L, Naas T, Nicolas D *et al.* Characterization of VIM-2, a carbapenem-hydrolyzing metal lo-β-lactamase and its plasmid and integron-borne gene from a *Pseudomonas aeruginosa* clinical isolate in France. *Antimicrob. Agents Chemother.* 44, 891–897 (2000).

36. Akiba M, Sekizuka T, Yamashita A *et al.* Distribution and relationships of antimicrobial resistance determinants among extended-spectrum-cephalosporin-resistant or carbapenem-resistant *Escherichia coli* isolates from rivers and sewage treatment plants in India. *Antimicrob. Agents Chemother.* 60 (5), 2972–2980 (2016).

37. Dortet L, Nordmann P, Poirel L. Association of the emerging carbapenemase NDM-1 with a bleomycin resistance protein in Enterobacteriaceae and *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 56, 1693–1697 (2012).

38. Markovska R, Stoeva T, Schneider I *et al.* Clonal dissemination of multilocus sequence type ST15 KPC-2-producing *Klebsiella pneumoniae* in Bulgaria. *APMIS.*123, 887–894 (2015).

39. Mendes RE, Kiyota KA, Monteiro J *et al.* Rapid detection and identification of metallo-β-lactamase-encoding genes by multiplex real-time PCR assay and melt curve analysis. *J. Clin. Microbiol.* 45(2), 544–547 (2007).

40. Nordmann P. Global spread of carbapenemase-producing Enterobacteriaceae. *Emerg. Infect. Dis.* 17(10), 1791–1798 (2011).

41. Leflon-Guibout V, Jurand C, Bonacorsi S *et al.* Emergence and spread of three clonally related virulent isolates of CTX-M-15-producing *Escherichia coli* with variable resistance to aminoglycosides and tetracycline in a French geriatric hospital. *Antimicrob. Agents Chemother.*48(10), 3736–3742 (2004).

42. Fernández-Martínez M, Miró E, Ortega A *et al.* Molecular identification of aminoglycoside-modifying enzymes in clinical isolates of *Escherichia coli* resistant to amoxicillin/clavulanic acid isolated in Spain. *Int. J. Antimicrob. Agents.* 46(2), 157–163 (2015).

43. Ayad A, Drissi M, de Curraize C *et al.* Occurrence of ArmA and RmtB aminoglycoside resistance 16S rRNA methylases in extended-spectrum β-lactamases producing *Escherichia coli* in Algerian hospitals. *Front. Microbiol.* 7, 1409 (2016).

44. Chen JY, Siu LK, Chen YH, Lu PL, Ho M, Peng CF. Molecular epidemiology and mutations at *gyrA* and *parC* genes of ciprofloxacin-resistant *Escherichia coli* isolates from a Taiwan medical center. *Microb. Drug Resist.*7(1), 47–53 (2001).

45. White PA, McIver CJ, Rawlinson, WD. Integrons and gene cassettes in the Enterobacteriaceae. *Antimicrob. Agents Chemother.*45(9), 2658–2661 (2001).

46. Al-Agamy MH. Phenotypic and molecular characterization of extended-spectrum β-lactamases and AmpC β-lactamases in *Klebsiella pneumoniae*. *Pak. J. Pharm. Sci.* 26(2), 291–298 (2013).

47. Magiorakos AP, Srinivasan A, Carey RB *et al.* Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.* 18, 268–281 (2011).
48. Baquero F, Martínez JL, Cantón R. Antibiotics and antibiotic resistance in water environments. *Curr. Opin. Biotechnol.* 19(3), 260–265 (2008).
49. Colomer-Lluch M, Jofre J, Muniesa M. Antibiotic resistance genes in the bacteriophage DNA fraction of environmental samples. *PLoS ONE* 6(3), e17549 (2011).
50. Miró E, del Cuerpo M, Navarro F, Sabaté M, Mirelis B, Prats G. Emergence of clinical *Escherichia coli* isolates with decreased susceptibility to ceftazidime and synergic effect with co-amoxiclav due to SHV-1 hyperproduction. *J. Antimicrob. Chemother.* 42(4), 535–538 (1998).
51. Waltner-Toews RI, Paterson DL, Qureshi ZA *et al.* Clinical characteristics of bloodstream infections due to ampicillin–sulbactam-resistant, non-extended- spectrum-beta-lactamase-producing *Escherichia coli* and the role of TEM-1 hyperproduction. *Antimicrob. Agents Chemother.* 55(2), 495–501 (2011).
52. Oteo J, Cercenado E, Cuevas Ó *et al.* AmpC β-lactamases in *Escherichia coli*: emergence of CMY-2-producing virulent phylogroup D isolates belonging mainly to STs 57, 115, 354, 393, and 420, and phylogroup B2 isolates belonging to the international clone O25b–ST131. *Diagn. Microbial. Infect. Dis.* 67 (3), 270–276 (2010).
53. Martín O, Valverde A, Morosini MI *et al.* Population analysis and epidemiological features of inhibitor-resistant-TEM-β-lactamase-producing *Escherichia coli* isolates from both community and hospital settings in Madrid, Spain. *J. Clin. Microbiol.* 48 (7), 2368–2372 (2010).
54. Caniça MMM, Lu CY, Krishnamoorthy R, Paul GC. Molecular diversity and evolution of bla TEM genes encoding β-lactamases resistant to clavulanic acid in clinical *E. coli*. *J. Mol. Evol.* 44 (1), 57–65 (1997).
55. Reguera JA, Baquero F, Pérez-Diaz JC, Martinez JL. Factors determining resistance to β-lactam combined with β-lactamase inhibitors in *Escherichia coli*. *J. Antimicrob. Chemother.* 27(5), 569–575 (1991).
56. Harris PNA, Ferguson JK. Antibiotic therapy for inducible AmpC β-lactamase-producing Gram-negative bacilli: what are the alternatives to carbapenems, quinolones and aminoglycosides? *Int. J. Antimicrob. Agents.* 40(4), 297–305 (2012).
57. Bréchet C, Plantin J, Sauget M *et al.* Wastewater treatment plants release large amounts of extended-spectrum β-lactamase-producing *Escherichia* coli into the environment. *Clin. Infect. Dis.* 58(12), 1658–1665 (2014).

•• **Describes ESBL-producing *E. coli* samples recovered from the environment focusing on the most important resistant determinants found in wastewater.**

58. Pfaller MA, Segreti J. Overview of the epidemiological profile and laboratory detection of extended-spectrum β-Lactamases. *Clin. Infect. Dis.* 42(Suppl. 4), S153–S163 (2006).
59. Chow JW, Fine MJ, Shlaes DM *et al. Enterobacter* bacteremia: clinical features and emergence of antibiotic resistance during therapy. *Ann. Intern. Med.* 115(8), 585–590 (1991).
60. Meir-Gruber L, Manor Y, Gefen-Halevi S *et al.* Population screening using sewage reveals pan-resistant bacteria in hospital and community samples. *PLoS ONE* 11(10), e0164873 (2016).
61. Girlich D, Poirel L, Nordmann P. CTX-M expression and selection of ertapenem resistance in *Klebsiella pneumoniae* and *Escherichia coli*. *Antimicrob. Agents Chemother.* 53(2), 832–834 (2009).
62. Washington II JA, Timm JA. Unclassified, citrate-positive member of the family Enterobacteriaceae resembling *Escherichia coli*. *J. Clin. Microbiol.* 4(2), 165–167 (1976).
63. Ishiguro N, Sato G. The distribution of plasmids determining citrate utilization in citrate-positive variants of *Escherichia coli* from humans, domestic animals, feral birds and environments. *J. Hyg.* 83(2), 331–344 (1979).

• **Describes rarely found citrate-positive *E. coli* strains.**

64. Lecointre G, Rachdi L, Darlu P, Denamur E. *Escherichia coli* molecular phylogeny using the incongruence length difference test. *Mol. Biol. Evol.* 15(12), 1685–1695 (1998).
65. Johnson JR, Delavari P, Kuskowski M, Stell AL. Phylogenetic distribution of extraintestinal virulence-associated traits in *Escherichia coli*. *J. Infect. Dis.* 183(1), 78–88 (2001).
66. Picard B, Garcia JS, Gouriou S *et al.* The link between phylogeny and virulence in *Escherichia coli* extraintestinal infection. *Infect. Immun.* 67(2), 546–553 (1999).
67. Bingen E, Picard B, Brahimi N *et al.* Phylogenetic analysis of *Escherichia coli* strains causing neonatal meningitis suggests horizontal gene transfer from a predominant pool of highly virulent B2 group strains. *J. Infect. Dis.* 177(3), 642–650 (1998).
68. Pupo GM, Karaolis DK, Lan R, Reeves PR. Evolutionary relationships among pathogenic and nonpathogenic *Escherichia coli* strains inferred from multilocus enzyme electrophoresis and *mdh* sequence studies. *Infect. Immun.* 65(7), 2685–2692 (1997).
69. Chakraborty A, Saralaya V, Adhikari P, Shenoy S, Baliga S, Hegde A. Characterization of *Escherichia coli* phylogenetic groups associated with extraintestinal infections in South Indian population. *Ann. Med. Health Sci. Res.* 5(4), 241–246 (2015).

70. Bae IK, Kim J, Sun JYH *et al.* Comparison of pulsed-field gel electrophoresis and repetitive sequence-based PCR methods for molecular epidemiological studies of *Escherichia coli* clinical isolates. *Indian J. Med. Res.* 140(5), 679–685 (2014).

• **Describes the use of pulsed-field gel electrophoresis in molecular epidemiological studies on *E. coli*.**

71. Casarez EA, Pillai SD, Di Giovanni GD. Genotype diversity of *Escherichia coli* isolates in natural waters determined by PFGE and ERIC-PCR. *Water Res.* 41(16), 3643–3648 (2007).

72. Sidjabat HE, Silveira FP, Potoski BA *et al.* Interspecies spread of *Klebsiella pneumoniae* carbapenemase gene in a single patient. *Clin. Infect. Dis.* 49(11), 1736–1738 (2009).

73. Alouache S, Estepa V, Messai Y, Ruiz E, Torres C, Bakour R. Characterization of ESBLs and associated quinolone resistance in *Escherichia coli* and *Klebsiella pneumoniae* isolates from an urban wastewater treatment plant in Algeria. *Microb. Drug Resist.* 20(1), 30–38 (2014).

74. Woerther PL, Burdet C, Chachaty E, Andremont A. Trends in human fecal carriage of extended-spectrum β-lactamases in the community: toward the globalization of CTX-M. *Clin. Microbiol. Rev.* 26 (4), 744–758 (2013).

75. Dolejska M, Frolkova P, Florek M *et al.* CTX-M-15-producing *Escherichia coli* clone B2-O25b-ST131 and *Klebsiella spp*. isolates in municipal wastewater treatment plant effluents. *J. Antimicrob. Chemother.* 66(12), 2784–2790 (2011).